N

N8 (Human)

► Tumor Protein D52 (TPD52)

Na^+/Ca^{2+}-K^+ Exchanger

► SLC24A Family (K^+-Dependent Na^+-Ca^{2+} Exchanger, NCKX)

Na^+/HCO_3^- Cotransporter NBCn1

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Synonyms

NBC2; NBC3; NBCn1; SLC4A7

Historical Background

NBCn1 is an electroneutral Na^+/HCO_3^- cotransporter encoded by *SLC4A7* gene, a member of the bicarbonate transporter family of solute carrier 4 (SLC4). In total, this family contains ten members: (1) three electroneutral anion exchangers AE1 (SLC4A1), AE2 (SLC4A2), and AE3 (SLC4A3); (2) five Na^+-coupled HCO_3^- transporters (NCBTs), including two electrogenic Na^+/HCO_3^- cotransporters NBCe1 (SLC4A4), NBCe2 (SLC4A5), two electroneutral Na^+/HCO_3^- cotransporters NBCn1 and NBCn2 (SLC4A10), and an electroneutral Na^+-driven Cl^-/HCO_3^- exchanger NDCBE (SLC4A8); (3) two less well-characterized members SLC4A9 and SLC4A11.

The association between the transport of Na^+ and HCO_3^- was reported in 1970s in epithelial cells from different systems, such as proximal tubules (Burg and Green 1977) and jejunum (Turnberg et al. 1970; Podesta and Mettrick 1977). In 1983, Boron and Boulpaep clearly conceptualized for the first time Na^+/HCO_3^- cotransporter based on their study with renal proximal tubules from Salamander (Boron and Boulpaep 1983). Proximal tubule is the major site for HCO_3^- reabsorption in the kidney. By microperfusion, Boron and Boulpaep demonstrated that the transport of HCO_3^- across the basolateral membrane of the proximal tubule is coupled to the transport of Na^+. Moreover, this Na^+-coupled transport of HCO_3^- is electrogenic and sensitive to inhibition by the stilbene disulfonate reagents, such as 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid (SITS) and 4,4-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS).

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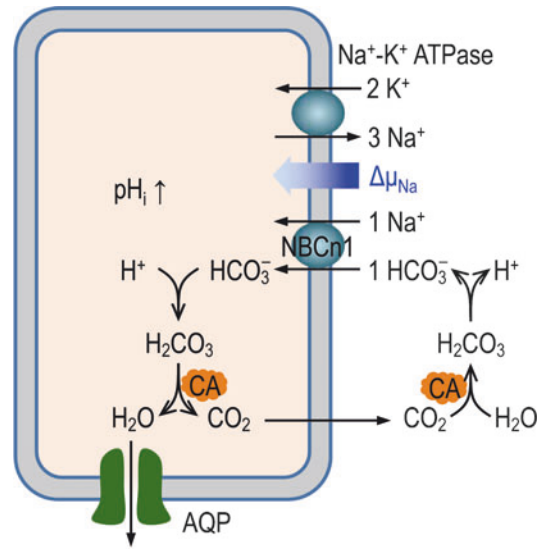
Following the discovery by Boron and Boulpaep, Na^+-coupled HCO_3^- transport was functionally characterized in many cell types from diverse systems, such as rat hepatocytes (Gleeson et al. 1989; Renner et al. 1989), retinal pigment epithelium of frog (la Cour 1989), bovine corneal endothelium (Bonanno and Giasson 1992), cardiac Purkinje fibers (Dart and Vaughan-Jones 1992), smooth muscle cells from rat arteries (Aalkjaer and Cragoe 1988; Aalkjaer and Hughes 1991) and guinea-pig ureter (Aickin 1994), rat hippocampal neurons (Schwiening and Boron 1994), and fibroblast cells (L'Allemain et al. 1985). Except for the Na^+-dependence, some of these HCO_3^- transport activities are also dependent on Cl^- (L'Allemain et al. 1985; Schwiening and Boron 1994).

In 1997, Romero et al. (1997) cloned, from the kidney of Salamander, the cDNA encoding the first Na^+-coupled HCO_3^- transporter, designated as NBCe1, literally meaning the first electrogenic Na^+-bicarbonate cotransporter. The sequence of NBCe1 is homologous to the then well-established Na^+-independent Cl^-/HCO_3^- exchangers AE1–3 of the SLC4 family. Based upon the successful cloning of NBCe1 by Romero et al., many more variants of NBCe1 and other NCBTs have been identified from a broad spectrum of tissues of different species.

NBCn1 is the first established electroneutral Na^+/HCO_3^- cotransporter. Ishibashi et al. (1998) identified from human retina a cDNA encoding NBCn1 (designated as "NBC2"). However, this clone was incomplete, missing sequences for the initial amino-terminus (Nt) of NBCn1, and contained some cloning artifact. In 1999, the Kurtz group identified, from human skeletal muscle and heart, the complete cDNA encoding NBCn1, designated as "NBC3" (Pushkin et al. 1999). In 2000, the Boron group identified the cDNAs of three different NBCn1 variants from rat artery (Choi et al. 2000). This last study functionally demonstrated that the protein is an electroneutral Na^+/HCO_3^- cotransporter and therefore designated it as NBCn1 ("n" meaning electroneutral) following the convention for the nomenclature of NBCe1 ("e" meaning electrogenic).

Molecular Function of NBCn1

NBCn1 is a secondary active transporter that mediates coupled movement of Na^+ and HCO_3^- across the plasma membrane. As shown in Fig. 1, the energy required to drive the operation of NBCn1 is derived from the electrochemical potential difference of Na^+ across the plasma membrane. Under physiological condition, the cell maintains an inwardly directed electrochemical gradient of Na^+ by the activity of Na^+-K^+-ATPase, a primary active transporter. This inwardly directed electrochemical gradient of sodium is used as the driving force for the transmembrane movement of many different solutes, e.g., proton, HCO_3^-, and glucose, by different secondary active transporters.



Na^+/HCO_3^- Cotransporter NBCn1, Fig. 1 Molecular function of NBCn1 in cells. NBCn1 mediates electroneutral Na^+/HCO_3^- influx at an apparent stoichiometry of 1 Na^+:1 HCO_3^- driven by the inwardly directed electrochemical gradient of Na^+ established by the primary active transporter Na^+-K^+-ATPase. On the intracellular side, HCO_3^- consumes one proton to generate CO_2 and H_2O, both of which can leave the cell via diffusion. On the extracellular side, CO_2 recreates H^+ and HCO_3^-. The latter can be recycled by NBCn1. The net effect of the whole process is equivalent to the extrusion of one proton from the cell. *CA* carbonic anhydrase, *AQP* aquaporin, $\Delta\mu_{Na}$ electrochemical driving force of Na^+ across plasma membrane

The cotransport of Na^+ and HCO_3^- carried by NBCn1 is electroneutral with an apparent stoichiometry of 1 Na^+ to 1 HCO_3^-. Therefore, the ion transport by NBCn1 carries no movement of net charge across the plasma membrane and has no effect on the membrane potential of the cells. Unlike the other Na^+-coupled HCO_3^- transporters of SLC4 family, NBCn1 is relatively insensitive to DIDS (Choi et al. 2000).

The HCO_3^- transport mediated by NBCn1 plays an important role in intracellular pH (pH_i) regulation. Under physiological conditions, NBCn1 functions as an acid-extruder by catalyzing the inward movement of HCO_3^- in the cell. As shown in Fig. 1, in the cytosol, HCO_3^- titrates proton to form H_2CO_3, causing a rise in pH_i. H_2CO_3 is then dissociated into H_2O and CO_2 under the influence of carbonic anhydrase (CA). H_2O and CO_2 flux out of the cell either through channels or by simple diffusion. In the extracellular space, the opposite process takes place to generate HCO_3^- and H^+. The net outcome of the whole process is the extrusion of a proton from the cell.

Structure of NBCn1

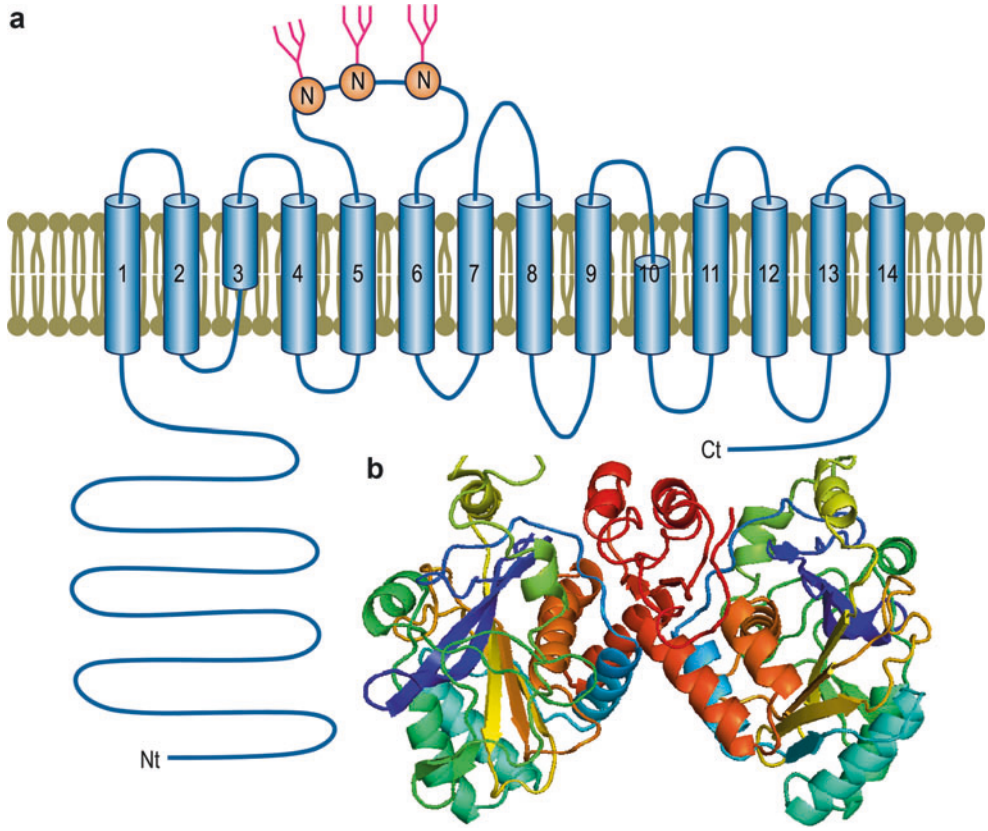
NBCn1 is an N-glycosylated integral membrane protein with an apparent molecular weight of ~180 kDa. As shown in Fig. 2a, NBCn1 contains a large amino-terminal (Nt) domain and small carboxyl terminal (Ct) domain that both localizes on the intracellular side. The transmembrane domain (TMD) contains 14 transmembrane segments (TMs). The Nt domain and the TMD each account for ~45% of the whole polypeptide, whereas the Ct domain accounts for the remaining 10% of the polypeptide.

The fine structure of NBCn1 remains to be resolved, although considerable amount of efforts have been made to explore the structures of the SLC4 family transporters during the past decades. Most of the studies were focused on AE1 (the first identified member of SLC4 family) and NBCe1 (the first identified Na^+-coupled member of SLC4 family) (see reviews Liu et al. 2015; Reithmeier et al. 2016). The crystal structures of both the Nt and TMD domains of human AE1 have been resolved (Zhang et al. 2000; Arakawa et al. 2015). Both the Nt and TMD of AE1 are dimers in the crystal structure, consistent with the idea long-held in the field that the SLC4 transporters are dimers in the plasma membrane.

As will be discussed in the next section, the Nt of NBCn1 contains two conserved regions: Nt-CR1 and Nt-CR2. Nt-CR1 and Nt-CR2 are highly homologous among all SLC4 members. In the crystal structure of the Nt domain of AE1, these two regions are intertwined to form a compact structure, representing the core of the Nt domain (Zhang et al. 2000; Arakawa et al. 2015). Figure 2b shows a model of the three-dimensional (3D) structure of the Nt domain of NBCn1 (lacking Nt-VR1 and cassette II, see next section) predicted by molecular modeling based upon the crystal structure of the Nt of human AE1.

The TMD of the transporter is the machinery responsible for the ion translocation across the plasma membrane. Each monomer in the dimer contains a functional unit for ion translocation. The TMD of NBCn1 shares ~42% of sequence identities with the TMD of AE1. Figure 3 shows a model of the 3D structure of the TMD of NBCn1 obtained by molecular modeling based upon the crystal structure of the TMD of human AE1. The crystallography study on human AE1 (Arakawa et al. 2015) reveals that the TMD of SLC4 transporters contains 14 transmembrane helices (TMs). The 14 TMs fall into two inverted repeats (TM1−7 vs. TM8−14), a structural feature present in a number of membrane channels and transporters (see review Liu et al. 2015). The two inverted repeats can be superimposed on each other upon appropriate transformation. The TMD of SLC4 transporters comprises of two structural domains: the so-called core domain and gate domain that are separated by a cleft. The core domain consists of TMs 1–4 and TMs 8–11, whereas the gate domain consists of TMs 5–7 and TMs 12–14 (Arakawa et al. 2015). During ion translocation, the core domain and the gate domain undergo conformational changes to allow alternating access to the substrate binding site that resides in about the center of the membrane.

One should note that there would be some fundamental differences in the molecular

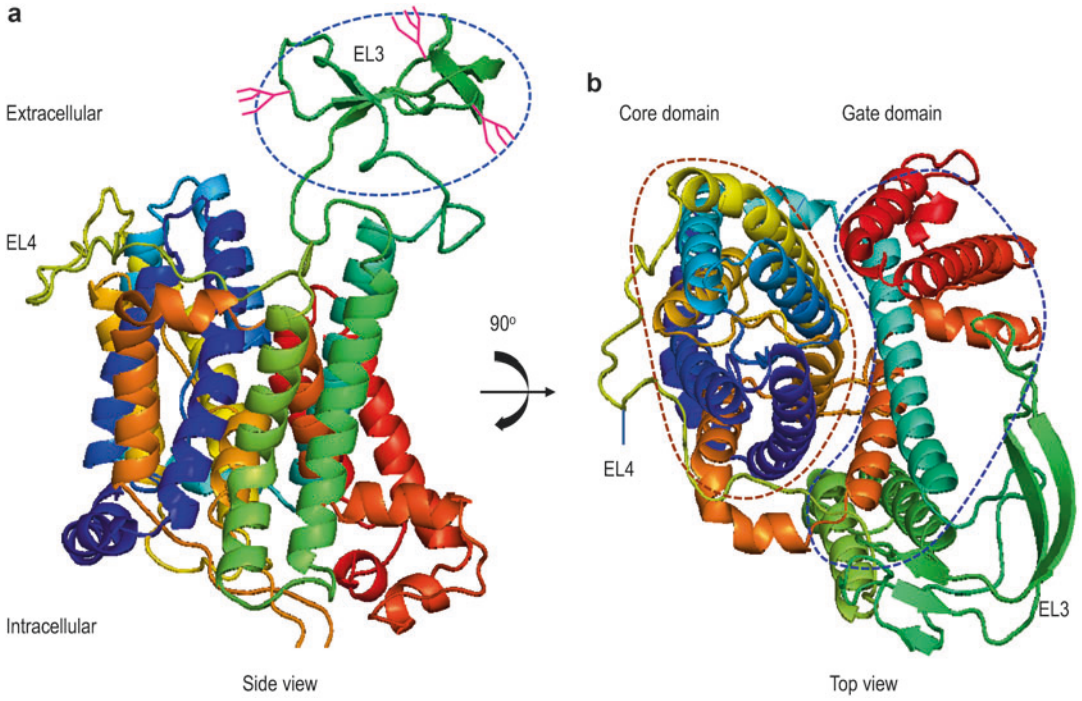


Na^+/HCO_3^- Cotransporter NBCn1, Fig. 2 Topology model of NBCn1 (a) and three-dimensional structure model of Nt domain of NBCn1 (b). The overall topology of NBCn1 is predicted based upon the crystal structure of the TMD of human AE1 (Arakawa et al. 2015). The three-dimensional structure of the Nt domain of NBCn1 is generated by molecular modeling based upon the crystal structure of AE1 Nt (PDB#: 1HYN). The molecular modeling was performed with the sequence of the Nt of human NBCn1-G (NCBI accession# NP_001245308.1, see Fig. 5) using the online tool SWISS MODEL from Protein Structure Bioinformatics Group at Swiss Institute of Bioinformatics (http://swissmodel.expasy.org/). The *pink trees* indicate the potential N-glycosylation sites on the third extracellular loop (EL3) of NBCn1

mechanisms underlying the ion translocation by AE1 and NCBTs including NBCn1. As Na^+-independent Cl^-/HCO_3^- exchanger, AE1 needs no binding site for Na^+. Moreover, it likely uses the same site for alternating binding of Cl^- and HCO_3^- during the ion translocation. However, NCBTs conduct cotransport of Na^+ and HCO_3^-. Therefore, in addition to a binding site for HCO_3^-, NCBT would contain a binding site for Na^+ distinct from that for HCO_3^-.

The TMD of NBCn1 contains some specific structural features different from the TMD of AE1. The third extracellular loop (EL3) of NBCn1 (and other NCBTs as well) is much larger than that of AE1. This EL3 contains three potential N-glycosylation sites (Figs. 2 and 3a). AE1 also contains an N-glycosylation site which resides on the EL4 of AE1. The role of the large EL3 of NBCn1 in ion translocation remains unknown.

The EL4 between TM7 and TM8 is of particular interest for the ion translocation machinery of NCBTs. As shown in Fig. 3b, the EL4 of NBCn1 exhibits a flexible structure connecting the core domain and the gate domain. This EL4 plays an important role in determining the electroneutrality vs. electrogenicity of NCBTs. Replacing EL4 of NBCe1 with that of NBCn1 abolishes the electrogenicity of NBCe1, whereas the opposite



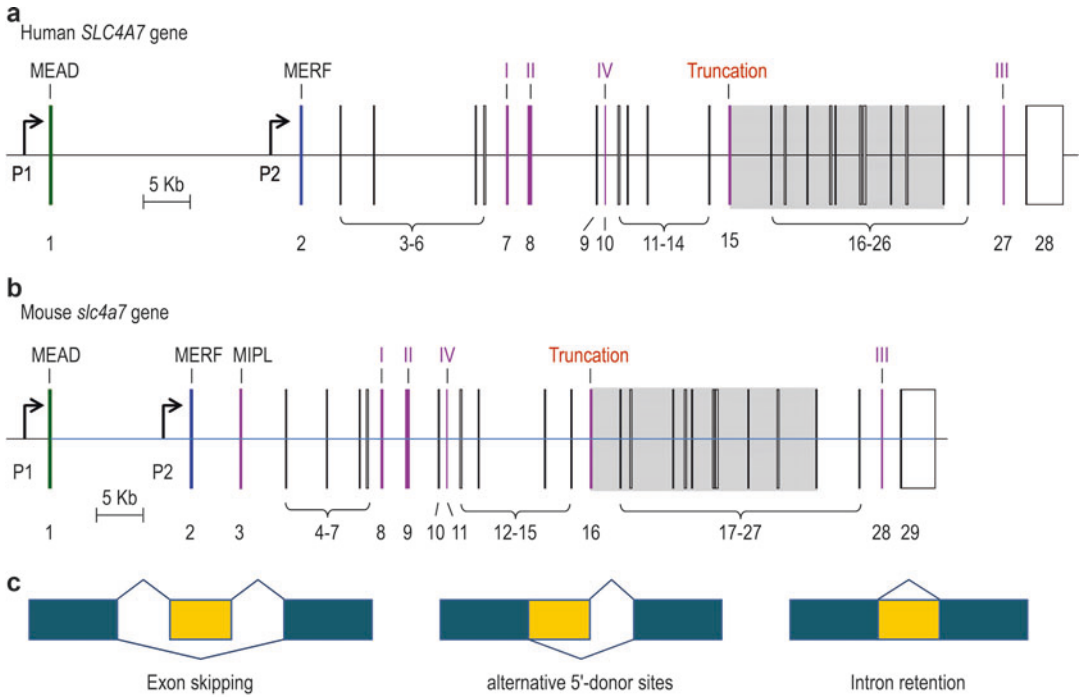
Na^+/HCO_3^- Cotransporter NBCn1, Fig. 3 Model of three-dimensional structure of NBCn1 TMD. The structure model is created by molecular modeling with human NBCn1 (NCBI accession# NP_003606.3) using the crystal structure of human AE1 (PDB# 4YZF) as template (Arakawa et al. 2015). Molecular modeling was performed using the online tool SWISS MODEL from Protein Structure Bioinformatics Group at Swiss Institute of Bioinformatics. The overall fold of NBCn1 TMD is very similar to that of human AE1. The EL3 of NBCn1 is predicted to have a fold with five antiparallel beta sheets. However, the accuracy of this prediction about the fold of EL3 remains to be tested due to its low-sequence homology with the counter region of AE1. The *pink trees* in the side view represent the potential N-glycosylation sites on EL3 of NBCn1. *EL3* the third extracellular loop, *EL4* the fourth extracellular loop

substitution converts an electroneutral transporter into electrogenic (Chen et al. 2011).

Structural Variations of NBCn1

Most mammalian genes often contain more than one promoter controlling the initiation of transcription. Moreover, most mammalian genes contain optional sequences that can be alternatively included in or excluded from the final messenger RNA during pre-RNA splicing. The optional sequences are termed as cassette exons herein. The presence of alternative promoters and cassette exons enables mammals to give rise to multiple expression variants from one single gene.

Shown in Fig. 4a and b are the structures of human *SLC4A7* and mouse *Slc4a7* genes, respectively. Both human *SLC4A7* and mouse *Slc4a7* contain two promoters, the distal promoter P1 and the proximal promoter P2. Human *SLC4A7* contains five known cassette exons (shown in purple). In mouse, the *Slc4a7* gene contains an additional cassette exon that appears to be species specific, i.e., not present in human genome. As shown in Fig. 4c, the mechanism of alternative splicing of the cassette exons in *SLC4A7* fall into three different categories: (1) exon skipping, such as exons 8, 10, 15, and 27 (in human *SLC4A7*), (2) alternative 5′-donor sites, such as exon 7 in human *SLC4A7* (exon 8 in mouse *Slc4a7*), (3) intron retention, such as exon 3 in mouse *Slc4a7* which



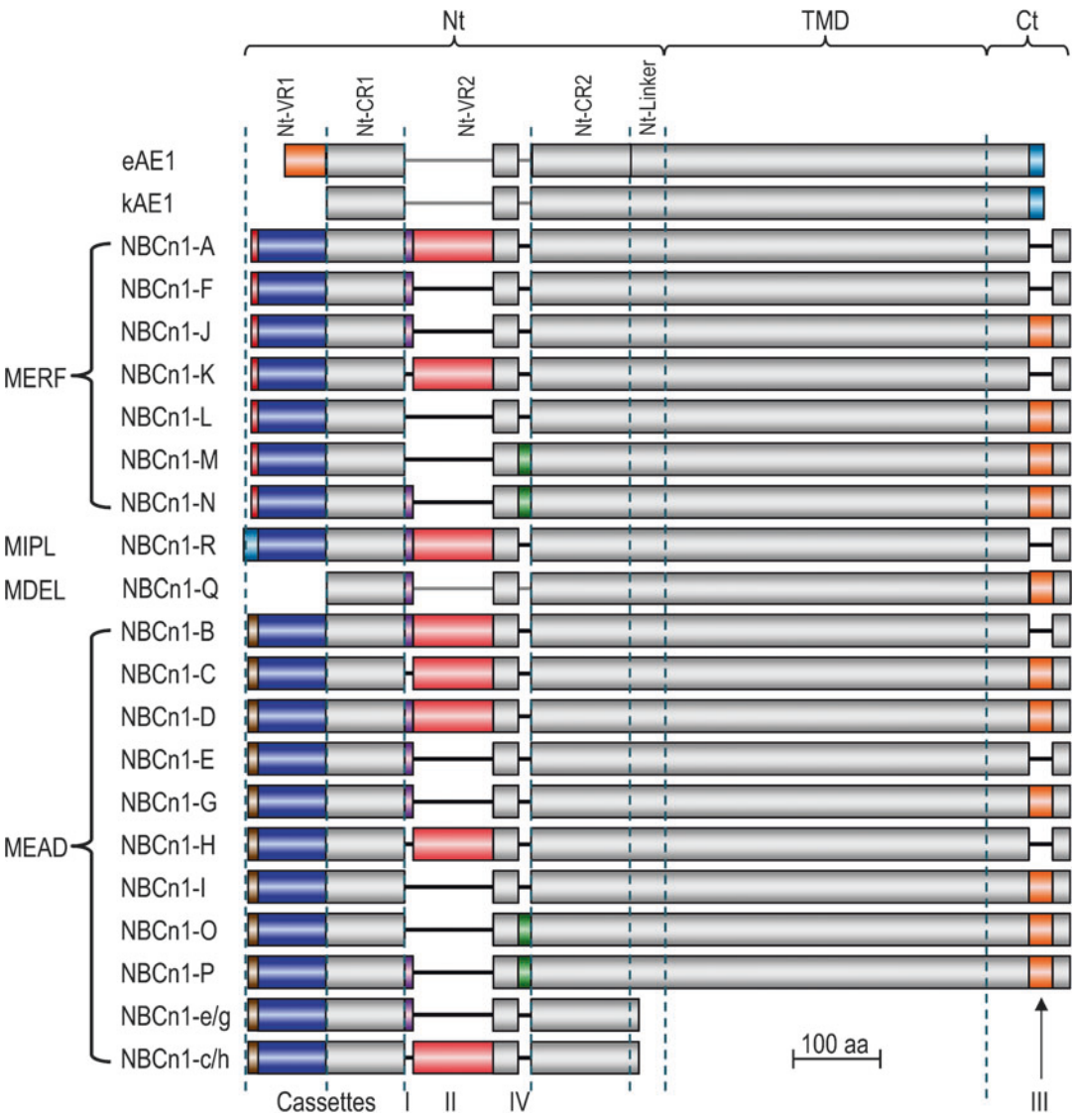
Na^+/HCO_3^- Cotransporter NBCn1, Fig. 4 Structures of human SLC4A7 (a) and mouse Slc4a7 genes (b). The human *SLC4A7* spans for ~110 kb at 3p22 in the genome, and the mouse *Slc4a7* spans for ~98 kb. P1 and P2 indicate the two promoters of *SLC4A7*/*Slc4a7*. Exon 3 in mouse *Slc4a7* appears species specific. Sequence homologous to mouse exon 3 is not identified in human *SLC4A7*. The exons in *grayed box* indicate the region encoding the TMD of NBCn1. *Purple* indicate the exons that can be alternatively spliced. Splicing-out exon 15 (exon 16 in mouse Slc4a7) causes the production of the isolated Nt domain of NBCn1 (see Fig. 5)

contains a cryptic intron. The entire exon 3 can be skipped in some transcripts of *Slc4a7*. Note that the transcription of *SLC4A7*/*Slc4a7* from alternative promoters P1 or P2 and the expression of their cassette exons are tissue specific.

As summarized in Fig. 5, the mammalian *SLC4A7* gene is able to produce at least 18 full-length NBCn1 variants plus two specialized products with the isolated Nt domain only. These NBCn1 variants contain four different extreme Nts. The variants with an extreme Nt starting with "MERF" or "MIPL" or "MDEL" are derived from promoter P2, whereas those starting with "MEAD" are derived from promoter P1. The Nt domain of NBCn1 contains three splicing cassettes: cassettes I, II, and IV, whereas the small Ct domain of NBCn1 contains one splicing cassette: cassette III. No structural variation is present in the TMD of NBCn1. The alternative Nts and splicing cassettes are collectively referred to as optional structural elements (OSEs) hereafter in the following discussion.

According to the localization of the OSEs, the Nt domain of NBCn1 can be divided into two variable regions (Nt-VR1 and Nt-VR2) and two conserved regions (Nt-CR1 and Nt-CR2; Fig. 5). The whole Nt domain is connected to the TMD by a linking peptide "Nt-linker." As discussed in the above section, the conserved regions Nt-CR1 and Nt-CR2 are highly homologous to the counterparts of other SLC4 members and form a structure representing the core domain of the Nt domain (Fig. 2b). The variable regions (Nt-VR1 and Nt-VR2) are appendages to this core domain. The structure of Nt-VR1 and Nt-VR2 and their relationships with the core domain of NBCn1 Nt remains unknown.

The OSEs play important roles in modulating the function of NBCn1. Firstly, some OSEs have profound effects on the intrinsic activity (the



Na^+/HCO_3^- Cotransporter NBCn1, Fig. 5 Expression variants of NBCn1. NBCn1 variants are alphabetically designated according to the order of discovery (for NCBI accession numbers, see review Liu et al. 2015). The variants with the same initial Nt sequence, e.g., those starting with "MEAD," are grouped together. Nt domain contains two variable regions Nt-VR1 and Nt-VR2 and two conserved regions Nt-CR1 and Nt-CR2. The Nt domain is connected to the TMD via a linker peptide "Nt-linker." Two AE1 variants (the erythrocytic variant eAE1 and the kidney variant kAE1) are included in the diagram for reference

transport activity per molecule) of NBCn1. Although the differences in the alternative Nts (MEAD vs. MERF, and likely MIPL) has little effect on the intrinsic transport activities of NBCn1, splicing cassettes II, III, and IV elicit strong stimulatory effect on the intrinsic transport activity of NBCn1 (Liu et al. 2013). Depending on the structural context, the intrinsic transport activity of one NBCn1 variant, e.g., NBCn1-N containing cassettes III and IV, can be five times higher than that of another, e.g., NBCn1-E lacking cassettes III and IV (Liu et al. 2013).

Secondly, some OSEs of NBCn1 contain binding sites for regulatory partners. One example is

Nt-VR1 which contains structural determinants for the binding of IRBIT (IP_3R binding protein released with IP_3), also known as S-adenosyl homocysteine hydrolase-like 1 (AHCYL1). The interaction of IRBIT can stimulate the activity of NBCn1 (Parker and Boron 2013). IRBIT can also interact with and inhibit the activity of inositol 1,4,5 trisphosphate (IP_3) receptor (IP_3R), a Ca^{2+} channel on endoplasmic reticulum that plays a critical role in cellular Ca^{2+} signaling (Yang et al. 2011). The interaction between IRBIT and IP_3R is abolished upon binding of IP_3 to IP_3R, a process that would cause a rise in intracellular Ca^{2+} concentration. Another well-studied example is cassette II which contains binding determinants for calcineurin A (CnA) (see review Parker and Boron 2013). Unique to NBCn1, this cassette contains 124 residues in human NBCn1 (123 aa in rodent NBCn1). Interaction of CnA with cassette II stimulates the transport activity of NBCn1 (Danielsen et al. 2013). CnA is a Ca^{2+}/-calmodulin-activated serine/threonine-specific phosphatase that plays an important role in the regulation of a series of channels and transporters in the cardiovascular system (Wang et al. 2014).

Note that, both signaling pathways involving IRBIT or CnA are related to Ca^{2+}. The relationship between NBCn1 and Ca^{2+} signaling in the cell is of interest. For more details, see discussion in section "NBCn1 in the Cardiovascular System."

NBCn1 in the Central Nervous System

NBCn1 plays important physiological and pathological roles in diverse systems. Discussed in this section is NBCn1 in the central nervous system (CNS). The role of NBCn1 in the kidney, cardiovascular system, and breast cancer will be discussed in the following sections.

CNS is likely the system where NBCn1 is most abundantly expressed in the body. Here, NBCn1 is predominantly expressed in neurons and the epithelial cells in choroid plexus. Together with other acid-base transporters, NBCn1 is involved in the maintenance of pH homeostasis in the brain.

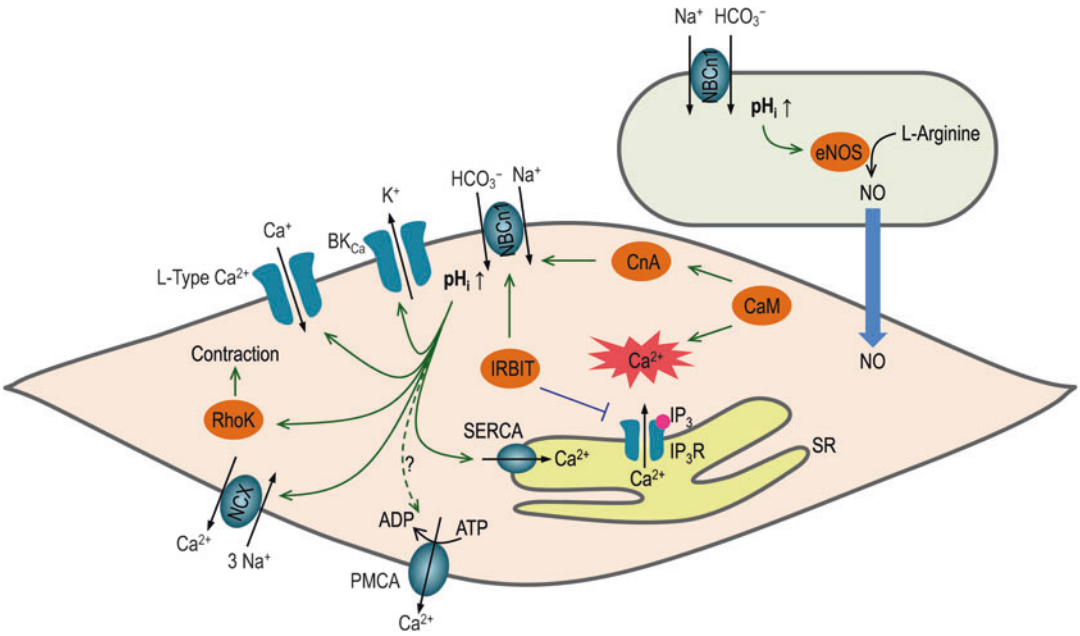
pH_i is a fundamental regulator of the excitability of CNS. It is generally appreciated that neurons in the CNS is stimulated by intracellular alkalosis and is inhibited by intracellular acidosis. On the other hand, neuronal activities, such as presynaptic transmitter release, $GABA_A$ receptor activities, action potential firing, can cause transients in both pH_i and extracellular pH (pH_o) in CNS. Thus, it is critically important to maintain the pH homeostasis in the nervous system.

Dysfunction of acid-base transporters is associated with multiple neural diseases, such as epilepsy, migraine, autism, and mental retardation. Specifically, *SLC4A7* is shown to be associated with drug addiction in human. In addition, genetic disruption of *Slc4a7* affects the development of retina and inner ears, resulting in blindness and auditory impairment in mouse, suggesting that NBCn1 plays an important role in sensory systems (Bok et al. 2003; Lopez et al. 2005).

NBCn1 in the Cardiovascular System

The physiological and pathophysiological significance of NBCn1 in the cardiovascular system is becoming increasingly recognized. In human, an SNP in *SLC4A7* gene is associated with increased risk of hypertension (Ehret et al. 2011). In mouse, genetic disruption in *Slc4a7* causes mild hypertension at rest, suggesting NBCn1 playing an important role in modulating the vascular tone (Boedtkjer et al. 2011). On the other hand, disruption of NBCn1 attenuates blood pressure increase induced by angiotensin II administration in mouse (Boedtkjer et al. 2011). NBCn1 appears to be the primary acid extruder expressed in vascular smooth muscle cells and heart endothelial cells. Knockout of NBCn1 causes mild intracellular acidosis in smooth muscle and endothelial cells under steady-state condition.

As shown in Fig. 6, the NBCn1-related phenotypes of the cardiovascular system might be the result of multiple different processes given the pH sensitivity of many soluble enzymes, membrane ion channels, and transporters in the blood vessel (see review Boedtkjer and Aalkjaer 2012). For example, the activities of the endothelial nitride oxide synthase (eNOS) and Rho kinase, myofilaments, and troponin complex in vascular smooth



Na^+/HCO_3^- Cotransporter NBCn1, Fig. 6 Role of NBCn1 in vascular system. NBCn1 is expressed in both endothelial cells and vascular smooth muscle cells (VSMCs). In the VSMCs, NBCn1 is regulated by IRBIT and calcineurin (CnA). Disruption in NBCn1 causes intracellular acidification, which in turn would affect the function of a number of proteins involved in the regulation of smooth muscle contraction. The activity of eNOS is reduced in endothelial cells due to knockout of NBCn1. *Green arrows* indicate stimulation, *blue line* indicates inhibition and *dashed line* indicates presumed effect. *BK_{Ca}* Ca^{2+}-activated potassium channel, *CaM* calmodulin, *IP_3* inositol 1,4,5 trisphosphate, *NCX* Na^+/Ca^{2+} exchanger, *IP_3R* IP_3 receptor, *SR* sarcoplasmic reticulum, *PMCA* plasma membrane Ca^{2+} ATPase

muscle cells are stimulated by elevating pH_i. In endothelial cells, eNOS is responsible for the production of nitric oxide (NO) from L-arginine. NO plays a critically important role in blood vessel relaxation. Rho kinase is involved in the regulation of the Ca^{2+} sensitivity of vascular smooth muscle cells, probably due to the phosphorylation and deactivation of myosin light chain phosphatase, which plays an important role in the development of hypertension in response to angiotensin II.

Raising pH_i stimulates the activity of the Ca^{2+}-activated potassium (BK_{Ca}) channel, which plays a pivotal role in modulating the excitability of vascular smooth muscle cells (VSMCs). Activation of the BK_{Ca} channel would render the VSMCs hyperpolarized and therefore inhibit smooth muscle contraction.

Excitation and contraction of VSMCs are accompanied with transients in intracellular Ca^{2+} content (Bolton 2006). The release and clearance of cytosolic Ca^{2+} are mediated by a group of Ca^{2+} channels and transporters on the plasma and sarco-endoplasmic membranes. The activities of these channels and transporters are affected by the changes in pH_i. For example, the sarcolemmal Na^+/Ca^{2+} exchanger (NCX), sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), and sarcolemmal L-type Ca^{2+} channels are stimulated by raising pH_i.

Consistent with the hypertension induced by disruption of *Slc4a7* in mouse, Boedtkjer et al. have shown that the activities of eNOS, Rho kinase, Ca^{2+}-activated potassium (BK_{Ca}) channel in mouse arteries are inhibited by intracellular acidosis derived from disruption of *Slc4a7* (Boedtkjer et al. 2011).

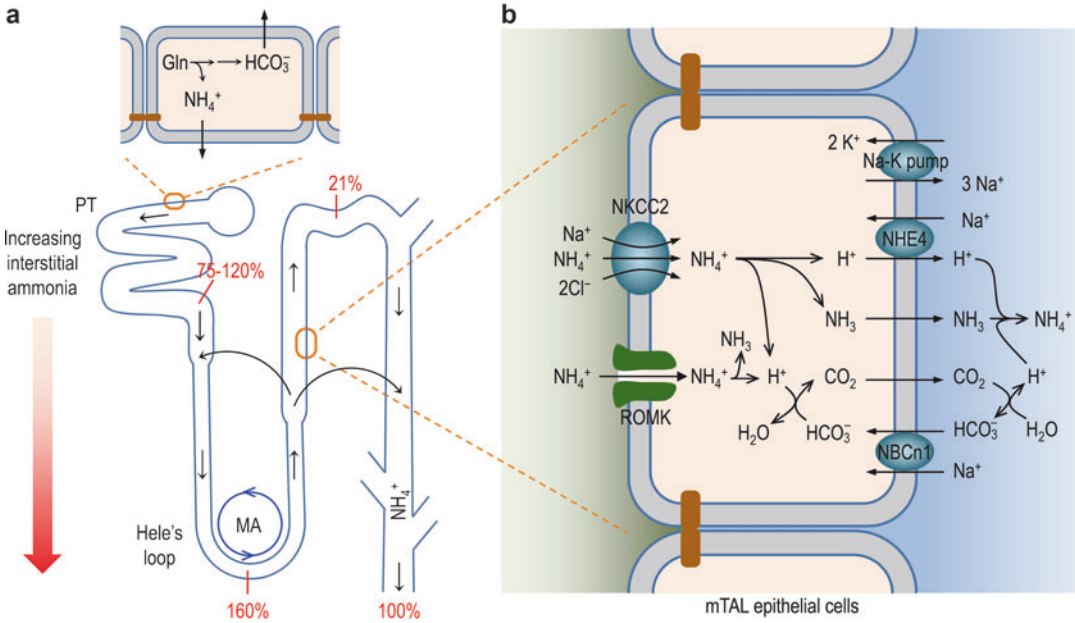
The regulation of pH_i and Ca^{2+} signaling is obviously reciprocally related. On one hand, as

discussed above, the activities of machineries involved in intracellular Ca^{2+} homeostasis is affected by changes in intracellular pH. On the other hand, the activity of acid-base transporters like NBCn1 is regulated by pathways related to Ca^{2+} signaling. As discussed in section "Structural Variations of NBCn1" and shown in Fig. 6, NBCn1 is stimulated by IRBIT and CnA, both of which are related to or affected by the intracellular Ca^{2+} signaling. The release of IRBIT from IP_3R relies on the binding of IP_3 to the Ca^{2+} channel IP_3R. In the meantime, the binding of IP_3 activates IP_3R, causing an intracellular Ca^{2+} transient due to the release of Ca^{2+} store in the SR lumen. The rise in the concentration of cytosol Ca^{2+} stimulates calmodulin (CaM), which in turn activates CnA, which in turn stimulates the HCO_3^- uptake by NBCn1.

NBCn1 in Renal Acid-Base Transport

The kidney plays a central role in maintaining the systemic acid-base balance in the body by reabsorbing bicarbonate in the renal filtrate and excreting net acid into the urine. The major site for bicarbonate reabsorption is the proximal tubule, where the electrogenic Na^+/HCO_3^- cotransporter NBCe1 plays an important role in HCO_3^- reabsorption. The role of NBCe1 in HCO_3^- reabsorption is not discussed here.

A large amount of the acid is excreted as ammonium in the urine (Weiner and Hamm 2007). As shown in Fig. 7a, the ammonium is primarily produced by glutamine metabolism in the proximal tubule epithelial cells. The ammonium is then secreted into the tubule lumen. A large fraction of the ammonium is reabsorbed



Na^+/HCO_3^- Cotransporter NBCn1, Fig. 7 Role of NBCn1 in ammonium transport in the kidney. (**a**) Overview of ammonium transport in the kidney. Ammonium is primarily derived from glutamine metabolism in the proximal tubule epithelial cells. In the Henle's loop, the ammonium undergoes a recycling process, resulting in medullary accumulation of ammonium. Numbers in *red* indicate the ammonium delivered to specific sites as percentage of the final ammonium excreted in the urine (see review Weiner and Hamm 2007). (**b**) Molecular mechanism for ammonium reabsorption by the epithelia in the mTAL. The luminal ammonium enters into the epithelial cells via the apical Na^+-K^+-Cl^- cotransporter NKCC or potassium channel ROMK. In the cell, NH_4^+ is dissociated to form NH_3 and H^+, resulting intracellular acidification. The basolateral NBCn1 mediates HCO_3^- uptake to attenuate the acidification. The NH_3 derived from NH_4^+ and the CO_2 derived from titration of HCO_3^- diffuse into the interstitial space

by the medullary thick ascending limb (mTAL) of the Henle's loop and secreted into the interstitial space; a small fraction of the ammonium is delivered to the distal convoluted tubule. While some of the ammonium secreted into the interstitial space might flux back into the descending limb of the Henle's loop, a large part eventually enters into the collecting duct and finally appears in the urine.

NBCn1 at the basolateral membrane of mTAL plays an important role in the ammonium reabsorption by the mTAL epithelial cells (Fig. 7b). Here, the uptake of ammonium from the tubular lumen could produce a profound acidification in the pH_i of the mTAL epithelial cells. NBCn1 could attenuate the intracellular acidification by mediating HCO_3^- influx to titrate the intracellular acid load due to the apical NH_4^+ influx. Moreover, the titration of NH_4^+ increases the inward driving force for NH_4^+ across the apical membrane and therefore promotes the NH_4^+ reabsorption by the mTAL.

NBCn1 and Breast Cancer

In solid tumors, the cancer cells undergo profound changes in cellular metabolism. The tumor tissues are usually poorly vascularized, resulting in inadequate blood supply and therefore hypoxic condition in the microenvironment of the tumors. On the other hand, the tumor tissues are metabolically highly active due to the high demand in energy. Compared to the normal tissues, tumor tissues have a much higher rate of aerobic glycolysis. The high rate of metabolism and increased aerobic glycolysis result in increased production of metabolic acids, e.g., lactate, from nonoxidative breakdown of glucose in the tumor tissues. This is the so-called Warburg effect (Webb et al. 2011).

As an adaptive response to the increased acid production, the acid-extruders, such as proton pumps, Na^+-H^+ exchangers, and monocarboxylate trasnporters, are often upregulated to promote acid clearance in the cancer cells. However, the poor vascularization in the tumor tissues impairs the disposal of the metabolic acids, resulting in an acidic microenvironment in the extracellular space in tumor tissues. The upshot is an outwardly-directed pH gradient in tumor tissues ($pH_i > 7.2$ vs. $pH_o = \sim 6.7–7.1$), which is in striking contrast to the inwardly directed pH gradient in normal tissues ($pH_i \approx 7.2$ vs. $pH_o \approx 7.4$) (Webb et al. 2011). The inverted pH gradient can promote tumor growth in several perspectives. The enhanced pH_i is stimulatory to the proliferation of tumor cells and is inhibitory to cell apoptosis. Moreover, the acidic pH_o promotes cell-matrix remodeling and therefore increases metastasis and evasion of tumor cells.

NBCn1 is a major acid extruder in breast cancer cells and plays an important role in breast cancer. Disruption of NBCn1 greatly delays the development of breast cancer in mouse (Lee et al. 2016). The expression of NBCn1 is upregulated in breast carcinomas and metastases (Boedtkjer et al. 2013). In breast cancer cells, the expression of NBCn1 is regulated by the signaling pathway involving receptor tyrosine kinase ErbB2 (see review by Gorbatenko et al. 2014). A constitutively active variant of ErbB2 truncated in the Nt is associated with increased risk of breast cancer. This Nt-truncated ErbB2 can greatly stimulate the transcription of *SLC4A7*. Interestingly, a single-nucleotide polymorphism (SNP) in the 3′-untranslated region of transcripts of human *SLC4A7* is strongly associated with increased risk of breast cancer in human. The effect of this SNP on the expression of NBCn1 in breast cancer remains unknown.

Summary

The electroneutral Na^+/HCO_3^- cotransporter NBCn1 is widely expressed in a wide spectrum of tissues. It plays an important role in the regulation of intracellular pH and the transepithelial transport of electrolytes. The physiological and pathological significance of NBCn1 has been emerging during the past years. The *SLC4A7* gene encoding NBCn1 has been associated with

multiple diseases, including breast cancer, hypertension, and drug addiction.

Although great progress has been made during the past years in understanding the structure and function, functional regulation, physiological and pathological roles of NBCn1, many issues remain unclear. The following questions are of particular interest for future study.

1. Molecular mechanism underlying the ion transport by NBCn1. TMD is the structural unit directly carrying out the ion translocation across the plasma membrane. The resolution of the crystal structure of AE1 TMD represents a major step forward towards understanding the structure and function of the SLC4 transporters. However, there are also major differences between the AE1 which is Na^+-independent and NBCn1 (other NCBTs as well) which is Na^+-dependent. What is the mechanism for the energy coupling underlying the cotransport of Na^+ and HCO_3^-? What is the structural mechanism for determining the stoichiometry of Na^+ and HCO_3^-?
2. Functional relationship between the Nt domain and TMD. The Nt domain is connected to TMD by a flexible linker. It has been well recognized that the Nt domain plays an important role in the regulation of the transporter. Specific structural elements (e.g., cassettes II and IV) in the Nt domain can stimulate the intrinsic activity of NBCn1. It remains unclear how the Nt domain affect the efficiency of the ion transport by the TMD. What is the physical interaction between the Nt domain and TMD of NBCn1?
3. Signaling pathways underlying the regulation of NBCn1. The activity of NBCn1 is stimulated by IRBIT and CnA. However, the signaling pathway underlying the regulation of IRBIT and CnA remains not so clear. What is the relationship between the regulation by the IRBIT pathway and that by the CnA pathway? As IRBIT and CnA are both related to Ca^{2+}, what is the role of Ca^{2+} signaling in the regulation of NBCn1?
4. Crosstalk between proton signaling and Ca^{2+} signaling. NBCn1 activity affects the dynamic concentration of protons in the cell. Fluctuation in cellular proton concentration affects the activities of the cellular machineries involved in the maintenance of cellular Ca^{2+} homeostasis. On the other hand, the Ca^{2+} signaling regulates the activity of NBCn1 via the CnA pathway. The crosstalk between proton signaling and Ca^{2+} signaling is of great interest.

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Na^+/K^+ Pump

▶ Na^+/K^+-ATPase

Na^+/K^+-ATPase

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Synonyms

Na^+/K^+ pump; Sodium pump; Sodium-potassium pump

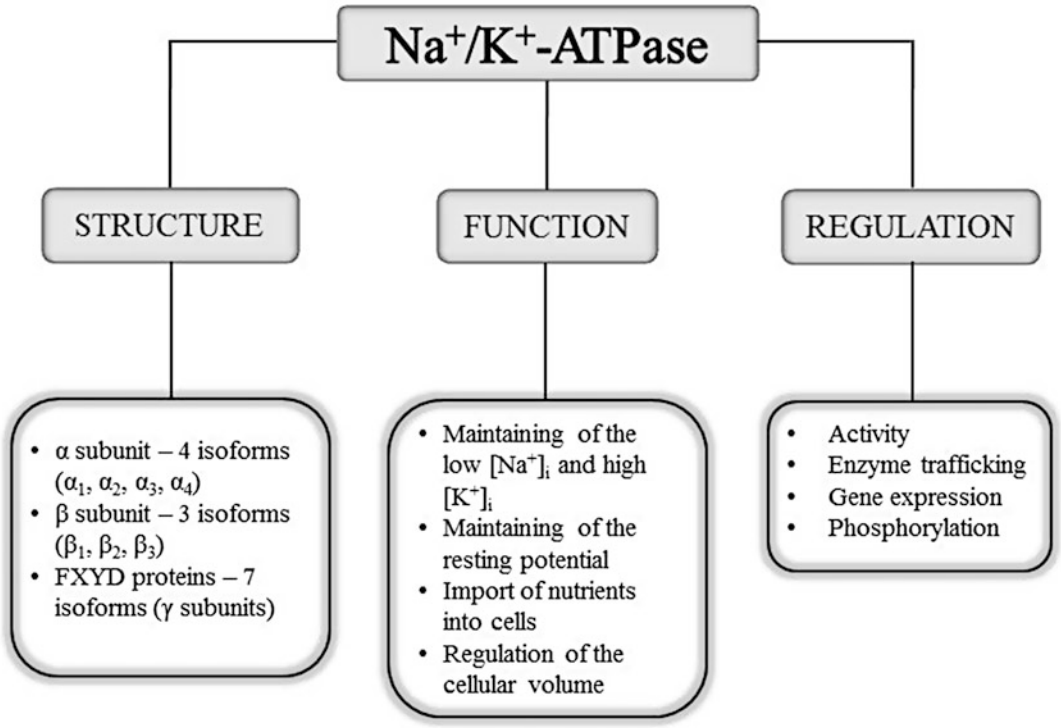
Historical Background

Danish scientist, Jeans C. Skou, was the first who suggested a link between transport of sodium (Na^+) and potassium (K^+) across the plasma membrane and adenosine-triphosphatase (ATPase) activity in 1950s. For the discovery of the Na^+/K^+-ATPase, Jeans C. Skou was awarded by the Nobel Prize in Chemistry in 1997. This discovery was important for understanding the reaction of excitable cells (nervous cells) to the stimuli and transmission of impulses. The Na^+/K^+-ATPase is a membrane protein and has a role in the active transport of Na^+ and K^+ ions across the plasma membrane. For this transport, Na^+/K^+-ATPase uses the energy derived from the process of hydrolysis of the terminal phosphate bond of ATP. During this process, the acyl phosphate forms as intermediate. The Na^+/K^+-ATPase helps maintaining resting potential and import of amino acids, glucose and other nutrients into cells and regulates cellular volume (Fig. 1).

Reduction in Na^+/K^+-ATPase levels is associated with obesity, apparently linked to hyperglycemic-hyperinsulinemia, which may repress or inactivate the enzyme. Decrease in cardiac Na^+/K^+-ATPase activity or protein concentration contributes to the deficiencies in cardiac contractility in animal models and has been documented in patients with heart failure (HF) (Schwinger et al. 2003; Obradovic et al. 2015). The Na^+/K^+-ATPase is also served as a receptor of digitalis steroids, and after their binding, Na^+/K^+-ATPase controls myocyte Ca^{2+} balance and cardiac contractility.

Na^+/K^+-ATPase belongs to the family of P-type ATPases, also including the closely related H^+,K^+-ATPase, Ca^{2+}-ATPases, heavy metal-transporting ATPases, and ATPases with lipid substrates and unknown substrates. The common characteristic of these ATPases is the formation of a covalent phosphoenzyme intermediate as a part of the catalytic mechanism. For each ATP hydrolyzed by the Na^+/K^+-ATPase, three internal Na^+ are exchanged for two external K^+ in a reaction cycle involving sequential binding and release of Na^+ and K^+ and transition between two major conformational states, the so-called E1 (Na-selective) and E2 (K-selective) (Therien and Blostein 2000).

The primary effect of the Na^+/K^+-ATPase is to maintain the low intracellular Na^+ and high intracellular K^+ concentrations required for a multitude of cellular functions. This occurs in several steps (Therien and Blostein 2000). Following binding of ATP to Na^+/K^+-ATPase, three Na^+ ions from the cytoplasm associate with the molecule. Transfer of a phosphate group (via hydrolysis of ATP) to the Na^+/K^+-ATPase results in a conformational change that creates an opening at the outside of the cell that allows the three bound Na^+ ions to be released. Following cleavage of the phosphate group, two extracellular K^+ ions then bind the Na^+/K^+-ATPase and are released inside the cell (Therien and Blostein 2000; Kaplan 2002). By maintaining the Na^+ gradient between intracellular and extracellular compartments, this enzyme has influence on many vital functions, like cell volume, absorption processes in kidney, and excitability in nerve and muscle. Transport of sugars and amino acids in tissues depends on proper activity of Na^+/K^+-ATPase.



Na^+/K^+-ATPase, Fig. 1 Structure, function, and regulation of Na^+/K^+-ATPase. Na^+/K^+-ATPase: sodium/potassium adenosine-triphosphatase, $[Na^+]_i$: intracellular concentration of sodium ions, $[K^+]_i$: intracellular concentration of potassium ions

Structure of Na^+/K^+-ATPase

The Na^+/K^+-ATPase is a universally expressed transmembrane protein of oligomeric structure, consisting of two main subunits α and β which are present in equal molar ratio (1:1) (Therien and Blostein 2000). In addition, other proteins such as FXYD proteins, also referred as γ subunits, are differently expressed in cell- and tissue-specific manner. The FXYD proteins have a role in stabilizing the Na^+/K^+-ATPase and regulating its function (Garty and Karlish 2006).

The large α subunit (∼110 kDa) contains 10 transmembrane domains (usually numbered as M1 to M10) and three large intracellular domains. These cytosolic domains catalyze transfer of phosphoryl group. The amino terminal tail and the second cytoplasmic peptide together form actuator domain or A domain required for dephosphorylation of the enzyme and for coupling ATP hydrolysis to ion transport through the membrane (M) domain. The large cytoplasmic loop connecting M4 and M5 forms two domains: the nucleotide-binding domain (N domain) responsible for ATP binding and the phosphorylation domain (P domain) which catalyzes transfer of the c-phosphoryl group to the enzyme (Therien and Blostein 2000; Kaplan 2002).

The smaller β subunit (∼35–55 kDa) is necessary for proper folding and insertion of the newly synthesized protein into the membrane. It is composed from one transmembrane segment, short cytoplasmic tail, and large glycosylated extracellular segment. In addition, β subunit affects the intrinsic ion binding and transport properties of the mature pump. Although β subunit is important

for the normal activity of the Na^+/K^+-ATPase, its role is still not completely understood (Therien and Blostein 2000; Kaplan 2002).

The auxiliary subunit of Na^+/K^+-ATPase is FXYD family of proteins. Seven different FXYD proteins (FXYD1 to FXYD7) have been identified in mammals. These proteins act as tissue-specific regulatory subunits of the Na^+/K^+-ATPase kinetic properties. FXYD represent a group of small proteins with a single transmembrane segment containing the invariant FXYD motif (after which they are named) in the extracellular domain, and two conserved glycine and a serine residues. FXYD proteins modify the affinity for Na^+, K^+, and ATP, influencing kinetics and transport properties of Na^+/K^+-ATPase. The conserved FXYD motif has an important role in stabilizing interactions between Na^+/K^+-ATPase and FXYD protein (Garty and Karlish 2006; Geering 2008).

Structurally, α and β isoforms exhibit 85% and 45% identity and display a tissue- and cell-specific distribution and a developmentally regulated pattern of expression. The Na^+/K^+-ATPase α subunit exists in four isoforms. The α_1 isoform is dominant and found in all cells, while α_2 and α_3 isoforms are present in smaller amounts and are differentially expressed in tissues between species (Geering 2008). In human heart α_1, α_2, and α_3 are expressed together with β_1 and very low levels of β_2 in a region-specific manner, while in rat heart only α_1 and α_2 subunits are expressed (Schwinger et al. 2003). The α_4 isoform is located in testis and regulates sperm motility (Yan and Shapiro 2016). Three isoforms of β subunits have been identified so far. Similar to the α_1 isoform, the β_1 isoform is expressed ubiquitously, while β_2 is mainly expressed in skeletal muscle and heart, and β_3 in testis and central nervous system (Suhail 2010).

The concentration of Na^+/K^+-ATPase in tissues varies greatly; a large difference exists between the lowest (i.e., 250–500 sites/cell in erythrocytes) and the highest (i.e., 11,000–12,000 pmol/g wet weight in the brain cortex) concentrations. The Na^+/K^+-ATPase could be pharmacologically modified by administrating drugs exogenously. For instance, Na^+/K^+-ATPase found in membranes of heart cells is an important target of ▶ cardiac glycosides (CG), ▶ inotropic drugs used to improve ▶ heart performance by increasing its force of contraction (Therien and Blostein 2000; Kaplan 2002) (Fig. 1).

Function and Regulation of Na^+/K^+-ATPase

Hormones and environmental factors influence Na^+/K^+-ATPase function through different mechanisms including regulation of activity, enzyme trafficking, gene expression, and phosphorylation (Therien and Blostein 2000; Al-Khalili et al. 2004; Sudar et al. 2008; Obradovic et al. 2014). Control and regulation of Na^+/K^+-ATPase activities via a direct influence on the kinetic properties of the enzyme that is already present in the plasma membrane occur within minutes to hours. The second mechanism of regulation means that new Na^+/K^+-ATPase subunits are delivered to the plasma membrane from intracellular stores (underneath the plasma membrane) when needed. Regulation of Na^+/K^+-ATPase subunit gene expression happens over days, while changes in the turnover rate of the existing Na^+/K^+-ATPase through its direct phosphorylation are still controversial. The main hormones that regulate the Na^+/K^+-ATPase in the above-mentioned ways are: aldosterone, insulin, androgen, thyroid hormone, and estrogen.

Cardiac glycosides inhibit Na^+/K^+-ATPase activity and subsequently increase the concentration of intracellular Na^+ ($[Na^+]i$) (Bagrov et al. 2009). The concentration of $[Na^+]i$ is regulated by balance of Na^+ influx and efflux mechanisms, and the Na^+/K^+-ATPase provides the only significant Na^+ efflux pathway (Kaplan 2002). The inhibition of Na^+/K^+-ATPase by endogenous CG in myocytes leads to an increased Na^+ concentration, followed by increased $[Ca^{2+}]i$ (via NCX). This increase in $[Ca^{2+}]i$ content triggers the release of Ca^{2+} from the sarcoplasmatic reticulum, resulting in increased heart contraction. The Na^+/K^+-ATPase also acts as a signal transducer activating a number of intracellular pathways. Different molecules including CG or hormones binding to the extracellular region of the α-subunit activate signaling cascade and regulate

different processes (Aperia 2007); unfortunately, this mechanism is still poorly understood. It has been reported that noninhibitory doses of ouabain triggered the Na^+/K^+-ATPase-dependent activation of the inositol 1,4,5-trisphosphate receptor (IP3R) via a direct interaction, resulting in increase of oscillatory $[Ca^{2+}]i$ content (Aperia 2007). Ca^{2+} oscillations appeared as the most versatile of all Ca^{2+} oscillations generated by the Na^+/K^+-ATPase–IP3R complex having a pleiotropic action on cell signals (Aperia 2007).

Short-term regulation of Na^+/K^+-ATPase activity includes changes in kinetic behavior of the Na^+/K^+-ATPase that already exist in the membrane (Therien and Blostein 2000). This mechanism of Na^+/K^+-ATPase regulation occurs within minutes to hours in response to changes in ion concentration, CG binding, and hormones action via PKA, PKC, Akt, PKG, or extracellular signal-regulated kinases 1 and 2 (Therien and Blostein 2000; Al-Khalili et al. 2004; Sudar et al. 2008; Obradovic et al. 2013). Although evidence suggests that phosphorylation/dephosphorylation of the Na^+/K^+-ATPase α-subunit mediated by protein kinases leads to changes in transport properties, this mechanism of regulation is still controversial (Therien and Blostein 2000; Fuller et al. 2013). Long-term regulation of Na^+/K^+-ATPase includes regulation of translocation of new Na^+/K^+-ATPase subunits from intracellular stores to the plasma membrane and transcription regulation of α- and β-subunit genes and de novo synthesis.

Environmental factors such as Na^+ and K^+ ions, ATP, and CG for which α-subunit of Na^+/K^+-ATPase contains the binding sites are responsible for instant regulation of Na^+/K^+-ATPase activity. Major determinant of Na^+/K^+-ATPase activity is the level of $[Na^+]i$, where $[Na^+]i$ of 70–100 mM causes maximal activity. On the other hand, increased intracellular K^+ ($[K^+]i$) reduces Na^+/K^+-ATPase activity because of its competition with $[Na^+]i$ for binding to the E1 form. During ion pumping cycle, the Na^+/K^+-ATPase undergoes large-scale domain rearrangements while transitioning between two conformational states, E1 and E2, and sufficient concentration of ATP is necessary to allow this process (Shinoda et al. 2009). Endogenous CG (also known as digoxin-like immunoreactive factors) are produced in mammals in a manner similar to the production of steroid hormones and are involved in the regulation of inotropy effect and blood pressure (Bagrov et al. 2009). In kidneys, CG bind to α-subunit and subsequently inhibit renal tubular Na^+/K^+-ATPase, which causes natriuresis and diuresis. However, CG binding to the Na^+/K^+-ATPase induces generation of reactive oxygen species (ROS) in heart and kidneys. This increase of ROS can induce $Na^{+/}K^+$-ATPase oxidation and its conformational changes and inhibition of activity, while ultimately may cause its degradation. The impact of ROS on Na^+/K^+-ATPase is particularly significant in several diseases which are characterized by the elevation of endogenous ROS level, including heart failure. Glutathionylation, reversible oxidative modification of the β_1 subunit of $Na^{+/}K^+$-ATPase, is another important mechanism of its regulation (Figtree et al. 2009). Additionally, glutathionylation of FXYD proteins is critical for reversal of β_1 subunit glutathionylation and $Na^{+/}K^+$-ATPase inhibition induced by exposure of myocytes to angiotensin II and chemical oxidants. Also, growing evidence suggests that binding of ouabain to the Na^+/K^+-ATPase in noninhibitory doses may initiate signal transduction and modulate cell proliferation, apoptotic threshold, cell to cell contact, and cell migration. One more and relatively new type of Na^+/K^+-ATPase regulation involves regulation of associated FXYD proteins (Garty and Karlish 2006). Phospholemman (PLM, FXYD1) is a protein responsible for regulation of the cardiac Na^+/K^+-ATPase. PLM contains PKA and PKC phosphorylation sites on its cytoplasmic C-terminal tail and responds to adrenergic and other hormonal signals. Unphosphorylated PLM tonically inhibits Na^+/K^+-ATPase by decreasing the affinity for $[Na^+]i$, and this inhibition is relieved by PKA-mediated phosphorylation of PLM at Ser^{68} and/or PKC-mediated phosphorylation of PLM at Ser^{63}, Ser^{68}, or Thr^{69} residues (Fuller et al. 2009). Stimulation of β_1/β_2-adrenergic receptors increases the level of cyclic adenosine-monophosphate (cAMP) and induces activation of PKA, which phosphorylates PLM at Ser^{68}. Activation of

PKA and, consequently, stimulation of the Na^+/K^+-ATPase via Ser^{68} PLM phosphorylation, limits $[Na^+]i$ and $[Ca^{2+}]i$ by favoring Ca^{2+} extrusion via NCX exchanger. Increased $[Na^+]i$ in the myocardium in heart failure favors more Ca^{2+} influx (through NCX) and better contractility in failing hearts. However, a chronic increase in $[Na^+]i$ and $[Ca^{2+}]i$ levels is also associated with dysfunctional cardiac hypertrophy and arrhythmogenesis. Stimulation of the Na^+/K^+-ATPase mediated by PLM phosphorylation may protect from Ca^{2+} overload, reducing the likelihood for triggered arrhythmias. In addition, phosphorylation of PLM Ser^{68} and cardiac Na^+/K^+-ATPase activity are negatively regulated by protein phosphatase 1 (PP1), which has been implicated in the regulation of cardiac β-agonist responses and contractility (El-Armouche et al. 2011). In failing human hearts, β-AR signaling is impaired, PLM Ser^{68} phosphorylation diminished, and PP1 potentially hyperactivated. CG are produced in mammals in a manner similar to steroid hormones from cholesterol and act as indirect regulators of cardiac contractility (positive inotropy) (Lingrel 2010).

Reduced Na^+/K^+-ATPase function seems to play a causal role in the development of cardiovascular (CV) diseases, probably due to the association of decreased Na^+/K^+-ATPase activity with other risk factors (e.g., obesity or impaired estradiol signaling) (Schwinger et al. 2003; Obradovic et al. 2013; Borović et al. 2016). Thus, the regulation of Na^+/K^+-ATPase activity and expression as well as the regulation of different Na^+/K^+-ATPase isoforms may be important for the treatment and possible prevention of CV diseases.

Summary

The Na^+/K^+-ATPase is a universally expressed membrane protein responsible for maintaining the low intracellular Na^+ and high intracellular K^+ concentrations required for multitude of cellular functions. Besides, the Na^+/K^+-ATPase helps maintaining resting potential, import of amino acids, glucose, and other nutrients into cells and regulates cellular volume. This enzyme has oligomeric structure consisting of two main subunits α and β and one auxiliary subunit FXYD. Subunits α and β are present in equal molar ratio and they combine to form a number of Na^+/K^+-ATPase isozymes (1:1), while FXYD proteins are present in cell and tissue-specific manner. Numerous hormones and environmental factors regulate Na^+/K^+-ATPase through several ways: by influencing Na^+/K^+-ATPase activity, changing subunit gene expression, regulating enzyme trafficking to the plasma membrane, and via phosphorylation of Na^+/K^+-ATPase which are present on membrane. The Na^+/K^+-ATPase found in the membrane of cardiomyocytes is an important target of CG, which improve heart performance by increasing contraction. Reduction of Na^+/K^+-ATPase activity/expression is associated with different pathophysiological conditions. Thus, increasing our understanding of the molecular mechanisms determining the regulation of Na^+/K^+-ATPase may help develop new strategies for the treatment of CV diseases.

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See Also

► FXYD1

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Nab2 (*S. cerevisiae, S. pombe, C. thermophilum*)

▶ ZC3H14

NaCH

▶ ENaC

nAChR

▶ Acetylcholine (Nicotinic) Receptor

N-Acylsphingosine Amidohydrolase 2

▶ Neutral Ceramidase

NAD^+ and $NAD(P)^+$ Nucleosidase

▶ CD38

NAD-Dependent Deacetylase Sirtuin-2

▶ SIRT2

NADE

► BEX3

NADPH-Thioredoxin Oxidoreductase

► Thioredoxin Reductase

NADP-Thioredoxin Reductase

► Thioredoxin Reductase

NaPi-IIa

► SLC34

NaPi-IIb

► SLC34

NaPi-IIc

► SLC34

Nasal Epithelial Sodium Channel

► ENaC

Natriuretic Peptide Receptor Type A (NPRA)

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Synonyms

ANPa; ANPRA; GCA; GC-A; GUC2A; GUCY2; NPR1 (natriuretic peptide receptor 1); NPRA; NPR-A

Historical Background

Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and urodilatin (URO) represent a family of cardiac, vascular, and renal-derived hormones that play an essential role on the regulation of blood pressure, intravascular volume, and electrolyte homeostasis in all mammals. Most of the biological actions of ANP, BNP, and URO are mediated by activation of the natriuretic peptide receptor type A (NPRA), also designated as guanylyl cyclase-A/ natriuretic peptide receptor type A (GC-A/NPRA). Binding of these natriuretic peptides to NPRA leads to activation of the particulate guanylate cyclase (pGC) catalytic domain which generates cGMP-dependent second messenger signaling cascade. An increased level of intracellular cGMP activates three different targets: cGMP-dependent protein kinases (PKGs), cGMP-dependent ion-gated channels

(CNGs), and cGMP-dependent phosphodiesterases (PDEs).

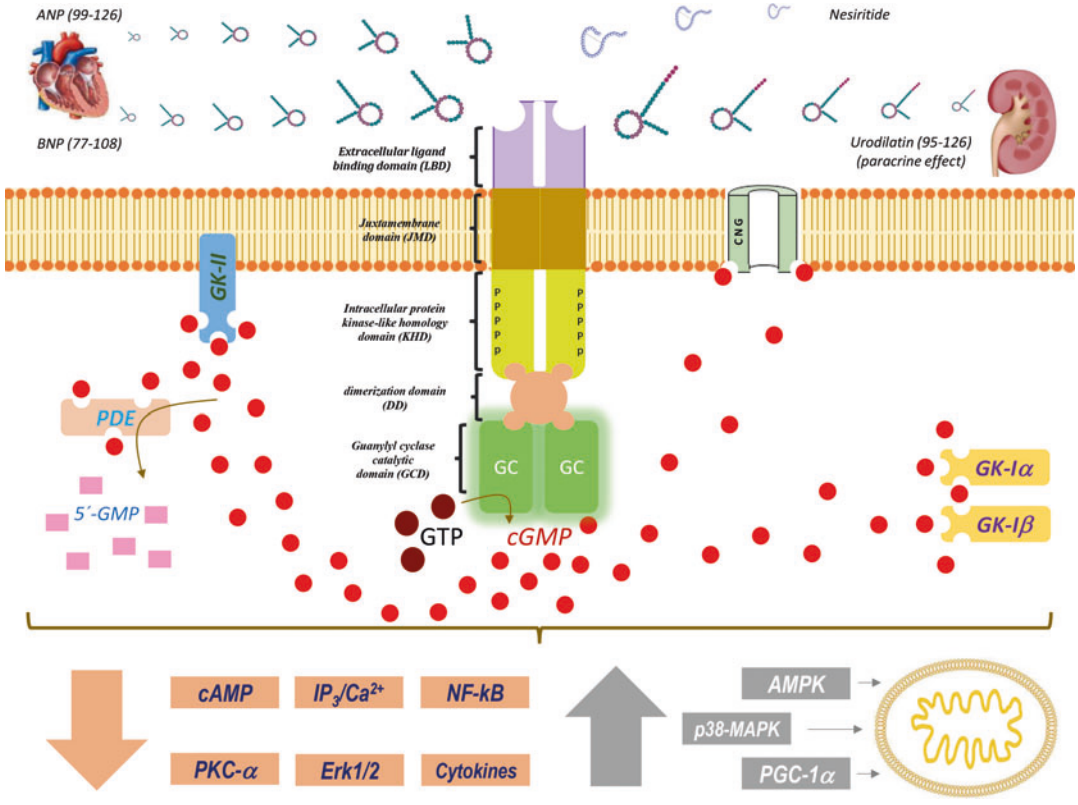
Gen Structure

Human NPRA is encoded by *Npr1* gene (OMIM 108960) which is located in chromosome 1 (q21–q22). Human NPRA gene is approximately 16 kilobases (kb), it contains 22 exons and 21 introns, and encodes for a peptide of 1061 amino acids and 135-kDa (Zhang et al. 2014).

NPRA Structure, Activation, Internalization, and Inactivation/Recyclation

ANP, BNP, and URO bind selectively to NPRA (Koller et al. 1993; Choi et al. 2011). NPRA is a 135-kDa transmembrane protein that constitutes a membrane guanylyl cyclase homodimeric receptor consistent with the guanylyl cyclase receptor family and composed by three domains: extracellular, transmembrane, and intracellular. The extracellular ligand binding domain (LBD) has a chloride-binding site near to the dimerization interface (Zhang et al. 2014) and has five glycosylation sites linked to nitrogen and three intramolecular disulfide bonds (Potter 2011). The transmembrane domain is represented by the juxtamembrane domain (JMD). The intracellular domain includes three highly conserved regions ordered as follows (from membrane to cytoplasm): the protein kinase-like homology domain (KHD) of 280 amino acids, the dimerization domain (DD), and the pGC catalytic domain (GCD) which involves 250 amino acids. The binding of two molecules of ANP causes the activation of NPRA, followed by the phosphorylation of five serines and two threonines near to the N-terminal site of the KHD (Potter 2011). Then, the KHD domain activates pGC catalytic domain by translation of ANP/BNP signals, with the consequent conversion of GTP to cGMP, which, in turn, can activate three different proteins: cGMP-dependent protein kinase I and II (GKI and GKII), PDEs, and CNGs. All of these nucleotides are related to the biological responses to the receptor ligands or agonists (Pandey 2015). It is also known that the phosphorylation state of one domain of these receptors via GKI and GKII determines their sensitivity to ligand (Garbers et al. 2006). cGKI and cGKII activation are linked to inflammation, cell growth, proliferation, and apoptosis. The dephosphorylation by PDEs route generates inactive 5′-nucleotide monophosphates through degradation of cyclic nucleotides, representing a way by which PDEs can regulate the intracellular signaling pathway of natriuretic peptides (Rukavina Mikusic et al. 2014). The cGMP-dependent signaling may also antagonize different pathways such as intracellular Ca^{2+} release, IP_3 formation, protein kinase C (PKC) activation and mitogen-activated protein kinases (MAPKs) and cytokine production (tumor necrosis factor-α and interleukin-6) (Pandey 2015). In adipose cells, PKG-mediated phosphorylation activates different target proteins involved in the initiation of lipolysis (Collins 2014 and Moro and Lafontan 2013).

The binding affinity of the NPs for NPRA is the following: ANP > BNP >> CNP. Once ANP or BNP has bound to NPRA, this receptor is internalized in a ligand-dependent manner. Internalization of NPRA is a complex process carried out by NPRA C-terminal domain, as well as by specific small peptide sequence motifs related to it, such as GDAY (Gly-Asp-Ala-Tyr) (Pandey 2015). After then, the plasmatic membrane receptor is removed by clathrin-coated vesicles and subject to inactivation/desensitization by dephosphorylation, a process that is also regulated by the agonist ANP (Pandey 2015). Therefore, the inactivated molecules of NPRA are redistributed to two different locations: most of them are degraded by lysosomes (75%), while a small portion (25%) is recycled to the plasma membrane and ready to bind new receptor molecules. NPRA and its mRNA are expressed in the kidney, lung, adipose, adrenal, brain, heart, testis, vascular smooth muscle, and endothelial tissues and play critical physiological and pathophysiological roles on several target cells and tissues to control cell growth, apoptosis, proliferation, and inflammation among other functions (Potter 2011) (Fig. 1).



Natriuretic Peptide Receptor Type A (NPRA), Fig. 1 NPRA structure, activation, and signaling pathway. *Grey up arrow*: activate; *orange down arrow*: inhibit

NPRA in Cancer

Recent research suggests that NPRA expression and signaling are present in different types of cancer like lung, prostate, gastric, and ovarian, being an important factor for tumor growth.

Related to the study of carcinogenic mechanism of NPRA, it has been demonstrated that preimplantation embryos and embryonic stem cells express NPRA. Though, using an RNAi directed to NPRA sequence, it could be identified phenotypic changes related to downregulation of pluripotency factors and upregulation of differentiation genes. NPRA expression seems to be necessary during implantation, angiogenesis, proliferation, and metastasis of tumor cells, representing a novel target for cancer therapy (Zhang et al. 2014). In this way, downregulation of NPRA by transfection of siRNA induced apoptosis and autophagy in prostate and gastric cancers, respectively (Li et al. 2016 and Wang et al. 2011). Moreover, NPRA deficiency protects C57BL/6 mice from lung, skin, and ovarian cancers and inactivates the expression of pro-inflammatory transcription factor NF-κB (Zhang et al. 2014). The fact that tumor burden is significantly reduced by therapy with siNPRA in mice open a new horizon in the study of NPRA for therapeutic purposes in cancer (Zhang et al. 2014).

NPRA in Hypertension and Cardiac Homeostasis

ANP through NPRA activation decreases blood volume and blood pressure by increasing water and salt excretion by the kidneys and by vasodilation of vascular smooth muscle. Several data suggest that impairment in the natriuretic peptide system plays a crucial role in the development of

essential hypertension (EH) (Zois et al. 2014). In this sense, a Japanese study revealed a functional deletion mutation of the 5′-flanking region of the *Npr1* gene that caused a reduction in gene transcriptional activity and conferred increased susceptibility to EH or left ventricular hypertrophy (LVH) (Nakayama et al. 2000). In addition, Rubattu et al. found that hypertensive patients with *Npr1* gene promoter allelic variant 11/12, had also significantly higher values of left ventricular mass index (LVMI). Stimulation of NPRA acts as an endogenous protective mechanism that prevents cardiac remodeling in failing hearts antagonizing the effects of ANG II and AT1 stimulation (Kilić et al. 2007). The fact that patients carrying the mutant alleles of *Npr1* gene had altered LVMI suggests the functional relevance of this receptor since promoter gene variants could reduce NPRA activity and be involved in ventricular remodeling in human EH (Rubattu et al. 2006).

NPRA activation and signaling are also important in the maintenance of cardiovascular homeostasis (Madhani et al. 2003). In this way, *Npr1* knockout mice at endothelial cells and smooth muscle arteries developed significant hypertension (Sabrane et al. 2005). In contrast, Kishimoto et al. have shown that *Npr1* knockout animals subject to selective transgenic changes in NPRA have reduced cardiomyocyte size, but not hypertension (Kishimoto et al. 2001). Moreover, *Npr1* gene deficiency has been related to atherosclerosis and cardiac hypertrophy (Alexander et al. 2003). Activation of NPRA by BNP showed antifibrotic effects as a consequence of its important role in the control of extracellular matrix production that leads to cardiac fibrosis. Because of these findings, BNP has been proposed as an oral therapy for cardiac diseases (Garbers et al. 2006). All these evidences indicate that genetic alterations that reduce NPRA signaling are directly involved in impairment of cardiovascular homeostasis and development of EH (Pandey 2011).

NPRA in the Kidney

ANP/NPRA complex exerts its intrarenal actions on glomeruli and renal tubules. NPRA activation increases glomerular filtration rate, renal plasma flow, and water and sodium excretion and also stimulates nitric oxide synthesis in proximal tubules (Pandey 2011; Rukavina Mikusic et al. 2014). Additionally, it has been reported indirect actions of NPRA activation through enhancing renal dopaminergic system. Fernández et al. demonstrated that renal ANP stimulation of NPRA results in an increase of dopamine bioavailability in renal tubular cells allowing the dopamine receptor D1 recruitment and the inhibition of Na^+-K^+-ATPase activity with the consequent reduction of sodium reabsorption and increasing natriuresis (Fernández et al. 2005). Furthermore, it has been reported by Choi et al. that URO acting through NPRA stimulates cGMP signaling cascade and leads to an increase in renal dopamine uptake and to a decrease of Na^+-K^+-ATPase activity in the outer and juxtamedullary cortex as well as in the medulla, improving natriuresis and therefore reducing sodium reabsorption (Choi et al. 2011). Renal NPRA activation may also act as a renoprotective pathway against inflammation. In this sense, renal activity of pro-inflammatory NF-κB and other cytokines is increased in a knockout mice model for *Npr1* gene (Rukavina Mikusic et al. 2014).

NPRA in Central Nervous System

NPRA in mammalian animal models was found in circumventricular organs of the central nervous system such as the vascular organ of the lamina terminalis, the choroid plexus, the area postrema, and the subfornical organ, and it was also found in the hypothalamus and astroglial cells of several central nervous system regions (Mahinrad et al. 2016).

It has been demonstrated that the complex ANP/NPRA is involved in the regulation of neuroinflammation. When ANP binds to NPRA in the macrophages activated by lipopolysaccharide (LPS), it inhibits pro-inflammatory transcription factors such as activator protein-1 and NF-kB causing the inhibition of IL-1β and nitrite production. It was also reported that ANP increased microglia phagocytic activity in the rat

hippocampal area and that the administration of human recombinant BNP could reduce inflammatory markers and microglial activation (Mahinrad et al. 2016).

ANP is also involved in the regulation of norepinephrine metabolism in the rat hypothalamus by acting as a negative neuromodulator by reducing the noradrenergic neurotransmission through activation of guanylate cyclase signaling (Vatta et al. 1999).

ANP was found to regulate aqueous humor production in the eye, and the presence of NPRA receptors were identified by immunohistochemistry in different layers of the retina. Kuribayashi et al. demonstrated that in the rats' retina, ANP acting through NPRA exerted neuroprotective actions against neurotoxicity induced by the intravitreal administration of N-methyl-D-aspartate (NMDA), which reduced dopamine levels. Moreover, the neuroprotective effect of ANP was inhibited by the administration of a dopamine D1 receptor antagonist (Kuribayashi et al. 2006).

NPRA in Fat Cell Metabolism and Skeletal Muscle

NPRA is abundantly expressed in adipose tissue and its activation induces lipolysis. This effect caused by ANP stimulation of NPRA in adipocytes is specific to primates, so it cannot be studied in other models such as rat, mouse, rabbit, or dog (Moro and Lafontan 2013). Through activation of GKI, NPRA promotes hormone-sensitive lipase-mediated triglyceride degradation and increases free fatty acid availability. By this mechanism, the natriuretic peptides enhance lipid oxidation into the β-oxidation pathway in the liver, skeletal muscle, and adipose tissue (Schlueter et al. 2014). The lipolytic response to NPRA is higher in large adipocytes than in small ones located in the same adipose tissue depot. Moreover, ANP and BNP acting through NPRA increase the expression of mRNA of adiponectin in human fat cells. Nonetheless, a high-fat diet in mice induces a downregulation of NPRA in brown and white fat.

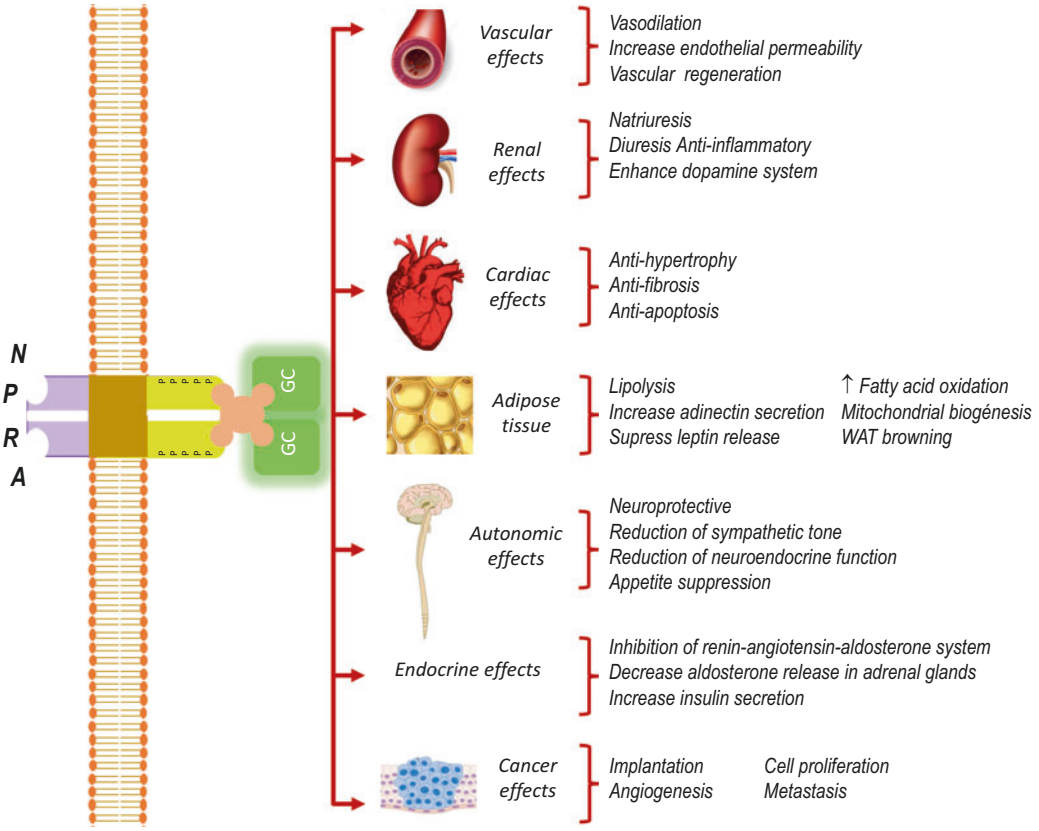
In the skeletal muscle tissue, the overexpression of BNP and cGKI by transgenic method in mice under high-fat diet induced a high level of body energy expenditure and fat oxidation with reduction of fat mass and an increased expression of mitochondrial oxidative genes. Using the same mice model, it was identified a protection pathway to insulin resistance induced by diet. Also, in obese patients, it was shown that NPRA expression in skeletal muscle cells was upregulated in response to aerobic exercise. All these data may suggest that intracellular signaling of cGMP triggered by ANP-BNP/NPRA interaction as well as its lipid mobilizing effect could contribute to mitochondrial biogenesis induced by exercise in human skeletal muscles (Moro and Lafontan 2013).

Also, it was recently demonstrated that energy-balanced control exert by NPRA activation resulted in appetite suppression. Furthermore, external administration of BNP to healthy subjects showed to increase satiety feeling, decreasing hunger and total ghrelin levels (Moro and Lafontan 2013).

NPRA in the Pancreas and Liver

ANP stimulation of NPRA in pancreatic beta islet cells increases insulin secretion by blockade of ATP-sensitive K^+ channel and intracellular Ca^{2+} augment. On the other side, knockout mice for *Npr1* exhibited a decreased secretion of insulin associated with a lower β-cell mass, resulting in a higher fasting blood glucose level compared to the wild type (Moro and Lafontan 2013).

As it was reported in the kidney, ANP may exert hepatoprotective actions against inflammation, since NPRA stimulation reduced NF-κB activity and TNF-α release by Kupffer cells and also reduced oxidative injury in response to ischemia-reperfusion or to LPS without losing the defense functions of those cells (Moro and Lafontan 2013) (Fig. 2).



Natriuretic Peptide Receptor Type A (NPRA), Fig. 2 Effects of NPRA activation in different tissues and organs

N

Therapeutic Implications of NPRA

The effect of the administration of exogenous natriuretic peptides has been evaluated by several clinical trials. Analogous molecules of ANP and BNP have been designed in order to reproduce the beneficial effects of NPRA activation in patients with chronic heart failure. In this context, different randomized and placebo-controlled trials demonstrated that the administration of nesiritide (a recombinant molecule of human BNP) improves the natriuresis and diuresis and elicits a significant decrease in plasma renin activity and aldosterone inhibition. Other natriuretic peptide analogs like M-ANP and cenderitide-NP are more resistant to degradation by metallopeptidase neprilysin, thereby increasing the half-time life to interact with NPRA (Díez 2016).

Summary

ANP, BNP, and URO bind to NPRA, a transmembrane receptor encoded in *Npr1* gene. NPRA activation leads to a second messenger cascade signaling commanded by cGMP which triggers activation of other enzymes such as GKI. Its mRNA is expressed, among others, in the heart, kidney, vascular smooth muscle, skeletal muscle, endothelial, adipose, and brain tissues, as well as in the adrenal, lung, and testis. NPRA plays critical physiological and pathophysiological roles on

several target cells and tissue system mechanisms, such as cell growth, apoptosis, proliferation, and inflammation. It is related to different processes like cancer, hypertension, cardiac remodeling and hypertrophy, atherosclerosis, renal abnormalities, neuroprotection, fat tissue and skeletal muscle metabolism, insulin secretion, and hepatoprotection. Although new knowledge have been updated in the last years, future studies with molecular biology techniques will provide complementary insights about how genetic alterations of NP receptors could be associated to the origin and development of human pathologies.

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Natriuretic Peptide Receptor Type B (NPRB)

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Synonyms

AMDM; ANPb; ANPRB; ECDM; GCB; GC-B; GUC2B; GUCY2B; NPR2 (natriuretic peptide receptor 2); NPRB; NPRBi; SNSK

Historical Background

The natriuretic peptides (NP) system is a family of peptides named A, B, and C-type natriuretic peptides (ANP, BNP, CNP) and urodilatin (URO), synthetized by the heart (ANP and BNP), kidney (URO), and vascular smooth muscle (CNP) but also by other cells widely dispersed in different organs and tissues (Koller and Goeddel 1992). The NP family plays an essential role on the regulation of blood pressure, the intravascular volume, and electrolyte homeostasis in mammals. Based on the fact that their main actions are exerted on regulation of the cardiovascular system, the NP are included within the big family of vasoactive peptides, together with angiotensin II, endothelins, bradikinin, and vasopressin among others (Padney 2015).

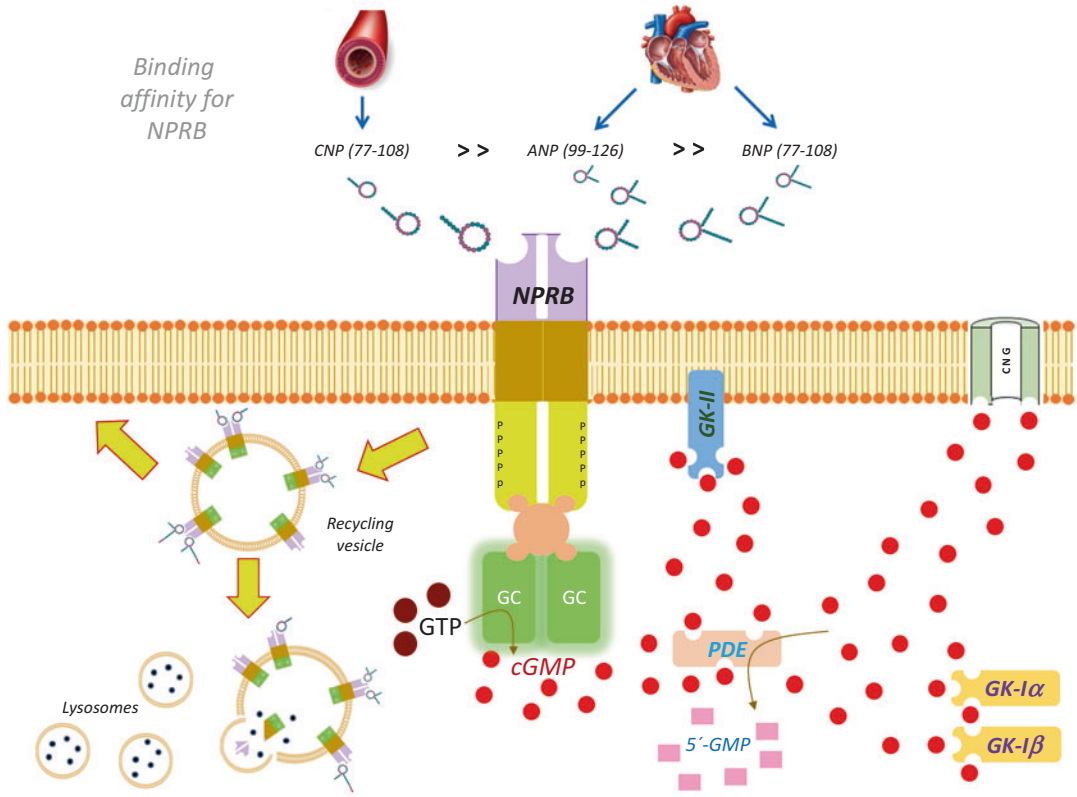
Binding of NP to either natriuretic peptide receptor type A (NPRA) or type B (NPRB) leads to activation of particulate guanylate cyclase (pGC) catalytic domain generating the second messenger cyclic GMP (cGMP) signaling cascade, which mediates most biological actions of these peptides. The phosphorylation state of one domain of these receptors via cGMP-dependent protein kinases I and II (cGKI and cGKII) determines their sensitivity to ligand (Garbers et al. 2006). The binding affinity of the NPs for NPRB is the following: CNP > ANP > BNP (Cantú et al. 2015).

Gene Structure

NPRB is encoded by *Npr2* gene (OMIM 108961) which is located in chromosome 9 (p12–21). Human NPRB gene is approximately 16.5 kilobases (kb), which contain 22 exons, 21 introns, and the junction between introns and exons follows the GT-AG patron. The gene encodes for a peptide of 1061 amino acids (Rehemudula et al. 1999).

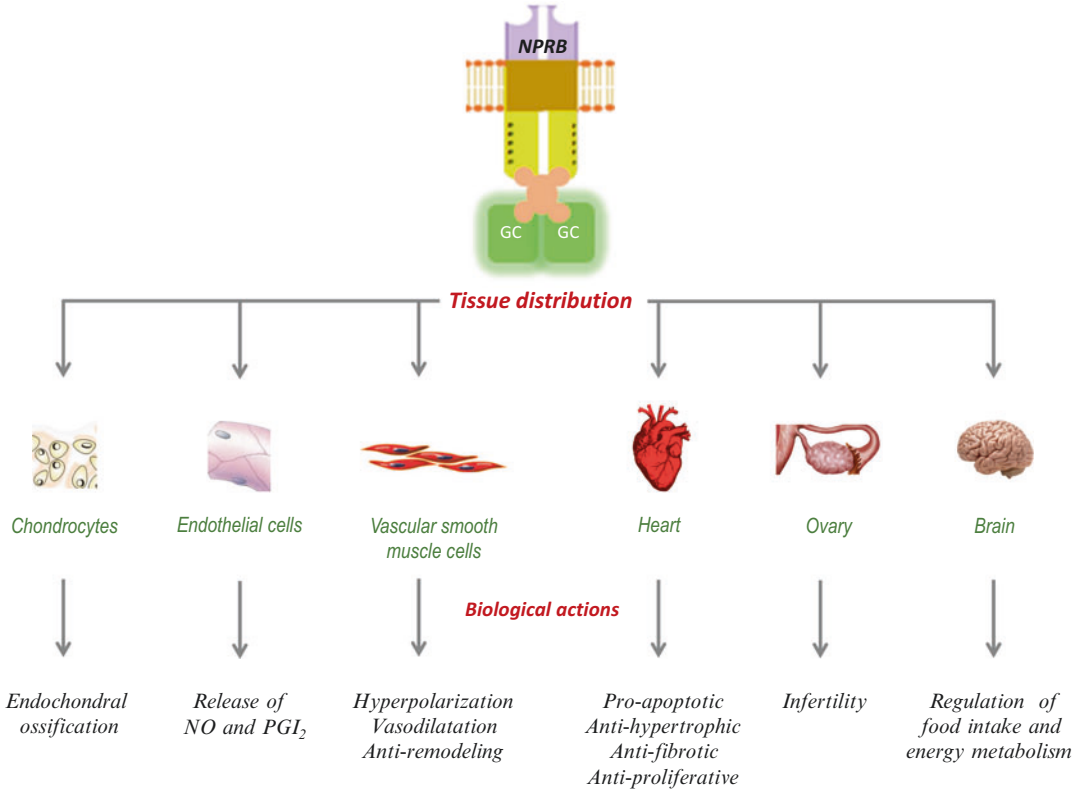
NPRB Structure, Activation, and General Actions

NPRB is a 135 kDa transmembrane protein that binds selectively to CNP (Pandey and Singh 1990) (Fig. 1). NPRB is a membrane guanylyl cyclase homodimeric receptor characterized by a general structure consistent to the guanylyl cyclase receptor family: an extracellular ligand-binding domain, highly glycosylated on asparagine residues, and three intramolecular disulfide



Natriuretic Peptide Receptor Type B (NPRB), Fig. 1 NPRB structure, activation, and signaling pathway

bonds (Garbers 1992). In basal conditions, it is highly phosphorylated while the loss of phosphate residues leads to its inactivation (Potter 2011). Its transmembrane domain comprises four highly conserved regions ordered as follows (from membrane to cytoplasm): the juxtamembrane domain (JMD), the protein kinase-like homology domain (KHD) of 280 amino acid, the dimerization domain (DD), and the pGC catalytic domain. Ligand binding stimulates pGC activity that converts GMP to cGMP, which in turn activates several target molecules, such as cGKI and cGKII, cyclic nucleotide-regulated ion channels, and cGMP-regulated phosphodiesterases (PDE) like PDE5. Not only extracellular ligand-binding is required for NPRB activation but also the phosphorylation of up to six residues within the KHD. NPRB has a high degree of similarity with NPRA: approximately 43% in the ligand-binding domain, 72% in the KHD, and 91% in the pGC domain (Garbers 1992; Khurana and Pandey 1993; Koller and Goeddel 1992; Pandey and Singh 1990; Schlueter et al. 2014). NPRB is internalized in a ligand-dependent manner once CNP binds to it. It is degraded by lysosomes and the recycled molecules go back to the plasmatic membrane to form new receptors (Padney 2015). The expression of NPRB takes place in brain, bones, chondrocytes, lungs, and ovary tissue, as well as in vascular smooth muscle cells and fibroblasts (Potter 2011) (Fig. 2). It has also been found in cardiac myocyte caveoles (Padney 2015). NPRB plays an important role in long bone growth by regulating



Natriuretic Peptide Receptor Type B (NPRB), Fig. 2 NPRB: distribution and biological effects

endochondral ossification. In the brain, the binding of CNP to NPRB was associated with central regulation of food intake and energy metabolism (Schlueter et al. 2014).

Npr2 Gene and Growth Disorders

CNP and its receptor NPRB are recognized as important regulators of longitudinal growth. Animal models allowed investigators to found the relation between CNP or NPRB genes (*Nppc* or *Npr2*, respectively) and the fundamental role of their products CNP/NPRB in endochondral ossification (Vasques et al. 2014).

It has been shown that height variability in healthy individuals is related to polymorphisms in two genes related to the CNP pathway. Bartels et al. demonstrated that a severe skeletal dysplasia characterized by dwarfism and short limbs called acromesomelic dysplasia type Maroteux (AMDM) is caused by biallelic mutations that generate the loss of function in *Npr2* gene. Furthermore, homozygous mutations produce a severe short stature and body disproportion (Vasques et al. 2014). It has also been found heterozygous mutations in *Npr2* gene that seem to be associated with mild and variable growth impairment without a distinct skeletal phenotype (Wang 2015). On the other hand, heterozygous gain-of-function mutations in *Npr2* were pointed out as a cause of tall stature, and a similar phenotype had been observed in an individual with overexpression of the CNP caused by a balanced translocation (Vasques et al. 2014).

Considering all the data available until today, CNP should be studied as a promising therapy for achondroplasia (ACH) and growth impairment

diseases. In fact, it is an ongoing investigation using a once-daily subcutaneous administration of a CNP analog called BMN-111, which has an extended half-life due to neutral endopeptidase resistance. Preclinical studies in mouse models of ACH using this new molecule showed improvement of dwarfism. In humans, a phase 2 multicenter and multinational trial has been initiated, with estimated study completion in 2017 (ClinicalTrials.gov Identifier: NCT02055157).

NPRB in Cardiovascular Diseases and Hypertension

CNP bound to NPRB promotes antiproliferative, proapoptotic, antifibrotic, and antihypertrophic effects on cardiomyocytes. It was observed that NPRB expression occurred at the site of vascular injury, and the administration of CNP after an arterial damage inhibited intimal proliferation, and therefore neointimal restenosis. The interaction between CNP and NPRB has been related to the progression of atherosclerotic lesions and was also found to reduce cardiac ischemia-reperfusion injury. All these beneficial effects could be associated with the inhibition of cardiac fibroblasts collagen synthesis by CNP, positioned this natriuretic peptide as a potent antihypertrophic agent through its interaction with NPRB (Garbers et al. 2006; Cantú et al. 2015).

It was described that CNP/NPRB induces vasorelaxation in large conduction vessels and small resistant arteries, and also inhibits smooth muscle proliferation, leading to a reduction in blood pressure. Studies conducted in spontaneously hypertensive rats with normal CNP blood levels demonstrated that alterations of NPRB were related to hypertension and vasorelaxation attenuation (Rahmutula et al. 2001).

Systematic screening of polymorphism of *Npr2* was conducted by Rahmutula et al. looking for an association of these changes to cardiovascular diseases. They identified a 9 bp insertion/deletion (I/D) in intron 18 that was not related to essential hypertension, contrary to an 11-repeat allele of the GT repeat polymorphism in intron 2. Also, and related to this polymorphism, they found a C to T transition at nucleotide (nt) 2077 in exon 11 but it was not associated with myocardial infarction (Rahmutula et al. 2001).

Npr2 Gene and Infertility

Several studies showed the relation between CNP and the granulosa cells of the ovarian cortex. In that tissue, CNP acting through its receptor NPRB inhibits the oocyte maturation. To confirm this observation, investigators demonstrated the administration of CNP to infertile female mice derived in ovarian growth. Furthermore, after this treatment and with the external supplement of gonadotropins, ovulation was successfully induced (Vasques et al. 2014).

In a particular natural mutation of *Npr2* occurring in female mice, it was observed no progression of the oocyte to the two-cell embryo stage due to premature meiotic resumption. Also, female mice lacking of NPRB showed infertility associated with the absence of estrus cycle and uterine atrophy with thickness reduction of endometrium and myometrium (Vasques et al. 2014).

Summary

CNP binds to NPRB, a transmembrane receptor encoded in *Npr2* gene. NPRB activation leads to a second messenger cascade signaling commanded by cGMP. Its mRNA is expressed in bones, chondrocytes, vascular smooth muscle cells, and fibroblasts as well as in ovary tissue. It is also expressed in brain and lungs. NPRB plays critical physiological and pathophysiological roles in long bone growth by regulating endochondral ossification, cardiovascular diseases, hypertension, and also in oocyte maturation. It has been shown that some polymorphisms are related to the development of essential hypertension and infertility, but above all mutations of *Npr2* gene are related to pathologies associated with growing diseases like AMDM and ACH. Although new knowledge have been updated in previous years,

in vitro assays, experiments in animal models, and clinical trials using molecular biology techniques will further provide complementary insights to better understand and characterize this receptor and its gene.

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Natriuretic Peptide Receptor Type C (NPRC)

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Synonyms

ANP-C; ANPRC; ANPR-C; C5orf23; GUCY2B; NPR3 (natriuretic peptide receptor 3); NPRC; NPR-C

Historical Background

The natriuretic system constitutes a family of cardiac- and vascular-derived hormones named Atrial Natriuretic Peptide (ANP), Brain Natriuretic Peptide (BNP), C-type Natriuretic Peptide (CNP), and Urodilatin (URO), which play an essential role on the regulation of blood pressure, intravascular volume, and electrolyte homeostasis in all mammals. Binding of natriuretic peptides (NPs) to either Natriuretic Peptide Receptor Type A (NPRA) or type B (NPRB) leads to activation of the particulate guanylate cyclase (pGC) catalytic domain which generates cGMP-dependent second messenger signaling cascade, mediating most of the biological actions of these peptides (Anand-Srivastava and Trachte 1993). NPs bind also to NPRC, which is considered a clearance receptor responsible for receptor-mediated degradation of these peptides. In addition, recent studies have revealed multiple effects of NPRC on different cells and organs, and most of these effects were enhanced by CNP stimulation rather than by ANP and BNP (Anand-Srivastava 2005).

Gene Structure

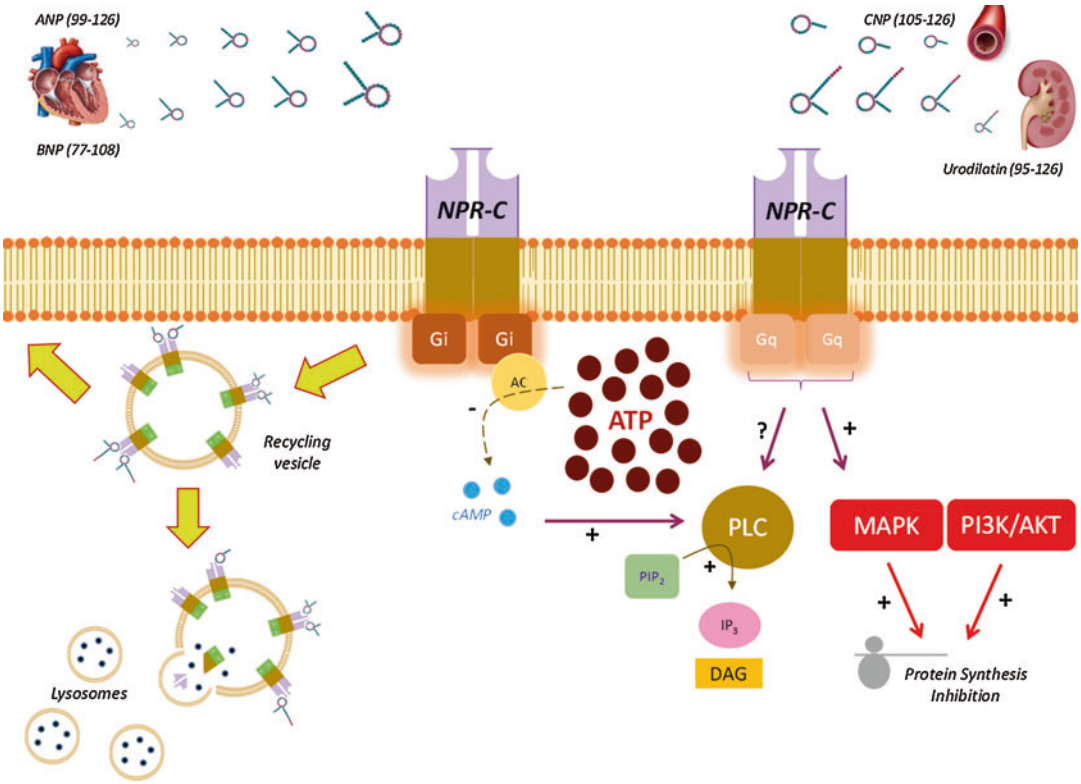
Human NPRC is encoded by *Npr3* gene (OMIM 108962) which localizes in chromosome 5(p14-p13). NPRC gene structure is approximately 75 kilobase pairs (kbp) and contains eight exons and seven introns. The exons size ranges from 88 bp (exon 7) to 769 bp (exon 1), and the introns size ranges from 1.5 kbp (intron 7) to 6.5 kbp (introns 2 and 3) (Rahmutula et al. 2002).

NPRC Structure and Localization, Signaling Pathways, and Biological Actions

NPRC is a disulfide-linked homodimer of a single transmembrane 64–66 kDa protein with a large extracellular region of about 440 amino acids of a single membrane-spanning domain and a short 37 amino acid cytoplasmic domain that has been reported to contain G protein-activating sequences to allow for inhibitory guanine nucleotide regulatory protein (Gi)-dependent signal transduction (Anand-Srivastava 2005). NPRC lacks the seven transmembrane motifs of typical G-protein-coupled receptors (GPCRs) and may therefore be considered as an atypical GPCR. Until today, two different subtypes of NPRC with molecular masses of 67 and 77 kDa have been identified. Although NPRC is considered as a clearance receptor, several studies indicate that NPRC receptor activation may trigger different intracellular signals. It has been reported that NPRC can be coupled to Gi protein and inhibit the adenylyl cyclase (AC) activity in the cell (Anand-Srivastava 2005). Additionally, this receptor can trigger phospholipase C (PLC) signaling pathway in rat parotid glands and aortic smooth muscle cells (Hirata et al. 1989; Bianciotti et al. 1998). Finally, NPRC can also activate Gqα/mitogen-activated protein kinase (MAPK)/PI3K and AKT pathways, which are involved in cell proliferation in vascular smooth muscle cells (Li et al. 2006) (Fig. 1).

The binding affinity of the NPs for NPRC is the following: ANP > BNP > CNP (Cantú et al. 2015). NPRC is the most widely and abundantly expressed NPR. It is present in the cardiac fibroblasts and myocytes, vascular smooth muscles cells (VSMC), kidney, cerebral cortex, brain striatum, hypothalamus, gastrointestinal smooth muscle, zona glomerulosa of the adrenal glands, bone, and chondrocytes. Also, it is the most expressed NPR in endothelial cells (Anand-Srivastava 2005).

NPRC first proposed action was the removal of NPs from circulation leading to their internalization and depuration from blood, considering it as a clearance receptor. After ligand binding, the NPRC-ligand complex undergoes endocytosis and then dissociates intracellularly, followed by hydrolysis of ligand in lysosomes and rapid recycling of the receptor back to the cell surface (Cohen et al. 1996; Nussenzveig et al. 1990). However, several studies indicate that NPRC plays additional roles implicated in NPs biological actions. In this way, novel NPs physiological effects have been described, particularly in the heart and vasculature. CNP and BNP acting through NPRC exert antiproliferative effects in cardiac fibroblasts. Also, CNP has been found to act as a NPRC-dependent endothelium-derived hyperpolarizing factor in the resistance vasculature regulating systemic blood pressure by hyperpolarization of VSMC and controlling local blood flow (Rose and Giles 2008). It has been proposed that these effects are mediated, at least in part, by AC inhibition and decreased cAMP levels. AC comprises three components: a receptor, a catalytic subunit, and Gs or Gi guanine nucleotide regulatory protein. The G proteins are transducers that transmit the signal from the hormone-occupied receptor to the catalytic subunit. The hormonal stimulation mediated by Gs results in increased formation of cAMP whereas the hormonal inhibition mediated by Gi results in decreased formation of cAMP (Anand-Srivastava 2005). NPRC has also been involved in the modulation of other signaling pathways. A possible cross-talk may exist between the AC and PLC pathways in VSMC, as the inhibition of AC and decreased levels of cAMP induced by NPRC activation contributed to stimulation of phosphatidylinositol (PI) turnover (Mouawad et al. 2004). It was demonstrated that when CNP binds to NPRC in pancreas, acini cells



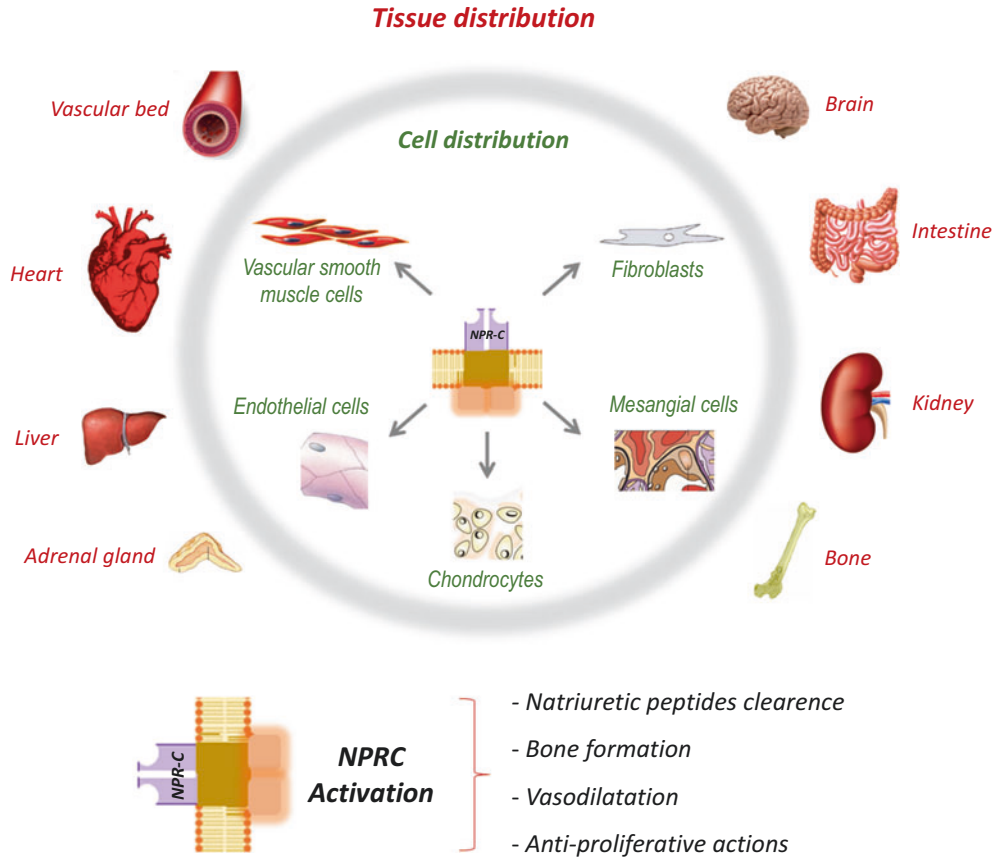
Natriuretic Peptide Receptor Type C (NPRC), Fig. 1 NPRC structure, activation, and signaling pathway. ANP: Atrial Natriuretic Peptide; BNP: Brain Natriuretic Peptide; CNP: C-type Natriuretic Peptide; Gi: G inhibitory protein; AC: adenylyl cyclase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophospate; Gq: Gq protein; PLC: phospholipase C; PIP2: phosphatidylinositol 4,5-bisphosphate; IP3: inositol triphosphate; DAG: diacylglycerol ; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3 kinase; AKT: protein kinase B. +: stimulation; -: inhibition; ?: unknow

stimulate the amylase release through PLC pathway (Sabbatini et al. 2007).

NPRC stimulation can also result in the activation of constitutive nitric oxide synthase (NOS) in gastrointestinal smooth muscle (Murthy et al. 1998). Additionally, NPRC was able to inhibit platelet-derived growth factor (PDGF) and endothelin-3-stimulated (MAPK) activity in astrocytes (Prins et al. 1996). Regarding its physiological actions elicited by NPs, NPRC activation inhibits endothelial and VSMC proliferation and also mediates the attenuation of cyclooxygenase 2 (COX-2) expression induced by ANP. NPRC activation by ANP has also been implicated in modulating endothelial permeability in coronary endothelial cells. It was reported that NPRC activation by BNP elicited antiproliferative actions in cardiac fibroblasts through a non-cGMP-mediated mechanism. Several effects of CNP seem to be related to NPRC signaling. In cardiac myocytes, NPRC stimulation by CNP induced inhibition of L-type Ca^{2+} influx. CNP antiproliferative effects in cardiac fibroblasts also seem to involve NPRC activation (Rubattu et al. 2010) (Fig. 2).

Regulation of NPRC Expression

It has been described that several hormonal factors can regulate NPRC expression. Endothelin 1, angiotensin II (Ang II), and arginine vasopressin (AVP) reduce the density and expression of NPRC in VSMC. The effect elicited by AVP has been associated with an attenuation of AC inhibition



Natriuretic Peptide Receptor Type C (NPRC), Fig. 2 NPRC localization and biological actions

mediated by NPRC. ANP has also been reported to regulate its own receptors. Experiments carried out in VSMC culture demonstrated that ANP elicited homologous downregulation of NPRC receptors. This effect depends on the degree of previous receptor occupation. Growth factors are also capable to regulate the expression of NPRC in VSMC and other tissues. In this way, fibroblast growth factors (FGF-1 and FGF-2) and PDGF-BB reduce NPRC mRNA expression in VSMC of pulmonary artery. On the other hand, transforming growth factor β1 (TGF-β1) showed to increase NPRC expression in murine thymic stromal cell line. Additionally, noradrenaline decreased ANP binding which was attributed to downregulation of the NPRC in cultured VSMC (Anand-Srivastava 2005). In the kidney, it was observed that dietary salt supplementation downregulates NPRC mRNA levels, suggesting a mechanism by which local ANP would facilitate natriuresis/diuresis and the maintenance of volume homeostasis (Nagase et al. 1997).

NPRC and Cardiovascular Diseases

Several evidences indicate a possible role of NPRC in the pathophysiology of some cardiovascular diseases (Cantú et al. 2015). An antihypertensive role has been proposed on the basis of alterations in NPRC function, amount, and tissue distribution in various models of hypertension. In this way, a dysfunction of NPRC signaling pathway has been found in spontaneously hypertensive rats (SHR), leading to impairment of NOS response to ANP with reduced nitric oxide (NO) availability, which in turn could help to explain the development of hypertension in this

model. Another study in SHR revealed that NPRC concentration in the kidney was higher in normotensive rats compared to Wistar-Kyoto (WKY) control rats, indicating a decrease in renal ANP availability and contributing to the hypertensive state (Rubattu et al. 2010). NPRC mRNA in aorta from stroke prone SHR (SP-SHR) but not NPRA was downregulated as compared to WKY control rats. The treatment of SP-SHR rats with an Ang II receptor type 1 (AT1 receptor) antagonist restored NPRC mRNA to levels similar to those found in control rats, suggesting that vascular NPRC downregulation may be mediated by Ang II. The downregulation of NPRC in aorta was also reported in Deoxycorticosterone acetate (DOCA)-salt hypertensive rats, whereas ANP plasmatic levels were elevated. These results suggest that higher plasma ANP levels could be responsible for NPRC downregulation in this model. The ANP-mediated inhibition of AC was significantly enhanced in heart and aorta from SHR compared to WKY rats. This inhibition was also attenuated in platelets from SHR and hypertensive patients (Anand-Srivastava 2005). Preliminary results revealed a potential role of NPRC in the pathogenesis of atherosclerosis. It has been reported an increase in NPRC expression in neointimal smooth muscle cells in patients receiving percutaneous coronary intervention, as a possible result of a complex response of neointima to injury. Molecular imaging technique demonstrated the presence of NPRC near the luminal surface of atherosclerotic plaques and in VSMC. Moreover, NPRC-dependent extracellular signal-regulated kinase (ERK 1/2) phosphorylation activated the vasoprotective effect of CNP, resulting in an augmentation of endothelial cell proliferation and inhibition of VSMC growth (Naruko et al. 2005). After myocardial infarction, NPRC expression is increased in infarcted and non-infarcted regions of the left ventricular wall while appears to be decreased in the kidneys and lungs. This decrease would reflect a mechanism to favor an increase in plasma concentration of NP in this pathological context. NPRC expression is also enhanced in heart failure as well as in platelets from patients with this condition. It has been postulated that this increase may be responsible for a reduction in ANP availability and for the existence of resistance to biological effects of this peptide in these patients (Rubattu et al. 2010). Conversely, both homozygotes and heterozygotes knockout mice for *Npr3* exhibit alteration in cardiovascular and renal functions, with reduced ability to concentrate urine, mild diuresis, blood volume depleted, and blood pressure values below normal levels. All these changes show that NPRC would regulate the local availability of NPs, according to specific local needs (Rukavina Mikusic et al. 2014).

There are several evidences linking mutations in *Npr3* and cardiovascular diseases. A study in 200,000 European descents showed an association between rs1173771 polymorphism in NPRC with hypertension. Moreover, several evidences indicate a potentially novel single nucleotide polymorphism (SNP) rs700926 in *Npr3* associated with coronary artery disease in Han Chinese population. In these studies, 11 SNPs of *Npr3* (rs700926, rs1833529, rs2270915, rs17541471, rs3792758, rs1833529, rs2270915, rs17541471, rs3792758, rs696831, and rs696831) were identified to be associated to coronary artery disease (Hu et al. 2016). Fox et al. found that four *Npr3* SNPs (rs700923, rs16890196, rs765199, rs700926) were associated to left ventricular dysfunction after coronary artery bypass grafting, and they were able to predict patient outcome when combined with *Npr3* SNPs. Although rs700926 near intron 1 of *Npr3* may not directly influence the NPRC mRNA expression, Fox et al. also found that mRNA expression level of NPRC in human peripheral blood leukocytes was significantly higher in individuals-patients carrying the polymorphism than those not carrying it. These findings are consistent with established effects of NPRC variant on coronary artery disease (Fox et al. 2009). Another study performed by Saulnier et al. in patients with type 2 diabetes found a consistent and significant association between the rs2270915 polymorphism of the *Npr3* and systolic blood pressure (SBP). Patients who did not carry the polymorphism had lower SBP values than carriers. The rs2270915 also influenced the response of SBP to salt reduction, given the fact that patients without carrying the polymorphism

showed a greater reduction of SBP after restriction of salt intake compared to carriers. The authors concluded that this genetic variation may affect pressure response to changes in dietary sodium (Saulnier et al. 2011).

NPRC and Hypoxia

NPRC, but not NPRA, gene expression is selectively downregulated in the lungs of rats and mice in response to hypoxia. This phenomenon is mediated by overexpression of tyrosine kinase-activating growth factors, such as acidic FGF-1 and occurs even in the absence of ANP gene expression. Selective downregulation of NPRC expression in lung in the setting of hypoxia may contribute to the increase in circulating ANP levels seen under hypoxic conditions and may enhance the vasodilator effects of ANP in the lung, thus modulating hypoxic pulmonary vasoconstriction/hypertension (Sun et al. 2001).

NPRC and Obesity

Adipocytes and adipose tissue have a high expression of NPRC. It has been described that NPRC expression is regulated positively by a high-fat diet and is suppressed by fasting (Rubattu et al. 2010). NPRC protein levels are markedly elevated during obesity. It is proposed that overexpression of NPRC would increase the clearance of NPs in adipose tissue, which could potentially contribute to reduced circulating levels of these peptides and predispose to hypertension in obese individuals (Collins 2014). Additionally, in obese hypertensive patients, weight reduction induced by fasting reduced blood pressure levels and increased diuresis and natriuresis (Rubattu et al. 2010).

NPRC and Bone Turnover

NPRC knockout mice resulted in skeletal abnormalities, characterized by hunched backs, dome-shaped skulls, decreased weight, and elongated femurs, tibias, metatarsal, digital bones, vertebral bodies, and body length. This model developed a skeletal-overgrowth phenotype, thus implicating a role for NPs in bone growth (Jaubert et al. 1999).

Summary

NPRC has traditionally been considered a clearance receptor of NPs responsible for receptor-mediated degradation of these peptides. In the last years, this view has been overcome by several studies showing evidence of NPRC multiple effects on different cells and organs and of the existence of a specific intracellular mechanism of action. These effects would be mediated by CNP stimulation rather than by ANP and BNP. A main role on vascular, cardiac, and metabolic physiology and on bone turnover has been proposed. Many studies indicate that alterations of NPRC function and structure as well as the existence of gene polymorphisms would be implicated in the pathophysiology of several diseases. Further investigation is needed to completely elucidate the physiological role played by NPRC and also to understand how NPRC alterations at gene and protein level could contribute to the development of pathological states as a cause of natriuretic system disruption.

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Natural Cytotoxicity Receptors (NCR)

▶ NK Receptor

Natural Killer Cell Group (NKG)

▶ NK Receptor

NBC2

▶ Na^+/HCO_3^- Cotransporter NBCn1

NBC3

▶ Na^+/HCO_3^- Cotransporter NBCn1

NBCe1

▶ NBCe1 Electrogenic Na^+-Coupled $HCO_3^-(CO_3^{2-})$ Transporter

NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter

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Synonyms

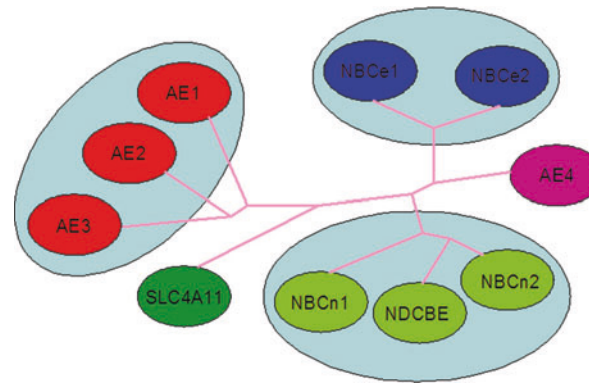
Electrogenic sodium bicarbonate cotransporter 1; NBCe1

Historical Background: SLC4 Gene Transporter Family

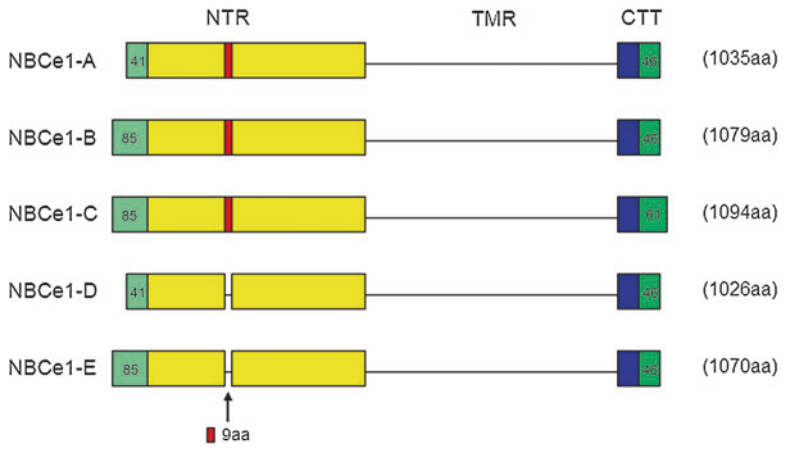
The NBCe1 transporter belongs to the *SLC4* gene family whose 10 members are homologous membrane transport proteins that differ in their ability to transport Na^+, Cl^-, HCO_3^-(CO_3^{2-}), H^+, NH_3, and water (Kurtz 2013; Parker and Boron 2013; Liu et al. 2015). AE1, AE2, and AE3 (*SLC4A1, −2, −3,* respectively) mediate the electroneutral exchange of Cl^- and HCO_3^-. The *SLC4A9* gene encodes AE4 has previously been reported to function as a Cl^-/HCO_3^- exchanger and electroneutral Na^+-HCO_3^- cotransporter, but has recently been shown to mediate electroneutral monovalent cation (Na^+/K^+)-dependent Cl^-/HCO_3^- exchange (Pena-Munzenmayer et al. 2016). NDCBE (encoded by *SLC4A8*) like AE1, AE2, and AE3 is an anion exchanger yet differs in that it couples the electroneutral transport of Na^+ and HCO_3^- (or CO_3^{2-}) in exchange for Cl^-. NBCn1 (*SLC4A7* gene) and NBCn2 (*SLC4A10* gene) transport Na^+-HCO_3^- electroneutrally. NBCn2 differs from NBCn1 in that a Cl^-/Cl^- exchange process is part of its transport cycle. NBCe1 (*SLC4A4* gene) and NBCe2 (*SLC4A5* gene) mediate electrogenic Na^+-HCO_3^- and/or CO_3^{2-} transport. *SLC4A11* is the only member of the SLC4 family that does not transport HCO_3^- or CO_3^{2-}. It is a multifunctional transporter that transports H^+ in a Na^+-coupled or independent mode and also mediates electrogenic H^+-NH_3 cotransport and water flux (Vilas et al. 2013; Zhang et al. 2015; Kao et al. 2016). Figure 1 shows a dendrogram of the SLC4 family demonstrating that in general, sequence similarity is predictive of a given transporter's functional properties.

NBCe1 Variants

NBCe1 was functionally originally identified in the salamander proximal tubule (Boron and Boulpaep 1983) and subsequently cloned from salamander kidney (Romero et al. 1997). The *SLC4A4* gene (Abuladze et al. 2000) encodes five NBCe1 variants (NBCe1-A-E) with NBCe1-D/E identified in mouse (Liu et al. 2011) (Fig. 2). These variants arise from two separate promoters and alternate slicing of exon 6 and exon 24 that results in differences in their N- and C-termini and the use of an alternate 9 aa cassette (Liu et al. 2011). All five variants have an identical transmembrane region but differ in their extreme



NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Fig. 1 SLC4 transporter dendrogram. Transporters with similar function tend to cluster based on the similarity of their amino acid sequence. The *SLC4A11* gene is a multifunctional transporter lacking an accepted protein name



NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Fig. 2 The five known NBCe1 variants are depicted (not to scale). These variants share three separate regions that include: (1) N-terminal region (NTR); (2) transmembrane region (TMR); and (3) C-terminal tail (CTT). The five variants share an identical TMR and differ in their NTR and CTT. NBCe1-A and NBCe1-D only differ in their NTR where the -D variant lacks a stretch of 9 aa (RMFSNPDNG). NBCe1-B and NBCe1-E also only differ in their NTR where the -E variant also lacks the same 9 aa cassette. NBCe1-B and NBCe1-C differ in their CTT where the -C variant has a unique CTT with a type I PDZ motif. NBCe1-D and NBCe1-E transcripts were originally detected in mouse reproductive tissues (Liu et al. 2011) and have not yet been demonstrated at the protein level

N- and C-termini (Kurtz 2013; Parker and Boron 2013; Liu et al. 2015). Of the variants that have been functionally characterized and studied at the protein level, NBCe1-A, NBCe1-B, and NBCe1-C mediate electrogenic Na^+-HCO_3^-(and/or CO_3^{2-}) transport but differ in their cellular localization, regulation, and intrinsic properties.

Examples of organs/cells where NBCe1 plays an important transport role is shown in Fig. 3. In the kidney, NBCe1-A is localized to the basolateral membrane of the proximal tubule S1 and S2 segments with minimal expression in the S3 segment (Abuladze et al. 1998b; Marino et al. 1999a). The transporter plays a key role in proximal tubule transepithelial bicarbonate absorption (Romero et al. 1997; Abuladze et al. 1998a; Maunsbach et al. 2000; Skelton et al. 2010). In extrarenal tissues, NBCe1-A has been localized at the protein level in the eye (Bok et al. 2001; Usui et al. 2001) and salivary gland (Brandes et al. 2007). NBCe1-A mRNA transcripts have been detected in nasal submucosal glands (Lee et al. 2005). NBCe1-B which was originally cloned from pancreas is more widely expressed that NBCe1-A, and contributes to transepithelial bicarbonate transport and intracellular and extracellular pH regulation in various tissues (Ishiguro et al. 1996a, b; Abuladze et al. 1998a; Marino et al. 1999; Gross et al. 2001a). The NBCe1-B variant that is identical to NBCe1-A in its transmembrane and C-terminal regions has a unique extreme N-terminus wherein 85 aa replace the 41 aa in NBCe1-A. The tissues in which NBCe1-B has been localized either at the transcript or protein level include intestine, gall bladder, submucosal and salivary glands, nasal mucosa, lung, heart, brain, eye, skeletal muscle, and ameloblasts (Abuladze et al. 1998a; Choi et al. 1999; Bok et al. 2001; Usui et al. 2001; Kristensen et al. 2004; Lee et al. 2005; Kreindler et al. 2006; Moser et al. 2007; Perry et al. 2007; Majumdar et al. 2008; Paine et al. 2008; Yu et al. 2009; Abdulnour-Nakhoul et al. 2011; De Giusti et al. 2011; Garciarena et al. 2013; Jalali et al. 2014; Namkoong et al. 2015). It should be noted that several of these studies did not distinguish whether NBCe1-C/−E were potentially expressed. NBCe1-C, originally cloned from rat brain (McAlear et al. 2006; Majumdar et al. 2008), has a unique type I PDZ motif in its C-terminus. NBCe1-D and NBCe1-E transcripts found in mouse reproductive tissues are identical

to NBCe1-A and NBCe1-B, respectively, except that in the cytosolic N-terminus they lack a nine amino-acid cassette (Fig. 2; Liu et al. 2011).

Charge Transport Stoichiometry Considerations and Anion Substrates: HCO_3^- Versus CO_3^{2-} Transport

The direction of NBCe1 transport in cells and epithelia is determined by the electrochemical driving force (μ) across the transporter. The charge transport stoichiometry and the membrane potential are the two major determinants of the overall electrochemical driving force given that in all cells the chemical gradient for Na^+ and base (HCO_3^-, CO_3^{2-}) is inward (extracellular to cytoplasm) (Kurtz et al. 2004). In the human kidney proximal tubule, it is widely assumed NBC1-A has a charge transport stoichiometry of 1:3 (1 Na^+: 1 HCO_3^-: 1 CO_3^{2-}) and that the value of μ is positive leading to cellular base efflux. In the rat proximal tubule in vivo a charge transport stoichiometry of 1:3 was reported (Yoshitomi et al. 1985); however, in the isolated perfused rabbit proximal tubule, a value that varied from 1.2–1:2.7 was reported that depended on the composition of the experimental solutions used (Seki et al. 1993; Müller-Berger et al. 1997). In *Necturus* proximal tubules in vivo, the charge transport stoichiometry could be decreased from 1:3 to 1:2 as a result of acutely increasing the PCO_2 (Planelles et al. 1993). Moreover, Gross et al. reported that the charge transport stoichiometry of NBCe1 could vary depending on the cell type in which the measurements were made (Gross et al. 2001b). In expression systems with excellent signal/noise and few technical artifacts, the charge transport stoichiometry of human NBCe1-A expressed in both human HEK293 cells and *Xenopus* oocytes is 1:2 (Lee et al. 2013; Zhu et al. 2013b). It is currently unknown whether the charge transport stoichiometry *in vivo* in humans can be modulated as has been reported in vitro with regards to changes in intracellular Ca^{2+} (Muller-Berger et al. 2001), an acute change in the PCO_2 (Planelles et al. 1993), and altered phosphorylation status (Gross et al. 2001c).

The NBCe1 charge transport stoichiometry of 1:2 is compatible with either Na^+-CO_3^{2-} cotransport (one anion interaction site) or 1 Na^+:2 HCO_3^- transport (two anion interaction sites). Given that NBCe1-B in secretory epithelia such as intestine, pancreas, and salivary glands and nonsecretory cells such as astrocytes normally mediates cellular Na^+-coupled base influx, it is assumed that the transporter has a charge transport stoichiometry of 1:2. A value of 1:2 has been measured in cultured pancreatic cells (Gross et al. 2001a). Zhu et al. reported that human NBCe1-A expressed in HEK-293 cells functioning with a 1:2 charge transport stoichiometry transports Na^+-CO_3^{2-} based on experiments that utilized NO_3^- as a surrogate for CO_3^- transport (Zhu et al. 2013b). Surface pH measurements in *Xenopus* oocytes also suggest in preliminary experiments that rat NBCe1-A functions as a Na^+-CO_3^{2-} cotransporter (Lee et al. 2011).

In native human proximal tubule cells it will be very difficult to determine the charge transport stoichiometry of NBCe1-A. It remains possible that proximal tubule cell-specific factors modulate the stoichiometry *in vivo* altering the electrochemical driving force across the transporter. Given that human NBCe1-A has a charge transport stoichiometry of 1:2 *in vitro* in expression systems with excellent signal/noise, the question has arisen as to whether a 1:2 charge transport stoichiometry is sufficient to drive Na^+-CO_3^{2-} efflux in vivo. Although the necessary human data is unavailable, it has been shown using data from rat proximal tubules that μ of NBCe1-A would have a positive value resulting in Na^+-CO_3^{2-} efflux (that is very sensitive to changes in small changes in μ) (Zhu et al. 2013b). Given these findings, if NBCe1 mediates Na^+-CO_3^{2-} transport, a change in its name to NCCe1-A, i.e., sodium carbonate cotransporter electrogenic 1-A would more accurately reflect the transported species.

NBCe1 Mutations: Proximal Renal Tubular Acidosis

Proximal renal tubular acidosis (pRTA) is a syndrome caused by several diseases that impair

proximal tubular bicarbonate absorption (Haque et al. 2012). The abnormality in proximal tubule bicarbonate transport can be isolated (Table 1) or associated with additional proximal tubule transport defects as part of Fanconi's syndrome (Table 2). Net transepithelial proximal tubule bicarbonate absorption (apical to basolateral direction) is mediated by the coupling of apical NHE3 mediated H^+ transport (to a lesser extent the apical H^+-ATPase) and basolateral NBCe1-A HCO_3^-(and/or CO_3^{2-}) transport (Fig. 3 (Boron 2006; Hamm et al. 2013). Bicarbonate absorption is enhanced by membrane bound carbonic anhydrase enzymes that catalyze the CO_2 hydration/dehydration reactions in the lumen and peritubular compartments, and cytoplasmic carbonic anhydrase that catalyzes the cytoplasmic conversion of CO_2 into H^+ and HCO_3^-. In the absence of other proximal tubule transport defects (Fanconi's syndrome), the only known cause of hereditary (autosomal recessive) pRTA is mutations in the *SLC4A4* gene that affect basolateral NBCe1-A mediated transport (Igarashi et al. 1999). Because the majority of mutations also affect other NBCe1

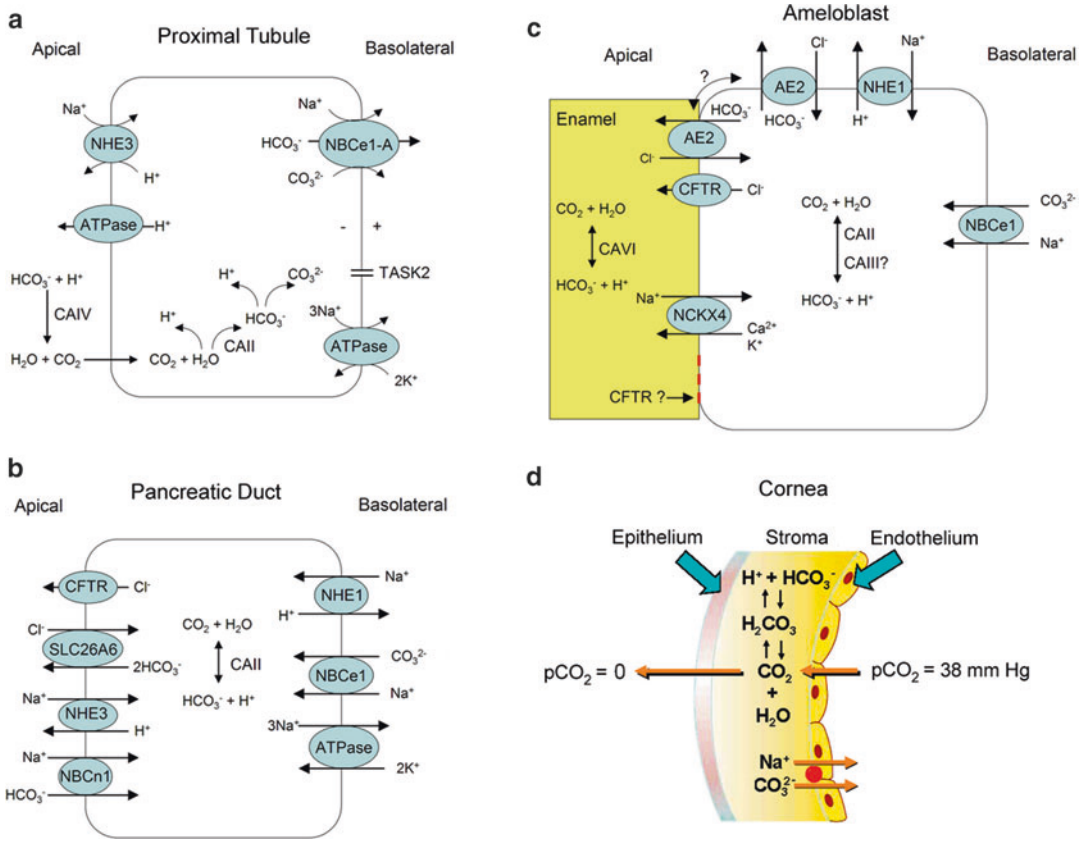
NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Table 1 Inherited Causes of Isolated pRTA and Associated Extrarenal Abnormalities

Gene	Protein	Inheritance	Renal phenotype	Extrarenal phenotype
CA2	CAII	Autosomal recessive	pRTA, dRTA, hypokalemia	Osteopetrosis involving axial skeleton, long bones with widening of metaphyses, and skull; growth defect; intracerebral calcification
[a]SLC4A4	NBCe1	Autosomal recessive	pRTA, hypokalemia	Glaucoma, cataracts, band keratopathy, increased serum amylase and lipase, enamel defects, intracerebral calcification, decreased IQ, growth defect
Unknown gene(s)	Unknown	Autosomal dominant	pRTA	Decreased radial bone density, thinner iliac cortices, subaortic stenosis, colomboma, growth defect

[a]Headaches have been reported in patients with the R510H, L522P, and R881C missense mutations, 2311delA, and a homozygous C-terminal 65 bp-del. In heterozygous patients with the 65 bp-del and the L522P mutations, headaches also been reported and have been attributed to a dominant negative effect

NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Table 2 Disorders Causing pRTA with Fanconi's Syndrome

Gene	Protein	Inheritance	Disease
ALDOB	Aldolase B	Autosomal recessive	Hereditary fructose intolerance
ARSA	Arylsulfatase A	Autosomal recessive	Metachromatic leukodystrophy
ATP7B	Cu^{++} transporting ATPase β peptide	Autosomal recessive	Wilson's disease
CLCN5	$2Cl^-/H^+$ exchanger	X-linked	Dent's disease 1
Complex IV	Cytochrome C oxidase	N/A	Cytochrome C oxidase deficiency
CTNS	Cystinosin	Autosomal recessive	Cystinosis
FAH	Fumarylacetoacetase	Autosomal recessive	Tyrosinemia type I
EEHADH	Peroxisomal bifunctional enzyme	Autosomal dominant	FRTS3
GALT	Galactose-1-phosphate Uridylyltransferase	Autosomal recessive	Galactosemia
MMAB	Methylmalonyl CoA mutase	Autosomal recessive	Methylmalonic acidemia
OCRL1	PIP2 5-phosphatase	X-linked	Dent's disease 2
OCRL1	PIP2 5-phosphatase	X-linked	Lowe's syndrome
PC	Pyruvate carboxylase	Autosomal recessive	Pyruvate carboxylase deficiency
SLC2A2	GLUT2	Autosomal recessive	Fanconi-Bickel syndrome



NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Fig. 3 Transport models of some of the cells/tissues that are involved in patients with pRTA mutations. (**a**) kidney proximal tubule, (**b**) pancreatic duct, (**c**) maturation stage ameloblast, (**d**) cornea

transcripts, patients have an extrarenal phenotype in addition to proximal renal tubular acidosis that includes cataracts, band keratopathy, glaucoma, mental retardation, basal ganglia calcifications, migraine headaches, tooth enamel defects, and short stature. The constellation of findings is diagnostic of patients with NBCe1 mutations.

Since Igarashi et al. first described two patients with homozygous NBCe1 mutations (Igarashi et al. 1999), a total of nine missense mutations (NBCe1-A numbering: R298S, S427L, T485S, G486R, R510H, L522P, A799V, R881C, and Q913R), two nonsense mutations (Q29X, W516X), and two frameshift deletions (2311 delA and a C-terminal tail 65 bp-del) have been reported (Table 3) (Kurtz 2013). The NBCe1-A-Q29X mutation involving the NBCe1-A N-terminus (Igarashi et al. 2001; Azimov et al. 2008) could also potentially involve NBCe1-D (Liu et al. 2011). The patient reported with the NBCe1-A Q29X mutation did not have band keratopathy or cataracts as do typical patients implying that mutant NBCe1-A does not cause these ocular abnormalities. Mice with congenital loss NBCe1 (all variants) have a more severe phenotype than humans with decreased survival, severe volume depletion, and colonic obstruction (Gawenis et al. 2007; Lacruz et al. 2010; Yu et al. 2016). A neurologic and ocular phenotype has not been reported in mice since these abnormalities either might become manifest after a longer period of time or are unique to humans. Interestingly, mice with loss of NBCe1 have impaired proximal tubule ammonia metabolism including a decrease in phosphate-dependent glutaminase and phosphoenolpyruvate

NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Table 3 [a]Proximal RTA caused by NBCe1 mutations

Mutation	Location	Classification	Effect of Mutation
Q29X	N-terminal region	Nonsense	NBCe1-A protein truncation
R298S	N-terminal region	Missense	Mistargeting: apical/basolateral membranes
			Abnormal interaction of the N- terminal region with the cytoplasmic region
S427L	TM1	Missense	Mistargeting: predominant apical membrane
			Abnormal helix packing
			Decreased G_{HCO3}
			Impaired I_{HCO3} reversal at −Vm
T485S	IL1	Missense	Altered ion interaction
			Electroneutral transport
G486R	TM3	Missense	Altered ion interaction
R510H	TM4	Missense	ER retention
W516X	TM4	Nonsense	Truncation: all NBCe1 variants
L522P	TM4	Missense	ER retention
2311 delA	IL4	Frameshift	Truncation: all NBCe1 variants
A799V	H4	Missense	Intracellular retention
			Decreased G_{HCO3}
			Bicarbonate-independent G_{cation}
R881C	EL5	Missense	ER retention
[b]Q913R	TM13	Missense	ER retention
65 bp-del	C-terminal tail	Frameshift	ER retention

[a]Numbered according to NBCe1-A amino acid sequence; G_{HCO3}: bicarbonate conductance; G_{cation}: cation conductance; I_{HCO3}: bicarbonate-dependent current; −Vm: negative plasma membrane voltages
[b]Reported in a compound heterozygote (R510H/Q913R) patient

carboxykinase and increased expression of glutamine synthetase (Handlogten et al. 2015). These data show that NBCe1 is essential for mediating proximal tubule bicarbonate absorption and also required for normal proximal tubule renal ammonia metabolism.

Patients with *SLC4A4* mutations have a disease process that mechanistically depends on the type of mutation involving the transporter. For example, nonsense/frameshift mutations result in absence of the full-length NBCe1 protein whereas missense mutations can either perturb the intrinsic function of the transporter and/or impair plasma membrane processing/targeting because of misfolded protein that is targeted to the endoplasmic reticulum (Table 3). Migraine headaches are an interesting manifestation in that they appear to be mutation specific and have been reported in patients with R510H, L522P, R881C, 2311 delA, and 65 bp-del mutations (Suzuki et al. 2010). It is of interest that headaches were also been reported in heterozygous family members of a patient with a 65 base-pair C-terminal deletion and the L522P mutations. The underlying pathophysiology is conjectural and in homozygotes may be caused by misfolded ER retained NBCe1-B in brain astrocytes resulting in NMDA-mediated neuronal hyperactivity, whereas in heterozygotes mutant-wild type NBCe1-B hetero-oligomers might be retained in the ER (Suzuki et al. 2010; Yamazaki et al. 2013).

Proximal renal tubular acidosis due to NBCe1 mutations is inherited in an autosomal recessive Mendelian fashion unlike patients with distal renal tubular acidosis caused by another member of the SLC4 family, AE1, where both autosomal recessive and dominant inheritance patterns have been reported (Batlle and Haque 2012). It is currently unknown whether individuals who are heterozygous carriers for NBCe1 mutations have subtle defects in proximal tubule bicarbonate

absorption and/or ocular and neurologic findings. Furthermore, there are no reports of patients with gain of function mutations although residues in NBCe1 have been identified whose substitution can stimulate NBCe1 transport (Abuladze et al. 2005). Yamazaki et al. studied several NBCe1 SNPs including E122G, S356Y, K558R, and N640I *in vitro* and reported that the function of K558R was decreased 41–47% (Yamazaki et al. 2011). It would be of interest to determine the potential impact of these SNPs on proximal tubule bicarbonate absorption and ocular and neurologic function in animal and human studies.

In addition to patients with NBCe1 mutations, a familial form of isolated pRTA has also been reported that appears to be inherited in an autosomal dominant Mendelian fashion. The extrarenal manifestations differ in that these patients have decreased bone density and short stature (Table 1) (Brenes et al. 1977; Lemann et al. 2000; Katzir et al. 2008). The cause of this syndrome is currently unknown as mutations in the coding region of proximal tubule H^+/base transport proteins including NBCe1, NHE3, NHE8, CAII, CAIV, CAXIV, PAT1(CFEX), NHERF1, and NHERF2 have been ruled out (Katzir et al. 2008). Whether mutations in intronic and promoter regions encoding any of these proteins are present has not yet been determined.

NBCe1 Mutations: Extrarenal Manifestations

Although some progress has been made, we currently lack a complete understanding of the cause of many of the extrarenal manifestations in patients for the following reasons: (1) NBCe1 dysfunction at the cellular level versus the effect of chronic systemic acidemia needs to be more precisely defined; (2) Additional work also needs to be done to determine the relative contribution of specific NBCe1 variants in affected cell types; and (3) The tissue distribution of each variant is not precisely known because several of the studies utilized antibodies that were unable to distinguish among certain variants i.e., NBCe1-B/-C/-E for example.

Patients with NBCe1 mutations have growth retardation that is not unique to this disorder in that decreased growth occurs in other pediatric diseases where metabolic acidosis is present from birth (Batlle and Haque 2012; Haque et al. 2012). Of interest, normal fetal blood is acidic in comparison to maternal blood, and it is conceivable that prior to birth the blood of babies with NBCe1 mutations might differ from normal (Spackman et al. 1963). A similar process might be occurring in other diseases where metabolic acidosis is present at birth.

Given the known expression of NBCe1 variants in the cortex and hippocampus (Majumdar et al. 2008), abnormal brain development and low IQ are more likely due to the loss of NBCe1 function in specific cell types in the brain. The role of the systemic acidemia is unclear but in general is not associated per se with this phenotype (Majumdar et al. 2008). Patients with NBCe1 mutations have calcified basal ganglia (Igarashi et al. 1994) as do patients with CAII mutations (Bosley et al. 2011). NBCe1 transcripts are expressed in the striatum and although conjectural, the calcification might be due to an elevated pH in this region due abnormal HCO_3^-(CO_3^{2-}) transport leading to calcium phosphate precipitation.

NBCe1 is expressed in several parts of the eye (Bok et al. 2001; Usui et al. 2001) and loss of its transport functions results in multiple abnormalities including band keratopathy, glaucoma, and cataracts. The mechanism for these abnormalities is poorly understood. With regards to band keratopathy, normal eyelid opening during blinking creates a brief loss of CO_2 acutely alkalizing the anterior corneal tear coat (Fig. 3). We have hypothesized that the elevated corneal pH would normally be decreased towards normal by an increase in endothelial cell NBCe1-B transport. In patients with NBCe1 mutations, defective NBCe1-B transport would prevent the corneal pH from being regulated towards normal resulting in calcium phosphate precipitation (band keratopathy) in the central cornea, the region of the cornea that is exposed during eyelid opening. The mechanism for lens cataracts in these patients is not known. Previous studies have shown that toad lens epithelial cells have electrogenic Na^+-

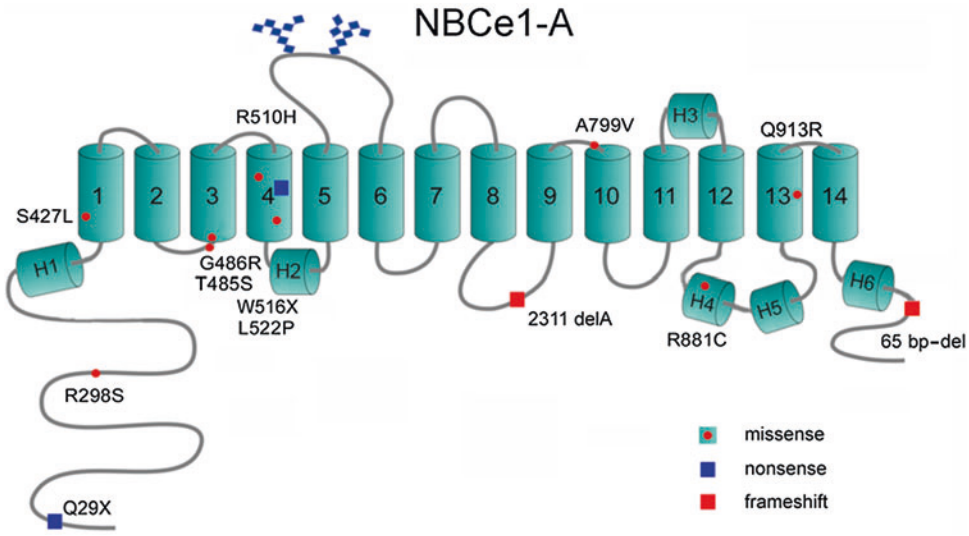
base transport (Wolosin et al. 1990) that is likely mediated by NBCe1-B (Bok et al. 2001). NBCe1-B is expressed in the rat lens epithelium (Bok et al. 2001), where it is possible that defective cellular HCO_3^-(and/or CO_3^{2-}) transport and the resultant abnormal lens pH alters lens transparency. NBCe1-B is expressed in the rat pigmented ciliary body (Bok et al. 2001), and Woloson et al. has demonstrated electrogenic Na^+-base transport process in rabbit ciliary body (Wolosin et al. 1993). How the loss of NBCe1 activity leads to glaucoma is also currently unknown.

In teeth, the extracellular matrix is calcified during enamel formation and this process mediated by ameloblasts is highly pH dependent. Patients with NBCe1 mutations have abnormal tooth enamel (Dinour et al. 2004) that has been attributed to abnormal pH regulation of the extracellular matrix due to loss of local ameloblast NBCe1 transport (Fig. 3) (Lacruz et al. 2010; Lacruz et al. 2012). Ameloblasts express several of the NBCe1 variants including NBCe1-B and/or -D/E and NBCe1-C protein (Paine et al. 2008; Jalali et al. 2014). Similar abnormalities have not been found in other patients with congenital or chronic metabolic acidosis suggesting that systemic acidemia and/or a decrease in salivary pH is not responsible for the phenotype in patients with NBCe1 mutations. Interestingly, however, in a study where mandibular E11.5 explants from $NBCe1^{-/-}$ mice were maintained in host kidney capsules of normal mice for 70 days, tooth enamel and dentin had morphological and mineralization properties similar to cultured $NBCe1^{+/+}$ mandibles. If these findings can be extrapolated to patients, the results suggest that the cause of the enamel phenotype is primarily to be due to abnormalities in systemic pH (Wen et al. 2014).

In the pancreas, NBCe1-B is localized to the ductal cell basolateral cell membrane where it plays a role in cellular HCO_3^-(CO_3^{2-}) influx and transepithelial HCO_3^-(CO_3^{2-}) secretion (Fig. 3) (Ishiguro et al. 1996a, b; Marino et al. 1999; Gross et al. 2001a; Satoh et al. 2003). Unlike in cystic fibrosis where transepithelial bicarbonate secretion is thought to be defective, the loss of NBCe1-B function does not lead to any clinically apparent ductal abnormalities perhaps because of the upregulation of additional basolateral membrane bicarbonate influx pathways (Park and Lee 2012). Patients with NBCe1 mutations have increased serum amylase and lipase suggesting that acinar cell function is perturbed despite the fact that in humans acinar cells have not been shown to express NBCe1; although NBCe1-B is expressed in rat acinar cells. In mice, loss of NBCe1 leads to a severe intestinal GI phenotype (Gawenis et al. 2007; Yu et al. 2016). NBCe1-B is expressed in the human intestine; however, the loss of the transporter in patients with NBCe1 mutations does not lead to an obvious phenotype suggesting the possibility that either compensatory transport mechanisms come into play, and/or NBCe1-B plays a less important transport role in the normal physiology of the human GI tract.

NBCe1 Mutations: Structure-Function Properties

Within the SLC4 transporter family, the structural properties of NBCe1-A and AE1 have been most thoroughly studied. The structural properties of NBCe1-A provide a topologic framework not only for other NBCe1 variants because they share an identical transmembrane region, but also potentially there are insights that can be applied to other Na^+-coupled SLC4 bicarbonate transporters. Fluorescence image moment studies and spatial intensity distribution analysis (SpIDA) have demonstrated that NBCe1-A is a dimer in the native kidney in situ (Sergeev et al. 2012). The dimerization state of the N-terminal region can potentially be modulated by changes in intracellular pH and/or bicarbonate (Gill 2012). Each monomer consists of 1035 amino acids (~140-kDa glycoprotein) and can independently transport ions (Kao et al. 2008). In addition, the dimeric state per se plays a role functionally (Chang et al. 2014). The topologic structure of NBCe1 is subdivided into three separate regions that include a long N-terminal cytoplasmic region, a large transmembrane region, and a shorter C-terminal cytoplasmic tail. Zhu et al. using cysteine scanning mutagenesis to analyze the



NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Fig. 4 (**a**) Linear topology of NBCe1-A. Known pRTA mutations are depicted (NBCe1-A numbering)

topologic properties of NBCe1-A showed that the transporter has 14 transmembrane segments (TMs) (Fig. 4). All NBCe1 variants have a large glycosylated extracellular loop between TMs 5 and 6 (Choi et al. 2003) that is not accessible to enzymatic digestion and is compactly folded within the protein (Zhu et al. 2015). An additional small extracellular loop is present between TMs 7 and 8. Both the N-terminal region and C-terminal tails of NBCe1 protein variants are located in the cytoplasm. It has been hypothesized that certain topologic features in NBCe1 might resemble the vGLUT and LeuT prokaryotic Na^+-coupled transporters (Yamashita et al. 2005; Watanabe et al. 2010; Zhu et al. 2010a, b).

N-Terminal Cytoplasmic Region

A preliminary crystal structure of the NBCe1-A N-terminal cytoplasmic region has been reported (Gill et al. 2015). Alignment with AE1 shows similar hydrophobic residues and predicted helical regions. Within the N-terminal cytoplasmic region two NBCe1 mutations have previously been identified: A Q29X nonsense mutation that was initially thought to be specific for NBCe1-A yet would be predicted to also truncate NBCe1-D; and a R298S mutation that is shared by all NBCe1 variants. The potential for mutation specific therapy was demonstrated for the first time in the context of the Q29X mutation (Azimov et al. 2008). This mutation results in a wt-CAG sequence encoding glutamine being replaced by a UAG stop codon causing premature truncation of the transporter (Igarashi et al. 2001). In HEK 293-H cells expressing the NBCe1-A-Q29X mutant, ribosomal read-through was induced by the aminoglycoside G418 resulting in the production of full-length functional NBCe1-A protein (Azimov et al. 2008). The findings in this study suggested the possibility of treating the ocular abnormalities in patients using topical aminoglycosides and avoiding their systemic toxicity. In the future, it may be possible to treat patients with stop codon mutations with less toxic more potent aminoglycosides that have a flexible N-1-AHB group ((*S*)-2-hydroxy-4-aminobutyl group at the N-1 position) (Nudelman et al. 2009). In addition, compounds such Ataluren (Peltz et al. 2013) that can also induce ribosomal read-through may prove efficacious.

The cytoplasmic N-terminal R298S mutation that is shared by all NBCe1 variants appears to be localized to a tightly folded region that has been hypothesized to form a "HCO_3^- tunnel" in the wild-type transporter but whose structure might be disrupted in the mutant (Igarashi et al. 1999;

Horita et al. 2005; Chang et al. 2008; Suzuki et al. 2010; Zhu et al. 2010b). Moreover, Zhu et al. have suggested that the N-terminal and transmembrane regions of the transporter normally interact with each other and have hypothesized that in the mutant the efficient delivery of HCO_3^-(and/or CO_3^{2-}) to the ion permeation pathway is impaired (Zhu et al. 2013a). NBCe1-A appears to differ from AE1 in that the latter does not have a substrate access tunnel in its N-terminal cytoplasmic region (Shnitsar et al. 2013). An additional mechanism may be involved in that other groups have shown that when expressed in MDCK cells, the NBCe1-A R298S and corresponding NBCe1-B R342S mutants do not traffic to the basolateral plasma membrane preferentially but are expressed on the apical and basolateral membranes (Li et al. 2005; Suzuki et al. 2008) suggesting that a targeting signal in the N-terminal region has been perturbed. Gill et al. have reported that R298 forms part of a putative substrate conduit near the dimer interface that is held together by hydrogen-bond networks (Gill 2012). Moreover, it was proposed that the R298S mutation results in a temperature alteration of the monomer–dimer equilibrium that can expose charged surfaces resulting in protein aggregation and precipitation (Gill et al. 2015).

TM1–14 (Transmembrane Region)

TM1 which contains 31 amino acids and is longer than a standard TM has an N-terminal cytosolic portion with a helical conformation that connects the cytoplasmic portion with the transmembrane portion (Zhu et al. 2013a). The extracellular C-terminal end of TM1 forms a component of the ion permeation pathway where Thr^{442} which is located within a ^{441}ITFGGLLG^{448} motif (NBCe1-A numbering) that is found in most SLC4 transporters is suggested to be part of an extracellular gate involved in ion entry (Zhu et al. 2009). TM1 contains several functionally important key residues that appear to line the ion permeation pathway including Ala^{428}, Ala^{435} in addition to Thr^{442} (Zhu et al. 2009). Substituting of Asp^{416}, Gln^{424}, Tyr^{433}, and Asn^{439} with cysteine causes misfolding of the transporter and intracellular retention. The cysteine scanning data are compatible with C-terminal end of TM1 having an open structure forming part of an aqueous-accessible cleft. These studies also revealed tight protein packing of the cytoplasmic pre-TM1 region.

The TM1 S427L pRTA mutation decreases transporter function by 90% resulting in pRTA. Of interest, the mutation also blocks the reversal of the direction of the transport current (Dinour et al. 2004). Ser427 has been localized to space-confined region in TM1 wherein the serine side chain hydrophobicity appears to be involved in helix packing and ionic interactions between helices (Zhu et al. 2013a). A conformational change in TM1 associated with a collapse/altered configuration of the ion permeation pathway has been hypothesized to be the cause of functional impairment associated with the S427L mutation. As in the R298S mutation, the S427L mutant when expressed in MDCK cells is mistargeted although in this instance, the mutant transporter is mislocalized to the apical membrane (Li et al. 2005).

Both the T485S mutation in intracellular loop 1 (IL1) and the adjacent G486R mutation in the beginning of TM3 independently cause pRTA (Horita et al. 2005; Suzuki et al. 2008, 2010; Zhu et al. 2010b, 2013b). Given the structural and chemical similarity between serine and threonine, the T485S mutation would not be predicted to cause pRTA and decrease overall base transport by approximately 50% (Horita et al. 2005; Suzuki et al. 2008, 2010; Zhu et al. 2010b, 2013b). Accordingly, Zhu et al. hypothesized that Thr^{485} must reside either in the aqueous ion permeation pathway or an ion interaction site (Zhu et al. 2013b). Thr^{485} was localized to an aqueous confined region, and the functional sensitivity to MTS reagents was substrate-dependent providing the first evidence that Thr^{485} is located in an ion interaction site. Whole cell patch clamp experiments of the wild-type transporter expressed in HEK-293 cells were compatible with NBCe1-A mediated electrogenic Na^+-CO_3^{2-} cotransport and a single anion interaction site (Zhu et al. 2013b). The T485S mutation converted NBCe1-A from an electrogenic to an electroneutral transporter compatible with the mutant mediating Na^+-HCO_3^- cotransport (Zhu et al. 2013b). Using

NO_3^- as a surrogate for CO_3^{2-} transport, the T485S mutant unlike the wild-type transporter failed to mediate Na^+-driven NO_3^- transport (Zhu et al. 2013b) suggesting that it favors HCO_3^- as a substrate. The adjacent G486R mutation was shown to perturb transporter function by altering the orientation of Thr^{485} (Zhu et al. 2013b).

In the context of the electroneutral T485S NBCe1-A pRTA mutation with an associated ~50% decrease in transport activity, the proximal tubule basolateral membrane electrical potential is no longer a driving and given that the ion gradients are inward (basolateral to cytoplasm), the transporter will initially mediate inward Na^+-base influx significantly impairing transepithelial bicarbonate absorption. Assuming that the threonine to serine substitution also decreases the functional activity of NBCe1-B/-C by ~50% and converts these variants to electroneutral transporters, a decrease in their cellular base influx activity would be predicted. In addition, mutant NBCe1-B/-C transport would be potentially further perturbed by the decrease in the extracellular bicarbonate concentration (due to renal bicarbonate loss).

TM4, where the R510H, W516X, and L522P pRTA mutations are located, is reported to act as a scaffolding helix that is essential for normal protein folding (Zhu et al. 2010b). In addition, this helix has potential stop transfer and signal anchor sequences (Igarashi et al. 1999; Horita et al. 2005; Li et al. 2005; Demirci et al. 2006; Suzuki et al. 2008, 2010; Zhu et al. 2010b; Yamazaki et al. 2013). The W516X nonsense mutation truncates the transporter prematurely and likely results in misfolded protein (Lo et al. 2011). In W516X knock-in mice, prenatal Ataluren therapy (induces ribosomal read-through) significantly increased NBCe1 protein abundance and activity, and increased postnatal survival although prenatal bicarbonate administration achieved a higher survival rate (Fang et al. 2015). Similarly, both the R510H and L522P mutations induce misfolding and ER retention (Igarashi et al. 1999; Horita et al. 2005; Li et al. 2005; Demirci et al. 2006; Suzuki et al. 2008, 2010; Zhu et al. 2010b; Yamazaki et al. 2013). Why R510H causes protein misfolding is unclear; however, it is possible that the size of the side chain and/or magnitude of the positive charge at this position is required for the ionic interaction between TM4 and its neighboring TMs. Since L522I and L522C are processed to the plasma membrane normally (Zhu et al. 2010b; Yamazaki et al. 2013), the proline substitution at this position is the likely cause of increased TM flexibility resulting in helix disruption, protein misfolding, and intracellular ER retention.

Asp^{555} in TM5 appears to prevent several anions from being transported nonspecifically by NBCe1-A (Yang et al. 2009). When glutamate is substituted at Asp^{555}, NBCe1-A also transports of Cl^-, NO_3^-, and SCN^- in addition to electrogenic Na^+-HCO_3^-(CO_3^{2-}) cotransport. The well-known stilbene inhibitor 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) also interacts with residues in TM5 where the inhibitor binds from the transporter's extracellular surface to a KKMIK motif (Lu and Boron 2007). It is currently unknown whether DIDS sterically blocks ionic interaction/permeation through the transporter or blocks ion transport by preventing substrate-induced conformational changes. At more positive membrane voltages, the apparent affinity between DIDS and NBCe1 is increased likely via voltage-dependent changes in transporter conformation (Yamaguchi and Ishikawa 2005; Lu and Boron 2007). DIDS is also able to inhibit the transporter function by interacting with an undermined intracellular binding site (Heyer et al. 1999). The site and mechanism of functional inhibition by other compounds including tenidap (Ducoudret et al. 2001), benzamil (Ducoudret et al. 2001), and S0859 (Ch'en et al. 2008) have not been investigated.

Two of the NBCe1 extracellular loops appear to play a structural/functional role. Located between TM5 and TM6, extracellular loop 3 (EL3) is the largest (83 amino acids) loop in NBCe1, a feature that is shared by other Na^+-driven SLC4 transporters. The loop contains four cysteine residues that are intramolecularly disulfided that form a highly ordered domain-like structure that is glycosylated (Zhu et al. 2015). It has been hypothesized that this region in EL3 may bind a ligand(s) thereby

regulating the function of the transporter analogous to the Cys-loop ligand-gated ion channel superfamily (Zhu et al. 2015). Extracellular loop 4 (EL4) located between TM7 and TM8 can interact with CAIX (Orlowski et al. 2012) and membrane bound CAIV (Alvarez et al. 2003). EL4 has also been reported to be involved in the ion transport stoichiometry and electrogenic properties of NBCe1 (Chen et al. 2011). Whether residues in EL4 are also involved in ion permeation/interaction thereby helping to determine the electrogenic properties of NBCe1 remains to be determined.

As shown in AE1 (Tang et al. 1999; Arakawa et al. 2015) TM8 was reported to also play an important role in NBCe1-A ion permeation (McAlear and Bevensee 2006). In a cysteine scanning study of TM8, the accessibility to pCMBS was altered by the presence of substrate ions and the stilbene inhibitor DNDS (4.4-dinitro-stilbene-2, 2′-disulfonate). Leu^{750} was found to be an important residue involved in ion permeation. It has recently been hypothesized that NBCe1-A-Asp754 interacts with Na^+ and that loss of Arg^{730} in AE1 (replaced by Ile^{803} in TM10 of NBCe1-A) may be necessary to allow Na^+ to permeate to its site of interaction (Reithmeier et al. 2016).

Deda et al. reported a patient with extrarenal K^+ loss due to diarrhea and vomiting causing severe acute hypokalemia, who otherwise had with the same phenotype as other patients with NBCe1 mutations (Deda et al. 2001). The patient had a A799V EL5 pRTA mutation significantly decreasing mutant NBCe1-A function (Horita et al. 2005; Suzuki et al. 2010; Zhu et al. 2010b). Interestingly, the mutant transporter had an associated HCO_3^--independent cation leak conductance (Parker et al. 2012). Given the expression of NBCe1 in skeletal muscle (sarcolemma and possibly t-tubules) during severe acute hypokalemia, a patient with the A799V mutation would be predicted to develop exacerbated muscle weakness (Parker et al. 2012).

The R881C Helix loop 4 (HL4) pRTA mutation is another example where the plasma membrane expression Xenopus oocytes and mammalian cells differs (Horita et al. 2005; Toye et al. 2006; Suzuki et al. 2010; Zhu et al. 2010b). When expressed in Xenopus oocytes, the plasma membrane expression of the R881C mutant transporter is decreased (Horita et al. 2005; Toye et al. 2006). The R881C mutant when expressed in mammalian cells is retained in the ER likely due to misfolding (Zhu et al. 2010a). Importantly, the loss of functional activity appears to be entirely due to abnormal plasma membrane localization (Toye et al. 2006).

Chen et al. using a TM swapping approach reported that TM6 and TM12 in their topology model form a "functional unit" that plays a role in plasma membrane localization (Chen et al. 2012). In these experiments, a chimera was made with TM6 and what was referred to as "TM12" by replacing these TMs with corresponding TMs from electroneutral NBCn1. Although plasma membrane processing was significantly decreased, the interpretation of the data is more complicated since residues from TM12, intracellular loop 6 (IL6), and TM13 were also swapped based on our most studied topological model (Zhu et al. 2015) rather than TM12 alone (Fig. 4). In particular, mixed chimeras per se can potentially fold improperly resulting in ER retention (Fujinaga et al. 2003) and residues in TM12 may play a role in helix packing (Zhu et al. 2010a).

Rather than TM12 alone, the entire C-terminal transmembrane region from TM10–14 appears to play an important role in helix packing and protein folding (Zhu et al. 2010a). The structure of NBCe1 is stabilized by residues clustered on the surface of the protein that form intramolecular hydrogen bonds. The loops connecting TMs11–14 are not exposed to the aqueous but rather are tightly folded in the protein. Met^{858} is bracketed by Pro^{857} and Pro^{858} and is the amino acid residue where TM12 bends abruptly into the lipid bilayer. Lys^{924} in TM13 likely contributes to helix packing by acting as a counter ion. Extracellular loop 7 (EL7; Thr^{926}-Ala^{929}) is minimally exposed to the aqueous media suggesting it is tightly folded in the transmembrane region (Zhu et al. 2010a).

A patient with the first compound heterozygous NBCe1 mutation (R510H/Q913R) was recently reported (Myers et al. 2016). The Q913R mutation was localized intracellularly as was a previously reported Q913C substitution in

TM13 likely to due to protein misfolding (Abuladze et al. 2005). In *Xenopus* oocytes, the mutant protein had normal activity but was associated with a HCO_3^- independent anion-leak whose clinical significance is unclear.

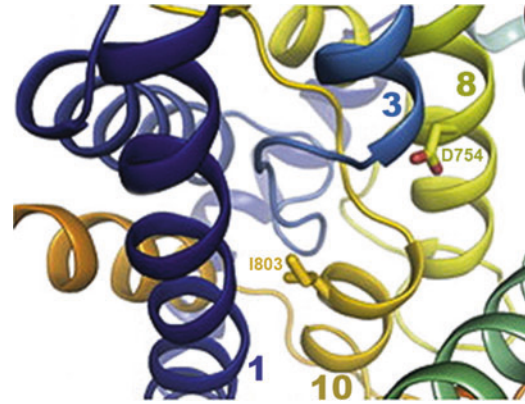
C-Terminal Cytoplasmic Tail

The C-terminal cytoplasmic tails belonging to each monomer in the NBCe1 dimer have stretches of strongly charged amino acids that may form a regulatory motif (Zhu et al. 2010a). Although the role(s) of the C-terminal tail is not precisely defined, there is evidence suggesting that it plays a role in membrane processing and targeting in that a 65 bp-del frame shift mutation causing pRTA truncates the C-terminal tail and in mammalian cells the mutated protein is retained in the ER (although not in *Xenopus* oocytes) (Suzuki et al. 2010). Furthermore, studies in MDCK cells suggest that a ^{1010}QQPFLSP^{1015} motif in the C-terminal tail functions as a basolateral targeting signal (Li et al. 2004).

Comparison of NBCe1 and AE1

The determination of the crystal structure of transmembrane region of the human AE1 dimer allows homology models of other SLC4 transporters based on sequence similarity to be generated (Arakawa et al. 2015; Reithmeier et al. 2016). NBCe1 has the identical predicted 7 + 7 topology as AE1 that is predicted given their sequence similarity. While AE1, AE2, and AE3 have a conserved Glu681 that acts as a H^+ binding site in AE1 required for sulfate transport, in NBCe1, Asp^{754} is the homologous residue that potentially helps coordinate Na^+ (Reithmeier et al. 2016) Fig. 5).

Cysteine scanning mutagenesis studies have detailed additional differences in their topologic features, which likely reflects their functional properties differences and molecular mechanisms of ion transport. These differences are in keeping with the well-characterized differences in the atomic structure of prokaryotic ion exchangers versus Na^+-coupled substrate transporters. The structural differences between NBCe1-A and AE1 likely play an important role in different



NBCe1 Electrogenic Na^+-Coupled HCO_3^-(CO_3^{2-}) Transporter, Fig. 5 Predicted NBCe1-A site for ion coordination (modified from Reithmeier et al. 2016) based on homology with the crystalized transmembrane region of AE1 (Arakawa et al. 2015; Reithmeier et al. 2016)

functional properties and ion substrate affinities. In NBCe1-A, for example, TM1 has been shown to play a role in forming part of the ion translocation pathway and residues in TM1 interact tightly with the cytoplasmic region (Zhu et al. 2009). The AE1-TM1, however, does not appear to be involved in ion permeation and the AE1 N-terminal cytoplasmic region does not appear to form a substrate access tunnel as in NBCe1-A (Shnitsar et al. 2013). In NBCe1-A, the large extracellular loop (EL3) is intradisulfided unlike AE1 and resistant to enzymatic digestion suggesting it is tightly folded and has been hypothesized to play an important a role in ligand binding (Zhu et al. 2015). The transmembrane region of NBCe1-A does not appear to have the reentrant loops that were previously reported in AE1 (Zhu et al. 2003; Zhu et al. 2010a). In NBCe1-A TMs13 and 14 are not involved in ion translocation as has been reported in AE1 (Zhu and Casey 2004; Zhu et al. 2010a).

NBCe1 Regulation

Intrinsic Autoregulatory Features

The structural differences in the N-terminal and/or C-terminal regions of the NBCe1 variants allow variant-specific functional regulation with various

cytoplasmic factors. McAlear et al. first reported that NBCe1-A N-terminus functions as an autostimulatory domain (ASD). How the ASD enhances NBCe1-A function remains unknown (McAlear et al. 2006). NBCe1-D has the same extreme N-terminal sequence and it would be of interest to determine its role as an ASD in this variant. Zhu et al. reported evidence for interaction between the transmembrane region and the NBCe1-A N-terminus (Zhu et al. 2013a). The functional relevance of this finding and whether the autostimulation of the transporter required interaction of specific residues in the extreme N-terminus with the intracellular transmembrane region requires further study. Unlike the NBCe1-A ASD, the identical N-terminus of NBCe1-B and NBCe1-C (and presumably the NBCe1–E variant) functions as an autoinhibitory domain (AID) (McAlear et al. 2006; Lee et al. 2012). The C-terminus in NBCe1-C may also either function as a separate AID or contribute to the N-terminal AID.

IRBIT

The second messenger IRBIT binds to the IP3 receptor (Ando et al. 2003) and when released from the receptor regulates ion channels and transporters (Ando et al. 2014). IRBIT stimulates NBCe1-B and NBCe1-C but not NBCe1- A (Shirakabe et al. 2006). Various residues/regions within the NBCe1-B N-terminus has been reported to contribute to IRBIT binding/regulation including: amino acids 1–18 and 37–62 (Shirakabe et al. 2006); an RRR motif (42–44) in the positively charged 37–65 region (Hong et al. 2013); T49 (Hong et al. 2013); and residues 4–16 (Lee et al. 2012; Seki et al. 2008) initially suggested that IRBIT functions by masking the NBCe1-B AID inhibition. Protein phosphatase-1 (PP-1) binds to IRBIT and blocks its stimulation of NBCe1-B function (Devogelaere et al. 2007). Using a mutated IRBIT that does not bind PP-1 (Lee et al. 2012) showed that the function of NBCe1-B coexpressed with the mutated IRBIT was greater than an N- terminally truncated NBCe1-B mutant lacking the N-terminal AID (Lee et al. 2012) suggesting that a mechanism in addition to masking the AID must underlie the effect of IRBIT.

WNK/SPAK

The WNK (with-no-lysine kinase)/SPAK (STE20/SPS1-related proline/alanine-rich kinase) pathway (Yang et al. 2011) inhibits NBCe1-B (Yang et al. 2011; Hong et al. 2013). WNK does not act as a kinase per se but binds to SPAK which phosphorylates Ser65 decreasing the plasma membrane expression of the transporter (Hong et al. 2013). The recruitment of protein phosphatase 1 (PP-1) by IRBIT counters the WNK/SPAK inhibition of the transporter (Yang et al. 2011; Lee et al. 2012; Hong et al. 2013). The phosphorylation of NBCe1-B-Thr^{49} that has previously been show to increase transporter activity (Gross et al. 2003) is required for both IRBIT and WNK/SPAK pathway regulation of the transporter (Hong et al. 2013). IRBIT and PIP_2 (see below) may compete for the same binding N-terminal site (positively charged N terminal region 37–65) (Hong et al. 2013). Whether IRBIT modulates NBCe1-B function via a change in plasma membrane expression likely depends on the cell type and specifically the activity of the endogenous WNK/SPAK pathway (Shirakabe et al. 2006; Lee et al. 2012; Yang et al. 2011).

Additional Factors Affecting the NBCe1 Phosphorylation State: AII, PKC, PKA/cAMP, ATP, and Src Kinase

The phosphorylation state of NBCe1 affects both its functional properties and plasma membrane localization. ANG II has a dose-dependent biphasic effect on NBCe1 function (Coppola and Frömter 1994a, b; Horita et al. 2002; Zheng et al. 2003; Perry et al. 2006). Mediated through AT_{1B} receptors, the inhibition of NBCe1-A via ANG II is mediated by Ca^{2+}-insensitive PKCε leading to decreased NBCe1-A plasma membrane localization (Perry et al. 2006, 2007). In experiments in *Xenopus* oocytes expressing NBCe1-A, Ca^{2+} shifts the ion transport stoichiometry from 1:2 to 1:3 that may be mediated by a change in PKC-dependent phosphorylation of the transporter (Muller-Berger et al. 2001). It has also been reported that PKA-dependent phosphorylation of NBCe1-A-Ser^{982} shifts the ion transport stoichiometry in the opposite direction from 1:3 to 1:2 (Gross et al. 2001c). In the extreme NBCe1-B N-terminus, cAMP-induced phosphorylation of

Thr^{49} increases transport without altering the ion transport stoichiometry (Gross et al. 2003). In addition, cAMP has also been reported to increase intestinal NBCe1-B function in part because of an increase in plasma membrane localization (Bachmann et al. 2003; Yu et al. 2009). A decrease in plasma membrane localization of both NBCe1-A and NBCe1-B is induced by both PKCs (PKCαβγ) and a novel PKCδ that participate in the constitutive and stimulated (carbachol) mediated endocytosis of the transporters (Perry et al. 2009) providing an additional modulatory pathway in salivary duct, ileum, and colon (Bartolo et al. 2009; Perry et al. 2009; Yu et al. 2009). In addition to phosphorylation via PKCs, it has been suggested that ATP can phosphorylate NBCe1-A via an as yet undefined protein kinase increasing transporter function (Muller-Berger et al. 2001). In submandibular glands, acute intracellular acidification stimulates NBCe1-B phosphorylation via Src kinase that results in increased plasma membrane localization of the transporter (Namkoong et al. 2015).

PIP_2; PIPKIα

PIP_2 activation of NBCe1-B and NBCe1-C involves a staurosporine-sensitive kinase (Thornell et al. 2012; Thornell and Bevensee 2015). PIP_2 also prevents the rundown of NBCe1-A in oocyte macropatches (and potentially NBCe1-D) (Wu et al. 2009). Hong et al. (2013) suggested that PIP_2 and IRBIT compete for a polycationic site arginines (42–44) in the NBCe1-B N-terminus. The exact PIP_2 binding site(s) in NBCe1 is unknown and there are additional cationic stretches in common to NBCe1 transporters where PIP_2 may bind.

It has recently been shown that IRBIT forms a signaling complex with members of the PIPK family that includes PIPK type Iα (PIPKIα) and type IIα (PIPKIIα) (Ando et al. 2015). Phosphatidylinositol 4-phosphate, Mg^{2+}, and/or ATP interfere with this interaction and binding experiments showed that IRBIT, PIPKIα, and NBCe1-B form a tertiary complex. The complex with PIPKIα is hypothesized to modulate the activity of NBCe1-B through changes in the local production of PIP_2.

Calcium/IP3

In *Xenopus* oocytes PIP_2 stimulation of NBCe1-B and NBCe1-C requires its hydrolysis to IP3 (Thornell et al. 2012). The function of NBCe1-B and NBCe1-C and not NBCe1-A is increased by a rise in intracellular Ca^{2+} (Thornell et al. 2012) that may be mediated through the N-terminal AID. An increase in intracellular Ca^{2+} by activating endogenous oocyte Gq-coupled receptors increases NBCe1-B expression and NBCe1-C function (Thornell et al. 2012). An increase in bath calcium in oocyte macropatch membranes (intracellular surface) shifted the charge transport stoichiometry from 1:2 to 1:3 (Muller-Berger et al. 2001).

Magnesium

Intracellular Mg^{2+} can inhibit of NBCe1-B function (Yamaguchi and Ishikawa 2008, 2012) and potentially NBCe1-C/−E. The effect may be mediated by the N-terminal AID, however, it is currently unknown whether Mg^{2+} and IRBIT compete for a common binding site or act independently. When expressed in *Xenopus* oocytes, Mg^{2+} induced NBCe1-A functional rundown via a mechanism that has been hypothesized to potentially be mediated by a Mg^{2+}-dependent phosphatase (5′-lipid phosphatase) that involved the dephosphorylation of PIP_2 to PIP (Wu et al. 2009). Mg^{2+} may also block the interaction of PIP_2 with the transporter which is compatible with the finding that polyvalent cations decrease the inhibitory effect of Mg^{2+} on NBCe1-A and NBCe1-B function (Yamaguchi and Ishikawa 2008; Yamaguchi and Ishikawa 2012; Wu et al. 2009). During an ischemic insult, inhibition of NBCe1 function by an elevation of intracellular Mg^{2+} may reduce cellular dysfunction perhaps via a change in intracellular Na^+ and/or pH (Wu et al. 2009).

Chloride

Intracellular Cl^- regulates NBCe1-B transport via two GXXXP-containing sites and regulation of NBCe1-A is mediated via single cryptic GXXXP motif (Shcheynikov et al. 2015). Under basal conditions, NBCe1-B is inhibited by a high Cl^- concentration via interaction with the low affinity GXXXP site, and IRBIT activation of

NBCe1-B unmasks a second high affinity Cl^- interacting GXXXP site. Changes in Cl^- concentration between 5 and 140 mM have no effect on NBCe1-A activity and deletion of residues 29–41 unmasked the cryptic GXXXP site that mediates the Cl^--dependent inhibition of NBCe1-A activity.

Carbonic Anhydrase

CA inhibition was shown to reduce the activity of NBCe1-A operating with 1:3 but not a 1:2 charge transport stoichiometry (Gross et al. 2002). In pull-down studies, the magnitude of acetazolamide mediated inhibition of NBCe1-A was shown to vary with the degree of CAII/NBCe1-A binding. C-terminal NBCe1-A ^{958}LDDV^{961} and ^{986}DNND^{989} motifs were proposed to be part of a single binding site (Pushkin et al. 2004). Using isothermal titration calorimetry, Gross et al. proposed that NBCe1 and cytoplasmic CAII interact at a high affinity biding site (160 nM) to form a transport metabolon (Gross et al. 2002). Alvarez et al. showed that expression of a catalytically impaired CAII mutant resulted decreased NBCe1-B function (Alvarez et al. 2003). Further studies in Xenopus oocyte expression systems provided additional support for interaction between CAII and NBCe1 (Pushkin et al. 2004; Becker and Deitmer 2007). Membrane-associated CAIV and CAIX were subsequently shown to interact with the extracellular surface of NBCe1-B at EL4 (dependent on Gly767) (Alvarez et al. 2003; Orlowski et al. 2012). Other studies were unable to demonstrate a functional interaction between NBCe1 and CAII (Lu et al. 2006; Piermarini et al. 2007; Yamada et al. 2011). Differences in techniques/preparations used among the various assays employed in these studies may account for the various findings reported. Mice with targeted disruption of CAII and patients' loss of function mutations in CAII do not have a severe proximal tubule bicarbonate wasting phenotype (Sly et al. 1985; Lewis et al. 1988). The finding that loss of CAII function results in a milder phenotype than loss of NBCe1 function in patients suggests that even if a functional coupling between CA proteins and NBCe1 exists in vivo, the interaction does not appear to be clinically significant. Moreover, patients with CAIV mutations do not have pRTA but instead have an ocular phenotype due to retinitis pigmentosa (RP17) (Rebello et al. 2004). Schueler reexamined the interaction between CA enzymes and NBCe1-A in *Xenopus* oocytes and showed that CAI, CAII, and CAIII stimulate NBCe1-A function that was mediated through carbonic anhydrase enzymatic activity and not intramolecular proton shuttling (Schueler et al. 2011).

STCH

In NBCe1-B, sp70-like stress 70 protein chaperone STCH interacts with residues 96–440 (distal to IRBIT interaction) inducing a significant increase in plasma membrane localization (Bae et al. 2013). Whether STCH also increases the plasma membrane expression of other variants is not known. Increased STCH-induced NBCe1-B plasma membrane localization may represent a novel regulatory pathway that certain cells possess to prevent cellular dysfunction during metabolic acidosis by increasing their ability to regulate intracellular pH more efficiently (Bae et al. 2013).

Hormonal Factors and Systemic Blood Pressure

PTH, dopamine, norepinephrine, and potentially changes in systemic blood pressure have been shown to modulate the expression of NBCe1. Specifically, PTH decreases NBCe1 activity in rats possibly via cAMP, whereas there is no effect on NBCe1 function in rabbits (Sasaki and Marumo 1991). Dopamine decreases NBCe1-A activity in rat and rabbit proximal tubules (Kunimi et al. 2000). In rats, chronic infusion of noradrenaline increases NBCe1 expression (Sonalker et al. 2008). In the SHR rat, NBCe1 protein expression is increased approximately twofold in comparison to control WKY rats (Sonalker et al. 2004); however, the causal relationships in this model are unknown.

Blood Chemistry Changes

Both $NaHCO_3$ and NaCl administration decrease the expression of NHE3 and NBCe1-A in the proximal tubule (Amlal et al. 2001). The change in NBCe1-A expression provides a potential

mechanism for ameliorating metabolic alkalosis by increasing renal bicarbonate excretion, and volume overload by increasing renal Na^+ excretion. K^+ depletion that is associated with metabolic alkalosis is accompanied by an increase in proximal tubule bicarbonate reabsorption (Roberts et al. 1955; Rector et al. 1964; Capasso et al. 1987). The latter finding is potentially due to increased proximal tubule expression of NBCe1-A (Amlal et al. 2000).

Miscellaneous Systemic Diseases

In a model of renal transplant rejection in rats, the expression of NBCe1-A is increased (Velic et al. 2004); however, NBCe1-A expression is decreased by the calcineurin inhibitor FK506 (Mohebbi et al. 2009). In lithium-induced distal renal tubular acidosis (dRTA), NBCe1-A expression is upregulated perhaps as a compensatory mechanism that increases proximal tubule bicarbonate reabsorption (Kim et al. 2003). In a ureteral obstruction model of hyperkalemic dRTA, NBCe1-A expression in the proximal tubule is decreased (Wang et al. 2008). Hypothyroidism in humans can be associated with incomplete dRTA, and in a hypothyroid model in rats, decreased NBCe1 abundance has been reported (Mohebbi et al. 2007). Concomitant NH_4Cl loading in hypothyroid rats increases NBCe1 expression (Mohebbi et al. 2007).

Summary

The present review summarizes our current understanding of the structure-function properties and regulation of the electrogenic sodium bicarbonate cotransporter NBCe1. NBCe1 encoded by the *SLC4A4* gene belongs to the SLC4 gene transporter family which share amino acid sequence homology and in general couple the transport of HCO_3^- (and/or CO_3^{2-}) to Na^+ and/or Cl^-. The mammalian *SLC4A4* gene encodes five NBCe1 variants (NBCe1-A-E) that share an identical plasma membrane region but differ in the sequence of their cytoplasmic N- and C-termini. Mutations in the transporter cause the kidney disease proximal renal tubular acidosis (pRTA) with associated neurologic and ocular abnormalities. These mutations prevent both normal plasma membrane targeting and/or alter the functional properties of NBCe1. This review highlights the structure-function properties and regulation of NBCe1, and its role in cellular acid-base physiology in health and disease.

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NBCn1

▶ Na^+/HCO_3^- Cotransporter NBCn1

NCAM

► NCAM1

N-CAM

► NCAM1

NCAM1

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Synonyms

CD56; D2; N-CAM; NCAM

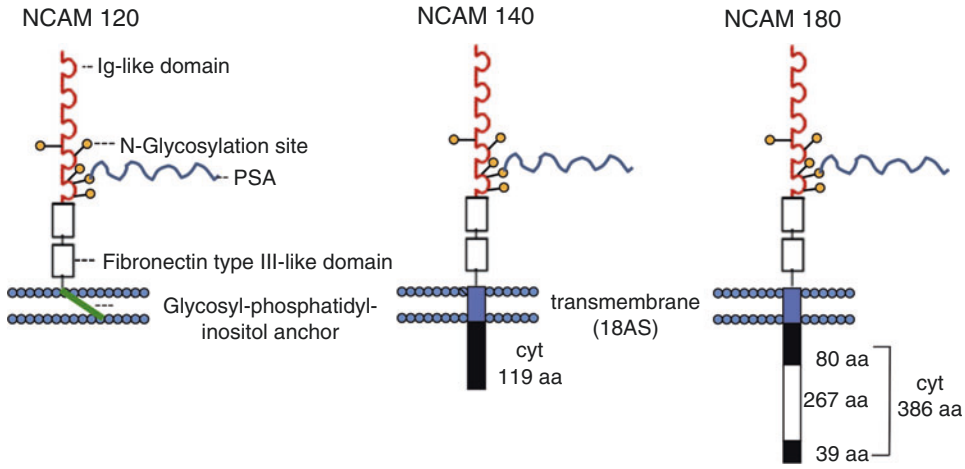
Historical Background

Cell–cell interactions via cell adhesion are the basis for the evolution of all multicellular organisms. The first experiments to understand cell adhesion were performed at the beginning of the last century by Wilson (1907), who dissociated sponges and allowed them to fuse and to reconstitute. Fifty years later, Townes and Holtfreter (1955) demonstrated that dissociated cells from amphibian embryos adhere to form random aggregates of their origin, with ectoderm forming an outer surface layer, endoderm forming a compact central ball, and mesoderm producing a loose array of cells. In 1963, Roger Sperry (1963) proposed that different cells bear distinct cell surface proteins that serve as markers or tags. One of these markers, the Neural Cell Adhesion Molecule (NCAM) was first described in 1974 by Elizabeth Bock (Jacque et al. 1974) and designated as D2 antigen. Three years later, the group of Edelman (Thiery et al. 1977) approved that NCAM mediates cell–cell adhesion and established it as one of the first cell adhesion molecules. When NCAM was sequenced and cloned during the mid-1980s, it became obvious that NCAM belongs to the immunoglobulin superfamily (Barthels et al. 1987) and that NCAM is not only responsible for static adhesion, but also transmits signals across the cell membrane. With the help of transgenic knockout techniques, Cremer and colleagues could demonstrate 1994 (Cremer et al. 1994) that NCAM is involved in learning and memory.

Structure and Function

NCAM is a glycoprotein belonging to the immunoglobulin superfamily. All members of this protein superfamily have in common the presence of at least one immunoglobulin-like domain, which received their name from immunoglobulins (antibodies). There are more than 750 proteins known with immunoglobulin-like domains. An immunoglobulin-like domain usually consists of 70–110 amino acids with a defined secondary structure including one disulfide bridge. NCAM consists of five immunoglobulin domains in its extracellular domain. Furthermore NCAM has two membrane proximal fibronectin type III (F3) homologous repeats. There exist three major isoforms of NCAM, which are generated by alternative splicing from one single gene (Barbas et al. 1988). The isoforms are named according their apparent molecular weight as NCAM120 (gylcosylphosphatidyl inositol anchor = GPI-anchored) or NCAM140 and NCAM180 (transmembrane-anchored). NCAM140 and NCAM180 differ in the intracellular domain in that NCAM180 has an additional insert of 267 amino acids (see Fig. 1). Although identified in the nervous system, NCAM is expressed in

Please note that there exist a close relative to NCAM, the Neural Cell Adhesion Molecule 2; also called OCAM or RNCAM. The overall similarity to NCAM is 45%. However, this entry is a summary only on the Neural Cell Adhesion Molecule.



NCAM1, Fig. 1 Schematic representation of the three major isoforms of NCAM

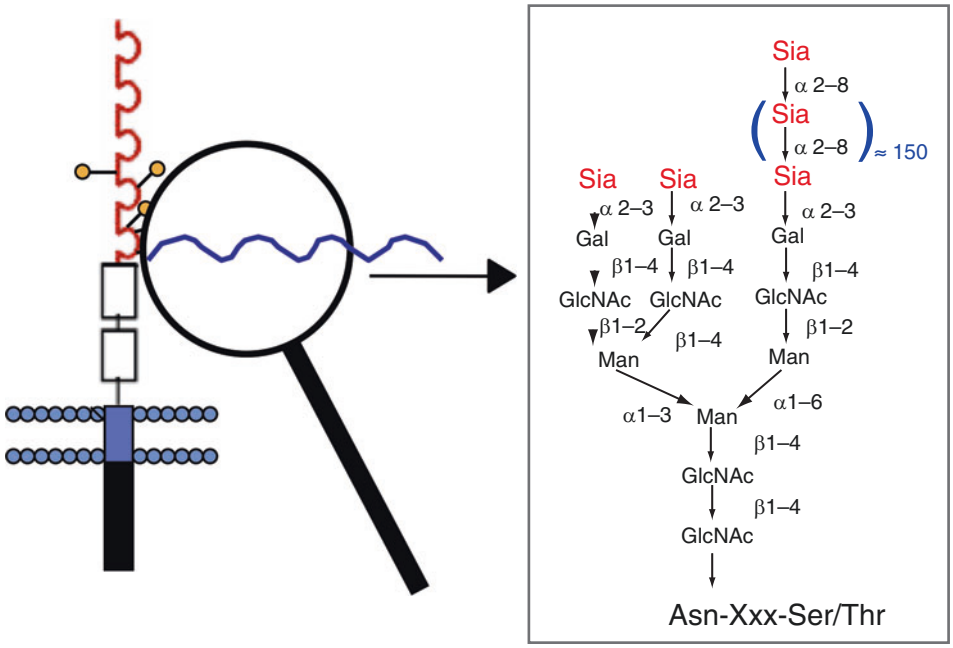
many other cell types or tissues, such as muscle cells or immune cells. This is underlined by the fact that NCAM corresponds to CD56 and is a marker of natural killer cells.

NCAM mediates preferentially homophilic NCAM–NCAM interactions, which are Ca^{2+}-independent. The homophilic NCAM–NCAM interactions mediate adhesion between neurons and neurons, neurons and glia cells, and between glia cells and glia cells. NCAM140 is known to be responsible for the fasciculation of axons or neurites and for promotion of axonal regeneration after injury. NCAM-deficient mice have defects in learning and memory and have a smaller brain compared to wild-type animals. Furthermore, the nervous system–specific NCAM180 is thought to stabilize cell–cell contacts at synapses. A recent model of NCAM–NCAM interaction suggests that two NCAMs form dimers on the cell curface (cis-interaction) and that these dimers bind to existing dimers on opposing cells (trans-interaction) via their Ig-domains 2 and 3 in anti-parallel orientation ("zipper"-like) (Soroka et al. 2003).

Posttranslational Modification

NCAM is notable for several posttranslational modifications. Like many other proteins involved in signal transduction, NCAM was demonstrated to be phosphorylated. NCAM contains numerous (depending on its isoform) potential phosphorylation sites on Serine or Threonine residues but only one on a Thyrosine residue. In addition, NCAM has five N-glycosylation sites and nearly 30% of its apparent molecular weight is represented by glycans. It is of special interest that NCAM bears a very unique type of glycosylation, namely, polysialylation (Finne et al. 1983). Polysialylation is characterized by the presence of up to 150 monomers of sialic acid on the outer chains of the N-glycans of the fifth immunoglobulin-like domain (see Fig. 2). Polysialylation on NCAM represents more than 95% of all polysialylation of a specific organism. The addition of polysialic acids introduce one negative charge per sialic acid and alter dramatically the function of NCAM. Polysialylated NCAM is much less adhesive compared to non-polysialylated NCAM. The expression of polysialic acid is strongly regulated. It is high during development and drops during lifetime. Adult mammals (including humans) express high levels of polysialylated NCAM only at sites of plasticity, such as hippocampus, the place of learning and memory.

Please note that there are several and sometimes confusing abbreviations for polysialic acid or polysialylated NCAM used in the literature (PSA = polysialic acid; E-NCAM = embryonic NCAM; polySia = polysialic acid).



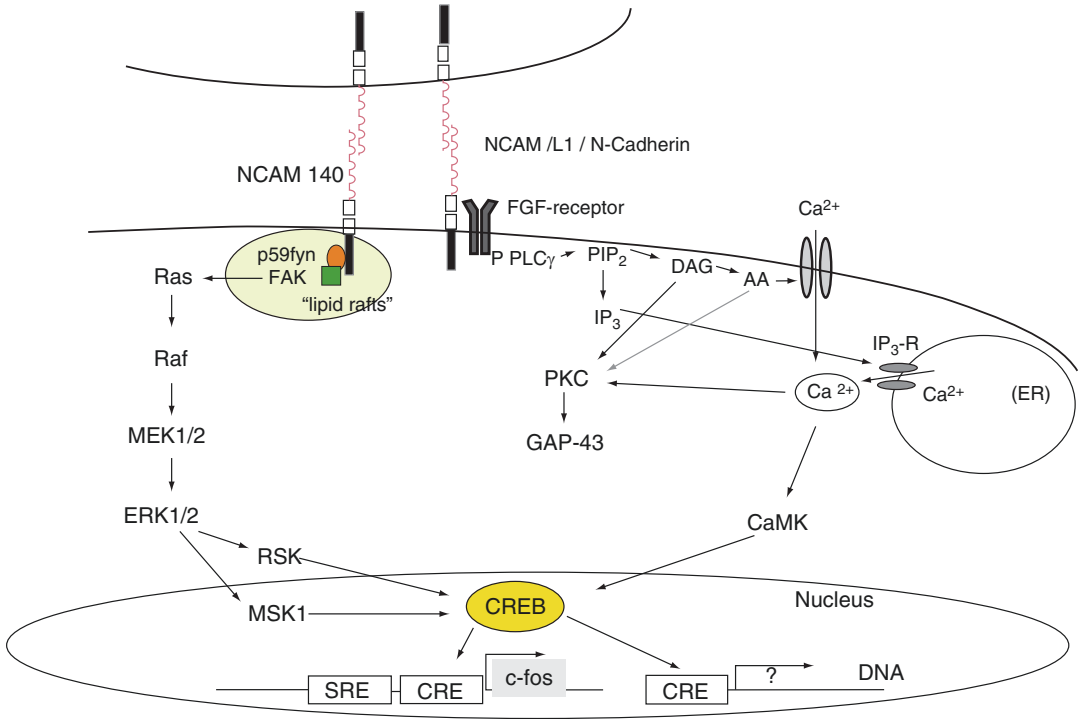
NCAM1, Fig. 2 Polysialylation of NCAM. Note that polysialylation of NCAM results in the introduction of more than 100 negatively charged sialic acids on a classical Aspargine-linked glycan structure

Signaling

The last two decades of research on NCAM-mediated signal transduction focuses mainly on NCAM-mediated promotion of neurite outgrowth, which will be discussed in the following. One central pathway of the NCAM-mediated signal transduction is the mitogen-activated protein kinase pathway (MAPK pathway). The MAPK pathway can be activated by two mechanisms: First, via the Fibroblast Growth Factor Receptor (FGF-receptor) (Doherty and Walsh 1996) and second via non-receptor kinases such as FAK and Fyn (Beggs et al. 1997).

The Mitogen-Activated Protein Kinase Pathway

It has been demonstrated that NCAM interacts directly with the FGF-receptor on one cell membrane (cis-interaction) via the F3 modules 1 and 2 (Kiselyov et al. 2003). NCAM binding is capable of activating the FGF-receptor upon NCAM–NCAM interaction. The FGF-receptor activation leads to recruitment of the adaptor proteins Shc and Grb-2, which then activate Ras as key enzyme of the MAPK pathway. Final target of the MAPK pathway is the transcription factor ▶ CREB (Jessen et al. 2001), which activates transcription of genes, which are responsible for neurite outgrowth. The NCAM-mediated FGF-receptor activation and neurite outgrowth can be inhibited by ATP, which could be explained by an overlapping ATP and FGF-receptor binding sites in the second F3 module of NCAM (Skladchikova et al. 1999). However, the also close polysialylation at the Ig module 5 of NCAM is essential for NCAM-dependent neurite outgrowth (Doherty et al. 1990). Another possibility to activate the MAPK pathway is via the recruitment of the non-receptor kinases FAK and Fyn. It has been demonstrated in several studies that NCAM is also associated with the receptor protein tyrosine phosphatase-alpha (RPTP-alpha). NCAM–NACM interaction leads to the activation of RPTP-alpha and to recruitment of Fyn and FAK, which then are responsible for further activation of the MAPK pathway (see Fig. 3).



NCAM1, Fig. 3 Simplified scheme of the NCAM-mediated signal transduction (For details see text or for review see Maness and Schachner (2007))

Phospholipase C and Calcium

NCAM-mediated signaling has been demonstrated to involve an increase in intracellular Ca^{2+}-concentration (Kolkova et al. 2000). Several studies suggested that phospholipase Cγ (PLCγ) is responsible for the NCAM-mediated increase in Ca^{2+}-concentration. Upon NCAM–NCAM interaction, PLCγ cleaves PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG). ▶ IP_3 Receptors binds to its intracellular receptor at the ER-membrane and releases Ca^{2+} from the ER store. Furthermore ▶ arachidonic acid (AA) might be released from DAG by the DAG lipase, which further activates Ca^{2+}-channels at the plasma membrane. All this leads to an increase of intracellular Ca^{2+}. The increased Ca^{2+} binds to calmodulin and this leads to the activation of the Ca^{2+}-calmodulin-dependent protein kinase II. Ca^{2+}-calmodulin-dependent protein kinase II phosphorylates several target proteins, which are involved in neurite outgrowth (see Fig. 3).

Cyclic Adenosine Monophosphate and Cyclic Guanosine Monophosphate

The involvement of the two second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in NCAM signaling has been demonstrated in several studies by the use of specific inhibitors (Shimomura et al. 1998). However, the exact role of the respective heterotrimeric G-proteins (in the case of cAMP) or nitric oxid synthases (in the case of cGMP) is not clear yet.

Summary

NCAM may transmit signals into the cell by several mechanisms. This is, like in many other cases, a complex network of signal transduction pathways. The MAPK pathway seems to be very crucial for NCAM-mediated signal transduction. However, little is known about relative roles of the

individual players and further research is necessary to elucidate the total network of NCAM signaling.

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N

NCDase

► Neutral Ceramidase

(Nck-Associated Protein-4 = SOCS7)

► SOCS

NCKX

► SLC24A Family (K^+-Dependent Na^+-Ca^{2+} Exchanger, NCKX)

NCLK

► CDK5

NcoA-1

► Steroid Receptor Coactivator Family

NCoA-2

► Steroid Receptor Coactivator Family

NDFIP1 and NDFIP2

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Synonyms

Ndfip1:
Bypass Sod1p Defects (Bsd2p) (*Saccharomyces cerevisiae*); Nedd4 family-interacting protein 1 (Ndfip1); Nedd4 family-interacting protein (Ndfip), dNdfip, DmeI, CG32177 (*Drosophila* Ndfip1); NEDD4 WW domain-binding protein 5 (human); Nedd4 WW domain-binding protein 5 (N4WBP5)

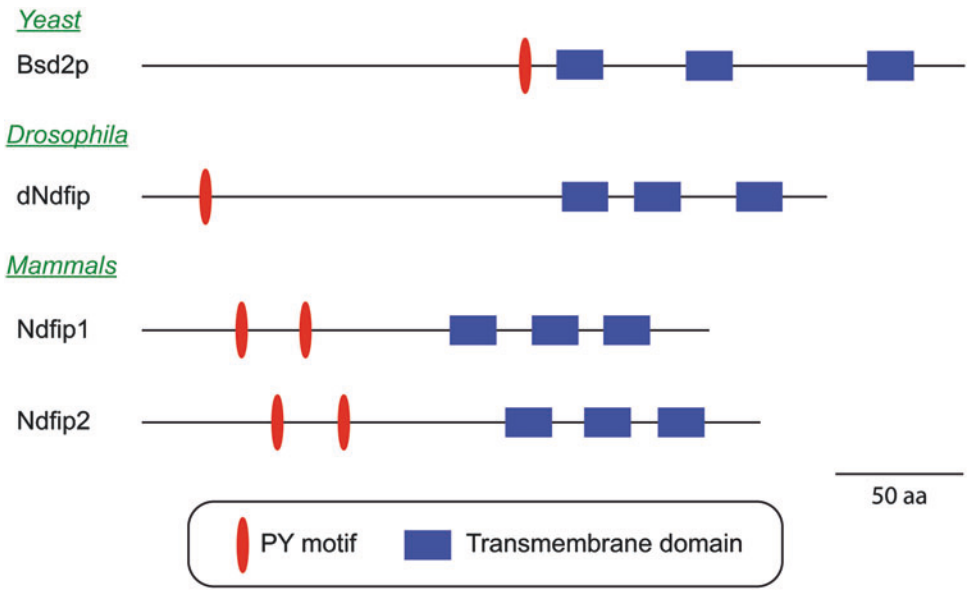
Ndfip2:
KIAA1165; Nedd4 family-interacting protein 2 (Ndfip2); NEDD4 WW domain-binding protein 5A (N4WBP5A)

Historical Background

Nedd4 family-interacting protein 1 (Ndfip1) was first identified in a far-western screen searching for proteins that interact with the WW domains of the Nedd4 ubiquitin ligase (Jolliffe et al. 2000). It was initially named Nedd4 WW domain-binding protein 5 (N4WBP5). Following this discovery, a closely related mammalian protein was identified (named N4WBP5A, now called Ndfip2) and further characterized as belonging to a small group of evolutionarily conserved proteins (Harvey et al. 2002), with a single homologue in yeast [Bsd2p (Hettema et al., 2004)] and *Drosophila melanogaster* [dNdfip (Dalton et al. 2011); Fig. 1]. Both Ndfip1 and 2 are ubiquitously expressed and interact with several members of the Nedd4 family of ubiquitin ligases (Harvey et al. 2002; Shearwin-Whyatt et al. 2004). Ndfip1 mostly localizes to the Golgi, whereas Ndfip2 localizes to late endosomes and Golgi (Harvey et al. 2002; Shearwin-Whyatt et al. 2004). Ndfips all contain three transmembrane domains, plus several proline-rich motifs (PY motifs), which enable the binding to WW domain-containing proteins (Fig. 1). Specifically, Ndfips are best known for their ability to bind to the Nedd4 family of ubiquitin ligases, thereby regulating their subcellular localization (Beck et al. 2015) and activation (Mund and Pelham 2009), as well as functioning as adaptors to mediate the interaction between the ligase and non-PY-containing substrates (Foot et al. 2008). This latter function facilitates the ubiquitination of substrates and promoting their appropriate trafficking within the cell.

Ndfips and Immunity

Not long after their initial characterization, an Ndfip1 knockout mouse was generated (Oliver et al. 2006), which paved the way for the discovery of the involvement of Ndfip1 in a number of novel mechanisms. These mice developed a severe inflammatory phenotype of the skin and lung, reminiscent of that of the Itch knockout mouse (Perry et al. 1998). Upon further analysis, it was determined that these mice had a complex dysregulation of T cell activation. Ndfip1 regulates the activity of the Nedd4 family ubiquitin ligase Itch in T cells, and thus in the absence of Ndfip1, Itch remains in an inactive state, preventing the downregulation of JunB (Fig. 2). This accumulation of JunB in activated T cells promotes T helper (Th) 2 maturation and proliferation, leading to inflammatory disease (Oliver et al. 2006). These mice also have fewer inducible regulatory T cells due to the accumulation of JunB, resulting in the production of IL-4 and

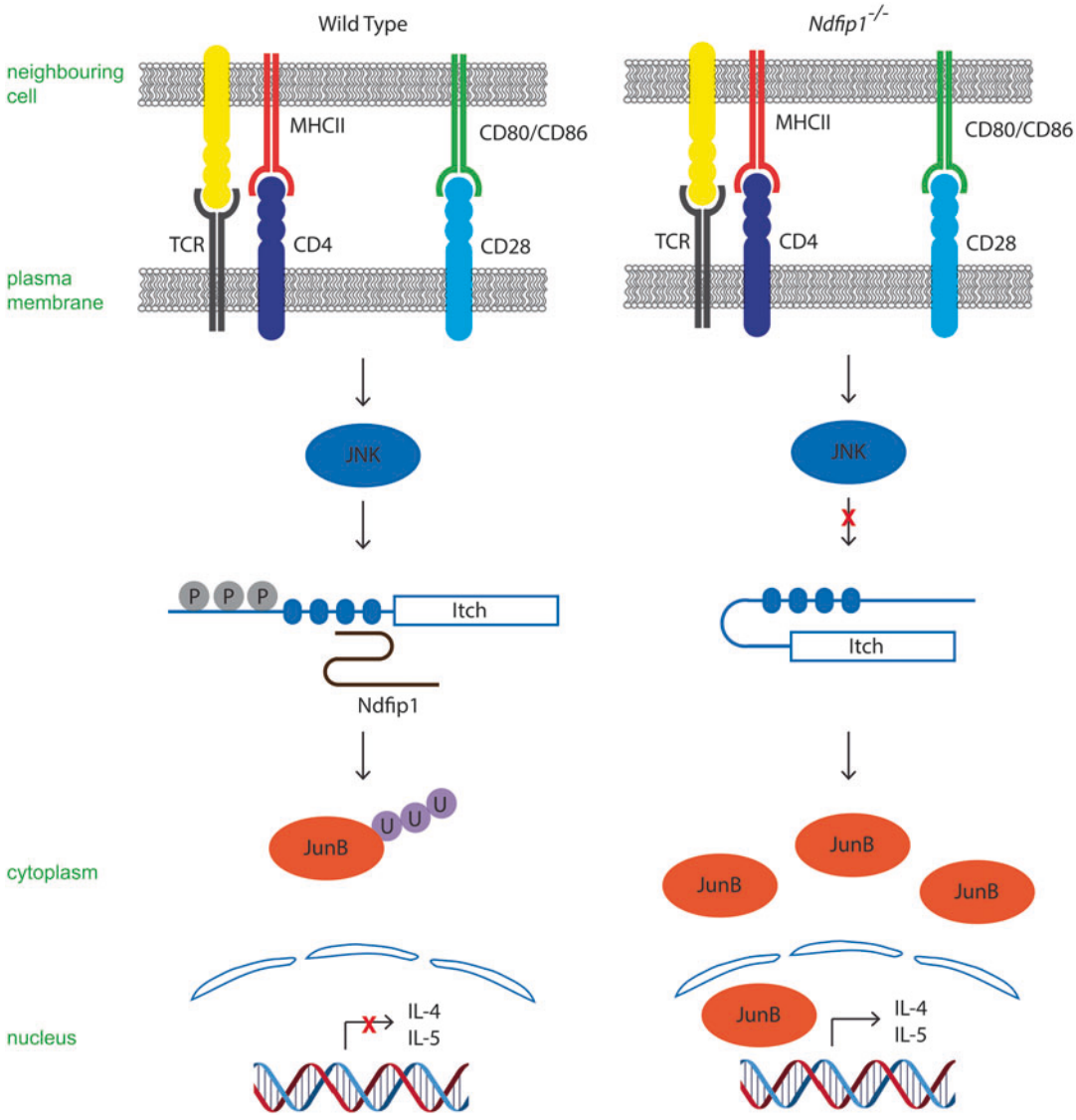


NDFIP1 and NDFIP2, Fig. 1 Primary structure of Ndfip family members is evolutionarily conserved. All Ndfip family members contain three transmembrane domains and either one (yeast, *Drosophila*) or two (mammals) PY motifs to enable binding to WW domain-containing substrates. There are two homologues in mammals (Ndfip1 and Ndfip2) but only one in *Drosophila* and yeast

IL-5 and the suppression of Foxp3 (Oliver et al. 2006; Beal et al. 2012). Ndfip1 has further been shown to be required for controlling the regulatory circuit that controls T cell activation through regulating IL-2 and CD25 levels to prevent T cells from becoming activated in the absence of costimulation by CD28 (Ramos-Hernandez et al. 2013). Additionally, Ndfip1 controls erroneous T cell activation by causing CD4+ T cells to exit the cell cycle prematurely and preventing differentiation into IL-4-producing cells (Altin et al. 2014). Ndfip1 has also been implicated in RIG-I-like receptor-mediated immune signaling, by controlling the levels of another Nedd4 family member Smurf1, which in turn regulates the degradation of the mitochondrial antiviral signaling protein MAVS (Wang et al. 2012). Recently, Ndfip1, along with the ubiquitin ligase Nedd4-2, has been shown to be involved as a negative regulatory axis in IgE-dependent mast cell activation (Yip et al. 2016). Combined, these studies indicate an essential role of Ndfip1 in immune cell function.

Ndfips and Brain Injury

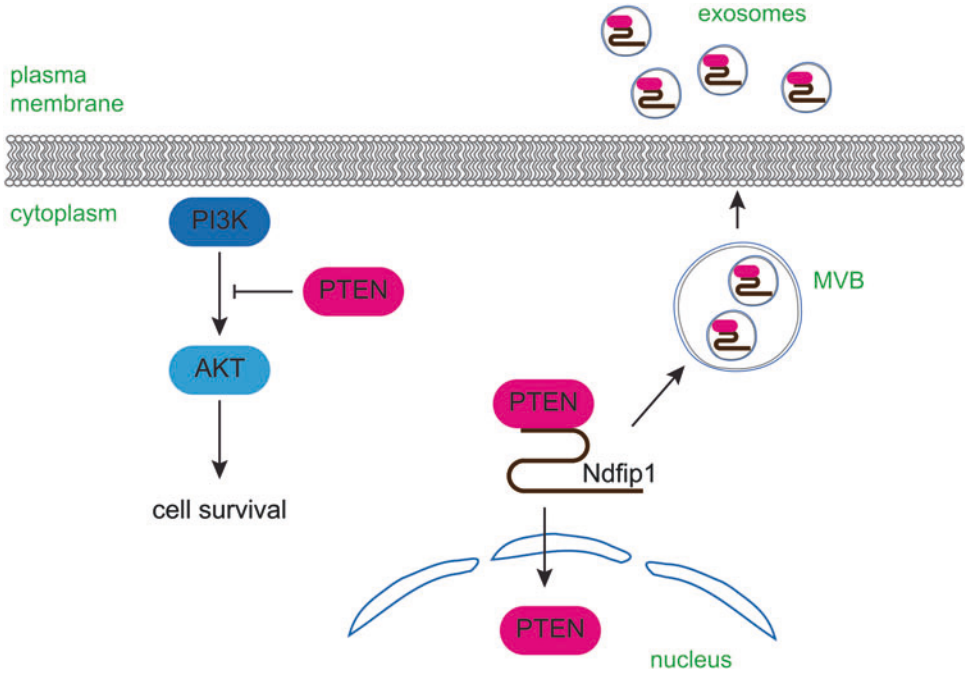
As well as its essential role in controlling the immune system, Ndfip1 has also been implicated in neuronal cell survival following traumatic brain injury. During an initial screen for genes expressed in the cortex following injury, Ndfip1 was found to be upregulated in the cells surrounding the site of injury, and this was confirmed in vitro, where overexpression of Ndfip1-protected cultured cortical neurons from growth factor starvation induced cell death (Sang et al. 2006). This has been attributed in part to the role of Ndfip1 in the nuclear import of PTEN, which is required for the activation of Akt and the promotion of cell survival signaling pathways (Howitt et al. 2012; Goh et al. 2014; Mund and Pelham 2010) (Fig. 3). Ndfip1 also controls PTEN levels in the cell by targeting it to a subset of extracellular vesicles called exosomes, which are released from the multivesicular body (MVB) into the extracellular space (Putz et al. 2012).



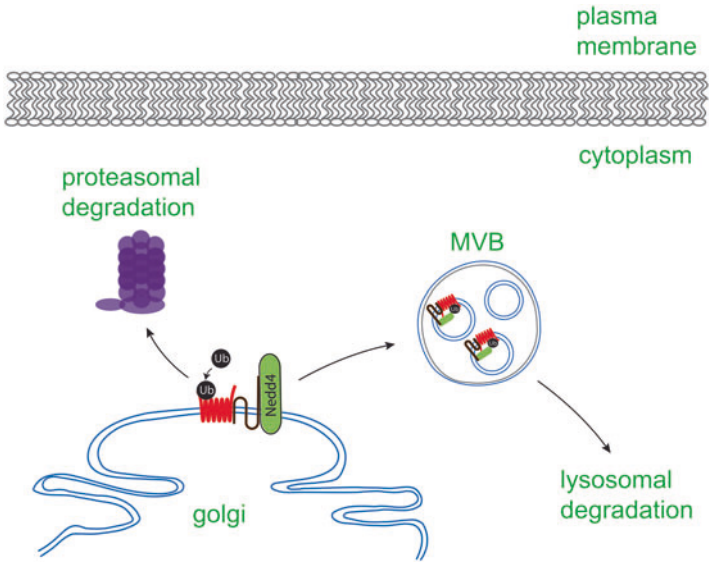
NDFIP1 and NDFIP2, Fig. 2 Ndfip1 is required for Itch-mediated regulation of JunB. Under normal conditions, activation of T cells leads to the binding of Itch to Ndfip1, which changes Itch conformation and allows phosphorylation by JNK. Phosphorylated Itch can then ubiquitinate JunB, leading to its degradation. In *Ndfip1*$^{-/-}$ mice, Itch remains in a closed conformation, inhibiting phosphorylation by JNK and therefore preventing the ubiquitination of JunB. The resulting accumulation of JunB causes an increase in specific cytokine expression, resulting in a hyperactivated T cell phenotype

Ndfips and Metabolism

In their role as adaptor proteins for Nedd4 family members, Ndfips control different metabolic processes to maintain body homeostasis. This was first discovered in yeast, where Bsd2p controls the divalent metal ion transporter Smf1p through the recruitment of the Nedd4 ubiquitin ligase Rsp5p (Hettema et al. 2004; Liu and Culotta 1999). This regulatory mechanism is conserved in mammals where both Ndfip1 and Ndfip2 control the regulation of the Smf1 homologue DMT1 via the recruitment of Nedd4 family members WWP2 and Nedd4-2, which is important for



NDFIP1 and NDFIP2, Fig. 3 Ndfip1 regulates PTEN nuclear import to promote neuronal survival following traumatic brain injury. PTEN normally acts by inhibiting the activation of Akt by PI3K. Following traumatic brain injury, Ndfip1 is upregulated to bind to PTEN and shuttle it into the nucleus where it is sequestered away from PI3K, allowing Akt to be activated and thus triggering cell survival pathways. Alternatively, Ndfip1 may target PTEN into MVBs for release in exosomes



NDFIP1 and NDFIP2, Fig. 4 Ndfips act as adaptors to mediate the ubiquitination of transmembrane proteins. For target proteins that do not contain PY motifs (such as DMT1), Ndfips act as adaptors to recruit the Nedd4 family of ubiquitin ligases (shown as Nedd4) to enable ubiquitination and subsequent degradation either by the proteasome or through the endosomal pathway to the lysosome

maintaining iron homeostasis in the mouse (Foot et al. 2008, 2011, 2016; Howitt et al. 2009) (Fig. 4). A similar role has been shown for the human ether-a-go-go-related gene (hERG) potassium channel, where both Ndfip1 and Ndfip2 recruit Nedd4-2 to subcellular compartments to facilitate the ubiquitination and subsequent degradation of hERG before it reaches the plasma membrane (Kang et al. 2015).

Ndfip1 has also been implicated in pancreatic function through its role in regulating JunB (Beck et al. 2015). In pancreatic β cells, expression of Ndfip1 resulted in ER stress due to both the degradation of JunB (a potent inhibitor of ER stress) and the inhibition of the unfolded protein response that serves to protect cells against ER stress. This then prevents insulin secretion, induces caspase activity, and leads to β cell death (Beck et al. 2015).

Summary

Ndfips are highly conserved proteins that primarily regulate the function of the Nedd4 family of ubiquitin ligases in their role in regulating substrates through ubiquitination. Generation of knockout mouse models has revealed the importance of these proteins both in maintaining normal homeostasis in physiological systems such as the immune system and iron regulation and during injury or disease as shown in the brain's response to traumatic brain injury. Given their ubiquitous expression pattern, it is likely that these proteins have as yet undiscovered roles in other tissues or systems.

See Also

- ▶ hNEDD4-2
- ▶ Nedd4

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Nebula

► Regulator of Calcineurin 1 (RCAN1)

Nebula, nla

► RCAN

NEDD4

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Synonyms

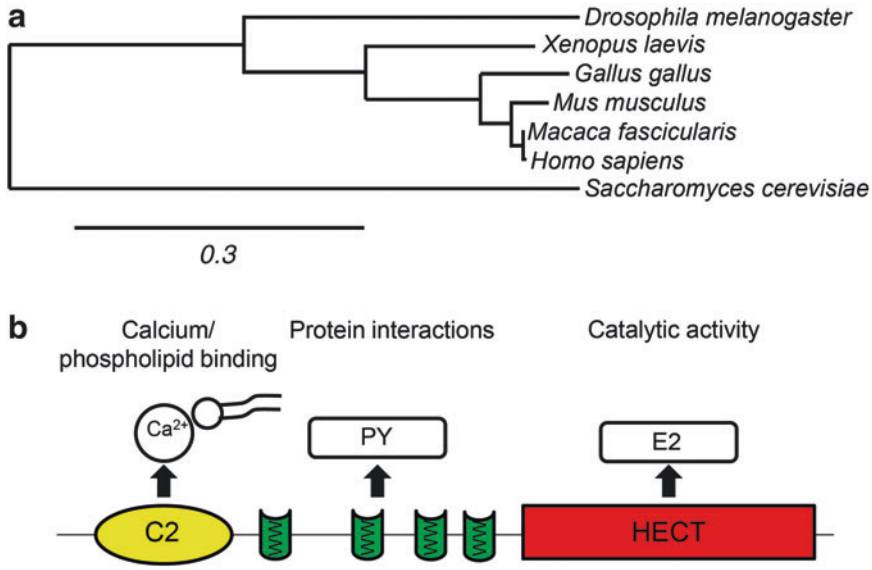
NEDD4-1; Neural precursor cell expressed developmentally downregulated 4; RPF1

Historical Background

NEDD4 (neural precursor cell expressed developmentally downregulated 4) was first identified in a screen for genes that are downregulated during development of the mouse brain (Kumar et al. 1992). Further characterization showed that mouse NEDD4 is a HECT type of ubiquitin ligase, comprising three WW domains (involved in protein-protein interactions) and a C2 calcium/phospholipid binding domain (Staub et al. 1996; Kumar et al. 1997). Discovery of the HECT domain gave the initial clues as to the function of NEDD4, as this domain was first identified in the papilloma virus oncoprotein E6-associated protein (E6-AP) and shown to be involved in the ubiquitination of p53 (Boase and Kumar 2015). The HECT family of ubiquitin ligases is found in all eukaryotic organisms, with NEDD4 the founding member of the NEDD4 family of HECT ligases, which contains nine human proteins (Scheffner and Kumar 2014).

NEDD4 Conservation, Structure and Expression

NEDD4 is found in all eukaryotes and is a highly evolutionarily conserved protein (Fig. 1a). The *NEDD4* gene encodes for a protein of about 120 kD in humans. All orthologs display a similar domain structure consisting of an N-terminal C2



NEDD4, Fig. 1 (**a**) Phylogenetic tree analysis of NEDD4 sequences from various species (analyzed using Phylogeny.fr: www.phylogeny.fr). Sequences were sourced from the NCBI protein database as follows: *Homo sapiens* (human): NP_006145.2, *Mus musculus* (mouse): NP_035020.2, *Macaca fascicularis* (monkey): XP_005559683.1, *Gallus gallus* (chicken): XP_015147366.1, *Xenopus laevis* (frog): NP_001084258.1, *Drosophila melanogaster* (fly): NP_996116.1, and *Saccharomyces cerevisiae* (yeast): AAC03223.1. (**b**) Structure of NEDD4. The C2 domain facilitates calcium-dependent phospholipid binding. The WW domains bind to PY or similar motifs in substrates and regulatory proteins. The HECT domain binds an E2 and acts as an acceptor of ubiquitin, which is then transferred to the target protein

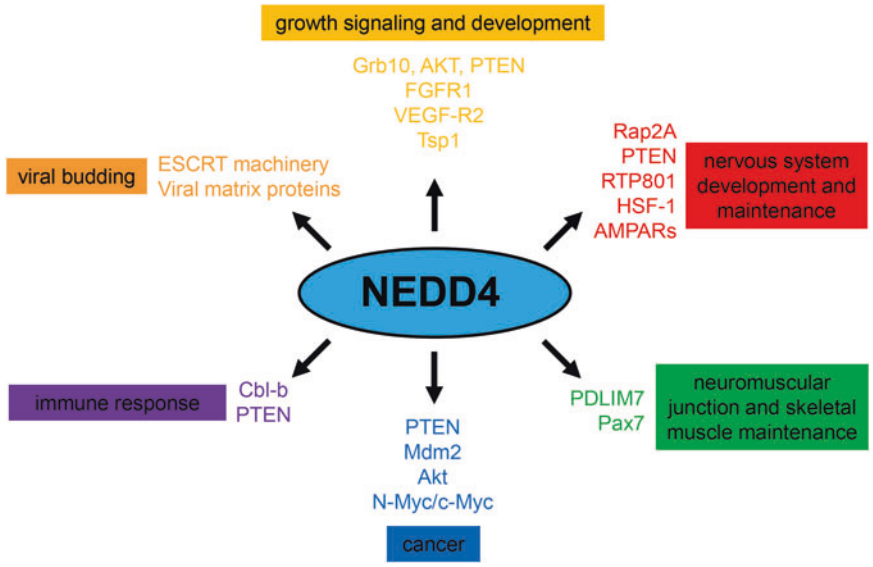
domain, 3–4 WW domains, and a C-terminal HECT domain (Fig. 1b) (Boase and Kumar 2015). The C2 domain functions to target proteins to phospholipid membranes in a calcium-dependent manner. The WW domains contain two conserved tryptophan (W) residues, separated by 21 amino acids. WW domains bind to PPXY (PY), LPXY or variations of these motifs in target/regulatory proteins and this is the main determinant of substrate specificity. The catalytic activity of NEDD4 is imparted by the C-terminal HECT domain which acts as an acceptor of ubiquitin to then transfer it onto substrates (Rotin and Kumar 2009). NEDD4 is expressed in most tissues and cell types and throughout animal development (Boase and Kumar 2015).

NEDD4 Functions

Ubiquitination is a three-step process that involves the formation of a thioester bond between ubiquitin and an internal cysteine residue of a ubiquitin-activating enzyme (E1) (Rotin and Kumar 2009). The ubiquitin is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2). Finally, ubiquitin ligases (E3s) facilitate the transfer of ubiquitin to one or more lysine residues in the target protein. HECT E3s, such as NEDD4, have a conserved cysteine residue that forms an intermediate thioester bond with the ubiquitin C-terminus before catalyzing substrate ubiquitination. The precise functions of NEDD4 are dictated by the substrates it targets for ubiquitination (discussed below, and see Fig. 2). There are over 60 NEDD4 targets identified to date, however only a few have been validated by in vivo studies (Boase and Kumar 2015). Most of the physiologically relevant functional information on NEDD4 is derived from mouse knockout studies.

Regulation of Development and Growth Signaling

NEDD4 knockout in mouse results in embryonic lethality (Cao et al. 2008). Close analysis of the



NEDD4, Fig. 2 The functions of NEDD4 are dictated by its substrate proteins. Examples of targets are shown for different physiologically relevant functions of NEDD4

first NEDD4$^{-/-}$ embryos generated revealed delayed embryonic development, reduced growth and body weight, and decreased mitogenic activity. This was attributed to reduced insulin growth factor 1 (IGF1) and insulin signaling caused in part by upregulation of Grb10, a negative regulator of IGF1 signaling (Cao et al. 2008). Other studies suggest that NEDD4 also regulates IGF1 and insulin signaling via targeting AKT and PTEN (Shi et al. 2014; Boase and Kumar 2015). Recently, it was demonstrated that mice haploin–sufficient for NEDD4 display insulin resistance, enhanced lipolysis, and protection from high-fat diet-induced obesity (Li et al. 2015).

Other growth factor signaling pathways shown to be regulated by NEDD4 include the fibroblast growth factor receptor 1 (FGFR1) (Persaud et al. 2011), and the vascular endothelial growth factor receptor 2 (VEGF-R2) via NEDD4 regulation of Grb10 (Murdaca et al. 2004). Other potential growth signaling pathways, not always validated in vivo, have been summarized previously (Boase and Kumar 2015).

Another independently generated NEDD4$^{-/-}$ mouse line has been reported to display embryonic lethality with heart defects (double-outlet right ventricle and atrioventricular cushion defects) and vasculature abnormalities (Fouladkou et al. 2010). This was attributed to increased levels of thrombospondin-1 (Tsp1), which is an inhibitor of angiogenesis, and shows a role for NEDD4 in regulation of heart development.

NEDD4 in the Nervous System

Mouse knockout studies indicate that NEDD4 has a critical function in dendrite formation and arborization in hippocampal and cortical neurons through the regulation of Ras-related protein 2A (Rap2A) (Kawabe et al. 2010). NEDD4 also plays an important role in cranial neural crest cell survival and craniofacial development (Wiszniak et al. 2016) and in nerve regeneration (Christie et al. 2012), although the mechanisms for this remain unclear. Given its role in the nervous system, it is not surprising that aberrant NEDD4 is linked to multiple neurological disorders. For example, lower levels of NEDD4 in Parkinson's disease (characterized by the abnormal accumulation of aggregates of α-synuclein protein in neurons) contribute to neuronal death by elevating levels of the proapoptotic protein RTP801 (Canal et al. 2016). In Alzheimer's disease, accumulation of the neurotoxic ß-amyloid induces synaptic alterations which involve, in part,

NEDD4-mediated ubiquitination of AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepro pionic acid receptors) to promote their internalization and weaken synaptic strength (Rodrigues et al. 2016). Additional evidence for the regulation of AMPAR trafficking by NEDD4 has been shown recently; for example, learning and memory are significantly impaired in NEDD4 heterozygous mice (Camera et al. 2016).

NEDD4 in the Neuromuscular Junction (NMJ) and Skeletal Muscle

NEDD4 is also implicated in regulating the formation and function of neuromuscular junctions (NMJ) (Liu et al. 2009). In a skeletal muscle specific NEDD4 knockout mouse, subjected to denervation induced skeletal muscle atrophy, heavier weights and larger fiber cross sectional area demonstrated a role for NEDD4 in the development of this atrophy (Nagpal et al. 2012). The mechanism for this is not known, but recent studies have identified NEDD4 targets including PDLIM7 (PDZ and LIM domain 7) that may contribute to the observed phenotype (D'Cruz et al. 2016). NEDD4 has also been shown to control skeletal muscle progenitor fate by regulating Pax7, leading to its degradation by the proteasome and promoting muscle differentiation (Bustos et al. 2015).

Role of NEDD4 in PTEN Regulation and Cancer

NEDD4 is frequently overexpressed in tumors and cancer cell lines (Zou et al. 2015). The tumor suppressor phosphatase PTEN has been reported to be a target of NEDD4-mediated ubiquitination and proteasomal degradation (Wang et al. 2007). Other findings suggest that NEDD4 modulates PTEN function both positively and negatively, depending on the level and localization of NEDD4. Importantly, PTEN levels and ubiquitination does not appear affected in NEDD4$^{-/-}$ mice, suggesting that this may be specific to certain conditions such as oncogenic stress (Cao et al. 2008). NEDD4 has also been associated with cancer independently of PTEN. For example, it has been shown to stabilize Mdm2, a ubiquitin ligase that exerts oncogenic activity primarily by suppressing p53, another prominent tumor suppressor (Zou et al. 2015). Furthermore, the elevated levels of NEDD4 may provide an additional mechanism to drive tumorigenesis in endometrial tumors, through regulation of PI3K/AKT signaling (Zhang et al. 2015). Conversely, NEDD4 ubiquitinates the oncoproteins N-Myc and c-Myc to target them for degradation (Liu et al. 2013). This effect is mediated by the direct binding of the histone deacetylase SIRT2 to NEDD4 to repress NEDD4 gene expression, thereby enhancing N-Myc and c-Myc expression. Hence, in this context decreased levels of NEDD4 contribute to cancer.

NEDD4 in the Immune Response

NEDD4 has been implicated in the regulation of T cell function. Due to the embryonic lethality of NEDD4$^{-/-}$ mice, in order to study this, fetal liver chimeras have been generated where only cells of hematopoetic origin are deficient in NEDD4 expression. T cells generated from these NEDD4$^{-/-}$ fetal liver chimeras developed normally, but proliferated less and produced less IL2, demonstrating that NEDD4 promotes the conversion of naïve to activated T cells (Yang et al. 2008). Mechanistically, NEDD4 has been shown to ubiquitinate Cbl-b (another E3 ubiquitin ligase), which plays a critical role in T cell activation (Guo et al. 2012). In the NEDD4 lacking T cells, higher levels of Cbl-b inhibited T cell activation by impeding the association of NEDD4 with PTEN, thereby suppressing PTEN activation. In addition, NEDD4$^{-/-}$ T cells were unable to provide support for B-cells to undergo immunoglobulin class switching (Yang et al. 2008).

Endocytosis and Viral Budding

Multiple proteins containing a ubiquitin interacting motif (UIM), such as γ2-adaptin, have been shown to interact with NEDD4, resulting in the targeting of proteins for ubiquitination to induce endocytosis (Boase and Kumar 2015). In addition, lysosomal proteins important for golgi to lysosome sorting including LAPTM5 (lysosomal associated protein transmembrane 5) have also been found to interact with NEDD4. Given its link to endocytosis, it is not surprising that many viruses also contain PY motifs

within their matrix proteins to facilitate interaction with NEDD4 and viral budding. Some examples include Human Immunodeficiency Virus (HIV), Influenza, Ebola, and the Epstein-Bar virus (Boase and Kumar 2015; Chesarino et al. 2015). In addition, components of the ESCRT machinery which are required for viral budding have also been shown to interact with NEDD4 (Boase and Kumar 2015).

Regulation of NEDD4

NEDD4 is regulated in a number of ways, including by accessory proteins, post–translational modifications (such as phosphorylation), oxidative stress, and auto-ubiquitination (Boase and Kumar 2015). Ndfip1 and Ndfip2 are adaptor proteins that contain three PY motifs which interact with NEDD4 to facilitate its binding to substrate proteins that do not contain their own PY motifs (Foot et al. 2011). Post–translationally, NEDD4 can be phosphorylated by multiple proteins, including casein kinase 1δ (which then allows its regulation by the $SCF^{\beta\text{-}TRCP}$ complex), and by c-Src to enhance its catalytic activity (Boase and Kumar 2015). Upregulation of NEDD4 has been reported by the Ras signaling pathway, and by the transcription factor FOXM1B (Forkhead box protein M1B) in response to oxidative stress (Boase and Kumar 2015).

Auto-ubiquitination also plays a major role in the regulation of NEDD4. In the absence of calcium, the enzymatic activity of NEDD4 is suppressed by its C2 domain binding to the HECT domain (Wang et al. 2007). Many factors have been shown to disrupt this autoinhibition, allowing NEDD4 to conduct its ubiquitin ligase activity. These include the presence of calcium, phosphorylation by c-Src, or when the adaptors proteins Ndfip1 and Ndfip2 bind the WW domains of NEDD4. Additional NEDD4 binding proteins such as p34 and Cbl-b also inhibit this autoubiquitination (Boase and Kumar 2015). Negative regulation of NEDD4 has been reported by the interferon inducible ISG15 and heclin (HECT ligase inhibitor) by preventing ubiquitin transfer or formation of thioester bonds. Other proteins shown to inhibit NEDD4 include the proto-oncogene ΔNp63α and PTEN (Boase and Kumar 2015).

Summary

NEDD4 is a member of the HECT E3 ubiquitin ligase family. It is highly conserved during evolution and is likely to be the most ancient member of the NEDD4 family. In vivo studies in mouse reveal the importance of this gene in the regulation of growth signaling and the immune response as well as development and function of the nervous system, muscle, and heart. In addition, there is much evidence implicating NEDD4 as a critical regulator of components and pathways leading to tumor initiation and progression. Given the pathological conditions involving misregulation of this protein, or its substrates, the opportunities for therapeutic interventions utilizing NEDD4 are extensive.

See also

- ▶ hNEDD4-2
- ▶ Insulin-Like Growth Factor Receptor Type I (IGF1R)
- ▶ Ndfip1 and Ndfip2
- ▶ PTEN

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Nedd4 Family-Interacting Protein (Ndfip), dNdfip, Dmel, CG32177 (*Drosophila* Ndfip1)

► NDFIP1 and NDFIP2

Nedd4 Family-Interacting Protein 1 (Ndfip1)

► NDFIP1 and NDFIP2

Nedd4 Family-Interacting Protein 2 (Ndfip2)

► NDFIP1 and NDFIP2

NEDD4 WW Domain-Binding Protein 5 (Human)

► NDFIP1 and NDFIP2

Nedd4 WW Domain-Binding Protein 5 (N4WBP5)

► NDFIP1 and NDFIP2

NEDD4 WW Domain-Binding Protein 5A (N4WBP5A)

► NDFIP1 and NDFIP2

NEDD4-1

► NEDD4

NEDD4–2

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Synonyms

hNEDD4-2; KIAA0439; NEDD4L; NEDL3

Historical Background

NEDD4–2 (neural precursor cell expressed, developmentally downregulated 4-like) encoded by the *Nedd4L* gene, belongs to the NEDD4 family of ubiquitin protein ligases. NEDD4 family members are HECT-type ubiquitin ligases (E3) that act at the final step of the ubiquitin cascade to accept ubiquitin from a ubiquitin-conjugating enzyme (E2) and transfer it to their cognate substrates. Ubiquitination of a protein often targets it for degradation; however it may also affect protein localization, trafficking, and recognition by signaling or regulatory complexes. The other members of the NEDD4 family include NEDD4, ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1, and NEDL2 (Rotin and Kumar 2009).

NEDD4–2 (originally submitted in the database as mouse KIAA0439 or human *NEDD4L* gene) is most closely related to the founding member, NEDD4, which was originally identified in the early embryonic central nervous system as a developmentally downregulated gene (Kumar et al. 1992). NEDD4 and NEDD4–2 share approximately 78% similarity, and while homologs of NEDD4 are found in all eukaryotes demonstrating high evolutionary conservation, NEDD4–2 likely arose much later by gene duplication as its homologs are only found in vertebrates (Harvey and Kumar 1999; Yang and Kumar 2010).

NEDD4–2 Structure and Expression

NEDD4–2 has distinctive modular domain architecture similar to other NEDD4 family members,

	Length (aa)	Comparison to Isoform 1	CCDS	Uniprot
Isoform 1	975		CCDS45872	Q96PU5-1
Isoform 2	911	356-419 missing	CCDS58632	Q96PU5-2
Isoform 3	871	356-459 missing	-	Q96PU5-3
Isoform 4	854	1-121 missing	CCDS45875	Q96PU5-4
Isoform 5	955	356-375 missing	CCDS45873	Q96PU5-5
Isoform 6	947	Alternative NH_2, 356-375 missing	CCDS59323	Q96PU5-6
Isoform 7	967	Alternative NH_2	CCDS45874	Q96PU5-7
Isoform 8	834	1-121, 356-375 missing	CCDS45876	Q96PU5-9

NEDD4–2, Fig. 1 Predicted structure of human NEDD4–2 proteins. Comparison of *hNEDD4–2* isoforms based on transcript data available on Uniprot and Ensembl databases. Isoform 1 is considered the canonical isoform. Variability between isoforms occurs at the NH2 terminus and/or within the WW domains. The putative serum- and glucocorticoid-regulated (sgk)1 phosphorylation site is shown as a *red arrowhead* (absent in isoforms 2, 3, 5, 6, and 8)

comprising an amino terminal Ca^{2+} phospholipid binding (C2) domain, four WW (protein–protein interaction) domains, and a HECT domain at the carboxyl terminus (Fig. 1) (Harvey and Kumar 1999). The WW domains generally bind PY (PPxY) or similar motifs in substrates and regulatory proteins. The number and position of WW domains vary among NEDD4 family members (generally 2–4), which are thought to contribute to substrate specificity and involvement in distinct biological processes.

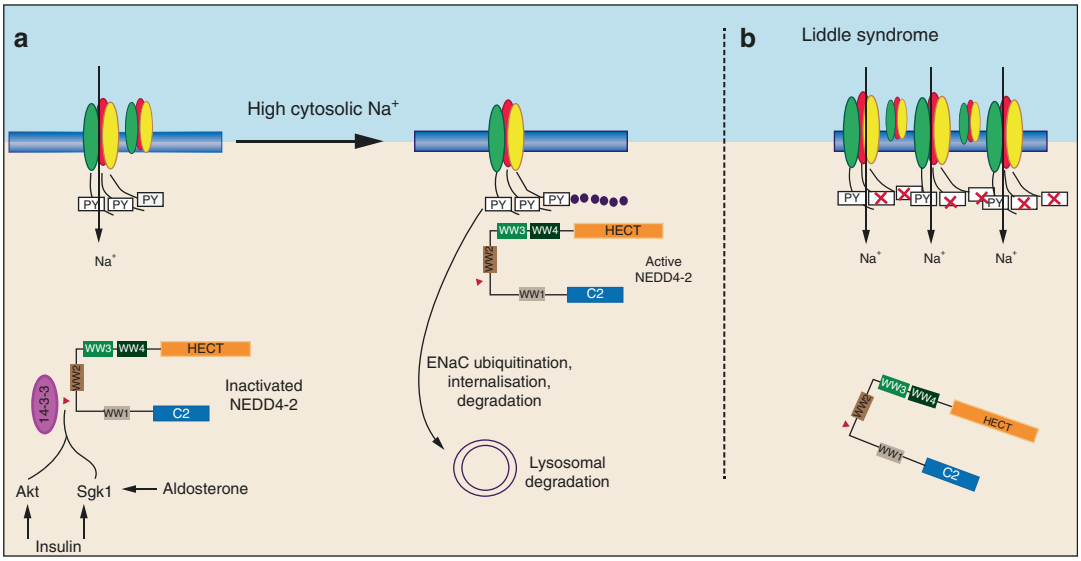
NEDD4–2 transcripts are present in many tissues, with particularly high expression in the liver, brain, heart, and lung (Araki et al. 2008; Harvey et al. 2001). The canonical isoform of *NEDD4–2* contains a C2 domain, all four WW domains, and a HECT domain; however, many alternatively spliced isoforms of *NEDD4–2* are predicted (Fig. 1). According to the Ensembl database, 18 protein-coding transcripts exist for human NEDD4–2/NEDD4L, including seven CCDS transcripts. Variability in the transcripts lie within the NH_2-terminal region (as some transcripts have, or lack the C2 domain), and also internally, varying the number of WW domains and sgk1 phosphorylation sites (Itani et al. 2003, 2005). In most tissues, NEDD4–2 (both mouse and human) appears as two protein bands of ~110–115 kDa, one of which may vary slightly in a tissue-specific manner (Fotia et al. 2003; Harvey et al. 2001; Itani et al. 2003, 2005; Ronzaud et al. 2013).

NEDD4–2 Functions

A number of proteins have been shown to be targets of NEDD4–2 (reviewed by Goel et al. (2015)); however, interactions with many of the putative targets have only been demonstrated in vitro and have not been validated using physiologically relevant animal models, such as in gene knockout mice. Here we focus on some key validated NEDD4–2 substrates.

Regulation of Sodium Homeostasis Through ENaC and NCC

The epithelial sodium channel (ENaC) plays an essential role in fluid and electrolyte homeostasis, and in kidney, it is necessary for Na^+ homeostasis and maintenance of blood pressure. In the lung,



NEDD4–2, Fig. 2 NEDD4–2 is critical regulator of ENaC. (**a**) Under conditions of high cytoplasmosmic Na^+, NEDD4–2 acts to downregulate ENaC by ubiquitination, triggering its removal from the membrane and degradation in the lysosome. NEDD4–2 binds to the PY motifs in the c terminus of α, β, and γ subunits of ENaC through its WW3 and WW4 domains and subsequently attaches ubiquitin molecules (*purple dots*), triggering degredation of ENaC in the lysosome. The function of NEDD4–2 can be inhibited by its phosphorylation by Sgk1 (serum- and glucocorticoid-regulated kinase 1) and Akt (PKB), under the control of aldosterone and insulin, and subsequent binding of 14-3-3. (**b**) Mutations or deletions in the PY motifs of β or γ ENaC, associated with Liddle syndrome, affect the binding of NEDD4–2. Subsequent accumulation of ENaC channels in the membrane increases Na^+ reabsorption and water uptake, resulting in hypertension

ENaC is responsible for normal fluid clearance from alveolar spaces and subsequently normal exchange of gases.

ENaC is composed of three subunits, α, β, and γ, each with two transmembrane domains, and cytoplasmic N and C termini. Each subunit contains a PY motif at its C terminus. Under conditions of high Na^+, ENaC is downregulated by direct binding of the WW domains of NEDD4–2 to PY motifs of ENaC, leading to its ubiquitination, removal from the membrane, and subsequent degradation (Harvey et al. 2001; Kamynina et al. 2001; Fotia et al. 2003) (Fig. 2).

In mixed genetic background, an apparently incomplete *NEDD4–2* knockout in mice results in slightly elevated ENaC expression in the kidney and a mild salt-sensitive hypertension (Shi et al. 2008). Importantly the hypertension can be partially rescued by Amiloride, a specific inhibitor of ENaC. A null allele of NEDD4–2 in C57Bl6 background is perinatal lethal (Boase et al. 2011). Most animals die at time of birth due to inability to inflate their lungs, and pups that are born will die approximately 20 days after birth with severe lung inflammation. The NEDD4–2 deficiency results in increased ENaC expression and activity in the lung, presumably causing premature clearance of fetal lung fluid and subsequent drying of epithelia in surviving animals (Boase et al. 2011). A lung-specific knockout of *NEDD4–2* also leads to perinatal lethality approximately 20 days after birth (Kimura et al. 2011); These mice present with a cystic fibrosis-like phenotype including airway mucus obstruction and inflammation, which was reversed by treatment with Amiloride (Kimura et al. 2011). These studies demonstrate the critical role of Nedd4–2 in regulating ENaC function.

The Na^+-Cl^- cotransporter (NCC) is expressed in the distal collecting duct of kidney and is important for regulation of Na^+ balance and blood pressure. NEDD4–2 binds to and ubiquitinates NCC, however, as NCC does not have a classical PY motif, the mechanism of interaction remains unclear. Regulation of NCC by

NEDD4–2 has been demonstrated to be critical for Na^+ absorption leading to an increase in blood pressure in mice with a kidney specific deletion of NEDD4–2 (Ronzaud et al. 2013). These mice also show increased levels of βENaC and γENaC, and renal outer medullary K^+ channel (ROMK) (Ronzaud et al. 2013). Treatment with thiazide was able to partially reverse the hypertension suggesting that NCC is also an important physiological target of NEDD4–2 (Ronzaud et al. 2013).

Regulation of Voltage-Gated Sodium Channels and Neuropathic Pain

Voltage-gated sodium channels (Na_vs) are expressed in many cell types, particularly in the nervous system. Na_vs mediate the influx of Na^+ in response to local depolarizing stimuli, which generates action potentials in electrically excitable cells. There are nine members of the Na_v family, seven of which contain PY motifs and can interact with the WW domains of NEDD4 and NEDD4–2 (Fotia et al. 2004); Several of these channels have been shown to be ubiquitinated and inhibited by NEDD4–2 (Fotia et al. 2004; van Bemmelen et al. 2004). In mouse cortical neurons, NEDD4–2 regulates Na_vs specifically in response to elevated intracellular Na^+, but does not affect steady-state Na_v activity (Ekberg et al. 2014). In animal models of neuropathic pain, a condition associated with hyperexcitability of neurons following nerve injury, NEDD4–2 is implicated in altered ubiquitination and regulation of Na_vs (Cachemaille et al. 2012; Laedermann et al. 2013; Ekberg et al. 2014).

Regulation of Mast Cell Function

Following antigen binding, crosslinking the IgE receptor on mast cells plays a critical role in the pathology of IgE-dependent allergic disorders. Recently NEDD4–2 and the adaptor NDFIP1 have been shown to limit the intensity and duration of such IgE-dependent signaling, through, in part, ubiquitination of phospho-Syk, a kinase required for downstream signaling (Yip et al. 2016). Thus NEDD4–2 is a negative regulator in IgE-dependent mast cell activity that plays a role in dampening the IgE-dependent allergic response.

Other Possible Targets and Functions

In vitro data implicate NEDD4–2 in the regulation of many other proteins, including several potassium and chloride ion channels, surfactant protein C, glutamate and dopamine transporters, EGFR, TGF-β receptor, WNT signaling, dopamine transporter, and divalent metal ion transporter (DMT1) (reviewed by Goel et al. (2015)). In addition, NEDD4–2 has also been show to play a role in nerve growth factor mediated functions through the regulation of TrkA (Arevalo et al. 2006; Yu et al. 2014; Yu et al. 2011). Despite a long list of potential substrates, most of the interacting proteins for NEDD4–2 have been discovered by in vitro methods, and more mechanistic and in vivo work is required before these functions of NEDD4–2 can be fully verified.

Regulation of NEDD4–2

Autoregulation

The WW domains within NEDD4–2 can weakly bind to the LPxY motif in its HECT domain. This interaction is thought to stabilize NEDD4–2 and prevent its autoubiquitination, resulting in more NEDD4–2 available to bind to its substrates, including ENaC (Bruce et al. 2008). In addition, deubiquitination of NEDD4–2 by USP2–45 is also known to maintain NEDD4–2 protein stability (Krzystanek et al. 2012; Oberfeld et al. 2011; Pouly et al. 2013).

Ndfip Adaptors

Proteins that don't have a PY motif require adaptor proteins to bind to the WW domains of NEDD4 E3s, including NEDD4–2. One such family of adaptor proteins is NDFIPs (NDFIP1 and NDFIP2). Both proteins have PY motifs to bind to the WW domains of NEDD4 family E3s (Harvey et al. 2002; Mund and Pelham 2009; Shearwin-Whyatt et al. 2006), and through NDFIP1, NEDD4–2 has been shown to regulate divalent metal ion transporter, DMT1 (Foot et al. 2008; Howitt et al. 2009); the water channel, aquaporin 2 (AQP2) (de Groot et al. 2014); and may also modulate NEDD4–2s regulation of ENaC (Konstas et al. 2002).

14–3-3 Proteins

In response to insulin and aldosterone signaling, NEDD4–2 is phosphorylated by kinases, SGK1 and AKT, which triggers its interaction with 14-3-3 proteins (Snyder et al. 2004; Bhalla et al. 2005; Lee et al. 2007). The binding of 14-3-3 to NEDD4–2 inhibits its function by preventing it from interacting with its substrates, such as the ENaC (Nagaki et al. 2006) (Fig. 2).

Clinical Significance

NEDD4–2/NEDD4L variants have been linked to salt-sensitive and essential hypertension in human subjects (Knight et al. 2006). In addition, mutations in β and γENaC, which disrupt or delete the PY motif in Liddle syndrome, abrogate the ability of NEDD4–2 to bind to and ubiquitinate ENaC. This results in accumulation of the functional ENaC on the cell membrane, triggering increased sodium reabsorption, which leads to increased reabsorption of water and consequentially hypertension (Foot et al. 2016). Based on mouse studies stated above, NEDD4–2 may also be important in IgE-dependent allergic disorders and controlling neuropathic pain.

Summary

NEDD4–2 is a member of the NEDD4 ubiquitin ligase family that comprises nine members of HECT type E3s. It is a close relative of NEDD4, which is highly conserved during evolution. Most of the known substrates of NEDD4–2 are membrane proteins, including ion channels and transporters. In particular, NEDD4–2 plays an essential role in sodium homeostasis in the lung and kidney through the regulation of ENaC, and the variants of NEDD4–2 gene are linked to human hypertension.

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NEDD4L

► NEDD4–2

NEDL3

► NEDD4–2

Nek1

► NEKs, NIMA-Related Kinases

Nek10

► NEKs, NIMA-Related Kinases

Nek11

► NEKs, NIMA-Related Kinases

Nek2

► NEKs, NIMA-Related Kinases

Nek3

► NEKs, NIMA-Related Kinases

Nek4

► NEKs, NIMA-Related Kinases

Nek5

► NEKs, NIMA-Related Kinases

Nek6

► NEKs, NIMA-Related Kinases

Nek7

► NEKs, NIMA-Related Kinases

Nek8

► NEKs, NIMA-Related Kinases

Nek9/Nercc1

► NEKs, NIMA-Related Kinases

NEKs, NIMA-Related Kinases

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Synonyms

Nek1; Nek2; Nek3; Nek4; Nek5; Nek6; Nek7; Nek8; Nek9/Nercc1; Nek10; Nek11

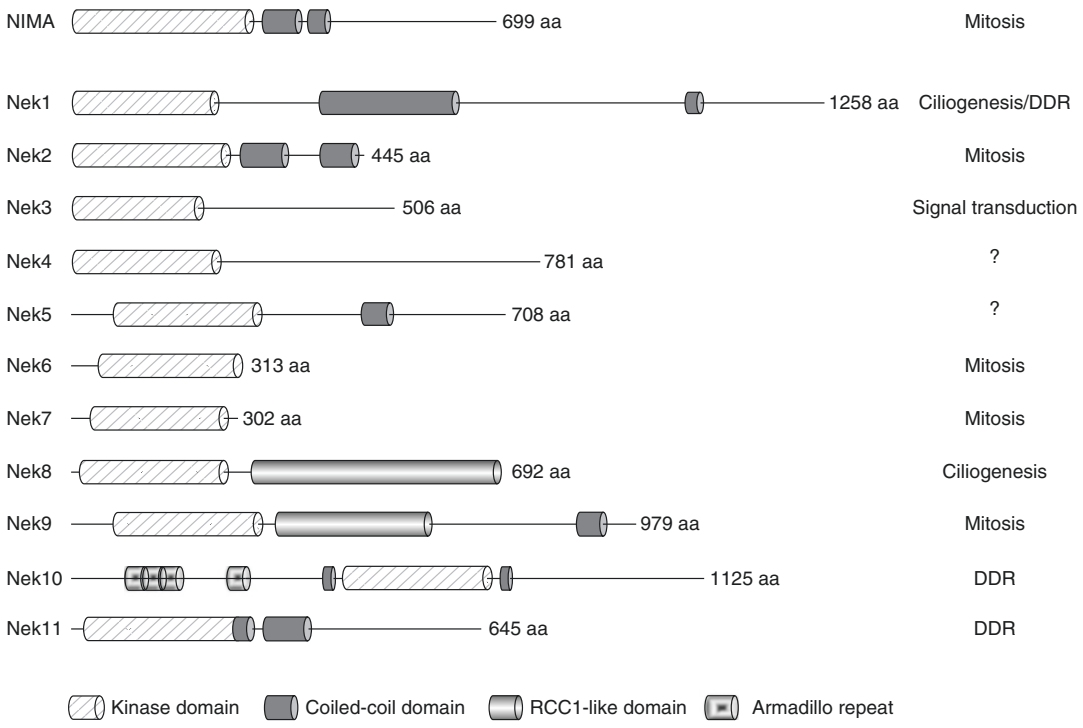
Historical Background

The *N*IMA-*r*elated *k*inase, or "Nek," family constitutes approximately 2% of all human kinases. They are related in sequence, as their name suggests, to NIMA (699 residues, 80 kDa), a serine/threonine protein kinase present in the filamentous fungus, *Aspergillus nidulans*. Ron Morris identified the gene, nimA, through analysis of a temperature-sensitive loss-of-function mutant that was *n*ever *i*n *m*itosis (nim) when cells were incubated at the restrictive temperature (Morris 1975). Loss of NIMA activity led to G2 arrest, while overexpression of NIMA drove cells into a premature mitosis from any point in the cell cycle (Oakley and Morris 1983; Osmani et al. 1988, 1991). Mechanistically, NIMA is likely to regulate multiple aspects of mitotic entry, with evidence that its activity is required for nuclear pore disassembly, relocalization of the master regulator, cdc2-cyclin B, to the nucleus and spindle pole body (SPB), and chromatin condensation. NIMA is subsequently degraded in an anaphase-promoting complex/cyclosome (APC/C)-dependent manner and this is necessary for mitotic exit (reviewed in O'Connell et al. 2003; O'Regan et al. 2007).

Aspergillus cells are syncytial and undertake a semi-closed mitosis. It may therefore have evolved control mechanisms that are specific for this form of cell division. However, an early suggestion that NIMA-related kinases might be conserved in other eukaryotes came from expressing *Aspergillus* NIMA in cells from diverse species, including humans, and observing cell cycle defects (Lu and Hunter 1995; O'Connell et al. 1994). With the complete sequencing of many genomes, it is now clear that kinases related to NIMA by sequence are indeed present in most eukaryotes (Parker et al. 2007). However, so far, only the NIM-1 protein from the highly related filamentous fungus, *Neurospora crassa*, has been proven to be a functional homologue of NIMA capable of rescuing an *Aspergillus nimA* mutant suggesting the possibility of functional divergence. Indeed, the budding yeast, *Saccharomyces cerevisiae*, and fission yeast, *Schizosaccharomyces pombe*, each have one NIMA-related kinase in their genome, Kin3 and Fin1, respectively, but these are not essential for mitotic entry. However, careful studies revealed that Fin1 does contribute to the timing of mitotic onset through regulating the localization of the polo-like kinase, Plo1, to the SPB, which in turn promotes activation of cdc2-cyclin B (Grallert and Hagan 2002). Fin1 also contributes to mitotic spindle formation and mitotic exit (Grallert and Hagan 2002; Grallert et al. 2004). Thus, Neks from different species do play roles in mitotic progression.

Surprisingly, some lower eukaryotes have many genes encoding Nek kinases. For example, the unicellular organisms *Chlamydomonas* and *Tetrahymena* have 10 and 39 Nek genes, respectively. The key to this expansion of Nek genes appears to lie in an alternative non-mitotic function for Nek kinases, that is, in ciliogenesis. *Chlamydomonas* produce two elongated cilia, or flagella, that allow the organism to swim in response to environmental stimuli. To date, only two of the *Chlamydomonas* Nek genes have been studied in depth, Fa2p and Cnk2p, but loss of either protein affects both flagella disassembly and cell cycle progression (Bradley and Quarmby 2005; Mahjoub et al. 2002). Meanwhile, *Tetrahymena* has hundreds of cilia that fall into different classes depending on their location and length, and all of the Neks tested so far in this organism localize to cilia and regulate cilia length (Wloga et al. 2006).

The human NIMA-related kinase family consists of 11 proteins, named Nek1 to Nek11, that are encoded by distinct genes (Fig. 1). Apart from Nek10, these share a common protein domain structure with an N-terminal catalytic kinase domain, containing all the signature motifs of a serine/threonine kinase, and a C-terminal regulatory domain that is highly variable in length and sequence. These differences in the non-catalytic regions contribute to the distinct patterns of expression, localization, activation, and regulation that are seen across this family. Functionally, though, studies performed across many systems



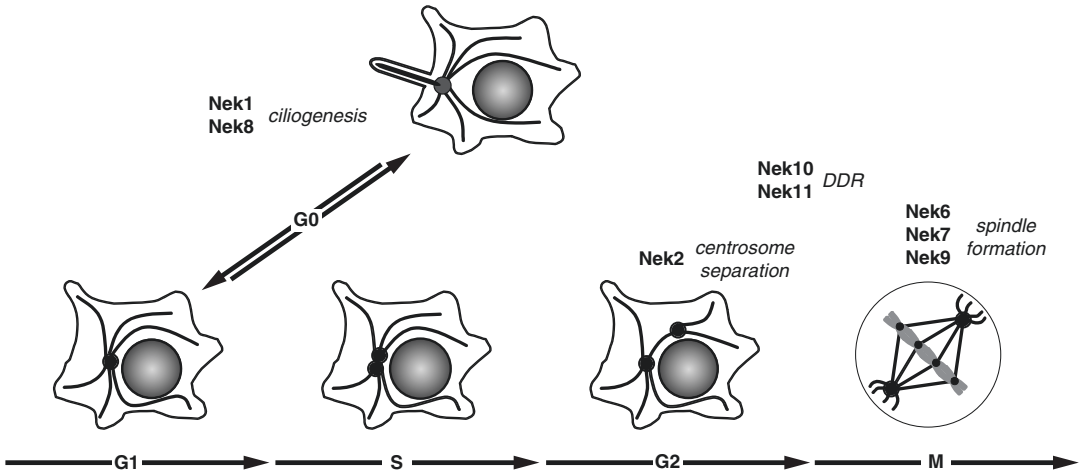
NEKs, NIMA-Related Kinases, Fig. 1 *The human NIMA-related kinase family.* The schematic diagram shows the domain organization of the 11 human NIMA-related kinases, Nek1 to Nek11, below that of the *Aspergillus* NIMA kinase. These generally have an N-terminal catalytic domain followed by a C-terminal non-catalytic region containing potential regulatory motifs. Several Neks have putative coiled-coil sequences; in the case of Nek2 the first of these is an atypical leucine zipper that promotes dimerization, whereas the second has been identified as a SARAH domain that mediates interaction with Hippo pathway components. The length of each kinase is indicated (amino acid number) together with its best understood function

including humans, support the hypothesis that the majority of Neks contribute in one way or another to cell cycle progression and/or ciliogenesis (O'Connell et al. 2003; O'Regan et al. 2007; Quarmby and Mahjoub 2005). Thus, altered expression or mutation of Neks can interfere with these key processes, implicating them in both human cancer and inherited ciliopathies.

Specifically, research on the human proteins has demonstrated a role for Nek2, Nek6, Nek7, and Nek9 in mitotic regulation, Nek10 and Nek11 in the DNA damage response, Nek1 and Nek8 in ciliogenesis, and Nek3 in signal transduction (Fig. 2). At the time of writing, little is known about the function of Nek4 and Nek5. A brief summary of findings to date on the regulation and function of each mammalian Nek is presented below.

Mitotic Neks: Nek2, Nek6, Nek7, and Nek9

Of all the human Nek kinases, **Nek2** is the most closely related by sequence to NIMA being 48% identical within the catalytic domain. For this reason, it is to date the most well-studied member of this family. It also shares a number of other important properties with NIMA, including cell cycle–dependent expression, APC/C-dependent degradation and localization to the centrosome, the higher eukaryotic microtubule organizing center and equivalent of the fungal SPB (Fry 2002; Hayward and Fry 2006). However, like the yeast Neks and unlike *Aspergillus* NIMA, human Nek2, as far as one can tell, is not essential for mitotic entry.



NEKs, NIMA-Related Kinases, Fig. 2 *Functions of NIMA-related kinases in the cell cycle*. The majority of human Neks play roles in cell cycle progression. A schematic view of cells progressing through the cell cycle with the point at which the Neks act and their best understood function is indicated. However, it should be stressed that each of these Neks is likely to have multiple functions beyond those indicated, for example, Nek6 and Nek7 are also implicated in nuclear pore complex disassembly and cytokinesis, while Nek1 may have functions in the DDR as well as ciliogenesis. The roles of Nek4 and Nek5 remain unknown at the time of writing, while Nek3 is involved in prolactin-mediated cell migration

Nek2 is a ubiquitous protein with at least three splice variants, Nek2A (445 residues; 51 kDa), Nek2B (384 residues; 44 kDa), and Nek2C (437 residues; 50 kDa; also called Nek2A-T). These all share the N-terminal kinase domain followed by a leucine zipper coiled-coil motif that promotes dimerization and activation. Nek2A then contains a second coiled-coil motif at its C-terminus. Nek2B is somewhat shorter than Nek2A and lacks the second coiled-coil, whereas Nek2C is identical to Nek2A apart from missing an 8 residue sequence that lies between the leucine zipper and C-terminal coiled-coil. The relative expression of the three variants differs depending on cell type and developmental stage, although generally Nek2A appears to be the predominant isoform. The major function of Nek2A is to regulate centrosome organization through the cell cycle, as described below. Similarly, Nek2B is required for assembly and maintenance of centrosome structure and this may be particularly important during early embryonic development as, in *Xenopus*, it is the only variant expressed at this stage (Uto and Sagata 2000). Interestingly, a role for Nek2B has been proposed in mitotic exit in human cells where its depletion leads to cytokinesis failure (Fletcher et al. 2005). The 8 residue deletion that leads to Nek2C creates a functional nuclear localization signal not present in Nek2A or Nek2B, raising the possibility of nuclear-specific functions for this variant (Wu et al. 2007).

Overall, Nek2 expression and activity are regulated in a cell cycle–dependent manner (Fry et al. 1995; Schultz et al. 1994). The protein is almost undetectable during G1 but accumulates abruptly at the G1/S transition and remains high until late G2. This is a combined result of transcriptional repression and APC/C-mediated degradation in G1 and transcriptional upregulation in S/G2 (Hayward and Fry 2006). However, Nek2A activity is further regulated by a number of key structural and sequence-specific features of the kinase. The N-terminal kinase domain contains a number of sites which undergo autophosphorylation upon dimerization through the leucine zipper (Rellos et al. 2007). It is likely that phosphorylation at least at some of these sites, particularly those in the activation loop, is required for kinase activation. The C-terminal non-catalytic domain also contains autophosphorylation sites, although the functions of these remain unclear. The C-terminal

coiled-coil motif has recently been defined as a putative SARAH domain, which enables interaction with the Hippo pathway proteins, hSav1 and the Mst2 kinase (Mardin et al. 2010). Mst2 acts as an upstream activator of Nek2, phosphorylating sites in the C-terminal region that regulate localization of Nek2 to the centrosome. The C-terminal domain also contains a site that mediates direct interaction of Nek2 with the phosphatase, PP1 (Helps et al. 2000). PP1 negatively regulates Nek2 through dephosphorylation, while Nek2 may be able to inhibit PP1 by phosphorylation. This creates a very sensitive bistable switch that allows rapid Nek2 activation, once PP1 activity starts to decrease at the onset of mitosis (Eto et al. 2002). Nek2 is also negatively regulated by the focal adhesion scaffolding protein, HEF1, although the mechanism is not known (Pugacheva and Golemis 2005). Finally, the C-terminal domain of Nek2 contains two destruction motifs that target it for APC/C-mediated degradation in early mitosis (Hayes et al. 2006).

Nek2 localizes to the centrosome throughout the cell cycle (Fry et al. 1998b). An additional fraction is present in the cytoplasm where it colocalizes with and is trafficked along microtubules (Hames et al. 2005). This localization is dependent on the region between the leucine zipper and SARAH domain that encompasses sites phosphorylated by Mst2 (Hames et al. 2005; Mardin et al. 2010). The primary role of Nek2 at the centrosome is as a regulator of centrosome cohesion. Overexpression of active Nek2 induces premature centrosome splitting during interphase (Fry et al. 1998b), while expression of inactive Nek2 or depletion by RNAi inhibits centrosome separation and promotes monopolar spindle formation (Faragher and Fry 2003; Fletcher et al. 2005; Mardin et al. 2010). Nek2 promotes loss of cohesion or "disjunction" of centrosomes through phosphorylation of C-Nap1 and, possibly rootletin and ▶ β-catenin (Bahmanyar et al. 2008; Fry et al. 1998a). These proteins, together with Cep68, form a flexible linker structure that extends between the proximal ends of the parental centrioles and which must be dismantled at the onset of mitosis to allow centrosome separation and spindle formation to occur. In addition to the role of Nek2 in centrosome separation, Nek2 has been implicated in regulating the microtubule organizing capacity of the centrosome through phosphorylation of Nlp and centrobin, chromatin condensation through phosphorylation of HMGA2, spindle checkpoint signaling through phosphorylation of Hec1, and nuclear pore complex disassembly through phosphorylation of Nup98 (Laurell et al. 2011; O'Regan et al. 2007).

Nek9 is a 979 residue protein of 113 kDa that, following its N-terminal catalytic domain, has a C-terminal regulatory region comprising a central Regulator of Chromosome Condensation 1 (RCC1)-like domain and a C-terminal coiled-coil motif. It is expressed throughout the cell cycle, but becomes phosphorylated and activated specifically in mitosis (Roig et al. 2002). Nek9 is subject to complex but, as yet, poorly understood regulation. It is thought that during interphase Nek9 adopts an autoinhibited conformation with the RCC1-like domain blocking access to the catalytic site. However, at the G2/M transition, a number of events lead to release of this conformation and activation of the kinase. These are likely to include dimerization, autophosphorylation (especially on T210 in the activation loop), and, potentially, activation by upstream kinases such as Cdk1 and Plk1 (Roig et al. 2002; Bertran et al. 2011). In terms of localization, Nek9 is mainly cytoplasmic, although it can also be found in the nucleus. However, its activated form appears to be specifically concentrated on spindle poles during mitosis (Roig et al. 2005).

Early functional studies on Nek9 by overexpression of wild-type or mutant constructs, or by inhibition through antibody microinjection, led to the hypothesis that Nek9 activity contributes to mitotic spindle formation (Roig et al. 2002). How exactly it does this remains unclear, but Nek9 can interact with the γ-tubulin ring complex (γ-TuRC) that nucleates microtubules both from the centrosome and within the spindle (Roig et al. 2005). Hence, one is tempted to speculate that Nek9 directly contributes to microtubule nucleation in mitosis. However, depletion of Nek9 from *Xenopus* egg extracts prevents the formation of spindles via either the centrosome- or chromatin-mediated pathways (Roig et al. 2005). This

suggests that Nek9 function is unlikely to be restricted to regulating the microtubule nucleating activity of γ-tubulin. Nek9 also interacts with BICD2, a protein associated with microtubule-dependent motor proteins, raising the possibility that BICD2 might target Nek9 to microtubules, as well as potentially being a substrate (Holland et al. 2002). Furthermore, the presence of the RCC1-like domain, together with the demonstration that the ▶ Ran GTPase can bind to Nek9, points to a potential role in Ran-mediated spindle formation (Roig et al. 2002). Some reports suggest that Nek9 could have functions outside of mitosis. For example, Nek9 was reported to associate with the FACT complex, a chromatin modifying complex involved in replication and transcription (Tan and Lee 2004). This could explain the presence of a nuclear fraction of Nek9 in interphase and, although not consistent with the timing of bulk Nek9 activation, it is possible that the FACT complex activates a restricted pool of Nek9 in the nucleus, which in turn might be inhibited by the adenovirus E1A protein.

One major route through which Nek9 almost certainly regulates mitotic spindle organization is through interaction, phosphorylation, and activation of two other NIMA-related kinases, Nek6 and Nek7. Indeed, Nek9 was first identified through its association with Nek6 (Roig et al. 2002). Subsequently, Nek9 was shown to phosphorylate sites within the activation loop of Nek6 and this, together with the high degree of similarity between Nek6 and Nek7, led to the proposal that these three Neks form a mitotic cascade in which Nek9 acts upstream of Nek6 and Nek7 (Belham et al. 2003). Lately, it has been demonstrated that Nek9 may also activate Nek7, and by analogy Nek6, through an allosteric mechanism independent of phosphorylation (Richards et al. 2009).

Nek6 and **Nek7** are the smallest family members being only 313 (36 kDa) and 302 (35 kDa) residues, respectively. As a result they comprise little more than a catalytic domain with only a short (30–40 residue) N-terminal extension. By sequence, they are highly related to each other, sharing 86% amino acid identity within the catalytic domain, although the N-termini of the two proteins are not conserved. Often considered as a pair because of their similarity, both Nek6 and Nek7 are, like their upstream activator Nek9, cell cycle regulated with maximal activity in mitosis. Moreover, while they give slightly different localization patterns, with Nek6 weakly associated to spindle fibers and Nek7 more concentrated on spindle poles, RNAi depletion experiments demonstrate that they are both essential not only for assembly of a robust mitotic spindle, but also potentially for completion of cytokinesis and cell abscission (Kim et al. 2007; O'Regan and Fry 2009; Yin et al. 2003; Yissachar et al. 2006). In support of a late mitotic role for these kinases, Nek6 activation, judged with a phosphospecific antibody against a key activation loop residue, peaks at the time of cytokinesis (Rapley et al. 2008), while mouse embryonic fibroblasts derived from Nek7$^{-/-}$ embryos show defects indicative of cytokinesis failure (Salem et al. 2010).

How these kinases regulate spindle formation and cytokinesis remains to be defined. However, despite their sequence similarity and requirement for both events, it is likely that their respective roles and substrates differ. Systems approaches have identified a large number of putative interacting partners and substrates for Nek6 (Ewing et al. 2007; Vaz Meirelles et al. 2010). These include proteins involved in chromatin condensation, microtubule binding, and nuclear pore complex organization. On this basis, Nek6 has been proposed to be a high confidence hub kinase with an expansive network of substrates involved in diverse cellular processes. Moreover, a number of these interactions depend on the N-terminal extension of Nek6 explaining why Nek6 and Nek7 may target different proteins (Vaz Meirelles et al. 2010). However, to date, the only substrates that have been studied in any detail for Nek6 and Nek7 are the kinesin motor, Eg5, which is phosphorylated by Nek6 promoting spindle pole separation (Rapley et al. 2008), and the nuclear pore complex component, Nup98, whose combined phosphorylation by Cdk1 and potentially multiple Neks, including Nek2, Nek6, and Nek7, promotes nuclear pore complex disassembly upon mitotic entry (Laurell et al. 2011).

Checkpoint Neks: Nek10 and Nek11

Nek10 is a protein of 1,125 residues in length (129 kDa) that is unique in this family, in that unlike the other Neks, Nek10 has its catalytic domain in the center of the protein with long N- and C-terminal non-catalytic regions. The catalytic domain is flanked by two coiled-coil motifs, while in addition the N-terminal region contains four armadillo repeats. Nek10 is one of the least characterized members of the family at the present time with, for example, no data yet on its localization. However, the first report into its function has placed Nek10 within the G2/M DNA damage checkpoint, suggesting that Nek10 is required for Erk1/2 activation in response to UV-induced damage (Moniz and Stambolic 2011). In unperturbed cells, Nek10 forms a trimeric complex, interacting with Mek1 via ▶ Raf-1. In response to UV treatment, Mek1 undergoes autophosphorylation and activation in a Nek10-dependent manner; this leads to phosphorylation of Erk1/2 and G2/M arrest. Importantly, though, UV treatment increases neither association of Nek10 with Raf-1 and Mek1, nor Nek10 activity, and there is no evidence that Nek10 directly phosphorylates Raf-1 or Mek1. Hence, it seems likely that other factors or events contribute to the mechanism by which Nek10 modulates Mek1 signaling.

Nek11 exists as at least four splice variants: Nek11 Long (Nek11L; 645 residues, 74 kDa), Nek11 Short (Nek11S; 470 residues, 54 kDa), and two isoforms that are present in databases but have yet to be reported, Nek11C (482 residues, 55 kDa) and Nek11D (599 residues, 69 kDa). Expression of at least the Nek11L isoform is cell cycle regulated being highest from S through to the G2/M phases of the cell cycle (Noguchi et al. 2002). Localization studies revealed that Nek11 localizes to the nucleus, and possibly nucleolus, in interphase cells, as well as spindle microtubules in prometaphase and metaphase cells (Noguchi et al. 2002, 2004). It was also suggested that Nek11 might be phosphorylated by the Nek2 kinase, converting Nek11 into an active conformation (Noguchi et al. 2004); however, this has yet to be verified. More convincingly, Nek11 is implicated in the DNA damage response (DDR). Nek11 activity is increased in response to stalled DNA replication and genotoxic stresses, such as ionizing radiation (IR), and this activation is blocked by caffeine, an inhibitor of the DDR kinases, ATM and ATR (Melixetian et al. 2009; Noguchi et al. 2002). The role of Nek11 in the G2/M DNA damage checkpoint appears to be a central one. Upon DNA damage, ATM and ATR phosphorylate and activate Chk1; this in turn activates Nek11 by phosphorylation of Ser273 (Melixetian et al. 2009). Both Chk1 and Nek11 then phosphorylate Cdc25A on residues that promote binding of the β-TrCP E3 ubiquitin ligase. Ultimately, this results in the proteasomal degradation of Cdc25A arresting the cell in G2. While this model is highly attractive, it has been argued that casein kinase 1, and not Nek11, is the major kinase that regulates the phospho-dependent recruitment of β-TrCP. It is also worth noting that, in *Xenopus*, Erk1/2 can target Cdc25A for degradation through the β-TrCP pathway following genotoxic stress. Clearly, further work is required to determine the relative importance of these kinases in mediating G2/M arrest in response to different forms of DNA damage.

Ciliary Neks: Nek1 and Nek8

Nek1 was the first Nek to be identified in mammals and is also the largest member of the family, being composed of 1,258 residues (145 kDa). It was initially reported to have dual serine-threonine and tyrosine kinase activity in vitro, although this is unlikely to be the case in vivo (Letwin et al. 1992). The first clue to its function came almost a decade later when, completely unexpectedly, mutations of the gene encoding Nek1 were found to be causative in two mouse models for polycystic kidney disease (PKD), named *kat* (for kidney, anemia, testis) and kat^{2J} (Upadhya et al. 2000; Vogler et al. 1999). Cystic kidney diseases are now known to be common hallmarks of ciliopathies, whereby the underlying defect is in the formation or function of the primary cilium. Indeed, this was the first indication

that Nek kinases may have some role in ciliogenesis. Since then, Nek1 has been localized to the primary cilia in a number of different cell types (Mahjoub et al. 2005; Shalom et al. 2008; White and Quarmby 2008). Here, it is proposed to negatively regulate ciliogenesis, as overexpression of wild-type and certain truncated forms of Nek1 inhibit ciliogenesis, whereas overexpression of mutants predicted to be catalytically inactive do not (White and Quarmby 2008). Interestingly, Nek1 may also be involved in cell cycle control and, specifically, the DDR. Like Nek11, Nek1 activity is elevated in response to IR and Nek1-deficient cells are sensitive to DNA damage (Polci et al. 2004). Furthermore, although to date no *bona fide* substrates have been identified for Nek1, a number of proteins involved in DNA double-strand break repair were found as Nek1 binding partners (Surpili et al. 2003).

Nek8 is a smaller protein than Nek1, being 692 residues in length (80 kDa). Interestingly, Nek8 shares a very similar domain organization to Nek9, with Nek8 also having an RCC1-like domain. On this basis, one might expect that Nek8 would have a role in mitosis. However, subsequent to identification of the mouse Nek1 PKD model, a missense mutation in the RCC1-like domain of Nek8 was identified in the *jck* mouse model of autosomal recessive juvenile PKD (Liu et al. 2002). Consistent with this, Nek8 was also found to localize to the primary cilia (Quarmby and Mahjoub 2005; Sohara et al. 2008). In fact, Nek8 concentrates in the proximal region of the cilia, known as the inversin compartment, a localization that is dependent on the inversin protein (Shiba et al. 2010). Inversin is a protein encoded by a gene that is mutated in the human childhood kidney disorder, nephronophthisis (NPHP). This disease is a typical ciliopathy with at least nine candidate Nphp genes identified so far. Importantly, Nek8 turns out to be Nphp9, thus implicating the Neks in human inherited disease for the first time (Otto et al. 2008). Mutations in Nek8 found in NPHP patients lead to loss of Nek8 localization from the cilia (Trapp et al. 2008). Nek8 has also been found to interact with ► polycystin-2 (PC-2), a causative gene for human autosomal dominant PKD (ADPKD), with abnormal phosphorylation of PC-2 detected in cells from the *jck* mouse (Sohara et al. 2008). To date, though, much still remains to be learnt about the cellular basis of renal cyst formation. Thus, it remains unclear whether defects in Nek1 and Nek8 cause cystic kidney disease directly, by interfering with the structure of the primary cilium itself, or more indirectly, by abrogating cilia-dependent signaling. It is also formally possible that these Neks somehow relay signals between the cilium, at the time when the cell is quiescent, and the mitotic spindle, once it reenters the cell cycle.

Signal Transduction Neks: Nek3

In contrast to most other Neks, there is no clear indication that **Nek3** (506 residues, 58 kDa) is a cell cycle–dependent kinase with conflicting reports over whether Nek3 expression in elevated in dividing or quiescent cells. Localization studies indicate that Nek3 is predominantly cytoplasmic, with no evidence to date that Nek3 associates with centrosomes (Tanaka and Nigg 1999). Unexpectedly, however, yeast two-hybrid and co-immunoprecipitation studies found a direct interaction between Nek3 and members of the ► Vav family of guanine nucleotide exchange factors that was enhanced in response to signaling from the prolactin receptor (Miller et al. 2005). These studies suggested that prolactin receptor stimulation induces Nek3 kinase activity causing Vav2 to interact with the kinase domain of Nek3 and become phosphorylated. Phosphorylated Vav proteins then activate downstream signaling targets involved in tumor progression. Indeed, overexpression of Nek3 potentiated prolactin-mediated cytoskeletal reorganization of cells; however, if Nek3 was depleted then cytoskeletal reorganization was attenuated, as was cell migration and invasion. This is therefore the first report of a Nek kinase being involved in growth-related signaling events. However, Nek3 has also been reported to more directly regulate cytoskeletal dynamics in neurons by altering levels of acetylated tubulin, thus raising the possibility of a role for Nek3 in neuronal disorders (Chang et al. 2009).

Nek4 and Nek5

Little research has been carried out on **Nek4** (781 residues, 90 kDa). However, a recent study implies that this kinase might also play a role in microtubule regulation, as changes in Nek4 expression led to altered sensitivity of cells to microtubule poisons (Doles and Hemann 2010). There is no specific published data yet on **Nek5** (708 residues, 81 kDa). Intriguingly, though, Nek5 was identified in a microarray analysis of FOXJ1 target genes. This transcription factor governs motile cilia assembly by regulating genes involved in cilia biogenesis and function, suggesting that Nek5 may contribute to ciliogenesis in multiciliated cells.

Nek Kinases and Cancer

There is increasing evidence implicating Nek kinases in cancer. Most commonly, this involves upregulated expression, although a few rare mutations have been identified in cancer genome screens. However, whether elevated expression or mutation contributes to the transformed phenotype remains an important but currently unanswered question. The current data on Nek kinases and cancer can be summarized as follows. Nek1 may be required to protect genome stability as cells deficient in Nek1 form tumors in mice (Chen et al. 2011); this would be consistent with it having a role in the DNA damage checkpoint. Nek2 expression levels are frequently upregulated in a wide range of cancer cell lines and primary tumors, and the Nek2 gene has been reported to be amplified in breast and gastric cancers (Hayward and Fry 2006; Kokuryo et al. 2007; Suzuki et al. 2010; Tsunoda et al. 2009). Nek3 is enriched in breast carcinomas (Miller et al. 2005, 2007), while the Nek3 gene is located in a chromosomal region that is frequently deleted in several types of human cancer and a polymorphism in the Nek3 gene is linked to prostate cancer. Nek4 appears to be frequently deleted in lung cancer, although interestingly this might make tumors more sensitive to particular microtubule poisons (Doles and Hemann 2010). The Nek6 gene is found at a locus for which loss of heterozygosity is associated with several cancers, while, like Nek2, the expression of Nek6 is widely elevated in cancer cell lines and tumors (Nassirpour et al. 2010). Nek7 is also overexpressed in breast, colon, and larynx cancers (Capra et al. 2006), and mutations in Nek7 have been identified in lung and ovarian cancers. Nek8, although primarily associated with ciliogenesis, is upregulated in some breast tumors. Although this could suggest that Nek8 has alternative functions in cell cycle control, a link between cilia-dependent signaling and tumorigenesis is beginning to emerge. A few point mutations have been identified in Nek9, although their relevance remains unclear, and a link between Nek9 expression and human cancers is not yet well established. On the other hand, Nek10 has been identified as a candidate breast cancer susceptibility gene and mutations of this kinase have been reported in several human cancers, including lung. Finally, Nek11 expression is increased during colorectal cancer development suggesting that this kinase too is implicated in cancer progression (Sørensen et al. 2010).

Summary

In summary, of the 11 human Nek kinases, Nek2, Nek6, Nek7, and Nek9 function in mitotic regulation, Nek10 and Nek11 in the DDR, Nek3 in signal transduction, and Nek1 and Nek8 in ciliogenesis. Currently, Nek4 and Nek5 have no assigned function. Hence, although these kinases are related to each other in sequence, it is clear that they are regulated in quite different manners and function in diverse processes. However, based on studies in both lower and higher eukaryotes, the common underlying theme is that many, albeit perhaps not all, Neks contribute to microtubule organization during cell cycle progression and/or ciliogenesis. Importantly, advances in understanding how this family contributes to cell cycle events make these kinases attractive targets for therapeutic interventions in human cancer. RNAi-mediated depletion of Nek2 and Nek6, for example, has been found to inhibit proliferation of cancer cell lines and tumor xenografts, and

selective pharmacological inhibitors are beginning to be generated (Hayward et al. 2010; Jeon et al. 2010; Jin et al. 2006; Kokuryo et al. 2007; Nassirpour et al. 2010; Qiu et al. 2009; Suzuki et al. 2010; Tsunoda et al. 2009; Whelligan et al. 2010; Wu et al. 2008). Hence, although a better understanding of the basic biology of NIMA-related kinases is still required, there is growing evidence that Neks could serve as important targets for the management of cancers. Finally, the identification of mutations in Nek8 as causative for an inherited human disease emphasizes the need for further research into the processes and pathways in which this family of kinases operate.

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Nemo-Like Kinase

▶ NLK

Nephropontin

▶ Osteopontin (*Spp1*)

Nerve Growth Factor

▶ NGF

Nerve Growth Factor (Beta Polypeptide)

▶ NGF

Nerve Growth Factor, Beta

▶ NGF

Nerve Growth Factor, Beta Polypeptide

▶ NGF

Nerve Growth Factor, Beta Subunit

▶ NGF

NESP55

▶ *GNAS* Complex Locus

Net1 (Neuroepithelial Cell Transforming Gene 1 Protein)

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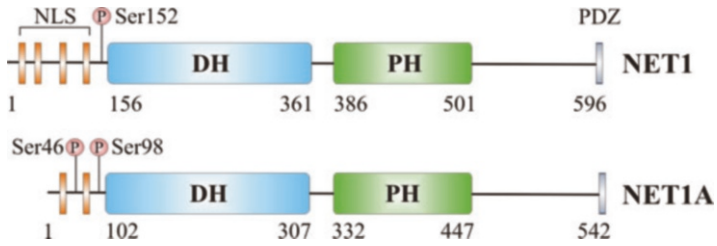
Synonyms

ARHGEF8; Net1A

Historical Background

Rho family small GTPases control multiple cell functions, including organization of the actin cytoskeletal, cell motility and invasion, and cell cycle progression. They act as molecular switches, cycling between their active GTP-bound and inactive GDP-bound states. When bound to GTP, Rho GTPases initiate intracellular signaling by binding to downstream proteins known as effectors (Jaffe and Hall 2005). The activation state of Rho GTPases is controlled by three families of proteins, known as GDP exchange factors (Rho GEFs), GTPase activating proteins (Rho GAPs), and guanine nucleotide dissociation inhibitors (Rho GDIs). Rho GEFs activate Rho proteins by stimulating the release of GDP, thereby allowing the binding of GTP (Rossman et al. 2005; Meller et al. 2005). Rho GAPs accelerate the intrinsic GTPase activity of Rho proteins to hydrolyze GTP to GDP



Net1 (Neuroepithelial Cell Transforming Gene 1 Protein), Fig. 1 Domain organization of Net1 proteins. Nuclear localization signal (NLS) sequences are shown in *orange*. The catalytic Dbl homology (DH) domain is shown in *light blue*. The pleckstrin homology (PH) domain is shown in *light green*. The C-terminal PDZ domain-binding site (PDZ) is shown in *dark blue*. Serine 152 is a negative regulatory Pak1 phosphorylation site (serine 98 in Net1A). Serine 46 is a negative regulatory AMPK phosphorylation site. *Numbers* refer to amino acids within human Net1 and Net1A

(Tcherkezian and Lamarche-Vane 2007). Rho GDIs sequester inactive, GDP-bound Rho GTPases in the cytosol, thereby maintaining and stabilizing a ready pool of Rho proteins for subsequent activation (DerMardirossian and Bokoch 2005). Within this regulatory network, it is the Rho GEFs that are primarily responsible for translating upstream signaling events into Rho protein activation.

During the mid-1990s a number of groups used NIH 3T3 cell focus formation assays to identify candidate oncogenes. In this type of screen, 3T3 cells are made to express a cDNA expression library derived from cancer cells and then tested for subsequent loss of contact inhibition. This can be observed through the formation of cell foci, which are essentially masses of transformed cells. The neuroepithelial transforming gene 1 (Net1) was discovered in this way using a cDNA library derived from neuroepithelioma cells (Chan et al. 1996). The Net1 cDNA initially cloned from this screen lacked the coding sequence for the first 145 amino acids of full length Net1, which was subsequently found to be critical to its transforming activity. NIH 3T3 cells expressing truncated Net1 exhibited anchorage-independent growth in soft agar assays and were tumorigenic when injected into nude mice. Thus, Net1 fit the requirements for a putative oncogene.

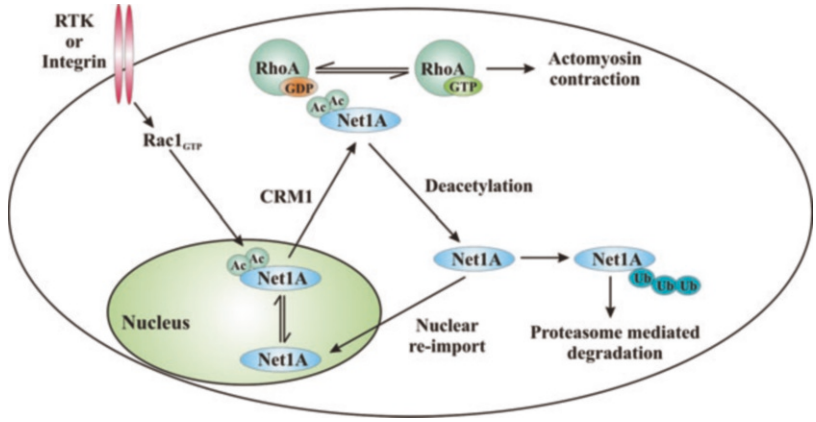
Since that time it has become appreciated that Net1 belongs to a large family of Rho GEFs containing tandem diffuse B cell lymphoma (Dbl) and pleckstrin homology (PH) domains. There are nearly 70 members within the Rho GEF family that exhibit different specificities for Rho proteins, distinct regulatory mechanisms, and unique tissue distributions (Rossman et al. 2005). Net1 was originally described as being widely expressed in humans, with lower levels of expression in the heart, brain, and pancreas (Chan et al. 1996). It was later shown that Net1 isoforms are also widely expressed in mice, with substantial expression in nearly all tissues except the brain, heart, and skeletal muscle (Zuo et al. 2014). Two isoforms of Net1 exist, Net1 and Net1A, which are identical except for their N-terminal regulatory domains (Fig. 1). The N-terminus of the longer Net1 isoform consists of an 85 amino acid span that contains two nuclear localization signal (NLS) sequences (Schmidt and Hall 2002), while the unique 31 amino acid portion of Net1A has no identified function. The rest of the N-terminus is shared between Net1 isoforms, and this region contains two additional NLS sequences (Song et al. 2015). The DH domains of Net1 proteins bind to RhoA, and in conjunction with the PH domain mediate RhoA activation. Although PH domains were originally characterized as phosphoinositide-binding domains, the PH domain of Net1 has not been shown to bind phospholipids. The shared C-terminus of Net1 isoforms is 95 amino acids in length and contains a C-terminal PDZ domain-binding site which mediates interaction with Dlg1 and Magi1b (Garcia-Mata et al. 2007; Carr et al. 2009; Dobrosotskaya 2001).

Regulation of Net1 Activity

Net1 isoform transcription is precisely regulated by alternative promoters which allows for their differential expression (Dutertre et al. 2010). For example, in MCF7 breast cancer cells estrogen potently stimulates Net1 transcription and weakly downregulates Net1A transcription. On the other hand, progesterone stimulates the transcription of both Net1 and Net1A in MCF7 and T47D breast cancer cells (Dutertre et al. 2010) (Richer et al. 2002). TGFβ has been reported to stimulate Net1A expression in HaCaT human keratinocytes and ARPE-19 retinal pigment epithelial cells by Smad2/3- and Erk1/2-dependent pathways (Shen et al. 2001; Papadimitriou et al. 2011; Lee et al. 2010). Moreover, Net1 isoform mRNAs are subject to downregulation by miR-24 in keratinocytes and miR-22 in K562 leukemia cells (Papadimitriou et al. 2011; Ahmad et al. 2014). Other studies have not differentiated between Net1 isoforms when assessing Net1 expression. For example, IL-2 stimulation increases Net1 expression in Kit 225 human lymphocytes (Mzali et al. 2005). Both ▶ TNFα and LPA stimulate Net1 expression in AGS gastric cancer cells (Leyden et al. 2006; Murray et al. 2008). RANKL stimulates Net1 expression in RAW264.7 mouse macrophages (Brazier et al. 2006). Sonic hedgehog stimulates Net1 expression in myocytes during limb bud development (Hu et al. 2012).

Once translated, the cellular activity of Net1 proteins is highly regulated by subcellular localization, posttranslational modification, and degradation. Net1 isoforms contain multiple NLS sequences in their amino-termini, and when overexpressed in cells they accumulate in the nucleus (Schmidt and Hall 2002; Song et al. 2015; Qin et al. 2005). Since RhoA activation occurs at the plasma membrane, this means that nuclear sequestration of Net1 isoforms is a mechanism to negatively regulate their activities. The longer Net1 isoform has not been reported to localize outside the nucleus. However, integrin ligation, LPA, TGFβ, and EGF have all been reported to cause cytoplasmic accumulation of Net1A (Papadimitriou et al. 2011; Murray et al. 2008; Carr et al. 2012, 2013). These observations suggest that only Net1A controls RhoA activation, which is supported by data indicating that knockdown of Net1A, but not Net1, impairs breast cancer cell motility and invasive capacity in vitro (Carr et al. 2013). Signaling pathways controlling Net1A cytoplasmic accumulation are not well defined. Integrin activation, LPA, and EGF all require activation of Rac1 for cytoplasmic localization of Net1A, and overexpression of constitutively active Rac1 potently stimulates Net1A cytoplasmic accumulation (Song et al. 2015; Carr et al. 2012, 2013). Net1A is also acetylated on two sites surrounding its second NLS, which is required for cytoplasmic localization following EGF stimulation (Song et al. 2015). Net1A export from the nucleus is likely to require the nuclear exportin CRM1, as treatment of cells with leptomycin causes the Net1 truncation mutant Net1ΔN, which still contains one NLS, to accumulate in the nucleus (Schmidt and Hall 2002). There is no identifiable nuclear export sequence (NES) in Net1 isoforms, suggesting that Net1A must bind to another protein for CRM1-mediated nuclear export. These data support a model in which ligand stimulation activates Rac1, which signals to Net1A to cause CRM1-mediated nuclear export. Reimport into the nucleus would be slowed by acetylation of the second of the two NLS sequences, thereby allowing cytoplasmic accumulation and RhoA activation (Fig. 2).

Once in the cytoplasm Net1A activity is negatively regulated by phosphorylation and proteasome-mediated degradation. Net1 is phosphorylated by the Rac and Cdc42-regulated kinase ▶ Pak1, which inhibits its catalytic activity (Alberts et al. 2005). Presumably Net1A is also phosphorylated by Pak1, as these phosphorylation sites are conserved between isoforms. Pak1 phosphorylates Net1 on serines 152, 153, and 538 in vitro, and on serine 152 in cells. Serines 152 and 153 are contained within an amino-terminal extension of the DH domain that is restricted to a subset of RhoA-specific GEFs (Alberts and Treisman 1998). Substitution of serines 152 and 153 with the phosphorylation mimetic glutamate inhibits the ability of Net1 to catalyze GDP exchange on RhoA in vitro and blocks the ability of expressed Net1ΔN to cause



Net1 (Neuroepithelial Cell Transforming Gene 1 Protein), Fig. 2 Regulation of the subcellular localization of Net1A. Exposure of cells to receptor tyrosine kinase (RTK) or integrin ligands stimulates Rac1, which then causes CRM1-mediated nuclear export of Net1A. Once in the cytoplasm Net1A stimulates RhoA activation, leading to actomyosin contraction. Acetylation of Net1A reduces its rate of nuclear re-import. Once deacetylated, Net1A is either imported into the nucleus or targeted for proteasome-mediated degradation

actin stress fiber formation in cells. Similarly, overexpression of constitutively active Pak1 blocks actin stress fiber formation caused by coexpression of wild type Net1, but not by a Net1 mutant containing alanine substitutions at serines 152 and 153 (Alberts et al. 2005). Net1A is also subject to phosphorylation on serine 46 by AMPK, which inhibits its ability to stimulate invadopodia formation (Schaffer et al. 2015).

The cellular activity of Net1A is tightly regulated by ubiquitylation (Carr et al. 2009; Papadimitriou et al. 2011). Net1 isoforms contain a C-terminal PDZ domain-binding site that mediates interaction with proteins within the Dlg1 family (Garcia-Mata et al. 2007; Carr et al. 2009). Interaction of Net1A with Dlg1 prevents its ubiquitylation in MCF7 breast cancer cells and increases its half-life from 25 min to 10^5 h. This effect is specific for Dlg1, since coexpression of the Net1-interacting PDZ domain protein Magi1b does not stabilize Net1A (Carr et al. 2009). The truncation mutant Net1ΔN is stable when expressed in MCF7 cells, indicating that the amino-terminus of Net1A is important for regulating its degradation (Carr et al. 2009). Interestingly, interaction of endogenous Net1A with Dlg1 in these cells is dependent on the formation of E-cadherin-mediated cell contacts, since disruption of these contacts causes a rapid and dramatic increase in Net1A ubiquitylation. Net1A is also degraded following integrin ligation, as treatment with the proteasome inhibitor MG132 prolongs cytoplasmic accumulation of Net1A following cell replating on collagen (Carr et al. 2012).

Regulation of Actin Cytoskeletal Organization by Net1

Net1 has been shown to act as a GEF for RhoA and RhoB, but not Rac1 or Cdc42 (Alberts and Treisman 1998; Srougi and Burridge 2011). When overexpressed in mouse fibroblasts, Net1 stimulates the formation of actin stress fibers, which is a hallmark of RhoA activation (Alberts and Treisman 1998). The N-terminal truncation mutant Net1ΔN is far more efficient at stimulating stress fiber formation than full length Net1 or Net1A, which is due to the enhanced cytoplasmic localization of Net1ΔN (Qin et al. 2005). Net1 isoforms have also been reported to mediate actin stress fiber formation in human keratinocytes and retinal pigment epithelial cells following stimulation with TGFβ (Shen et al. 2001; Papadimitriou et al. 2011; Lee et al. 2010). In addition, Net1A promotes actin polymerization and focal adhesion maturation in MCF7 cells during adhesion (Carr et al. 2012). Similar to other

RhoA-subfamily GEFs, the ability of Net1 isoforms to stimulate actin stress fiber formation is dependent on downstream activation of the RhoA effector kinases ▶ ROCK1 and ▶ ROCK2 (Tran et al. 2000).

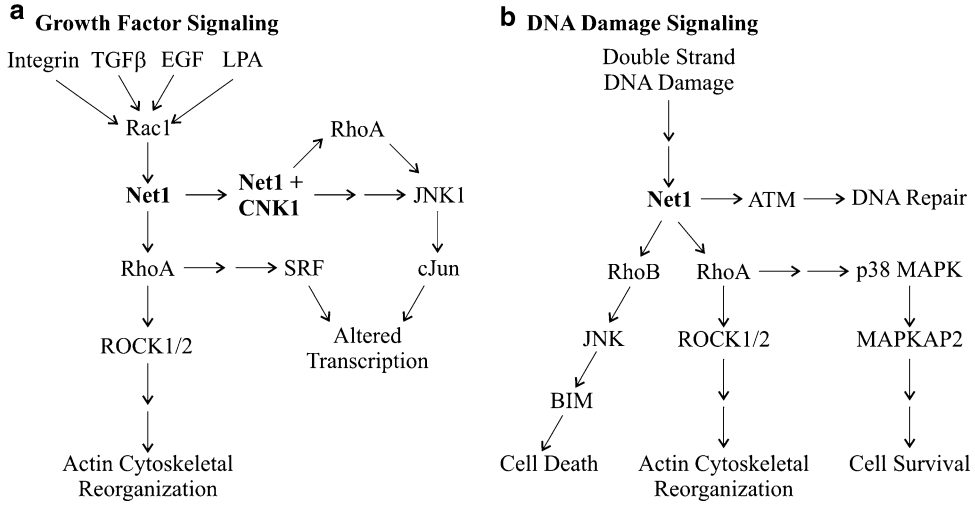
Additional Physiological Roles of Net1

Net1 plays important roles during development and differentiation. For example, in chicken epiblasts Net1A expression is required for RhoA activation at the basal surface and maintenance of the integrity of the basement membrane (Nakaya et al. 2008). During gastrulation, epiblasts undergo an epithelial to mesenchymal transition (EMT) that is accompanied by loss of Net1A expression on the basal membrane, and enforced expression of RhoA or Net1A in these cells prevents basement membrane breakdown and EMT (Nakaya et al. 2008). Similarly, overexpression of xNet1A in *Xenopus* embryos inhibits gastrulation movements (Miyakoshi et al. 2004). Long-term treatment of HaCaT keratinocytes with TGFβ, which causes EMT, also downregulates Net1A expression (Papadimitriou et al. 2011). These data indicate that cells must downregulate Net1A for EMT to occur. Alternatively, Net1A expression is strongly upregulated during mouse osteoclast differentiation in vitro, and inhibition of Net1A expression prevents cell fusion that is required for osteoclast formation (Brazier et al. 2006). All of these processes are associated with dramatic changes in actin cytoskeletal organization or altered cell motility, both of which are RhoA-regulated events.

Recently it has been shown that Net1 expression is required for mouse mammary gland development during puberty (Zuo et al. 2014). Mice with a *Net1* deletion are viable and have offspring at normal Mendelian ratios, but experience a delay in mammary gland development. This is characterized by reduced invasion of milk ducts into the mammary fat pad and reduced branching complexity of the ductal tree. This is due to blunted RhoA signaling to the actomyosin contractile apparatus and reduced expression of estrogen receptor α. Although the ductal tree eventually fills the fat pad, ductal branching remains incomplete (Zuo et al. 2014).

By regulating the activation of RhoA and MAP Kinases, Net1 also impacts transcription factor activation. For example, overexpression of the N-terminal deletion mutant Net1ΔN in NIH 3T3 cells stimulates serum response factor (SRF) activation. This requires activation of both RhoA and the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) pathway (Alberts and Treisman 1998). In Hela cells, Net1ΔN expression stimulates SAPK/JNK activation through interaction with the scaffold protein connector enhancer of KSR 1 (CNK1), which results in efficient activation of the transcription factor c-Jun (Jaffe et al. 2005). Stimulation of c-Jun activity by Net1ΔN requires both RhoA and SAPK/JNK activation, and stimulation of c-Jun activity by the extracellular ligand LPA requires endogenous CNK1 expression. However, it is unknown whether LPA-stimulated c-Jun activity also requires endogenous Net1. Net1 also interacts with CARMA1 and CARMA3 to stimulate NFκB activation in Jurkat T cells and HEK293 cells, respectively (Vessichelli et al. 2012). This effect is specific for ligands such as PMA/ionomycin, LPA, and IL1β, but not TNFα (Fig. 3a).

Net1 plays a role in DNA damage signaling (Fig. 3b). Net1 is dephosphorylated on the negative regulatory site serine 152 following exposure of Hela cells to ionizing radiation (IR) (Guerra et al. 2008). In addition, RNAi-mediated knockdown of Net1 expression prevents RhoA activation and sensitizes these cells to IR-induced apoptosis. RhoA activation by Net1 may occur exclusively in the nucleus (Dubash et al. 2011). Net1 knockdown also blocks activation of p38 MAPK and its downstream substrate MAPKAP2, both of which are required for cell survival following IR exposure (Guerra et al. 2008). Net1, along with the Rho GEF Ect2, has also been shown to stimulate RhoB activation after IR exposure in MCF7 cells, an event which promotes cell death (Srougi and Burridge 2011). However, whether Net1 promotes cell survival or death after IR is controversial, since another group found that Net1 knockdown protected MCF7



Net1 (Neuroepithelial Cell Transforming Gene 1 Protein), Fig. 3 Regulation of cell signaling by Net1. Net1 proteins respond to diverse stimuli to contribute to intracellular signaling following exposure to (**a**) extracellular growth factors or (**b**) double-strand DNA damage

cells from IR-mediated cell death (Oh and Frost 2014). In this study it was also found that inhibition of Net1 expression reduced ATM activation and H2AX phosphorylation. Surprisingly, overexpression of wild type or catalytically inactive Net1A also suppressed ATM activation. Expression of constitutively active RhoA or RhoB did not affect ATM. These data suggest that Net1A controls DNA damage signaling in a Rho GTPase independent manner.

Net1 and Cancer

Net1 was originally identified as a potential oncogene in mouse fibroblasts (Chan et al. 1996), and subsequent work suggests that it may play an important role in human cancer. For example, Net1 transcripts are overexpressed in human gastric and hepatocellular cancers, as well as gliomas (Leyden et al. 2006; Shen et al. 2008; Tu et al. 2010). In gastric cancer this may be due to alternative polyadenylation which shortens the 3′ UTR of Net1, resulting in enhanced expression (Lai et al. 2015). In estrogen receptor positive breast cancer patients, coexpression of Net1 protein with alpha6beta4 integrin is predictive of decreased distant metastasis-free survival (Gilcrease et al. 2009). This study did not distinguish between Net1 isoforms. In a retrospective analysis of breast cancer patients, high expression of Net1 mRNA, but not Net1A, was prognostic for decreased metastasis-free survival (Dutertre et al. 2010).

Net1 isoforms appear to contribute to cancer cell function in an isoform-specific manner, such that the longer Net1 isoform primarily contributes to proliferation while the shorter Net1A isoform contributes to motility. For example, siRNA knockdown of both Net1 isoforms in AGS gastric cancer cells inhibits their proliferation as well as their ability to invade a Matrigel extracellular matrix (Leyden et al. 2006; Murray et al. 2008). However, only knockdown of Net1, but not Net1A, inhibits MCF7 cell proliferation (Dutertre et al. 2010). This may be a due to a role for the Net1 isoform in controlling mitotic progression (Menon et al. 2013). On the other hand, knockdown of Net1A, but not Net1, inhibits motility and invasion of human breast cancer cells (Carr et al. 2013). Accordingly, Net1A, but not Net1, interacts with focal adhesion kinase (FAK) during cell adhesion and colocalizes with FAK in an actomyosin-dependent manner. During Matrigel invasion, Net1A is required for amoeboid, RhoA-dependent, but not integrin-driven, mesenchymal invasion (Carr et al. 2013).

Overexpression of Net1A also drives invadopodia formation, which is inhibited by phosphorylation on serine 46 by AMPK (Schaffer et al. 2015). Taken together these data suggest that Net1 isoforms may contribute to human cancer. Future studies in mouse models of cancer will be required to assess whether the functions of each isoform identified in cancer cells in vitro will apply to tumorigenesis or metastasis in vivo.

Summary

Net1 is a Rho GEF that is specific for the RhoA subfamily of small G proteins. Two isoforms exist in most cells which exhibit differential subcellular distributions and may play distinct roles in the cell. The activities of Net1 proteins are tightly regulated by phosphorylation and ubiquitylation. Net1 proteins play important roles in development and also contribute to cellular differentiation. Net1 proteins may also be aberrantly expressed in human cancers and contribute to cancer initiation and/or progression.

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Net1A

► Net1 (Neuroepithelial Cell Transforming Gene 1 Protein)

Neural Precursor Cell Expressed Developmentally Downregulated 4

► NEDD4

Neural Wiskott–Aldrich Syndrome Protein

► N-WASP

Neural-Specific Protein-Tyrosine Phosphatase

▶ Striatal-Enriched Protein-Tyrosine Phosphatase (STEP)

Neuroendocrine Secretory Protein 55

▶ *GNAS* Complex Locus

Neurofibromin 2

▶ Merlin (*NF2*)

Neurofibromin 2 (Bilateral Acoustic Neuroma)

▶ Merlin (*NF2*)

Neurofibromin 2 (Merlin)

▶ Merlin (*NF2*)

Neurogenin1

▶ Neurogenins

Neurogenin2

▶ Neurogenins

Neurogenin3

▶ Neurogenins

Neurogenins

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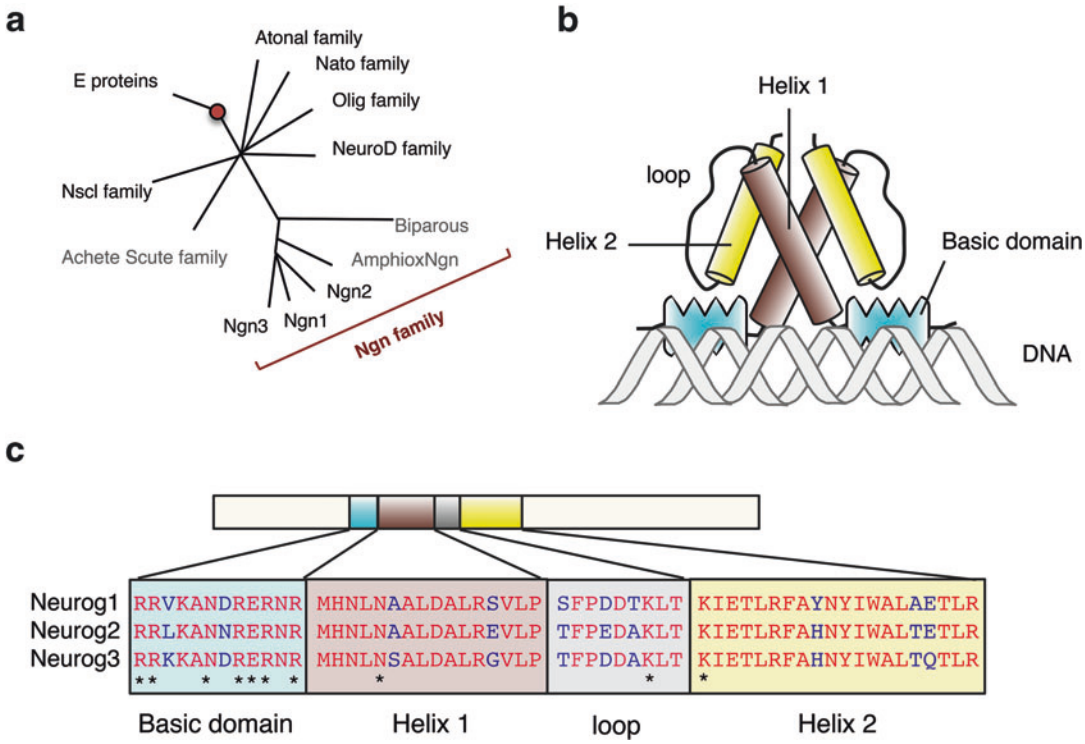
Synonyms

Neurogenin1 [Neurog1; Ngn1; Math4C; bHLHa6; Neurod3]; Neurogenin2 [Neurog2; Ngn2; Math4A; bHLHa8; Atoh4]; Neurogenin3 [Neurog3; Ngn3; Math4B; bHLHa7; Atoh5]

Historical Background

Neurogenins (Neurog or Ngn) belong to a family of proneural proteins that play essential roles in cell fate determination during neurogenesis (Fig. 1a). They belong to a large class of basic helix-loop-helix (bHLH) transcription factors, which contains a conserved helix-loop-helix dimerization domain and a basic region providing DNA-binding activity (Fig. 1b) (Guillemot 1999). Proneural bHLH genes are both necessary and sufficient to initiate neuronal differentiation in a cell-autonomous manner and they are expressed at the correct time and place to select neural precursors for neuronal commitment.

They were initially identified in the fruit fly *Drosophila*, following the discovery of mutant flies lacking specific subsets of external sensory organs. Analysis of this mutant led to the identification of the *achete scute* (*as-c*) gene complex, which encodes for proteins that share sequence similarity with each other and with other transcription factors containing the bHLH domain. Subsequently, another proneural gene in Drosophila, *Atonal* (*ato*) was identified. Atonal mutant flies lack a complementary set of sensory organs, namely internal mechanosensory transduction organs, suggesting that proneural genes not only provide neural competence to ectodermal cells but also encode neuronal subtype information (Guillemot 1999).



Neurogenins, Fig. 1 Structure, function, and conservation. (**a**) Phylogenetic tree of proneural basic helix-loop-helix (bHLH) transcription factors, based on protein sequence similarities in the bHLH domain. The tree is showing the main families of vertebrate genes; members of the Neurogenin family are indicated. (**b**) Schematic representation of the bHLH domain. Dimerization between two factors occurs via the helix-loop-helix (HLH) domain, whereas the basic region contacts the DNA. (**c**) Alignment of Neurog1/2/3 amminoacidic sequence showing high degree of conservation in the bHLH region (aminoacids in *red*). *Asterisks* indicate DNA-binding residues

These works paved the way for the identification of many vertebrate genes that are related to the Drosophila *asc* and *ato* and account for the specification of most of the neuronal cell types present in the mammalian nervous system. The first Neurogenin member (Neurog1) was cloned in 1996 by PCR screening using degenerate oligonucleotide primers based on sequence conservation with other bHLH proteins (Ma et al. 1996). A similar PCR screening subsequently identified two additional Neurog members, Neurog2 and Neurog3, which together with Neurog1, define a novel subfamily of Atonal-related mouse genes (therefore also called Mammalian atonal homologs, Math4A, B, and C) (Sommer et al. 1996). Independently, Neurog2 was also identified in a yeast two-hybrid screen through its ability to interact with another mammalian bHLH factor (Guillemot 1999).

By analogy with the proneural genes in Drosophila, vertebrate Neurogs were predicted to have proneural activities during mammalian neurogenesis since the time of their discovery in the late 1990s. However, it is only in the subsequent 20 years that researchers have extensively characterized the requirements of Neurog activity for the generation of various neuronal subtypes and have disclosed novel functions in the specification of nonneuronal cells.

Biochemical Properties of Neurogenins

Neurogenins are characterized by the presence of specific residues in their bHLH domain, which distinguish them from other bHLH factors. The amminoacidic sequence in the bHLH of the three Neurog members is highly conserved, sharing

82% identity (Fig. 1c), in comparison, for example, to the 67% identity shared between the bHLH domain of Neurogs and another Atonal-related factor NeuroD (Bertrand et al. 2002). The high level of conservation in the bHLH domain reflects the similarities in biochemical properties, including the dimerization with cofactors, the binding to DNA, and the regulation of transcription.

Dimerization and DNA-Binding Properties

Like other bHLH factors, Neurogs bind DNA as heterodimeric complexes (Fig. 1b). The HLH domain mediates Neurog binding to the dimerization partners, such as the ubiquitously expressed bHLH proteins, or E proteins, which are encoded by the Drosophila daughterless (Da) gene or by the vertebrate E2A (with the two alternative products E12 and E47), HEB, and E2-2 genes. Since dimerization is a requirement for DNA binding, factors that interfere with Neurog-E protein interaction can impair Neurog functions. The ID (Inhibitor of differentiation) proteins, which have an HLH domain but lack the basic DNA-binding region, can bind E proteins and sequester them away from Neurogs. Therefore, by competition for E-protein binding, ID proteins inhibit Neurog activity (Bertrand et al. 2002).

Upon dimerization with E proteins, Neurogs use the basic domain to bind DNA sequences; the majority of the residues that directly contact the DNA lie within the basic region, with three additional residues present in the HLH domain (Fig. 1c). The conserved DNA sequence bound by Neurogs contains a core hexanucleotide motif, originally found in the immunoglobulin k-chain enhancer (kE2 motif) and mainly known as E-box (Bertrand et al. 2002).

Regulation of Transcription

Given their role as transcriptional regulators, Neurogs promote differentiation by ultimately activating a series of target genes, whose expression is upregulated by Neurog activity through direct Neurog binding to promoter or enhancer regions. Identification of target genes is crucial to understand the mechanism through which Neurogs operate during neurogenesis and although progresses have been made, little is known about the transcriptional program activated by Neurogs on a genome-wide scale. Several genes have been proposed to be direct Neurog targets, because their expression was deregulated in loss-of-function and gain-of-function experiments; however, it is not clear whether they were directly bound by Neurog or activated further downstream in the transcriptional cascade (Gohlke et al. 2008; Serafimidis et al. 2008). Chromatin immunoprecipitation (ChIP) experiments can directly test whether a genomic region is bound by a transcription factor and when analyzed in combination with gene expression studies, they help identify bona fide target genes. In this way, a few Neurog targets have been characterized, including Neurod1 and Insm1, which are of particular interest since they are involved in neuronal subtype determination in the brain and also in endocrine cell differentiation in the pancreas (Masserdotti et al. 2015; Mellitzer et al. 2006). In more recent years, the identification of additional direct targets also provided crucial insights into novel functions of Neurog2 that extend beyond neuronal specification activities (Heng and Guillemot 2013), such as neuronal migration. For example, the small GTP-binding protein Rnd2 is a Neurog2 direct target that is activated in newborn neurons to control cytoskeletal remodeling and neuronal migration in the cerebral cortex (see below for details).

As well as acting as transcriptional activators at specific promoters and enhancers, Neurogs can also modify the chromatin around their target genes by recruiting epigenetic modifiers. There is indeed evidence that a subunit of SWI-SNF chromatin-remodeling complex, Brg1, interacts with and mediates the transcriptional activity of Neurog1 in Xenopus embryos and teratocarcinoma P19 cells (Heng and Guillemot 2013). Moreover, Neurog1 and Neurog2 have been shown to recruit the transcriptional coactivators p300/CBP and PCAF within multiprotein complexes that exert histone acetyltransferase activity and promote activation of specific genes for example in motoneuron precursors.

In addition to activating neuronal genes, Neurogs promote neuronal differentiation by inhibiting alternative fates. However, there is no

clear evidence that Neurogs can act as transcriptional repressors, but the inhibitory activity is likely to be an indirect effect. For example, Neurog1 inhibits astrocyte fate in the cerebral cortex by sequestering CBP/p300/Smad1 transcriptional complex away from STAT, thus preventing the activation of glial genes (Heng and Guillemot 2013).

Neurogenin Role in Lineage Specification

Neurog1 and Neurog2 in Neurogenesis

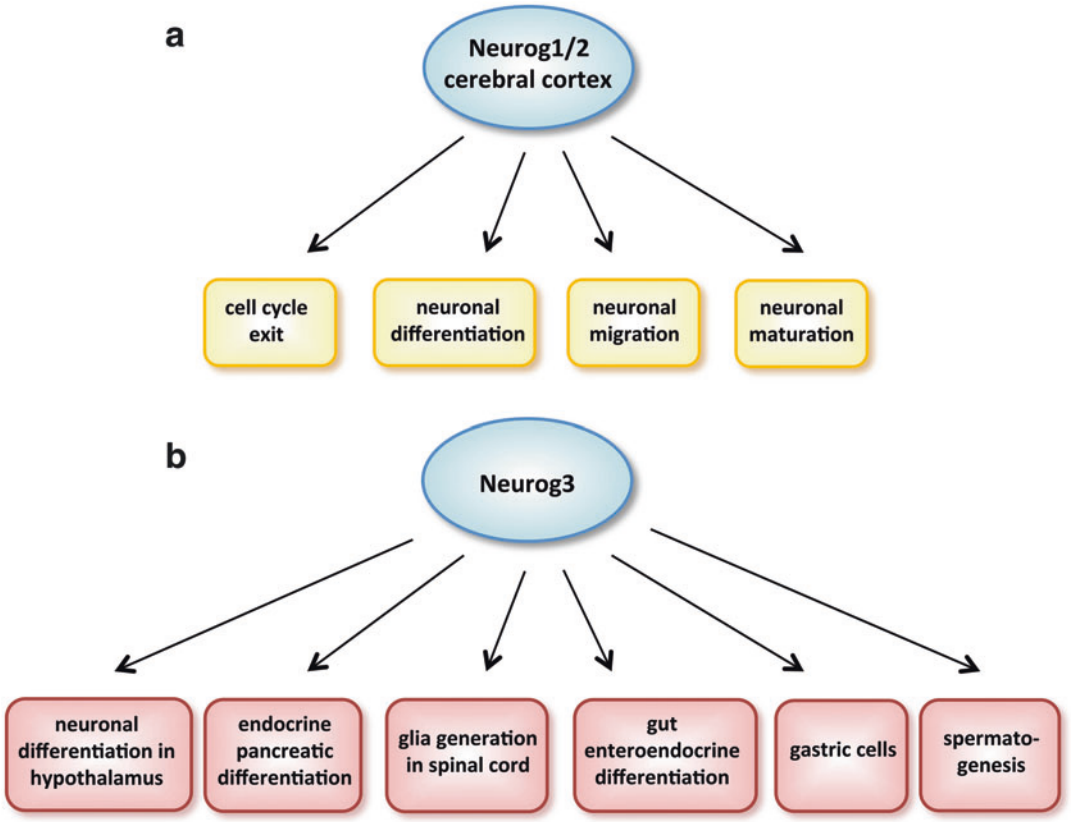
Neurogs are expressed in several regions of the embryonic and adult brain, where they control the generation of specific neuronal subtypes. In particular, the developing cerebral cortex represents the site in which Neurog activities have been predominantly investigated. Neurog1 and 2 are both expressed in the dorsal cortical epithelium, where they instruct both pan-neuronal and cortical-specific identity to dorsally located neural progenitors and promote the generation of cortical pyramidal neurons (Azzarelli et al. 2015; Heng and Guillemot 2013). While Neurog1 knockouts do not exhibit overt phenotype in the cerebral cortex, Neurog2 knockouts show reduced number of early born neurons that occupy deep cortical layers, a phenotype that is exacerbated in Neurog1; Neurog2 double knockouts. Moreover, the neurons that are generated in the absence of Neurogs are not properly specified, because they express some ventral markers, at the expenses of dorsal markers typical of excitatory glutamatergic neurons (Fode et al. 2000; Heng and Guillemot 2013).

Neurog2 role in instructing a cortical glutamatergic neuron phenotype has also been demonstrated by in vitro experiments, in which overexpression of Neurog2 in neural stem cell cultures or in glia cells drives the generation of neurons exhibiting characteristics typical of this lineage (Heinrich et al. 2010). Furthermore, Neurog2 exhibits a similar subtype specification activity in the dentate gyrus of the hippocampus and in the subependymal zone of the adult rodent brain, where it promotes the generation of glutamatergic granule neurons and olfactory neurons. However, the specificity of the neuronal sublineage properties induced by Neurog2 strongly depends on the cellular context, because ectopic Neurog2 expression in the ventral division of the embryonic brain is not sufficient to impart full dorsal identity to ventral progenitors.

The activity of Neurog2 in the cerebral cortex is not only important for the specification of glutamatergic neurotransmitter phenotype, but it also regulates later stages of neuronal development, such as mode of migration, axonal projection, and dendritic morphologies (Heng and Guillemot 2013; Heng et al. 2008) (Fig. 2a). Neurog2 knockout displays clusters of cells in ectopic cortical positions, which result from migration defects. Accordingly, acute deletion of Neurog2 by in utero electroporation of shRNA or Cre in Neurog2 conditional mice revealed abnormal migration with neurons accumulating in the intermediate zone between the proliferative areas and the cortical plate where mature neurons are located. Mechanistic insights showed that Neurog2 regulates migration by activating the transcription of an atypical small GTP-binding protein, Rnd2, which then suppresses the levels of active RhoA and promotes cytoskeletal remodeling necessary for the transition of migratory cells out of the intermediate zone (Heng et al. 2008).

In keeping with Neurog role in the regulation of multiple aspects of cortical neuron development, Neurog also controls the pattern of axonal projections. Neurog2 knockout has abnormal corpus callosum and Neurog2 silencing decreased contralateral projections of upper layer neurons, while redirecting some of the projections toward the lateral cortex or subcortical regions of the same hemisphere, which are typical of lower layer neuronal subtypes. Therefore, through the coordinated regulation of the distinct aspects of cortical neuron development, Neurogs contribute to the early steps of circuit formation and to the overall cortical architecture.

Neurog1 and 2 are also expressed in neural progenitor populations in other regions, including the spinal cord and the peripheral nervous system (PNS), where they play important function in the



Neurogenins, Fig. 2 Neurog functions. (**a**) Scheme of Neurog1 and 2 functions in the developing cerebral cortex. (**b**) Summary of Neurog3 functions in different tissues

specification of both pan-neuronal and subtype-specific properties. For example, Neurog2 expression in the ventral half of the spinal cord contributes to the specification of motoneurons, whereas during PNS development Neurogs specifically activate sensory markers and promote the generation of several cranial and spinal sensory ganglia (Bertrand et al. 2002).

Neurog3 in Neuroendocrine Differentiation

Neurog3 is generally expressed in regions of the brain where Neurog1 and Neurog2 are absent or less abundant. Particular attention has been dedicated to Neurog3 exclusive expression in specific subregions of the hypothalamus, which is a portion of the ventral brain composed by several small nuclei with important neuroendocrine and metabolic functions. In this area, Neurog3 plays a fundamental and nonredundant role in the specification of pro-opiomelanocortin (POMC)+ neurons, which control feeding behavior. Indeed, reduction in the number of POMC+ neurons is observed in defined nuclei of the Neurog3 knockout hypothalamus and leads to obesity due to increased food intake and decreased energy expenditure (Anthwal et al. 2013).

Interestingly, Neurog3 is also the only Neurog member present in regions outside the nervous system, such as the gut, stomach, spermatogonia, and embryonic pancreas, thus playing unique functions in these territories (Fig. 2b). In the developing pancreas, for example, Neurog3 specifies the distinct endocrine cell types that will form pancreatic islets. These cells secrete hormones like insulin and glucagon directly into the bloodstream and control glucose homeostasis. Due to their important role in glucose metabolism, loss or dysfunction of these cells and, in particular,

deficiency in insulin-producing β-cells leads to life-threatening diseases, such as diabetes. As expected from the important role of Neurog3 in the specification of endocrine cells, Neurog3 knockout animals are deprived of endocrine pancreatic cells and die perinatally of diabetes (Gradwohl et al. 2000). In addition to the role in the endocrine pancreas, Neurog3 also specifies cells with endocrine properties in the stomach and gut, thus suggesting that the genetic program activated downstream of Neurog3 may exhibit some degree of conservation in different cell types.

Neurogenin Role in Cell Reprogramming

As a consequence of their powerful activity in cell fate specification, Neurogs have been used to drive in vitro differentiation or transdifferentiation of stem cells and somatic cells into cell types that are lost or damaged during diseases. Consistent with the proneural role of Neurog2 in the embryonic cortex, forced Neurog2 expression in postnatal mouse astrocyte is sufficient to generate neurons that express markers typical of cortical glutamatergic neurons and form excitatory synaptic contacts (Heinrich et al. 2010; Masserdotti et al. 2015) (Fig. 3a). Moreover, direct reprogramming of human fibroblasts into subtype-specific neurons of the dopaminergic or cholinergic lineages has been achieved through the combinatorial expression of Neurog2 with other subtype-specific transcription factors that act as fate determinants (Fig. 3a). Neurog2-mediated generation of specific neuronal subtypes could be particularly beneficial to replace neurons in neurodegenerative disorders like Parkinson's disease, which are characterized by selective degeneration of a single neuronal population.

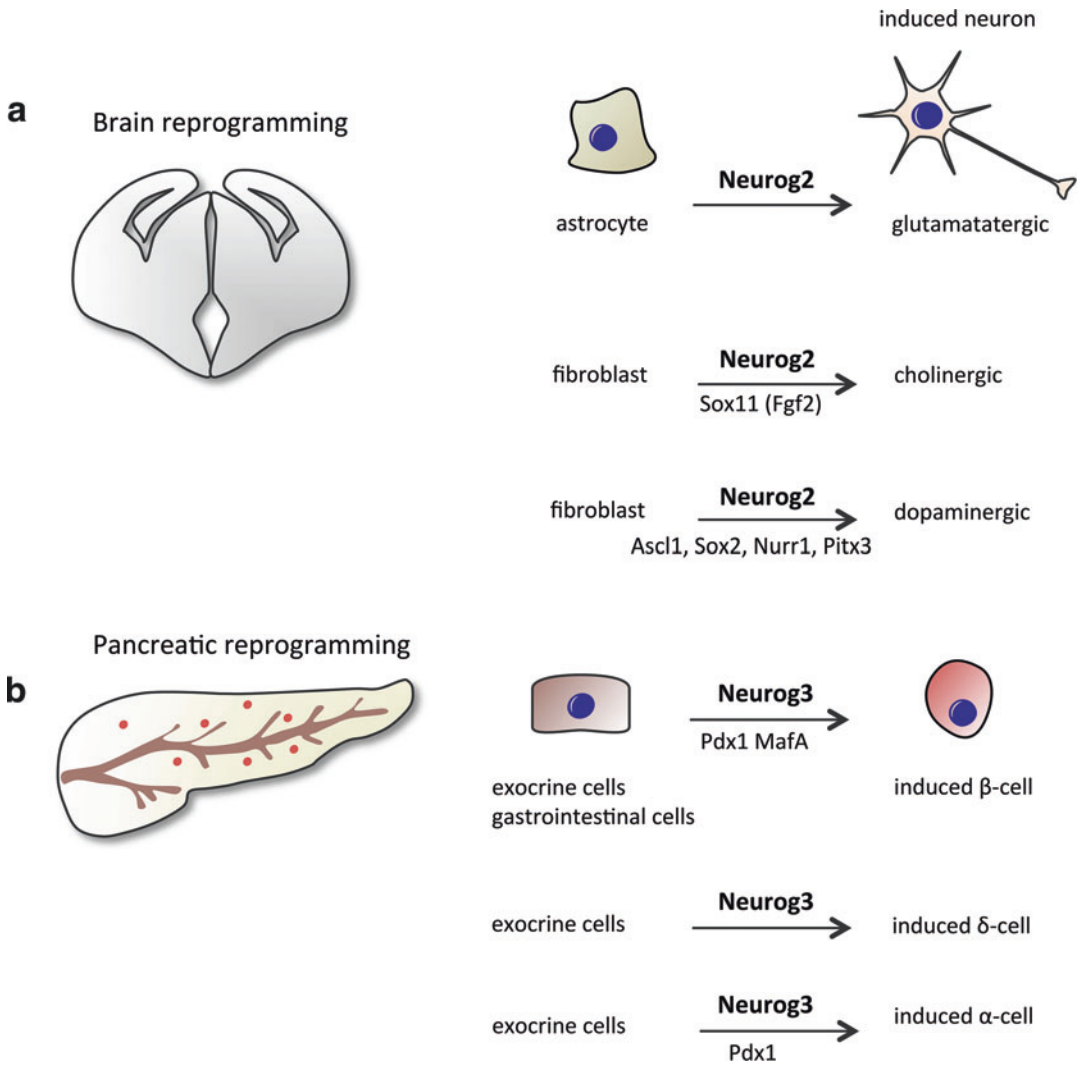
While Neurog2 drives neuronal differentiation from various sources, Neurog3 induces the generation of endocrine pancreatic cells, including insulin-producing β-cells, which are lost in diabetes. Ectopic expression of Neurog3 in cells of the exocrine pancreas, such as acinar or ductal cells, is able to reprogram them into the endocrine lineage. However, the specific endocrine subtypes obtained strongly depends on the presence of additional transcription factors, with the combination of Neurog3, Pdx1, and MafA being specifically required for the generation of insulin-positive cells (Zhou et al. 2008) (Fig. 3b). Therefore, controlled expression of Neurogs in human stem cells or somatic cells might be sufficient to generate in vitro cell types of interest to either study human diseases or to replace lost or damaged tissues.

Regulation of Neurogenin Activity

Lateral Inhibition

The expression of Neurogs during embryonic development is restricted to only a subset of progenitor cells at a given time. This salt-and-pepper distribution is due to a process of lateral inhibition mediated by Notch signaling, whereby progenitor cells expressing Neurogs upregulate the Notch ligand Dll1, which results, on adjacent cells, in Notch signaling activation and repression of Neurog expression (Fig. 4a). Through this mechanism, Neurogs drive cell-autonomous differentiation, while signaling to neighboring cells to remain progenitors, thus preventing premature depletion of the progenitor pool by extensive differentiation.

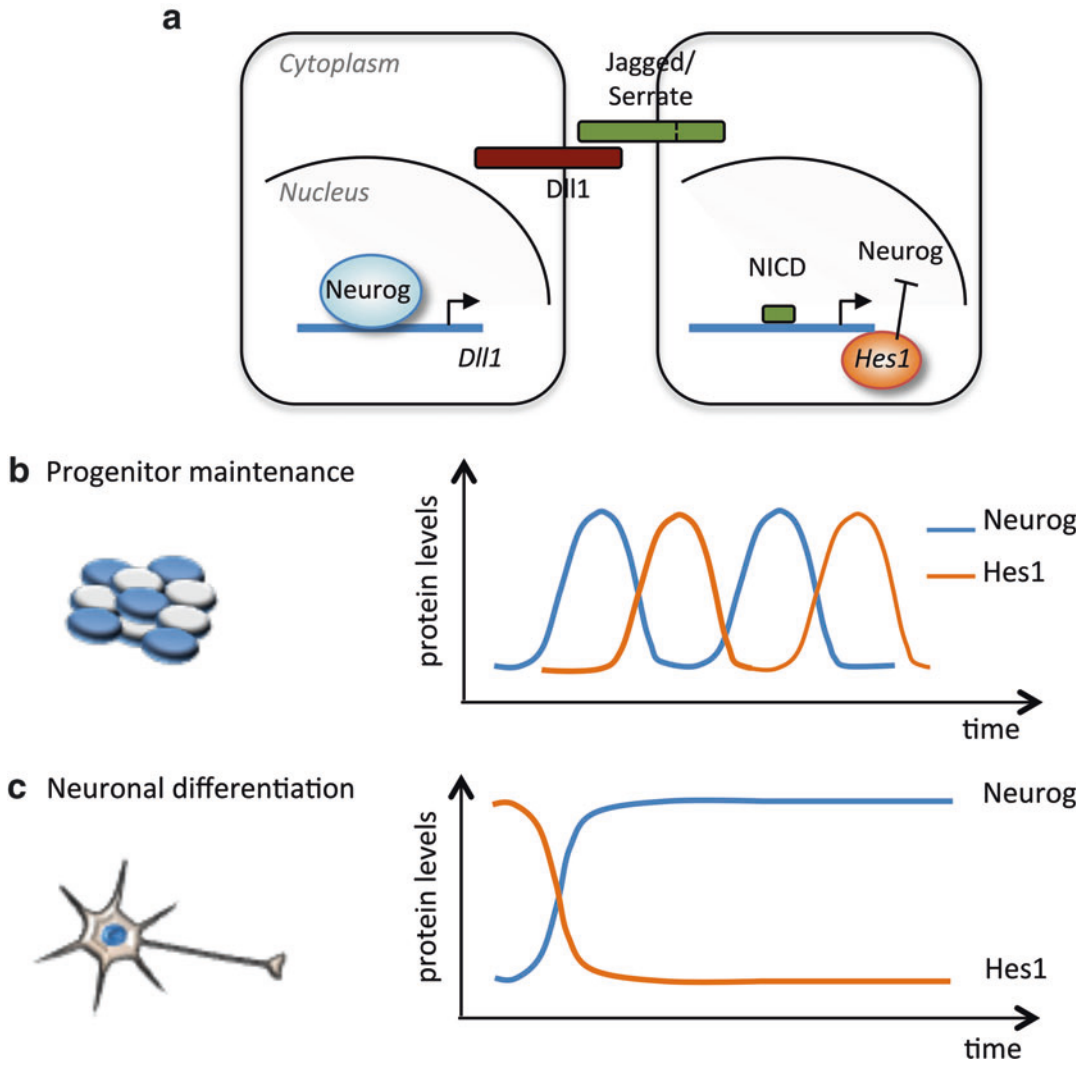
More recently, work from the laboratory of Ryoichiro Kageyama has shown that this process is more dynamic than previously thought. Indeed, the expression of Neurog2 in neural progenitors has been found to oscillate out of phase with the expression of the ▶ Notch signaling effector Hes1 (Shimojo et al. 2008), with a period of 2–3 h (Fig. 4b). The fast dynamics of this process indicate that Neurog-Hes oscillation may occur several times during cell cycle, which in neural progenitor range from 18–24 h. Therefore, Neurog transient and oscillatory expression may still be compatible with cycling progenitors, whereas sustained Neurog expression and Hes1 downregulation mark the transition from proliferating progenitor to differentiating neurons (Fig. 4c).



Neurogenins, Fig. 3 Neurogs in cell reprogramming. (**a**) Neurog2 mediates reprogramming of nonneuronal somatic cells into neurons of various sublineages. (**b**) Neurog3 transdifferentiates cells of the gastrointestinal tract, such as exocrine pancreatic cells, gastric, and intestinal cells into hormone-secreting endocrine pancreatic cells

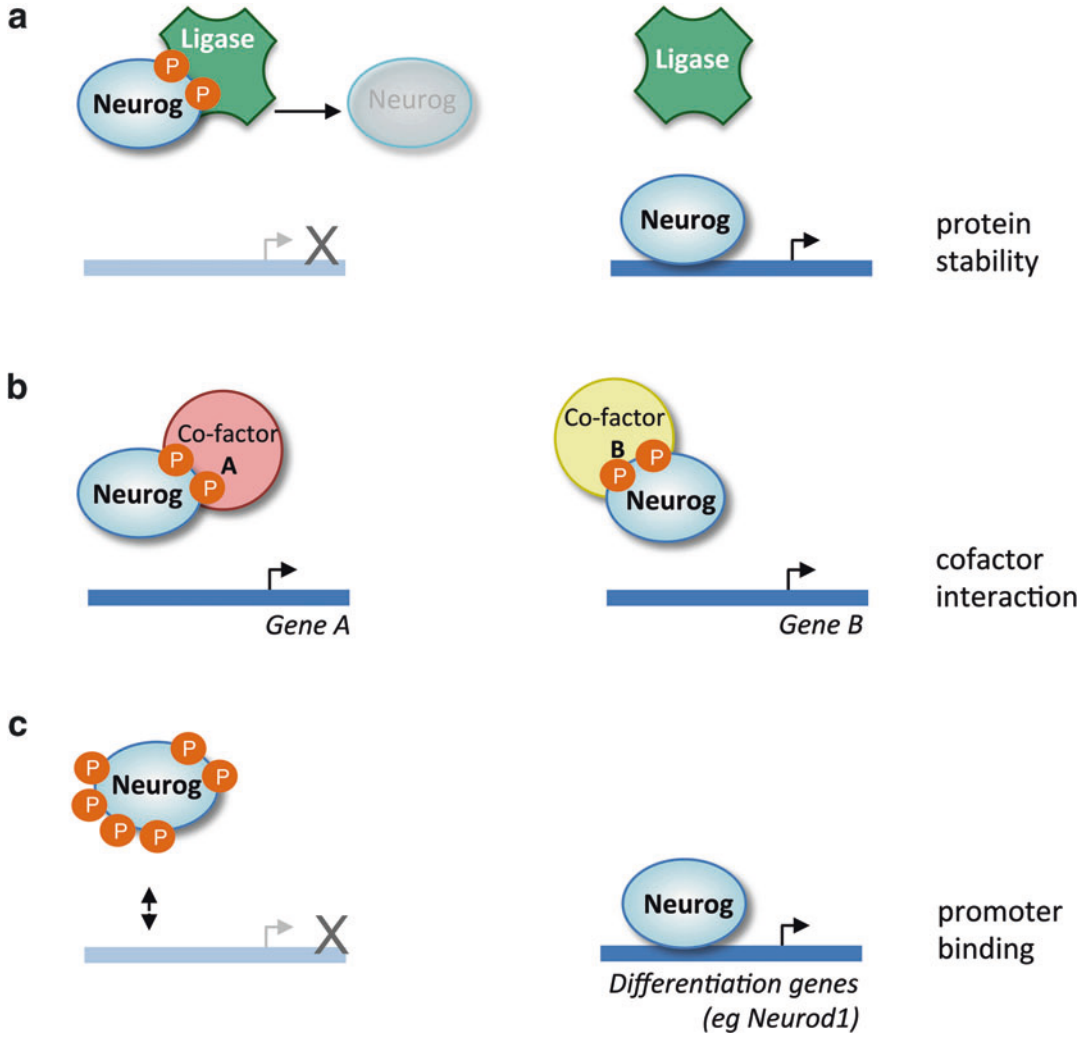
Phosphorylation

Consistent with the oscillatory model, events that alter the pattern of Neurog expression, for example, by stabilizing or destabilizing the short-lived Neurog proteins, ultimately determine changes in cell fate decisions. It has been shown that Neurog3 phosphorylation on two Serines, S183 and S187, by glycogen synthase kinase GSK3β promotes the association with the E3 ubiquitin ligase Fbxw7, which in turn marks Neurog3 for degradation (Fig. 5a) (Sancho et al. 2014). Phospho-mediated recruitment of E3 ubiquitin ligases by Neurog3 is crucial to maintain normal endocrine differentiation in the pancreas, since preventing this phosphorylation increases Neurog3 protein half-life and results in expansion of the endocrine cell compartment.



Neurogenins, Fig. 4 Lateral inhibition with Notch signaling. (**a**) Mechanisms of bHLH lateral inhibition with Notch signaling effectors. Neurogs induce the expression of Dll1, which activates Notch receptor on neighboring cells. Activation of Notch receptor results in Notch intracellualr domain (NICD) release and translocation to the nucleus, where it activates the transcription of Notch effectors like Hes1. Hes1 inhibits Neurog expression. (**b**) Hes1-Neurog oscillation maintains progenitor porliferation. (**c**) Sustained Neurog expression over time, and concomitant decrease of Hes1, marks neuronal differentiation

Neurog2 is also highly phosphorylated on multiple sites, although alteration in protein stability due to phosphorylation has not been reported yet. Phosphorylation of Neurog2 has been shown to regulate the ability of Neurog to interact with additional transcription factors (see next section) or to bind target gene promoters (Fig. 5b, c). For example, in the developing cerebral cortex, Neurog2 is phosphorylated on multiple Serine-Proline (SP) sites by cyclin-dependent kinases (CDKs), which are highly expressed in cycling progenitors (Ali et al. 2011). CDK-mediated phosphorylation on 9 SP sites has been shown to diminish the ability of Neurog2 to specifically bind regulatory regions of differentiation genes, such as Neurod1 and



Neurogenins, Fig. 5 **Neurog regulation by phosphorylation.** (**a**) Phosphorylated Neurog interacts with E3 ubiquitin ligases that in turn mark Neurog for degradation. Preventing Neurog phosphorylation increases protein stability. (**b**) Site-specific phosphorylation of Neurog favors the interaction with a particular cofactor over another. Neurog-cofactor interaction promotes the activation of a precise subset of genes. (**c**) Multisite phosphoryaltion of Neurogs limits the abiltiy to bind regulatory regions of downstream target genes

Neurod4 (Fig. 5c). Therefore, as development progresses and neurogenesis declines, the rise in Neurog phosphorylation correlates with a decrease in Neurog proneural activity.

Interaction with Cofactors

The ability of Neurogs to initiate distinct genetic programs in different cellular contexts raises the question of how they achieve specificity for target genes. Studies on Neuorg2, which specifies distinct neuronal sublineages in different regions of the nervous system, provide crucial insights into the mechanisms of target gene diversification.

Context-dependent functions of Neurog2 result, at least in part, from the differential expression of additional transcription factors, which confer target specificity by binding to specific DNA sequences in the vicinity of the E-box. In ventral spinal cord progenitors, Neurog2 interacts with LIM-homeodomain transcriptional

complexes via the adaptor protein NLI to promote the expression of the motoneuron determination gene Hb9 (Heng and Guillemot 2013). Likewise, Neurog2 binding to the same bridging factor NL1 seems to be required in the cerebral cortex for Neurog2 interaction with a different homeodomain transcription factor, thus resulting in coactivation of cortical neuron genes (Heng and Guillemot 2013).

Interestingly, the interaction with cofactors is highly dynamic and often requires the presence of posttranslational modifications on Neurog proteins. For example, Neurog2 interaction with NL1 in the spinal cord requires the phosphorylation of Neurog2 on Serine 231 and 234 by GSK3β. Whether this site-specific phosphorylation is required for Neurog2-NL1 interaction also in the cerebral cortex is not known, but, in this context, Neurog2 phosphorylation has been shown to facilitate the formation of Neurog2-E protein heterodimers at the expenses of Neurog2-Neurog2 homodimers, which exhibit a different potency in activating cortical neuronal genes (Li et al. 2012). Therefore, understating the context-dependent activity of Neurogs is a fundamental question that may provide crucial insights into how Neurogs specify distinct cell types.

Summary

The discovery of Neurogs strongly contributed to understanding how different cell types originate during embryonic development, especially in the nervous system. Although the role of Neurogs in cell fate specification has been extensively studied, important questions remain unanswered. For example, the exact function of Neurogs in instructing subtype-specific properties is still in its infancy, as is the identification of direct transcriptional target genes on a genome-wide scale. Recent advances in genomic and transcriptomic techniques will definitely contribute to the identification of the transcriptional programs activated by Neurogs. Such data will also help to understand how the same Neurog specifies distinct cell types in different tissues. Deciphering how Neurogs achieve target specificity is also crucial in view of the expanding field of cell reprogramming, which aims at changing cell fate by manipulating Neurog expression or activity. Indeed, it is important to control that Neurog overexpression selectively activates genes for the intended lineage and generates only the specific cell type of interest. Furthermore, the recent discovery that Neurogs undergo several posttranslational modifications provides a more amenable way to alter the activity of endogenous Neurog proteins. As biological and genomic research continues to reveal novel Neurog functions and novel regulatory mechanisms of Neurog activity, the impact of Neurog-mediated reprogramming in regenerative medicine will also grow.

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Neurokinin-1 Receptor

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Synonyms

Neurokinin-1 receptor (NK-1R); Substance P receptor; Tachykinin 1 receptor (TACR1).

Historical Background

The undecapeptide substance P (SP) belongs to the tachykinin peptide family (hemokinin 1 (HK-1), neurokinin A (NKA), and B (NKB) are also included in this family). This family of peptides exerts many biological actions through three receptors named neurokinin-1, neurokinin-2, and neurokinin-3. The neurokinin-1 receptor (NK-1R) shows a preferential affinity for SP/HK-1, NK-2R for NKA, and NK-3R for NKB (Pennefather et al. 2004). The affinity of the NK-1R for NKA and NKB is, respectively, 100- and 500-fold lower than for SP (Gerard et al. 1991). SP, after binding to the NK-1R, exerts many biological actions (Muñoz and Coveñas 2014). The NK-1R protein is encoded by the *TACR1* gene (Takeda et al. 1991). In humans, this gene is located on chromosome 2 and spans 45–60 kb, and it is contained in five exons (Gerard et al. 1991). The NK-1R belongs to the 1 (rhodopsin-like) G protein-coupled

receptors (GPCRs) family (also known as seven-transmembrane domain receptors, 7TM receptors or serpentine receptors).

Distribution and Isoforms of the NK-1R

The distribution of the NK-1R is widespread. This receptor has been located in both central and peripheral nervous systems, in the immune system (leucocytes, lymphocytes, monocytes, macrophages), gastrointestinal tract, lung, placenta, thyroid gland, and skin, platelets and endothelial cells, etc. (O'Connor et al. 2004; Muñoz et al. 2010; Muñoz and Coveñas 2014). Moreover, an overexpression of the NK-1R has been reported in cancer cells in comparison with normal cells (Muñoz and Coveñas 2013). This receptor has been located in many human cancer cells such as glioma, neuroblastoma, retinoblastoma, melanoma, hepatoblastoma, osteosarcoma, cholangiocarcinoma, and oral, gastric, colon, pancreatic, lung (small and non-small cells), breast, and endometrial carcinomas (Muñoz and Coveñas 2013). In tumor cells, the NK-1R was observed in both cytoplasm and nucleus.

The NK-1 receptor contains 407 amino acids and has a molecular mass of 46 kDa (Hopkins et al. 1991). It is a seven-transmembrane-helix receptor showing three extracellular (E1, E2, and E3) and three intracellular (C1, C2, C3) loops with the possibility for a fourth loop (C4), due to the palmitoylation of cysteine, seven-transmembrane domains (TM I–VII), and an amino-terminal (extracellular) and a carboxy-terminal (cytoplasmic) domain (Pennefather et al. 2004; García-Recio and Gascón 2015). SP binds to residues 178–183 (Val-Val-Cys-Met-Ile-Glu) located in the middle of the second extracellular loop (E2): a covalent link occurs between the peptide and the methyl group of a methionine residue (Met-181) (Kage et al. 1996) (Fig. 1), whereas C3 is responsible for the binding to the G protein. The C-terminus contains serine/threonine residues, which, once phosphorylated, cause desensitization of the receptor when it is repeatedly activated by SP (DeFea et al. 2000).

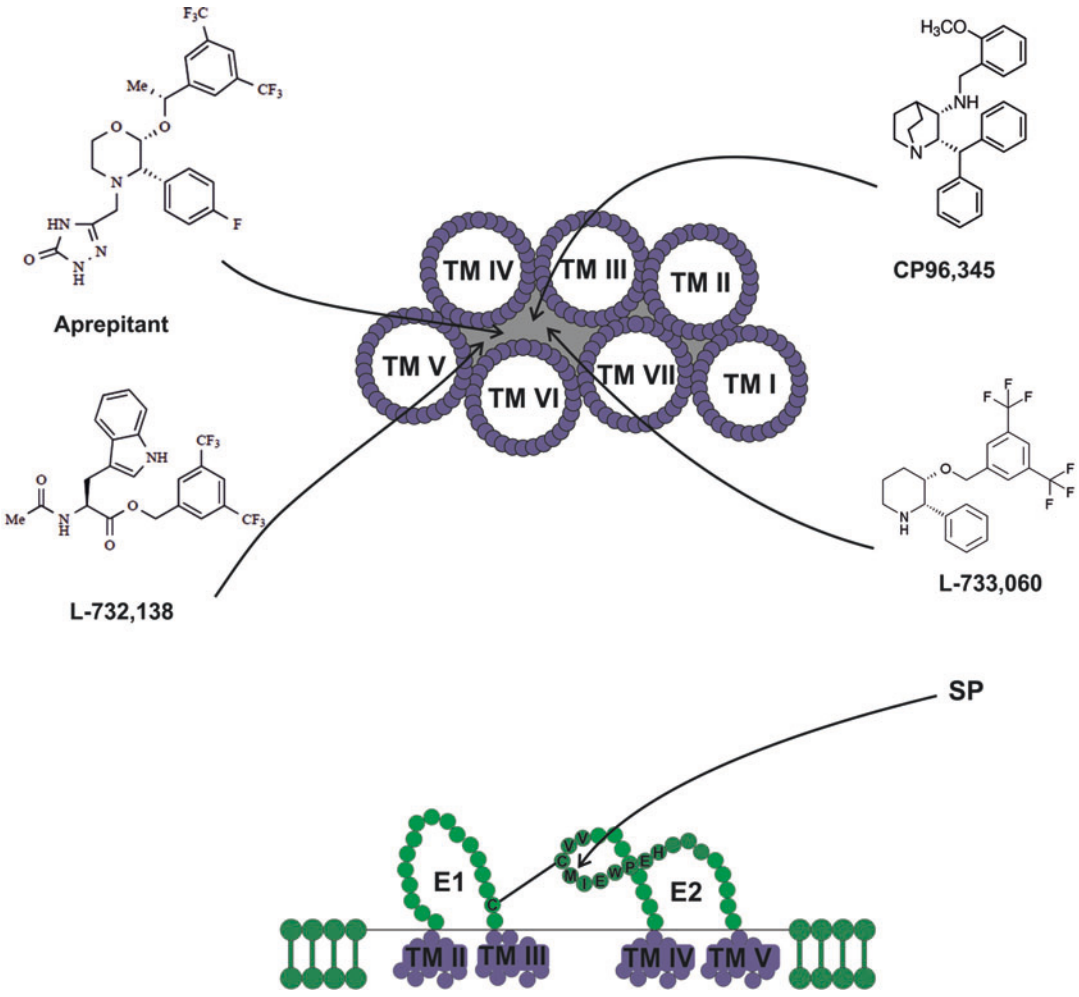
Two isoforms of the NK-1R have been reported: the full-length and the truncated forms. The first (fl-NK-1R) contains 407 amino acids and the second (tr-NK-1R) 311 amino acids (the last 96 amino acids at the C-terminus are lost) (Fong et al. 1992).

The loss of certain C-terminal serine and threonine residues is important for G protein-coupled receptor kinase (GRK) interaction and β-arrestin recruitment for subsequent receptor internalization (DeFea et al. 2000). The tr-NK-1R could be able for prolonging the responses after the binding of ligands because its desensitization and internalization are affected. Due to the different structure of both isoforms, they have a different functional significance, differing in cell signaling capability (Douglas and Leeman 2010). In fact, in colitis-associated cancer, the expression of the tr-NK-1R (but not of the fl-NK-1R) predicted the progression from quiescent colitis to a high-grade dysplasia and cancer (Gillespie et al. 2011). It has been also reported that the tr-NK-1R was overexpressed in human hepatoblastoma cell lines, whereas negligible levels of this receptor were found in human fibroblasts and in nonmalignant HEK-293 cells (Berger et al. 2014).

NK-1R Mechanism of Action: Cell Signaling

SP, after binding to the GPCR NK-1R, induces a change in the Gα subunit, allowing it to exchange GTP for GDP and permitting the dissociation of the Gβγ dimer, inducing the signaling cascade. The hydrolysis of GTP returns the Gα subunit to its inactive state, allowing again the trimeric formation with the Gβγ subunit (Neer 1994) (Fig. 2). There are five subtypes of Gα (Gαs, Gαi, $G\alpha q_{11}$, Gα12/13, Gαo) which are associated to the following signaling pathways:

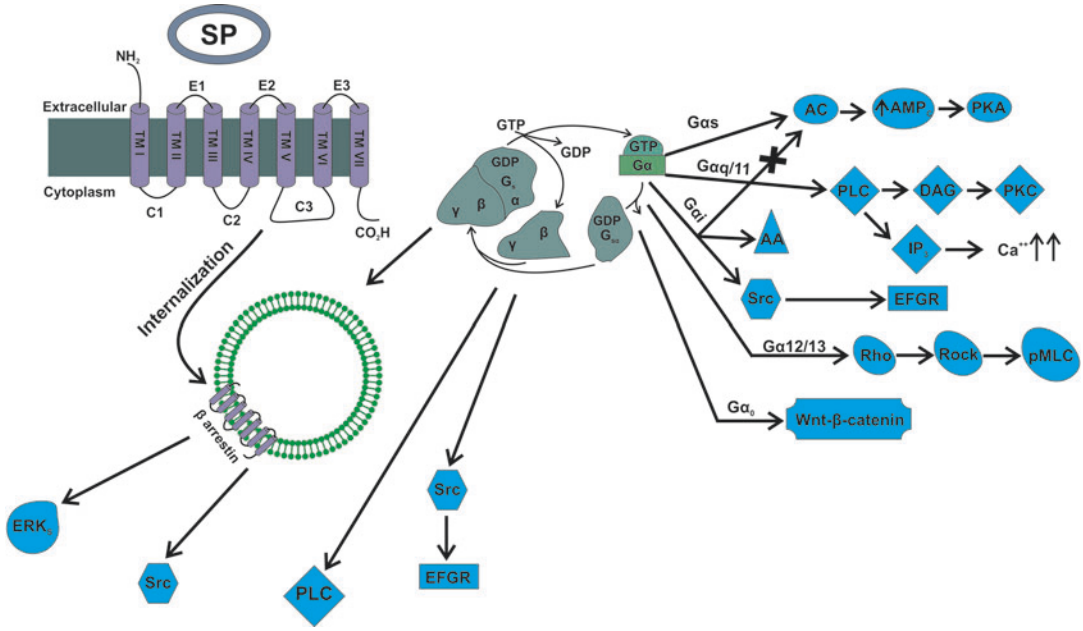
1. Gαs. This subunit produces the activation of the second messenger adenylate cyclase (AC) which catalyzes the conversion of ATP into cyclic adenosine monophosphate cytoplasmic (cAMP): by increasing the level of



Neurokinin-1 Receptor, Fig. 1 Binding sites for SP and NK-1R antagonists

cAMP the activation of protein kinase A (PKA) occurs (Laniyonu et al. 1988).

2. Gαi. It is involved in the inhibition of various types of AC. The increase in the extracellular signal-regulated kinases (ERK)1/2 phosphorylation is through a $G\alpha_i$ pathway, and it is mediated by mitogen-activated protein kinase kinase (MEK)1/2. Moreover, $G\alpha_i$ stimulates the release of [^{3}H]araquidonic acid (Garcia et al. 1994; Lee et al. 2009).
3. $G\alpha q_{11}$. The effector of the $G\alpha q_{11}$ pathway is the phospholipase C-β (PLCβ), which catalyzes the cleavage of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP2) into the second messengers inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG). IP3 acts on IP3 receptors located in the membrane of the endoplasmic reticulum (ER) eliciting the release of Ca^{2+} from the ER, whereas DAG diffuses along the plasma membrane where it may activate a ser/thr kinase called protein kinase C (PKC). Because many isoforms of PKC are also activated by an increase in the intracellular level of Ca^{2+}, both pathways can converge on each other through the same secondary effector (Nakajima et al. 1992).
4. Gα12/13. It could regulate changes in cytoskeletal rearrangement when cells are prepared



Neurokinin-1 Receptor, Fig. 2 SP, after binding to the GPCR NK-1R, induces many cell signaling pathways

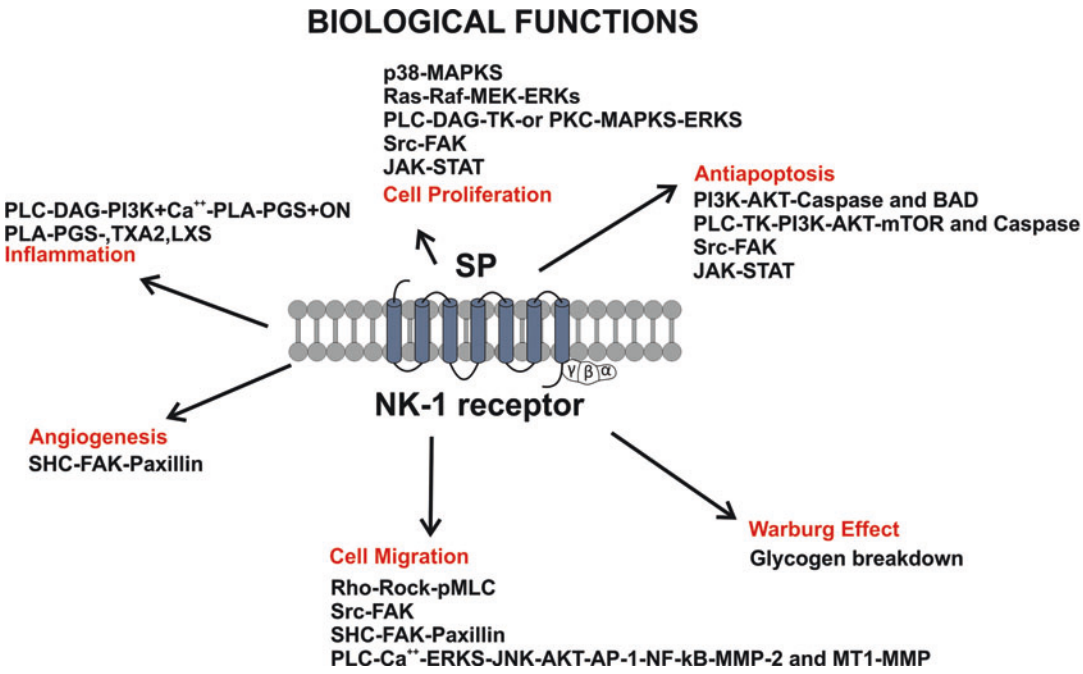
to migrate. These changes depend on the activation of the Rho/Rock signaling pathway which directly modulates the myosin regulatory light chain (MLC). This phosphorylated protein is associated with the formation of small spherical outgrowths arising from the plasma membrane known as bubbles or blebs, in a process known as blebbing (Meshki et al. 2009).

5. Gαo. NK-1R activates Gαo in Sf9 cells (Nishimura et al. 1998). This subunit downstreams the GPCR Frizzled (Fz) signal. Gαo is crucial for the activation of Wnt-β-catenin signaling pathway (Malbon 2005).

The Gβγ subunit activates effectors such as PLCβ, adenylyl cyclases, PI3K, K^{++} ion channels, and Src (Malbon 2005) (Fig. 2). β-arrestin is a member of the mitogen-activated protein kinase (MAPK) signaling pathway, and originally it was involved to mediate receptor uncoupling and internalization; however, it is currently known that β-arrestin is required for the activation of ERK1/2 by GPCRs. In the case of proteinase-activated receptor 2 (PAR2), β-arrestin forms a complex with the internalized receptor, Raf-1, and ERK1/2, retaining in the cytosol the activated kinases. This complex prevents the proliferative effects associated with the translocation of ERK1/2 into the nucleus, regulating the mitogenic potential of a given signal (DeFea et al. 2000). A different β-arrestin complex, containing the β2-adrenergic receptor (β2-AR) and the tyrosine kinase Src, also leads to the activation of ERK1/2 (Luttrell et al. 1999). The associations between trimeric G proteins and second messengers lead a cascade of intracellular events that cause a particular response, depending on the cell type.

Biological Actions of the NK-1R

1. Cell proliferation (Fig. 3). SP, after binding to the NK-1R expressed in glioma cells, induced mitogenesis and the incorporation of [^{3}H]thymidine into the DNA and potently induced c-myc mRNA (Luo et al. 1996). Moreover, SP trough NK-1R activates MAPK cascade, including ERK1/2 and p38MAPK. These pathways are often activated under different



Neurokinin-1 Receptor, Fig. 3 By triggering many cell signaling pathways, the NK-1R regulates several biological functions: inflammation, cell proliferation, angiogenesis, and cell migration

conditions and can lead to both growth and apoptosis. The most commonly studied mechanism by which GPCRs activate MAPK is the release of G-protein βγ subunits which recruit components of the Ras-dependent cascade, such as SHC, GRB2, and Src, leading to the activation of Raf-1 and MAPK 1, a specific activator of ERK1/2 (Esteban et al. 2006). DAG activated by $G\alpha q_{11}$ diffuses along the plasma membrane where it may activate a Ser/Thr PKC.

2. Cell migration (Fig. 3). This mechanism is regulated by neuropeptides/classical neurotransmitters (e.g., SP, noradrenaline, dopamine). Adrenoceptor, D_2 receptor, or NK-1R antagonists inhibit the migration of tumor cells (Lang et al. 2004). SP, via the NK-1R, induces changes in cellular shape (e.g., melanoma or carcinoma cells), including blebbing, which is crucial in cell movement/spreading and in cancer cell infiltration (Meshki et al. 2009). Rho-associated protein kinase (Rock) is also involved in these changes, and, in glioma cells, it has been reported that SP induced the phosphorylation of p21-activated kinase (PAK) and an increased phosphorylation of the myosin regulatory light chain kinase (MLCK), but this was not observed in human non-tumor cells (Meshki et al. 2011).
3. Angiogenesis (Fig. 3). SP, after binding to the NK-1R located in endothelial cells, promoted angiogenesis (Ziche et al. 1990). Moreover, SP-induced calcium increase activates calcium PKC isoforms. PKC is involved but is not mandatory for VEGF induction. SP stimulates phosphorylation of both ERK and c-Jun N-terminal kinase (JNK MAP kinases), which can be activated by PKC-dependent and PKC-independent mechanisms. Activation of these MAPKs leads to activation of the AP-1 transcription factor, a heterodimer of c-Fos and c-Jun (Theoharides et al. 2010)
4. Antiapoptotic effect (Fig. 3). SP, via the NK-1R, stimulates cell proliferation and inhibits apoptosis by a mechanism involving the formation of a β-arrestin-dependent scaffolding complex that includes internalized NK-1R, Src, and the MAPK ERK1/2 (DeFea

et al. 2000). The blockade of the NK-1R by NK-1R antagonist inhibited the basal kinase activity of Akt, increased apoptosis, and caused the cleavage of caspase-3 and the proteolysis of poly (ADP-ribose) polymerase. In the NK-1R-mediated Akt phosphorylation, it is known the total involvement of phosphatidylinositol 3-kinase (PI3K) and the non-receptor tyrosine kinase Src. the partial involvement of the epidermal growth factor receptor (EGFR), and the no involvement of MAPK/ERK (Akazawa et al. 2009).

5. Warburg effect (Fig. 3). SP, via the NK-1R, stimulates the breakdown of glycogen and increases the intracellular Ca^{2+} concentration, but this was completely blocked by the NK-1R antagonist CP-96,345 (Medrano et al. 1994). The Warburg effect occurs because most cancer cells predominantly produce energy by means of a high rate of glycolysis followed by lactic acid fermentation. Growing tumor cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin; this occurs even if oxygen is plentiful. SP/NK-1R from tumor cells produces glycogen breakdown, and the glucose obtained increases the metabolism of these cells (Muñoz and Coveñas 2013).
6. Inflammation (Fig. 3). SP is a main mediator in neurogenic inflammation (O'Connor et al. 2004) and, via the NK-1R, triggers the activation of the transcription factor NF-κB that controls the expression of cytokines (Lieb et al. 1997). SP stimulates human peripheral blood monocytes to produce inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)α (Lotz et al. 1988), and increases the release of TNFα and IL-10 by human macrophages and monocytes (Ho et al. 1996).

NK-1R as a Therapeutic Target and NK-1R Antagonists as New Drugs

There are two types of NK-1R antagonists: peptide NK-1R antagonists and non-peptide NK-1R antagonists. They are a heterogeneous group of compounds that, after binding specifically to the NK-1R, block the pathophysiological actions mediated by SP. Peptide NK-1R antagonists (L-amino acids are replaced by D-amino acids in the SP molecule) are also known as SP analogue antagonists. They are rapidly degraded by peptidases, exert toxic effects, and are not brain penetrant. Non-peptide NK-1R antagonists include many compounds with different chemical compositions but showing similar stereochemical features (the affinity for the NK-1R); they are not degraded by peptidases and are brain penetrant (Muñoz and Coveñas 2013). To date, two non-peptide NK-1R antagonists are used in clinical practice: aprepitant (Emend) and its intravenous administered prodrug fosaprepitant (Ivemend). They are used for the treatment of nausea and vomiting. Non-peptide NK-1R antagonists and SP bind to different sites of the NK-1R: the peptide binds to the extracellular ends of the transmembrane helices, and the antagonists bind more deeply between the III-VII transmembrane domains (Muñoz and Coveñas 2013) (Fig. 1).

The SP/NK-1R System: Clinical Significance

1. This system is involved in chemotherapy-induced nausea and vomiting (CINV). NK-1R antagonists improve CINV (Hesketh et al. 1999).
2. Pruritus. A novel antipruritic strategy has been reported by targeting the NK-1R with aprepitant (Ständer et al. 2010).
3. Antidepressant. In the limbic system, the SP/NK-1R system is involved in depression. Aprepitant showed the same antidepressant activity as paroxetine, and the side effects exerted by this non-peptide NK-1R antagonist were similar to placebo (Kramer et al. 1998).
4. Viral infection. The SP/NK-1R system is involved in viral fusion and transcription (e.g., HIV, viral myocarditis), whereas NK-1R antagonists exerted an antiviral action (Wang et al. 2007; Robinson et al. 2009). In a clinical assay, these antagonists improved the biological biomarkers, and it has been suggested that increased doses of NK-1R

antagonists could exert higher antiviral effects against HIV (Barrett et al. 2016).

5. Inflammatory diseases. The SP/NK-1R system is involved in neurogenic inflammation, and NK-1R antagonists exert anti-inflammatory effects (O'Connor et al. 2004; Muñoz and Coveñas 2014).
6. Cancer progression. The SP/NK-1R system promotes cancer progression. SP induces tumor cell proliferation, has an antiapoptotic effect, stimulates angiogenesis, and induces tumor cell migration for invasion and metastasis. NK-1R antagonists counteract all aforementioned mechanisms (Muñoz and Coveñas 2013).

Summary

The NK-1R belongs to the GPCRs family, and it is widely distributed by the body, including cancer cells (in which it is overexpressed). The preferred endogenous ligands for the NK-1R are SP and HK-1. The tr-NK-1R is overexpressed in cancer cells and in tumors induced by inflammatory processes. SP, via the NK-1R, regulates many cell signaling pathways involved in inflammation, mitogenesis, glycogen breakdown, antiapoptotic effect, angiogenesis, and cell migration. The NK-1R is also involved in many pathophysiological actions: nausea and vomiting, pruritus, viral infection, inflammation, and cancer progression. NK-1R antagonists can counteract these pathophysiological actions exerting antiemetic, antipruritic, antiviral, anti-inflammatory, and antitumor effects. The NK-1R could be considered a new and promising target in many diseases, and non-peptide NK-1R antagonists could be used for numerous therapeutic interventions.

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Neurokinin-1 Receptor (NK-1R)

▶ Neurokinin-1 Receptor

Neuronal CDC2-Like Kinase

▶ CDK5

Neuronal Nicotinic Acetylcholine Receptor

▶ Acetylcholine (Nicotinic) Receptor

Neuronal Nicotinic Receptor

▶ Acetylcholine (Nicotinic) Receptor

Neuronal Tryptophan Hydroxylase

▶ Tryptophan Hydroxylase 2

Neuropolypeptide H3

▶ PEBP-1

Neurotactin

▶ CX3CL1

Neurotensin Receptor (NTSR)

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Synonyms

NTR; NTS

Historical Background

Neurotensin was originally isolated from bovine hypothalamus by Carraway and Leeman in 1973 and was identified as a 13 amino acid peptide (Carraway and Leeman 1973). It was named "neurotensin" in view of its hypotensive activity. Neurotensin is produced by neurons in the central nervous system (CNS) and N cells of the gastrointestinal tract. Neurotensin acts as a hormone and a neurotransmitter or neuromodulator in the periphery and in the CNS, respectively. Neurotensin is involved in the regulation of a number of physiological functions such as analgesia, neurodevelopment, neurodegeneration, thermal regulation, metabolic regulation, pituitary hormone secretion, gastrointestinal motility, and inflammation. It was later discovered that neurotensin is also present in several types of tumors and is implicated in tumorigenesis and tumor progression. Thus, signaling pathways downstream of neurotensin could be a potential drug target for the treatment of a variety of diseases. However, we had to wait 17 years to see the first receptor for neurotensin (neurotensin receptor 1 (NTSR1)). Subsequently, two additional subtypes of the neurotensin receptor (NTSR2 and NTSR3) and a candidate for NTSR4 have been identified. Development of subtype-selective neurotensin analogs facilitated investigations of the role of neurotensin receptors in mediating the effects of neurotensin.

Neurotensin Receptor Subtypes

Neurotensin exerts its effects primarily through two neurotensin receptor subtypes, NTSR1

Neurotensin Receptor (NTSR), Table 1 Tissue distribution of neurotensin receptors

NTSR1	NTSR2	NTSR3/sortilin	SorLA/LR11 (NTSR4)
CNS	CNS	CNS	CNS
Anterior pituitary	Anterior pituitary	Thyroid gland	Kidney
Autonomic nervous system	Upper gastrointestinal tract	Heart	Ovary
Enteric nervous system	Heart	Adrenal gland	Testis
Gastrointestinal tract	Pancreas	Pancreas	Lymph node
Carotid body		Adipose tissue	
Liver		Skeletal muscle	
		Placenta	
		Testis	

and NTSR2. NTSR1 is a high-affinity (Kd = 0.1–0.3 nM) neurotensin receptor which is not sensitive to levocabastine, a nonpeptide H1 histamine antagonist. NTSR2 has a low affinity (Kd = 3–5 nM) for neurotensin and is sensitive to levocabastine. Both NTSR1 and NTSR2 are 7-transmembrane G-protein-coupled receptors (GPCRs). The human *NTSR1* gene, encoding a 418 amino acid protein, is located on the long arm of chromosome 20 (20q13). The human *NTSR2* gene encodes a 410 amino acid protein and is located on chromosome 2 (2p25.1). Separate NTSR subtypes display distinctive tissue distribution patterns (Table 1). NTSR1 is distributed widely throughout the CNS and is also found in the small and large intestines as well as the liver. NTSR2 is located more diffusely in the CNS than NTSR1. Two additional receptors have been shown to bind neurotensin among other ligands. Both receptors are single transmembrane domain receptors of the type I family. The NTSR3 is the intracellular sorting protein sortilin that interacts with multiple ligands including neurotensin, low-density lipoprotein (LDL), lipoprotein lipase (LPL), and progranulin. The human sortilin 1 (*SORT1*) gene, encoding an 831 amino acid protein, is located on the short arm of chromosome 1 (1p21.3-p13.1). NTSR3/sortilin, like NTSR1, binds neurotensin with high affinity once converted to its mature form upon cleavage. Sorting protein-related receptor (SorLA, also known as LR11) has been proposed as a fourth neurotensin receptor. The human *SorLA* gene, encoding a 2214 amino acid peptide, was mapped to chromosome 11 (11q 23.3–24). NTSR3/sortilin and SorLA/LR11 are located primarily in the CNS but also in nonneuronal tissues (Table 1, Vincent et al. 1999; Dobner 2005; St-Gelais et al. 2006).

Signal Transduction of NTSR Receptors

Stimulation of NTSR1 leads to the activation of multiple signaling pathways. The major signal transduction event associated with the activation of NTSR1 is the stimulation of phospholipase C (PLC), which is responsible for the production of inositol 1,4,5-triphosphate, mobilization of Ca^{2+}, and activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a member of mitogen-activated protein kinase (MAPK) in a variety of tissues and cells. Neurotensin-induced ERK1/2 activation is also mediated via the activation of small GTPase Ras protein and epidermal growth factor receptor (EGFR) in human colonial epithelial cells. NTSR1 activation stimulates nuclear factor kappaB (NF-κB) which is dependent on intracellular calcium release. Neurotensin activates the Rho family proteins RhoA, Rac1, and Cdc42, and neurotensin-induced NF-κB activation, but not ERK1/2 activation, is mediated through the activation of RhoA, Rac1, and Cdc42. Little is known about the signaling pathways activated by the other three receptor subtypes. When NTSR2 is challenged with neurotensin, internalization of receptor–ligand complexes occurs through the activation of ERK1/2. Stimulation of NTSR3/sortilin activates ERK1/2 and phosphoinositide 3-kinase (PI3K)-dependent pathways in

microglial cell lines (Martin et al. 2005; Zhao and Pothoulakis 2006; Ferraro et al. 2009). Homodimer and/or heterodimer of GPCRs play important roles in receptor trafficking, agonist binding, and signal transduction. Heterodimerization/oligomerization alters receptor functions by forming new receptor complexes that exhibit ligand-binding properties distinct from monomeric receptors. NTSR1 and NTSR2 can form heterodimers, and NTSR2 suppresses neurotensin-induced NTSR1 activity (Hwang et al. 2009). Additionally, NTSR1 forms heterodimers with other receptors such as apelin receptor and kappa opioid receptor. Formation of these heterodimers may provide additional functional resources to the cells.

Physiological Relevance of CNS NTSR Signaling

Neurotensin-containing neurons are neuroanatomically associated with the brain dopamine system, and neurotensin acts as a neuromodulator of dopaminergic transmission in several areas of the brain, including the nigrostriatal and mesolimbic pathways. Neurotensin increases the activity of dopaminergic neurons and dopamine release by antagonizing dopamine D_2 receptor function through NTSR1-mediated increases in intracellular Ca^{2+} and an interaction between NTSR1 and D_2 receptors. Neurotensin levels are increased, and NTSR1 mRNA levels are lower in the substantia nigra of Parkinson's disease patients than in controls. Treatment with neurotensin analogs reduces tremor and muscle rigidity in animal models of Parkinson's disease. These findings argue for the possibility that enhanced signaling through the substantia nigra NTSR1 supplements dopaminergic agonists to augment the function of the remaining dopaminergic neurons in Parkinson's disease. However, activation of NTSR1 by neurotensin enhances N-methyl-D-aspartate (NMDA)-induced increase in extracellular glutamate levels. The neurotensin-mediated potentiation of NMDA receptor signaling may be mediated by phosphorylation of the NMDA receptors by protein kinase C. Glutamate is the major excitatory neurotransmitter, and the excessive activation of glutamate receptors, especially NMDA receptors, has been postulated to contribute to the neuronal injury. In cultured dopaminergic neurons, neurotensin enhances the neurotoxic effects of glutamate on dopaminergic neurons via NTSR1 activation. Thus, treatment with selective NTSR1 antagonists may be beneficial in improving the symptoms of Parkinson's disease (Antonelli et al. 2007; Mustain et al. 2011). Abnormal neurotensin-NTSR1 signaling has been also found in the brain of Alzheimer's disease patients. Regardless of whether CNS neurotensin-NTSR1 signaling is beneficial or deleterious in neurodegenerative processes, this signaling pathway is likely to be involved in the etiology of neurodegenerative diseases.

Centrally administered neurotensin causes a variety of effects similar to those exhibited by antipsychotic drugs. Reduced signaling capacity through NTSR1 contributes to psychotic symptom in schizophrenia. Cerebrospinal fluid levels of neurotensin are lower in schizophrenic patients than in control subjects, whereas increased levels of neurotensin are associated with improvement in symptoms during treatment. The density of neurotensin receptors (primarily NTSR1) is decreased in the intermediate entorhinal cortex of schizophrenia patients. Both NTSR1-deficient mice and NTSR2-deficient mice show schizophrenia-like signs, such as amphetamine-induced hyperlocomotion and lower basal glutamate levels. The lack of neurotensin causes diminished prepulse inhibition (PPI), a schizophrenia-like sign, while NTSR1 deficiency does not alter PPI and NTSR2-deficient mice have elevated PPI (Mustain et al. 2011). These findings support the idea that enhanced neurotensin-NTSR signaling is beneficial in improving schizophrenia symptoms. It is presently unclear what NTSR subtype is involved in this process. Brain-region-specific alterations in neurotensin-NTSR signaling may play a role in the etiology of schizophrenia.

Neurotensin has analgesic effects that are μ-opioid independent. Although NTSR2 was initially considered as a main receptor subtype mediating the antinociceptive effect of neurotensin,

NTSR1 is also involved in neurotensin-induced analgesia. Both NTSR1 and NTSR2 are required for different aspects of neurotensin-induced analgesia, and these two receptors modulate the pain-induced behavior responses by suppressing activity of distinct spinal and/or supraspinal neural circuits (Dobner 2005; St-Gelais et al. 2006; Roussy et al. 2009).

CNS neurotensin-NTSR signaling plays a role in neuroendocrine function via modulation of the activity of the hypothalamus–pituitary axis. Neurotensin stimulates secretion of gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) and inhibits thyroid-stimulating hormone (TSH) secretion. Hypothalamic action of neurotensin increases prolactin secretion, while neurotensin action on the adenohypophysis causes a reduction in prolactin secretion. The direct hypothalamic effect of neurotensin in reducing prolactin secretion may be mediated by dopamine release into the portal system. It is unclear whether these effects of neurotensin are mediated via NTSR1 and/or NTSR2. Both receptors are expressed in the hypothalamus and anterior pituitary. Neurotensin has been implicated in the mediation of the preovulatory positive feedback of estradiol on GnRH release via NTSR2 activation. NTSR2 also mediates neurotensin-induced increase in CRH and corticosterone secretion in response to stress (Lafrance et al. 2010; Stolakis et al. 2010; Mustain et al. 2011).

Neurotensin participates in the regulation of metabolism. Central administration of neurotensin reduces food intake via NTSR1. NTSR1 mediates effects of other anorexigenic hormones, leptin and xenin. NTSR1 is also involved in hedonic feeding by mediating the hypothalamic leptin – mesolimbic dopamine system (Opland et al. 2013). Genetic deletion of NTSR2 did not cause any metabolic abnormalities in mice, while NTSR1-deficient mice have increased body weight, food intake, and adiposity, implicating NTSR1 in metabolic regulation (Mustain et al. 2011).

Neurotensin contributes to thermoregulation. CNS action of neurotensin reduces body temperature. NTSR1-deficient mice have increased body temperature and do not respond to the hypothermic effect of neurotensin, implicating neurotensin-NTSR1 signaling in thermal control.

Physiological Relevance of Peripheral NTSR Signaling

The effects of neurotensin in the gastrointestinal tract are mediated by both neural and hormonal mechanisms. Neurotensin elicits a relaxing effect on duodenal longitudinal muscle and induces contraction of the ileal muscle and the proximal colon. These neurotensin-induced alterations in gastrointestinal motilities were blocked by NTSR1 antagonist and were absent in NTSR1-deficient mice. Neurotensin-related peptide, xenin, also affects gastrointestinal motility partly via activation of NTSR1. These findings indicate that neurotensin regulates gastrointestinal motility via NTSR1 (Zhao and Pothoulakis 2006).

Neurotensin-NTSR1 signaling is associated with the progression and differentiation of tumors. NTSR1 is expressed in tumors of the ovary, pancreas, colon, prostate, and breast. High NTSR1 expression is associated with a larger tumor size, and the size of tumor becomes smaller in the presence of NTSR1 antagonist. In human breast cancer cell lines, functionally expressed NTSR1 receptor coordinated a series of transforming events, including cellular migration and invasion. The *NTSR1* gene is a target of oncogenic pathways known to activate genes involved in cancer cell proliferation and transformation. When NTSR1 is challenged with neurotensin, a variety of signaling pathways are activated. For example, activation of NTSR1 promotes growth of colon tumors through NF-κB activation. Neurotensin also increased the activity of PI3K signaling by increasing specific microRNA species via NTSR1 in colon epithelial cells. During prolonged neurotensin agonist stimulation, NTSR1 receptors are recycled to the cell surface, leading to the long-term activation of ERK1/2, an oncogenic signaling pathway. Activation of NTSR1 by neurotensin leads to the activation of EGFR/EGFR2 (also known as HER2) signaling, contributing to breast cancer growth and

metastasis. Neurotensin–NTSR1 signaling enhances epithelial-to-mesenchymal transition and promotes tumor metastasis by activating the Wnt/β-catenin signaling pathway in hepatocellular carcinoma (Ye et al. 2016). These findings place NTSR1 at a major nexus between neurotensin and tumorigenesis.

Physiological Relevance of Non-GPCR NTSRs

Whereas both NTSR3/sortilin and SorLA/LR11 are capable of binding neurotensin, the physiological relevance of these receptors is largely unclear. NTSR3/sortilin is predominantly expressed in regions of the CNS. Autism is a neurodevelopmental disorder and microglia are activated in the brains of patients with autism. NTSR3/sortilin is expressed in microglia and mediates neurotensin-induced chemokine release from human microglia through activation of mammalian target of rapamycin (mTOR) signaling (Patel et al. 2016). Thus, activation of NTSR3/sortilin may be involved in the pathogenesis of autism. NTSR3/sortilin binds not only neurotensin but also other ligands such as the receptor-associated protein (RAP), LPL and nerve growth factor precursor (proNGF) (St-Gelais et al. 2006). It is thus possible that NTSR3/sortilin subserves non-neurotensin-related functions in the CNS. Recent studies suggest that NTSR3/sortilin plays a role in the regulation of cognition, metabolism, and pathogenesis of cardiovascular disease. Levels of NTSR3/sortilin mRNA and protein were reduced in adipose tissue and skeletal muscle of obese mice and patients. NTSR3/sortilin promotes the formation of insulin-responsive glucose transporter 4 (GLUT4) storage vesicles in adipocyte cell line. Palmitate reduces the expression of NTSR3/sortilin in adipocytes and skeletal muscle cells (Tsuchiya et al. 2010). NTSR3/sortilin deficiency protects mice from high-fat diet-induced metabolic impairments by improving insulin sensitivity (Rabinowich et al. 2015). These findings suggest that NTSR3/sortilin plays a role in glucose homeostasis by modulating GLUT4 trafficking in adipose tissue and skeletal muscle and mediates FFA-induced insulin resistance in these tissues. Additionally, NTSR3/sortilin is also present in several types of cancer cells (St-Gelais et al. 2006; Bakirtzi et al. 2011; Mustain et al. 2011). However, the precise role of NTSR3/sortilin in cancer is presently unknown.

It has been suggested that SorLA/LR11 regulates the processing of the amyloid precursor protein and levels of SorLA/LR11 are reduced in the brains of Alzheimer disease patients, suggesting a possible role of SorLA/LR11 in the pathogenesis of this disease (Andersen et al. 2005).

Summary

Neurotensin exerts diverse actions through NTSR receptors in the CNS and in the periphery. Alterations in NTSR signaling have been implicated in a wide range of pathologic conditions such as schizophrenia, Parkinson's disease, metabolic disorders, and cancer. Signaling pathways involving NTSR receptors and their downstream mediators could be potential drug targets for the treatment of these impairments. Pharmacological tools and genetically engineered animal models enabled us to manipulate NTSR signaling, and we have begun to elucidate specific role for each NTSR subtype. Creation of animal models with cell type-specific ablation of specific NTSR subtype should help clarify the role of NTSR subtypes in the mediation of neurotensin action. Development of additional subtype-selective agonistic and antagonistic neurotensin analogs that cross the blood–brain barrier may offer new avenues for the treatment of disorders associated with altered NTSR receptor signaling.

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Neutral CDase

▶ Neutral Ceramidase

Neutral Ceramidase

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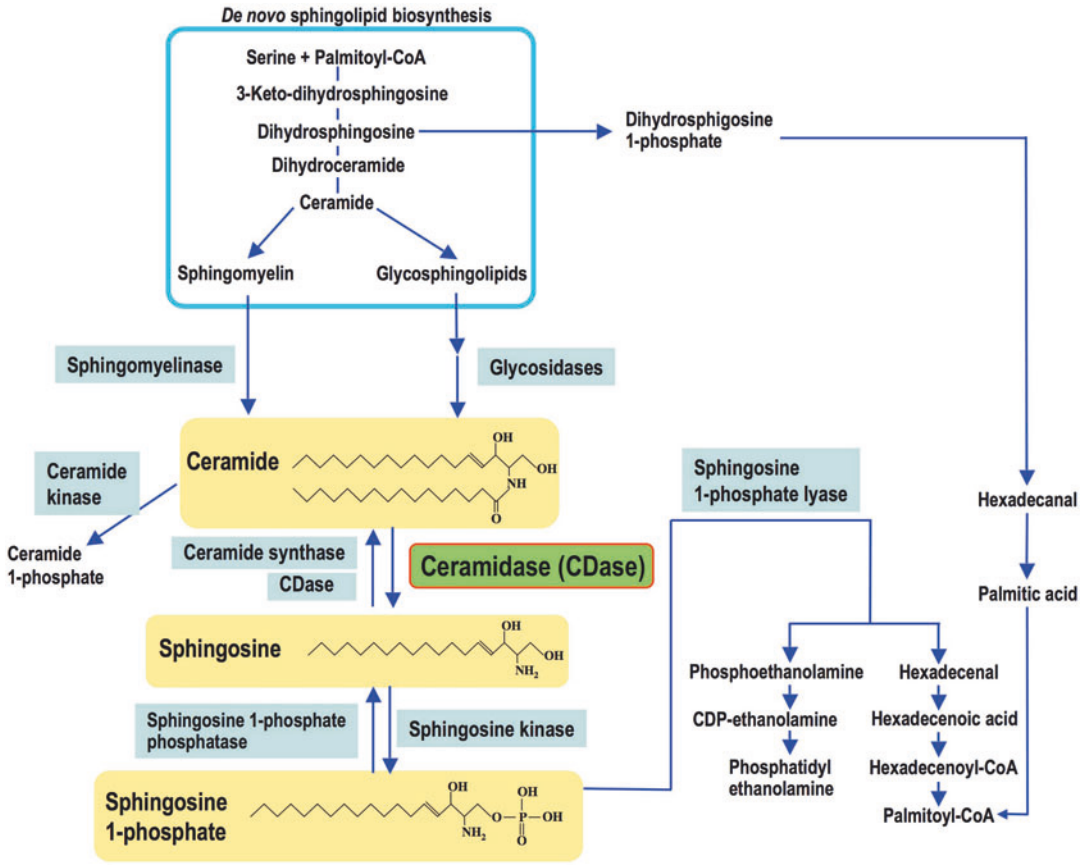
Synonyms

ASAH2; *N*-acylsphingosine amidohydrolase 2; NCDase; Neutral CDase; Nonlysosomal ceramidase

Historical Background

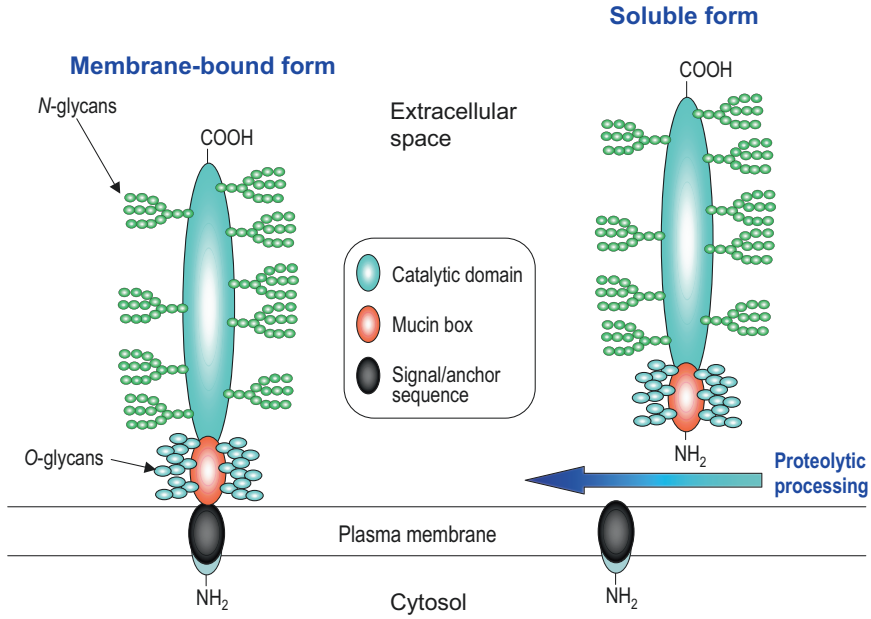
Ceramidase (CDase) is an enzyme that hydrolyzes the *N*-acyl linkage between a fatty acid and a sphingoid base in ceramide (Cer) and is widely



Neutral Ceramidase, Fig. 1 Pathways of *de novo* biosynthesis and degradation of sphingolipids

distributed from bacteria to mammals. Cer comprises the hydrophobic portion of complex sphingolipids including sphingomyelin and glycosphingolipids. The most abundant sphingoid base in mammalian tissues is sphingosine (Sph), which has a *trans*-double bond between the C4 and C5 positions, and Sph can be phosphorylated by Sph kinases and converted to Sph 1-phosphate (S1P). Cer, Sph, and S1P have been shown to function as lipid signaling molecules that regulate various signal transduction systems. Sph cannot be synthesized via the *de novo* sphingolipid biosynthesis pathway; that is, the formation of the C4–C5 *trans*-double bond by dihydroceramide desaturase, DES1, occurs after *N*-acylation of dihydrosphingosine by Cer synthases, and thus Sph does not arise as an intermediate of *de novo* sphingolipid biosynthesis (Fig. 1). Therefore, Sph can be only generated through hydrolysis of Cer by CDase. This means that the generation of S1P also completely depends on CDase. Therefore, hydrolysis of Cer by CDase is crucial for sphingolipid (Cer/Sph/S1P)-mediated signal transduction.

In earlier studies, the presence of isoforms of CDase in animal tissues was found, these isoforms differing in their catalytic pH optima, acid, or neutral/alkaline (Gatt 1963; Yavin and Gatt 1969). Acid CDase is involved in catabolism of Cer in lysosomes, and point mutations of the enzyme cause Farber disease, in which Cer is accumulated in lysosomes (Ito et al. 2014). The presence of enzyme activity of CDase exhibiting an optimum pH between neutral and alkaline pH was first found in rat brain (Yavin and Gatt 1969). Several lines of evidence have indicated that the



Neutral Ceramidase, Fig. 2 Membrane topology and secretion of neutral CDase**.** Neural CDase is expressed at plasma membranes as a type II integral membrane protein. The enzyme is partly secreted into the extracellular space after cleavage of the NH_2-terminal signal/anchor sequence

activity of neutral/alkaline CDase, which is detected in plasma membrane fractions, is upregulated by growth factors and cytokines (Ito et al. 2014); thus, it was thought that the enzyme is involved in regulation of signal transduction systems via modulation of the balance of Cer, Sph, and S1P levels. In 1998, the neutral CDase gene was cloned from *Pseudomonas aeruginosa* (Okino et al. 1999). The purified enzyme catalyzes both hydrolysis and condensation of fatty acids to Sph to generate Cers. Following the identification of bacterial neutral CDase, homologs of the enzyme were purified from mammalian tissues, and their genes were cloned (Mitsutake et al. 2001; Tani et al. 2000b). In mammalian tissues, neutral CDase can exist as both membrane-bound and soluble forms. The soluble form is generated posttranslationally via proteolytic processing of the NH_2-terminal region including the signal/anchor sequence (Tani et al. 2007) (Fig. 2). It should be noted that neutral CDase is widely distributed in microorganisms, insects, plants, and vertebrates and that its primary structure is highly evolutionally conserved (Tani et al. 2000b). Alkaline CDase exhibiting an alkaline pH optimum (pH 8.5–9.5) was first identified in budding yeast *Saccharomyces cerevisiae*, and it was found that human has three types of alkaline CDase, which are localized at the ER/Golgi (Ito et al. 2014). The primary structures of these three CDases (acid, neutral, and alkaline) are completely different, and thus CDases comprise three independent families (Ito et al. 2014).

Enzymatic Properties of Mammalian Neutral CDase

The pH dependency of neutral CDase purified from mouse liver is quite broad; that is, although the enzyme exhibits the highest activity around pH 7.5, weak enzyme activity is observed under acidic (pH 4.0) and alkaline (pH 10.0) conditions (Tani et al. 2000a). The enzyme hydrolyzes the *N*-acyl linkage of various species of Cers but not glycosphingolipids or sphingomyelin. Cers containing Sph (d18:1) are hydrolyzed much

faster than ones containing dihydrosphingosine (d18:0). Cers containing phytosphingosine (t18:0) are strongly resistant to hydrolysis by the enzyme. Interestingly, neutral CDase hydrolyzes a fluorescent analogue of Cer, C12-NBD-Cer, much faster than radioisotope-labeled natural Cers. Thus, C12-NBD-Cer is now recognized as a useful artificial substrate for measurement of neutral CDase activity. The enzyme activity is significantly enhanced by the addition of detergents. Sodium cholate has the strongest effect; that is, 1.0% of the detergent increases the enzyme activity approximately fourfold in comparison with the level in the absence of the detergent. The activity of neutral CDase is strongly inhibited by Cu^{2+}, Zn^{2+}, and Hg^{2+}; however, EDTA does not affect the enzyme activity (Tani et al. 2000a).

Neutral CDase catalyzes not only hydrolysis of Cers but also Cer synthesis involving sphingoid bases and fatty acids (El Bawab et al. 2001; Tani et al. 2000a). The reverse reaction does not require coenzyme A, indicating that the reaction mode is completely different from that of Cer synthases. The enzyme exhibits the reverse activity in a narrow pH dependency in a neutral range as compared with that of the hydrolysis activity. Although 1.0% sodium cholate is most favorable for the hydrolysis activity of the enzyme, the reverse activity is almost completely abolished in the presence of the detergent. The reverse activity is inhibited by glycerophospholipids, especially phosphatidic acid and cardiolipin. The physiological significance of the reverse activity remains unclear; however, it is worth noting that neutral CDase can synthesize Cer *in vivo* when overexpressed in HEK293 cells (El Bawab et al. 2001).

Conformation and Catalytic Mechanism of Neutral CDase

In 2009, the crystal structure of neutral CDase from *Pseudomonas aeruginosa* was determined (Inoue et al. 2009). The enzyme is composed of two domains, a NH_2-terminal domain harboring an active site and an immunoglobulin-like COOH-terminal domain. A Zn^{2+}-binding site at the center of the NH_2-terminal domain functions as the active center of the enzyme, and the reaction mechanism is similar to that of the Zn^{2+}-dependent carboxypeptidase Y. The reverse activity of neutral CDase also proceeds in a Zn^{2+}-dependent manner. The Mg^{2+}/Ca^{2+} binding site is located at the interface between the NH_2-terminal and COOH-terminal domains, and it is suggested that Mg^{2+}/Ca^{2+} may be involved in stabilization of the interaction between the two domains. These catalytic mechanisms are well conserved in mammalian neutral CDases; however, crystal structure analysis of human neutral CDase revealed that eukaryotic neutral CDases have a deep hydrophobic active site pocket stabilized by a eukaryotic-specific subdomain, which is not present in bacterial neutral CDases, and this pocket enables steric exclusion of sphingolipids with larger head groups and specific recognition of the small hydroxyl head group of Cers (Airola et al. 2015).

Subcellular Localization and Tissue Distribution of Mammalian Neutral CDase

Mammalian neutral CDase has a signal/anchor sequence at the NH_2-terminus and is distributed at plasma membranes as a type II integral membrane protein, and thus the catalytic region faces the extracellular space (Tani et al. 2007) (Fig. 2). In addition, the enzyme is partly secreted into the extracellular space after cleavage of the NH_2-terminal signal/anchor sequence, which means that the enzyme functions as both membrane-bound and soluble proteins (Fig. 2). Mammalian neutral CDases have 9–10 *N*-glycosylation sites in their catalytic region and are highly *N*-glycosylated. In addition, the enzymes have a highly *O*-glycosylated domain (mucin box) that follows the signal/anchor sequence, and this domain plays an important role in the plasma membrane localization of the enzyme. Although the precise mechanism by which the mucin box contributes to plasma membrane localization of the enzyme is unknown, one possible explanation is

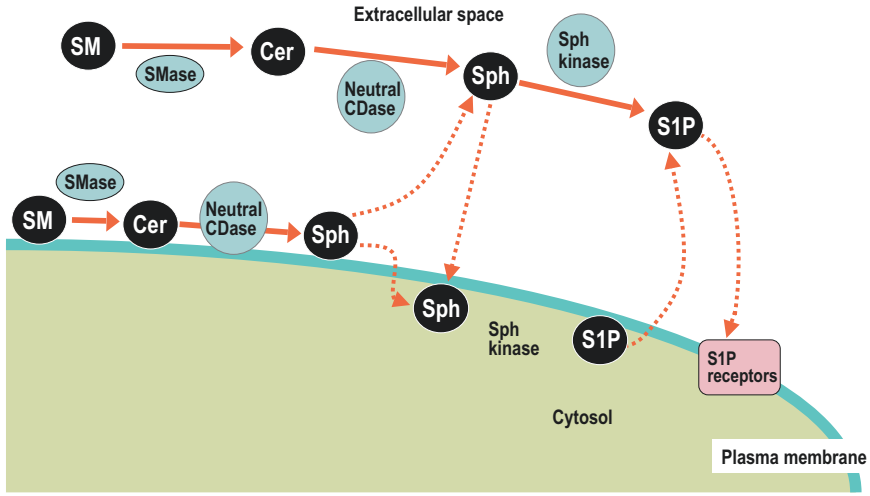
that *O*-glycans on the mucin box prevent the cleavage of the signal/anchor sequence by unknown proteases. It is worth noting that zebra fish neutral CDase also has a mucin box and is localized at plasma membranes (Yoshimura et al. 2004); however, the enzymes from *Pseudomonas aeruginosa*, *Aspergillus oryzae*, and *Drosophila melanogaster* do not have mucin box and function as secretory proteins (Ito et al. 2014). This may suggest that the structure of the mucin box in neutral CDases was acquired during evolution for localization of the enzyme at plasma membranes as a type II integral membrane protein.

In mouse tissues, mRNA expression of neutral CDase is ubiquitously detected; however, the strongest expression is observed in the small intestine including the duodenum, jejunum, and ileum (Kono et al. 2006; Tani et al. 2000b). In rat kidney, neutral CDase is distributed at the apical membranes of proximal tubules, distal tubules, and collecting ducts, whereas in the liver the enzyme is mainly detected as a soluble protein and is distributed among endosome-like organelles in hepatocytes (Mitsutake et al. 2001). Although mouse kidney neutral CDase has *O*-glycans, the liver enzyme does not have both the signal/anchor sequence and the mucin box, suggesting that the posttranslational modification around the NH_2-terminal region of the enzyme occurs in a tissue-specific manner and affects the intracellular distribution of the neutral CDase (Ito et al. 2014).

The subcellular localization of human neutral CDase is somewhat controversial; that is, the enzyme is localized to mitochondria when the enzyme fused to GFP at the NH_2-terminus is overexpressed in HEK293 and MCF7 cells, whereas the enzyme fused to GFP at the COOH-terminus is distributed at plasma membranes of HEK293 cells (Ito et al. 2014). This discrepancy in the intracellular distribution of the enzyme may arise from the use of overexpression systems; thus, the precise intracellular distribution of human neutral CDase *in vivo* remains to be determined. It should be noted, however, that neutral CDase contributes to production of Sph in mouse brain mitochondria (Novgorodov et al. 2014).

Involvement of Mouse Neutral CDase in Hydrolysis of Cer at the Outer Leaflet of Plasma Membranes and the Extracellular Space

Overexpression of mouse neutral CDase in Chinese hamster CHOP cells does not cause any changes in the Cer, Sph, and S1P levels; however, CHOP cells overexpressing neutral CDase exhibit a decrease in the Cer level and transient increases in the Sph and S1P levels as compared with mock-transfected cells when cells are treated with bacterial sphingomyelinase (SMase), which is used as a tool for the hydrolysis of cell-surface sphingomyelin to increase the amount of free Cer in the plasma membranes (Tani et al. 2007). This indicates that cell-surface Cer produced by extracellular bacterial SMase is hydrolyzed by the neutral CDase. The neutral CDase-mediated Sph generation occurs at the outer leaflet of plasma membranes, whereas the subsequent S1P generation occurs mainly inside the cell following the incorporation of the generated Sph. Both plasma membrane-localized neutral CDase (type II integral membrane protein) and the secreted enzyme (soluble form) are involved in hydrolysis of Cer on the cell surface. Furthermore, in serum, the secreted neutral CDase is able to produce Sph from Cer that has been generated from lipoprotein-bound sphingomyelin by bacterial SMase. The Sph generated in the serum is converted to S1P by extracellular Sph kinase, which is secreted from vascular endothelial cells via a nonclassical secretory pathway, and the generated S1P can bind to cell-surface S1P receptor $S1P_1$ (Tani et al. 2007). Collectively, these lines of evidence suggest that the neutral CDase is involved in the metabolism of Cer at the plasma membrane and in the extracellular space and that the enzyme can regulate S1P-mediated signaling through the generation of extracellular S1P (Fig. 3). It should be noted that the cell-surface generation of Sph via neutral CDase is involved in the generation of S1P in human platelets, which are one of the important sources of blood S1P (Tani et al. 2007).



Neutral Ceramidase, Fig. 3 Scheme for metabolic pathways of Cer involving neutral CDase at the outer leaflet of the plasma membrane and in the extracellular space. *SMase* sphingomyelinase, *Sph kinase* sphingosine kinase, *SM* sphingomyelin, *Cer* ceramide, *Sph* sphingosine, *S1P* sphingosine 1-phosphate

Physiological Functions and Regulation of Neutral CDase in Mammalian Tissues and Cells

In 2006, Kono et al. reported the establishment of neutral CDase gene knockout mice (Kono et al. 2006). The neutral CDase null mice have a normal life span and do not exhibit clear abnormal phenotypes. Although loss of neutral CDase does not cause significant changes of the Cer and Sph levels in most tissues, neutral CDase null mice exhibit an increase in the C16-Cer level in the jejunum and decreases in the Sph levels in the jejunum, ileum, and colon. Neutral CDase is detected in the intestinal tract along the brush border and intestinal contents, and neutral CDase null mice are defective in intestinal digestion of dietary Cer; thus it is suggested that neutral CDase is involved in the catabolism of dietary sphingolipids (Kono et al. 2006). Intestinal neutral CDase activity is increased in patients with ulcerative colitis, and neutral CDase null mice exhibit an increase in systemic inflammation in a mouse model of colitis; thus it is suggested that neutral CDase may be involved in protection against inflammatory bowel disease (Snider et al. 2012). In contrast, in colon cancer cells, inhibition of neutral CDase causes an increase in the Cer level, which induces apoptosis and autophagy (Garcia-Barros et al. 2016). Furthermore, neutral CDase null mice are protected from colon carcinogenesis induced by azoxymethane, a potent carcinogen of colon cancer, suggesting that neutral CDase is involved in the regulation of initiation and development of colon cancer. Thus, neutral CDase may become a novel target for colon cancer therapy (Garcia-Barros et al. 2016).

It has been reported that growth factors, cytokines, and glucocorticoids increase the activity and/or expression level of neutral CDase in hepatocytes and renal mesangial cells, and it is suggested that neutral CDase has cytoprotective effects against inflammatory stimuli in these cells. In contrast, in rat renal mesangial cells, an increase in nitric oxide production, which is associated with inflammatory disorders, induces degradation of neutral CDase and subsequently an increase in the Cer level (Ito et al. 2014).

Several lines of evidence have indicated that neutral CDase is involved in protection from apoptosis. For example, INS-1 cells, a rat β-cell line, are protected from cytokine-induced apoptosis by an increase in the expression level of neutral CDase (Zhu et al. 2008). Furthermore,

downregulation of neutral CDase is involved in enhancement of apoptosis. In human keratinocytes, upregulation of the expression level of neutral and acid CDases caused by low-dose UVB results in protection from UVB-induced apoptosis, whereas high-dose UVB causes downregulation of these enzymes, which enhances UVB-induced apoptosis (Uchida et al. 2010). On the other hand, occasionally, neutral CDase positively regulates cell death; that is, in traumatic brain injury, activation of neutral CDase and reduced activity of Sph kinase 2 promote the production of Sph in mitochondria and, consequently, secondary brain injury (Novgorodov et al. 2014). In addition, mouse embryonic fibroblasts lacking neutral CDase are protected from nutrient- and energy-deprivation-induced necroptosis via autophagy and mitophagy (Sundaram et al. 2016). The opposite effects of neutral CDase on cell death may be attributed to the fact that the enzyme is involved in not only a decrease in the Cer level and an increase in the S1P level via Sph kinases, which suppress cell death, but also an increase in the Sph level, which enhances cell death.

Several transcription factors involved in regulation of the neutral CDase expression level have been identified. The first exon of the mouse liver and kidney neutral CDase genes is different from that in the brain, suggesting that the promoter region of the gene is different in each tissue, and transcriptional regulation of the gene occurs in a tissue-specific manner (Okino et al. 2002). Hepatocyte nuclear factor-4α (HNF-4α) and glucocorticoid receptor functionally bind to the liver- and kidney-type promoter regions, respectively. Human neutral CDase promoter does not show any significant similarity with the mouse neutral CDase promoter and has functional transcriptional response elements that bind to transcription factors, including AP-1, NF-Y, AP-2, Oct-1, and GATA. In HEK293 cells, serum stimulates the neutral CDase expression level via the c-Jun/AP-1 signaling pathway (Ito et al. 2014). Both mouse and human neutral CDase promoters do not have TATA and CAAT boxes, typical features of a housekeeping gene.

Summary

This review summarizes the current understanding of the structure and cellular and molecular functions of neutral CDase. The genetic information of neutral CDase is highly conserved in organisms from bacteria to mammals. In prokaryotes, neutral CDase is only found in specific bacteria, such as *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (Okino et al. 1999). Although they cannot synthesize sphingolipids by themselves, the enzyme is thought to be involved in host infection by these bacteria (Ito et al. 2014). In contrast, eukaryotic organisms synthesize sphingolipids by themselves, and neutral CDases play important roles in the catabolism of Cer and regulation of sphingolipid-mediated signaling. Interestingly, the physiological importance of neutral CDase seems to differ among organisms. For example, the deletion of neutral CDase is embryonic lethal in fruit fly, *Drosophila melanogaster*; however, neutral CDase null mice do not exhibit notable phenotypic defects under normal physiological conditions (Ito et al. 2014; Kono et al. 2006). One possible explanation for this is that acid and/or alkaline CDases can complement the loss of neutral CDase because the neutral enzyme is a pan-CDase in fruit fly; however, mammals have acid and alkaline CDases (Ito et al. 2014). However, the subcellular localization of neutral CDase is quite different from those of acid and alkaline enzymes; that is, a neutral enzyme is plasma membrane-bound and secretory protein, whereas acid and alkaline enzymes are localized at lysosomes and the ER/Golgi, respectively. This means that transport of Cer, a substrate for CDases, between organelles is critical for the complementation of functions among CDases. Thus, understanding how Cer is transported between organelles will become an important issue for elucidation of the functional interaction of these CDases.

The balance of the cellular contents of Cer/Sph/S1P is thought to regulate diverse cellular responses, such as apoptosis, proliferation, and cell differentiation, and thus CDases play crucial roles in various signaling systems through

regulation of the balance of these lipid signaling molecules. Recent studies indicated the possibility that neutral CDase will become a promising target for cancer therapy because inhibition of neutral CDase suppresses colon carcinogenesis through increased apoptosis of the cancer cells (Garcia-Barros et al. 2016). In this case, neutral CDase is involved in suppression of apoptosis; however, occasionally, neutral CDase positively regulates cell death (Novgorodov et al. 2014; Sundaram et al. 2016). Like neutral CDase, SMases and Sph kinases also play pivotal roles in regulation of the balance of the Cer/Sph/S1P contents and subsequently cell death and survival; thus, for elucidation of the functional complexity of neutral CDase, comprehensive understanding of the regulation of SMases and Sph kinases, together with neutral CDase, is required.

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Nexin, Plasminogen Activator Inhibitor Type 1

▶ SerpinE1

NF2

▶ `Merlin (*NF2*)

NFAT

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Synonyms

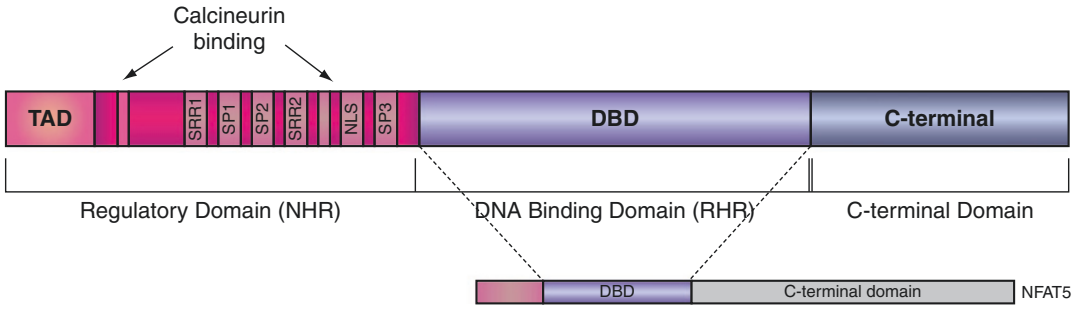
NFATc1 (Nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 1; NFAT2; NFATc, NFAT cytoplasmic); NFATc2 (Nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 2; NFAT1; NFATp, NFAT preexisting); NFATc3 (Nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 3; NFAT4; NFATx); NFATc4 (Nuclear factor of activated T-cells cytoplasmic calcineurin dependent 4, NFAT3); NFAT5 (Nuclear factor of activated T-cells 5; TonEBP, Tonicity-responsive enhancer binding protein; OREBP, Osmotic response element-binding protein)

Historical Background

The gene encoding for the first nuclear factor of activated T cells (NFAT) protein was cloned more than 20 years ago and termed NFATp, as it was shown to be "preexistent" in the cytosol of resting T cells. In activated T cells, NFATp interacted with the transcription factors Fos and Jun in the nucleus to induce the expression of interleukin (IL-) 2 (Jain et al. 1993). Soon after, new members of the NFAT family were isolated, and the characterization of the calcineurin-mediated dephosphorylation and activation of NFAT proteins identified them as the targets of the immunosuppressive effects of cyclosporine A. Although initial efforts were focused on studying the role of NFAT proteins in the regulation of T cell activation, it soon became clear that members of this ubiquitously expressed family of transcription factors were involved in the regulation of a multitude of programs of development and differentiation in many cell types and tissues. This chapter reviews the current understanding of the mechanisms that regulate the activity of NFAT proteins and the functions that these proteins have in different cells and tissues.

Family and Structure

The NFAT family of transcription factors comprises five different members: NFATc1 (also known as NFATc or NFAT2), NFATc2 (NFATp or NFAT1), NFATc3 (NFAT4 or NFATx), NFATc4 (NFAT3), and NFAT5. The activation of NFATc1, NFATc2, NFATc3, and NFATc4 is regulated by the calcium/calmodulin-activated phosphatase calcineurin; NFAT5 is the only NFAT protein that is not regulated by calcium (Macian 2005). NFAT5, which was also identified as the tonicity-responsive enhancer-binding protein, has been shown to regulate the expression of osmoprotective genes in mammalian cells in response to osmotic stress (Aramburu et al. 2006). All NFAT family members have a conserved DNA-binding domain, which shares structural homology with the Rel domain found in the NF-κB family of transcription factors. The DNA-binding domain confers specific DNA binding and mediates interactions with many transcriptional partners, including Fos and Jun proteins (Chen et al. 1998). With the exception of NFAT5, NFAT proteins have also an N-terminal



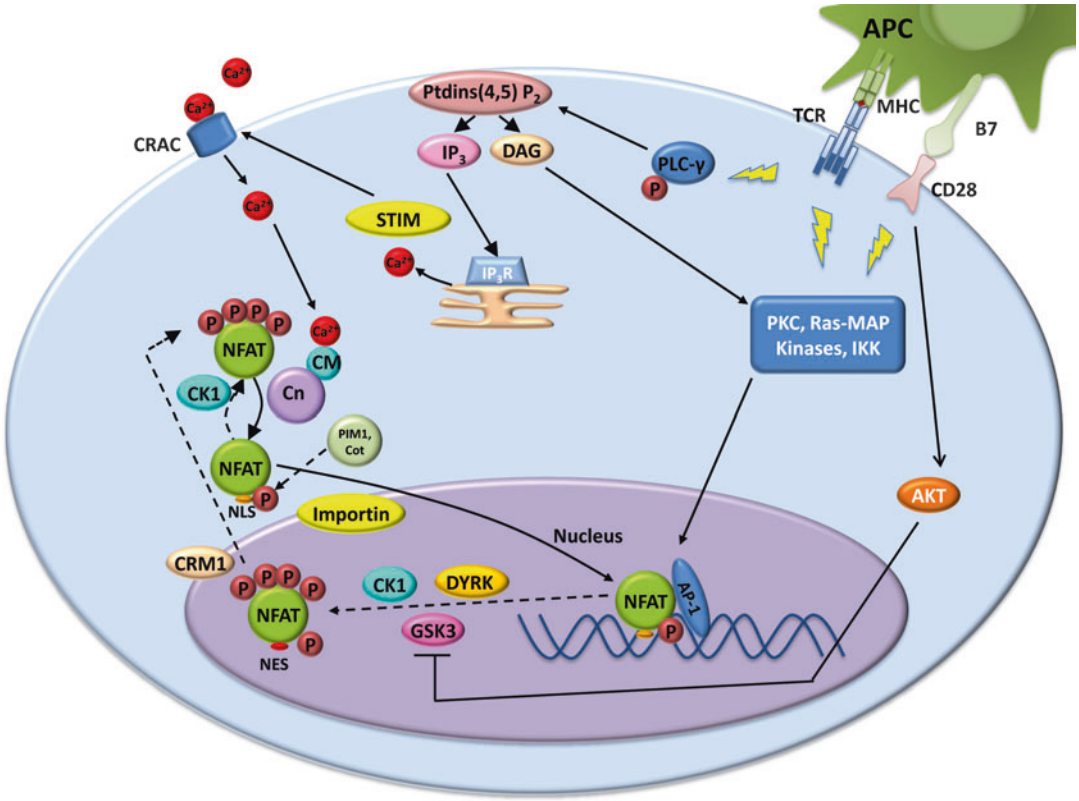
NFAT, Fig. 1 NFATc1, c2, c3, and c4 contain a regulatory domain (NHR or NFAT-homology domain), which comprises a transactivation domain (TAD), several target phosphorylation motifs for NFAT-kinases (SRR1, SRR2, SP1, SP2, and SP3), calcineurin-binding sites, and the nuclear localization signal (NLS); and a DNA-binding domain (DBD or Rel-homology region, RHR), which also contains residues required to interact with Fos and Jun proteins. NFAT5 shares a conserved DNA-binding domain but differs in the rest of its structure for the other NFAT family members

regulatory domain, known as the NFAT homology region (NHR). The NHR contains transactivation and regulatory domains, which include interaction sites for calcineurin and several kinases that regulate NFAT activation by modifying the phosphorylation status of multiple serine-containing motifs (Fig. 1) (Hogan et al. 2003). NFAT5 also contains a Rel domain that represents a conserved DNA-binding and dimerization domain but it lacks an NHR (Lopez-Rodriguez et al. 2001). The C-terminal region is not conserved among the different NFAT proteins and has been shown to contain sites that may allow interactions with other proteins.

Regulation

NFAT activation is mainly regulated by its subcellular localization. The net result of the rate of nuclear import and export of NFAT proteins, which is controlled by their phosphorylation status, determines the overall level of activation of NFAT. Other mechanisms have also been described to contribute to the fine regulation of the transcriptional activity of NFAT.

Regulation by calcium and calcineurin: Engagement of calcium-coupled receptors, such as the T cell receptor (TCR), induces the activation of the calcium/calmodulin-dependent phosphatase calcineurin, which binds NFAT proteins and directly dephosphorylates them, inducing their translocation into the nucleus. In T cells, where this complex regulation has been better characterized, engagement of the TCR induces activation of the phospholipase Cγ, which hydrolyzes phosphatidylinositol 4,5-biphosphate into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds IP_3 receptors in the endoplasmic reticulum and induces calcium release from intracellular calcium stores. STIM proteins sense this depletion and activate calcium entry through interaction with ORA1, an integral component of the calcium-release activated calcium (CRAC) channels in the plasma membrane, causing a further increase in the intracellular calcium levels (Oh-hora and Rao 2008). In response to the increase in the intracellular calcium concentration, calcineurin is activated, binds to and dephosphorylates NFAT proteins, which are heavily phosphorylated and localized in the cytosol in resting cells (Fig. 2). At least 13 different phosphorylation sites located in serine-rich motifs and SPxx-repeat motifs in the regulatory domain are dephosphorylated by calcineurin. This causes a conformational change in NFAT that exposes a nuclear localization signal, allowing NFAT import into the nucleus, where it binds specific sites and cooperates with other transcription factors to activate the expression of distinct sets of genes (Hogan et al. 2003).



NFAT, Fig. 2 *NFAT regulation by calcium/calcineurin and NFAT-kinases in activated T cells*. Pathways involved in the activation of NFAT by nuclear import and modulation of its transcriptional activity are represented with *solid arrows*, whereas the pathways involved in NFAT nuclear export or cytosolic retention are depicted with *dashed arrows*. *APC* antigen presenting cell, *TCR* T cell receptor, *MHC* major histocompatibility complex, *PLC-γ* phospholipase Cγ, *P* phosphate, Ptdins(4,5)P_2, phosphatidylinositol 4,5-biphosphate, *IP3* inositol-1,4,5-triphosphate, *DAG* diacylglycerol, *IP3R* inositol-1,4,5-triphosphate receptor, Ca^{2+} calcium, *CRAC* calcium release activated calcium channel, *STIM* stromal interaction molecule, *NFAT* nuclear factor of activated T cells, *CM* calmodulin, *Cn* calcineurin, *CK1* casein kinase1, *NLS* nuclear localization signal, *NES* nuclear export signal, *PIM1* protooncogene serine/threonine-protein kinase 1, *Cot* cancer Osaka thyroid oncogene 1, *DYRK* dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1, 2, *GSK3* glycogen synthase kinase 3, *CRM1* exportin 1, *JNK* C-Jun N-terminal kinase, *AP-1* activator protein 1, *PKC* protein kinase C, *IKK* inhibitor of kappa B kinase

NFAT-kinases: Phosphorylation of NFAT proteins is required to promote nuclear export and to maintain cytosolic localization in resting conditions. Several kinases have been reported to be responsible for the phosphorylation of different serine-containing motifs in NFAT. Casein kinase1 (CK1) binds the N-terminal region of NFAT and regulates its nuclear export and cytosolic retention through phosphorylation of a serine-rich motif. Glycogen synthase kinase 3 (GSK3) phosphorylates serine-proline motives in NFATc2 and NFATc1, promoting NFAT nuclear export. These phosphorylation sites appear to be created by previous priming by cyclic-AMP dependent protein kinase A (PKA)-mediated phosphorylation of NFAT. Activation of the AKT kinase negatively regulates GSK3 and prolongs NFAT residence time in the nucleus. The dual-specificity tyrosine-(Y)-phosphorylation-regulated kinases DYRK1A and DYRK2 also regulate NFAT nuclear export through phosphorylation of an SP motif that primes for subsequent phosphorylation by CK1 or GSK3 (Muller and Rao 2010). LRRK2 may also phosphorylate NFAT proteins and contribute to the regulation of NFAT activation (Liu et al. 2011). Interestingly, it has been shown that many of

these kinases, including CK1 and GSK3, exist in a complex with a noncoding RNA repressor of NFAT (NRON) that inhibits nuclear import of NFAT by facilitating interactions of those kinases with NFAT (Sharma et al. 2011). Recently, in response to IL-7, JAK3 has been reported to induce phosphorylation of NFATc1 in $CD4^{-}CD8^{-}$ thymocytes; however, in this case, phosphorylation of this NFAT protein induces its translocation into the nucleus in a calcineurin-independent manner (Patra et al. 2013).

Transcriptional regulation of NFAT: NFATc1A is an isoform of NFATc1 that is regulated at a transcriptional level by an autoregulatory loop under the control of an inducible NFAT-dependent promoter. In T cells, it has been shown that the activation of constitutively expressed isoforms of NFATc1 and NFATc2 induces the expression and accumulation of this inducible NFATc1A isoform (Serfling et al. 2006).

Posttranslational regulation: Several posttranslational mechanisms that contribute to the regulation of NFAT activity have been described. Sumoylation of NFAT is an NFAT nuclear retention mechanism, which might also regulate NFAT transcriptional activity. Evidence has also been presented supporting that poly(ADP-ribose) polymerase-1 (PARP-1) interacts with NFATc1 and NFATc2, regulating NFAT nuclear export and transcriptional activity (Muller and Rao 2010). Furthermore, NFATc2 has also been shown to be ubiquitinated by the E3 ubiquitin ligase MDM2 in breast cancer cells, which leads to proteasome-mediated degradation (Mancini and Toker 2009).

Transcriptional Activity

NFAT proteins form transcriptional complexes, in which they cooperate with other transcription factors to activate or repress the expression of specific genes. This allows cells to integrate calcium signaling with other signaling pathways to regulate the expression of specific programs of gene expression. Initially identified as the nuclear component of the NFAT activity that was responsible for the expression of IL-2 in activated T cells, activator protein 1 (AP-1) complexes are the best characterized NFAT partners and are one of the main transcription factors that interacts with NFAT during T cell activation. In response to the engagement of the TCR and costimulatory receptors, calcium signaling and the Ras-MAPK pathway converge in the activation of NFAT and Fos and Jun proteins, which form the AP-1 complex. The DNA-binding domain of NFAT interacts with AP-1, forming a quaternary complex on DNA that activates the expression of activation-induced genes, including numerous cytokines (Macian et al. 2001). The number of transcription factors that have been identified to cooperate with NFAT has grown in the last few years. In many cases, these interactions occur in specific cells of tissues and are responsible for the regulation of different programs of activation, differentiation, or development. NFAT proteins are also able to form homodimers. These complexes bind to κB-like sites that contain two tandem NFAT-binding sites separated by one or two bases. Dimers formed by NFATc2 have been implicated in regulating the expression of genes that induce a hyporesponsive state in T helper cells. In addition, NFAT5 needs to form homodimers to bind to DNA and exert its transcriptional activity (Lopez-Rodriguez et al. 2001; Baine et al. 2009).

Functions

The innate immune system is our first line of defense against invading pathogens and, therefore, it must react quickly and efficiently. Cell types involved in this process include eosinophils, neutrophils, mast cells, and macrophages. NFAT proteins have been shown to be expressed in all these cell types and serve a pivotal role in transforming molecular signals to expression of genes. Ligation of pattern recognition receptors in myeloid cells by microbes can lead to NFAT activation in different cells of the innate immune system, including neutrophils, dendritic cells, and macrophages. Indeed, fungal infections that are commonly seen in cyclosporine A-treated individuals appear to be linked to inhibition of NFAT-mediated gene expression in neutrophils.

Interestingly, not only calcineurin regulated members of the NFAT family of transcription factors but also NFAT5 participate in the regulation of gene expression in response to pattern recognition receptors in cells that participate in the innate immune response. NFAT expression also plays an integral part of the mast cell response and survival, as ligation of the Fcε receptor results in an influx of intracellular calcium, causing mast cells to release histamine-containing granules and produce cytokines (Muller and Rao 2010; Fric et al. 2012).

The adaptive immune response is highly specific and efficient at targeting pathogens and infected cells for elimination. The activation of effector cells is governed by the recognition of an antigen by the B cell receptor (BCR) or its presentation in the context of an MHC molecule to cognate TCRs on T cells. NFAT has been most extensively studied in T cells, although the participation of NFAT in the regulation of B cells has also been characterized. NFAT proteins are activated in B cells in response to BCR engagement and participate in the induction of the programs of gene expression that regulate B cell activation and differentiation. In T cells, coordinated engagement of the TCR and the costimulatory receptor CD28 allows NFAT to cooperate with other transcription factors, such as Fos and Jun, to induce the expression of many genes that are required to ensure effective T cell activation (Macian 2005; Muller and Rao 2010). However, in the absence of costimulation, TCR triggering causes NFAT nuclear localization, which, without the opportunity to cooperate with Fos and Jun, forms homodimers that regulate the expression of genes that maintain T cells in a tolerant hyporesponsive state termed anergy (Baine et al. 2009). The involvement of NFAT proteins in the regulation of immune tolerance is also mediated through their participation in the development of regulatory T cells (Treg), a distinct population of T cells that express the transcription factor FoxP3 and have the capacity to suppress the activation of other immune cell populations. NFAT proteins not only regulate the expression of FoxP3 but also cooperate with this transcription factor to activate (CTLA-4, CD25, and GITR) or inhibit (IL-2) the expression of genes in Treg and, therefore, regulate the differentiation and function of these suppressor cells (Wu et al. 2006).

Development of thymocytes is also dependent on NFAT activity. In the thymus, immature thymocytes cells rearrange both the α and β chains of the TCR and mature from a double negative (CD4-CD8-) thymocytes into a double positive thymocyte that expresses both CD4 and CD8 coreceptors. As mentioned above, NFATc1 is crucial in the regulation of this process (Patra et al. 2013). Double positive thymocytes then undergo a rigorous process that involves being positively selected for the presence of a TCR that can interact with MHC molecules but negatively selected for self-reactive TCR that can recognize self-antigens. The lack of calcineurin activity in immature thymocytes leads to a block in positive selection, suggesting that NFAT proteins are also involved in this step of thymocyte development. Supporting this fact, NFATc2 and NFATc3 appear to be responsible for regulating the thresholds of signal transduction that govern positive selection in the thymus (Gallo et al. 2007).

CD4+ T cells can differentiate into many different effector subsets, such as Th1, Th2, or Th17, a process that is regulated by the cytokine profile of the extracellular environment. In all these differentiation events, NFAT proteins regulate the expression of specific genes that help define the particular T cell subtype. The ability of NFAT to direct opposite programs of differentiation depends on the activation of transcriptional copartners. For instance, T-bet is an integral cotranscriptional partner for NFAT in the development of Th1 cells and the secretion of their signature cytokine IFN-γ. Likewise, GATA3–NFAT interactions are necessary for IL-4 expression and maturation into Th2 cells. Similarly, the presence of IL-6 in the extracellular milieu induces the expression of RORγt, which synergizes with NFAT in the expression of IL-17, IL-21, and IL-22 to allow Th17 differentiation (Muller and Rao 2010). As mentioned above, NFAT proteins participate in the regulation of Treg generation and have also been implicated in the differentiation of follicular helper T cells (Wu et al. 2006; Martinez et al. 2016).

Outside of the immune system, NFAT expression has been reported in almost all tissues, although the expression of any individual NFAT protein is often limited to specific tissues. For instance, whereas NFATc1, NFATc2, and NFATc3 are expressed in cells of the immune system and in many nonlymphoid cells and tissues, NFATc4 expression has not been reported in immune cells. An increasing number of reports have characterized the role that different NFAT proteins have as regulators of development, differentiation, and function in many cells and tissues, including skeletal muscle differentiation, myocardial hypertrophy, control of heart valve formation, vascular development, cartilage formation, neuronal development, and the regulation of stem cell quiescence (Wu et al. 2007). The expression of different NFAT proteins is developmentally regulated in skeletal muscle and these proteins control progression from immature precursors to mature myocytes and contribute to the specification of muscle fiber type. NFAT proteins also regulate cartilage growth and bone remodeling. The role that NFAT proteins play in the regulation of osteoclast differentiation has been amply documented. In these cells, RANKL-mediated activation of NFATc1 directs the expression of a set of genes required for osteoclast differentiation. Initial characterization of a mouse model that lacked expression of NFATc1 unequivocally showed that NFATc1 played a key role in the formation of the heart's valves. In the adult heart, NFAT proteins partner with members of the GATA and MEF2 families of transcription factors to regulate myocardial hypertrophy. NFATc3 and NFATc4 are expressed in perivascular mesenchymal cells, which regulate the assembly of blood vessels during embryogenesis, and mice that lack those NFAT proteins present an abnormal vascular development. Vascular endothelial growth factor is a major activator of NFAT proteins in endothelial cells and engagement of its receptor leads to the activation of NFAT-dependent genes such as COX2. NFAT proteins also regulate neuronal axon growth and are essential for neuronal development and the differentiation of Schwann cells. NFAT has also been shown to control beta cell growth in the endocrine pancreas and regulate insulin-signaling pathways and adipogenesis. NFATc1 has also been implicated in the maintenance of stem cell quiescence in the skin follicle by repressing the expression of the cell cycle kinase CDK4 (Wu et al. 2007; Sitara and Aliprantis 2010).

NFAT proteins are key regulators in the control of cell development and differentiation in part by modulating proliferation and cell death. As such, altered NFAT signaling has been described in cancer cells. As expected due to their pivotal role in lymphocyte development, dysregulated NFAT activity have been associated with several forms of B and T cell lymphoma and leukemia. Furthermore, NFAT proteins have been shown to be involved in the regulation of specific properties of different cancers. For instance, NFATc2 and NFAT5 appear to positively regulate migration and invasion of breast cancer cells. NFAT proteins have also been proposed to regulate tumor-associated angiogenesis and may directly activate the expression of oncogenes, such as *Myc* (Mancini and Toker 2009; Muller and Rao 2010).

Pharmacological Modulation of NFAT Activity

Pharmacological agents used to modulate NFAT activity have been tremendously beneficial in the clinic. These agents, which inhibit the activation of T cells and act as immunosuppressants, are widely used in therapies ranging from treatment of autoimmune disease to prevention of organ transplant rejection. However, their widespread use is limited by their costly side effects, such as nephrotoxicity. The activation of NFAT is dependent on its dephosphorylation, and, therefore, the phosphatase calcineurin has been an important area of focus for inhibitor development. Nevertheless, the specificity of NFAT inhibition is limited by the fact that NFAT is not the only target of calcineurin. Cyclosporine A and FK506 are the most widely used and studied suppressors of NFAT activity. Both are calcineurin inhibitors whose mechanism of action is quite similar. They bind separate intracellular peptidyl prolyl isomerases (cyclosporine A-cyclophilin; FK506-

FKBP12). These complexes then bind to distinct regions of calcineurin and inhibit its phosphatase activity. The fact that these compounds not only affect NFAT but also the activity of other endogenous targets also limits their use as specific NFAT inhibitors (Li et al. 2011). The search for more specific inhibitors has centered on targeting specifically the interaction of NFAT and calcineurin. Calcineurin-binding sites on NFAT have been mapped to the N-terminal regulatory domain and include the amino acid sequence SPRIET. A closer analysis of this sequence among NFAT family members revealed the consensus-binding sequence PxIxIT, which laid the groundwork for the discovery using combinatorial libraries of the highly potent VIVIT peptide (Hogan et al. 2003). This peptide has been successfully used in mouse models of graft rejection and tumor progression. The major benefit of the VIVIT peptide is in its higher specificity for the inhibition of NFAT function; however, this sequence is still conserved in other proteins, such as AKAP79, Cabin1, or MCIP1, that have calcineurin-binding activity. Due to the severe side effects caused by long-term administration of cyclosporine A or FK506, finding inhibitors with more specificity for NFAT has been also pursued. Disrupting the ability of NFAT to bind DNA or enhancing the nuclear export of NFAT has been explored. As binding partners and gene targets of NFAT are being discovered, further inhibitors with higher specificity could be developed, which should lead to a higher degree of precision to specifically block NFAT activity.

Summary

Members of the NFAT family of transcription factors have been established as crucial regulators of numerous programs of development, differentiation, and activation in many cell types and tissues. NFAT activation is induced by the engagement of calcium-coupled receptor. This eventually leads to the translocation of NFAT into the nucleus, where it cooperates with several transcriptional partners so that signals that emanate from different inputs can be integrated to ensure specific regulation of distinct programs of gene expression. Circuits of regulation that fine-tune NFAT activation have also been characterized and are likely to play important roles in the regulation of NFAT activity. Given the wide range of tissue expression of NFAT proteins, it is clear that novel functions and targets of these transcription factors still remain to be discovered. The identification of specific functions for individual NFAT family members and the characterization of the differential spatial and temporal expression of specific NFAT proteins during unique programs of development should also enhance our understanding on how NFAT-regulated programs of development are orchestrated. The development of new, highly specific inhibitors of NFAT activity to resolve the inherent toxic effects associated with the use of calcineurin inhibitors is an ongoing challenge that could have great clinical impact. Given the increasing amount of evidence that implicates NFAT signaling in oncogenesis and cancer progression, these new therapeutic approaches could prove of great value not only to suppress NFAT-regulated immune responses but also to design new interventions for the treatment of certain types of cancer and other pathologies controlled by this family of transcription factors.

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NFAT5

▶ NFAT

NFATc1

▶ NFAT

NFATc2

▶ NFAT

NFATc3

▶ NFAT

NFATc4

▶ NFAT

NFE2L2

▶ Nrf2 (NF-E2-Related Factor2)

NF-E2-Related Factor 2

► Nrf2 (NF-E2-Related Factor2)

NF-Kappa-B Inhibitor Zeta

► IkBz

NFKBIZ

► IkBz

NFPR

► Formyl Peptide Receptor

NF-κB Essential Modulator

► IKK (IκB Kinase) Complex

NF-κB Family

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Synonyms

Nuclear factor-kappa-B; Nuclear factor of kappa light polypeptide gene enhancer in B cells

List of Discussed NF-κB Family Members and Regulatory Molecules

NFκB1: p105, p50, KBF1, EBP-1
RelA: p65, NFKB3
RelB: I-REL
REL: c-Rel
NFκB2: p100, p52; LYT10
Bcl-3: BCL4, D19S37
NFKBIA: IκBα, IKBA, MAD-3, NFKBI
NFKBIB: IκBβ, IKBB, TRIP9
NFKBIE: IκBε, IKBE
NFKBIZ: IκBζ, IKBZ, INAP, MAIL
CHUK: IKKα, IKBKA, IKK1, IKKA, NFKBIKA, TCF16
IKBKB: IKKβ, IKK2, IKKB, NFKBIKB
IKBKG: IKKγ, NEMO, AMCBX1, FIP-3, FIP3, Fip3p, IP, IP1, IP2, IPD2, IKKAP1, IKKG
IKBKE: IKKε, IKKE, IKKI
MAP3K14: NIK, HS, HSNIK
TNFAIP3: A20, OTUD7C, TNFA1P2
OTUD7B: CEZANNE
CYLD: HSPC057, CDMT, CYLD1, CYLDI, EAC, MFT1, SBS, TEM, USPL2

Historical Background: Discovery and Structure

NF-κB (nuclear factor-kappa-light-chain-enhancer of activated B cells) is a collective term for a family of transcription factors. NF-κB has a complex regulation, modulates the expression of a wide set of genes, both by promoting and by suppressing gene expression and biological responses and is involved in a variety of diseases. The dysfunction of NF-κB is associated with inflammatory disease, cardiovascular injury, cancer, diabetes, kidney injury, viral infections, and human genetic disorders, among others (Kumar et al. 2004).

NF-κB was identified as a protein that bound to a specific decameric DNA sequence (ggg ACT TTC C), within the intronic enhancer of the immunoglobulin kappa light chain in mature B and plasma cells but not pre B cells (Sen and Baltimore 1986). NF-κB DNA-binding activity was induced by a variety of exogenous stimuli,

was independent from de novo protein synthesis, and was bound to several DNA sequences.

NF-κB family members share structural homology with the retroviral oncoprotein v-Rel, resulting in their classification as NF-κB/Rel proteins (Gilmore 2006). There are five NF-κB proteins in the mammalian NF-κB family: RelA (p65), RelB, c-Rel, p50 (NFκB1, generated from p105), and p52 (NFκB2, generated from p100). All of them may form homo- and heterodimeric complexes. The most common and best characterized active form is the RelA/p50 heterodimer (Hayden and Ghosh 2004).

Each member of the NF-κB family has a conserved N-terminal region termed the Rel homology domain (RHD). The Rel homology domain mediates the DNA binding, dimerization, and nuclear transport of the NF-κB proteins (Li and Verma 2002). However, the transcription activator domain (TAD) necessary for target gene expression is present only in the carboxyl terminus of RelA, c-Rel, and RelB subunits. Large precursors, p105 and p100, undergo processing to generate mature p50 and p52, respectively. The p50 and p52 NF-κB subunits do not contain transactivation domains. However, they participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel (Li and Verma 2002). The p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators.

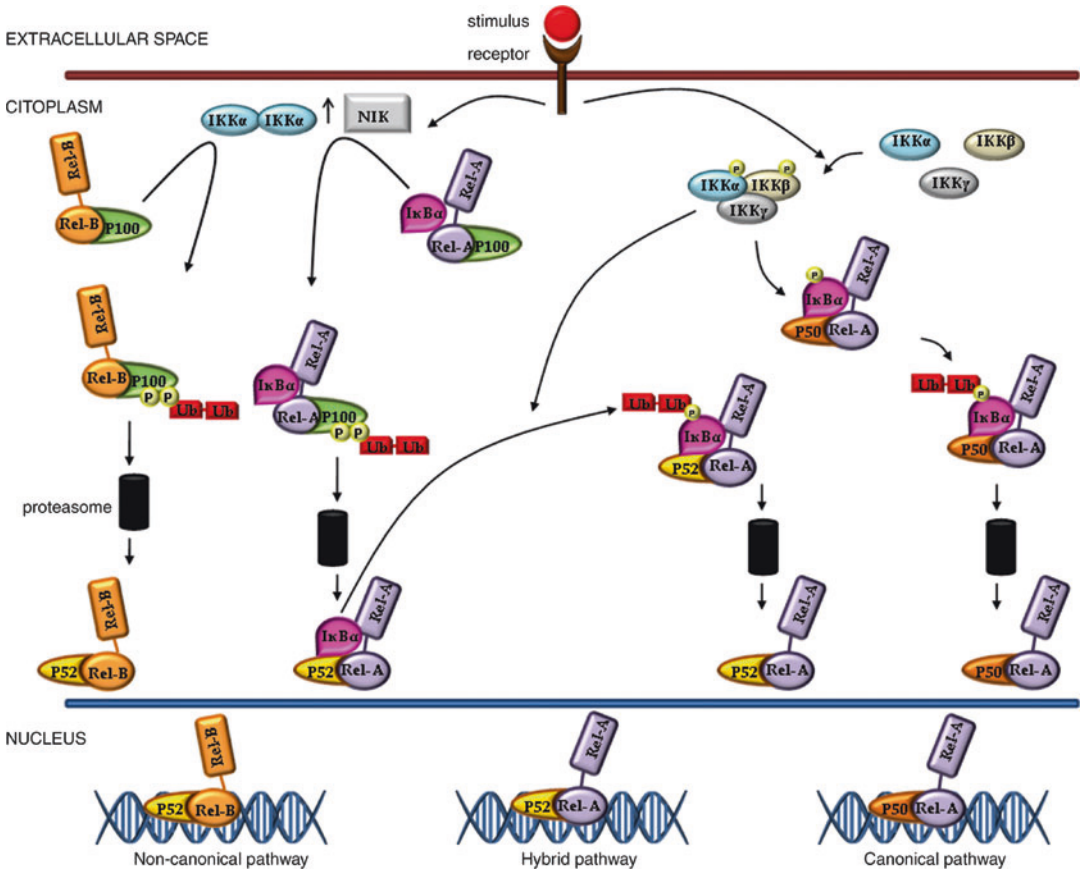
NF-κB Activation

Activation of NF-κB requires a number of discrete steps (Fig. 1). There is a long list of known inducers of NF-κB activity, including many inflammatory cytokines such as TNF-α superfamily cytokines and IL-1, T cell activation signals, growth factors, reactive oxygen species (ROS), bacterial lipopolysaccharide (LPS), and other microbial products that activate toll-like receptors (TLRs) and other stress inducers (http://people.bu.edu/gilmore/nf-kb/inducers/index.html).

In the cytoplasm of almost all cell types, inactive NF-κB is associated with inhibitory κB proteins (IκBs) that regulate NF-κB nuclear translocation and DNA binding (Sanz et al. 2010a). IκBs are a class of inhibitor proteins that contain an N-terminal regulatory domain, followed by multiple copies of a sequence called ankyrin repeats and a COOH-terminal PEST domain that is important in regulating IκB turnover. The ankyrin repeats mediate the association between IκBs and NF-κB dimers and mask the nuclear localization signals (NLS) of NF-κB proteins, thus preventing nuclear translocation and keeping them in an inactive state in the cytoplasm. The most important IκBs are IκBα (associated with transient NF-κB activation), IκBβ (involved in sustained activation), and IκBε. Additional IκBs are Bcl-3, NFκBiz, p100, and p105. Bcl-3 and NFκBiz do not induce cytoplasmic retention of NF-κB, but regulates gene expression as transcriptional co-activators for p50 and p52 homodimers in the nucleus.

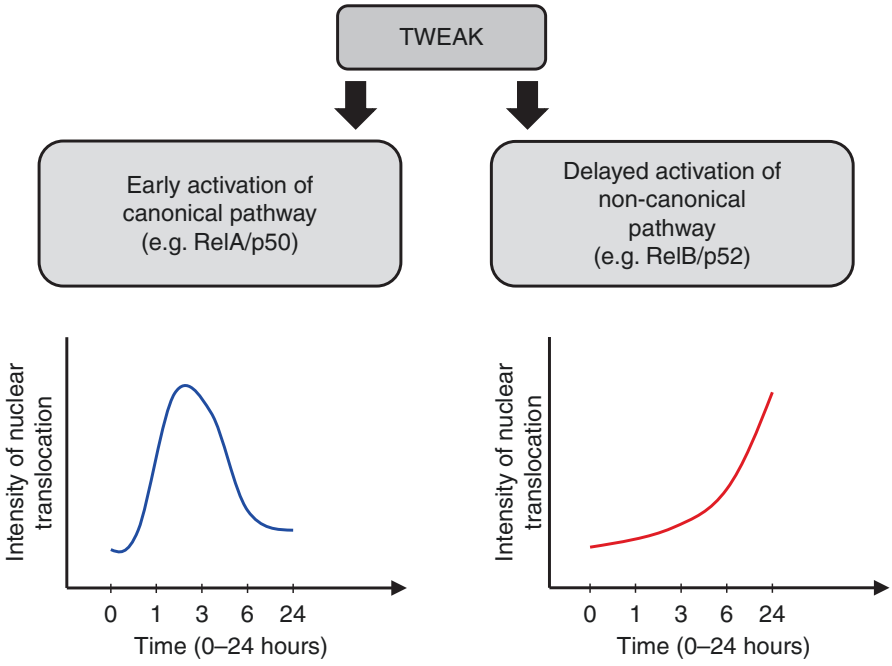
The phosphorylation, ubiquitination, and subsequent degradation by the 26S proteasome of IκB proteins are a key step in NF-κB activation that release active NF-κB (Karin and Delhase 2000). NF-κB activation is initiated by signal-induced phosphorylation of IκBs, mediated by a high-molecular-weight complex that contains a serine-specific IκB kinase (IKK). IKKs have 52% amino acid identity and a similar structural organization, which includes kinase, leucine zipper, and helix-loop-helix domains. IKKs form both homo- and heterodimers. Gene disruption studies of IKK genes in mice indicate that IKKβ is the critical kinase involved in activating the NF-κB pathway, while IKKα likely plays an accessory role (Yamamoto and Gaynor 2001). The IKK complex consists of three subunits, including the kinases IKKα and IKKβ (catalytic subunits also called IKK-1 and IKK-2, respectively) (Sanz et al. 2010a), and the regulatory nonenzymatic subunit IKK-γ (NEMO, NF-κB essential modulator) (Sanz et al. 2010a). IKKs are key convergence steps for multiple signaling pathways that lead to NF-κB activation.

There is a temporal and selective control of NF-κB target gene activation. Some genes are transcribed following a short stimulation of NF-κB. These include negative regulators of NF-κB activity such as IκBα and inflammatory



NF-κB Family, Fig. 1 A representation of canonical and noncanonical NF-κB pathway activation. In the canonical pathway, IKK activation leads to proteasomal IκB degradation, which allows nuclear migration of RelA/p50 and other complexes. In the noncanonical pathway, NIK and IKKα recruitment leads to proteasomal processing of p100 to p52, allowing nuclear migration of RelB/p52 complexes. p100 may also retain RelA, c-Rel, and p50 in the cytoplasm. Thus, proteasomal processing of p100 to p52 also generates RelA/p52, c-Rel/p52, or p52/p50 complexes. IκB molecules weakly sequester RelB/p52 complexes, and they are free for nuclear translocation upon p100 processing. In the hybrid pathway, noncanonical p100 processing generates RelA/p52 and c-Rel/p52 complexes that are retained in the cytosol by IκB proteins. Classical pathway degradation of IκB proteins allows nuclear migration of these complexes. *P* phosphorylation, *Ub* ubiquitin

cytokines such as IL-6 and MCP-1. Other genes are transcribed only when NF-κB activation occurs for at least 1 h, such as cell-surface receptors, adhesion molecules, and some chemokines such as RANTES/CCL5 (Hoffmann et al. 2002). DNA accessibility contributes in part to these temporal patterns. Cells respond sensitively to time-varying inputs in complex and dynamically changing signaling environments to achieve efficient gene expression regulation. As an example, NF-κB dynamics synchronize with oscillating TNF signal and become entrained, leading to significantly increased NF-κB oscillation amplitude and mRNA output compared to non-entrained response. This allows cells to achieve efficient gene expression in dynamically changing signaling environments. (Kellogg and Tay 2015). A switchlike response in NF-κB activity implies the existence of a threshold in the NF-κB signaling module. As an example, the switch mechanism for NF-κB activation in B cell receptor (BCR) signaling includes MAGUK protein 1 (CARMA1, CARD11), TAK1 (MAP3K7), and IKKβ (Shinohara et al. 2014).



NF-κB Family, Fig. 2 Temporal pattern profile of TWEAK-induced canonical and noncanonical NF-κB pathway activation in renal proximal tubular epithelial cells. TWEAK is one of a handful of TNF superfamily cytokines that activates both the canonical NF-κB pathway, leading to early transient nuclear translocation of RelA/p50, and the noncanonical NF-κB pathway, leading to delayed nuclear translocation of RelB/p52

N

NF-κB activation and nuclear translocation can proceed either through the classical/canonical pathway, that is a rapid and transient response of stimuli involving mainly RelA/p50, or the alternative/noncanonical NF-κB pathway, that involves slow activation of the RelB/p52 heterodimer leading to prolonged activation of NF-κB target genes (Sanz et al. 2010a) (Figs. 1 and 2). These pathways require different IKK complexes, activate different NF-κB complexes, and may have different target genes. While many stimuli have been shown to activate the canonical NF-κB pathway, the list of noncanonical NF-κB pathway activators is more limited (Poveda et al. 2013).

Canonical pathway: The classical NF-κB pathway is triggered by most of the stimuli that activate NF-κB. The activated IKK complex IKKα, IKKβ, and NEMO phosphorylates two specific serines near the N terminus of IκBα to trigger its ubiquitin-dependent degradation by the 26S proteasome, allowing nuclear migration of RelA/p50 and other NF-κB dimers (Haas 2009).

Noncanonical pathway: The alternative pathway results in nuclear translocation of the heterodimer RelB/p52 leading to prolonged activation of NF-κB target genes (Senftleben et al. 2001). Only a small number of stimuli are known to activate NF-κB via this pathway, including TNF superfamily members such as TWEAK, but also lymphotoxin-α, BAFF, or RANKL (Sanz et al. 2010b). This pathway requires IKKα phosphorylation by NF-κB-inducing kinase (NIK, MAP3K14), a serine/threonine kinase that binds to TRAF2. Activation of the noncanonical NF-κB pathway involves degradation of an inhibitory protein, TNF receptor-associated factor 3 (TRAF3). The deubiquitinase OTUD7B is a pivotal regulator of signal-induced noncanonical NF-κB activation through deubiquitination and stabilization of TRAF3 (Hu et al. 2013). IKKα phosphorylates p100 leading its polyubiquitination and promoting the proteasomal processing to p52, freeing the active p52/RelB dimers that migrate to the nucleus (Senftleben et al. 2001).

The noncanonical IKKε (IKKi) is a serine/threonine kinase inducible by inflammatory mediators that activates IRF-7; phosphorylates IκBα, NF-κB p65, and c-Rel; and is required for activation of an NF-κB complex containing p52 and p65 (Wietek et al. 2006; Harris et al. 2006). However, mice bearing a deletion of the IKKε gene activate NF-κB normally in response to LPS.

Hybrid pathways: The hybrid pathway of NF-κB activation requires the contribution of both pathways: the NF-κB complex is generated by the alternative pathway and is activated by the classical pathway (Sanz et al. 2010a). MAP3K14 may also stimulate the canonical NF-kB pathway.

Finalization of NF-κB Activation

NF-κB activation is regulated by negative feedback loops, which control the duration of NF-κB nuclear localization in response to a stimulus. Negative feedback mechanisms include NF-κB-dependent induction of IκBα, A20, and Cezanne. Newly formed IκBα sequesters NF-κB subunits and terminates transcriptional activity unless a persistent activation signal is present. Deubiquitinating enzymes CYLD (cylindromatosis gene), A20, and Cezanne induced by proinflammatory signaling can block IKK activation by removing polyubiquitin chains (Van der Heiden et al. 2010). Inactivation of DNA-bound NF-κB requires copper metabolism Murr1 domain-containing (COMMD) proteins, that regulate the ubiquitination pathway, and acts in conjunction with CCDC22 to direct the degradation of IκB proteins (Starokadomskyy et al. 2013).

The "NF-κB-p62-mitophagy" pathway is a macrophage-intrinsic regulatory loop through which NF-κB restrains its own inflammation-promoting activity and orchestrates a self-limiting host response that maintains homeostasis and favors tissue repair by restraining NLRP3-inflammasome activation (Zhong et al. 2016).

Target Genes

NF-κB complexes regulate the transcription of multiple genes related to inflammation, immunity, apoptosis, cell proliferation, and differentiation. At http://people.bu.edu/gilmore/nf-kb/ the reader can find updated lists of NF-κB gene targets classified by function as cytokines/chemokines and their modulators, immunoreceptors proteins involved in antigen presentation, cell adhesion molecules, acute phase proteins, stress response genes, cell-surface receptors, regulators of apoptosis, growth factors, ligands and their modulators, early response genes, transcription factors and regulators, viruses, enzymes, and others.

While NF-κB frequently promotes gene transcription, it may also function as a repressor of gene expression. Thus, anti-inflammatory cytokines may induce the synthesis of nuclear-located atypical IκB proteins, which bind to DNA-bound NF-κB dimers and repress transcription of inflammatory genes (Ghosh and Hayden 2008). Additional mechanism involves competition of RelA with co-activators of transcription, posttranslational modifications of RelA, and posttranslational modification of histones near the NF-κB target genes (Sanz et al. 2010a).

No clear-cut differences in DNA-binding sequences have been observed for the different NF-κB complexes, and considerable promiscuity is thought to occur.

The Role of NF-κB Signaling in Disease

Studies in knockout mice have revealed specific functions of each NF-κB family proteins in the regulation of disease. Deletion of RelA or IKKβ genes in mice causes embryonic lethality due to excess apoptosis in the liver indicating that their function is indispensable during development (Beg et al. 1995; Li et al. 1999). On the other hand, mice lacking RelB are immunodeficient but develop normally to adulthood (Sha et al. 1995). Mice lacking c-Rel or p52 have defective immune functions (Caamaño et al. 1998).

Dysregulated activation of the NF-κB pathway is involved in the pathogenesis of a number of human diseases. The NF-κB family controls multiple processes, including immunity, inflammation, cell survival, differentiation and proliferation, and regulates cellular responses to stress,

hypoxia, stretch, and ischemia. Activation of the NF-κB pathway is involved in the pathogenesis of chronic inflammatory disease, kidney disease, atherosclerosis, infections, neurological diseases, cancer, and aging, among others. Evidence for the involvement of NF-κB in disease often comes from functional studies in experimental cell and animal models as well as descriptive data from animal models and human samples. We will provide some examples of NF-κB involvement in disease processes.

Inflammation

NF-κB is involved in the pathophysiology of autoimmune and inflammatory disorders, such as rheumatoid arthritis, asthma, and others.

NF-κB is activated in the inflamed synovium of rheumatoid arthritis patients as well as in the synovium of animal models in this disease. Intra-articular gene transfer of IKKβ into the joints of normal rats resulted in synovial inflammation, while dominant-negative adenoviral IKKβ construct ameliorated the severity of arthritis. NF-κB "decoy" or RelA antisense oligodeoxynucleotides prevented the development of arthritis in rats (Neurath et al. 1996).

Asthma is characterized by the lung infiltration of inflammatory cells, increased NF-κB, and is evident in biopsies from asthmatic patients (Kumar et al. 2004). Treatment with steroids decreases NF-κB activity in mice, cultured cells (Kumar et al. 2004), and in asthmatic patients as well as reducing the symptoms of the disease.

Kidney Disease and Aging

The role and regulation of NF-κB in kidney disease was recently reviewed and includes functional studies showing improvement of kidney disease outcomes when targeting NF-κB in experimental models (Sanz et al. 2010a). In tubular cells, NFκBiz protein downregulation, as observed in experimental AKI, increases inflammatory responses while protecting from inflammation-induced apoptosis and also prevents klotho downregulation (Poveda et al. 2016). More recently, MAP3K14 activity-deficient aly/aly (MAP3K14 aly/aly) mice are protected from AKI, and this appears to depend on kidney cell MAP3K14 deficiency that limits tubular cell inflammatory responses (Ortiz et al. 2017). Many drugs used in kidney disease target NF-κB, including steroids, calcineurin inhibitors, drugs targeting the renin-angiotensin system, and statins, but no functional studies of therapies specifically targeting NF-κB are available in humans. The recent observation that inflammation downregulates klotho mRNA expression and protein via NF-κB activation provides a link between inflammation, kidney disease, NF-κB, and accelerated aging (Moreno et al. 2011). Klotho is a kidney-secreted hormone with antiaging properties and klotho knockout mice die prematurely from accelerated aging. Indeed, this may represent a wider phenomenon of NF-κB-mediated suppression of cell- or tissue-protective genes, since NF-κB also mediated the inflammation-induced downregulation of the mitochondria biogenesis regulator PGC1α (Ruiz-Andres et al. 2016). In addition, DNA damage drives aging in part through NF-κB activation involving RelA and IKK, and this was prevented by IKK/NF-κB inhibitors (Tilstra et al. 2012).

Cardiovascular Disease

NF-κB has been linked to both cardiovascular health and disease. NF-κB can protect cardiovascular tissues from injury or contribute to pathogenesis depending on the cellular and physiological context.

Atherosclerosis is a chronic lipid-driven inflammatory disease characterized by accumulation of lipids in arterial walls, which can lead to a heart attack or stroke. Recruitment of monocytes and their extravasation into the subendothelial space, a key event in atherogenesis, is regulated by NF-κB. Activated NF-κB has been identified in situ in human atherosclerotic plaques (Collins and Cybulsky 2001).

One consequence of atherosclerosis is tissue ischemia. Ischemia-reperfusion alters oxygen availability leading to NF-κB activation through proinflammatory cytokines and endogenous ligands for TLRs. Blocking NF-κB using pharmacological inhibitors or decoy oligonucleotides can reduce myocardial infarction in animal models. However, in a murine myocardial infarction model NF-κB activation was essential for the

protection of cardiomyocytes from apoptosis via induction of cytoprotective genes (Van Der Heiden et al. 2010). This illustrates the "good and evil" aspects of NF-κB.

Human Immunodeficiency Virus (HIV) and Other Infections

Most viruses encode proteins that are capable of activating NF-κB. HIV infection induces NF-κB activation that allows evading the immune response. HIV has two NF-κB binding sites called long terminal repeat (LTR) that are involved in viral transcription. NF-κB activation by viral infection is required for viruses to induce proliferative responses, like expression of cyclin D1, replicate their genetic material, and induce pathogenic responses.

In the response to bacterial infections, the M1 to M2 macrophage reprogramming that develops during LPS tolerance depends on shifting the balance between active p65-p50 and inhibitory p50-p50 NF-κB pathways (Rackov et al. 2016). RelA is essential for stress-induced transcriptional remodeling in the liver and the subsequent activation of the acute phase response, whose functional role includes compartmentalization of local infection, decreasing bacterial infection-induced death (Quinton et al. 2012). Several mutations in NF-κB genes cause immune deficiency. IKBKB mutations encoding IKK2 were recently reported to induce deficiency of innate and acquired immunity characterized by hypogammaglobulinemia or agammaglobulinemia, peripheral blood B cells and T cells almost exclusively of naive phenotype and absent regulatory T cells and γδ T cells (Pannicke et al. 2013).

Neurological Diseases

NF-κB is associated with antiapoptotic as well as proapoptotic mechanisms. Consistent with its role in regulating apoptosis, NF-κB serves as a cell survival role in stressed neurons through the upregulation of antiapoptotic and antioxidant genes. NF-κB activation may also be involved in the initiation of neuritic plaques and neuronal apoptosis during the early phases of Alzheimer's disease, whereas mature plaque types show mainly reduced NF-κB activity (Kumar et al. 2004).

Cancer

A variety of dysregulations of NF-κB activation has been described in cancer cells and thought to be of pathogenic relevance. NF-kB may have a dual role in oncogenesis. Loss of the tumor-suppressor function of RelA in the early stages of Kras-driven pancreatic neoplastic transformation was associated with a protumorigenic tumor microenvironment. By contrast, in advanced stages of Kras-driven murine pancreatic ductal adenocarcinoma, RelA enhanced tumor progression (Korc 2016). Constitutive NF-κB activation is involved in some forms of cancer, and inhibition of NF-κB abrogates cell proliferation in these tumors (Kumar et al. 2004). Genes encoding RelA, c-Rel, p105/p50, and p100/p52 proteins are located within regions of the genome involved in oncogenic rearrangements or amplifications. Oncogenic fusions between RELA and C11orf95 are frequent in supratentorial ependymomas and result in fusion proteins that are translocated spontaneously to the nucleus to activate NF-κB target genes and rapidly transform neural stem cells (Parker et al. 2014). Mutations that can lead to tumors include those that inactivate IκB proteins as well as amplifications of genes encoding NF-κB. In tumor models, NF-κB is activated in tumor cells in response to chemotherapy, and inhibition of NF-κB by viral expression of IκB leads to enhancement in the apoptotic response of the chemotherapy.

In cancer cells, negative feedback loops are overridden through unclear mechanisms to sustain oncogenic activation of NF-κB signaling. Overexpression of miR-30e* directly represses IκBα expression, while miR-182 directly suppressed cylindromatosis (CYLD), another NF-κB negative regulator, leading to prolonged NF-κB activation in cancer (Song et al. 2012).

NF-κB activation may also contribute to cancer complications. Serum factors from cachectic mice and patients induce Pax7 expression in an NF-κB-dependent manner, and this impairs the regenerative capacity of myogenic cells in the muscle microenvironment to drive muscle wasting in cancer (He et al. 2013).

Therapeutic Targeting of NF-κB

The identification of NF-κB as a key player in the pathogenesis of disease suggests that NF-κB-targeting drugs aimed at blocking NF-κB activity might be effective in the clinic. Suppression of NF-κB activation has potential therapeutic applications. In fact, some well-known commercially available drugs, such as glucocorticoids, nonsteroidal anti-inflammatory drugs, and calcineurin inhibitors, modulate NF-κB activity.

Repression of NF-κB-dependent gene expression is one of the major elements of immunosuppression and anti-inflammation by glucocorticoids. Glucocorticoids induce IκBα synthesis and enhance the cytosolic retention of NF-κB in monocytes and lymphocytes (Yamamoto and Gaynor 2001). However, glucocorticoids block NF-κB activation by different mechanisms in different cell types.

Nonsteroidal anti-inflammatory drugs are used in the treatment of chronic inflammatory disease. Most of them also target NF-κB. Both aspirin and salicylate inhibit NF-κB activation in patients with chronic inflammatory conditions by inhibiting ATP binding to IKKβ.

Cyclosporin A (CsA) and tacrolimus (FK-506) are calcineurin-inhibitor immunosuppressive agents used in organ transplantation to prevent rejection. Both inhibit the NF-κB pathway in lymphocytes by distinct mechanisms: preventing IκBα degradation and translocation of c-Rel from the cytoplasm to the nucleus, respectively (Yamamoto and Gaynor 2001). By contrast, nephrotoxicity is in part mediated by recruitment of NF-κB-mediated inflammatory responses in tubular cells (González-Guerrero et al. 2013).

Proteasome inhibitors may prevent NF-κB activation function by reducing IκB degradation. There are a variety of proteasome inhibitors, some in clinical use, like bortezomib.

Since oxygen radical species promote NF-κB activation, drugs with antioxidant properties may inhibit NF-κB activation.

A variety of other approaches has been used to inhibit NF-κB activation in cell culture or experimental animal models (http://people.bu.edu/gilmore/nf-kb/inhibitors/index.html). These include small molecules, siRNA, oligodeoxynucleotides, degradation-resistant IκBs, peptide-siRNA nanocomplexes targeting specific NF-κB subunits, and other specific NF-κB inhibitors, whose efficacy has been shown in animal models of inflammatory disease. Since NF-κB may have dualistic roles in disease, specific NF-κB inhibition might result in unintended side effects. As an example, NF-κB may promote both cell survival and inflammation in cells stimulated with certain cytokines, such as TNF or TRAIL, and NF-κB inhibition in these circumstances may result both in less secretion of inflammatory mediators and cell death.

Summary

NF-κB is a term used for a family of transcription factors composed of homo- or heterodimeric DNA-binding protein complexes that may be activated in response to a wide variety of stimulus inducing cell stress. NF-κB, in turn, promotes the transcription or repression of a wide array of genes involved in many key cell biology processes. As a result, NF-κB contributes to the pathogenesis of many diseases. Specific NF-κB inhibition is promising in experimental animal models, but experience in humans is limited. By contrast, many commonly used drugs may target NF-κB directly or indirectly as one of several mechanisms of action.

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NF-κB, Nuclear Factor KappaB

► Inhibitor of KappaB

NGF

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Synonyms

Beta-nerve growth factor; Beta-NGF; HSAN5; Nerve growth factor; Nerve growth factor (beta polypeptide); Nerve growth factor, beta; Nerve growth factor, beta polypeptide; Nerve growth factor, beta subunit; Ngfb; NGFB

Historical Background

Nerve growth factor (NGF) was discovered by Rita Levi-Montalcini, who revealed that mouse sarcomas transplanted into chicken embryos secrete a factor into the blood that induces sensory and sympathetic nerve growth (Levi-Montalcini 1952; Levi-Montalcini 1987). She was awarded the Nobel Prize in 1986 for this work (Abott 2009). NGF is indispensable for the prenatal growth of sensory and sympathetic nerves. Two NGF receptors have been identified, one with high affinity and the other with low affinity for NGF (Landreth and Shooter 1980); these were later named tropomyosin receptor kinase (Trk) A and p75 neurotrophin receptor (p75NTR), respectively. NGF was also identified as a key molecule in the development of basal forebrain cholinergic neurons (Chen et al. 1997). In adults, NGF plays an important role in the protection and survival of both peripheral and central nervous system neurons and in the generation of pain sensation (Marlin and Li 2015).

NGF is synthesized initially as a glycosylated precursor molecule called pro-NGF, which is cleaved intracellularly by furin and extracellularly by plasmin, thus generating mature NGF (Khan and Smith 2015). Pro-NGF binds to p75NTR and sortilin, and also to TrkA with relatively low affinity, promoting apoptosis in neurons to counter the effect of NGF (Bradshaw et al. 2015). The physiological roles of pro-NGF, however, are under investigation.

In addition to NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 were discovered later as other neurotrophins that induce nerve growth.

NGF/TrkA Signaling

NGF is composed of approximately 120 amino acids and is expressed in various cells, such as neurons, Schwann cells, epithelial cells, smooth muscle cells, fibroblasts, and immune cells (Bandtlow et al. 1987, Otten and Gadient 1995). In neural and nonneural tissues, NGF expression is high in the dorsal root ganglion, heart, and spleen and is relatively low in the sympathetic ganglia, brain, spinal cord, sciatic nerve, liver, muscle, kidney, and lung (Yamamoto et al. 1996). NGF binding to the extracellular domain of TrkA causes dimerization of TrkA, which is also expressed in various tissues, such as the dorsal root ganglion, sympathetic ganglia, and spleen, and to a lesser degree in the brain, spinal cord, kidney, and spleen (Yamamoto et al. 1996).

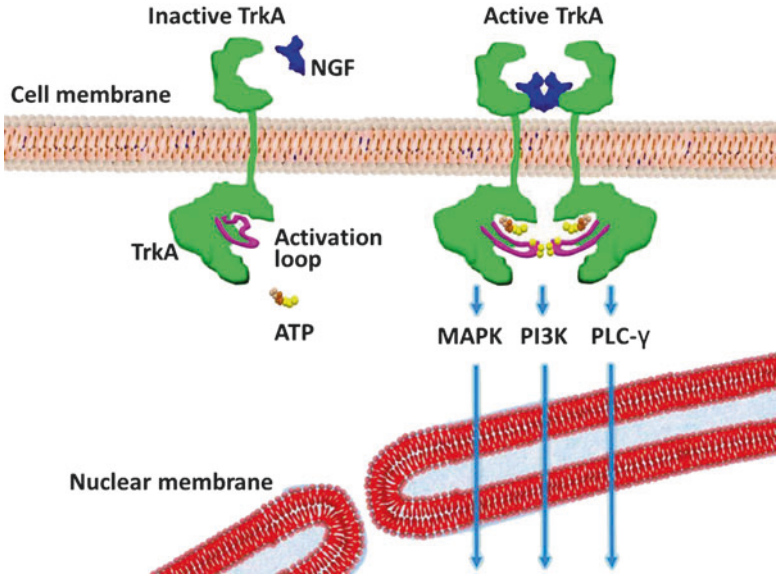
TrkA, a high-affinity NGF receptor, possesses tyrosine kinase activity. The activation loop of TrkA is wedged in the center of the enzymatic activity site in the inactive state, and in this state, three amino acid residues, Asp-Phe-Gly (DFG motif), in the activation loop prevent adenosine triphosphate (ATP) from entering the site, consequently suppressing the tyrosine kinase activity (Cunningham and Greene 1998; Artim et al. 2012). When the NGF dimer binds to the TrkA dimer, the activation loop is released from the center of the enzymatic activity site, and then TrkA uses ATP and autophosphorylates tyrosine residues on the contralateral activation loop (Wiesmann and de Vos 2001). NGF leads to autophosphorylation of five different tyrosine residues in the intracellular portion of TrkA that are located in the juxtamembrane domain and the C-terminal domain, including three tyrosine residues in the activation loop. Activation of TrkA then activates major signaling cascades including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ) pathways, transmitting signals into the nucleus (Khan and Smith 2015) (Fig. 1).

Tissues that express NGF undergo peripheral nerve innervation during development and maintain nerve cell integrity in the adult (Marlin and Li 2015). After the NGF dimer binds to the TrkA dimer at the distal axon terminal of neurons, NGF/TrkA complexes are endocytosed with Rab5, and then NGF/TrkA-containing endosomes are retrogradely transported in the axon along microtubules with dynein and Rab7, supporting the survival and growth of neurons (Bucci et al. 2014; Marlin and Li 2015). During retrograde transport, MAPK activation is sustained by NGF/TrkA in endocytic vesicles to recruit dynein for retrograde transport (Mitchell et al. 2012) (Fig. 2).

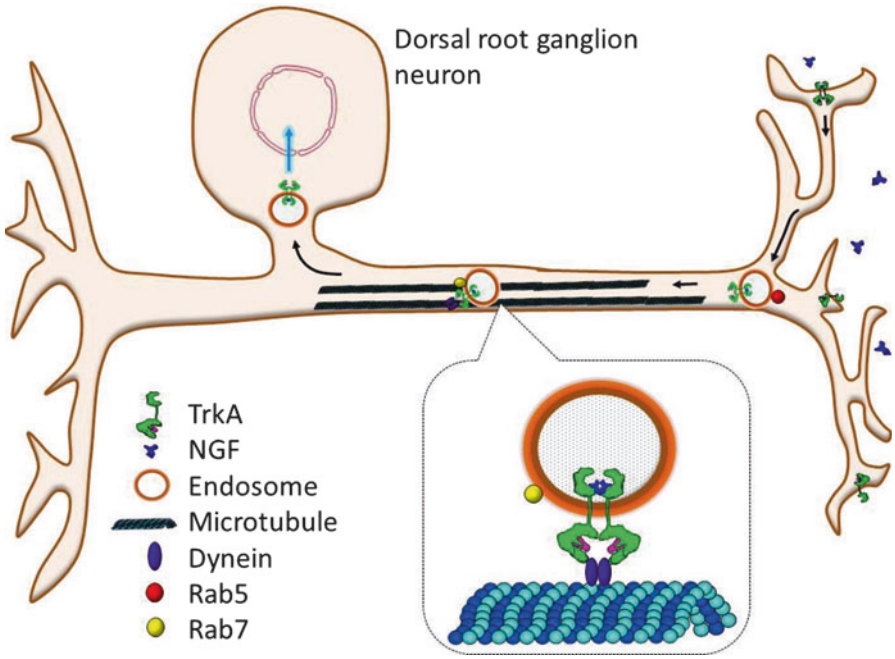
NGF and Pain

Congenital insensitivity to pain is a rare disorder in which patients do not sense pain. As NGF/TrkA signaling during the fetal period plays a pivotal role in the growth of nerve fibers that transmit pain sensations, a mutation in the gene encoding TrkA or NGF leads to a defect in the physiological actions of prenatal NGF and causes this type of hereditary sensory and autonomic neuropathy (Indo et al. 1996; Miranda et al. 2002; Einarsdottir et al. 2004).

On the other hand, NGF induces pain during adulthood (Hirose et al. 2016). NGF is secreted from immune cells with other inflammatory mediators in inflamed tissues (Basbaum et al. 2009). When tissue injury occurs, NGF binds to TrkA, phosphorylating sodium channels and transient receptor potential vanilloid 1 (TRPV1) at the sensory nerve endings, thus upregulating the



NGF, Fig. 1 Summary of NGF/TrkA signaling. In inactive TrkA, the activation loop (*purple-colored* loop) directly occludes the space where ATP binds to the active site of the kinase. NGF binding to TrkA, however, induces dimerization of TrkA and transition from an inactive form to an active form in which ATP can access the active kinase sites. In active TrkA, three tyrosine residues in the activation loop are phosphorylated (*yellow balls*), and two other autophosphorylated tyrosine residues activate mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ) pathways



NGF, Fig. 2 Retrograde axonal transport of the NGF/TrkA complex. At the axon terminal, NGF induces formation of NGF-activated TrkA, which is endocytosed and retrogradely transported along microtubules towards the soma with the microtubule-dependent motor protein dynein. Rab5 regulates the formation of early endosomes, and Rab7 regulates the transport of late endosomes

expression of TRPV1, substance P, sodium channels, and calcitonin gene-related peptide. These changes induce peripheral sensitization and cause a hypersensitive reaction (hyperalgesia) in response to nociceptive pain (Khan and Smith 2015; Hirose et al. 2016). When peripheral nerve injury occurs, NGF is released from Schwann cells, satellite glial cells, and invading immune cells, increasing the expression of BDNF in both the dorsal root ganglion in the peripheral nerve and the spinal dorsal horn to induce central sensitization during neuropathic pain (Khan and Smith 2015). Therefore, analgesics that suppress NGF/TrkA signaling, such as anti-NGF antibody, are promising candidates for clinical use for the treatment of refractory pain (Hirose et al. 2016).

Chronic lower back pain, including both nociceptive and neuropathic pain, is one of the most common types of chronic pain, with a prevalence as high as 10–20%. Intervertebral disk degeneration is a major cause of chronic lower back pain. During degeneration, activated proteases, such as metalloproteinases, degrade the extracellular matrix of intervertebral disks, producing endogenous molecules that act as danger-associated patterns (DAMPs). The DAMPs activate toll-like receptors (TLRs) on intervertebral disk cells. Activated TLRs increase the expression of NGF and BDNF, which primarily induce aneural innervation of the disks and contribute to the development of chronic pain (Krock et al. 2016).

Potential Clinical Applications of NGF

Although inhibitory agents of NGF activity may be novel pain killers as mentioned above, clinical trials of NGF itself also have been performed to treat peripheral neuropathies, central nervous system diseases, and ocular and skin diseases. As dysregulation of NGF function occurs in peripheral neuropathies, recombinant human NGF (rhNGF) is expected to be useful for treating diabetic polyneuropathy, human immunodeficiency virus-associated peripheral neuropathy, and chemotherapy-induced peripheral neurotoxicity. Side effects of rhNGF such as hyperalgesia and arthralgias, however, have been observed (Aloe et al. 2012).

As NGF plays an important role in the development and survival of basal forebrain cholinergic neurons (Chen et al. 1997), NGF application, using gene therapy or intracerebroventricular infusion because of impermeability of NGF through the blood-brain barrier, was performed to treat Alzheimer's disease (AD), Parkinson's disease, and traumatic brain injury (Aloe et al. 2012). Although the healthy functions of basal forebrain cholinergic neurons depend on a supply of NGF that is cleaved from pro-NGF by plasmin, pro-NGF accumulates in the cerebral cortex due to impaired metabolism of NGF in AD. Therefore, correcting NGF metabolism may also be a potential therapeutic strategy for treating AD in the future (Iulita and Cuello 2014).

Keratinocytes produce NGF to regulate skin homeostasis. Topical mouse NGF (mNGF) has been used in clinical trials to treat skin ulcers. In the visual system, NGF modulates the principal functions of conjunctival epithelial and goblet cells, induces corneal epithelial cell proliferation and differentiation, and regulates the development and differentiation of the retina and optic nerve in addition to promoting the survival and recovery of retinal ganglion cells (Lambiase et al. 2011). mNGF in eye drops was used in clinical trials to treat neurotrophic keratitis, dry eye, and glaucoma (Aloe et al. 2012; Lambiase et al. 2011).

Summary

NGF is synthesized initially as pro-NGF, which is cleaved into NGF intracellularly and extracellularly. After the NGF dimer binds to the TrkA dimer at distal nerve endings, NGF/TrkA complexes are endocytosed and transported retrogradely to the soma. Activated TrkA induces activation of MAPK, PI3K, and PLC-γ pathways.

NGF is expressed not only in neural tissues but also in nonneural tissues. During the prenatal period, NGF plays a pivotal role in the growth of sensory and sympathetic nerves and the development of basal forebrain cholinergic neurons. After birth, the role of NGF is the protection and

survival of peripheral and central nervous system neurons and the development and maintenance of nociceptive or neuropathic pain. NGF also regulates homeostasis in skin and ocular systems. Therefore, NGF/TrkA signaling inhibitors suppress pain caused by inflammation or nerve injury. On the other hand, NGF may be a useful therapeutic agent for peripheral neuropathies, central nervous system diseases, and ocular and skin diseases.

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NGFB

► NGF

NGFI-B/Nur77 Beta-Type Transcription Factor Homolog

▶ NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

NGFRAP1

▶ BEX3

NHERF

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Synonyms

EBP50 (Ezrin-radixin-moesin-Binding phosphoprotein 50 kDa); NHERF (N^+/H^+ Exchange Regulating Factor); SLC9A3R1 (Solute carrier family 9 member 3 regulator 1)

Historical Background

The functional presence of NHERF-1 was implicated in the late 1980s from a series of studies of inhibition of Na^+/H^+ exchange in rabbit kidney brush border membrane by cyclic AMP-dependent protein kinase (PKA). It took almost another decade before the cloning and molecular identification of NHERF-1 (Weinman et al. 1995). Shortly after, NHERF-2 was cloned from a yeast two-hybrid screen as an NHE3 interacting protein and was initially named E3KARP based on its ability to mediate PKA-dependent inhibition of Na^+/H^+ exchanger 3 (NHE3) in PS120 fibroblasts (Yun et al. 1997). NHERF-3 was initially identified as a protein that is upregulated in low dietary phosphate and was later shown to interact with the type II Na/P_i cotransporter (Npt2a) (Gisler et al. 2001). NHERF-4 was shown to interact with the intestinal receptor guanylyl cyclase C to inhibit the catalytic activity of the receptor in response to heat-stable enterotoxin (Scott et al. 2002).

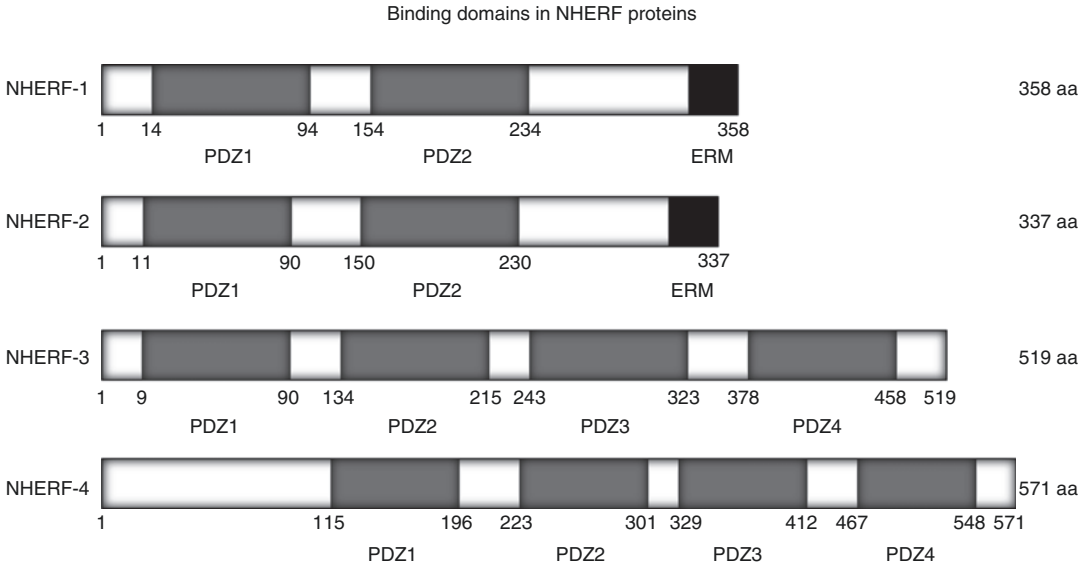
Introduction

The NHERF family consists of four related proteins that are present in the brush border membrane of the mammalian intestine, colon, and renal proximal tubules. These proteins contain 2 or 4 PSD-95/Dlg/ZO-1 (PDZ) domains (Fig. 1) (Weinman et al. 1995; Yun et al. 1997; Gisler et al. 2001; Scott et al. 2002). NHERF-1 and NHERF-2 contain two PDZ domains as well as an ezrin-radixin-moesin-merlin (ERM) binding domain at the carboxyl terminus (Lamprecht et al. 1998). NHERF-3 and NHERF-4 have four PDZ domains without ERM binding domain (Gisler et al. 2001; Scott et al. 2002). PDZ domain interaction with their interacting ligands or proteins generally occurs at the ligand COOH terminus although non-canonical interaction with an internal motif has been reported. NHERFs are able to interact with multiple proteins through their PDZ domains, including transporters, channels, transmembrane receptors, and other cytoskeleton proteins localized at or below the plasma membrane (Table 1). NHERFs play significant roles in maintenance and regulation of a broad range of cellular functions in a variety of tissues through the interactions with multiple target proteins.

NHERF Basics

Localization

NHERF-1 and NHERF-2 are expressed in a broad range of tissues and organs (Yun et al. 1997). NHERF-3 and NHERF-4 show highest expression in the kidney and gastrointestinal tract (Gisler et al. 2001; Scott et al. 2002). Immunofluorescent confocal microscopic analysis of NHERF proteins shows that NHERF proteins have different subcellular localization in polarized epithelial cells. NHERF-1 and NHERF-3 are



NHERF, Fig. 1 Binding domains in NHERF proteins. Four members of NHERF family (NHERF1-4) are shown with PDZ domains and ERM binding regions. Numerical numbers denote lengths of amino acids

located in the brush border membrane under basal conditions. The brush border localization of NHERF-2 was shown, but it is predominantly in the intermicrovillar clefts just below the brush border membrane. NHERF-4 is primarily distributed in the cytosol as well as in the subapical region, but not in the brush border membrane (Donowitz et al. 2005). NHERF expression in non-epithelial cells is less well documented, but the expression of NHERF-1 and NHERF-2 in neurons and astrocytes, where these proteins show a membranous expression, has been reported.

Regulation of NHERF

NHERF-1 is regulated by phosphorylation. NHERF-1 is constitutively phosphorylated on Ser 289 by G protein–coupled receptor kinase 6 (GRK 6), which enhances oligomerization of NHERF-1 (He and Yun 2010). Furthermore, NHERF-1 is phosphorylated by the cyclin-dependent kinase Cdc2 at Ser279 and Ser301, which impair oligomerization, and at S77 by protein kinase C, which interferes with parathyroid hormone (PTH)-induced signaling (Weinman et al. 2007). In contrast to NHERF-1, NHERF-2 does not appear to be regulated by phosphorylation (Lamprecht et al. 1998). Both NHERF-1 and NHERF-2 have been shown to form homotypic as well as heterotypic dimers. Dimerization of NHERF is thought to affect their interaction with other proteins. NHERF-3 mRNA expression is regulated by peroxisome proliferator-activated receptor alpha (PPARα), a ligand-activated transcription factor that plays an important role in the regulation of lipid homeostasis (Tachibana et al. 2008). Regulatory mechanism of NHERF-4 has not yet been studied.

Association with Cell Surface Proteins

Transporters and Channels

The majority of functional characterization of the NHERF family came from heterologous expression of these proteins. In addition to the importance of NHERF-1 and NHERF-2 in regulation of NHE3 by PKA, these studies have provided evidence for the importance of NHERFs for trafficking, membrane retention, and dimerization of the cystic fibrosis transmembrane regulator (CFTR) (Singh et al. 2009). Although initial

NHERF, Table 1 Interactions of NHERF proteins with ligands and other proteins

NHERF proteins	Domains	Binding partners	Authors
NHERF-1	PDZ1	β2-adrenergic receptor (β2AR)	Hall et al. (1998)
		Platelet-derived growth factor receptor (PDGFR)	Maudsley et al. (2000)
		κ opioid receptor	Li et al. (2002)
		Parathyroid hormone receptor	Sneddon et al. (2003)
		5-HT4a serotonin receptor	Joubert et al. (2004)
		Epidermal growth factor receptor (EGFR)	Lazar et al. (2004)
		Cystic fibrosis transmembrane conductance regulator (CFTR)	Short et al. (1998)
		Trp 4 & 5 calcium channels	Tang et al. (2000)
		Na^+/phosphate cotransporter (Npt2a)	Gisler et al. (2001)
		Phosphatase and tensin homologue (PTEN)	Takahashi et al. (2006)
		G protein-coupled receptor kinase 6A	Hall et al. (1999)
		Phospholipase C β-1,2 & 3	Tang et al. (2000)
	PDZ2	NHE3	Weinman et al. (1995)
		H^+ ATPase	Breton et al. (2000)
		Yes-associated protein (Yap65)	Mohler et al. (1999)
		β-catenin	Shibata et al. (2003)
NHERF-2	PDZ1	PDGFR	Maudsley et al. (2000)
		CFTR	Sun et al. (2000)
		Trp 5 calcium channel	Embark et al. (2004)
	PDZ2	β2AR	Hall et al. (1998)
		PDGFR	Takahashi et al. (2006)
		Lysophosphatidic acid 2 receptor (LPA_2)	Oh et al. (2004)
		Lysophosphatidic acid 5 receptor (LPA_5)	Lin et al. (2010)
		NHE3	Yun et al. (1997)
		PTEN	Takahashi et al. (2006)
		Cyclic GMP kinase II	Cha et al. (2005)
		PLCβ-3	Hwang et al. (2000)
		Protein kinase C (PKC)	Lee-Kwon et al. (2003)
		Serum glucocorticoid regulated kinase (SGK) 1 & 3	Yun et al. (2002)
		Podocalyxin	Takeda et al. (2001)
NHERF-3	PDZ1	CFTR	Wang et al. (2000)
		NHE3	Gisler et al. (2003)
		Renal urate anion exchanger (URAT1)	Anzai et al. (2004)
	PDZ2	Proton-coupled peptide transporter (PEPT2)	Kato et al. (2004)
		Intestinal anion exchanger down-regulated in adenoma (DRA)	Gisler et al. (2003)
	PDZ3	NPT 1 & 2	Gisler et al. (2003)
		PEPT2	Kato et al. (2004)
		CFTR	Wang et al. (2000)
	PDZ4	Organic cation transporter, novel (OCTN) 1 & 2	Kato et al. (2005)
		NHERF 1 & 2	Gisler et al. (2003)
NHERF-4	PDZ1	OCTN 1 & 2	Kato et al. (2005)
	PDZ2	OCTN 1 & 2	Watanabe et al. (2006)
	PDZ3	Guanylyl cyclase C	Scott et al. (2002)
	PDZ4	Epithelial Ca2+ channel, transient receptor potential cation channel, subfamily V, member 6 (TRPV6)	Kim et al. (2007)
NHERF-1 NHERF-2	ERM	Ezrin, Radixin, Moesin, and Merlin	Murthy et al. (1998)

studies suggested redundancy in the functions of the NHERF family, studies of rodents that are genetically targeted to delete one or more of the NHERF family members have helped to reveal distinct physiological roles of NHERF proteins. NHERF-1 is essential for cAMP- and parathyroid hormone (PTH)-induced inhibition of renal NHE3, but not intestinal NHE3 (Weinman et al. 2005). Ablation of NHERF-1 decreases forskolin-induced secretion of bicarbonate by CFTR (Singh et al. 2009). NHERF-1 is also essential for the recruitment of Npt2a to the brush border membrane of renal proximal tubule cells (Weinman et al. 2005). NHERF-2 appears to play a dual role in regulation of NHE3. Glucocorticoid- or lysophosphatidic acid (LPA)-mediated stimulation of NHE3 is dependent on the presence of NHERF-2 (He and Yun 2010). Similarly, NHERF-2 is necessary for inhibition of NHE4 by cyclic GMP kinase II (cGKII), protein kinase C (PKC), and Ca^{2+}-mediated signaling (He and Yun 2010). NHERF-3 ablation in mouse colon abolishes cAMP- and Ca^{2+}-induced inhibition of NHE3 (Donowitz et al. 2005). NHERF-3 is also involved in the localization of organic cation/cartinin transporter (OCTN2, Slc22a5) and H^+/dipeptide transporter (PepT1, Slc15a1) in the brush border membrane (Sugiura et al. 2008). NHERF-4 activates NHE3 via a Ca^{2+}-dependent mechanism (Zachos et al. 2008).

G Protein–Coupled Receptors

NHERF proteins interact with several G protein–coupled receptors (GPCRs), including the β_2-adrenergic receptor (β_2-AR), κ-opioid receptor, PTH type 1 receptor (PTH1R), P2Y receptor, and lysophosphatidic acid receptor (Ritter and Hall 2009). The first insight into the role of NHERF in GPCR-mediated signaling came from the finding that agonist-promoted association of NHERF-1 with the carboxyl terminus of β_2-AR displays NHERF-1 from NHE3 blocking the inhibition of NHE3 by PKA (Ritter and Hall 2009). Evidence shows that NHERF-1 regulates β_2-AR trafficking by regulating agonist-promoted recycling of receptor proteins, which can be perturbed by interruption of NHERF-1 binding, and hence directing the receptor to lysosome (Ritter and Hall 2009). In addition, the NHERF proteins regulate GPCR-mediated signaling through selective recruitment of signaling proteins, including phospholipase C and G proteins, which could potentiate or redirect G protein–mediated signaling (Mahon et al. 2002).

Receptor Tyrosine Kinases

In addition to GPCRs, NHERFs associate with receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR). The binding of NHERF-1 to the carboxyl terminus of PDGFR potentiates receptor activity only when NHERF-1 is allowed to oligomerize (Maudsley et al. 2000). Evidence shows that the interaction between PDGFR and NHERF-1 can be disrupted by phosphorylation of the carboxyl terminus of PDGFR by GRK2 (Hildreth et al. 2004). Recent study showed that NHERF-1 facilitates actin cytoskeletal reorganization mediated by PDGFR (Theisen et al. 2007). Unlike PDGFR, EGFR lacks the carboxyl terminal PDZ binding sequence, but yet it was shown that EGFR interacts with NHERF-1 involving a non-canonical internal PDZ binding motif (Lazar et al. 2004). This interaction appears to stabilize EGFR at the cell surface by restricting EGF-induced receptor degradation, which causes EGFR to remain longer at the cell surface.

NHERF as a Signaling Molecule

Cellular Signals

PDGFR is activated through dimerization and autophosphorylation upon ligand binding. NHERF-1 dimers enhance dimerization of PDGFR to potentiate mitogenic signals transduced by extracellular signal-regulated kinase (Erk) 1/2. Similarly, transient receptor potential 4 (TRP4) calcium channel associates with phospholipase C (PLC) β isoforms to activate protein kinase C signals by binding to NHERF PDZ1 domain. NHERF-1 has significant importance in PTH-mediated signaling as evidenced by the defective PTH signaling in NHERF-1-deficient mice (Weinman et al. 2005). NHERF-1 modulates PTH

signaling by affecting PTH receptor recycling, membrane retention, and desensitization.

In addition to the regulation of the membrane receptors and channels, NHERF-1 interacts with Akt and inhibits PKA-mediated Erk1/2 activation by decreasing the stimulatory effect of 14–3-3 binding to B-Raf (Wang et al. 2008).

Cancer

Overexpression of NHERF-1 in breast cancer cells and the transcriptional regulation of NHERF-1 by estrogen suggested a potential role of NHERF-1 in cancer. In addition, unpublished data in our lab show the elevated expression of NHERF-2 in colon adenocarcinoma. However, the mechanism and effects of NHERFs in tumorigenesis are unclear. The interaction of NHERF-1 with PDGFR and EGFR appears to suggest an oncogenic role of NHERF-1. In addition, NHERF-1 expression is elevated in hepatocellular carcinoma where NHERF-1 complexes with β-catenin to promote Wnt signaling (Shibata et al. 2003). On the other hand, NHERF-1 or NHERF-2 recruits phosphatase and tensin homolog (► PTEN) tumor suppressor to restrict the activation of the PI3K (Georgescu et al. 2008). Therefore, NHERFs appear to play a dual role in tumorigenesis and their role in cancer requires additional studies.

Summary

From the uncertain identity as a cofactor of cAMP-induced regulation of NHE3, NHERF proteins have firmly rooted their identity as the major molecular scaffolds. The role of the NHERF proteins extends beyond the regulation of ion transporters. Growing evidence links NHERF to cancer, inflammatory diseases, hypertension, and neurological disorder. However, the functional roles and the underlying mechanisms of NHERF-mediated regulation are incompletely understood. A combination of biochemical and cellular approaches along with physiological studies using animal models lacking one or more of the NHERF proteins should advance the understanding of the physiological and pathophysiological functions of the NHERF proteins.

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NHERF (N^+/H^+ Exchange Regulating Factor)

▶ NHERF

Nicotinic Acetylcholine Receptor

▶ Acetylcholine (Nicotinic) Receptor

Nicotinic Receptor

▶ Acetylcholine (Nicotinic) Receptor

NIL-2-A

▶ ZEB1 (Zinc Finger E-Box Binding Homeobox 1)

NimO (*Aspergillus*)

▶ Dbf4

NINAC (p174, p132)

▶ Myosin III

NIP

▶ GIPC

NIPK

▶ Tribbles

NK Receptor

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Synonyms

Killer immunoglobulin-like receptors (KIR); Killer lectin-like receptors (KLR); Natural cytotoxicity receptors (NCR); Natural killer cell group (NKG)

Historical Background

Natural Killer (NK) cells are a major component of the innate immune system, providing surveillance against infected or transformed cells without the requirement of prior host sensitization. NK cells were first discovered by their cytotoxic potency against tumor cells (Kiessling et al. 1975), and their importance was demonstrated early on in herpes viral infections, usually mild or localized, which become severe and life-threatening in NK-deficient patients. NK cell effector functions are governed by the balance of potentially opposing signals from a diverse array of both activating and inhibitory receptors on the cell surface that are not rearranged from germ-line receptor segments, unlike B and T cell receptors (BCRs, TCRs) on lymphocytes. While NK cells are now well characterized in terms of their origin, differentiation, and receptor repertoire, advances are still being made in NK cell receptor modulation, ligand recognition, and signaling and activation mechanisms which greatly enrich our understanding of NK cell biology: an important component of a complex immune system that remains incompletely understood.

Function and Signaling

Because of their potent cytolytic activity, the function of NK cells needs to be tightly regulated to limit potential autoreactivity. Molecular events driving activation occur at the NK immune synapse (NKIS), the surface of contact between NK and potential target cells. The NKIS comprises an array of molecular interactions including receptor–ligand pairs, adhesion molecules, signaling adaptors, signaling effectors, membrane lipid rafts, and cytoskeletal molecules (MacFarlane and Campbell 2006). NK cells kill sensitive target cells by release of cytotoxic granules that contain perforin and granzymes; perforin polymerizes and forms a transmembrane pore that allows the delivery of granzymes and associated molecules into the cytosol where they initiate various apoptotic death pathways. NK cells express a variety of receptors that serve either to activate or to suppress their cytolytic activity (Lanier 2005). NK cell surface receptors are commonly divided into activating and inhibitory receptors, but they can also be structurally classified into C-type lectin-like receptors and immunoglobulin (Ig)-type receptors.

The functional response of an NK cell is the result of the integration of signals transduced by the set of activating and inhibitory receptors engaged upon target cell interrogation (Bryceson and Long 2008). The majority of known activating receptors transduce signals through their association with one or two of four transmembrane-spanning accessory proteins: the DNAX-activating proteins with molecular weights of 10 kDa (DAP10) or 12 kDa (DAP12), ▶ CD3ζ, or the Fcε-receptor γ-chain (FcεRIγ; Table 1). All four accessory adaptor proteins exist as disulfide-linked homodimers, but CD3ζ and FcεRIγ can also form disulfide-linked heterodimers. Electrostatic interactions link activating receptors and accessory adaptor molecules through either an arginine or lysine residue in the receptor or an aspartic or glutamic acid residue on the accessory protein. The cytoplasmic domains of DAP12, CD3ζ, and FcεRIγ all contain one or more immunoreceptor tyrosine-based activation motifs (ITAMs) containing a sequence signature: YxxL/I (x denotes any amino acid). Two of these motifs are typically separated by six to eight amino acids in the tail of the molecule [YxxL/Ix(6–8)YxxL/I]. Receptor–ligand binding leads to phosphorylation of tyrosine

NK Receptor, Table 1 Ectodomain structure, ligand information and endodomain signaling motifs are catalogued for a series of NKRs

Activating	Structure	Ligand	Signaling
NKG2D	C-type lectin homodimer	MICA/B, ULBP1-6	DAP10
NKG2C/E-CD94	C-type lectin heterodimer	HLA-E	DAP12
CD16	Ig monomer	IgG	FcεRIγ, CD3ζ
NKp46 (NCR1)	Ig monomer	Influenza hemagglutinin	FcεRIγ, CD3ζ
NKp44 (NCR2)	Ig monomer	Influenza hemagglutinin	DAP12
NKp30 (NCR3)	Ig monomer	B7H6, BAT3, viral pp65	FcεRIγ, CD3ζ
KIR2DS1	Ig monomer	HLA-Cw2,4,5,6 (C2 epitope)	DAP12
KIR2DS4	Ig monomer	HLA-A, C	DAP12
KIR3DS1	Ig monomer	HLA-Bw4?	DAP12
KIR2DL4	Ig monomer	HLA-G?	FcεRIγ, 1 ITIM
2B4	Ig monomer	CD48	ITSM, SAP
NTB-A	Ig monomer	NTB-A	ITSM, SAP
CRACC	Ig monomer	CRACC	ITSM, SAP
DNAM-1	Ig monomer	CD112, CD155	Protein kinase C
NKp80	C-type lectin homodimer	AICL	Undefined
Inhibitory	Structure	Ligand	Signaling
NKG2A/B-CD94	C-type lectin heterodimer	HLA-E	1 ITIM
KIR2DL1	Ig monomer	HLA-Cw2,4,5,6 (C2 epitope)	2 ITIM
KIR2DL2	Ig monomer	HLA-Cw1,3,7,8 (C1 epitope)	2 ITIM
KIR2DL3	Ig monomer	HLA-Cw1,3,7,8 (C1 epitope)	2 ITIM
KIR3DL1	Ig monomer	HLA-A, B (Bw4 epitope)	2 ITIM
KIR3DL2	Ig homodimer	HLA-A3, A11	2 ITIM
LIR1 (ILT2)	Ig monomer	HLA class I, HLA-G, UL18	4 ITIM
LIR2 (ILT4)	Ig monomer	HLA-G	3 ITIM
NKR-P1A	C-type lectin homodimer	LLT1	1 ITIM
LAIR1(CD305)	Ig monomer	Collagen	2 ITIM
CEACAM1	Ig monomer	CEACAM1, CEA	2 ITIM
SIGLEC7	Ig monomer	α2,8 disialic acid	1 ITIM
SIGLEC9	Ig monomer	α(2,3)- & α(2,6)-disialic acid	1 ITIM + 1 ITSM
IRp60	Ig monomer	Undefined	4 ITIM

residues within these motifs by membrane proximal Src kinase family molecules. This in turn leads to recruitment of protein tyrosine kinases (PTKs) of the spleen tyrosine kinase (Syk) family, such as Syk and ζ-associated protein of 70 kDa (▶ ZAP-70). Subsequent downstream signaling pathways can involve phosphatidylinositol 3 kinase (▶ PI3K), Vav, phospholipase Cγ (PLCγ), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK). PLCγ activation leads to intracellular calcium flux. Activation of PLCγ, PKC, Vav, and PI3K, as well as elevation of cytosolic calcium concentrations is an important event for initiation of the cytolytic functions by NK cells. Activation of ERK is important for granule-mediated cytotoxicity and ▶ IFN-γ production.

Alternatively, the cytoplasmic signaling domain of DAP10 contains a YINM motif, which may lead to the association of DAP10 with PI3K or the adaptor molecule Grb2, as opposed to Syk or ZAP70. Significant differences have emerged that distinguish DAP10-mediated activation signals from those downstream of ITAMs (Billadeau et al. 2003). This may lead to divergent effector functions: DAP10 can stimulate cytotoxicity, but not IFN-γ production by NK cells, whereas DAP12 can trigger both responses. Multiplicity of activating signals results in

complex functional cross talk, leading to the integration of different receptor–ligand interactions directing NK-mediated cell killing.

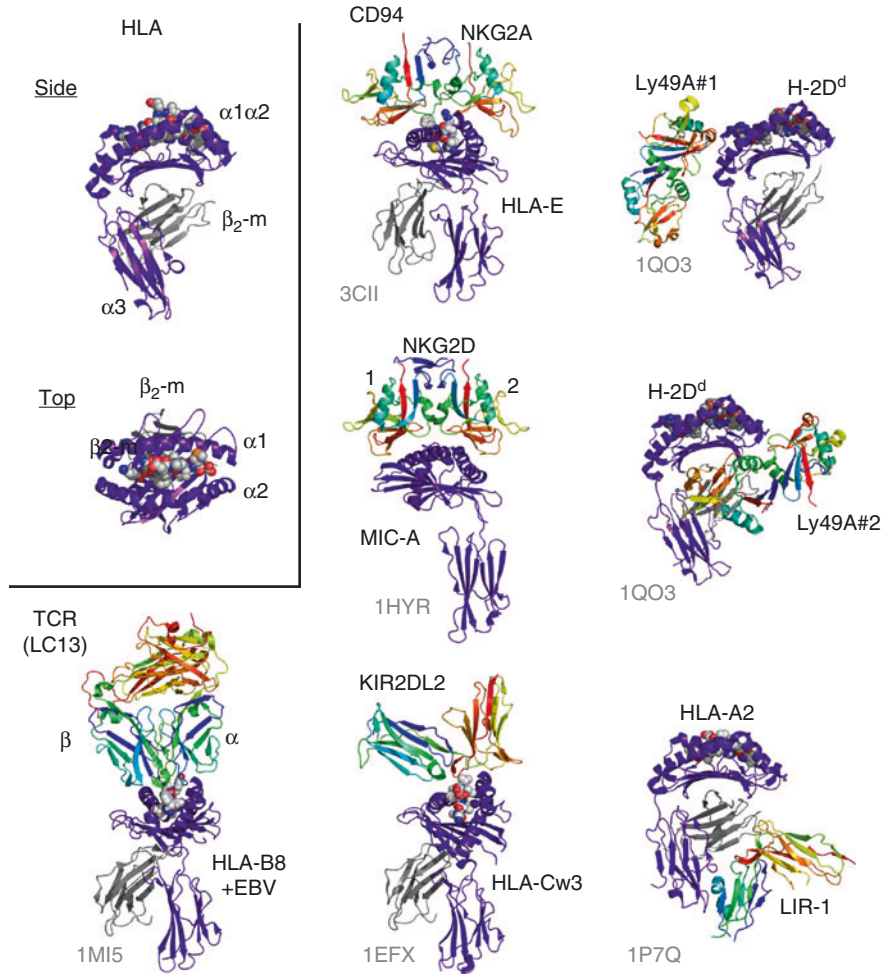
NK cells are also activated in response to interferons or macrophage-derived cytokines, including IL-2, IL-12, and IL-15. Activated NK cells in turn release cytokines such as TNF-α and IFN-γ, which can promote cellular resistance to infection and influence adaptive immunity resulting in infection clearance. NK cells also express the Fc receptor (FcR) molecule (CD16), an activating receptor that binds the Fc portion of antibodies. This allows NK cells to target cells against which a humoral response has been mobilized and to lyse cells through antibody-dependent cellular cytotoxicity (ADCC).

Although the extracellular domains of NK cell inhibitory receptors are diverse, the cytoplasmic signaling motifs of these transmembrane receptors are remarkably similar. The cytoplasmic tails of inhibitory receptors have one or more copies of conserved sequence motifs (V/IxYxxL/V) known as immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Binding of ligands to inhibitory receptors activates ▶ Src family kinases that phosphorylate ITIM tyrosine residues, leading to the recruitment of other enzymes, such as SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2, or the inositol phosphatase, ▶ SHIP. These tyrosine phosphatases are able to dephosphorylate protein substrates of tyrosine kinases linked to activating NK cell receptors. Recruitment and activation of SHP-1 by some inhibitory receptors has been functionally implicated in delivery of the dominant inhibitory signal in human NK cells. NK cells tolerate normal, autologous cells by sensing appropriate levels of surface-expressed self-MHC class I molecules that induce an inhibitory signal. Infection or transformation often alters MHC class I cell surface expression, typically as part of a strategy to evade T cell responses, which is then detected by NK cells. This "missing-self" hypothesis was first proposed by Karre and colleagues (Karre et al. 1986) and subsequently demonstrated by Yokoyama and colleagues (Karlhofer et al. 1992). MHC class I–mediated inhibition is therefore crucial to the role played by NK cells.

Specificities and Recognition Mechanisms: Inhibitory Receptors

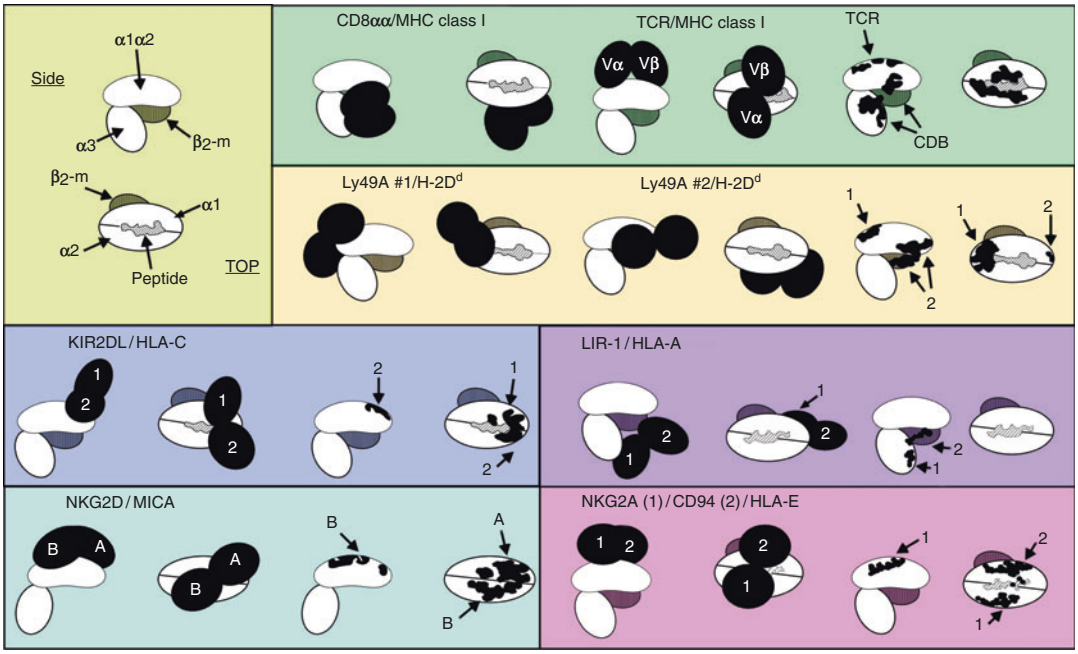
Major inhibitory NK receptors in humans include inhibitory isoforms of the Killer Immunoglobulin Receptors (KIRs, also CD158), the lectin-like receptor CD94/NKG2A, and the Leukocyte Immunoglobulin-like Receptors (LIRs, also CD85), which all recognize classical or nonclassical MHC class I molecules, and some less well-characterized inhibitory receptors that bind non-MHC class I molecules, such as the lectin-like receptor Natural Killer Receptor P1 (NKR-P1, also KLRB1 or CD161). In mice, the KIRs are replaced with the structurally distinct, C-type lectin-like Ly49 family of NK receptors. Inhibitory receptors that recognize classical and nonclassical MHC class I proteins limit signals through activating receptors. In this way, recognition of normal MHC class I expression dominantly suppresses NK attack of normal cells, whereas a lack of self-MHC class I shifts the balance toward targeted cell killing. Structural and functional studies of inhibitory Ly49 and H-2 complexes provided among the first detailed insights into NK cell recognition (Tormo et al. 1999). The homodimeric Ly49A receptor binds to H-2D^d at two distinct sites, one of which involves the α1 and α2 domains of MHC class I, whereas the second interaction site spans the underside of the platform domain (α1 and α2), α3 domain and β_2-microglobulin (Figs. 1 and 2). The second site is considerably more extensive than the first site and also overlaps the CD8 binding site on MHC class I molecules. The first binding site is consistent with *trans* interaction between NK and target cells where the second, more extensive contact site is likely a regulatory *cis* interaction between Ly49 and class I molecules on the same NK cell. Both binding sites are distinct from that of the TCR.

The highly polymorphic KIR family of human NK receptors consists of at least fifteen active genes and two pseudogenes. KIRs are either activating or inhibitory and contain either two or three extracellular Ig-like domains (named D0, D1, and D2). KIR nomenclature is based on the number of extracellular Ig domains (2D versus 3D) and the



NK Receptor, Fig. 1 NK receptor–ligand complex structures. Ribbon representations of an αβ TCR/MHC class I complex and several NK receptor–ligand structures. MHC class I molecules are shown in *purple*. Receptors are colored in *rainbow spectrum*; β_2m is shown in *gray*. Class I–bound peptides are shown in *solid spheres* and colored by element. PDB accession codes for the coordinate files used to generate the figure are indicated in *gray*

size of their cytoplasmic tails. Receptors possessing an ITIM-containing long cytoplasmic tail are designated by an L, whereas activating receptors with a short cytoplasmic tail are designated by an S. The cytoplasmic region of activating receptors does not contain an ITIM, but instead contains a positively charged residue that can interact with adaptor molecules such as DAP12. The KIRs are known to show allotypic specificity toward different HLA alleles (Table 1). The best characterized KIR–ligand interactions come from two complex crystal structures of two-domain inhibitory KIRs: KIR2DL2/HLA-Cw3 and KIR2DL1/HLA-Cw4 (Boyington et al. 2000; Fan et al. 2001). KIR2DL1/2 binds to HLA-C with a similar docking orientation as TCRs, with the D1/D2 domains contacting the α1/α2 helices of the MHC peptide–binding cleft and positions P7 and P8 of the bound peptide (Figs. 1 and 2). The KIR2DL1/2/HLA-C interface is dominated by charge complementarity and is mediated by six loops; three loops from D1 contact the α1 helix and bound peptide while one hinge loop and two loops from D2 contact the α2 helix. Allotypic specificity of KIR2D toward HLA-C alleles is determined by a dimorphism at



NK Receptor, Fig. 2 NK receptor–ligand complex schematics. Schematic representations of an αβ TCR/MHC class I complex and several NK receptor–ligand structures to highlight interaction surfaces. Each row shows two views of a receptor–ligand complex, first showing the organization of domains in the complex (receptor domains in *black*, labeled where a distinction between domains is significant; MHC class I ligand heavy chains in *white* and β_2m in *dark gray*). The arrangement of domains in the ligands is detailed in the inset; the approximate solvent-accessible surface area of the bound peptide, if present, is shown as a *cross-hatched* area. The two *right*-most columns show approximate footprints of receptors and co-receptors on the ligands as *black hatches*, labeled by receptor component, subsite or domain as appropriate

residues 77 and 80 of HLA and a reciprocal dimorphism in KIR2D at position 44. For example, KIR2DL1 (M44) will recognize the C2 epitope (N77/K80) of HLA-C, whereas KIR2DL2 (K44) will recognize the C1 epitope (S77/N80). In the absence of any KIR3D structures, a KIR3DL1/ligand complex model has been proposed, but remains to be confirmed. Analogous to the KIR2D complex structure, the D1/D2 domains contact the α1/α2 helices of HLA and position 8 of the bound peptide. The D0 domain contacts the α1 helix, stabilized by hydrophobic contacts with D1 and D2, consistent with the finding that D0 contributes to avidity. KIR3DL1 is known to recognize HLA-A and -B molecules with the Bw4 epitope (residues 77–83). Several lines of evidence also point to the importance of residue 80 of the Bw4 epitope for KIR interaction.

Besides receptors for classical MHC class I molecules, NK cells can indirectly gauge MHC class I expression on target cells through the NKG2x/CD94 receptor complexes. The NKG2x/CD94 receptors, which include both the inhibitory NKG2A/CD94 and activating NKG2C/CD94 receptors, contain C-type lectin-like ectodomains and recognize HLA-E, a nonclassical MHC class I molecule that presents MHC class I leader peptides. As with the KIR and Ly49 receptors, the inhibitory NKG2A/CD94 receptor binds the ligand with a higher affinity than the activating NKG2C/CD94 receptor. There are few conformational changes in either CD94-NKG2A or HLA-E upon binding, which reflects a typical "lock and key" recognition in innate receptor–ligand interactions (Petrie et al. 2008). A patch of amino acids (residues 167–170) of NKG2A account for the

approximately sixfold-higher affinity of the inhibitory NKG2A/CD94 receptor compared to its activating counterpart. These residues do not contact HLA-E or peptide directly but instead form part of the heterodimer interface with CD94. An evolutionary analysis reveals that residues at the CD94 interface have evolved under positive selection, suggesting that the evolution of these genes is driven by an interaction with pathogen-derived ligands. Consistent with this possibility, data show that NKG2C/CD94, but not NKG2A/CD94, weakly but specifically binds to the CMV MHC-homologue UL18 (Kaiser et al. 2008).

NK cells can also be inhibited by members of the LIR (or immunoglobulin-like transcript (ILT)) family. Some members of LIRs (including LIR1-3) are inhibitory receptors with intracellular ITIM motifs, while others (including LIR6-7) are activating receptors associated with adaptor proteins for signaling. LIR-1 and LIR-2 are receptors for a broad range of class I molecules, including nonclassical molecule HLA-G. HLA-G is highly expressed in the trophoblast, and the inhibitory function of LIR-HLA-G interaction has been implicated in immunological tolerance of the fetus. The LIRs show a distinct recognition mode with regard to its class I HLA-A2 or nonclassical MHC HLA-G ligand (Figs. 1 and 2). Both complex structures of HLA-A2 bound to LIR-1 and HLA-G bound to LIR-2 (Willcox et al. 2003; Shiroishi et al. 2006) showed the LIR binds at a site bridging the $\alpha 3$ and $\beta_2 m$ domains. The overall orientation of the MHC molecules with regard to LIR receptors resembles somewhat the *cis* binding site of Ly49A on H-2D^d, though none of the LIR domains are in contact with the underside of the platform domain of the MHC molecules (Figs. 1 and 2). The LIR-1 binding site on HLA-A2 overlaps with the CMV UL18 binding site. Binding sites on LIR-1 and LIR-2 similarly comprise residues at the interdomain hinge and a patch at the tip of domain 1. The overall geometry of the LIR receptor–MHC ligand complex is most consistent with a *trans* interaction involving recognition of an MHC class I protein on a target cell by a LIR receptor on an opposing NK cell.

Although the binding of an inhibitory receptor to MHC class I represents the major mechanism for inhibition and prevention of NK cell autoreactivity, there are other inhibitory receptors which bind to non-MHC class I ligands. NKR-P1 binds to lectin-like LLT1 molecules in humans. More information on the regulation of NKR-P1 ligands expression is required to determine the functional implications of this receptor–ligand interaction. Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) is an inhibitory receptor that binds to collagen and is widely expressed on immune cells. Several members of the sialic acid binding Ig-like lectin (Siglec) family of receptors, which bind sialyl groups with various specificities, carry ITIMs in their cytoplasmic tail. Presumably, Siglec molecules function as inhibitory receptors; yet, the biological significance remains elusive.

Specificities and Recognition Mechanisms: Activating Receptors

Activating receptors include ▶ NKG2D, CD16, natural cytotoxicity receptors (NCRs), activating KIRs, the signaling lymphocyte-activation molecules (SLAM) family of receptors, and DNAX accessory molecule-1 (DNAM-1). One of the best characterized NK-activating receptors is NKG2D (Gonzalez et al. 2006). It is a type II integral membrane protein associated with the adaptor protein DAP10. NKG2D binds several ligands, including MHC class I chain–related A/B (MICA/B) and the UL16-binding proteins, 1-6 (ULBP1-6). Expression of these ligands is upregulated in a variety of epithelially derived tumors and virally infected cells. Currently, the mechanisms that upregulate NKG2D ligand expression on cancer cells are not well understood. While NKG2D provides an important defense mechanism against tumors and viral infection, it can also contribute to autoimmunity. For example, MICA and MICB are dramatically upregulated in rheumatoid arthritis synoviocytes and are capable of activating autoreactive autologous T cells in an NKG2D-dependent manner.

MICA/B and ULBP1-6 are class I homologs that are not encoded by genes in the MHC complex and do not function as peptide-binding structures to present antigens to T cells. Multiple crystal structures of the receptor alone and three complexes (human NKG2D/MICA, NKG2D/ULBP3, and murine NKG2D/Rae-1β) show that NKG2D interacts with its MHC class I homologs in a manner very similar to how TCRs interact with classical MHC class I molecules (Strong and McFarland 2004; Figs. 1 and 2). Unlike KIR and Ly49A interactions at the first binding site, the NKG2D binding sites are much less dominated by charge–charge interactions. Both homodimer-related binding sites on NKG2D contribute approximately equally to the interactions in both complexes, reflecting a binding site that has evolved to bind multiple target sites without side-chain rearrangements. The considerable recognition degeneracy of NKG2D, accommodating structurally divergent, polymorphic families of ligands, is enabled not by a conformationally plastic binding site ("induced fit" or "conformer selection"), but rather by a "rigid adaptation" mechanism.

Another NK-activating receptor is the low-affinity receptor for IgG, CD16, which mediates ADCC and signals through adaptors containing cytoplasmic ITAMs. Several receptors, which activate antibody-independent, natural cytotoxicity are also associated with ITAM-containing signaling adaptors (Table 1). These receptors include NKp30, NKp44, and NKp46, which are referred to as NCRs (Biassoni 2009). CD16, NKp30, and NKp46, all use both FcεRIγ and CD3ζ as signaling adaptors. NKp44, on the other hand, partners with DAP12. Both ▶ NKp46 and NKp44 have been reported to bind viral hemagglutinin on infected cells while their cellular ligands have not been identified. NKp46 also contributes to enhanced killing of mitotic cells by NK cells, suggesting a role of NK cells in controlling the expansion of rapidly dividing cells. A recent study identified a tumor cell surface molecule that belongs to the B7 family that triggers NKp30-mediated activation of human NK cells (Brandt et al. 2009). This molecule, designated as B7H6, was not detected in normal human tissues but was expressed on human tumor cells. The expression of stress-induced self-molecules such as MICA/B and B7H6 associated with cell transformation serves as a mode of cell recognition in innate immunity. NKp30 also mediates killing of immature dendritic cells by NK cells. A nuclear factor, human leukocyte antigen-B-associated transcript 3 (BAT3), was also described as a ligand for NKp30. This ligand is released from tumor cells and plays an important role in tumor rejection in a multiple myeloma model via NKp30-mediated cytotoxicity. In addition, the main tegument protein (pp65) of human cytomegalovirus (HCMV) was proposed as an exogenous viral ligand as it inhibits NKp30-mediated cytotoxicity.

The specificity of the activating KIR family members has not been extensively characterized as compared with those of the inhibitory ones (Lanier 2005). KIR2DS1 and KIR2DS4 recognize HLA-C or HLA-A molecules weakly and KIR3DS1 is thought to recognize HLA-B with the Bw4 epitope, while ligands for other activating KIR receptors are undefined. The high sequence similarity between several pairs of activating and inhibitory KIR suggests they arose by gene duplication. However, in general, the activating KIRs either do not bind the cognate HLA class I, or bind with an affinity much weaker than that of the paired inhibitory KIR. The activating KIR receptors all have short cytoplasmic tails, and they transduce signals and trigger function through the ITAM-bearing adaptor DAP12. The exception is KIR2DL4. It has a functional ITIM in its cytoplasmic tail, yet it also associates with the FcεRIγ adaptor protein, a feature for an activating receptor. Despite the presence of an ITIM, ligation of KIR2DL4 with its specific monoclonal antibody (mAb) leads to cytotoxicity and IFN-γ production in IL-2-activated NK cells. Soluble HLA-G has been described as its ligand.

Other activating receptors signal through motifs in their own cytoplasmic tail, or through pathways that have not been well characterized. 2B4, NK-T-B-antigen (NTB-A), and CD2-like receptor-activating cytotoxic cells (CRACC), all belong to the SLAM family of receptors (Veillette

2006). They are predominantly expressed on hematopoietic cells and regulate both innate and adaptive immunity. They are members of the Ig superfamily that consist of multiple Ig-like domains in the extracellular region, a transmembrane segment, and a cytoplasmic domain containing multiple tyrosine-based motifs. Comparing with other ITAM-based activating receptors, SLAM receptors have distinct activation pathways. In humans, activation through these receptors is accompanied by the phosphorylation of unique TxYxxV/L/I sequence–based immunoreceptor tyrosine-based switch motifs (ITSMs) in their cytoplasmic tails and the recruitment of SLAM-associated protein (SAP) as adaptors. SAP couples SLAM family receptors to FYNT, a Src-related PTK that is expressed by hematopoietic cells. In mice, however, 2B4 apparently functions as an inhibitory receptor. It is unclear if 2B4 itself can independently trigger effector functions or rather serves as a costimulatory receptor. The strongest evidence supporting an activating role in human NK cells comes from the demonstration that transfection of CD48, its ligand, into certain NK-resistant target cells renders them susceptible to NK-mediated cytotoxicity and triggers the production of IFN-γ by the human NK cells. Such action can be blocked by anti-CD48 mAb and anti-human 2B4 mAb. Adaptor protein SAPs appears to play important roles in SLAM receptor functions. In patients with certain mutations in SAP, NK cells can no longer be activated through the 2B4 receptor. Furthermore, immature human NK cells express 2B4 receptors that mediate inhibitory function, presumably because these immature NK cells lack SAP. 2B4 may, therefore, be a multifunctional receptor in both human and mice, displaying different functions depending on factors such as NK cell differentiation and activation. The complex crystal structure between the N-terminal domains of mouse 2B4 and CD48 reveals that they interact in a mode related to, yet distinct from, that of the homophilic NTB-A dimer of the same SLAM family. Binding is accompanied by the rigidification of flexible 2B4 regions containing most of the polymorphic residues across different species and receptor isoforms (Velikovsky et al. 2007).

Other activating NK receptors, such as DNAM-1 and NKp80, have been implicated in various immune functions. DNAM-1 receptor is a member of the Ig superfamily that is expressed in a variety of human lymphocytes including NK cells. It has been implicated in cell adhesion and in triggering NK- and T-cell-mediated cytolysis. CD112 and CD155, which are components of cellular adherens junctions, have been identified as ligands for DNAM-1. These ligands are frequently upregulated, and interaction between DNAM-1 on NK cells and CD112 and CD155 on tumor cells augments NK cell–mediated cytotoxicity and cytokine production. The interaction of NK cell receptors with members of the nectin-like molecules indicates the importance of adhesion in the triggering of NK cell function. Physical association of DNAM-1 with LFA-1 is necessary for NK and T cell triggering. NKp80 is another NK-activating receptor with unknown signaling properties. The cellular ligand of NKp80 was recently identified as activation-induced C-type lectin (AICL). AICL is expressed on myeloid cells, and is upregulated by inflammatory stimuli. Thus, NKp80–AICL interactions may be important for NK cell-myeloid cell cross talk during inflammation.

Tumor and Viral Evasion Mechanisms

Tumors use several mechanisms to evade the immune system, including NK-mediated responses. Aberrant loss of the activating receptor NKG2D in cancer is a key mechanism of immune evasion (Groh et al. 2002). Production of soluble, secreted NKG2D ligands or growth factors, such as TGF-β released from tumors, is a mechanism for downregulating NKG2D expression. TGF-β may also play an important role in the conversion of normal T cells into regulatory T cells (T_{reg}), a population of T cells that is found in high levels in the tumor microenvironment. T_{reg} cells reduce NKG2D expression, suppress NKG2D-mediated NK cell cytotoxicity, and accelerate the progression of tumors.

Although the expression of MICA/B may result in tumor elimination, MICA shedding, through directed proteolysis from the surface of tumor cells, into the plasma is a common characteristic of many epithelially derived tumors. The process of shedding is mediated by interactions between MICA and a protein disulphide isomerase, endoplasmic reticulum protein 5 (ERp5 or PDI-P5). ERp5 forms a transitory complex with MICA and reduces an inaccessible disulfide bond in the α3 domain, which must induce a conformational change that is essential for the proteolytic cleavage of MICA (Kaiser et al. 2007).

Epigenetic repression of NKG2D ligand transcription, such as limiting the accessibility of promoter sequences to transcription factors or micro-RNA-induced repression of gene expression, is another tumor evasion mechanism. Transcription of MICA/B and ULBP1 is mainly regulated by ubiquitous transcription factors including Sp1, Sp3, and NFY. However, the binding of these transcription factors to their promoters may be regulated by the histone deacetylases (HDACs) and the structure of chromatin. HDACs remove the acetyl groups of histone to form compacted chromatin, which restrains the accessibility of promoter sequences to transcription factors. HDACs are overexpressed in many cancer types, and they participate in the repression of numerous proteins with tumor-suppression activities (Lopez-Soto et al. 2009). Micro-RNAs may also target the MICA/B 3′-untranslated region to repress expression (Stern-Ginossar et al. 2007).

Viral immune modulatory mechanisms also manipulate NK receptor and ligand expression (Wilkinson et al. 2008), and virus-derived decoy ligands for inhibitory receptors are used as viral evasion mechanisms. HCMV encodes an inhibitory decoy glycoprotein ligand, UL18, which is structurally similar to MHC class I proteins, including a peptide-binding groove and association with host β_2-microglobulin. UL18 is used to evade NK detection by binding to the inhibitory NK receptor LIR-1 with high affinity. Selective upregulation of inhibitory MHC class I antigens is another viral evasion mechanism; viral proteins that bind MHC class Ib proteins and upregulate their expression, while differentially downregulating CD8+ T cell activating MHC class Ia antigens, include HCMV-encoded UL40, US2, US11, and HIV-encoded Nef. A nine amino acid sequence (VAMPRTLIL) in HCMV UL40, identical to HLA-C leader sequences, binds to HLA-E and allows its surface expression when MHC class I expression is repressed by other viral mechanisms. On the other hand, HCMV-encoded US2 and US11 have been shown to selectively downregulate HLA-A surface expression without interfering with HLA-E expression. Similar evasion mechanisms are also observed with the HIV-encoded Nef protein, which directs HLA-A and B for lysosomal degradation while sparing HLA-C and HLA-E.

Viral proteins that directly interact with NK-activating ligands are also deployed by viruses to evade NK surveillance by sequestering and retaining ligands intracellularly. HCMV UL16 sequesters the NKG2D ligands MICB, ULBP1, and ULBP2 and downregulates their surface expression. Another HCMV-encoded protein, UL142, uses similar mechanism to downregulate MICA. Various serotypes of human adenovirus also encode proteins that are used to modulate surface expression of NK-activating receptors: The E3/19 K protein sequesters MICA/B proteins in the ER, downregulating surface expression. Expression of NK-activating ligands is also modulated by virus-derived micro-RNAs. The HCMV-encoded miRNA, hcmv-miR-UL112, selectively downregulates the NKG2D ligand MICB (Stern-Ginossar et al. 2007). Viruses also manipulate components of the host ubiquitin pathway to protect infected cells from NK responses. The KSHV-encoded immune evasion proteins, K3 and K5, have E3 ubiquitin ligase activity, possess a variant RING domain, and downregulate surface expression of NKG2D ligands MICA/B by ubiquitination of conserved lysine residues in the cytoplasmic tail of MICA/B which result in its trafficking from the cell surface to an intracellular compartment.

Summary

NK cells are capable of recognizing and destroying multiple pathogenic cell targets. This

broad specificity is mediated by multiple activating and inhibitory surface receptors and intracellular signal transduction molecules. NK receptors are regulated in a spatial and temporal fashion at the NK synapse where adhesion molecules and NK receptors interact with their ligands on the target cell. Healthy cells expressing MHC class I will engage NK inhibitory receptors and thus be spared, whereas infected or transformed cells, that have an aberrant expression of MHC class I and cell stress markers, become susceptible to attack. Significant progress has been made in the identification of many activating and inhibitory receptors, their ligands, and signaling pathways. However, many receptors, such as NCRs and activating KIRs, have poorly defined or unknown ligands. The signaling mechanisms of receptors such as NKp80 and many of the mechanisms governing the regulation of receptor expression also remain unknown. Furthermore, the interplay between the complex array of activating and inhibitory receptors leading to NK cell function needs to be defined. Future studies aimed toward identifying unknown ligands and understanding the dynamic interactions between receptor signaling and its effect on cellular function will help in the understanding of NK cell biology and the role that NK cells play in the immune system.

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NKG2D

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Synonyms

CD314; Killer cell lectin-like receptor subfamily K; Member 1 (KLRK1)

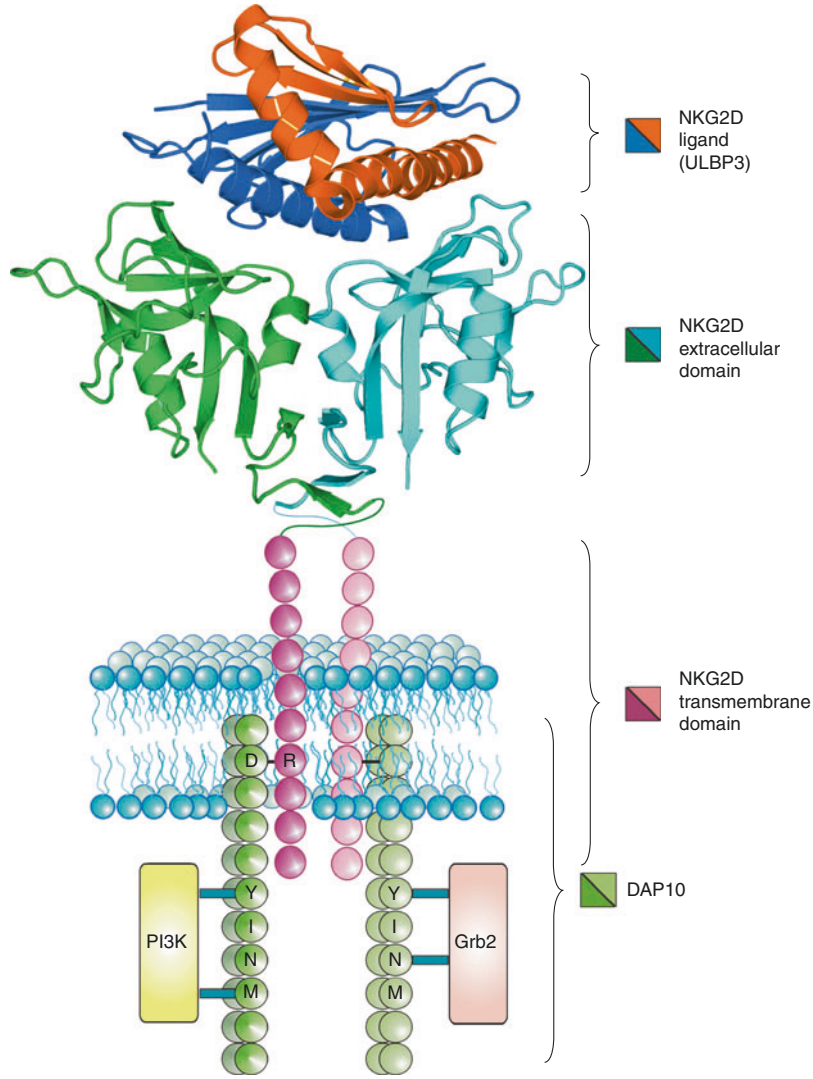
Historical Background

Natural killer (NK) cells are key components of the innate immune response and can mediate cellular cytotoxicity without previous antigen exposure. The mechanisms underlying NK cell activation have been uncovered in a stepwise fashion from the early 1980s (for a detailed review of the field and its development see (Lanier 2008)). The role of the CD16 FcγRIII cell surface receptor in antibody-dependent NK cell activation was described in 1983. In 1986, Karre and colleagues proposed the "missing self-hypothesis" hypothesis and demonstrated the presence of inhibitory NK cell surface receptors responsive to the self-MHC (major histocompatibility complex) molecules on normal host cells. The search for antibody-independent NK cell activating receptors led to the identification of the NKG2 family of NK cell receptors in 1991. Among these was NKG2D, a molecule that shared limited homology with other NKG2 family members and was expressed in both NK and T cells as a predicted type 2 transmembrane C-type lectin receptor. In 1994, Bahram et al. identified two polymorphic genes encoding molecules related to classical MHC class I molecules which they named major histocompatibility complex I chain-related genes A and B (MICA, MICB) (Bahram et al. 1994). In 1999, NKG2D was identified as the activating receptor for MICA and MICB (Bauer et al. 1999).

NKG2D is recognized as a dominant activating NK cell receptor, capable of activating NK cells (Bauer et al. 1999), and influencing T cell activation. It is expressed constitutively in human NK cells, γδ T cells, and $CD8^+$ T cells and may also be found in some invariant natural killer T (iNKT) cell and $CD4^+$ T cell populations. In mice, NKG2D is constitutively expressed in NK cells, but is only expressed in $CD8^+$ T cells when they have been activated. NKG2D has been identified in a wide range of mammals, including nonhuman primates, pigs, cattle, and rats.

NKG2D Structure

Human NKG2D is a 316 amino acid type 2 transmembrane C-type lectin-like protein encoded by the KLRK1 gene (Killer cell Lectin-like Receptor subfamily K-1) within the NK gene complex at chromosome 12p13.2–12.3. Structural studies show that human NKG2D exists as a dimer and lacks functional calcium coordinating carbohydrate binding domains (Fig. 1) (Li et al. 2001). Each NKG2D monomer contains two β sheets and two helical elements, with four intrachain disulphide bonds. Murine NKG2D is structurally similar to human NKG2D (Wolan et al. 2001), but unlike human NKG2D, it exists in two isoforms: a long isoform of 232 amino acids (NKG2D-L, isoform A) and a short 219 amino acid isoform (NKG2D-S, isoform B) (Lanier 2008).



NKG2D, Fig. 1 Human NKG2D ligand binding and signaling. The NKG2D extracellular domain and ULBP3 are shown in cartoon form based on the crystal structure of the complex. Each NKG2D molecule interacts with a DAP10 dimer through an association between an arginine residue (R) in the transmembrane region of NKG2D and an aspartate residue (D) in the transmembrane domain of DAP10. Upon ligand binding, signaling from NKG2D is transmitted through DAP10. Key interactions with DAP10 include binding of the p85 subunit of PI3K to the YXXM motif in DAP10, while Grb2 recognizes YXNX, following the phosphorylation of DAP10 by src family tyrosine kinases

The cytoplasmic portion of human NKG2D does not possess intrinsic signaling capability. Instead, a charged amino acid (arginine) in the transmembrane portion of NKG2D interacts with an aspartate residue in the adaptor molecule DAP10 (DNAX-activation protein 10). Each NKG2D molecule binds to a DAP10 dimer in this fashion to form a hexameric structure capable of signal transduction. The YINM motif in the cytoplasmic region of DAP10 recruits downstream signaling molecules. DAP10 lysine 84 is also necessary for ubiquitinylation and intracellular signaling (Quatrini et al. 2015). Murine NKG2D interacts with both DAP10 and DAP12, an ITAM (immunoreceptor tyrosine-based activation motifs)-containing adaptor molecule, through similar transmembrane region interactions. Both long and short NKG2D isoforms are capable of interacting with and signaling through either DAP10 or DAP12 in vitro, but in vivo the long form of NKG2D only interacts with DAP10 (O'Callaghan 2009). Human NKG2D does not interact with DAP12.

NKG2D Signaling

Signal transduction from NKG2D is complex (Fig. 1). An important early event in the

NKG2D-DAP10 signal cascade is tyrosine phosphorylation of the YINM motif in DAP10 by the src family tyrosine kinase; Lck and src kinase inhibitors abrogate downstream signal transduction. The phosphorylated YINM motif recruits further intermediates: the p85 subunit of ▶ PI3K (phosphatidylinositide-3 kinase) and the adaptor protein Grb-2 (growth factor receptor-bound protein 2). Both binding events are essential for signal transmission. The GNEF (guanine nucleotide exchange factor) Vav1, which plays an important role in actin reorganization, is recruited to Grb-2, where it is phosphorylated and activated. From here, signal transmission diverges, but it is known that Vav1 acts in part by binding the γ2 isoform of phospholipase C (PLCγ2), which among other downstream targets activates MAPK8 (mitogen-activated protein kinase 8, JNK1). In contrast, MAPK1 (ERK2) appears to be an important downstream target of PI3K. Ubiquitinylation of the NKG2D-DAP10 complex is also essential for internalization of the complex, degradation, and signal transduction via ERK1/2 (Quatrini et al. 2015). These signaling pathways are reviewed by (Lanier 2008).

While human NKG2D does not interact with DAP12, NKG2D-DAP12 signaling remains an important consideration in murine NKG2D models. Unlike DAP10, NKG2D-DAP12 signaling is dependent on the ITAM domain of the cytoplasmic portion of DAP12. Engagement of ligand by NKG2D leads to the phosphorylation of two tyrosine residues in the DAP12 ITAM by src family tyrosine kinases. This enables recruitment of the syk tyrosine kinase, which is necessary for NKG2D-DAP12 function. It is likely that the downstream components of this pathway are similar to those in other ITAM-associated receptors, which have been studied in greater detail in different contexts.

NKG2D Ligands

A striking characteristic of NKG2D is the wide range of distinct ligands with which it can productively interact. Such ligand diversity indicates that NKG2D has evolved as an important receptor receiving input from the various stimuli which act through the upregulation of the different ligands. For further detailed review, see Mistry and O'Callaghan (2007) and Lanier (2015).

Humans express eight distinct functional NKG2D ligands: MICA and MICB (major histocompatibility complex I chain-related genes A and B) and the UL16 binding proteins (ULBP) 1–6. MICA and MICB are distant homologues of classical MHC class I molecules. Both are highly polymorphic, a feature of much interest, with 105 and 42 known alleles, respectively, identified to date (http://hla.alleles.org/alleles/classo.html). MICA and MICB are transmembrane molecules with MHC class I-like α1, α2, and α3 domains. However, in contrast to classical MHC class I molecules, they do not associate with β2-microglobulin, and the cleft separating the α2 and α3 domains is closed and does not bind peptides.

In contrast, the ULBPs have limited polymorphism and lack an α3 domain. ULBP 1–3 were originally identified as proteins that were bound by the cytomegalovirus protein UL16 and that interacted with NKG2D to induce NK cell cytokine secretion and cytotoxicity. Seven related gene sequences were subsequently described in the same region of chromosome 6q. Three of these encode functional molecules, ULBP 4 (RAET1E, LETAL), ULBP 5 (RAET1G), and ULBP 6 (RAET1L). ULBPs 1–3 and ULBP 6 are GPI-linked cell surface proteins, while ULBP 4 and 5 exist principally in transmembrane forms. A GPI-linked splice variant of ULBP 5 has been described, while ULBP 4 has three functional splice variants, each encoding transmembrane receptors.

Mice do not have MICA or MICB genes. There are three families of murine NKG2D ligands: the retinoic acid early (RAE) 1 family including α, β, γ, δ, and ε; H60 a, b, and c; and murine ULBP-like transcript 1 (Mult1). RAE1 α, β, and γ were described in 1996 as glycophosphatidyl-inositol (GPI)-linked cell surface molecules of unknown function, isolated from retinoic acid-treated mouse embryonal carcinoma cells. The murine NKG2D ligands show limited homology with murine classical MHC class I molecules and

have an α1, α2 domain structure which does not present peptide and does not associate with β2 microglobulin. H60a, H60b, and Mult1 are transmembrane proteins, while H60c is a GPI-linked cell surface protein. The binding reactions between murine NKG2D and its ligands have been studied in detail, and binding affinities vary distinctly between NKG2D and its various ligands ((O'Callaghan et al. 2001); (Mistry and O'Callaghan 2007)).

The ligands for NKG2D are expressed on some normal cells, including gastrointestinal epithelial cells, and activated T cells, but are primarily displayed in pathological settings, notably in response to viral infection, some intracellular bacterial infections, and in many cancers. While several stimuli, broadly described as physiological stressors, are known to increase cell surface expression of NKG2D ligands on target cells, the mechanisms underlying regulation of NKG2D ligand expression remains poorly understood (Mistry and O'Callaghan 2007).

NKG2D Function

NKG2D may play significant roles in viral immunity, tumor immunity, autoimmune diseases, and transplant rejection (Lanier 2015). These roles are mediated through specific cellular processes, especially the induction of natural killer cell cytotoxicity and cytokine production. An understanding of the role of NKG2D in T cell costimulation is evolving.

Natural cytotoxicity. Natural cytotoxicity involves the coordination of several complex cellular processes including cytoskeletal reorganization, immune synapse formation, and perforin translocation. Freshly isolated human NK cells are capable of mounting a cytotoxic response to a target expressing NKG2D ligands without further experimental manipulation. Signaling through NKG2D can override inhibitory signaling through the interaction of HLA-E with CD94/NKG2A (Bauer et al. 1999) although in vivo NK cell activation is likely to reflect a broad balance between activating and inhibitory signaling. NKG2D may mediate TCR-independent cytotoxicity in IL-2-treated $CD8^+$ T cells, γδ-TCR T cells, and some $CD4^+$ T cell populations, but the in vivo significance of this is less certain and remains to be firmly established.

Cytokine and chemokine production. Early studies suggested that isolated ligation of NKG2D on NK cells and γδ-TCR T cells is sufficient to induce secretion of cytokines, primarily IFNγ, TNFα, and to a lesser extent, GMCSF and TNFβ (Fauriat et al. 2010). In one study, isolated human NKG2D ligation induced little cytokine secretion, but IFNγ and TNFα, prototypical NK cell cytokines, in addition to MIP-1α, MIP-1β, and RANTES, were produced when NKG2D was triggered together with CD16 (FCγRIII), natural cytotoxicity receptors, 2B4, or LFA-1(Fauriat et al. 2010) .

Costimulation. CD28 is the prototypical TCR costimulatory molecule, signaling through the p85 subunit of PI3K, which is recruited to a YMNM motif in the cytoplasmic domain of CD28. Given this shared signaling pathway with NKG2D, it was hypothesized that NKG2D might costimulate TCR-dependent T cell activation. While some early studies using cytokine-supported freshly isolated naïve ($CD62L^+$, $CD45RO^-$) human αβ TCR $CD8^+$ T cells provided evidence suggestive of NKG2D-mediated co-stimulation (Maasho et al. 2005), a direct comparison of the co-stimulating capacities of NKG2D and CD28 found that NKG2D ligation was insufficient to mediate costimulation in contrast to CD28 ligation (Ehrlich et al. 2005). Further studies suggest that NKG2D activation can costimulate effector $CD8^+$ αβ T cells, while CD28 signaling is necessary for naïve T cell costimulation (Rajasekaran et al. 2010). This functional divergence is also suggested by differential intracellular signaling pathway activity following CD28 or NKG2D activation (McQueen et al. 2016).

Murine knockouts. NKG2D knockout mice have provided further insights into NKG2D function (Guerra et al. 2008; Zafirova et al. 2009). Crossbreeding of NKG2D deficient mice with mice which develop prostate cancer or lymphoma demonstrates earlier and more rapid progression of these cancers in NKG2D-deficient mice (Guerra et al. 2008). In this lymphoma model, NKG2D signaling is necessary for NK cell

mediated lymphoma cell death (Belting et al. 2015). Furthermore, NK cells from NKG2D-deficient mice have a diminished ability to kill NKG2D ligand expressing target tumor cells (Zafirova et al. 2009). NK cell development also appears to be affected, with more rapid NK cell division, and enhanced cytokine responses to NK cell stimulation (Zafirova et al. 2009).

NKG2D Regulation

Several factors modify the cell surface expression of NKG2D (Mistry and O'Callaghan 2007; O'Callaghan 2009). Cytokines appear to be the principle regulators of NKG2D cell surface concentration on both NK cells and T cells. Altering NKG2D cell surface density may alter the influence of NKG2D on cellular activation. TGFβ decreases cell surface NKG2D expression in NK cells, while IL-21 decreases NKG2D cell surface expression in both NK cells and $CD8^+$ T cells. IL-15 increases NKG2D expression on $CD8^+$ T cells, $CD4^+$ T cells, and NK cells, while IL-2, IL-12, and IFNα have been shown to increase NKG2D expression on NK cells.

Excessive exposure to NKG2D ligands is reported to lower NKG2D cell surface concentrations in both physiological and pathological settings. In pregnancy, syncytiotrophoblast cells of the placenta produce exosomes containing soluble NKG2D ligands, which decrease NK cell NKG2D cell surface expression and reduce NK cell cytotoxicity (Hedlund et al. 2009).

At the transcriptional level, binding of the STAT3 transcription factor to a consensus site upstream of the NKG2D receptor gene is important for NKG2D receptor expression in both mouse and human cells (Zhu et al. 2014).

Summary

NKG2D is a homodimeric C-type lectin-like molecule which acts as an activating receptor in NK cells and can play an activating and potentially costimulating role in γδ T cells and $CD8^+$ T cells, and some $CD4^+$ T cell and iNKT cell populations. It is widely conserved in mammals, suggesting an important immune role. It is a promiscuous receptor with multiple diverse ligands, which are now well characterized in humans and mice. Future work is needed to further elucidate NKG2D signaling mechanisms, define functional interactions between NKG2D and other immune signaling molecules, and identify regulatory mechanisms that govern NKG2D ligand expression in target cells.

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NKp46

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Synonyms

CD335

Historical Background

NK cell function is finely regulated by a series of inhibitory or activating receptors. The inhibitory receptors, specific for major histocompatibility complex (MHC) class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of MHC class I (e.g., tumor cells). In the absence of sufficient signaling by their HLA class I-specific inhibitory receptors, human natural killer (NK) cells become activated and display potent cytotoxicity against cells that are HLA class I negative. This indicates that the NK receptors responsible for the induction of cytotoxicity recognize ligands on target cells different from HLA class I molecules. These receptors have been termed natural cytotoxicity receptors (NCR) and include NKp46, together with NKp44 and NKp30 (Bottino et al. 2000). A direct correlation exists between the surface density of NCR and the ability of NK cells to kill various target cells (Sivori et al. 1999). Importantly, mAb-mediated blocking of these receptors has been shown to suppress cytotoxicity against most NK-susceptible target cells. However, the process of NK-cell triggering during target cell lysis may also depend on the concerted action of NCR and other triggering receptors, such as NKG2D, or surface molecules, including 2B4 and NKp80, that appear to function as co-receptors rather than as true receptors.

Distribution and Function of NKp46 on NK Cells

NKp46 is a surface molecule of 46 kDa expressed by all human NK cells (including the $CD56^{bright}$ $CD16^{-}$ subset) irrespective of their state of activation. It is mostly confined to NK cells and its engagement induces a strong activation of NK-mediated cytolysis, Ca^{++} mobilization, and cytokine release (Moretta et al. 2001; Sivori et al. 1997).

In healthy donors, within the $CD56^{dim}$ NK cells, two distinct NK cell subsets (termed NCR^{dull} and NCR^{bright} NK cell subsets) can be often distinguished on the basis of the different

surface densities of NKp46 and NKp30 molecules. The NCR surface density directly correlates with the magnitude of the NK-mediated natural cytotoxicity and provides a rational explanation for the clonal heterogeneity of NK cells in killing target cells (Sivori et al. 1999). In particular, in healthy donors, most of the terminally differentiated $CD56^{dim}$ NK cells (characterized by the $CD56^{dim}KIR^{+}NKG2A^{-}CD57^{+}$ phenotype) display a lower surface expression of NCRs (Bjorkstrom et al. 2010; Lopez-Verges et al. 2010).

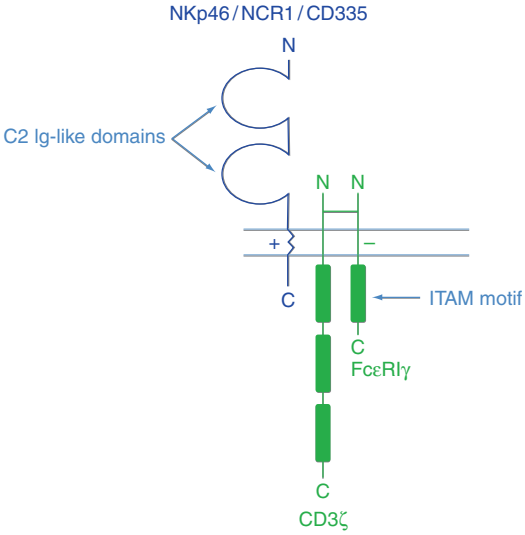
On the other hand, $CD56^{bright}$ NK cells are characterized by higher NKp46 surface expression as compared to $CD56^{dim}$ NK cells.

More recently, in $HCMV^{+}$ individuals a further small reduction of the NKp46 and NKp30 expression has been described in memory-like NK cells that are characterized by a highly differentiated surface phenotype, $CD56^{dim}CD16^{bright}LIR\text{-}1^{+}KIR^{+}NKG2A^{-}NKG2C^{+}CD57^{+}$, and by acquisition of some hallmarks of adaptive immunity, that is, clonal expansion, enhanced effector function (i.e., a functional enhancement in terms of ADCC and IFN-γ production), longevity, as well as given epigenetic modifications (Muntasell et al. 2013; Rölle and Brodin 2016).

More recently, in $HCMV^{+}$ individuals, a novel NK cell subset has been identified within the fully mature NK cells. This NK cell subset is characterized by the expression of the inhibitory PD-1 receptor and marked downregulation of NKp30 and NKp46. Indeed, the NCRs expression of $PD\text{-}1^{+}$ NK cells is maximally reduced as compared to that of $PD\text{-}1^{-}$ NK cells contained within the highly differentiated $KIR^{+}NKG2A^{-}CD57^{+}$ NK cell subset (Pesce et al. 2016).

Molecular/Biochemical Characterization of NKp46

NKp46 is encoded by the NCR1 gene, which maps in the telomeric region of "leukocyte receptor complex" (LRC) on human chromosome 19q13.42 (Kelley et al. 2005; Pessino et al. 1998). The NCR1 gene is conserved during speciation, at least in rodents, bovine, and primates (Biassoni 2008).



NKp46, Fig. 1 *NKp46 structure*. NKp46 is a type I glycoprotein belonging to the Ig-superfamily. It contains, in its transmembrane portion, a positively charged amino acid involved in the association with signal-transducing molecules characterized by negatively charged residues in their transmembrane portion and tyrosine-based motifs in their cytoplasmic tails

NKp46 is a type I transmembrane glycoprotein belonging to the Ig superfamily. It is characterized by two extracellular Ig-like domains of the C2 type (about 190 aa), connected by a short peptide (25 aa) to the transmembrane domain (about 20 aa) and by a small cytoplasmic domain (about 30 aa) (Fig. 1). The amino-terminal portion of the transmembrane region of NKp46 contains the positively charged amino acid arginine, which likely forms a salt bridge with the aspartic acid residue located in a similar topological context of the transmembrane domains of CD3ζ or FcεRIγ. The cytoplasmic tail does not contain immunoreceptor tyrosine-based activation motifs (ITAM), typically involved in the activation of the signal cascade(s) (Moretta et al. 2001; Pessino et al. 1998). NKp46 can transduce positive signaling thanks to its association with CD3ζ or FcεRIγ, two small polypeptides characterized by short extracellular portions and by cytoplasmic tails containing three and one ITAM, respectively, that become tyrosine phosphorylated upon receptor engagement. In NK cells, CD3ζ and FcεRIγ are also involved in intracellular signaling via

CD16 and have been shown to be phosphorylated by tyrosine kinases of the ► Src family, such as p56lck, followed by the recruitment of Syk and ► ZAP-70 tyrosine kinases (Moretta et al. 2001).

The crystal structure of NKp46 indicates a certain degree of structural conservation with KIR2DL2, CD89, glycoprotein VI platelet collagen receptor, LILRB1 (LIR-1/ILT2/CD85j), and LILRB2 (LIR2/ILT4/CD85d) molecules, which are encoded by genes that map close to each other on chromosome 19q13.42 (Ponassi et al. 2003).

NKp46 Ligands

The identification of the NKp46 ligand(s) on cancer cells is still being investigated. At this regard, it has been suggested that membrane-associated heparan sulfate proteoglycans (HSPGs) might be involved in the recognition of tumor cells by NKp46 and NKp30 (Bloushtain et al. 2004). These results have been not confirmed by other studies, at least for NKp30-mediated killing (Warren et al. 2005). Moreover, since heparan sulfate is also found on normal cells that are not killed by NK cells, it is likely that it does not represent a specific ligand of NKp46 or NKp30. Indeed, heparan sulfate is already known as a co-ligand for different growth factors, chemokines, lipid-binding proteins, and adhesion proteins (Capila and Linhardt 2002).

NKp46 is also involved in the killing of virus-infected cells, and it has been described to recognize the hemagglutinin of influenza virus (IV-HA) and the hemagglutinin-neuraminidase of Sendai virus (SV-HN) (Arnon et al. 2004; Gazit et al. 2006; Mandelboim et al. 2001). This recognition seems to depend on the sialylation of NKp46 receptor, in particular on α2,6-linked sialic acid carried by Thr-225 residue (located into the membrane proximal domain of NKp46) (Arnon et al. 2004). The Thr-225 of NKp46 plays a critical role in interaction not only with viral hemagglutinins, but also with unknown tumor cellular ligands, probably via different mechanisms not involving its sialylation (Arnon et al. 2004).

Other authors have suggested that vimentin, expressed on the surface of *Mycobacterium tuberculosis*–infected monocyte cell lines, mediates NKp46 binding to these cells, and contributes to their lysis (Garg et al. 2006).

It has been suggested that the positively charged amino acids K133, R136, H139, R142, and K146 present in the membrane proximal domain may be involved in ligand interaction. Indeed, mutagenesis of these amino acids on NKp46 to noncharged amino acids decreases the affinity of interaction with tumor cells at least 10–100 times, without affecting the interaction with viral ligands (Zilka et al. 2005).

NKp46 on T Cells

Although the cell-surface expression of NKp46 is the most selective marker for human and mouse NK cells described so far, there are non-NK populations that also express NKp46, including discrete subsets of γδ T cells in mice and subsets of intraepithelial lymphocytes in patients with celiac disease. In various chronic infectious and inflammatory diseases (such as celiac disease or CMV infection), human CTLs can aberrantly express NKp46 as well as other activating NK receptors, such as NKG2C or NKp44. These activating NK receptors, thanks to their association with ITAM-bearing molecules (DAP12, CD3ζ, or FcεRIγ), could interfere with normal T cell tolerance mechanisms by both inducing the expansion and unleashing the effector function of CTL, independently of TCR signaling (Meresse et al. 2006).

NKp46 surface expression has been shown also on transformed Sezary T cells. Surprisingly, in these cells, mAb-mediated engagement of NKp46 (that in NK cells functions as a triggering receptor) has been shown to result in inhibition of the CD3-mediated activation process (Bensussan et al. 2011).

NKp46 and ILCs

NK cells are one of the components of a broad family of innate lymphoid cells (ILCs). Different from T-cells and B-cells, ILCs are a group of

lymphocytes lacking recombination activating gene (RAG)-dependent rearranged antigen receptors (Diefenbach et al. 2014). ILCs represent a heterogeneous family of cells characterized by distinct patterns of cytokine production and lineage-specific transcription factors (Vacca et al. 2016). Cytotoxic-ILC population is represented by NK cells (Seillet et al. 2016), whereas helper-ILC populations are constituted by ILC1, ILC2, and ILC3 (Diefenbach et al. 2014).

ILC3 and NK cells share some phenotypic characteristics, primarily in term of NKp46 or other NCRs (Vacca et al. 2016). In particular, inside ILC3 cells, which are characterized by the expression of the transcription factor RORγt, both NCR^- and NCR^+ subsets can be distinguished. Indeed, in mice a subset of $CCR6^-$ ILC3 cells is characterized by the expression of NKp46 and in humans a consistent fraction of ILC3 cells derived from tonsils and gut lamina propria expresses NKp44, NKp46, and NKp30, although to lower levels compared to human NK cells (Killig et al. 2014). Human NCR^+ ILC3 cells homogenously express CCR6 (Cella et al. 2009) and are mainly located in tonsils and intestinal lamina propria, where they contribute to epithelial homoeostasis and host defense against extracellular pathogens (Montaldo et al. 2014). When human NCR^+ ILC3 were identificated, they were named NK22, because of their expression of NCRs and production of IL22 (Satoh-Takayama et al. 2008; Cella et al. 2009; Vivier et al. 2009), but more recently, according to the ILC nomenclature, their name was changed to NCR^+ ILC3 (Spits et al. 2013). Importantly, IL22 production by human NCR^+ ILC3 cells can occur upon engagement of NKp44 (Hoorweg et al. 2012; Glatzer et al. 2013). IL22 regulates the crosstalk between epithelial cells, immune cells, and the commensal microflora, and it instructs epithelial cell functions both in the maintenance of the barrier function and in inducing antimicrobial resistance (Dudakov et al. 2015).

Differently from murine $CCR6^-$ NCR^+ ILC3 cells, human NCR^+ ILC3 do not express T-bet or IFN-γ ex vivo, although expression can be induced after in vitro culture (Cella et al. 2009; Glatzer et al. 2013; Killig et al. 2014).

Recently, the biology of innate producers of IL-22 in mouse conditional deletion models targeting NCR^+ ILC3 but preserving B and T cells has been deeply analyzed. These data revealed the redundant role of murine NCR^+ ILC3 in the control of *C. rodentium* infection in immunocompetent hosts and a selective role of murine NCR^+ ILC3 in cecum homeostasis (Rankin et al. 2016).

Summary

NKp46 is a surface molecule of 46 kDa expressed by all human NK cells irrespective of their state of activation. It is mostly confined to NK cells and its engagement induces a strong activation of NK function. In particular, NKp46 plays an important role in the killing of virus-infected and tumor cells. The identification of the NKp46 ligand(s) on cancer cells is still being investigated. Indeed, it is important to understand whether NKp46 ligand(s) downmodulation or loss can be involved in tumor resistance to NK-mediated cytolysis. Moreover, this identification will allow designing strategies to increase tumor susceptibility to NK-mediated killing by manipulating the NKp46 ligand(s) expression at the tumor cell surface. Although NKp46 is the most selective marker for human and mouse NK cells described so far, there are non-NK populations that also express NKp46, including discrete subsets of γδ T cells in mice, human CTLs in various chronic infectious and inflammatory diseases, human transformed Sezary T cells, and mouse/human $ROR\gamma t^+$ ILC3 cell subsets.

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NLK

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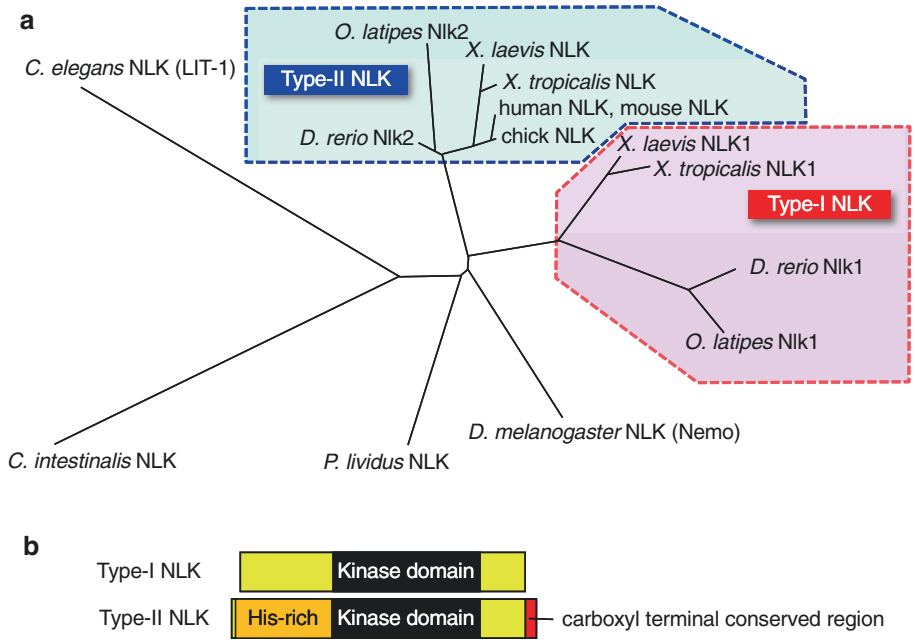
Synonyms

Nemo-like kinase

Historical Background

The protein kinase NLK was originally identified as *nemo*, a gene involved in *Drosophila melanogaster* eye development. *D. melanogaster* compound eyes are made up of hexagonal units called ommatidia. A mutation in *nemo* affects the movement of ommatidia cells, causing them to acquire a squared shape (Choi and Benzer 1994) which is reflected in the name "nemo," Korean for square. In 1998, Brott et al. isolated the mouse homolog of *nemo* and named it "Nemo-like kinase (NLK)." Meneghini et al. (1999) and Rocheleau et al. (1999) found that the *Caenorhabditis elegans loss-of-intestine-1* (*lit-1*) mutant, which lacks the endoderm, has a mutation in a homolog of NLK. This homolog phosphorylates Posterior pharynx defect protein 1 (POP-1) to relieve POP-1-mediated inhibition of gene expression required for endoderm formation. POP-1 is a homolog of the mammalian T-cell factor/lymphoid enhancer factor (Tcf/Lef), a key component of the Wnt/β-catenin signaling pathway. Ishitani et al. (1999) also discovered that mammalian NLK could phosphorylate and inhibit Tcf/Lef in human embryonic kidney 293 (HEK293) cells. This was the first discovery of a molecular function of NLK.

NLK is evolutionarily conserved from *C. elegans* to humans (Fig. 1). Invertebrates, chickens, and mammals possess only one NLK gene, whereas amphibians (*Xenopus laevis*/African clawed frog and *X. tropicalis*/Western clawed frog) and fish (*Danio rerio*/zebrafish and *Oryzias latipes*/medaka) possess two NLK genes, type-I NLK (NLK1/Nlk1) and type-II NLK (NLK2/Nlk2). Type-II NLK, but not type-I NLK, contains conserved regions at the N-terminus (histidine (His)-rich) and C-terminus (Fig. 1b) (Ota et al. 2012; Ishitani and Ishitani 2013). All NLK proteins exhibit high homology to mitogen-activated protein kinases (MAPKs). Mouse NLK (type-II) exhibits a 54.5% amino acid similarity to mouse MAPK1 (Brott et al. 1998). Interestingly, typical MAPKs, including MAPK1, contain a characteristic MAPK-activating phosphorylation sequence, Thr-Xxx-Tyr (TXY), in the activation loop, whereas NLKs do not have a TXY motif in



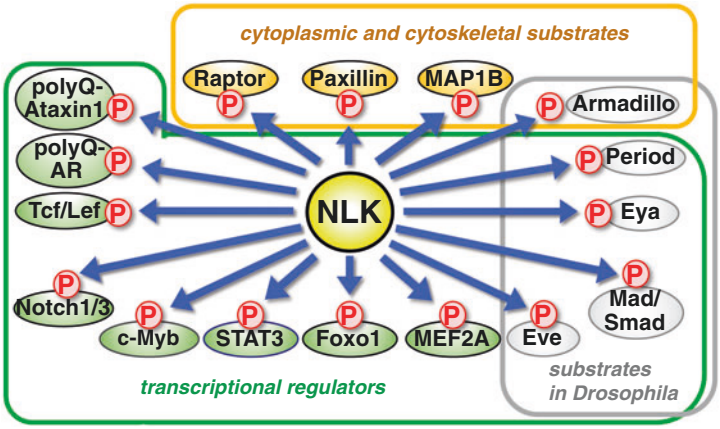
NLK, Fig. 1 NLK protein family. (**a**) Phylogenetic analysis of NLK homologs obtained by comparing amino acid sequences. Vertebrate type-I and type-II NLKs are shown in *blue* and *red*, respectively. (**b**) Schematic diagrams of type-I and type-II NLKs. Note that type-II NLKs, but not type-I NLKs, have conserved histidine-rich (His-rich) and C-terminal regions, which are indicated by *orange* and *red boxes*, respectively

their activation loop. Instead, they harbor a Thr-Xxx-Glu (TQE) sequence at an analogous site (Brott et al. 1998). Therefore, NLK is considered an atypical MAPK.

Roles of NLK-Mediated Phosphorylation in the Nucleus

Given the similarity between NLK and MAPKs, NLK is thought to function as a proline (Pro)-directed kinase that phosphorylates proteins on a serine (Ser) or threonine (Thr) residue immediately preceding a Pro. In practice, NLK phosphorylates Ser and Thr residues of Ser-Pro and Thr-Pro sequences on a variety of signaling molecules, such as the Tcf/Lef family of proteins, Notch1, and c-Myb (Fig. 2; Ishitani et al. 2003a, 2010; Kanei-Ishii et al. 2004; Ota et al. 2012). However, the exact consensus target sequence of NLK has not been characterized.

As mentioned above, *C. elegans* POP-1, a member of the Tcf/Lef protein family, was identified as the first NLK substrate. NLK phosphorylates and regulates vertebrate Tcf/Lefs. Vertebrates have four Tcf/Lef members (five in zebrafish), including Tcf7 (Tcf1), Tcf7L1 (Tcf3), Tcf7L2 (Tcf4), and Lef1. NLK phosphorylates Tcf7L1, Tcf7L2, and Lef1 on conserved Ser/Thr-Pro sequences (e.g., Thr155-Pro156 and Ser166-Pro167 on human Lef1). Although Tcf7 contains such conserved Ser/Thr-Pro sequences, NLK is unable of phosphorylating it (Ishitani et al. 1999, 2003a; Ota et al. 2012). NLK-mediated Tcf/Lef phosphorylation positively or negatively regulates Tcf/Lef activity. In human embryonic kidney 293 (HEK293) and cervical cancer (HeLa) cells, overexpression of NLK reduced DNA binding and inhibited the transcriptional activity of Tcf7L2 and Lef1 (Ishitani et al. 1999, 2003a; Ota et al. 2012). In addition, Yamada et al. (2006) reported that NLK promoted NLK-associated RING finger protein (NARF)-mediated ubiquitination and the subsequent proteasomal degradation of Tcf7L2 and Lef1 in HEK293 cells. On the contrary, NLK is essential for the



NLK, Fig. 2 NLK targets. Vertebrate cytoplasmic and cytoskeletal NLK substrates are indicated in *orange*, and transcriptional regulators phosphorylated by NLK are indicated in *green*. *Drosophila* Nemo substrates are indicated in *gray*

activation of Lef1-mediated transcription in neural progenitor cells (NPCs) and related cells. In NPC-like mammalian cell lines, rat pheochromocytoma tumor (PC12) cells, mouse neuroblastoma (neuro-2a) cells, and zebrafish midbrain NPCs, histone deacetylase HDAC1 binds to Lef1 and inhibits its transcriptional activity. NLK-mediated Lef1 phosphorylation stimulates the dissociation of Lef1 from HDAC1, thereby promoting Lef1 transcriptional activity. Such positive regulation promotes the proliferation of zebrafish midbrain NPCs and consequent midbrain size expansion. Consistent with this finding, knockdown of zebrafish type-II NLK, Nlk2, reduced Lef1 activity and cell proliferation in the developing midbrain, whereas co-knockdown of HDAC1 reversed this effect (Ota et al. 2012).

A biochemical screening for NLK substrates yielded the Notch signaling transcription factors, Notch1 and Notch3 (Ishitani et al. 2010). NLK phosphorylates the intracellular domain of Notch1 (Notch1-ICD) on seven Ser residues within conserved Ser-Pro motifs. Phosphorylation at these sites prevents the formation of a ternary complex between Notch1-ICD, the DNA-binding factor CSL (CBF-1, Suppressor of Hairless, and LAG-1) and a member of the Mastermind family of transcriptional coactivators. Consequently, Notch1-ICD-mediated gene expression is inhibited in a variety of mammalian cultures, including HEK293, HeLa, neuro-2a, PC12, and the colorectal cancer SW480 cells. NLK can also regulate Notch1 activity in vivo. In zebrafish, Notch1 signaling maintains NPCs in an undifferentiated state, preventing their commitment to neurons. Knockdown of zebrafish type-I NLK, Nlk1, enhanced Notch1 signaling and blocked NPC differentiation in the zebrafish neural plate. Accordingly, Nlk1-mediated downregulation of Notch1 signaling was likely to be essential for proper neurogenesis in the zebrafish neural plate (Ishitani et al. 2010). A recent report showed that NLK-mediated Notch1 inhibition contributed to natural killer cell development (Cichocki et al. 2011). Thus, NLK negatively regulates Notch1 signaling in vivo. In contrast, the role of NLK-mediated Notch3 phosphorylation remains unclear, although Ishitani et al. (2010) previously reported that NLK could promote Notch3 activity in a kinase-dependent manner in neuro-2a cells.

NLK can phosphorylate other families of transcriptional regulators, including c-Myb, signal transducer and activator of transcription 3 (STAT3), Forkhead box protein O1 (Foxo1), and MEF2A (Fig. 2). c-Myb is a transcription factor that regulates hematopoietic stem cell proliferation and differentiation. NLK promotes c-Myb degradation via phosphorylation of 15 Ser/Thr-Pro motifs in monkey kidney CV-1 cells (Kanei-Ishii et al. 2004). Kurahashi et al. (2005) showed that NLK could phosphorylate the c-Myb-related

protein A-Myb, thus promoting the dissociation of A-Myb from the transcriptional coactivator CBP and attenuation of A-Myb-dependent transcription. STAT3 is an important transcription factor that mediates cytokine signaling. NLK phosphorylates STAT3 on Ser-727 to promote downstream interleukin-6 (IL-6) signaling in human hepatocellular carcinoma (HepG2) cells (Kojima et al. 2005). Ohkawara et al. (2004) reported that *X. laevis* type-I NLK, NLK1, regulated mesoderm formation via STAT3 Ser-727 phosphorylation. Kim et al. (2010) showed that NLK phosphorylates Foxo1, which controlled the expression of genes involved in apoptosis, cell cycle, stress response, longevity, DNA repair, and glucose metabolism. NLK-mediated phosphorylation negatively regulates the transcriptional activity of Foxo1 by promoting its nuclear export in monkey kidney (Cos-1) cells. Satoh et al. (2007) reported that *X. laevis* NLK1 phosphorylated the MEF2A transcription factor to control embryonic anterior head formation. Interestingly, Ota et al. (2011) showed that NLK1 overexpression could induce the phosphorylation of key regulators of translational control, including Pumilio1, Pumilio2, and cytoplasmic polyadenylation element-binding protein (CPEB) in *X. laevis* oocytes, suggesting that NLK may phosphorylate not only transcriptional but also translational regulators.

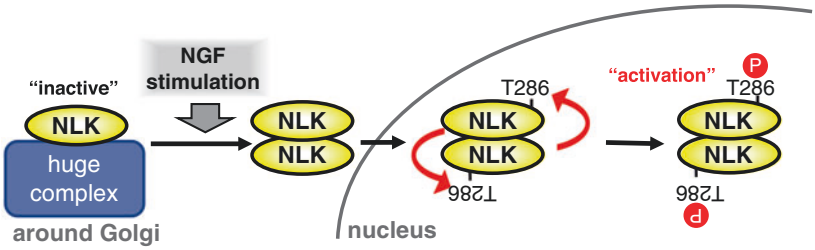
Recent *D. melanogaster* genetic analyses have revealed that Nemo phosphorylates a variety of transcriptional regulators, such as even-skipped (Eve), mothers against decapentaplegic (Mad), eyes absent (Eya), and period (Fig. 2). The phosphorylation of the Eve homeobox transcription repressor contributes to embryonic segment patterning (Braid et al. 2010). Mad is the *D. melanogaster* homolog of the bone morphogenetic protein (BMP) signaling transcription factor Smad. Mad phosphorylation negatively regulates BMP signaling during wing development (Zeng et al. 2007). Eya is a transcriptional regulator essential for eye development, which is activated by Nemo-dependent phosphorylation (Morillo et al. 2012). Period is a circadian clock component, whose stability is enhanced by Nemo-dependent phosphorylation (Chiu et al. 2011).

Occasionally, NLK can work without phosphorylating its substrate. NLK binds to ATF5, a member of the ATF/CREB protein family of transcription factors, thus stabilizing ATF5 in a kinase-independent manner downstream of IL-1 signaling (Zhang et al. 2015). Finally, NLK stabilizes and activates the transcription factor p53, albeit without phosphorylating it, in response to DNA damage (Zhang et al. 2014). Thus, NLK plays a variety of roles in the nucleus.

Roles of NLK-Mediated Phosphorylation Outside the Nucleus

Given that in many mammalian cell lines, exogenous NLK localizes mainly to the nucleus (Brott et al. 1998; Ishitani et al. 2009), nuclear functions of NLK have been well studied. Interestingly, our immunostaining analyses revealed that endogenous NLK localized to the cytoplasm and not to the nucleus in neuro-2a, PC12, HEK293, and HeLa cells. Moreover, stimulation with nerve growth factor (NGF) not only promoted the translocation of endogenous NLK into the nucleus and leading edges of the cell, but also induced the enzymatic activation of NLK (Ishitani et al. 2009; T.I. and S.I. unpublished observations). These findings suggest that NLK functions not only in the nucleus but also in the cytoplasm and near the plasma membrane. Consistent with this idea, Ishitani et al. (2009) showed that NLK phosphorylated the focal adhesion adaptor protein paxillin on Ser-126 and the microtubule-associated protein 1B (MAP1B) at the leading edge of NGF-treated PC12 cells (Fig. 2). *D. melanogaster* Nemo localizes to the plasma membrane and phosphorylates the β-catenin homolog armadillo during eye development (Fig. 2; Mirkovic et al. 2011), indicating that invertebrate NLK functions also near the plasma membrane. However, because mammalian NLK cannot phosphorylate β-catenin (Ishitani et al. 2003a; T.I. and S.I. unpublished observations), the relationship between NLK and β-catenin does not appear to be evolutionarily conserved.

Recently, Yuan et al. (2015) have reported that NLK plays an important role in stress response in



NLK, Fig. 3 Mechanisms of NLK activation. NGF signaling stimulates the dissociation of NLK from a large Golgi complex, inducing its dimerization and consequent autophosphorylation on Thr-286

the cytoplasm. In nutrient-rich conditions, the Rag1 GTPase binds to and activates the mechanistic target of rapamycin (mTORC1) and promotes cell growth by stimulating transcription, translation, and anabolism. To counteract osmotic and oxidative stress, NLK phosphorylates Raptor, an mTORC1 subunit, on Ser-863 (Fig. 2) and disrupts the interaction between Rag1 and mTORC1, thus inhibiting mTORC1 activation.

Regulation of NLK Activity

As described above, NLK is an atypical MAPK. The activity of typical MAPKs is tightly regulated, and overexpression is insufficient to activate them. MAPK kinase (MAPKK)-dependent phosphorylation of Thr and Tyr residues in the TXY motif is required to activate typical MAPKs. However, overexpression is sufficient for activation of NLK kinase (Brott et al. 1998; Ishitani et al. 2011), suggesting that NLK does not require the intervention of upstream kinases. Given that the negatively charged glutamic acid can mimic a phosphorylated amino acid, it is possible that Ser/Thr kinase(s)-mediated phosphorylation of Thr-286 in the TQE sequence leads to NLK activation. Ishitani et al. (2011) showed that exogenous NLK formed homodimers and then autophosphorylated Thr-286 in a *trans*-manner in HEK293 cells. Substitution of Thr-286 with a valine or phosphatase treatment reduced NLK kinase activity. The conserved C-terminal domain is required for NLK homodimerization. Mutation of Cys-425 in the C-terminal domain of NLK prevented NLK dimerization and hampered its kinase activity (Ishitani et al. 2011). Thus, NLK can be self-activated via homodimerization. Ishitani et al. (2011) also reported that inactive endogenous NLK was trapped in large heterologous complexes around the Golgi in unstimulated PC12 cells. Addition of NGF stimulated the release of NLK from the complex, its homodimerization leading to autophosphorylation on Thr-286 and subsequent activation (Ishitani et al. 2011) (Fig. 3). However, the detailed nature of the large Golgi complex remains unclear.

In addition to NGF, several extracellular factors have been identified as NLK activators. Endogenous NLK can be activated by epidermal growth factor (EGF) or Wnt-3a in PC12 cells (Ishitani et al. 2009; Ota et al. 2012) and by Activin A or Wnt-5a in HEK293 cells (Ohkawara et al. 2004; Ishitani et al. 2003b). Exogenous NLK can be activated by treatment with IL-6 or transforming growth factor-β (TGF-β) in HepG2 cells (Kojima et al. 2005) and by osmotic or oxidative stress in HEK293 cells (Yuan et al. 2015). Several intracellular modulators of NLK activity have also been identified. One of these is TAK1 MAP3K, which promotes the autophosphorylation and activation of NLK/LIT-1 in *C. elegans* (Meneghini et al. 1999; Ishitani et al. 1999). TAK1 appears to activate NLK indirectly. Kanei-Ishii et al. (2004) reported that TAK1 phosphorylated and activated homeodomain-interacting protein kinase 2 (Hipk2), which then bound to and activated NLK. Ohnishi et al. (2010) reported that p38 MAPK phosphorylated and activated NLK downstream of TAK1. Although TAK1 is involved in Wnt-5a- and IL-6-dependent activation of NLK (Ishitani et al. 2003b; Kojima et al. 2005), NGF and EGF activate NLK in a TAK1-independent manner

through Ras GTPase (Ishitani et al. 2009; T.I. and S.I. unpublished observations). Kim et al. (2012) recently described dimerization partner 1 (DP1) as an inhibitor of NLK. DP1 binds directly to and inhibits NLK, consequently enhancing Wnt/β-catenin signaling in *X. laevis* early embryos.

NLK is regulated at the protein level by noncoding RNAs. MicroRNAs miR-181a and miR-181b reduce NLK protein levels and prevent NLK-mediated suppression of Notch signaling, thereby promoting human natural killer cell development in vitro (Cichocki et al. 2011). In hepatocellular carcinoma (HCC) cell lines, miR-181 family members (miRNA-181a/b/c/d) and miR-101 negatively regulate NLK expression (Ji et al. 2009; Shen et al. 2014). In addition, miR-288a inhibits NLK expression in rat cardiomyocyte (H9C2) cells (Huang et al. 2016). Furthermore, a cancer-related long noncoding RNA, HOTAIR, has been shown to inhibit NLK transcription in glioma cells (Zhou et al. 2015). Because the regulation of NLK enzymatic activity is relatively loose, organisms may have evolved a variety of systems to control NLK expression.

To better understand NLK function and regulation, it would be advantageous to develop specific chemical inhibitors against NLK. At present, NLK activation can be blocked only with lithium chloride and lithium carbonate (Ishitani et al. 2009; T.I. and S.I. unpublished observations). However, lithium is not a specific inhibitor, and development of specific NLK inhibitors is awaited.

Physiological Roles of NLK

The NLK gene family plays important roles in central nervous system (CNS) development in vertebrates. *X. laevis* NLK1 regulates anterior brain formation through MEF2A phosphorylation in early embryos (Satoh et al. 2007). *D. rerio* Nlk1 controls neural plate anterior-posterior patterning via positive regulation of Wnt/β-catenin signaling (Thorpe and Moon 2004) and promotes the differentiation of NPCs into neurons by blocking Notch signaling (Ishitani et al. 2010). In contrast, *D. rerio* Nlk2 contributes to midbrain size expansion through Lef1 phosphorylation and consequent stimulation of Wnt/β-catenin signaling (Ota et al. 2012). Kortenjann et al. (2001) reported that type-II NLK-deficient mice suffered from various neurological abnormalities such as cerebellar ataxia, indicating that type-II NLK might contribute to brain development in mammals. Thus, type-I NLKs seem to control early brain (neural plate) development, whereas type-II NLK appears to regulate the later stages.

Mouse type-II NLK is also involved in non-neural tissue development, including lung morphogenesis, adipogenesis, and hematopoiesis. Ke et al. (2016) reported that type-II NLK-deficient mice became cyanotic because of lung maturation defects, exhibiting elevated vascular endothelial growth factor (VEGF) expression, and small and compressed alveoli (Ke et al. 2016). Kortenjann et al. (2001) showed that bone marrow adipogenesis was enhanced, whereas the number of hematopoietic cells was reduced in type-II NLK-deficient mice.

NLK in Disease

A number of studies have reported a correlation between cancer development and NLK expression or activity. NLK negatively regulates the expression of androgen receptor (AR) and the nuclear steroid hormone receptor Nurr1, both of which correlate with prostate cancer progression. Consistent with this, NLK expression has been found to decrease during prostate cancer progression (Emami et al. 2009; Wang et al. 2016). Similarly, NLK expression is inversely correlated with glioma grade (Cui et al. 2011; Sa et al. 2015). NLK can negatively regulate Wnt/β-catenin signaling through Lef1 phosphorylation and suppress mesenchymal activity of glioma cells; however, in high-grade gliomas in which NLK expression is very low, Wnt/β-catenin signaling and mesenchymal activities are upregulated (Sa et al. 2015). Thus, NLK exhibits tumor suppressor activities in this type of cancer.

By contrast, in HCCs, upregulation of NLK enhances the expression of cyclin D1, a core component of cell cycle regulation. NLK RNAi has been reported to reduce HCC viability (Jung et al. 2010). In addition, Mendes-Pereira et al. (2012) showed that NLK RNAi reduced the viability of PTEN-deficient breast, colorectal, bladder, and ovary cancer, as well as in melanoma cells. Consequently, NLK represents a potential therapeutic target for these tumor types, whose treatment would benefit from the existence of specific chemical NLK inhibitors.

A recent study using a mouse disease model has revealed that NLK is involved in polyglutamine diseases, including spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxia type 1(SCA1). Polyglutamine diseases represent a group of neurodegenerative disorders caused by expanded CAG repeats encoding a long polyQ tract in the corresponding proteins. SBMA is caused by polyQ-expanded AR (polyQ-AR), resulting in motor neuron degeneration and muscle bulk loss. SCA1 is caused by polyQ-expanded Ataxin1 (polyQ-Ataxin1) and leads to cerebellum and motor neuron degeneration. Interestingly, Lim and coworkers at Yale University reported that NLK phosphorylated polyQ-AR and polyQ-Ataxin1 (Fig. 2). Moreover, loss of one copy of the NLK gene rescued neuropathological phenotypes in SBMA and SCA1 model mice (Ju et al. 2013; Todd et al. 2015). Although the mechanisms by which NLK promotes SBMA and SCA1 are not fully understood, NLK may be a potential target for SBMA and SCA1 therapy.

Summary

NLK is an evolutionarily conserved atypical MAPK, which plays multifaceted roles in invertebrate and vertebrate development. NLK controls gene expression and cytoskeletal architecture by phosphorylating and regulating a variety of cell signaling components and cytoskeleton-associated proteins. As a result, it contributes to cell growth, proliferation, differentiation, and morphological changes during early embryonic patterning, brain development, lung morphogenesis, adipogenesis, and hematopoiesis. Although activated NLK phosphorylates many substrates, it is unlikely that it does so simultaneously. Instead, NLK probably phosphorylates and regulates specific substrates depending on the context. However, the detailed mechanisms of such selective regulation are still not clear. What is partly known is the process of NLK activation: NLK is released from a large Golgi complex in response to upstream stimulation, leading to dimerization and self-activation via autophosphorylation on Thr-286. However, the nature of this large complex and the mechanism by which NLK dissociates from it remain unclear, and these will be the focus of future studies. Recent reports have shown that NLK inhibition can ameliorate tumorigenesis and neurodegenerative diseases. Although specific chemical inhibitors against NLK have not been reported yet, they may be a useful tool not only in basic biological research but also in the treatment of multiple diseases.

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N-Lysine Methyltransferase SMYD

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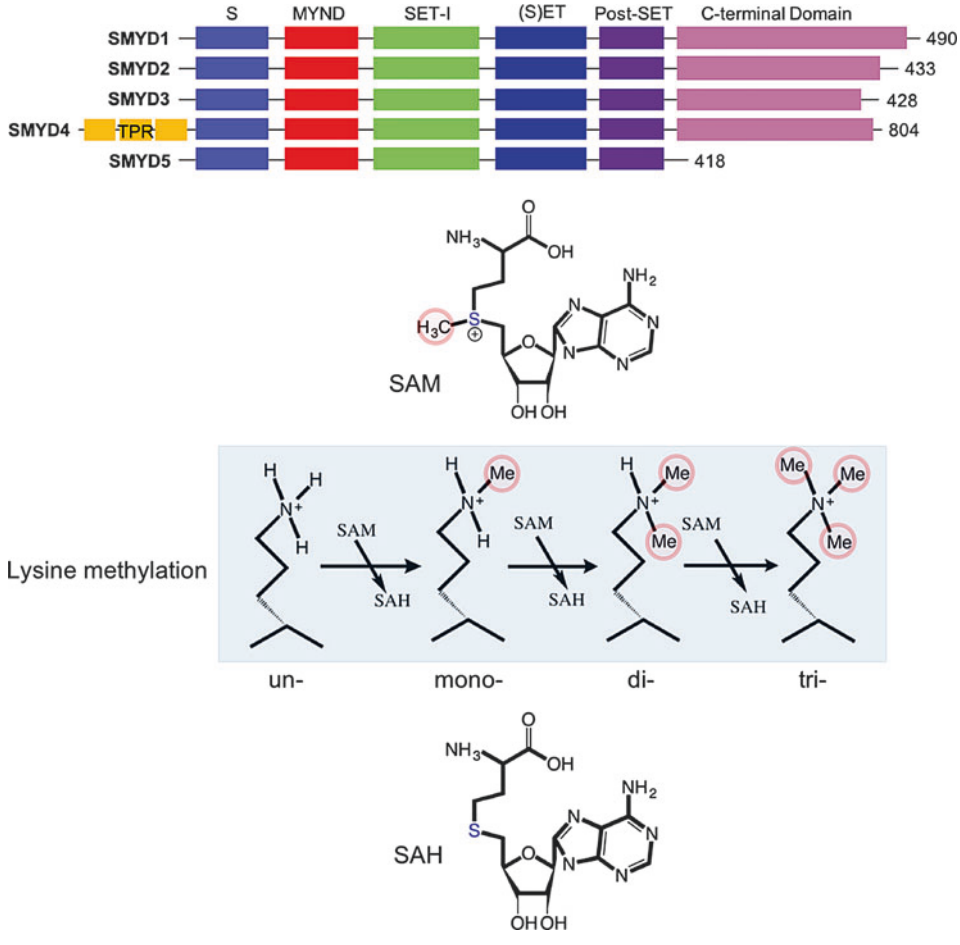
Synonyms

AdoMet: S-adenosylmethionine; MYND: myeloid, Nervy, and DEAF-1; SET: *Drosophila* Su

(var)3–9 and "Enhancer of zeste"; SMYD: SET and MYND domain-containing proteins; TPR: tetratricopeptide repeat

Historical Background

SMYD proteins are a family of histone and protein lysine methyltransferases containing a SET domain interrupted by a MYND zinc finger motif (Spellmon et al. 2015) (Fig. 1). The SET domain is evolutionally conserved in N-lysine methyltransferases and responsible for the methyltransferase activity (Sirinupong et al. 2010). The MNYD domain is a protein–protein interaction module involved in transcriptional cofactor recruitment (Spellmon et al. 2015). The SMYD family achieves the methyltransferase activity by adding methyl groups to target lysine residues using S-adenosylmethionine (AdoMet) as a donor substrate (Fig. 1). Recent studies show the SMYD protein family to be involved in cancer proliferation, epigenetic transcription regulation, the immune system, and muscle and heart development (Gottlieb et al. 2002; Hamamoto et al. 2004; Tan et al. 2006; Stender et al. 2012; Mazur et al. 2014). The SMYD family can be grouped into three classes: SMYD3

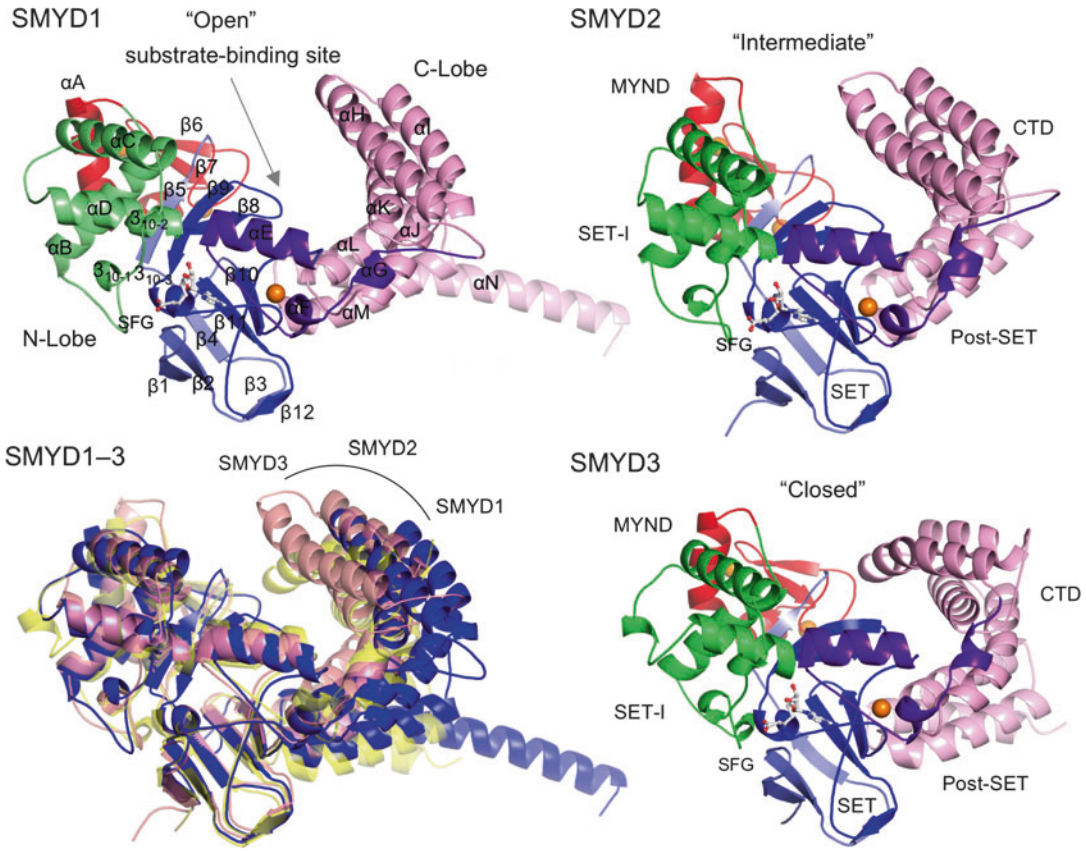


N-Lysine Methyltransferase SMYD, Fig. 1 Domain structure of the SMYD protein family (*top*) and AdoMet-dependent protein lysine methylation (*bottom*). SAM, AdoMet (S-adenosylmethionine); SAH, AdoHcy (S-adenosylhomocysteine); Me, methyl group. Lysine residue can be methylated three times: mono-, di-, and trimethylation

(including SMYD1 and SMYD2), SMYD4, and SMYD5 (Calpena et al. 2015) (Fig. 1). The SMYD3 class contains a TPR (tetratricopeptide repeat)-like C-terminal domain in addition to the SET and MYND domains. SMYD4 class contains an additional TPR motif at the N-terminus. SMYD5 class does not contain any TPR domains, and the C-terminal portion of SMYD5 is replaced by a glutamic acid-rich tract. The SMYD3 class is the most studied class within the SMYD family. To date, SMYD3 class is shown to be involved in muscle and heart development, proliferation of cancer cells, and estrogen receptor-mediated gene expression (Spellmon et al. 2015). Both SMYD4 and SMYD5 classes are poorly studied. It is shown that SMYD4 is associated with breast cancer, neuropathy, and muscle and heart development (Thompson and Travers 2008). SMYD5 is found to play a role in the immune response and regulate inflammatory gene expression through histone H4 K20 methylation (Stender et al. 2012).

Crystal Structure

SMYD1, SMYD2, and SMYD3 protein structures with cofactors and substrates have been solved (Sirinupong et al. 2010; Sirinupong et al. 2011; Jiang et al. 2014). SMYD4 and SMYD5 structures are yet to be determined. All solved SMYD structures show a bilobal structure (Fig. 2). The N-terminal lobe is split into four



N-Lysine Methyltransferase SMYD, Fig. 2 Crystal structure of SMYD proteins. SMYD1–3 is a structural superposition of SMYD1, SMYD2, and SMYD3. The S-sequence, MYND, SET-I, core SET, post-SET, and CTD (C-terminal domain) are depicted in *light blue*, *red*, *green*, *blue*, *purple*, and *pink*. Secondary structures, α-helices and β-strands, are labeled and numbered according to their position in the sequence. The cofactor analog sinefungin, SFG, is depicted by *balls* and *sticks*. Zinc ions are depicted by *orange spheres*

domains: SET, MYND, SET-I, and post-SET. The SET domain is stationed in the center of the N-terminal lobe in proximity to the C-terminal lobe. The C-terminal lobe is organized into seven up-down helices mimicking the structure of TPR repeats. The SET domain is approximately 130 amino acids long, being split into two sections. The small region of the SET domain is the S-sequence, which contributes to cofactor binding. The core SET domain is the large region of the SET domain, and this, together with SET-I and post-SET, contributes to both cofactor binding and substrate binding. In SMYD, the post-SET is made of a three-α-helix bundle surrounding a zinc atom coordinated by four cysteine residues. The MYND and SET-I domain are an insertion region between the SET domain strands β5 and β8. The MYND domain is able to bind proline-rich peptides and may also contribute to DNA binding in SMYD3 (Spellmon et al. 2015). The exact nature of DNA binding in SMYD3 is unknown. The deletion of the MYND does not affect the methyltransferase activity of SMYD2 in vitro suggesting the MYND domain is dispensable in methylation.

The difference in the positions of the N- and C-terminal lobes in SMYDs results in open and closed structures (Fig. 2). SMYD1 has the most open conformation with the substrate-binding site widely exposed. SMYD3 is found to have the most closed conformation. SMYD2 has an intermediate conformation between SMYD1 and SMYD3. Due to the unique protruding C-terminal helix, SMYD1 structure shows an open-ended "wrench" shape (Fig. 2). The absence of this helix in SMYD2 and SMYD3 results in a clamshell-like structure.

Protein Lysine Methylation

The SMYD protein family methylates both histone and nonhistone proteins (Hamamoto et al. 2004; Huang et al. 2006; Tan et al. 2006; Zhang et al. 2013; Mazur et al. 2014). Histone methylation links the SMYD family to the epigenetic process. Histone methylation is the process in which methyl groups are transferred to amino acids of histone proteins in nucleosomes. Increasing or decreasing transcriptional events can occur due to the methylation of histones; this is dependent on which amino acids are methylated. The histone methylation by SMYD proteins contributes to the regulation of chromatin remodeling and gene accessibility. SMYD1–3 methylate H3K4 which is a methylation site that promotes active transcription (Hamamoto et al. 2004; Sirinupong et al. 2010). The SMYD family does not have an effect on global H3K4 methylation levels but impacts selective promoter regions (Hamamoto et al. 2004; Tan et al. 2006). SMYD2 is found to dimethylate H3 K36 in vitro and repress transcription (Spellmon et al. 2015). The SMYD family also regulates gene expression by recruiting other transcriptional regulators. SMYD1 binds directly to class I and class II histone deacetylases (HDAC) which repress transcription (Gottlieb et al. 2002). SMYD3, being a member of an RNA polymerase complex, interacts with RNA polymerase II and RNA HELZ and plays an important role in transcriptional regulation (Hamamoto et al. 2004).

SMYD proteins are known to methylate nonhistone proteins, such as p53, VEGFR1, retinoblastoma (Rb), and heat shock protein-90 (Hsp90) (Abu-Farha et al. 2011; Jiang et al. 2014). The methylation of these nonhistone proteins expands the role of the SMYD protein family beyond the epigenetic process. The protein methylation may change protein function. SMYD3 methylation of VEGFR1 increases its kinase activity. Methylation of different target lysine residues on Rb and p53 by SMYD2 represses their apoptotic activity (Huang et al. 2006). The protein p53 functions as a tumor suppressor and plays an important role in the suppression of cancer. VEGFR1 is a protein that promotes vasculogenesis and angiogenesis. The protein Rb is a tumor suppressor and serves as a recruiter for chromatin remodeling enzymes. The methylation of these proteins has linked the SMYD protein family to tumorigenesis, cell signaling, and DNA damage response. SMYD2 can also methylate Hsp90 (Abu-Farha et al. 2011). The target lysine residues K209 and K615 are located on the nucleotide-binding domain and dimerization domain, respectively. Hsp90 is a

homodimeric, ubiquitous, and essential chaperone composed of three functional domains: the nucleotide-binding domain, middle domain, and dimerization domain. Hsp90 is involved in heat shock response, signal transduction, steroid signaling, and tumorigenesis. The methylation of Hsp90 by SMYD2 may have the potential to regulate Hsp90 dimerization and ATPase activity, thereby impact its chaperone activity and stress response.

Heart Development

The SMYD protein family plays critical roles in heart development. Deletion of SMYD1 in mice interferes with cardiomyocyte maturation and proper formation of the right ventricle (Gottlieb et al. 2002). With the SMYD1 deletion, the mice embryo fails to form the right ventricle and dies around 10.5 days post coitum (dpc). In zebrafish, deletion of SMYD1 results in no heart contraction and severe myofibril disorganization in cardiac muscles (Tan et al. 2006). SMYD2 is most expressed in the newborn heart. Deletion of SMYD2 has no apparent effect on mice (Spellmon et al. 2015). However, the knockdown of SMYD2 in zebrafish leads to impaired cardiac performance and defective myofibril organization in cardiac muscles (Spellmon et al. 2015). Deletion of SMYD3 in zebrafish produces a cardiac defect (Fujii et al. 2011). SMYD3 knockdown produces pericardial edema with abnormal expression of three heart chamber markers including cmlc2, amhc, and vmhc. The current results have provided supporting evidence of the importance of the SMYD protein family in heart development.

Muscle Development

The SMYD protein family plays crucial roles in muscle development. SMYD1 is specifically expressed in muscle cells under the regulation of myogenic transcriptional factors including the MyoD and Mef2 families and the serum responsive factor (Spellmon et al. 2015). Deletion of SMYD1 in zebrafish leads to complete blockage of myofibril organization in skeletal muscles, producing paralyzed embryos (Tan et al. 2006). All key sarcomere structures are disrupted in SMYD1-depleted zebrafish, including actin, myosin, and M-lines. SMYD1 is also expressed in extraocular muscles controlling movement of the eye and cranial cephalic muscles in zebrafish. SMYD2, SMYD3, and SMYD4 also play a role in muscle development (Spellmon et al. 2015). Knockdown of SMYD2 results in defective myofibril organization at Z-lines and I-bands. However, SMYD2 knockdown does not lead to complete disruption of the muscle developmental process. SMYD3 knockdown in zebrafish embryos leads to abnormal expression of myogenic markers including MyoD (Fujii et al. 2011). A recent study shows that SMYD3 is related to skeletal muscle atrophy (Proserpio et al. 2013). By regulating the expression of the myostatin gene, SMYD3 inhibits myogenesis and muscle cell growth and differentiation. SMYD4 is found in the visceral, cardiac, and somatic muscle precursors in *Drosophilia Melanogaster* during late embryogenesis (Thompson and Travers 2008). Knockdown of SMYD4 results in eclosion failure, causing disruptions at the late pupal stage. The eclosion failure is likely due to defective muscle development due to the necessity of abdominal muscles to escape the pupal case.

Cancer

The SMYD protein family is a contributing factor to the development of cancer. Overexpression of SMYD2 and SMYD3 has a tumor-promoting effect (Spellmon et al. 2015). SMYD2 is widely overexpressed in many cancers. In leukemia, the expression level of SMYD2 is almost eight times higher than normal bone marrow controls. The high expression level of SMYD2 is associated with low survival rates in leukemia patients. SMYD2 overexpression is also associated with esophageal squamous cell carcinoma (Spellmon et al. 2015). Knockdown of SMYD2 in esophageal squamous cell carcinoma inhibits tumor cell growth, with overexpression of SMYD2, and

promotes proliferation. SMYD2 connects to cancer possibly through methylation of nonhistone targets. As stated earlier with the ability to methylate p53 and Rb, SMYD2 could contribute to the dysregulation of these tumor suppressor proteins. Protein p53 regulates the cell cycle; with a mutation it will lead to overgrowth and tumor formation. Protein Rb is also a tumor suppressor gene that prevents cell overgrowth. Any dysregulation of these proteins could lead to cancerous effects.

SMYD3 is overexpressed in over 15 types of cancers (Spellmon et al. 2015). Overexpression of SMYD3 upregulates a number of genes corresponding to oncogenes, homeobox genes, and genes that contribute to the cell cycle. It is shown that SMYD3 upregulates the expression of the matrix metalloproteinase MMP-9 through H3K4 trimethylation. MMP-9 is involved in tumor progression and metastasis by stimulating cell migration, tumor invasion, and angiogenesis. Suppression of SMYD3 results in reduced MMP-9 gene expression. Methylation of nonhistone substrates also connects SMYD3 to cancer. MAP3K2 methylation by SMYD3 upregulates MAP kinase signaling contributing to the tumorigenesis of Ras-driven carcinomas (Mazur et al. 2014). Ras is a family of oncogenes that is activated in a large fraction of human cancers. The methylation of VEGFR1 by SMYD3 may promote angiogenesis and enable the cancerous tumor with sufficient access to blood and oxygen consumption (Spellmon et al. 2015).

SMYD4 has been associated with breast cancer (Spellmon et al. 2015). SMYD4 represses the expression of the platelet-derived growth factor receptor (PDGFR-α) gene in breast cancer cell lines. High expression of PDGFR-α promotes tumorigenesis. This suggests a role of SMYD4 as a tumor suppressor gene in breast cancer. Recent studies show several somatic mutations in SMYD proteins, which include missense, nonsense, insertions, and deletions (Kudithipudi and Jeltsch 2014). Some of these mutations target the catalytic SET domain and could lead to gain or loss of function. A mutation in SMYD4 could induce the same effects as SMYD2 or SMYD3, leaving it without a tumor suppressor effect. Therefore, the somatic mutation of the SMYD protein family may represent additional pathways for its involvement in the development of cancer.

Other Findings

There is supporting evidence of the SMYD protein family playing a role in the immune system and estrogen receptor (ER)-mediated expression. SMYD5 is shown to work in the genetic program of the immune response in *Drosophilia* and vertebrates (Stender et al. 2012). The methylation of histone H4K20 by SMYD5 represses the inflammatory response by the suppression of toll-like receptor 4 (TLR-4)-mediated expression in macrophages. The role of other SMYD family members in immunity is unknown, though they are all expressed in macrophages (Stender et al. 2012). SMYD3 is involved in estrogen receptor-mediated expression through the H3K4 methyltransferase activity (Kim et al. 2009). SMYD3 directly interacts with the ligand-binding domain of ER, functions as a cofactor, and upregulates ER activity. SMYD3 is required for ER-regulated gene transcription in estrogen signaling pathways. Downregulation of SMYD3 represses the expression of ER target genes and reduces the H3K4 methylation level at their promotor regions. ER is a sequence-specific transcription factor that regulates a cascade of gene targets whose products mediate the initiation, development, and metastasis in breast cancer cells. Overexpression of SMYD3 has been found in breast cancer tissues and breast cancer cell lines (Spellmon et al. 2015). This, together with the regulatory role of SMYD3 in ER-mediated gene transcription, has established a potential mechanistic link between SMYD3 and breast cancer.

Additional roles of the SMYD protein family await to be explored. SMYD5 expression is high in brain, skeletal muscle, digestive system, and salivary glands (Uhlen et al. 2010). Immunohistology staining shows SMYD5 is present in squamous epithelium, gallbladder, kidney tubule, and intestinal lumen. Knockout of SMYD5 in mice shows elevated hyperactivity possibly demonstrating an uncharacterized neuropathic disorder (Ayadi et al. 2012). SMYD2 may

have multiple functions. SMYD2 knockout mice show significant phenotypes in the behavior, reproductive system, renal system, immune system, and digestive and alimentary system (Uhlen et al. 2010). No significant phenotype is observed for SMYD3 knockout mice, but its gene expression is found to be associated with aging and autoimmune diseases such as rheumatoid arthritis.

Summary

SMYD protein family contributes to one of the seven classes of SET domain-containing lysine methyltransferases (Calpena et al. 2015). It contains five protein members: SMYD1, SMYD2, SMYD3, SMYD4, and SMYD5. These proteins can be grouped into three classes based on domain structures: SMYD3 (containing SMYD1 and SMYD2), SMYD4, and SMYD5. All SMYD proteins contain a SET domain interrupted by a MYND zinc finger. They also contain a C-terminal TPR motif, with the exception of SMYD5, which does not contain a TPR, and SMYD4 which contains two TPR regions. The TPR and MYND domains are key regulators in protein–protein interactions. The SET domain is required for methylation of both histone and nonhistone proteins. The SMYD protein family plays important roles in many cellular processes such as the cell cycle, chromatin remodeling, transcription, and signal transduction. SMYD proteins contribute to epigenetic changes during myogenesis and cardiomyocyte differentiation. However, there is much to be discovered regarding this functionally important protein family. SMYD proteins are of therapeutic interest due to the growing list of diseases linked to SMYD overexpression or dysfunction. Continuing knowledge of the SMYD protein family could provide valuable drug targets against cancer and cardiovascular disease.

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NM_020820 (NCBI Reference Sequence for *Homo sapiens* Prex1 cDNA) / NM_177782 (NCBI Reference Sequence for *Mus musculus* Prex1 cDNA)

▶ P-Rex1

NM1

▶ Nuclear Myosin I

NMI

▶ N-Myc and STAT Interactor (NMI)
▶ Nuclear Myosin I

NMMHC-II

▶ Nonmuscle Myosin II

NMT

▶ NMT (*N*-Myristoyltransferase)

NMT (*N*-Myristoyltransferase)

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Synonyms

Myristoyl CoA: protein *N*-myristoyltransferase; NMT; *N*-myristoyl transferase

Historical Background

Lipid modification is one of the most common posttranslational modifications in eukaryotic cells that take place either at or near the amino

terminus or the carboxy terminus of the proteins (Resh 2013). This process is sequence specific and classified as myristoylation, palmitoylation, prenylation, and glycosylphosphatidylinositol according to the identity of the attached lipid. The structural changes resulting from these modifications have effects on the stability, cellular location, and biological activity of the proteins (Resh 2013). These processes are becoming increasingly important for the study of cancer, as several key oncoproteins require this type of "posttranslational maturation" for their biological activity and for their ability to transform cells. Among these lipid modifications, protein myristoylation is known to be very important which refers to the covalent attachment of myristate, a 14-carbon saturated fatty acid, to the *N*-terminal glycine residue of a number of eukaryotic proteins (Rajala et al. 2000a). Protein myristoylation is catalyzed by the enzyme myristoyl CoA:protein *N*-myristoyltransferase, which is ubiquitously distributed among eukaryotes including humans (Rajala et al. 2000a; King and Sharma 1992). This enzyme often exists as isoforms and is very specific for the transfer of myristate in vivo (Rajala et al. 2000a). Myristoylation occurs in at least 0.5% of eukaryotic proteins suggesting an important role in the growth and survival of cells whereas myristate is $<1\%$ of the fatty acid pool (Resh 2012).

N-myristoyl transferase (NMT) exists in two major isoforms NMT-1 and NMT-2 in all mammalian species, both of which are highly conserved (Giang and Cravatt 1998). NMT-1 and NMT-2 have an overall sequence identity of 76–77% with most divergence being at their N terminus (Giang and Cravatt 1998). While NMT-2 appears as a single 65 kDa protein, NMT-1 exists as four distinct isoforms ranging from 49 to 68 kDa in size (Giang and Cravatt 1998). The smaller isoform of 416 amino acids is catalytically active and there is no functional requirement for the longer isoform. The extended N-terminus is located close to the myristoyl-CoA binding site as well as the peptide-binding site suggesting that this region plays an important role in the coordinated control of the catalytic activity of NMT-1 (Kumar and Sharma 2015). The N-terminal extensions of the human NMT-1 may also play a role in targeting the enzyme to ribosomes. A recent observation is that the N-terminal region in the catalytic module of NMT-1 functions as a regulatory control segment (Kumar and Sharma 2015). A comparison of the activity of NMT1 and NMT2 toward a small panel of substrate peptides in in vitro experiments reveals that the isozymes have similar, but distinguishable relative selectivity (Kumar and Sharma 2015). It was found that NMT-2 is not active in embryonic stem cells but its level increases during development. The distribution of both isozymes is found in normal tissue. The selective knockdown of the expression of NMT1, NMT2, or both isozymes in human colon cancer HT-29 cells and ovarian carcinoma SKOV-3 cells using small interfering RNAs (siRNA) shows that siRNA sequences unique to each NMT message selectively reduce the expression of the NMT1 or NMT2 isozyme by $>90\%$ for at least 72 h and had shown that NMT1 and NMT2 have both redundant and unique effects on protein processing, apoptosis, and cell proliferation (Ducker et al. 2005). Protein modifications have been identified to play essential roles in various oncogenic proteins and modified signal transduction pathways of cancer (Selvakumar et al. 2007). Myristoylation of proteins is known to be involved in the pathogenesis of cancer (Selvakumar et al. 2007). Consequently, NMT has been proposed as a molecular target in anticancer drug design (Das et al. 2012).

The Role of *N*-Myristoyltransferase in Cancers

Colon Cancer

NMT was found to be involved in the posttranslational modification of c-Src and also overexpressed in colon tumor cells (Selvakumar et al. 2007). Inhibition of Src myristoylation in colon cancer cell lines prevents the localization of the kinase to the plasma membrane, resulting in decreased colony formation and cell proliferation (Shoji et al. 1990). As NMTs and Src are overexpressed in colonic tumors (Selvakumar

NMT (*N*-Myristoyltransferase), Table 1 *N*-Myristoyltransferase activity in colonic mucosal homogenates and azoxymethane-induced rat colonic tumors (Magnuson et al. 1995)

Tissue	No.	*N*-Myristoyltransferase activity[a]	
		U/mg protein	U/g tissue
Normal mucosa from control rats	3	0.34 ± 0.02	7.76 ± 0.89
Normal-appearing mucosa from rats given injections	7	0.43 ± 0.08	8.86 ± 1.86
Colonic tumors	35	1.69 ± 0.15[b]	47.99 ± 5.06[b]

[a]Mean ± SE
[b]Significant difference from normal and normal-appearing mucosa ($P < .05$)

NMT (*N*-Myristoyltransferase), Table 2 *N*-Myristoyltransferase activity in azoxymethane-induced rat colonic tumors (Magnuson et al. 1995)

Tumor stage	No.	*N*-Myristoyltransferase activity[a]	
		U/mg protein	U/g tissue
Adenomatous polyp	5	2.32 ± 0.38	60.43 ± 12.5
A	8	1.39 ± 0.20	31.19 ± 5.67
Bl	10	2.11 ± 0.32	65.39 ± 12.59
B2	10	1.35 ± 0.02	40.80 ± 6.52
C2	2	0.90 ± 0.43	33.03 ± 15.09

[a]Mean ± SE

et al. 2007), NMT inhibitors have a potential role to play in colon cancer therapeutics.

An elevated level of NMT activity in the azoxymethane-induced rat model of colonic tumors was observed when compared with normal-appearing adjacent mucosa and normal mucosa (Table 1) (Magnuson et al. 1995). However, polyps and tumors of stage B1 had the highest NMT activity (Table 2). These observations suggest that NMT activity is elevated during the early stage of colonic carcinogenesis. It is noteworthy that elevated NMT activity was also observed in all these adenocarcinomas and was predominantly found in the cytosolic fraction (Magnuson et al. 1995). Elevated NMT activity was observed close to the rectum which is an area that carries a poor prognosis in cancer (Magnuson et al. 1995). Furthermore, NMT activity was evaluated in Crohn's disease colons or volvulus and found to be similar to normal mucosa, suggesting that NMT is specific to neoplastic cells and not to cells undergoing inflammatory or non-cancerous changes (Magnuson et al. 1995).

Elevated NMT activity during carcinogenesis may be due to the higher demand for myristoylation of various proteins/oncoproteins (src, ras, etc.) which are overexpressed and activated during tumorigenesis. Among several proteins in the intestine that are myristoylated, tyrosine kinases of the src family are the most studied. The levels of the myristoylated tyrosine kinases, $pp60^{c\text{-}src}$ and $pp60^{c\text{-}yes}$, are several folds higher in colonic preneoplastic lesions and neoplasms compared with normal colon cells (Selvakumar et al. 2007). Differential expression of $pp60^{c\text{-}src}$ has been observed in colonic tumor-derived cell lines (Selvakumar et al. 2007) and in colonic polyps prone to developing cancer (Selvakumar et al. 2007). Higher levels of cytoskeletal-associated $pp60^{c\text{-}src}$ protein tyrosine kinase activity have been observed in intestinal crypt cells along with higher expression of $pp60^{c\text{-}yes}$ in the normal intestinal epithelium. Studies have revealed that $pp60^{c\text{-}src}$ is overexpressed in human colon carcinoma and it has enhanced kinase activity in progressive stages and metastases of human colorectal cancer (Selvakumar et al. 2007). In colonic cell lines, blockage of $pp60^{c\text{-}src}$ *N*-myristoylation results in depressed colony formation and reduced proliferation (Magnuson et al. 1995). Earlier, it has been shown that src kinase activity is positively regulated by myristoylation and the nonmyristoylated c-Src exhibited reduced kinase activity (Raju et al. 1997).

An elevated expression of NMT in colon cancer cell lines was observed which correlates with high levels of c-Src (Giang and Cravatt 1998). The overexpression of NMT in colorectal cancer has implications with regard to the development of chemotherapeutic agents. However, from the above study it is not clear whether there is an increase in the production of NMT or the increased activity is due to a conformational change in the pre-existing enzyme. In addition, the increased NMT activity may be due to the

removal of an inhibitor or the presence of an activator. Therefore, the expression of NMT in normal colonic mucosa and adenocarcinomas from human colorectal surgical specimens were studied by immunoblotting and its localization was further confirmed by immunohistochemistry (Raju et al. 1997). In both normal mucosa and colorectal adenocarcinoma, NMT-1 with a molecular mass of 48.5 kDa was identified when probed with an antihuman NMT antibody (Fig. 1a). These findings were further confirmed by immunohistochemical studies which showed stronger cytoplasmic localization in colorectal adenocarcinomas compared to normal colonic mucosa. In addition the mucosal sections taken distant from the tumor showed only a mild reactivity (Fig. 1b). The transitional mucosa in the vicinity of the cancers did, however, stain more than normal mucosa albeit not to the same degree as the tumors (Fig. 1c).

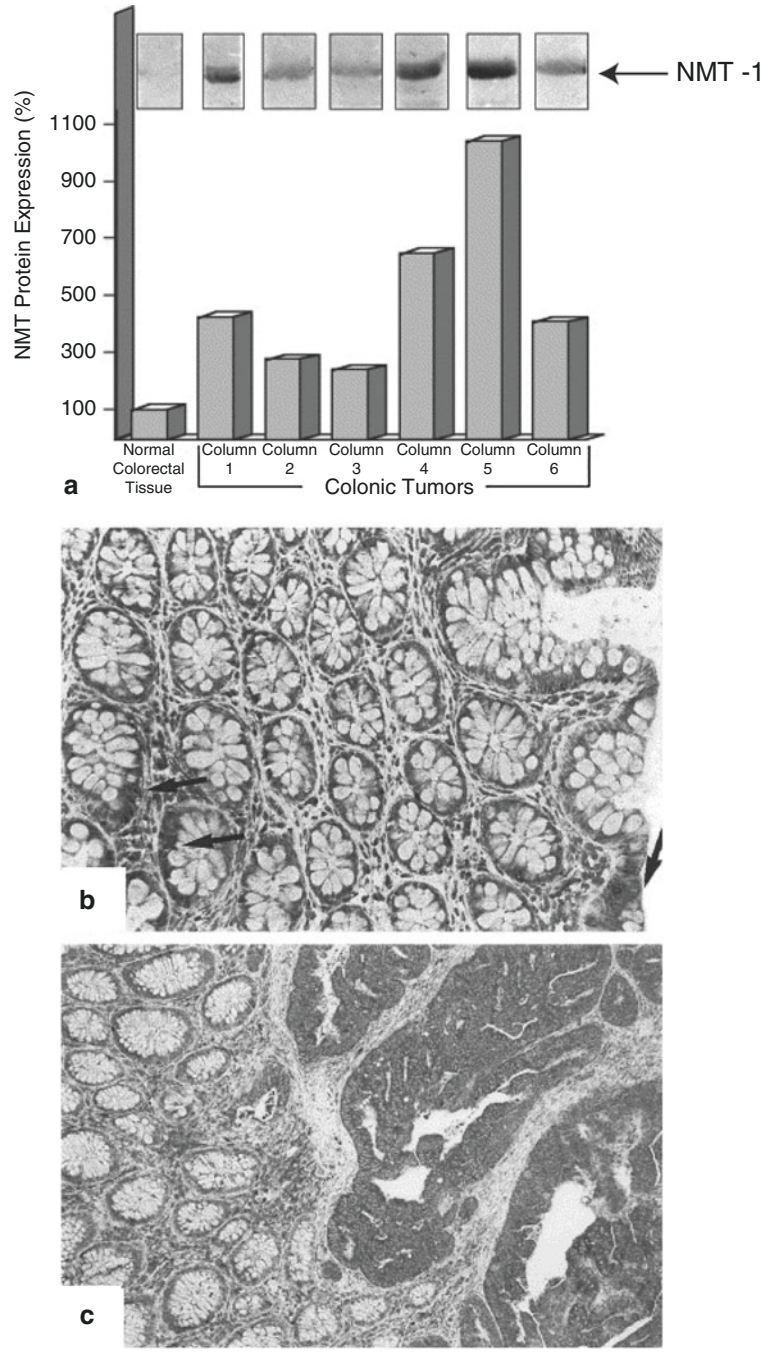
Since NMT is most abundant in the colon cancer tissue, a limitation of assessing the activity and protein expression of NMT for prognostic/diagnostic purposes is difficult because endoscopic biopsy must be performed to obtain the tumor tissue. An investigation of blood samples from colorectal cancer bearing rats and cancer patients in our laboratory showed that the NMT activity in the peripheral blood mononuclear cells (PBMC) of tumor bearing rats was significantly higher compared to PBMC control rats (Fig. 2) (Shrivastav et al. 2007b). NMT activity was also higher in the bone marrow of tumor bearing rats compared to normal bone marrow (Fig. 2). However, the highest activity was observed in the bone marrow macrophages of tumor-bearing rats (Fig. 2a). In addition, Western blot analysis studies revealed that there is overexpression of NMT-1 in the PBMC and in the bone marrow of tumor bearing rats compared to that of control rats (Fig. 2b).

Furthermore, immunohistochemical analysis revealed weak to negative staining for NMT-1 in PBMC of controls (Fig. 3a, b) whereas strong positivity was observed in the PBMC of colon cancer patients (Fig. 3c, d). We also observed that NMT-1 remained cytoplasmic in the control bone marrow mononuclear cells (Fig. 4a) whereas NMT-1 localized mostly in the nuclei of the bone marrow mononuclear cells of colon cancer patients (Fig. 4b). The difference in NMT-1 expression and its altered localization in the bone marrow of the tumor bearing host suggests that NMT-1 is a potential novel marker for diagnostic purposes.

Our laboratory had reported for the first time a high expression of NMT-2 in human colorectal tumors compared to normal tissues (Fig. 5a) (Selvakumar et al. 2006). The quantitative analysis demonstrated a significant increase in NMT-2 expression in human colorectal tumor tissues compared to normal mucosa (Fig. 5b). However, higher expression of NMT-2 was observed in polyps (Fig. 5, lanes P1 and P2). Furthermore, we observed the expression of NMT-2 in various human colon cancer cell lines (Colo320, SW480, SW620, HT29, DLDI, WiDr, HCT15, and HCT116). It is interesting that NMT-2 expression was higher in Colo320 cells compared to other cell lines (Fig. 5c). However, the expression of NMT-2 in HCT15 was poorly observed (Fig. 5c). These results indicate that the NMT-2 gene is upregulated during molecular events that take place during the malignant formation of colon cancer. The higher expression of NMT-2 was also reported in rat hepatoma cells by dioxin toxicity and the inducible level of NMT-2 was a direct consequence of Ah receptor activation [8] (Selvakumar et al. 2007).

Gallbladder Carcinoma

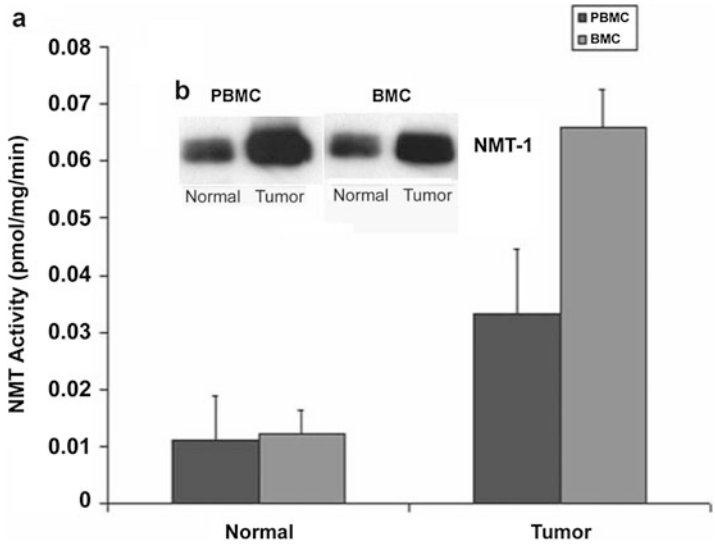
We reported for the first time that NMT-1 and NMT-2 protein expression is higher in colorectal adenocarcinomas than the adjacent nonmalignant mucosa (Raju et al. 1997; Shrivastav et al. 2007; Selvakumar et al. 2006). Then we extended our investigation on NMT expression in human gallbladder carcinoma (Rajala et al. 2000b). Gallbladder carcinoma is a rare yet often fatal cancer. Over 90% of gallbladder carcinomas are adenocarcinomas. Advanced local and regional disease is usually present at the time of diagnosis (Rajala et al. 2000b). When we analyzed gallbladder carcinoma cases, 60% of the gallbladder carcinomas demonstrated moderate to strong cytoplasmic positive for NMT in the invasive carcinoma with



NMT (*N*-Myristoyltransferase), Fig. 1 Western blot analysis of *N*-myristoyltransferase. (**a**) normal colorectal mucosa tissue and columns 1–6, colorectal tumor tissue samples; quantitative analysis of the NMT-1 protein band (see *arrow*) from Western blot was carried out using Imaging software (NIH at http://rsb.info.nih.gov/nih-image/download.html); (**b**) expression of NMT in colorectal adenocarcinoma in normal mucosa far removed from tumor showing a mild degree of focal staining (see *arrows*), and (**c**) transitional mucosa showing a mild to moderate degree of diffuse reactivity compared to the strong tumor reactivity on the right (Raju et al. 1997)

increased intensity in the invasive component, while 40% of the cases were observed as negative. The in situ component demonstrated that the cytoplasmic staining was from mild to moderate in 67% of the cases whereas normal gallbladder mucosa revealed weak to negative cytoplasmic staining (Fig. 6).

The increased expression of NMT in p53 mutant cases suggested that wild-type p53 may have a negative regulatory effect on NMT gene

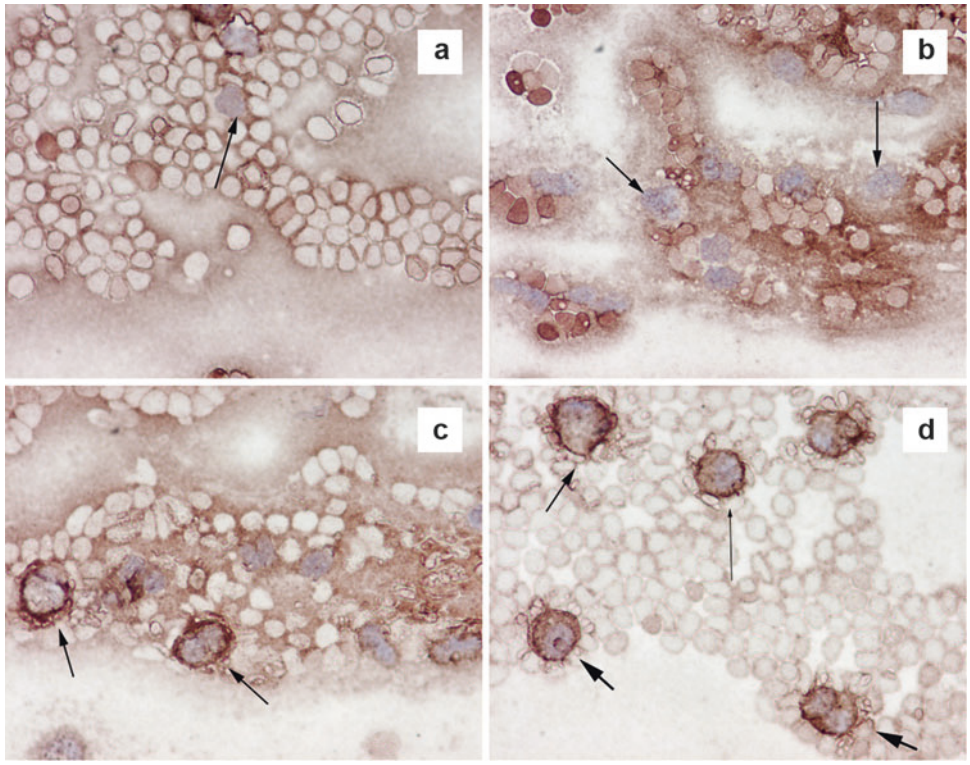


NMT (*N*-Myristoyltransferase), Fig. 2 *N*-myristoyltransferase activity in peripheral blood mononuclear cells (*PBMC*) and bone marrow cells (*BMC*) of normal and colorectal tumor bearing rats. (**a**) Isolated PBMC from peripheral blood of control or tumor bearing rat were assessed for NMT activity. Values are mean ± SD of three independent experiments. (**b**) Western blot analysis of PBMC and BMC of normal and colorectal tumor bearing rats (Shrivastav et al. 2007b)

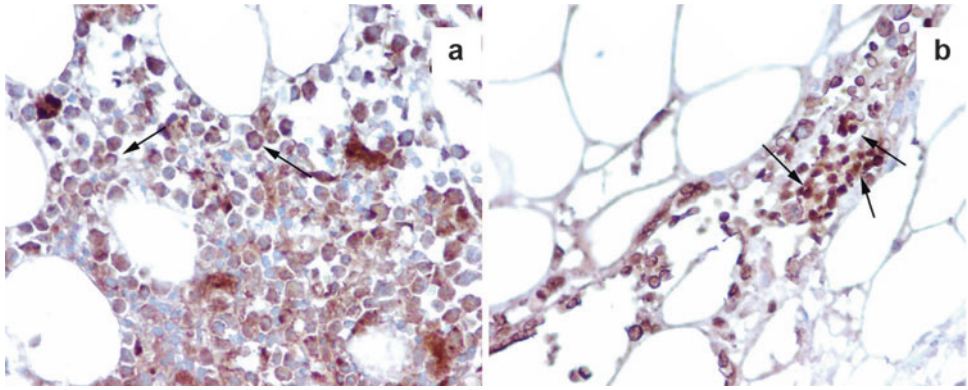
expression. NMT has been shown to be associated with a ribosomal subcellular fraction. The incidence of gallbladder carcinoma is higher in older age groups suggesting that hormonal imbalance may play a major role (Rajala et al. 2000b). Previous studies of prostate carcinoma indicate the greater abundance of NMT mRNA in hormone refractory cells than in hormone sensitive cells (Selvakumar et al. 2007). Src proteins of hormone sensitive cells were exclusively cytoplasmic, consistent with an absence of myristoylation, and suggested a regulatory role of hormones in NMT regulation (Selvakumar et al. 2007). Isolation of the NMT promoter and transfection studies in gallbladder cell lines would give a better understanding of the transcriptional regulation of NMT expression. Moderate to strong p53 staining was observed in 63% of the cases of the in situ components and 80% of the invasive components. Though the in situ staining of p53 was unrelated to the clinical outcome, moderate to strong staining of the invasive component as observed in 50% of the cases was associated with a mean survival time of 8.8 months. Our study shows that tumors with increased expression of p53 and NMT were associated with poor clinical outcomes as evidenced by their mean survival times. However, such a trend should be evaluated in larger numbers in order for this premise to be considered an independent prognostic marker. Since we observed a high expression of NMT in rat, human colon cancer, and human gallbladder cancer, we further investigated this enzyme expression in human brain tumors.

Brain Tumors

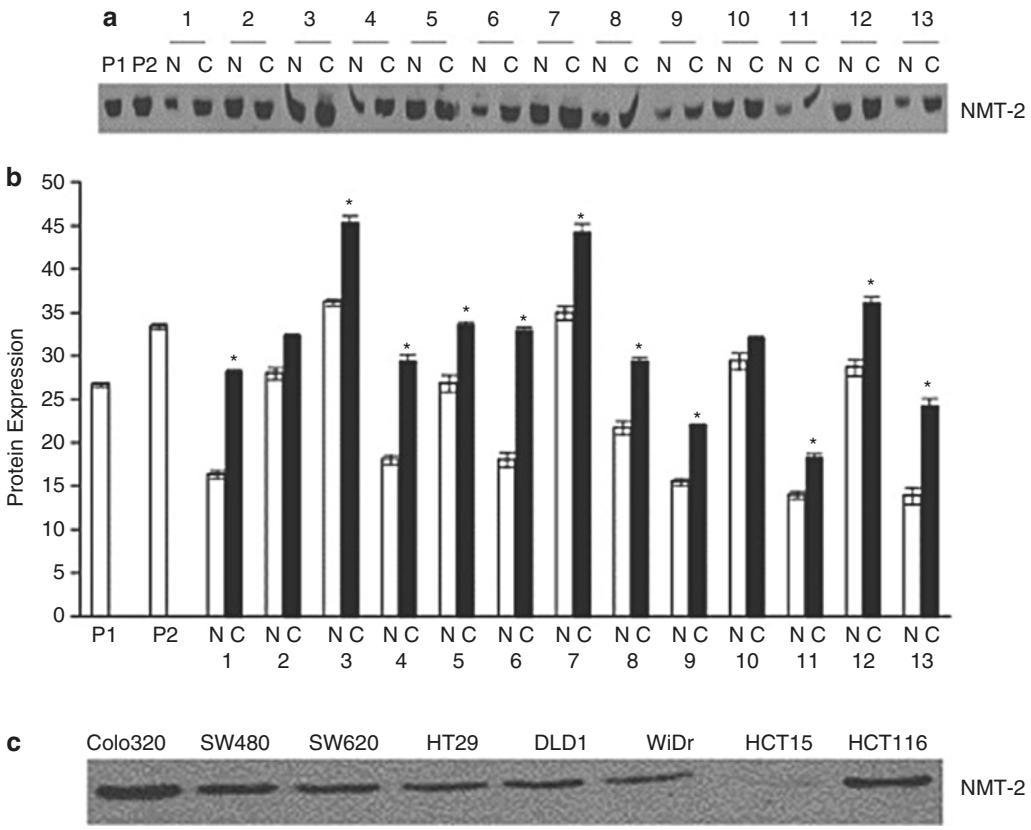
Brain tumors are the most common malignant solid tumor in childhood, and the incidence among adults is slightly higher in males than the females (~3.7 and ~2.6 per 100,000 males and females, respectively). The long-term disease free survival for children and adults with highly malignant brain tumors is poor. c-Src is a substrate for NMT and is known to be activated/overexpressed in various cancers and is increased in human malignant gliomas (Selvakumar et al. 2007). We reported for the first time a high NMT activity as well as protein expression of NMT-1 and NMT-2 in human brain tumors (Lu et al. 2005). Glial-derived neoplasms (gliomas) are the most



NMT (*N*-Myristoyltransferase), Fig. 3 Immunohistochemical analysis of PBMC of normal and colon cancer patients. (**a**) negative staining of lymphocytes (see *arrow*); (**b**) shows negative staining of monocytes (see *arrows*) in peripheral blood smear of control; (**c**) peripheral blood smear of colon cancer patients show positive staining of macrophages (*arrows*) and (**d**) peripheral blood smear of colon cancer patients show positive staining of neutrophil (*fat arrows*), lymphocyte (*lean long arrow*), and macrophages (*arrow*) (Shrivastav et al. 2007b)



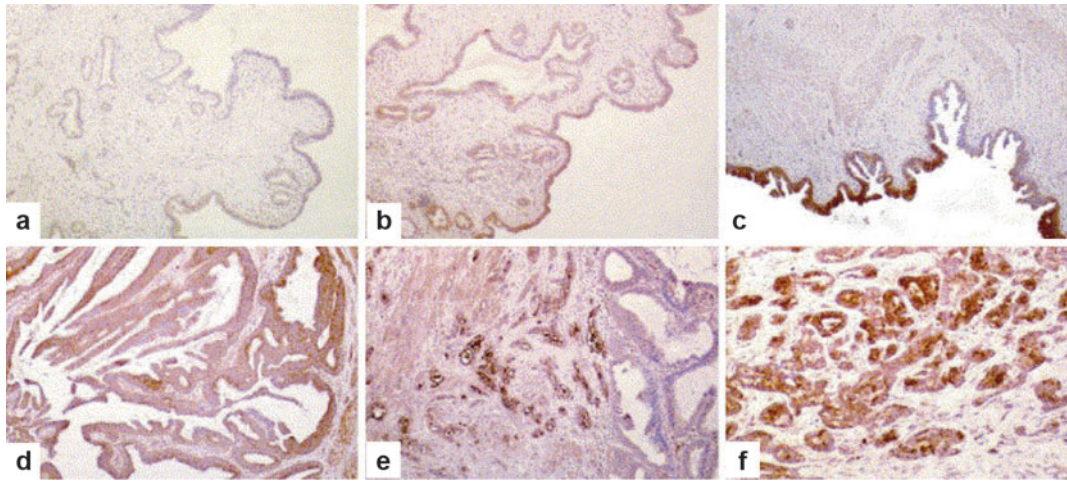
NMT (*N*-Myristoyltransferase), Fig. 4 Immunohistochemical analysis of bone marrow of normal and colon cancer patients. (**a**) NMT staining is mostly cytoplasmic in bone marrow of control (see *arrow*); (**b**) intense nuclear (and some cytoplasmic) staining for NMT is observed in the bone marrow of colon cancer patient (see *arrow*) (Shrivastav et al. 2007b)



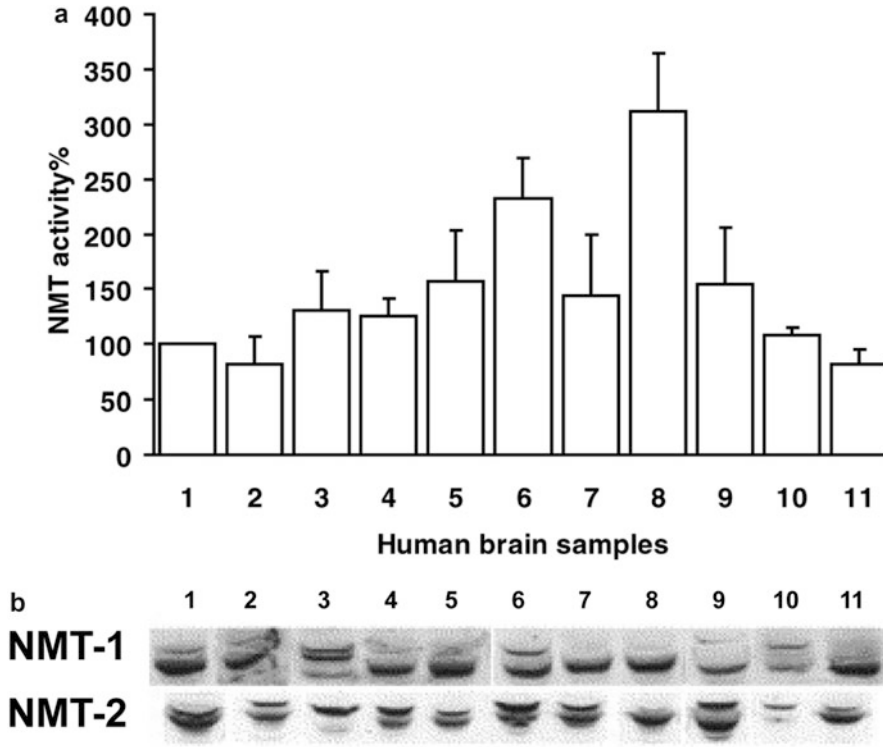
NMT (*N*-Myristoyltransferase), Fig. 5 Western blot analysis of *N*-myristoyltransferase-2 in human colorectal carcinoma. (**a**) Western blot analysis of human colorectal polyps (lanes P1, P2), normal (*N*), and tumor tissue (*C*). (**b**) Quantitation analysis of Western blot analysis was carried out using image software (NIH at http://rsb.info.nih.gov/nih-image/download.html). The data presented are representative of at least three separate experiments. Statistical significance was determined using Student *t* test analysis; $^{*}P < 0.05$. (**c**) Western blot analysis of various human colon cancer cell lines (Selvakumar et al. 2006)

aggressive brain tumor type, and account for 44% of all primary brain tumors. The most aggressive glial neoplasm is glioblastoma multiforme and this tumor type accounts for more than half of the primary brain tumors diagnosed in North America (Selvakumar et al. 2007; Lu et al. 2005).

At the present time, for diagnostic purposes, brain tumor sections stained with hematoxylin and eosin and an array of immunohistochemical stains using glial and neuronal marker proteins (glial fibrillary acidic protein, neuronal specific enolase, etc.) are scrutinized and categorized by light microscopy. Identification of tumor cells for diagnostic purposes is currently largely limited to light microscopy at the cellular level. Interestingly, we observed higher NMT activity in WHO grade 1 patients compared with others of higher WHO grades (Fig. 7a, lanes 5–9 vs lanes 10, 11). These observations were further supported by Western blotting using monoclonal antibodies against NMT-1 and NMT-2 and suggested both forms of NMT (NMT-1 and NMT-2) protein expressions were observed when compared to normal brain tissues (Fig. 7b). It revealed that these genes are upregulated as part of a series of molecular events that take place during carcinogenesis in the brain. Earlier it has been reported that the ubiquitous form of $pp60^{c\text{-}src}$ was expressed in malignant human glioma cell lines (Selvakumar et al. 2007). In anaplastic astrocytoma biopsy samples, focal adhesion kinase (FAK) is expressed and the activity of Src kinase is elevated as is the activity of Src kinase



NMT (*N*-Myristoyltransferase), Fig. 6 Expression of *N*-myristoyltransferase in gallbladder mucosa. (**a**) normal mucosa; (**b**) with weak positive staining; (**c**) carcinoma in situ with moderate staining; (**d**) papillary carcinoma with moderate staining; (**e**) invasive carcinoma with strong positive staining and negative overlying mucosa and (**f**) higher power of invasive carcinoma (Rajala et al. 2000b)



NMT (*N*-Myristoyltransferase), Fig. 7 *N*-myristoyltransferase activity and Western blot analysis of NMT in human brain tumor samples. (**a**) NMT activity; lanes 1–3, normal human brain tissue; lane 4, not WHO graded; lanes 5–9, WHO grade 1; lane 10, WHO grade 2; lane 11, WHO grade 4. The data were expressed as the mean ± SD of three samples in each group. (**b**) Western blot analysis of NMT-1 and NMT-2 in brain tumor tissue. Lanes 1–3, normal human brain tissue; lane 4, not WHO graded; lanes 5–9, WHO grade 1; lane 10, WHO grade 2; lane 11, WHO grade 4 (Lu et al. 2005)

associated with FAK (Selvakumar et al. 2007). C-yes (proto-oncogenepp62$^{c\text{-}yes}$) activities and protein levels were elevated in human melanoma and melanocyte cell lines (Selvakumar et al. 2007). Since these proteins were shown to be elevated in human glioma and melanoma cell lines (Selvakumar et al. 2007), it follows that N-myristoylation is required in order to facilitate the biological functions. This upregulation in gene expression suggests a role in the carcinogenic pathway as well as a possible therapeutic target for future study.

Oral Squamous Cell Carcinoma

Currently, oral squamous cell carcinoma (OSCC) is the most prevalent malignant neoplasm of the head and neck region. The recent increases in these numbers are attributed to the human papilloma virus (types 16 and 18) (Selvakumar et al. 2007), associated cancers of the tonsils and base of the tongue. Since increased expression and activity of NMT in various cancers suggest its involvement in carcinogenesis, we investigated the presence of NMT in oral cancer. Previously, it had been reported that NMT activity is found to be 2.5 fold greater in cancerous tissues over normal tissue samples from the same patient (Shrivastav et al. 2007a). In the same study, NMT was found in nuclear and cytoplasmic localizations in tumor samples compared to normal samples where the enzyme is primarily cytoplasmic (Shrivastav et al. 2007a). It is known that NMT will redistribute to the nucleus in ischemic cardiac cells under stress (Rajala et al. 2002). Epithelial cells involved in OSCC undergo a similar stress response with increasing tumor load, possibly providing an explanation for nuclear-bound NMT (Shrivastav et al. 2007a). The increased NMT activity could be due to the increased demand for myristoylation of oncoproteins and other diverse proteins involved in the multistep process of oncogenesis (Selvakumar et al. 2007). Myristoylated tyrosine kinases pp60$^{c\text{-}src}$ and pp60$^{c\text{-}yes}$ are known to be elevated in carcinomas of the colon, possibly creating an increased demand for NMT (Selvakumar et al. 2007). Secondly, atypical myristoylation of proteins which are not normally myristoylated could occur as a result of neoplastic changes (Selvakumar et al. 2007). This has been demonstrated with the protein p21 Ras, which results in transformation activity or with H-Ras and K-Ras where cellular localization and MAP kinase activation are affected (Selvakumar et al. 2007). It is still unknown how early overexpression of NMT occurs in OSCC but there is the potential for a role as a biomarker for diagnosis and the possibility as a targeted therapeutic agent.

Breast Cancer

A correlation has been established between NMT activity in mammary epithelial cells and proliferative ability (Clegg et al. 1999). Immunohistochemical analysis of grades 1, 2, and 3 ductal carcinomas of the breast cancer tissue array display a stepwise increase in staining (Shrivastav et al. 2009). While these tissues showed strong staining, normal breast tissue displays low or negative staining (Shrivastav et al. 2009). Furthermore, immunohistochemical analysis was carried out in breast cancer patients and revealed NMT positivity for malignant breast tumor whereas no or low NMT staining was observed in normal breast tissue (Table 3). Src, a substrate for *N*-myristoylation, has been observed to be 4–20 folds higher in breast cancer tissue when compared to controls (Selvakumar et al. 2007). Therefore it is possible that the overexpression of NMT is due to the high demand of the myristoylation of Src. NMT could prove to have overlapping functions and NMT-1 is critical for tumor cell proliferation suggesting that isoform-specific inhibitors could be a viable biomarker in the diagnosis of breast cancer.

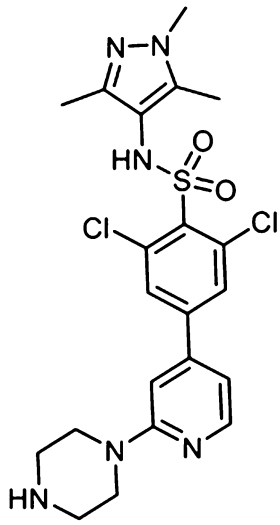
NMT Inhibitors as Cytotoxic Agents

Anticancer properties of various structurally diverse NMT inhibitors have been reviewed (Das et al. 2012). Among them, *N*-heterocyclic benzenesulphonamides have shown remarkable NMT inhibitory as well as anticancer properties. Recently a selective NMT inhibitor (Selvakumar et al. 2007) which was originally discovered as

NMT (*N*-Myristoyltransferase), Table 3 Summary of NMT positivity in female breast cancer (Shrivastav et al. 2009)

Age (years)	Pathology diagnosis	Grade	Type	Positivity (%)[a]	Intensity[a]	ER[b]
60	IDC NOS[c]	II	Malignant	>75	Strong	_
40	IDC NOS	I	Malignant	15–20	Moderate	+
52	IDC NOS	I	Malignant	35–50	Strong	_
38	IDC NOS	II	Malignant	>75	Strong	_
51	IDC NOS	II	Malignant	>75	Moderate	_
64	IDC NOS	III	Malignant	>75	Strong	_
48	IDC NOS	II	Malignant	>75	Strong	+
38	Medullary carcinoma	_	Malignant	50–75	Strong	_
49	Medullary carcinoma	_	Malignant	15–20	Weak	+
58	Mucinous adenocarcinoma	_	Malignant	50–75	Strong	_
54	Apocrine carcinoma	_	Malignant	35–50	Strong	_
60	Fibro-fatty tissue of No 01		Normal	_	Negative	_
38	Breast tissue of No 36	_	Normal	_	Negative	_
58	Breast tissue of No 38	_	Normal	5–10	Weak	+
54	Breast tissue of No 40	_	Normal	_	_	+

[a]NMT
[b]Estrogen receptor staining (*ER*)
[c]*IDC NOS* infiltrating ductal carcinoma, not otherwise specified. Immunohistochemical staining results were evaluated in a semiquantitative manner as follows: number of breast cancer cells positive: rare (1–5); 5–10%; 15–25%; 25–35%; 35–50%; 50–75%; >75%. Staining intensity was evaluated as negative, weak, moderate, or strong. Grade I or well-differentiated: cells appear normal and are not growing rapidly. Grade II or moderately differentiated: cells appear slightly different from normal. Grade III or poorly differentiated: cells appear abnormal and tend to grow and spread more aggressively (Shrivastav et al. 2009)



NMT (*N*-Myristoyltransferase), Fig. 8 The structure of NMT inhibitor showing potent cytotoxic properties

an inhibitor of *Trypanosoma brucei* NMT demonstrated dose-dependent inhibition of *N*-myristoylation that is cytotoxic in a time-dependent manner in HeLa cells (Fig. 8) (Thinon et al. 2016). Inhibition of *N*-myristoylation had resulted in a complete killing of HeLa cells. NMT inhibition in HeLa cells show that cells die through apoptosis following or concurrent with accumulation in the G1 phase. In this study, a quantitative proteomics analysis to map protein expression changes for more than 2700 proteins in response to treatment with the NMT inhibitor in HeLa cells showed that downregulation of proteins involved in cell cycle regulation and upregulation of proteins involved in the endoplasmic reticulum stress and unfolded protein response, with similar results in breast (MCF-7, MDA-MB-231) and colon (HCT116) cancer cell lines.

Summary

Protein myristoylation is a key biochemical process which plays an important role in the functioning of many cell proteins. Our extensive studies on NMT activity and expression in different human cancers reveal that NMT is over

expressed in cancer cells compared to non-malignant cells. These results demonstrate the role and significance of protein myristoylation in various cancers and had triggered our interests in developing NMT as a diagnostic marker. NMT inhibitors have shown potent anticancer properties which warrant their potential as future anticancer drugs. In the light of these results, further studies are required to examine a wide range of other tumors and the corresponding normal tissues for NMT activity to support the role of NMT in tumor progression. Secondly, indepth investigation of the role of NMT-1 and NMT-2 in tumorigenesis will contribute to further understanding of the mechanisms of tumor progression. In conclusion, the development of NMT inhibitors as future anticancer drugs warrants substantial potential which needs further research.

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N-Myc and STAT Interactor

► N-Myc and STAT Interactor (NMI)

N-Myc and STAT Interactor (NMI)

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Synonyms

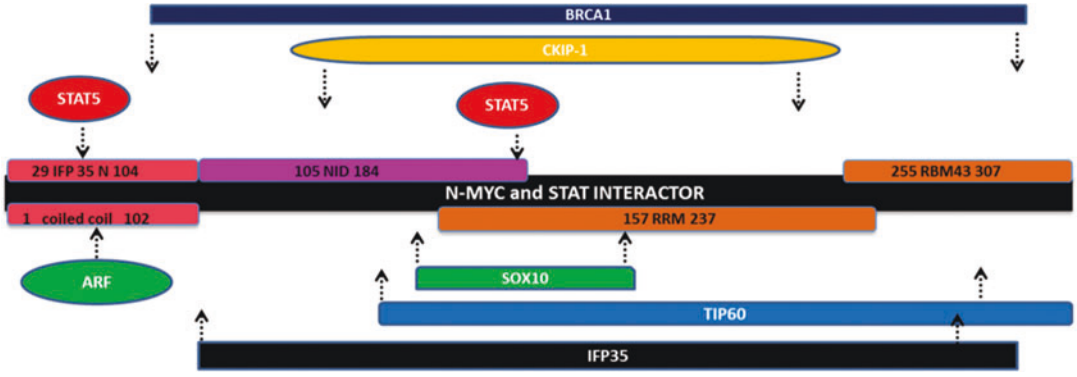
N-myc and STAT interactor; N-myc interactor; NMI

Historical Background

N-myc interactor, NMI, was first characterized as an interactor of c-Myc and n-Myc using a yeast two-hybrid screen. Since its initial discovery, NMI has been studied for its roles in viral pathologies and more extensively for its role in cancer progression. However, literature describing the role of NMI function in normal physiology is relatively unknown aside from inferences from its roles in various pathologies. NMI is a protein mostly studied for its associations with known transcription factors. In humans, expression profiling has determined that NMI is expressed in all fetal tissues except in the brain and primarily in the adult spleen, liver, and kidneys. The human NMI gene is located on chromosome 2q23 and contains three exons that can form four alternatively spliced mRNA transcripts; two with no retained introns code for protein products (Bao and Zervos 1996). NMI's expression is largely cytoplasmic; however, it has been detected in the nucleus by multiple studies. Many studies have detailed a growing list of known interactors. As such, the 38 kDa protein has been proposed to be an adapter molecule with different functions depending on cellular context. These protein-protein interactions occur across all functional domains of NMI. The first hundred amino acids were initially identified to comprise a coiled-coil domain with homology to the *C. elegans* protein CEF59; more recently the N-terminal sequence of NMI is predicted to contain a leucine zipper motif in an alpha-helical configuration similar to that of IFP35 (interferon-inducible protein 35). The NID domain is referred to as an NMI/IFP35 (NID) domain for its homology to IFP35 which is important for protein-protein interactions and cellular localization. The most recent predicted domains are an RNA recognition motif RRM_NMI as well as the RRM_SF, both of which are a part of the RNA-binding motif superfamily (Fig. 1).

NMI Interactors

NMI effects on cellular function seem to be a result of its various interactions with other molecules most of which are transcription factors. The first evidence of this function was discovered by NMI interaction studies demonstrating that NMI binds with all STATs besides STAT2 and phosphorylated or active STAT5b. Furthermore, studies detailing the promoter activity of STAT5 or STAT1 target genes have been shown to be greatly enhanced with NMI interaction (Zhu et al. 1999). Additionally, histone acetyltransferases CBP and p300 have also been implicated as additional binding partners, such that NMI may enhance the interaction between STATs and CBP/p300 (Zhu et al. 1999). NMI was shown to interact with another epigenetic writer, the acetyltransferase Tip60, through multiple domains including its unique NID domain (Zhang et al. 2007). NMI has also been found to be associated with p65 which enhances interaction between p65 and HDACs thereby inhibiting acetylation of p65 and down-regulating p65 transcriptional activity (Hou et al. 2016). Moreover, recent studies have further demonstrated NMI as transcriptional regulator of STAT signaling mediated through interactions with



N-Myc and STAT Interactor (NMI), Fig. 1 NMI has multiple functional domains reported to be important for its function. There is an N-terminal domain which has been termed as both the coiled-coil domain and IFP35 domain with highly similar homology to IFP35. The NID domain is thought to be important in protein-protein interaction and may be involved in determining cellular localization. Lastly, there are two putative RNA-binding motifs which have yet to be fully characterized. Multiple studies have determined a variety of NMI-interacting proteins most of which have been shown to influence a variety of cellular functions particularly in the context of cancer

heat shock family member Hsp105B. Interaction between NMI and Hsp105B enhanced expression of Hsp70 in a manner dependent on STAT3 (Saito et al. 2014). These studies suggest that NMI recruits epigenetic modifiers and facilitates their binding with transcription factors to enhance transcription of downstream target genes.

Additional evidence for NMI's involvement in modulation of transcription was defined by its interaction in a novel tricomplex between NMI, c-myc, and BRCA1. BRCA1 is essential for maintaining genome stability via its vital roles in DNA double-strand break repair by homologous recombination. BRCA1 is also known to transcriptionally repress another interactor of NMI, MYC. It was demonstrated that alone BRCA1 or NMI did not alter c-Myc transcriptional activation of telomerase reverse transcriptase expression; yet through sequestration of MYC, the complex effectively decreased the transcriptional activity of hTERT (Li et al. 2002).

The discovery of Sox10 protein as an NMI-binding partner has further demonstrated the importance of NMI as a modulator of transcription factor activity. Sox10 is well-known for its role in neural crest development and was shown to colocalize with NMI in the nucleus of glioma cell lines. The Sox family of transcription factors is evolutionarily conserved and classified by their HMG box which contains a DNA-binding and DNA-bending domain. They are essential for embryonic developmental processes and control many cell fate decisions including programming of embryonic stem cells. NMI was unable to change Sox10 localization; however, the study showed that NMI increased Sox-mediated transcription of its target gene myelin protein zero. Yet, the activity of the dopachrome tautomerase promoter, a second Sox10 target gene that contributes to pigment production in melanocytes, was unchanged by NMI. This suggests that NMI does not act as a global cofactor for transcriptional upregulation, but that genomic context as well as other factors may play a role in dictating NMI's effect on transcription. Moreover, it is more probable that NMI functions as a part of a large protein complex to elicit these functions in combination with Sox10 as suggested by previous studies with BRCA1 and c-Myc (Schlierf et al. 2005).

As the name suggests, NMI binds to more than just STATs. NMI was discovered in a yeast two-hybrid screen using the bHLH region of NMYC as bait. Further investigation has shown that the bHLH region of NMYC is not necessary for interaction with NMI, and the regions that bind between NMYC and CMYC differ. NMI binds to the DNA-binding region of MYC, while it binds to the acidic central region of the MYCN protein (Bao and Zervos 1996; Bannasch et al. 1999).

Interaction studies have continually demonstrated the role of NMI in stabilization of protein partners within the cytoplasm. NMI was able to block proteasomal degradation of ARF by binding ARF at the same location necessary for ubiquitin ligase interaction. Furthermore, knocking down NMI led to destabilization of ARF. NMI induction upon cellular stress with IFN alpha, cisplatin, or methylmethanesulfate led to its nuclear translocation and stabilization of ARF. NMI overexpression in these cells induced CHK2 activation and G2/M cell cycle arrest especially after DNA damage (Li et al. 2012). In addition to ARF, NMI stabilizes the IFP35 protein through interaction. The N-terminal coiled-coil domain of NMI is necessary, but not sufficient to inhibit proteasomal degradation of IFP35. IFP35 in turn was able to stabilize NMI expression; however, the effect on IFP35 by NMI was more pronounced (Zhou et al. 2000).

Regulation of NMI

Early studies identified that NMI is regulated at the transcriptional level through induction downstream of inflammatory cytokines IFNγ, IFNα, and IL-2. Downstream mediators such as STAT1 and STAT5 are most likely responsible for binding the NMI promoter; furthermore, analysis of the ENCODE data in the USCS portal reveals putative STAT regulatory elements in the NMI sequence. It is interesting that NMI in turn facilitates the activity of these factors allowing for a robust response after cytokine stimulation. Interferon treatment results in high molecular mass complex (HMMC) formation of 300–400 kDa, visualized as NMI/IFP35 speckles (NIS), facilitated by the dephosphorylation of IFP35. Other interactors of NMI such as C-Myc, N-Myc, and c-fos are not thought to be contained in the HMMC (Zhou et al. 2000). Molecular analysis confirmed that the fractionation properties of NMI change after treatment with IFNγ, indicating a shift in subcellular location. Multiple groups have shown that induction of NMI leads to its translocation into the nucleus allowing it to impact STAT-mediated transcription. However, other investigators have found that induction of NMI using cytokines leads to reorganization of NMI protein into puncta within the cytoplasm. Ets-1 was also shown to be an upstream regulator of NMI; it increased mRNA and protein of NMI when expressed in MCF-7 breast cancer cells. The Ets family of transcription factors are highly conserved among species and have documented roles in physiologic and pathologic processes (Jung et al. 2005). Although the Ets motif exists in the NMI gene sequence, the mechanism of upregulation has not been extensively explored; it is likely that it is at the transcriptional level because NMI mRNA expression increased. The study also did not confirm whether Ets-1 directly stimulated expression of NMI; therefore, it is possible that Ets-1 induces the expression of some other factor that in turn upregulates NMI.

Expression profiles of NMI have been most widely studied in the context of disease, namely, cancer. To date studies of NMI have not outlined genetic alterations such as deletions or mutations responsible for changes in expression. Most notably, NMI expression is significantly decreased in late stage breast cancer suggesting a transient down-regulation of NMI at the transcript level (Devine et al. 2014). Additionally, NMI expression both at the transcript and protein level has also been shown to be inversely correlated with disease progression in gastric cancers (Hou et al. 2016). Conversely, studies in glioblastoma utilizing three independent data cohorts suggest elevated expression of NMI was linked to poor prognosis and survival. Furthermore, this study was the first to suggest a possible epigenetic regulation of NMI transcript; however, detailed analysis was not preformed to address this possibility (Meng et al. 2015). Studies aimed to specifically address the regulatory networks responsible for modulation of NMI expression demonstrated posttranscriptional regulation of NMI by the micro-RNA family 29 in breast cancer cell lines as well as clinical samples. microRNAs are small noncoding RNAs that target a wide range of mRNA three prime untranslated regions resulting in inhibited translation or mRNA degradation. MiR-29 targeting of the NMI phenocopies previously reports

demonstrating various effects on downstream effector pathways (Rostas et al. 2014).

Posttranslational regulation of the NMI protein has not been extensively investigated. The only study to report proteasomal degradation of the NMI protein found that interaction with the severe acute respiratory syndrome coronavirus protein P6 led to ubiquitination of NMI and subsequent degradation (Cheng 2015). NMI is a relatively labile protein with a half-life of approximately 8 h, and this half-life may be extended through interaction with a variety of factors. Zhang and colleagues reported that interaction between Tip60 and NMI in the cytoplasm led to stabilization of the NMI protein. Interaction of the two proteins was not dependent on acyltransferase activity; however, the mechanism of stabilization remains to be discovered as they were unable to show that Tip60 acetylated NMI. They did however show that a different acetyltransferase, p300, acetylated NMI leading to increased levels of expression (Zhang et al. 2007).

Functions in Immunology and Viral Response

It is intuitive that NMI plays a role in the host's viral response based on its ability to influence JAK/STAT signaling as well as the fact that various cytokines such as IFNγ, IFNα, and IL-2 influence expression of NMI. NMI has been proposed to facilitate apoptosis through interaction with FMDV 2C, a key foot-and-mouth disease viral protein in swine. The formation of protein complexes was dependent on expression of NMI whereby both proteins colocalize within the endoplasmic reticulum. Furthermore, host cell apoptosis was shown to be dependent on NMI expression in FMDV-infected cells (Wang et al. 2012). In other viral studies, NMI was shown to regulate production of interferon in response to viral-mediated upregulation of NMI. In these studies, modulation of NMI expression had an inverse effect on interferon production, and Nmi transgenic mice displayed impaired induction of interferon upon viral infection. IRF7, which is a key mediator of this response, was also shown to interact with NMI causing degradation of IRF7 leading to the decreased production of interferon (Wang et al. 2013). Severe acute respiratory syndrome coronavirus protein 6 has also been shown to interact with NMI and mediate proteosomal degradation of NMI thereby halting interferon production and promoting viral survival. Irrespective of effects on interferon signaling, NMI is also able to directly inhibit prototype foamy virus replication. NMI was found to interact with the regulatory protein Tas and sequester it in the cytoplasm inhibiting transactivation of prototype foamy virus' long terminal repeat and internal promoter (Hu et al. 2014). Collectively, these limited studies have suggested an important role for NMI in viral immune response.

NMI Modulation of Cancer Signaling

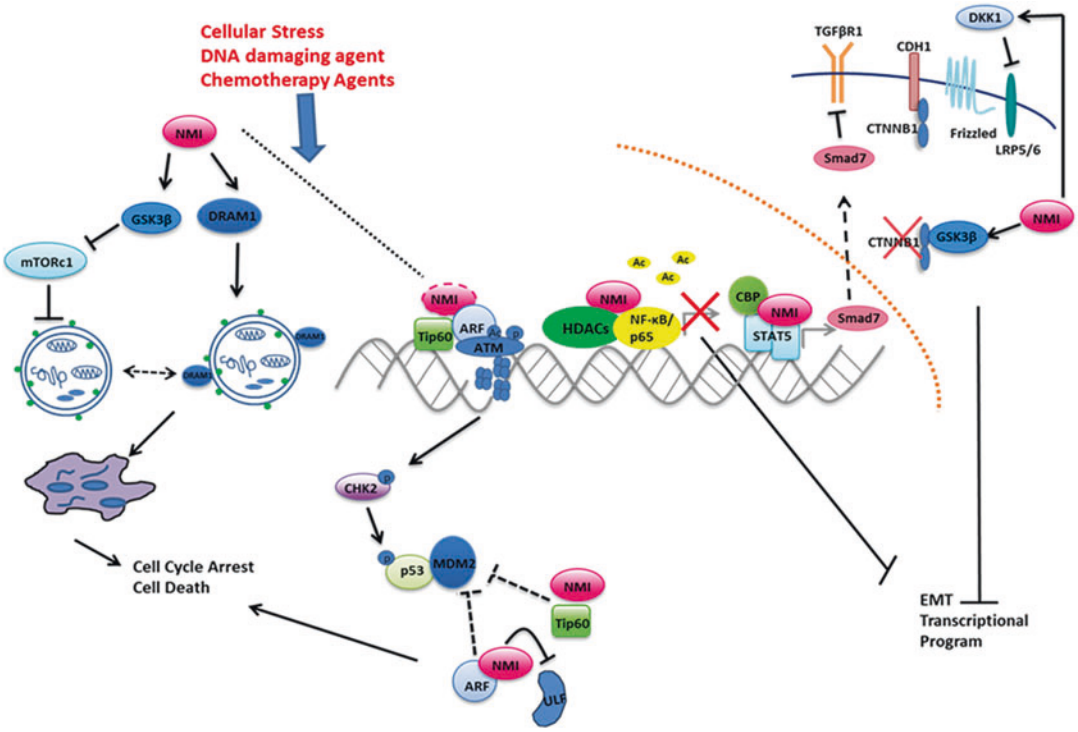
Although studies of NMI in the context of normal physiology have been very limited, a wider scope of studies from the perspective of cancer has elucidated various roles of NMI in cancer signaling. The role of NMI in modulating the activity of oncogenic transcription factors suggests that NMI is an important protein in cancer development. Some of the first evidence for NMIs involvement in cancer was defined by its interaction in a novel tricomplex between NMI, c-myc, and BRCA1. Through sequestration of MYC, the complex effectively decreased telomerase reverse transcriptase expression, a gene essential for immortalization of cancer cells (Li et al. 2002). A number of studies have been aimed at elucidating the role of NMI in breast cancer. Surveys of breast tumor specimens from the TCGA show little copy number variation or mutation of NMI; however, some independent studies have identified SNPs in the NMI gene present in patient breast cancer as well as ovarian cancer specimens. NMI protein level has been reported to be significantly reduced with increasing stage of breast cancer primary tumors with the lowest expression being seen in patients with metastatic disease. Moreover, NMI mRNA levels decrease with the grade of clinical breast cancer specimens suggesting that loss of NMI facilitates

progression to aggressive metastatic disease (Devine et al. 2014).

Metastasis of epithelial cancers is a complex process that employs epithelial-mesenchymal transition (EMT), a developmental phenomenon whereby epithelial cells undergo a change in phenotype allowing them to lose their canonical morphology and invade into surrounding tissue. Silencing NMI in breast cancer cells with an inherent epithelial phenotype causes a morphological change concomitant with increased expression of master EMT regulators ZEB2 and SLUG and subsequent change in EMT marker profiles such as E-cadherin and keratin-18. NMI antagonized TGFβ signaling, a pathway known to promote EMT, through the upregulation of the inhibitory Smad, Smad7. Through enhancing STAT5-mediated transcription, NMI increased the levels of Smad7 and reversed mesenchymal phenotypes in breast cancer cells (Devine et al. 2014). Negative regulation of EMT in cancer progression via NMI was further solidified by separate work detailing suppression of the NFkB signaling pathway in gastric cancer. NMI expression was found to augment the EMT process in gastric cancer as previously described in breast cancer; however, mechanistically, NMI was found to interact with p65 attenuating NFkB signaling. Exhaustive studies have clearly defined a major role for NFkB in cancer progression and EMT. NFkB signaling is mediated through translocation of p65 to the nucleus where it functions as a transcription factor. Furthermore, posttranslational modification of p65 is paramount in controlling its transcriptional activity either through differential phosphorylation or acetylation. NMI expression was found to enhance the interaction between p65 and HDACs resulting in decreased acetylation of p65 blocking transcriptional activity ultimately blocking the EMT process (Hou et al. 2016). Together, these studies define a prominent role for NMI as a master regulator of the EMT process via a complex signaling network (Fig. 2).

NMI has been implicated as key regulatory node in the signaling pathways which are major drivers of tumor progression. Some of the earliest reports demonstrated the effect of NMI on Wnt signaling in breast tumor growth. DKK1, the secreted inhibitor of Wnt signaling, was shown to be significantly upregulated by increasing expression of NMI (Fillmore et al. 2009). Independently, DKK1 has been shown to restrict tumor growth in breast cancer. Wnt signaling has roles in the normal physiology of breast development and maturation, but is also seen to be dysregulated in breast cancer. This pathway begins at the cell surface, where Wnt ligands bind to a member of the Frizzled family of receptors and either the LRP5 or LRP6 co-receptor. This tricomplex formation leads to the stabilization of cytosolic β-catenin, a transcriptional cofactor that can enter the nucleus, bind to a member of the TCF/LEF protein family, and subsequently activate transcription of Wnt target genes (Fig. 2). β-catenin accumulation is seen in breast carcinomas and is associated with poor prognosis. In breast cancer, deregulation of the Wnt pathway is not due to mutation of the intracellular components as in colorectal cancers. Instead, many breast cancers are augmented at other levels of signaling such as overexpression of LRP6 co-receptor and the FDZ7 receptor, upregulation of secreted Wnt ligands, and silencing of Wnt inhibitors SFRP-1 and WIF1. Re-expression of NMI in low-expressing breast cancer cell lines lead to increased levels of DKK1 and concomitantly reduced levels of beta-catenin and MYC, known downstream targets of Wnt signaling. In the same study, re-expression of NMI in tumor xenograft models reduced tumor growth *in vitro* and *in vivo* as well as invasion and migration of tumor cells (Fillmore et al. 2009). NMI has a multifaceted effect on the Wnt signaling pathway through another interactor, casein kinase 2-interacting protein-1 (CKIP-1 or PLEKHO1). CKIP-1 is also able to interact with casein kinase 2, a protein with noteworthy involvement in the canonical Wnt signaling pathway. CK-2 phosphorylates and inhibits Dishevelled, a negative regulator of Wnt signaling, and also phosphorylates and stabilizes β-catenin, which ultimately enhances the transcription of Wnt target genes. How CKIP-1 affects CK-2 and whether its interaction with NMI directly influences Wnt signaling are questions that remain unanswered.

Early studies mostly examined characteristics of NMI in hematologic cells; however, a consistent



N-Myc and STAT Interactor (NMI), Fig. 2 NMI has broad cellular functions that impact multiple cell signaling pathways and functions. Most of NMI functional impacts have been studied in the context of cancer progression. NMI functions have been linked to multiple signaling pathways, such as Wnt, TGFb, mTOR, and NFkB. NMI has been reported to activate GSK3B and inhibit mTOR signaling as well as upregulate DRAM1, both of which induce autophagic cell death. Furthermore, interactions with multiple regulators of the p53/MDM2/ARF pathway allow NMI to functionally impact cell cycle arrest. NMI is also a master regulator of the EMT program through multiple signaling nodes such as NF-KB, Wnt, and TGFβ. NMI interacts with proteins that involved at key regulatory nodes in each of these pathways thereby modifying their functions and ultimately mediating effects on cell death, cell cycle arrest, EMT, and ultimately cancer progression

functional role for NMI in hematologic malignancies has not been identified as of yet. One study discovered that interaction between STAT5, NMI, and N-myc mediates repression of MEF2C leading to increased apoptosis in T-cell acute lymphoblastic leukemia (Nagel et al. 2011). Others have identified the apoptosis-inducing protein, apoptin, as yet another interactor of Nmi from a human leukocyte library. Apoptin is encoded by the chicken anemia virus which infects hematopoietic cells in young chickens leading to anemia and eventually cell death. Interestingly, apoptin was found to selectively induce apoptosis in tumorigenic cell lines but not non-transformed non-tumorigenic cells. It was observed that apoptin localized to the nucleus in malignant cells but remained cytoplasmic in normal cells (Nagel et al. 2011). Nuclear localization, through its intrinsic NLS, of apoptin allows it to interact with the anaphase-promoting complex/cyclosome, causing G2/M cell cycle arrest and apoptosis. Although the nature of the interaction between NMI and apoptin is not well understood, the observation suggests that NMI may play a role in tumor-specific apoptosis.

In contrast to the findings in epithelial cancers, NMI expression in gliomas indicates poor patient prognosis and has been found by one group to independently predict overall survival and progression-free survival. NMI mRNA as well as protein was most highly expressed in stage 4 glioblastoma multiforme, the most common advanced stage of glioma. NMI does not increase in expression with grade; however, GBM has significantly higher expression when compared to normal

controls. *In vitro* and in xenografts, the study found that NMI impacts GBM cell line proliferation through its effect on G0/G1 cell cycle arrest, and this effect was proposed to be due to NMI binding with STAT1 (Meng et al. 2015). However, it is unclear whether NMI is a driving force behind GBM progression or it is a passenger that provides a good prognostic indicator.

Most recently studies have aimed to examine a role for NMI in mediating cell death in response to chemotherapy in breast cancer. Re-expression of NMI sensitized breast cancer cell lines to DNA-damaging agents such as doxorubicin and cisplatin. Moreover, the role of NMI in sensitization to chemotherapeutic agents was determined to be via induction of autophagy, otherwise termed programmed cell death II. NMI expression in breast cancer cell lines induced autophagic vacuole formation indicative of elevated levels of basal autophagy as determined through enhanced LC3 processing and increased Beclin and p62 expression. Functionally, NMI regulates autophagy by activation of GSK3β which downstream mediates inhibition of mTOR signaling, a known inhibitor of autophagy. In addition to inhibition of mTOR signaling, NMI influences autophagic cell death through upregulation of a vital autophagy component, DRAM1. Silencing of NMI in cancer cell lines was found to dramatically decrease the expression of DRAM1. Additionally, cells silenced for NMI were not able to upregulate DRAM1 in response to cytotoxic stress with cisplatin. Furthermore, knockdown of DRAM1 attenuates the effect of NMI on autophagic cell death mediated in response to chemotherapeutic agents (Fig. 2) (Metge et al. 2015).

Overall, NMI appears to play an essential role in progression or development of a variety of cancers. Loss of NMI in epithelial cancers such as breast cancer leads to unrestricted TGFβ and Wnt signaling pathways possibly through decreased activity of STAT5 and STAT1, respectively, causing increased invasiveness, growth, and potentially metastasis. Conversely, in cancers that arise from cell types of mesenchymal origin, such as glioblastoma, NMI expression seems to correlate with aggressiveness of disease.

Summary

NMI is an inducible protein that can be regulated by a variety of cytokines. Initially, described as an interactor of c-Myc and N-Myc as well as STAT, a broader role for NMI in cellular signaling has emerged. Most notably NMI has been found to be important in viral pathologies as well as in cancer. NMI appears to play a prominent role in regulation of multiple cell signaling pathways (Wnt, TGFb, mTOR, NFkB) but also in transcriptional regulation, not as a transcription factor itself, but through its vast protein-protein interaction complexes. Although the most extensive body of work has been done from the perspective of cancer biology, it has become increasingly clear that NMI function spans multiple signaling networks and cellular processes. Further studies are needed to unravel the complex cross talk between the various signaling pathways that are modulated by NMI. It will be of continued interest to further studies on this diverse molecule due to its multifaceted involvement in cancer progression and other relevant diseases.

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N-Myc Interactor

► N-Myc and STAT Interactor (NMI)

N-Myristoyl Transferase

► NMT (*N*-Myristoyltransferase)

Nociceptin Opioid Receptor (NOP)

► Opioid Receptor

Nonlysosomal Ceramidase

► Neutral Ceramidase

Nonlysosomal Cysteine Protease

► Calpain

Nonmuscle Filamin

► Filamin A

Nonmuscle Myosin II

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Synonyms

Heavy chain: NMMHC-II; MHCII; zipper (in *Drosophila*). Three isoforms are expressed in mammalian cells, also known as: (1) MHCII-A;

myosin-9= MHA= FTNS= EPSTS= BDPLT6= DFNA17= NMMHCA= NMHC-II-A= NMMHC-IIA; (2) MHCII-B=myosin-10= NMMHCB= NMMHC-IIB; (3) MHCII-C-=myosin-14= DFNA4= DFNA4A= FP17425= MHC16= MYH17= NMHC II-C= NMHC-II-C= PNMHH= myosin. Arabic numbers are now used too, e.g., myosin 2

Regulatory light chain: RLC; MLC; MRLC; MYRL; spaghetti squash (in *Drosophila*). Three isoforms are expressed in mammalian cells, also known as: (1) SM (smooth muscle)-RLC= MYL9= LC20= MLC-2C= MLC2= MRLC1= MYRL2; (2) MYL12A= HEL-S-24= MLC-2B= MLCB= MRCL3= MRLC3= MYL2B; (3) MYL12B= MLC-B=MRLC2

Essential light chain: MYL6= ELC= MLC= MLC-ALK= MLC1 (in *Drosophila*)

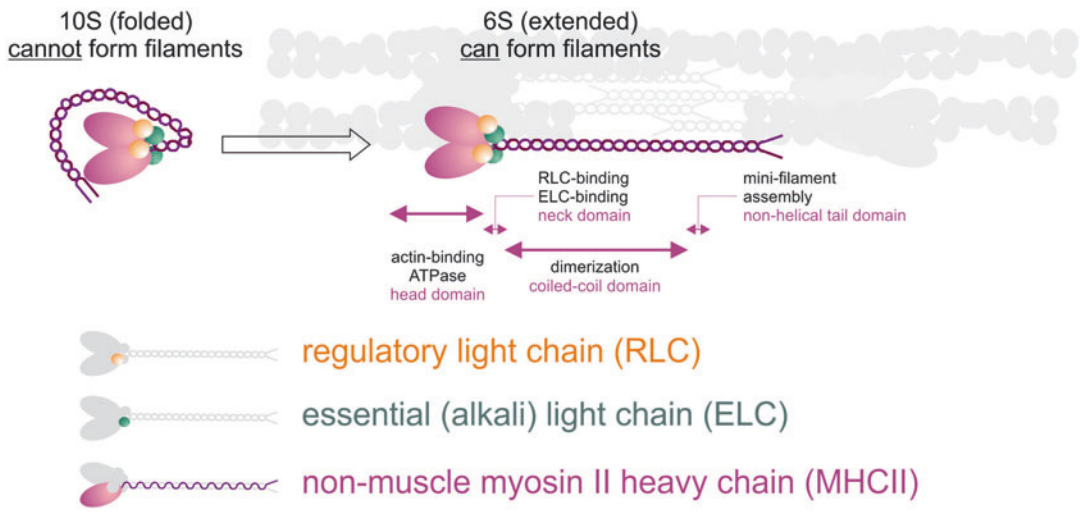
Functional unit (2×HC+2×RLC+2×ELC): NMII, NMMII, cytoplasmic myosin II

Historical Background

The discovery of nonmuscle myosin II (NMII) is linked to the story of muscle contraction (reviewed in Szent-Gyorgyi 2004). It started in 1864, when Köhler purified a viscous substance ("myosin") from muscle, which he thought related to the contractile properties of muscle. In 1939, myosin was identified as an ATPase made of two major components, myosin and actomyosin. This led to the seminal idea that myosin and actin formed two types of interacting filaments. In the 1950s, proteolytic studies revealed the main functional parts of myosin: a heavy meromyosin that interacted with actin and contained the ATPase activity and a light meromyosin that enabled the formation of myosin filaments. The myosin light chains were also identified around this time. These experiments, together with crystallographic and functional evidence of the different bands that comprise the muscle sarcomere, allowed Huxley to propose that muscle contraction was due to the sliding of actin and myosin filaments with respect to each other. The concept that myosin and ATP existed in different binding states was incorporated into this early model to formulate the myosin swinging cross-bridge model, which is the core of the function of muscle (and nonmuscle) myosin II activity (Huxley 1969). This model was the culmination of a hundred years of investigation, and it also spurred the interest of many cell biologists and physiologists in contractile events observed in nonmuscle cells, for example migrating cells and clot formation and retraction, and also in morphogenetic movements, for example gastrulation. This led to the rapid identification of nonmuscle myosin II (NMII) in platelets (Adelstein et al. 1971) and also in amoebae (Pollard and Korn 1973a, b). NMII is a ubiquitous protein expressed in all mammalian tissues as well as in simple organisms. Like muscle myosin II, NMII is endowed with ATPase activity and the ability to form filaments with actin. The main mechanism of action of NMII is also similar to that of muscle myosin II. It involves an ATP-powered conformational change of the myosin head (Spudich 2001). Such movement is conjoined with its interaction with actin. The conformational change of the myosin head while tethered to the actin produces the movement of the filament or exerts tension on it. This property has positioned NMII at the center of the mechanotransduction field as the major force and tension generator inside nonmuscle cells (Aguilar-Cuenca et al. 2014). In this chapter, we outline the major mechanisms controlling NMII ATPase and actin-binding activity and its assembly to form filaments as well as the emerging picture of downstream pathways that are regulated by NMII-generated mechanical force.

NMII Structure and Assembly

The multimolecular structure of NMII can be seen in Fig. 1. Briefly, the functional NMII unit comprises a hexamer made of two intertwined heavy chains (MHCII) that interact with each other through a long coiled-coil central domain. The actin-binding and Mg^{2+}-actin-dependent ATPase (head) domain are located near the N-terminus. The head and coiled-coil domains are linked by a neck region that acts as a movable hinge during the conformational changes that support myosin head swinging. It also serves as the binding site of



Nonmuscle Myosin II, Fig. 1 Structure of the NMII hexamer. *Top,* image represents the two conformations of the NMII hexamer, 10S, which is assembly-incompetent, and the 6S, assembly-competent form. The 6S form is represented associated to a myosin filament and actin filaments (shaded). *Bottom,* color-coded nomenclature of the image

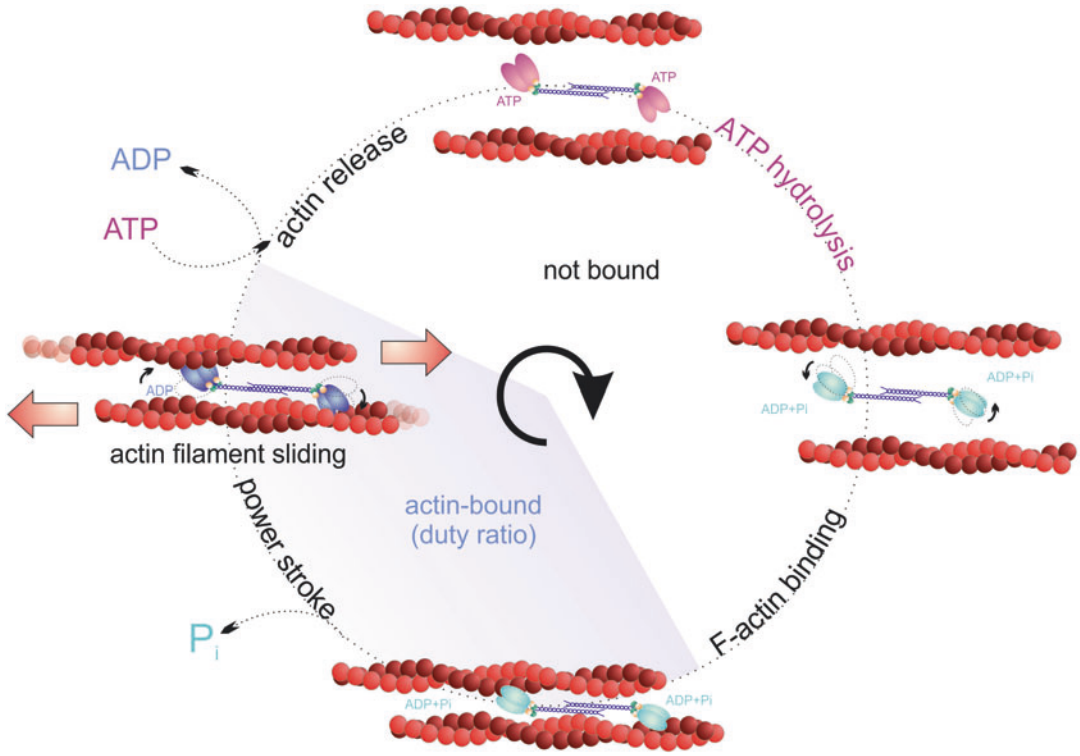
four light chains. Two of these chains (ELC) serve a structural purpose. The other two chains (RLC) control the ATPase function of the head domain of the heavy chain (see below). NMII exists in two conformations: an assembly-incompetent form, folded (10S) that can extend into an assembly-competent form (6S) that can bind actin (reviewed in detail in Cremo and Hartshorne (2007)). The 10S→6S conversion is regulated by an off/on switch in the regulatory chain constituted by two residues (Thr18/Ser19) that can be phosphorylated (ON) or not (OFF). This phosphorylation also promotes the ATPase activity of the heavy chain in the assembled form. Several kinases can phosphorylate one or both residues (see below). Additional mechanisms exist, for example phosphorylation of Ser1 and Ser2 by PKC. Only one isoform of the ELC has been described in mammals, encoded by *Myl6*, Gene ID: 4637, chromosome 12. (Note: All the gene identities and chromosome number correspond to the human molecules). Conversely, there are three mammalian variants of the RLC that can interact with NMII: one is the product of the gene *Myl9* (Gene ID: 98932, chromosome 2), which encodes the smooth muscle variant of RLC; the other two are the products of the genes *Myl12A* (Gene ID: 10627, chromosome 18) and *Myl12B* (Gene ID: 103910, chromosome 18), which are bona fide nonmuscle RLCs. Finally, three mammalian isoforms of the heavy chain are encoded by different genes: *Myh9* encodes the isoform II-A (Gene ID: 4627, chromosome 22); *Myh10* encodes the isoform II-B (gene ID: 4628, chromosome 17); and *Myh14* encodes the isoform II-C (Gene ID: 79784, chromosome 19). Nomenclature-wise, the heavy chain isoform determines the type of NMII hexamer, defining three paralogs: NMII-A, NMII-B, and NMII-C. There is no definitive evidence of a selective binding of different RLC isoforms to the neck of the three heavy chain isoforms. This is not unexpected due to the almost complete sequence identity of the RLC isoforms.

Regarding the coiled coil and nonhelical tail domain, these regions are thought to support the lateral interaction of the NMII hexamer with the equivalent region of other NMII hexamers to assemble antiparallel filaments (mini-filaments) that contain multiple hexamers (Ricketson et al. 2010), forming proto-sarcomeric structures in many cell types, particularly epithelial and mesenchymal cells. The size and stability of these mini-filaments depend of the isoform of the heavy chain. Although small filaments with a

mixed composition have been described in cultured cells (Beach et al. 2014; Billington et al. 2015), many NMII filaments present in live cells contain only one type of hexamer. However, assemblies of different NMII isoforms can assemble along the same actin bundles, forming a characteristic stippled pattern observed by confocal and super-resolution microscopy (Vicente-Manzanares et al. 2007; Beach et al. 2014).

Mechanism of Filament Sliding and Force Generation

NMII-based actin filament sliding can be described in terms of the swing cross-bridge model (Spudich 2001). This model is shown in Fig. 2. Briefly, ATP-bound NMII is free, that is, not associated with the actin filament. When ATP is hydrolyzed by the Mg^{2+}-actin-dependent ATPase activity of the head domain, NMII binds to the actin filament. The release of the resulting phosphate triggers a conformational movement that causes the sliding of the actin filament with respect to the NMII (10 nm/step on average). The exchange of ADP for ATP restarts the cycle. Mechanical work at a cellular level is caused by the concerted movement of many bundles of actin filaments propelled by multiple NMII filaments. A very important concept is the duty ratio, which can be defined as the fraction of time during which NMII remains bound strongly to actin in the ATPase cycle. The different isoforms display markedly different duty ratios (reviewed in Heissler and Manstein 2013). NMII-A displays the lowest duty ratio (0.05–0.1), which means



Nonmuscle Myosin II, Fig. 2 The swinging cross-bridge model. Image depicts the cycle of NMII interaction with ATP and actin filaments. NMII in purple represents ATP-bound NMII (not bound to actin); light blue represents the intermediate ADP+Pi-bound state; and dark blue represents the actin-bound, ADP-containing state. The image also includes the exchange of ADP for ATP and release of the hydrolyzed phosphate. Note that for representation purposes, the cycle is represented as four symmetric stages, but the actual amount of time the NMII spends in one or another conformation is isoform-dependent (see text for details)

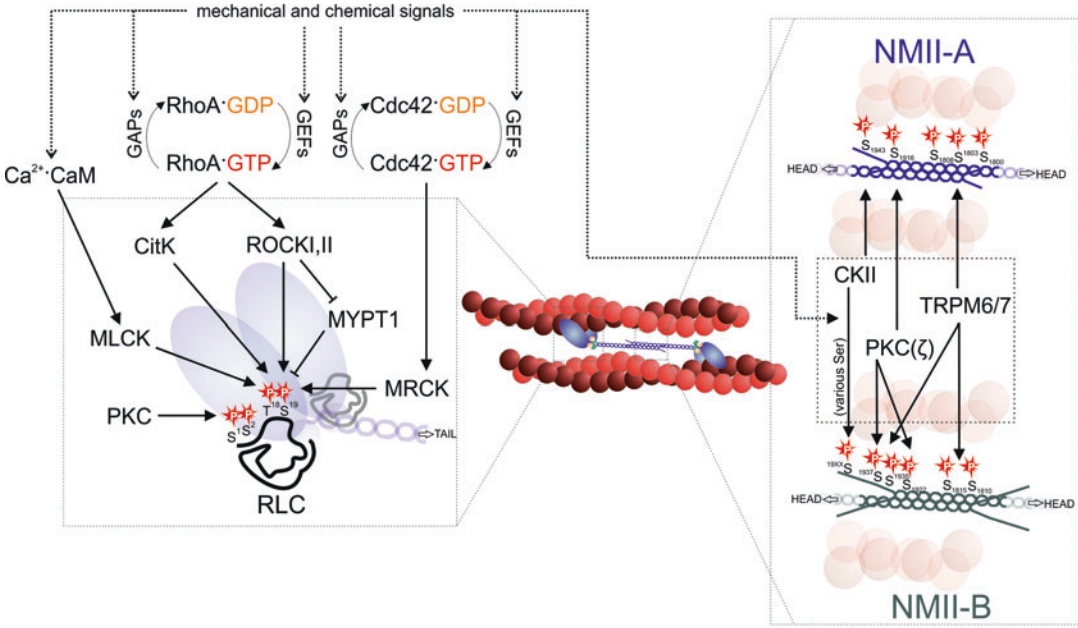
NMII-A remains strongly bound to actin 5–10% of the time. This is similar to the duty ratio of muscle MII. Conversely, NMII-B has a much higher duty ratio (0.2–0.4), which likely represents an adaptation to bear tension, rather than to produce force. Finally, the duty ratio of NMII-C is splicing-dependent, but intermediate between NMII-A and NMII-B (Heissler and Manstein 2011). An interesting property is that the duty ratio of the NMII isoforms is load-dependent, which means that high load increases their duty ratio, particularly NMII-B (Kovacs et al. 2007). Duty ratio and the stability of NMII filament assemblies (see next section) are the two main properties that explain the different cellular functions of the isoforms.

Two major regulatory checkpoints of NMII exist. One is the direct control of the actin binding/ATPase activity/ conformational movement of the NMII hexamer, which mainly takes place at the head and neck regions, including the RLC. The other is the control of the assembly state of the NMII hexamers (NMII filament growth/ disassembly equilibrium) as well as their subcellular localization. These two levels of control constitute the core of this review and are described in the following section.

Regulation of NMII Assembly and Activity

The major mechanism of regulation of the ATPase motor activity and filament assembly of NMII is through Ser/Thr phosphorylation. Several kinases have the potential to phosphorylate multiple sites in the RLC and the MHCII (Fig. 3). NMII serves as an endpoint of multiple signaling pathways that control NMII in different ways through the phosphorylation of various residues.

RLC phosphorylation is the central regulatory event that controls NMII activity. The regulatory hotspot of the RLC is Ser19, which is responsible for the extension of the 10S NMII hexamer into the 6S assembly-competent form (Rosenfeld et al. 1994). It also promotes the activation of the motor ATPase activity of the NMII (Adelstein and Conti 1975). Additional phosphorylation at Thr18 further increases the actomyosin enzymatic activity and favors filament formation. In live cells, NMII phosphorylated in both Thr18 and Ser19 mainly appears in thick, stable actomyosin bundles, whereas NMII phosphorylated only in Ser19 is predominantly located in thinner, more dynamic filaments (Vicente-Manzanares and Horwitz 2010). Different kinases promote the phosphorylation of the RLC in Thr18 and Ser19. A major activating pathway emerges from the small Rho GTPase RhoA. When bound to GTP, RhoA binds to and activates ROCK (RhO-associated Coiled-coil Kinase). ROCK may phosphorylate RLC directly on Ser19 (Amano et al. 1996). However, the main function of ROCK in this context is the phosphorylation and inactivation of MYPT1, which is the catalytic subunit of a phosphatase specific for the RLC of NMII. In this manner, ROCK triggers NMII activation by promoting RLC phosphorylation directly and also by preventing its dephosphorylation. In addition, MYPT1 can be regulated in other ways, for example it can be phosphorylated and inhibited by ZIPK (Ichikawa et al. 1996). Also, the Arf GEFs Big1 and Big2 regulate its incorporation into complexes, thereby controlling its access to NMII (Le et al. 2013). CitK (citron kinase) is a RhoA-dependent cell cycle-related kinase that phosphorylates both Ser19 and Thr18 and controls the mitotic function of NMII (Yamashiro et al. 2003). Another Rho family member, Cdc42, activates MRCK (Myotonic dystrophy-Related Coiled-Coil Kinase), which also phosphorylates RLC on Thr18 and Ser19 (Leung et al. 1998). NMII is also directly regulated by calcium through the activation of the myosin light chain kinase (MLCK). This kinase is activated by Ca^{2+}-calmodulin binding, and it phosphorylates RLC preferentially on Ser19 (Sellers et al. 1981). These kinases constitute a spatially segregated regulatory network, by which NMII is differentially activated throughout the cell. For example, MRCK and MLCK activate NMII closer to the leading edge (Totsukawa et al. 2004; Tan et al. 2008), whereas ROCK drives the activation of NMII in thick actomyosin bundles, which define the trailing edge of the cell (Totsukawa et al. 2004). Additional regulatory



Nonmuscle Myosin II, Fig. 3 Regulation of the phosphorylation of the RLC (conformational extension and ATPase activity of the heavy chain) and of the heavy chain tail domain (filament assembly). Throughout the image, S indicates Ser residues and T denotes Thr residues, followed by their position in the corresponding RLC (*left*) or MHCII (*right*). *Left*, regulation of the RLC. Please refer to the text for details. CitK, citron-kinase; ROCK, RhO associated Coiled coil Kinase; MLCK, Myosin Light Chain Kinase; MYPT1, MYosin Phosphatase SubuniT1; MRCK, Myotonic dystrophy-Related Coiled-coil Kinase; PKC, protein kinase C; Ca2.CaM, calcium-calmodulin; GAP, GTPase Activating Protein; GEF, GTP Exchange Factor. *Right*, regulation of the NMII coiled coil and non-helical domain. *Top right*, tail of NMII-A. *Bottom right*, tail of NMII-B. CKII, casein kinase-II; TRPM6/7, Transient Receptor Potential Melastatin

sites exist in the RLC, for example Ser1/Ser2. The phosphorylation of these Ser by PKC inhibits the function of NMII by decreasing the binding of NMII to actin filaments (Nishikawa et al. 1984; Komatsu and Ikebe 2007).

Regarding the control of NMII assembly into filaments, this is spatially mediated by the last loops of the α-helix coiled-coil rod domain and C-terminal nonhelical tail of the MHCII (∼last 200 amino acids of the MHCII). These regions interact laterally (mainly in an antiparallel fashion) with binding motifs in other NMII hexamers to mediate filament assembly. This interaction relies on intermolecular electrostatic interactions between negatively and positively charged regions that reside in this region of the MHCII. Importantly, this region contains most regulatory residues that underlie isoform specificity, thereby promoting homotypic interactions. This means that many mini-filaments contain only one type of paralog. Several kinases can regulate this activity, including TRPM (transient receptor potential melastatin)-6 and -7, members of the PKC (protein kinase C) family and CK2 (casein kinase 2). Regulation of these residues through phosphorylation introduces negative charges in selected regions of the different isoforms, which potentially destabilizes the electrostatic interactions between the chains and generally causes NMII disassembly and actomyosin filament instability that leads to cytoskeletal reorganization (Ricketson et al. 2010). TRPM7 also regulates NMII through the regulation of Mg^{2+} homeostasis (Stritt et al. 2016). These phosphorylations control the interaction of NMII with additional proteins. For example, NMII-A interacts with S100A4 (Mts1, metastatin-1) through a binding motif that comprises the last loops of the

coiled-coil domain and the nonhelical tail domain. This interaction is regulated by phosphorylation of Ser1943 of NMII-A. Although this residue is not within the S100A4-NMIIA binding interface, its phosphorylation and the subsequent addition of negative charge may alter the conformation of the C-terminus of NMII-A, thereby preventing the interaction (Dulyaninova et al. 2005). Another interacting protein is Lgl (Lethal giant larvae), which is a tumor suppressor protein that binds to the C-terminal of both MHCII-A and -B under the control of aPKCζ and inhibits NMII-A assembly in live cells (Dahan et al. 2012). Myosin binding protein H (MYBPH) can directly interact with NMII to inhibit cell migration (Hosono et al. 2012). Additional regulators of NMII include various tropomyosin isoforms. Although muscle tropomyosin inhibits muscle myosin II binding to actin filaments, nonmuscle tropomyosin isoforms have variable effects on NMII function, depending on the tropomyosin and NMII isoform (Barua et al. 2014). Also, supervillin and anillin can bind NMII and control its function in cell division (Smith et al. 2013).

Pharmacological Control of NMII Function

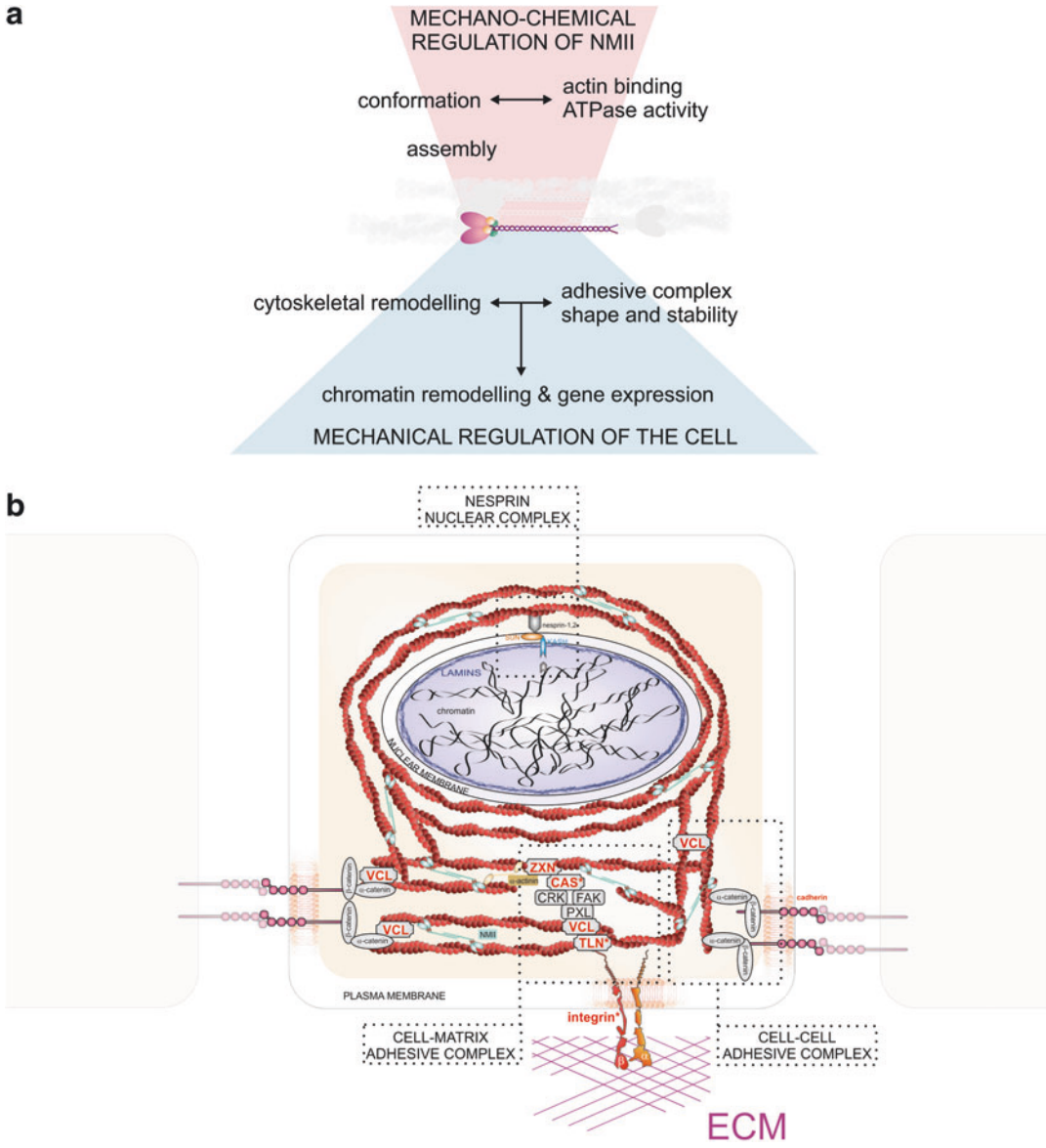
A specific NMII inhibitor exists, blebbistatin (Straight et al. 2003). The (−) isomer of blebbistatin blocks the ATPase activity of NMII (Shu et al. 2005). In this manner, blebbistatin initially blocks contraction (the power stroke does not occur) and later detaches NMII from actin (Kovacs et al. 2004). Blebbistatin is not isoform-specific, and although its original form was phototoxic upon blue or UV light illumination (Kolega 2004), a recent derivative, para-nitroblebbistatin, is not (Kepiro et al. 2014). Pharmacological interventions are usually carried out at the regulatory level. For example, there are several specific inhibitors for ROCK, for example Y27632 and HA-1077 (fasudil) (Uehata et al. 1997). The latter is used in several countries to treat vasospasm, stroke, and cognitive decline in Alzheimer's patients. There is an inhibitor for MLCK, ML-7. Inhibition of RhoA activity by ADP-ribosylation with C3 exoenzyme from *Clostridium botulinum* phenocopies many of the effects of direct NMII inhibition (Ridley and Hall 1992), but it has additional effects due to the role of RhoA as a controller of additional pathways involved in gene expression (Hill et al. 1995). Pharmacological activation of NMII can be elicited using a toxin produced by the Japanese sponge *Discodermia calyx*, calyculin A. This is a phosphatase inhibitor with moderate to low specificity for MYPT1 (Ishihara et al. 1989). On the other hand, no specific inhibitors of NMII assembly have been described yet. A recent study has identified a small molecule, 4-hydroxyacetophenone (4-HAP), which stabilizes NMII-B assemblies (Surcel et al. 2015), but the mechanism of such an effect remains unclear at present.

NMII-Dependent, Force-Sensitive Pathways

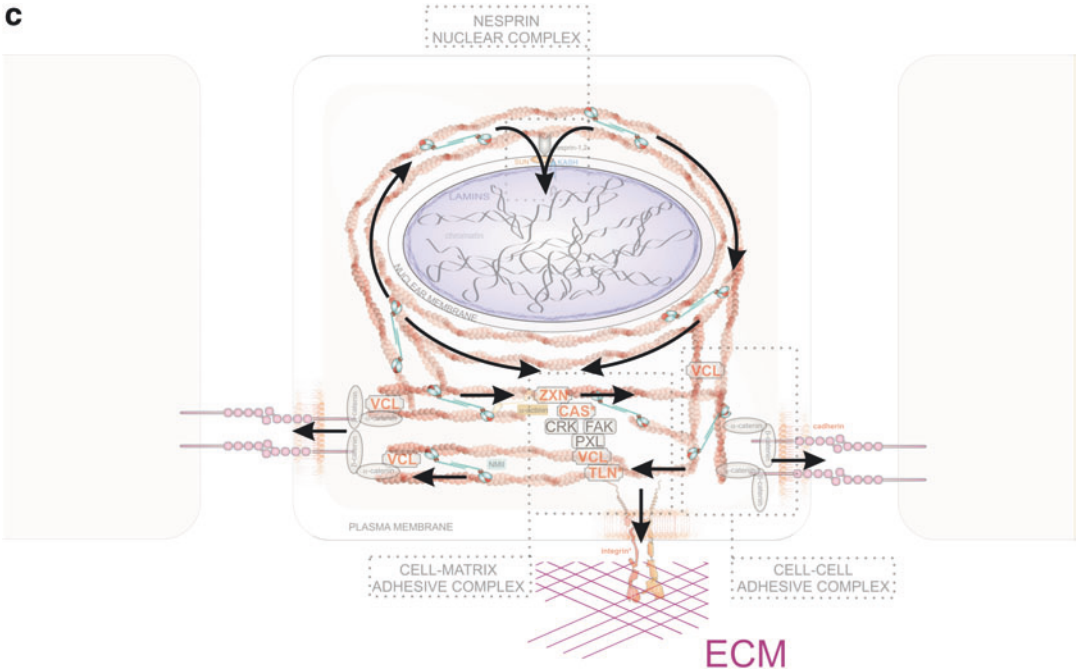
In addition to its position as a signaling endpoint, NMII activity also triggers many signal transduction pathways that contain mechano-reactive components, that is molecules that change their properties (binding, conformation, activity, etc.) in response to the application of mechanical force (Fig. 4a). This has been best illustrated in the case of cell-matrix adhesions. NMII activation generates mechanical work that increases the size of cell-matrix adhesions. This is due to several mechanisms occurring simultaneously. One is the lateral bundling of actin fibers, which brings diverse adhesion proteins into molecular proximity, triggering their interaction and thus increasing the number of molecules in cell-matrix adhesions ("clustering"). The other mechanism involves the conformational change of specific adaptors in response to mechanical work. One example is talin (TLN). When mechanically stretched, talin exposes a cryptic vinculin-binding site (del Rio et al. 2009), recruiting vinculin (and hence actin) to focal adhesions. Another example is p130CAS. This adaptor has a cryptic Tyr that is a substrate for Src only when p130CAS is stretched mechanically (Sawada et al. 2006). There is evidence of

mechanically linked conformational or clustering changes to several components of cell-matrix adhesions, including integrins, zyxin (ZXN), and others (Yoshigi et al. 2005;Friedland et al. 2009) (shown in red in Fig. 4b). Cell-cell cadherin-based adhesions are also subject to mechanical regulation. For example, E-cadherin binds actin in a force-dependent manner through α-catenin (Buckley et al. 2014). In this manner, the forces generated by NMII are transmitted to cell-cell and cell-matrix adhesions, controlling their composition, interacting strength, and dynamics **(**Fig. 4c**)**.

Mechanical regulation of gene expression is another point of great interest. Several transcription factors are activated in response to NMII-dependent mechanical signals, for example Yap/Taz or Nkx2.5 (Piccolo et al. 2014; Dingal et al. 2015). The precise mechanism of activation



Nonmuscle Myosin II, Fig. 4 (continued)



Nonmuscle Myosin II, Fig. 4 NMII is a signaling endpoint and an initiator of mechano-responsive signaling. (**a**) In red, the mechano-chemical regulation of NMII controls its conformation and actin binding and ATPase activity as well as its assembly into filaments of variable stability. In blue, the forces and work generated by the sliding of actin filaments driven by NMII promotes cytoskeletal remodeling, diverse effects on the molecular composition and stability of adhesive complexes (with other cells and the extracellular matrix) and even the modulation of gene expression by mechanical effects on the conformation of nuclear DNA. (**b**) Mechano-responsive cellular elements. The center of the image represents a cell that establishes cell-matrix adhesions (at the *bottom*) and cell-cell contacts (*left* and *right*, as indicated). Throughout the figure, NMII is represented in blue. Cell-matrix adhesive complex contain integrins (as indicated, in *red*/*orange*), TLN (talin), VCL (vinculin), PXL (paxillin), FAK (Focal Adhesion Kinase); CAS (p130CAS); ZXN (zyxin) and α-actinin (represented in pink as single anti-parallel arrays). Cell-cell adhesions contain cadherins (as indicated, in *pink*), vinculin and α- and β-catenin. The figure also depicts the nucleus enveloped in an actin-rich structure. In the nucleus, note the nesprin complex (nesprin/SUN/KASH), which connects the cytoskeleton and nucleoskeleton, and can transmit NMII-generated forces to chromatin through its interaction with nuclear lamins (sub-nuclear, *dark blue*). Molecules marked with an asterisk (*) change their conformation in response to force. Molecules in red have been shown to transmit forces through them independent of conformational changes. (**c**) The paths of the force. Figure is as in (**b**). *Black arrows* represent NMII-generated forces and how they are driven through actomyosin filaments to acting points, particularly cell-cell and cell-matrix adhesions, which control cell-cell interactions and matrix (ECM) remodelling, respectively. Forces are also transmitted to the nucleus, driving mechanically-regulated chromatin extension

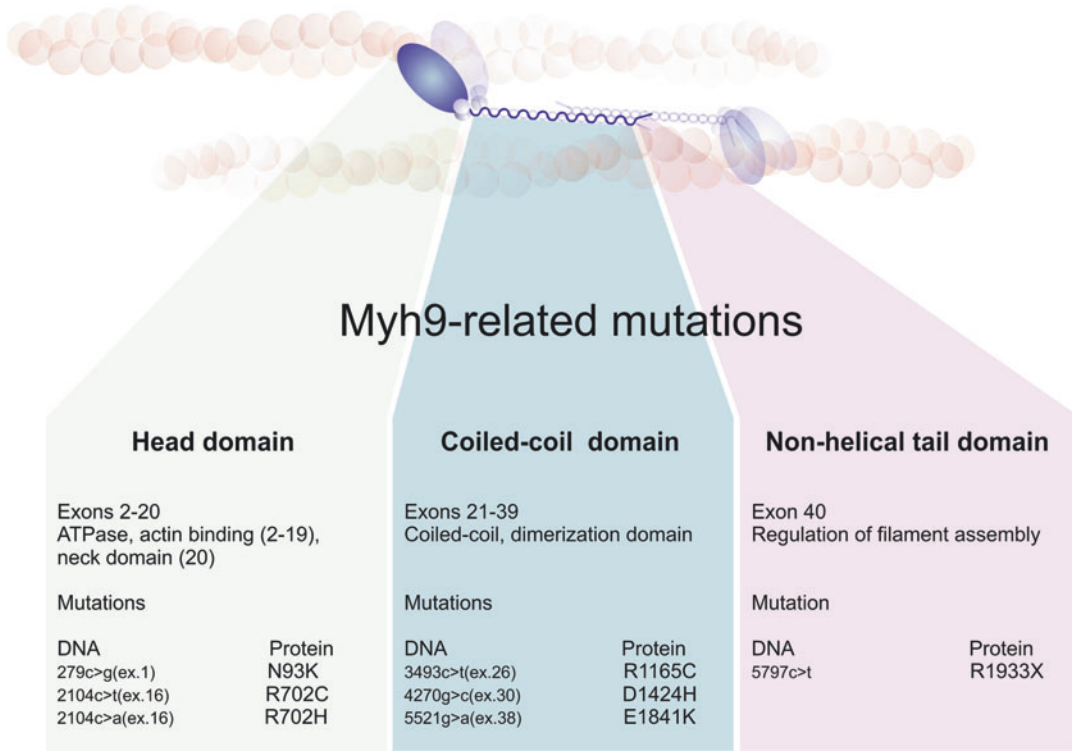
is still unclear, but it requires the translocation of the transcription factor from the cytoplasm to the nucleus, which is promoted by NMII-dependent forces. It also requires the binding of the transcription factor to its DNA targets in the nucleus, which depends on the degree of chromatin extension. Chromatin extension could be controlled by NMII-dependent forces through the connection of the cytoplasmic cytoskeleton to the nucleoskeleton through nuclear nesprin/SUN/KASH complexes (Fig. 4b, c). The connection of nucleoskeleton proteins to chromatin suggests that these forces may expose loops of condensed chromatin DNA, which would become accessible to mechano-sensitive transcription factors, enabling the expression of specific genes in response to mechanical cues. This is how stiff environments are thought to promote stem cell differentiation into osteoblasts: a stiff substrate would activate NMII, which would transmit

mechanical forces to chromatin through nuclear complexes, exposing DNA binding sites related to genes involved in osteogenesis. Although the mechanisms that control transcription factor shuttling in and out of the nucleus in response to mechanical forces are still unclear, some mediators have been identified in specific cell types, for example E-cadherin/β-catenin in epithelia (Benham-Pyle et al. 2015).

Mechanically driven gene expression is clearly an area of rapid development that aims to define the mechano-responsive elements in the nucleus that drive chromatin exposure under different mechanical stimuli. It is interesting to note that some of the mechano-responsive transcription factors identified so far, for example Yap/Taz and Nkx2.5, are also implicated in morphogenetic pathways that control the growth of tissues to their proper size. This suggests that the relationship between the shape of individual cells and tissues as a whole is conjoined by the convergence of mechanical signals and genetic responses.

Myh9/Myh14 Syndromes

Several genetic diseases are associated to mutations to the human *Myh9* gene (Seri et al. 2000). These include the May-Hegglin anomaly and Epstein, Fechtner, and Sebastian syndromes, which are now collective referred as *Myh9*-related diseases. Mutations affect different domains of the molecule (Fig. 5); hence, they compromise different aspects of NMII-A function, from ATPase activity (mutations to exons 2–20) to dimerization (exons 21–39) to mini-filament assembly (exon 40). These syndromes have several specific clinical manifestations, which accumulate over time. The most common clinical manifestation of these syndromes is macrothrombocytopenia, which is a



Nonmuscle Myosin II, Fig. 5 Some Myh9-specific mutations cause disease in humans. Panel includes mutations to the head domain (in *green*), the coiled-coil domain (in *blue*) and the single mutation ascribed to the junction between the non-helical and coiled-coil domain (in *red*). The Table embedded in the figure describes the DNA mutations with its corresponding exon and the result of the mutation in terms of protein sequence

shortage of mature platelets and the appearance of giant platelets in the bloodstream (Althaus and Greinacher 2009). Other manifestations include Döhle-like neutrophil inclusions, deafness, nephropathy, and cataracts. Döhle-like inclusions are likely aggregates of protein that cannot form filaments, and they appear in all of these syndromes except Epstein's. The etiology of the other alterations is less clear, but it seems related to mechanical compromise and/or impaired renewal of cells that control mechanically active processes, for example sound transmission in the ear (deafness), glomerular filtration in the nephrons (nephropathy due to glomerulosclerosis), or light transmission in the eye (cataracts). Interestingly, mutations in the head and rod domain of mouse *Myh9* gene cause similar defects to those observed in *Myh9* patients (Zhang et al. 2012).

A mutation in the gene *Myh10* has been recently identified, with a phenotype consisting of intrauterine growth restriction, microcephaly, developmental delay, failure to thrive, congenital bilateral hip dysplasia, cerebral and cerebellar atrophy, hydrocephalus, and congenital diaphragmatic hernia (CDH) (Tuzovic et al. 2013). This mutation phenocopies some aspects of mice strains carrying loss-of-function mutations to this gene (Ma et al. 2004). Finally, autosomal dominant hearing impairment (DFNA4) is associated to a mutation in *Myh14*. *Myh14* encodes the heavy chain isoform II-C. This isoform is expressed in the cochlea and several mutations are associated to DFNA4, including 20c>a = S7X (stop mutation in the motor domain); 1126 g>t = G376C and 2176c>a = R726S (both in the motor domain); and 2926c>t = L976F (in the coiled-coil domain) (Donaudy et al. 2004).

Summary

Nonmuscle myosin II has emerged as a central protein at the interface between chemistry and physics in terms of the control of the cellular behavior. This is mainly due to its ability to generate mechanical force and actin filament work, as well as its actin cross-linking properties. Two major mechanisms of control exist, related to the actin-binding and ATPase activity of the molecule as well as the stability of the NMII subunits in filaments. In addition to the human syndromes associated to single-site mutations in the MHCII, NMII plays central roles in cancer and inflammation as it controls cancer cell division, the physical integrity of tumor masses, their dissemination, and their implantation at distant sites from the original tumor. It also determines the mechanical properties of the extracellular microenvironment, which may promote, or inhibit, the development of these diseases. Finally, NMII also participates in leukocyte proliferation and migration, that is, the systemic response of multicellular organisms against external aggression or anomalous growth. Although too central to be a useful pharmacological target to treat disease so far, its complex regulation together with its ubiquity in terms of cellular function and tissue expression makes NMII a crucial mediator of most cellular responses and a lynchpin of the biology of complex organisms.

Acknowledgments We regret the large number of original articles that were left out due to space constraints. Dr. Vicente-Manzanares would like to thank Prof. Robert S. Adelstein, Dr. Mary Anne Conti and Dr. Xuefei Ma for critical reading of the manuscript and their advice, help and warm friendship over the years. This work was funded by grant SAF2014-54705-R from MINECO. Miguel Vicente-Manzanares is a Ramon y Cajal Assistant Professor (RYC2010-06094).

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Nonmuscle Myosin Light Chain Kinase

► MYLK (Myosin Light Chain Kinase)

Nonvoltage-Gated Sodium Channel 1

► ENaC

NOT

► NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

Notch (Notch1, Notch2, Notch3, Notch4)

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Synonyms

TAN1; Translocation-associated notch homolog

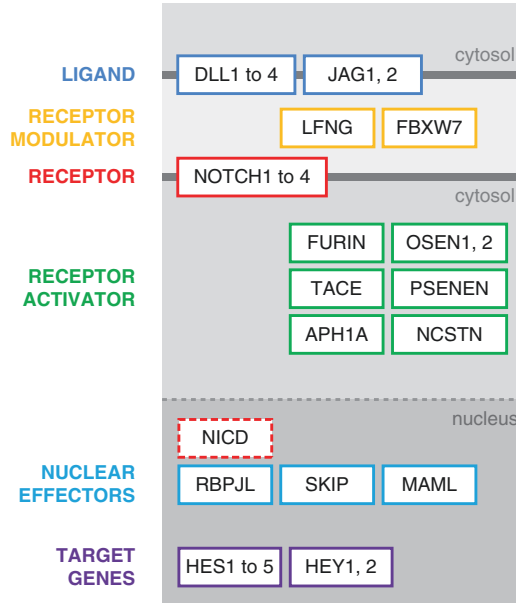
Historical Background

Notch mutants in *Drosophila melanogaster* were originally reported in 1919 (Morgan and Bridges 1919). Notch was named based on the *Drosophila melanogaster* mutants that exhibited irregular notches of missing tissue at the tips of their wing blades. This phenotype was caused by heterozygous loss-of-function mutations in a gene subsequently named "Notch" that was cloned in 1983 (Artavanis-Tsakonas et al. 1983).

Notch and Notch Signaling

Notch proteins are single-pass transmembrane receptors that mediate cell-to-cell communications for the regulation of cell fate decisions during developmental stages and also adult life. Notch receptors are highly conserved in throughout evolution and are found in a diverse range of organisms from worms to humans. The Notch family includes four receptors, NOTCH1, NOTCH2, NOTCH3, and NOTCH4. These receptors have an extracellular domain that contains multiple epidermal growth factor (EGF)-like repeats and an intracellular region that contains a RAM domain, ankyrin repeats, and a C-terminal PEST domain (Fig. 1).

The Notch receptor is synthesized as a 300-kDa precursor and is cleaved in the trans-Golgi compartment. The resulting extracellular and lumenal N-terminal fragment and the transmembrane domain and intracellular domain C-terminal fragment are assembled into the mature heterodimer receptor through a non-covalent linkage. The extracellular and lumenal portion of Notch undergoes extensive N- and O-linked glycosylation during synthesis and secretion, which is critical for proper folding of the receptor and its subsequent interactions with ligands (Fortini 2009).



Notch (Notch1, Notch2, Notch3, Notch4), Fig. 1 Notch activation and signaling pathway

Canonical Notch signaling is activated by binding of a ligand from the DSL family, that includes Delta and the Serrate/Jagged subfamily that is located on the adjacent cell's surface. Ligand binding activates proteolysis of Notch between gly1743 and val1744 (termed site 3 or S3) by γ-secretase. One fragment is the Notch intracellular domain (NICD) that is translocated to the nucleus. NICD functions in transcriptional regulation of the Hairy/E(spl) family (*Hes* genes in mammals, *her* genes in zebrafish), that codes for inhibitory basic helix-loop-helix (bHLH) transcriptional regulators that control many different secondary targets, including Notch ligand genes and the *Hes/her* genes themselves. The other fragment is the Notch extracellular domain (NECD) that is endocytosed by the ligand-expressing cell.

Lateral inhibition is a feedback loop in which adjacent and developmentally equivalent cells assume completely different fates. Binding of Delta and Notch across the two cells in the opposite direction generates transcriptional feedback. Delta expression by the signal-sending cell is stronger than by the signal-receiving cell, therefore, Notch signaling by the signal-receiving cell is activated. This signaling results in transcription of *Hes/her* genes, thereby producing an achaete-scute complex (AS-C) to block expression of differentiation genes and Delta transcription. Decreased Delta expression by the signal-receiving cell diminishes the Delta-Notch binding from the signal-receiving to signal-sending cell, thus inactivating Notch signaling by the signal-sending cell. As a result, the *Hes/her* genes are not transcribed by the signal-sending cells, enhancing differentiation and upregulating Delta expression. The final outcome of this feedback loop is that the signal-receiving cell maintains strong expression of the Notch receptor Notch expression and Notch signaling through *Hes/her*-dependent transcriptional feedback, while the signal-sending cell maintains strong ligand expression and repressed Notch signaling through AS-C-dependent transcriptional feedback (Lewis et al. 2009).

Physiological Roles of Notch Signaling

Notch and Development and Differentiation

The Notch pathway, with TGF-β, Wnt, and Hedgehog, is a representative pathway that regulates developmental and differentiation gene expression programs, so that at the correct time and place, cells with the same propensity for a particular cell fate can give rise to daughters that exhibit differences in morphology and protein expression.

A variety of strategies are used to achieve developmental goals. One strategy is to form gradients of signaling proteins either on cell surfaces or in extracellular spaces that help determine cell fates when they activate cellular receptors. These signaling proteins are known as morphogens. Another strategy is to utilize hierarchical sequences of gene expression so that over time different progeny will become different types of cells. Notch signaling is involved with these strategies to achieve proper cell development and differentiation (Beckerman 2005).

Somitogenesis

Notch signaling coordinates and synchronizes the cell clocks of individual cells when somites form. Segmentation and antero-posterior polarity of somites are formed under the control of Notch signaling and its associated pathways.

Somites are musculoskeletal segments, derived from presomitic mesoderm (PSM). Wnt and FGF are produced at the tail end of the PSM and spread to the anterior portion, generating a morphogen gradient. Anteriorly, somites form in response to lowered Wnt and FGF levels, and at the tail PSM grows caudally, expanding the embryo. This growth is a rhythmic process that results in segmentation, and originates from cell-intrinsic oscillation. The oscillation is paced by autoregulation of *Hes/her* genes, which results in a negative feedback loop. Delays in transcription and translation within the feedback loop determine the period of oscillation.

Blocking of Notch signaling disrupts segmentation. However, Notch signaling does not produce oscillation itself, but rather functions in the coordination. Even Notch-dissociated cells show oscillating expression, though in a less regular pattern (Lewis et al. 2009).

Mesp2 is a central mediator of Notch signaling in the somite mesoderm, generating antero-posterior polarity in the presumptive somite via a complex signaling network involving Dll1- and Dll3-Notch signaling and the Notch regulator presenilin 1 (Kuan et al. 2004).

Neural Development

Notch signaling controls neural cell fates, both the early-fate decisions between neural and epidermal and the late-fate decisions between different subtypes of neural cells (Cau and Blader 2009). Proneural clusters are developed in neural cells or epidermal cells. Following several iterations of the lateral inhibition feedback loop as cluster communication, only one cell of the cluster downregulates the Notch pathway and becomes a

neural precursor. The remaining cells are blocked from reaching the neural fate and, are either reselected during a second wave of neurogenesis or secondarily adopt an epidermal fate.

Notch is also required during the specification between two different neural subtypes. These binary decisions can either involve sister cells, as during the formation of *Drosophila* sense organs, or cells that are not linearly related, such as the R3 and R4 photoreceptors of the *Drosophila* eye. Once specified as a neural progenitor, the sensory organ precursor (SOP or pI) divides to generate two cells, pIIa and pIIb, which communicate via Notch. Subsequent divisions generate the four cells of the sensory organs, as well as a glial cell that undergoes apoptosis.

Hematopoietic System

Canonical Notch signaling is essential for the generation of definitive embryonic hematopoietic stem cells, but is dispensable for their maintenance during adult life (Sandy and Maillard 2009). Notch controls several early steps of T-cell development, as well as specific cell fate and differentiation decisions in other hematopoietic lineages. In addition, emerging evidence indicates that Notch is a potent, context-specific regulator of T-cell immune responses, which includes several disease models relevant to patients.

Notch signaling intensity varies throughout T-cell development. The Notch target genes *Deltex1* and *Hes1* are expressed at very low levels in bone marrow hematopoietic stem cells (HSCs). Upon arrival in the thymic environment, early T progenitors (ETPs) strongly upregulate the expression of Notch target genes. Expression levels of Notch target genes gradually increase during development from the ETP stage to the double negative 3a (DN3a) stage. After the β-selection checkpoint, during which Notch signaling is significantly downregulated, the intensity of Notch signaling steadily decreases from the DN3b stage to the CD4+/CD8+ double positive (DP) stage. Thymic single positive (SP) CD4+ and CD8+ T cells, as well as naive peripheral T cells, express low amounts of Notch target genes. Upon T-cell activation in the periphery, Notch signaling increases sharply in a context-dependent manner.

Notch and Cancer

The highly conserved Notch signaling pathway plays pleiotropic roles during embryonic development and is important for the regulation of self-renewing tissues (Koch and Radtke 2007). The physiological functions of this signaling cascade range from stem cell maintenance and influencing cell fate decisions of slightly differentiated progenitor cells, to the induction of terminal differentiation processes, all of which are recapitulated in different forms of cancers. Although Notch signaling is mainly associated with oncogenic and growth-promoting roles, depending on the tissue type, it can also function as a tumor suppressor.

Oncogenic Function

The first data describing the oncogenic consequences of aberrant Notch signaling in solid tumors were derived from animal studies characterizing a frequent insertion site, named int3, of the mouse mammary tumor virus (MMTV). The int3 site was later identified as the Notch4 locus. MMTV insertions have also been found in the Notch1 locus, albeit with a lower frequency. Together, these result on aberrant Notch signaling and mouse mammary tumorigenesis lead to the question of how significant aberrant Notch signaling is for human breast cancer. To date, only correlative evidence for the involvement of Notch signaling in human breast cancer is available.

Medulloblastoma has primarily been associated with aberrant ▶ sonic hedgehog signaling (Shh), which induces N- ▶ MYC expression. The primitive nature of medulloblastoma tumor cells and the fact that Notch signaling is involved in the maintenance of neural stem and progenitor cells motivated several groups to investigate the potential role of Notch in medulloblastoma. Expression studies using primary medulloblastoma tumor samples showed increased mRNA expression of NOTCH2, but not of NOTCH1. Increased expression of the target gene *Hes1*

correlated with a poor patient survival prognosis. Blocking of Notch signaling resulted in increased apoptosis and a reduction of viable cell in tumor cells.

While the causative role of aberrant Wnt signaling for the development of colorectal cancer is well established, it is currently less clear whether Notch signaling might have a similar oncogenic function within the gut. Since gene expression profiles of crypt cells and colorectal cancer cell lines appear to be very similar, colorectal cancer cells may represent the transformed counter part of crypt cells. Since Notch is a gate keeper of crypt cells, it is likely that Notch and Wnt signaling occur simultaneously in adenomas and crypt cells. Indeed, expression of the Notch target gene *Hes1* has been observed in adenomas of APC^{Min} mice, as well as in primary human colorectal tumors.

Notch signaling has been shown to play an important role during embryonic pancreas development by maintaining an undifferentiated precursor cell type. Notch receptors, ligands, and downstream targets, such as *Hes1*, were found to be upregulated in preneoplastic lesions, as well as in invasive pancreatic cancers in humans and mice. This suggests that Notch signaling in pancreatic cancers might be an early event leading to the accumulation of undifferentiated precursor cells.

Global gene expression profiling and immunohistochemistry have revealed the expression of multiple Notch receptors and ligands in primary lesions of human malignant melanomas, therefore, expanding the list of possible pathways involved in melanoma development. Subsequent studies using established melanoma cell lines showed that pharmacological blocking of Notch signaling can have growth suppressive effects. The oncogenic function of Notch signaling within these cell lines was linked to increased ► β-catenin-mediated signaling, as well as increased MAPK and AKT signaling.

Historically, human NOTCH was identified at the chromosomal breakpoint of a subset of T cell lymphoblastic leukemias/lymphomas containing a t(7;9)(q34;q34.3) chromosomal translocation. The translocation fuses the 3′ portion of NOTCH1 to the T-cell receptor Jb locus. This translocation results in a truncated NOTCH1 protein (N1ICD) that is constitutively active and aberrantly expressed. However, this seminal discovery did not reveal the full oncogenic potential of the truncated version of N1ICD. Less than 1% of all human T-cell leukemias or lymphomas contain this translocation. However, more importantly, aberrant Notch signaling was subsequently found in several human leukemias and lymphomas that lacked genomic rearrangements, signifying that upregulated Notch signaling might have a common role in human leukemogenesis.

Definitive proof for a central role of NOTCH1 in human T-cell acute lymphoblastic leukemia (T-ALL) cell lines came from a recent study that identified somatic activating mutations in the NOTCH1 receptor independent of the t(7;9) translocation. These mutations were detected in more than 50% of human T-ALL cases. Additionally, these mutations were found in all previously defined T-ALL subtypes.

Notch signaling was constitutively active in human clear cell renal cell carcinoma (CCRCC) cell lines. Blocking Notch signaling attenuated proliferation and restrained anchorage-independent growth of CCRCC cell lines and inhibited growth of xenotransplanted CCRCC cells in nude mice. Notch1 knockdown was accompanied by elevated levels of the negative cell-cycle regulators p21(Cip1) and/or p27(Kip1). Moreover, Notch1 and the Notch ligand Jagged1 were expressed at significantly higher levels in CCRCC tumors than in normal human renal tissues. Growth of primary CCRCC cells was attenuated upon inhibition of Notch signaling.

In adults, blood vessels in most organs are quiescent, but the growth of solid tumors requires specific embryonic signaling pathways to direct new blood vessels to grow around and into the tumor. VEGF is important in this process. Notch signaling has a strong effect on angiogenesis as seen in gain- or loss-of-function studies of Notch1, Notch1/4, Jagged1, Dll1, Dll4, Hey1/Hey2, and Presenilins (PS1 and PS2) (Iso et al. 2003; Li and Harris 2005).

By examining the whole-genome transcriptome profile of a xenograft model of breast

cancer and its metastasis form to brain, activation of Notch signaling was found to be crucial in brain metastasis. Over 2,000 genes were differentially expressed in brain metastatic cells, which included various metastasis-related genes and many genes related to angiogenesis, migration, tumorigenesis, and cell-cycle regulation. Interestingly, the Notch signaling pathway was activated in correlation with increased Jag2 mRNA expression, activated NICD, and NICD/CLS promoter-luciferase activity. Increased migration and invasion of brain metastatic cells as compared with primary breast cancer cells were inhibited by inactivation of Notch signaling using DAPT, a γ-secretase inhibitor, and RNAi-mediated knockdown of Jag2 and Notch1 (Nam et al. 2008; Jeon et al. 2008).

Tumor Suppressor Function

Instead of maintaining progenitor cells in an undifferentiated state, or influencing their cell fate decisions, in some tissues such as skin, prostatic epithelium, hepatocellular carcinoma, and small-cell lung cancer, Notch can also induce differentiation that is associated with growth suppression (Koch and Radtke 2007). However, the growth inhibitory role of Notch has mainly (with the exception of the prostate) been based on activated Notch1 overexpression studies. Thus, further experiments are needed in these tissues and cancer types to clarify whether Notch indeed has tumor suppressive functions.

Cancer Stem Cells (CSCs)

The strongest evidence to date for a role of Notch in CSCs is in breast cancer, embryonal brain tumors, and gliomas. γ-secretase inhibitors (GSIs) abolish the formation of secondary mammospheres from a variety of human breast cancer cell lines, as well as in primary patient specimens. In breast ductal carcinoma in situ (DCIS), the ability to form multilineage spheroids termed "mammospheres," indicators of stem-like cells, is dramatically decreased by GSIs, Notch-4 monoclonal antibodies, or Gefitinib. This finding suggests cooperation between epidermal growth factor receptor (EGFR) and Notch-4 in DCIS "stem cell" maintenance. There is evidence for a feedback loop between Her2/Neu and Notch, which may maintain CSCs in Her2/Neu-overexpressing tumors. Sansone and colleagues showed that in mammospheres from human breast cancers, IL-6 induces Notch-3 signaling, increases expression of Jagged-1, and, through Notch-3, promotes a hypoxia-resistant phenotype. The same group described the p66Shc-Notch-3 pathway as essential for maintaining the hypoxia-resistant phenotype of human breast cancer mammospheres. Fan and colleagues showed that Notch inhibition selectively depletes medulloblastoma CSCs as determined by CD133-high status or dye exclusion. The same group has described very similar findings in glioblastoma CSCs. Importantly, in gliomas, Notch confers radioresistance to CSCs. GSI treatment selectively enhanced radiation-induced death of glioma CSCs, but not bulk glioma cells. This effect was replicated by Notch-1 or Notch-2 knockdown, and was accompanied by AKT inhibition and reduced Mcl-1 expression. Other malignancies are being actively investigated. A role of Notch, STAT3, and TGF-β in hepatocellular carcinoma CSC maintenance has been suggested. In Gemcitabine-resistant pancreatic carcinoma cells, EMT (epithelial-mesenchymal transition) is associated with activation of Notch signaling, potentially linking Notch to the "Weinberg model" of stemness acquisition through EMT and to treatment resistance. Inhibition of Notch signaling through GSIs or Delta-4 monoclonal antibodies decreased the number of CSC and/or their tumorigenicity in some preclinical models (Pannuti et al. 2010).

Drug Development

The Notch signaling pathway in various cancers can be targeted at various levels, including receptor-ligand binding, release of NICD, as well as the coactivator complex. A promising strategy to block receptor-ligand binding employs inhibitory antibodies directed against Jagged1 or DLL4. Blocking DLL4 led to dysfunctional neovascularization and inhibition of tumor growth. The most promising results have been achieved using small-molecule inhibitors of the γ-secretase complex (GSI) that prevented the release of

NICD. A phase I clinical trial using the GSI MK0752 inhibitor was initiated in 2005. The third protein component of the Notch signaling complex that may possibly provide a suitable drug target is the coactivator complex consisting of CSL, MAML, and CBP/p300. Small inhibitory peptides acting as dominant negative forms of MAML or CLS decrease the transcriptional activation of target genes (Koch and Radtke 2007).

Even targeting developmental pathways such as Notch, will most likely not give the elusive "magic bullet," and will require the development of rational drug combinations. Such cocktails will be made possible only through a thorough understanding of cross-talk between Notch and other developmental and nondevelopmental pathways that may play roles in CSCs in specific malignancies. Our knowledge is rapidly evolving, but there is evidence to support some combinations of treatments. The following examples are not meant to be all-inclusive. However, these classes of agents are reasonable candidates for combination with Notch inhibitors: (1) Inhibitors of the PI3-kinase-AKT-mTOR pathway; (2) ▶ NF-κB inhibitors; and (3) Her2/Neu inhibitors, platinum compounds, EGFR inhibitors, and Hedgehog inhibitors. In breast cancer, a newly discovered feedback loop between Notch and ERα supports combining Notch inhibitors with antiestrogens. Antiestrogens plus GSI and Hedgehog-inhibitors plus GSI combinations are being investigated in ongoing clinical trials. In the case of the Hedgehog inhibitor-GSI combination, anti-CSC effects are being specifically measured.

Ultimately, the best use of Notch inhibitors and other CSC-targeted agents will be in the context of personalized medicine. To that end, it must be determined: (1) which cancers and specific cancer subtypes contain Notch-dependent CSCs; (2) what role specific components of Notch signaling play in these CSCs; (3) what pathways cross-talk with Notch in specific CSCs; and (4) how Notch activity can be measured in CSCs from individual patients (e.g., in biopsy material).

The design of clinical trials for CSC-targeted agents will have to consider that anti-CSC effects will not necessarily translate into rapid tumor volume changes. Disease-free or recurrence-free survival will be the most informative endpoints. For situations when this would require prohibitively long follow-ups, it will be important to develop accurate surrogate biomarkers that reflect anti-CSC effects. These may include spheroid formation assays, flow cytometry, and molecular tests, but posttreatment tumor tissue will be required in most cases. A question of potentially great interest is whether it is possible to assess CSC numbers or the relative "stemness" of individual tumors by studying CTCs. These cells can be isolated from patient blood by several methods, one of which is US Food and Drug Administration approved. Although these trials may be challenging, the payoff may be novel treatments that eliminate or greatly reduce treatment resistance in a broad range of malignancies (Pannuti et al. 2010).

Notch and Genetic Diseases

Loss of function of Notch pathway components can cause inherited genetic diseases such as Alagille syndrome, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and spondylocostal dysostosis (SCD) (Fiuza and Arias 2007).

Mutations in the *Jagged1* gene are responsible for Alagille syndrome, which is normally diagnosed in the first 2 years of life. This is an autosomal dominant mutation that causes defects in bile duct formation leading to liver problems, and is also responsible for kidney, eye, heart, and skeleton development problems. The great diversity of the disease presentation suggests that other factors may influence the outcome, such as genetic properties of Notch signaling regulators.

Mutations in human Notch 1 and 3 are responsible for CADASIL syndrome. These mutations lead to an autosomal vascular disorder resulting in the loss of the arteriolar vascular smooth muscle cells that are substituted by granular eosinophilic material. One specific feature of CADASIL syndrome is its late onset around the age of 45 years. This disease is linked with a variety of symptoms ranging from migraines and subcortical ischemic strokes to progressive dementia and premature death.

SCD is a family of diseases that results in vertebral defects. Essentially, SCD is caused by mutations in Dll3 resulting in rib defects that lead to abnormalities in vertebral segmentation and trunk size. Understanding the mechanisms of Notch signaling regulation is crucial in the development of therapeutic approaches for the treatment of these diseases.

Notch and Other Diseases

Alzheimer's Disease

Notch is expressed by neurons in the adult brain where it is present at particularly high levels in the hippocampus. The prospect that Notch is the substrate of γ-secretase/presenilin and plays a role in learning and memory suggests a potential link between Notch signaling and the pathogenesis of Alzheimer's disease (Woo et al. 2009). In postmitotic neurons, Notch proteins interact with PSs (presenilins) and with APP (amyloid precursor protein), which have roles in the memory deficits associated with Alzheimer's disease. In some cases, mutations in the genes encoding APP, PS1, and PS2 are responsible for early-onset Alzheimer's disease. The phenotype of PS1 deletion mice is similar to that observed in Notch knockout mice. The PS1/PS2 double knockout phenotype is even more similar, suggesting closely related functions for these proteins. Indeed, knockouts of any one of several c-secretase components cause developmental abnormalities that are similar to those caused by Notch 1 and Notch 2 knockouts.

Glomerular Disease

Albuminuria associated with sclerosis of the glomerulus affects millions of people and leads to a progressive decline in renal function. Activation of the Notch pathway, which is critical to glomerular patterning, contributes to the development of glomerular disease. Expression of the intracellular domain of Notch1 (ICN1) was increased in glomerular epithelial cells in diabetic nephropathy and in focal segmental glomerulosclerosis. Conditional in vivo re-expression of ICN1 exclusively in podocytes caused proteinuria and glomerulosclerosis. In vitro and in vivo studies showed that ICN1 induced apoptosis of podocytes through the activation of p53. Genetic deletion of a Notch transcriptional partner (Rbpj) specifically in podocytes or pharmacological inhibition of the Notch pathway (with a c-secretase inhibitor) protected rats with proteinuric kidney diseases (Niranjan et al. 2008).

Connection to Other Signaling Pathways

The *Drosophila* Disheveled gene, which encodes a component of the Wingless signaling pathway, interacts antagonistically with Notch and one of its ligands, Delta. Notch1 activation induced p21 in differentiating mouse keratinocytes. The induction was associated with the targeting of Rbpjk (RBPSUH) to the p21 promoter. Notch1 also activated p21 through a calcineurin-dependent mechanism acting on the p21 TATA box-proximal region. Notch signaling through the calcineurin/▶ NFAT pathway also involved calcipressin and Hes1.

Oncogenic Ras activates Notch signaling. Wild-type Notch1 is necessary to maintain the neoplastic phenotype in Ras-transformed human cells in vitro and in vivo. The oncogenic effect of NOTCH1 on primary melanoma cells was mediated by ▶ beta-catenin, which was upregulated following NOTCH1 activation. Inhibiting ▶ beta-catenin expression reversed NOTCH1-enhanced tumor growth and metastasis.

Microarray studies of the mouse presomitic mesoderm transcriptome demonstrated that the segmentation clock drives the periodic expression of a large network of cyclic genes involved in cell signaling. Mutually exclusive activation of the Notch-fibroblast growth factor (▶ FGF) and Wnt pathways during each cycle suggested that coordinated regulation of these 3 pathways underlies the clock oscillator. Another study identified two clusters, the first cluster contains the known cyclic genes of the Notch pathway: Hes1, Hes5, Hey1, Id1, and Nrarp, a direct target of Notch signaling. In the same cluster as the Notch pathway were members of the FGF-MAPK pathway, including Spry2 and Dusp6. The second cluster of periodic

genes contained genes cycling in an opposite phase to the Notch- ▶ FGF cluster. This cluster included a majority of the cyclic genes associated with Wnt signaling, including Dkk1, c ▶ Myc, Axin2, Sp5, and ▶ Tnfrsf19.

NOTCH and MYC regulate two interconnected transcriptional programs containing common target genes that regulate cell growth in primary human T-cell lymphoblastic leukemias. In bone marrow progenitor cells and T-cell acute lymphoblastic leukemia (T-ALL) cell lines, constitutively active NOTCH1 transcriptionally activated the NFKB pathway via the IKK complex, thereby causing increased expression of NFKB target genes.

Expression of NOTCH1 in human keratinocytes was under the control of ▶ P53. NOTCH1 suppressed tumor formation through negative regulation of ROCK1/ROCK2 and MRCK-α (CDC42BPA), which are effectors of small RHO GTPases implicated in neoplastic progression.

Some T-ALL cells show resistance to γ-secretase inhibitors that act by blocking NOTCH1 activation. Using microarray analysis, ▶ PTEN was identified as the gene most consistently downregulated in γ-secretase inhibitor-resistant T-cell lines. Studies in normal mouse thymocytes indicated that Notch1 regulated ▶ Pten expression downstream.

Genes in the Notch pathway were expressed in mature podocytes in humans and in rodent models of diabetic nephropathy and focal segmental glomerulosclerosis.

Summary

Notch activity via cell–cell contacts generates molecular differences between adjacent cells. The Notch pathway can mediate both instructive and lateral signaling in neural differentiation and tumorigenesis. Many Notch regulatory processes have been identified, but are not yet truly characterized. Notch activity regulation by ligand inhibitory effects is well described, but its mechanism of action is still unclear. The role and mechanisms of Notch and ligand trafficking are not well understood. CSL-independent Notch signaling remains undefined, both as a molecular pathway and in its effects. Further work is necessary to understand Notch signaling in all its complexity, and to provide insight into how to tackle Notch signaling in a more specific way to better approach different clinical contexts.

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NOXA, PMAIP (Phorbol-Myristate-Acetate-Induced Protein), APR (Adult T Cell Leukemia-Derived PMA-Responsive)

► BCL-2 Family

Npn3

► Sulfiredoxin

NPR1 (Natriuretic Peptide Receptor 1)

► Natriuretic Peptide Receptor Type A (NPRA)

NPR2 (Natriuretic Peptide Receptor 2)

► Natriuretic Peptide Receptor Type B (NPRB)

NPR3 (Natriuretic Peptide Receptor 3)

► Natriuretic Peptide Receptor Type C (NPRC)

NPRA

► Natriuretic Peptide Receptor Type A (NPRA)

NPR-A

► Natriuretic Peptide Receptor Type A (NPRA)

NPRB

► Natriuretic Peptide Receptor Type B (NPRB)

NPRBi

► Natriuretic Peptide Receptor Type B (NPRB)

NPRC

► Natriuretic Peptide Receptor Type C (NPRC)

NPR-C

► Natriuretic Peptide Receptor Type C (NPRC)

NPT2a

► SLC34

NPT2b

► SLC34

NPT2c

► SLC34

Nr0b2

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Synonyms

Nuclear receptor subfamily 0 group B member 2; SHP; SHP1; Small heterodimer partner

Historical Background

The nuclear receptor small heterodimer partner (SHP, Nr0b2) was originally identified in 1996 as a transcriptional repressor based on its protein-protein interactions with several nuclear receptor superfamily members such as retinoid receptors, the thyroid hormone receptor, and the orphan receptor MB67 (Seol et al. 1996). The SHP gene consists of two exons and one intron located at human chromosome 1p36.11, mouse chromosome 4 D2.3, and rat chromosome 5q36. Since no endogenous ligand for SHP has been identified so for, SHP is classified as an orphan nuclear receptor. However, SHP is present in a variety of tissues. In mice, SHP is abundant in the gallbladder and liver while expressed at lower levels in the brainstem, cerebellum, adrenal, pancreas, stomach, duodenum, jejunum, ileum, colon, kidney, ovary, testis, and heart. In humans, SHP expression is detectable in the liver, heart, pancreas, kidney, spleen, small intestine, adrenal gland, and stomach. In humans, the loss of function mutations in the SHP gene increases the morbidity risk of obesity and type 2 diabetes in Japanese populations (Nishigori et al. 2001).

SHP Is a Transcription Repressor

SHP contains the dimerization and ligand-binding domain (LBD) common in other nuclear receptor family members but lacks the conserved DNA binding domain (DBD) (Seol et al. 1996). SHP binds to other nuclear receptors through two functional LXXLL-related motifs (also called nuclear receptor box) and acts as a transcriptional coregulator. To date, many nuclear receptors can interact with SHP proteins including liver receptor homolog-1 (LRH-1), hepatocyte nuclear factor 4 (HNF4), estrogen-related receptors (ERRs), liver X receptors (LXRs), peroxisome proliferator-activated receptors (PPARs), glucocorticoid receptor (GR), estrogen receptors (ERs), thyroid hormone receptor beta (TRβ), retinoic acid receptor α (RARα), farnesoid X receptors (FXRs), pregnane X receptors (PXRs), constitutive androstane receptors (CARs), androgen receptors (AR), nerve growth factor IB (NGFI-B, Nur77), and retinoid X receptors (RXRs) (Zhang et al. 2011).

Three mechanisms appear to explain the inhibitory function of SHP on the transcriptional regulation of nuclear receptor target genes. In the first proposed mechanism, the binding of SHP to nuclear receptors induces a direct competition for coactivators such as p300 and cAMP response element-binding protein (CREB) binding to nuclear receptors. This manner of SHP-mediated transcriptional inhibition appears pronounced in ERs, RXRs, LRH-1, HNF4, ARs, LXRs, ERRs, GRs, Nur77, and FoxO1 induced transcription. In the second proposed mechanism, the binding of SHP to nuclear receptors triggers the recruitment of corepressors to DNA. For example, the SHP protein recruits a common repressor E1A-like inhibitor of differentiation 1 (EID1) to DNA. Recently, a conserved EID1 binding site at the N-terminus of SHP protein was identified, where EID1 mimics helix H1 and becomes an integral part for the SHP protein LBD fold (Zhi et al. 2014). The identification of the SHP-EID1 complex and integral structural motifs is important as it reveals a protein interface that regulates SHP repressive function. The binding of SHP to nuclear receptors in the third proposed mechanism induces the dissociation of the SHP-nuclear receptors complex from the DNA. For instance, the interaction between SHP and nuclear receptor RAR results in the inhibition of RAR-RXR heterodimer and RAR-PXR heterodimer binding

to DNA. Interestingly, most studies have revealed SHP as a transcriptional repressor; however, SHP activates nuclear factor-κB (NF-κB) and upregulates the transcriptional activity of PPARγ.

SHP in Bile Acid Synthesis

Bile acids are the end products of cholesterol catabolism in the liver and account for 50% of the daily turnover of cholesterol. The synthesis of bile acids requires the coordinated actions of many enzymes in the hepatocytes including the cytochrome P450 enzyme cholesterol 7 α-hydroxylase (CYP7A1)-initiated neutral pathway in the endoplasmic reticulum and the sterol 27-hydroxylase (CYP27A1)-initiated alternative acidic pathway in the mitochondria. After synthesis, bile acids are secreted into the gallbladder for storage and mixed with phospholipids and cholesterol. Upon ingestion of a meal, cholecystokinin from the small intestine induces gallbladder contraction, releasing micelle bile acids into the intestine to aid food digestion and absorption of fats and lipid-soluble vitamins. Approximately 95% of bile acids are recycled via portal circulation by which bile is reabsorbed from the distal ileum and transported back into the liver; only 5% of bile acid is eliminated in the feces. This small amount of loss is replenished via de novo synthesis of bile acids in the liver.

Bile acids are important signaling molecules in the regulation of lipid, glucose, and energy metabolism. However, because of detergent-like toxic properties, excessive accumulation of bile acids can cause cell damage leading to inflammation and fibrosis in gastrointestinal tract. Therefore, bile acid homeostasis is tightly controlled by a coordinated regulation of genes in bile acid biosynthesis, uptake, and efflux in the liver and ileum. SHP has been implicated as a key repressive regulator in all of these processes. For instance, bile acids bind to the bile acid receptor, FXR, leading to the induction of SHP which in turn represses LRH-1 and HNF4α activation of CYP7A1, resulting in the repression of bile acid synthesis (Goodwin et al. 2000; Lu et al. 2000). In this process, SHP coordinately recruits chromatin-modifying enzymes such as mSin3A-Swi/Snf chromatin remodeling complex, histone deacetylase SIRT1, and G9a methyltransferase to the CYP7A1 promoter, leading to the transcriptional repression of CYP7A1. Additional studies show that both the c-Jun N-terminal kinase (JNK) and PXR pathways are involved in CYP repression by bile acids in *Shp*-deficient animals (Wang et al. 2002). In addition, bile acid-activated FXR also induces expression of FGF15/19 (FGF15 is the mouse homolog of human FGF19) in the small intestine and the secreted FGF15 represses hepatic CYP7A1 transcription through FGF receptor 4 (FGFR4) (Inagaki et al. 2005). These studies demonstrate that the gut-liver signaling pathway regulated by FXR/FGF15/FGFR4 synergizes with liver FXR/SHP in the maintenance of bile acid homeostasis. Moreover, studies show that bile acids regulate posttranslational modifications of SHP protein, including protein phosphorylation and ubiquitination. Recently, Kim et al. found that the nucleoporin ran-binding protein 2 (RanBP2/Nup358) colocalizes with SHP at the nuclear envelope region and mediates SUMO2 modification of SHP protein at K68 in response to bile acids. Such modification facilitates SHP protein nuclear transportation and interaction with repressive histone modifiers to inhibit BA synthetic genes (Kim et al. 2016). Taken together, these studies indicate that SHP is a critical transcriptional repressor for bile acid synthesis and has an indispensable role in maintaining bile acid homeostasis.

SHP in Lipid Metabolism

Lipid production and clearance are also tightly controlled by a complex network of transcriptional programs regulated by a large number of nuclear receptor family members, many of which SHP interacts with. For instance, SHP augments nuclear receptor PPARγ transactivation and induces hepatic lipid accumulation. Transgenic mice with hepatocyte-specific SHP overexpression display increased hepatic lipid accumulation which is mediated by the increase of fatty acid and triglyceride biosynthesis (Boulias

et al. 2005). In line with these observations, deletion of SHP in obese leptin-deficient mice (ob/ob) prevents the development of nonalcoholic fatty liver through both increasing lipid secretion and decreasing de novo fatty acid synthesis and uptake (Huang et al. 2007). On the other hand, a dominant role of SHP in lipid synthesis has been reported as well. For example, bile acids and FXR agonists inhibit lipogenic gene SREBP-1c expression which is mediated through the upregulation of SHP transcription. Another study shows LRH-1 stimulates the transcription of fatty acid synthesis gene, FAS, and this response is completely blocked by SHP overexpression (Matsukuma et al. 2007). The SHP gene promoter contains an E box (CACGTG) element that can be activated by core circadian clock components CLOCK-BMAL1 (circadian locomotor output cycles kaput, brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1) to maintain the rhythmic expression of SHP in the liver. Microsomal triglyceride transfer protein (MTP) is a dedicated chaperone for biosynthesis of apolipoprotein B (apoB) that is required for the secretion of very low-density lipoprotein (VLDL) into the circulation. CLOCK and SHP coordinately regulate circadian changes of MTP that contributes to plasma lipid circadian rhythmicity (Pan et al. 2010). Disruptions in circadian rhythms might interfere with SHP regulation on MTP and, subsequently, induce dyslipidemia. These findings help to explain why night shift workers have increased risk for developing metabolic syndrome. In summary, these studies establish a critical role of SHP in modulating hepatic lipid synthesis and transport.

SHP in Glucose Metabolism

Glucose production and usage is mainly controlled by the balanced secretion and actions of insulin, glucagon, epinephrine, cortisol, and growth hormone. Accumulated evidence indicates SHP has a major function in inhibiting hepatic gluconeogenesis. Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) are two critical enzymes in hepatic gluconeogenesis. SHP is demonstrated to repress G6Pase and PEPCK gene expression through direct protein interactions with multiple nuclear receptors and transcriptional factors including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), hepatocyte nuclear factor-3 (HNF3), HNF6, and C/EBPα (CCAAT/enhancer-binding protein alpha) (Park et al. 2007). SHP is also involved in the regulation of glucose metabolism by transcription factor forkhead box protein O1 (FOXO1), the basic helix-loop-helix protein BETA2/NeuroD, and the aryl hydrocarbon receptor (AHR)/nuclear translocator (ARNT). In addition, bile acids inhibit the expression of G6Pase, PEPCK, and fructose 1, 6-bis phosphatase (FBP1) through the FXR-SHP nuclear receptor cascade, and the absence of this repression was observed in $Fxr^{-/-}$ and $Shp^{-/-}$ mice. These findings suggest that bile acid-associated FXR-SHP nuclear receptor cascade could be a novel target in treating insulin resistance and type 2 diabetes. Metformin is an antidiabetic drug widely used for the treatment of type 2 diabetes. Metformin induces SHP expression through AMP-activated protein kinase (AMPK) pathway, and the activation of SHP subsequently inhibits PEPCK and G6Pase gene expression, which suggests that SHP is indispensable for anti-diabetes function of metformin.

SHP in Inflammation

For the first time, in 2007, Li et al. introduced a link between SHP and anti-inflammation. In the study, FXR ligand, GW4064, was found to increase SHP expression and inhibit interleukin IL-1β-induced production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in vascular smooth muscle cells (Li et al. 2007). Since then, additional studies contributed to elucidate the association of SHP and inflammation suppression. For instance, SHP has been found to inhibit toll-like receptor (TLR)-induced inflammatory responses in innate immune cells through a biphasic interaction with two cytoplasmic partners of TLR4 pathway, TRAF6 and NF-κB subunit p65 (Yuk

et al. 2011). Recently, SHP was shown to directly affect the inflammasome, a large multimeric protein complex important for the activation of caspase-1 and maturation of the proinflammatory cytokines interleukin (IL)-1β and IL-18. SHP directly binds to the NLRP3 inflammasome and negatively regulates its activation to prevent excessive inflammatory responses (Yang et al. 2015). These studies indicate that SHP may also play an important role in the inhibition of inflammation.

SHP in Cancer

Accumulating evidence points to the involvement of SHP in the development of cancers, particularly hepatocellular carcinoma (HCC). In human HCC specimens, the expression of SHP is markedly diminished due to the SHP promoter hypermethylation-induced epigenetic silencing (He et al. 2008). Furthermore, an association study on SHP expression and HCC patient survival reveals SHP as a positive prognostic factor. Particularly, HCC patients with low SHP expression combined with a high expression of CDK4, MCM5, EXOCS1, CCNB1, BUB3, or BCL2L2 indicate a poor prognosis with a low chance of survival. Additional studies in $Shp^{-/-}$ mice demonstrate that SHP has a tumor suppressor function. For instance, $Shp^{-/-}$ mice at 12–15 months of age develop spontaneous HCC, which is strongly associated with increases of cyclin D1 expression and cell proliferation (Zhang et al. 2008). Yes-associated protein (YAP) plays a critical role in promoting hepatocyte proliferation and survival during embryonic liver development and hepatocellular carcinogenesis. YAP activation associates with spontaneous liver cancer formation in $Fxr^{-/-}Shp^{-/-}$ double knockout mice. Additional studies show that SHP acts as a pivotal cell death receptor that targets mitochondria and binds to antiapoptotic protein Bcl-2. Such binding, consequently, leads to the disruption of Bcl-2/Bid interaction and cytochrome C release, resulting in the activation of apoptosis. Therefore, loss of SHP in liver cancer results in defective apoptosis that enhances liver cancer development.

DNA methyltransferase 1 (DNMT1) plays a critical role in maintaining CpG methylation. DNMT1 upregulation and its associated aberrant gene silencing of tumor suppressors are frequently observed in human HCC. Recent studies revealed SHP as a potent repressor of DNMT1 transcription either through directly inhibiting nuclear receptor ERRγ-induced DNMT1 transcription or through indirectly inhibiting DNMT1 expression by antagonizing metal-responsive transcription factor-1 (MTF-1) (Zhang et al. 2012). These studies demonstrate that loss of SHP leads to the upregulation of DNMT1 that could further result in aberrant tumor suppressor gene silencing in HCC and drive the HCC development. SHP is also downregulated in many types of cancer, including adrenal cortex, cerebellum, kidney, skin, and thyroid, suggesting that SHP may function as a common tumor suppressor.

Summary

SHP is originally identified as a unique nuclear receptor that contains a putative ligand-binding domain but lacks a DNA binding domain. Thus, the transcriptional corepressor relies heavily on protein-protein interactions with multiple nuclear receptors and transcriptional factors to regulate diverse metabolic processes including bile acid synthesis, cholesterol and lipid metabolism, and glucose homeostasis. No endogenous ligand of SHP is known so far. However, bile acid-mediated activation of FXR or FGF15/FGF19 signaling induces SHP expression and increases its protein stability. The loss of function mutation of SHP has been linked to an increased morbidity risk of obesity and diabetes in Japanese populations. Connections between SHP and major regulatory pathways such as inflammation and apoptosis are still being elucidated. Particularly, the antitumor role of SHP in liver cancer development is well addressed in that it is now known that the SHP gene is hypermethylated and silenced in human hepatocellular carcinoma. Such a decrease in SHP

leads to upregulation of DNMT1 that further enhances the methylation and silencing of SHP and other tumor suppressor genes. Additionally, the long-term repression of SHP induces cell proliferation through the activation of CyclinD1 and defective apoptosis through loss of inhibition on antiapoptotic protein, BCL2. Furthermore, the loss of SHP in hepatocytes results in increased bile acid levels leading to YAP activation, which also drives liver cancer development. The accumulating insights of SHP and its numerous roles in overall homeostasis reveal SHP may be a promising therapeutic target of several metabolic diseases and liver cancer. However, major challenges for future research such as identifying endogenous SHP ligands and developing specific synthetic SHP ligands for pharmaceutical applications still remain.

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NR1B1

▶ Retinoic Acid Receptors (RARA, RARB, and RARC)

NR1B2

► Retinoic Acid Receptors (RARA, RARB, and RARC)

NR1B3

► Retinoic Acid Receptors (RARA, RARB, and RARC)

NR1C1

► Peroxisome Proliferator-Activated Receptor (PPAR)

NR1C1 (Nuclear Receptor Subfamily 1, Group C, Member 1)

► Peroxisome Proliferator-Activated Receptor Alpha (PPAR-Alpha)

NR1C2

► Peroxisome Proliferator-Activated Receptor (PPAR)

NR1C3

► Peroxisome Proliferator-Activated Receptor (PPAR)

NR3C2

► Mineralocorticoid Receptor

NR3C2VIT

► Mineralocorticoid Receptor

NR3C4 (Nuclear Receptor Subfamily 3, Group C, Member 4)

► Androgen Receptor (AR)

NR4

► IL-4 and IL-13 Receptors

NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

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Synonyms

HZF-3; Immediate-early response protein NOT; NGFI-B/Nur77 beta-type transcription factor homolog; NOT; Nuclear receptor related 1; Nuclear receptor subfamily 4 group A member 2; Nurr1; Orphan nuclear receptor NURR1; RNR1; T-cell nuclear receptor NOT; TINUR; Transcriptionally inducible nuclear receptor; Transcriptionally inducible nuclear receptor related

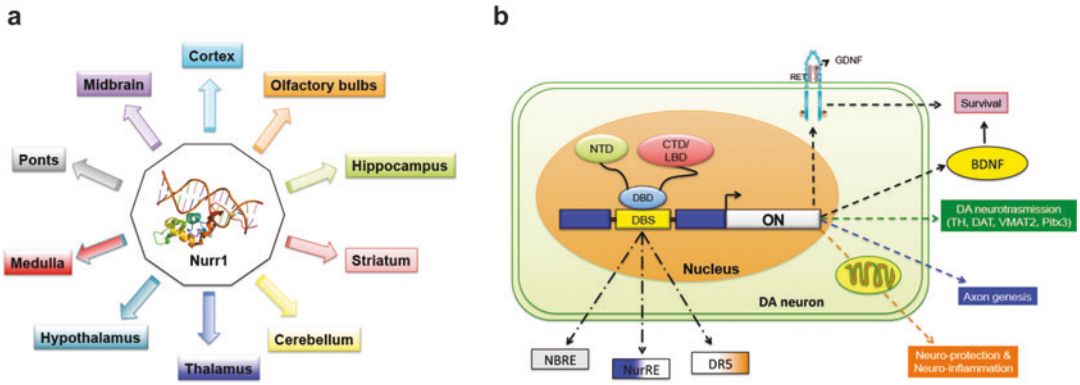
Historical Background

Orphan nuclear receptor Nurr1, also known as nuclear receptor subfamily 4, group A, member 2 (NR4A2), together with Nur77 (NR4A1) and Nor1 (NR4A3), is a member of the steroid/thyroid hormone nuclear receptor superfamily. Unlike the other nuclear receptors, Nurr1 is an immediate early gene, and its transcription can be induced by various stimuli such as depolarization, cAMP, inflammation, hormones, calcium, and growth factors (Volpicelli et al. 2004). Structurally, Nurr1 lacks a hydrophobic pocket for ligand binding and might function as ligand-independent nuclear receptor (Wang et al. 2016). The transcriptional regions of Nurr1 (AF1 and AF2, respectively) are localized at the N- and C-terminal, and the DNA-binding domain (DBD) is localized centrally, while the ligand-binding domain (LBD) is in the C-terminal part of the protein. The DBD is highly conserved among the nuclear receptor family members and is composed of two zinc finger modules able to bind nerve growth factor-inducible ß-binding response element (NBRE) as monomer or homodimer, Nurr response element as homodimer (NurRE), or DNA response elements composed of direct repeats spaced by five nucleotides (DR5) as heterodimer with retinoid X receptor (RXR).

NR4A2 is widely expressed throughout the brain and is present in telencephalic structures such as the cortex and hippocampus, although it is most well studied for its effects on dopaminergic (DA) neurons (Fig. 1a).

In mice, *Nurr1* expression is detected in the ventral midbrain at embryonic day 10 (E10), and its expression is reduced in the postnatal stages but is maintained into adulthood. It has been shown to regulate several aspects of post-mitotic development. It regulates key genes involved in dopaminergic (DA) neurotransmission, including tyrosine hydroxylase (TH), vesicular monoamine transporter 2 (VMAT2), DA transporter (DAT), bicoid-related Pitx3 (Volpicelli et al. 2012) RET, which encodes for the coreceptor for the glial cell line-derived neurotrophic factor (GDNF) family associated to the GDNF family receptor alpha (GFRas), neuropilin, and brain-derived neurotrophic factor (Fig.1b; BDNF, Volpicelli et al. 2007).

Nurr1 null mice die soon after birth for agenesis of mesDA neurons (Zetterstrom et al. 1997).



NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2), Fig. 1 (**a**) Nurr1 expression in adult mouse brain areas. (**b**) Schematic representation of Nurr1 structure e functions. Nurr1 presents centrally the DNA-binding domain (*DBD*) and the N- and C-terminal domain, respectively. The DBD is able to bind nerve growth factor-inducible ß-binding response element (*NBRE*) as monomer or homodimer, Nur response element as homodimer (*NurRE*), or DNA response elements composed of direct repeats spaced by five nucleotides (DR5) as heterodimer with retinoid X receptor (*RXR*). Nurr1 protein promotes survival by RET witch encodes for the coreceptor for the glial cell line-derived neurotrophic factor (*GDNF*) family associated to the GDNF family receptor alpha (*GFRas*), and brain-derived neurotrophic factor (*BDNF*) regulates genes involved in DA neurotransmission such as tyrosine hydroxylase (*TH*), vesicular monoamine transporter 2 (VMAT2), dopamine transporter (*DAT*), and bicoid-related Pitx3 or influences axon genesis and has a role in neuroinflammation and neuroprotection

Brains of heterozygous animals ($Nurr1^{+/-}$), apparently healthy, contained reduced dopamine levels and seem to have an increased susceptibility to toxic stress including mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the proteasome inhibitor lactacystin and methamphetamine. Conditional gene targeting of *Nurr1* in mice, both in late-differentiating DA neurons and in adult brain, has provided definitive evidence of a *Nurr1* role in the maintenance and survival of DA neurons. Briefly, *Nurr1* ablation at late stages of mDA neuron development by crossing Nurr1 with mice carrying Cre under control of the DAT locus showed a rapid loss of striatal DA, loss of mDA neuron markers, and neuron degeneration. Instead, Nurr1 mice, conditionally ablated in adult DA neurons by tamoxifen treatment of conditional Nurr1 gene-targeted mice expressing the $CreER^{T2}$ enzyme under the *DAT* gene regulatory sequences, exhibit progressive DA pathology associated with modest reduction of DA neuronal markers in ventral midbrain and striatum, reduced striatal DA, impaired motor behaviors, and dystrophic axons and dendrites, a hall-marker in early human PD (Cheng et al. 2010). Thus, in mice the adult ablation of Nurr1 recapitulates early features of PD and supports the idea that loss of function of Nurr1 might contribute to PD.

Nurr1 and Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by the progressive loss of DA neurons in the *substantia nigra pars compacta* and striatal deficiency (Decressac et al. 2013). The main motor and non-motor symptoms of PD, including bradykinesia, rigidity, resting tremor, and postural instability, seriously impair the patient's quality of life. Although the pathogenesis of PD is not still clearly understood, it is known that environmental and genetic factors contribute to PD. It is already known that the exposure to environmental toxins, such as MPTP, paraquat, and rotenone has been found to increase the risk of developing PD. Until now, at least 15 causal genes have been identified to be related to PD, such as α-synuclein, parkin, LARRK2, PINK, and DJ1 and Nurr1 (Verstraeten et al. 2015). PD is characterized by the presence of Lewy bodies containing aggregated and misfolded α-synuclein in patients' nigral DA neurons. In addition, mitochondrial dysfunction, reactive oxygen species, neuroinflammation, and autophagy or proteasome system impairment are considered as major pathogenic contributors to PD.

Even if mutations in *Nurr1* have not been identified as major genetic risk factors for PD, patients with familiar and sporadic PD polymorphism (7048-7049insG) in intron 6 of *Nurr1*, affecting *Nurr1* splicing, have been found (Zheng et al. 2003; Liu et al. 2012). In addition, in sporadic PD patients, three other variations in exon 1 (−253C>T, −223C>T, and –309C>T) were found. Even in familial PD, two mutations (−291delT and –245 T>G) in the noncoding exon 1 within the 5′untranslated region of NURR1 have been reported. Only one coding missense mutation in exon 3 of Nurr1 (709C>G) has been identified in a patient with nonfamilial PD. This mutation markedly attenuates Nurr1-induced transcriptional activation.

Downregulation of Nurr1 expression has been found in postmortem human brain tissue of sporadic PD patients with α-synuclein inclusions specifically in SN of DA neurons. In addition, an age-related decline of the number of Nurr1-expressing DA neurons has been noted in the aging human brain. In addition, data from conditional knockout mice seem to indicate *Nurr1* expression could be linked to development and progression of PD pathology and, in particular, in the development of early functional and degenerative changes that are seen in affected DA neurons. Interestingly, Nurr1 was also identified as a potential peripheral PD biomarker downregulated in peripheral blood lymphocytes of PD patients, independent of medication, disease severity, or duration. These observations deserve further exploration in large cohorts of well-characterized patients, particularly those in early stage of PD.

Recent work shows that the modulation of *Nurr1* expression could be used as an effective

treatment in PD. Given the dual role of *Nurr1* in both the development and maintenance of mDA neurons and protection from death induced by inflammation, Kim and collaborators (Kim et al. 2015) found that two antimalarial drugs, amodiaquine and chloroquine, stimulate the transcriptional function of Nurr1 through physical interaction with its ligand-binding domain. The two compounds, administered to 6-hydroxydopamine lesioned rats, a model of PD, significantly improved behavioral deficits without any detectable signs of dyskinesia. However, since these antimalaric drugs are widely used, a survey on PD patients that have used these drugs is still lacking, to confirm if the benefits in humans are similar to those reported in the rat model.

A new *Nurr1* transactivator, IRX4204 retinoid X receptor (RXR) agonist, activates cellular RXR-Nurr1 signaling and promotes substantia nigra DA neuron survival *in vitro*, suggesting that it could be used in PD prevention and/or treatment.

Nurr1 Function in Axon Genesis

Topoisomerase IIβ (*Top IIβ*) is downregulated in Nurr1 null mice. *Top IIβ* promoter shows two binding sites for Nurr1. Suppression of *Top IIβ* expression in mesencephalic cultures affects DA neuron neurite elongation and collapses the growth cone. Therefore, *Nurr1* might influence the processes of axon genesis in dopaminergic neurons via the regulation of *Top IIβ* expression (Xin Heng et al. 2012).

Nurr1 Neuroprotection and Inflammation

Excitotoxicity, oxidative stress, and mitochondrial dysfunction have been implicated in the pathology of many neurodegenerative disorders including PD (Decressac et al. 2013). An important strategy for neuroprotection is the regulation of the transcriptional expression of neuroprotective genes, including anti-apoptotic factors and scavengers of reactive oxygen species (ROS). In response to pathophysiological effectors such as hypoxia, oxidative stress, excitotoxicity, and ischemia, the transcription factor cyclic AMP-responsive element-binding protein (CREB) is activated. CREB-induced transcription factors or cofactors may contribute to activate a gene expression program essential for neuronal protection.

In the CNS, pathological stimuli such as ischemia, seizures, and focal brain injury, associated with CREB activation, robustly induce Nurr1 expression. Thus, in neurons exposed to excitotoxic and oxidative stress Nurr1 is a mediator of CREB-dependent neuroprotective responses and is also a regulator of neuroprotective genes. Moreover, synaptic *N*-methyl-D-aspartate (NMDA) receptor activation induces CREB-mediated neuroprotection and leads to upregulation of Nurr1.

Thus, Nurr1 might be important for DA neuron maintenance and survival via neurotrophic signaling regulation. Indeed, NMDA receptor-induced *Nurr1* expression promotes survival effect by binding BDNF promoter (Barneda-Zahonero et al. 2012; Volpicelli et al. 2007). In addition, the expression of the GDNF tyrosine kinase receptor RET also depends on Nurr1 during development. Conditional ablation of GDNF, RET, or Nurr1 in mice results in a progressive pathological changes resembling to early stages of PD, suggesting that the preservation of GDNF-RET-Nurr1 pathway is important for nigral DA neuron integrity and function. Data published by Decressac and co-workers demonstrate that α-synuclein might interfere with GDNF signaling via downregulation of Nurr1 and its transcriptional target RET, not only in α-synuclein overexpression models but also in human PD. Nurr1 overexpression was able to restore GDNF signaling, affected in α-synuclein-overexpressing DA neurons and provides protection of nigral DA neurons against α-synuclein toxicity, also in the absence of exogenous GDNF (Decressac et al. 2012).

Nurr1 seems to have both constitutive and inducible anti-inflammatory activity in monocyte/macrophage lineage immune cells, as well as in astrocytes and microglia, where it functions to mitigate the release of pro-inflammatory cytokines and neurotoxic factors. The anti-

inflammatory activity is regulated toward the NFkB signaling pathway in response to inflammatory stimuli such as tumor necrosis factor (TNF-α) and bacterial lipopolysaccharide (LPS; Saijo et al. 2009).

In brain glial cells, the anti-inflammatory effects of Nurr1 are mediated by docking to NFkB-p65 on target inflammatory gene promoters, followed by recruitment of the CoREST corepressor complex, resulting in clearance of NFkB-p65 and transcriptional repression. Since microglia activation and the increased levels of pro-inflammatory mediators might contribute to PD pathology, these studies suggest that Nurr1 protects against neuronal loss by limiting the production of neurotoxic mediators by microglia and astrocytes.

However, Nurr1 also may play a pro-inflammatory role. It is expressed at elevated levels in inflamed joint tissues from patients with arthritis. Nurr1 expression can be induced by inflammatory mediators in resident and infiltrating immune cells and promotes synovial hyperplasia by increasing proliferation of synoviocytes and inducing transcription of matrix-degrading enzymes and pro-inflammatory mediators (McCoy et al. 2015). In fact, an increased expression of Nurr1 in the synovium of patients with rheumatoid arthritis has been shown, suggesting a pro-inflammatory role for Nurr1 in the pathogenesis of rheumatoid arthritis (Murphy et al. 2001).

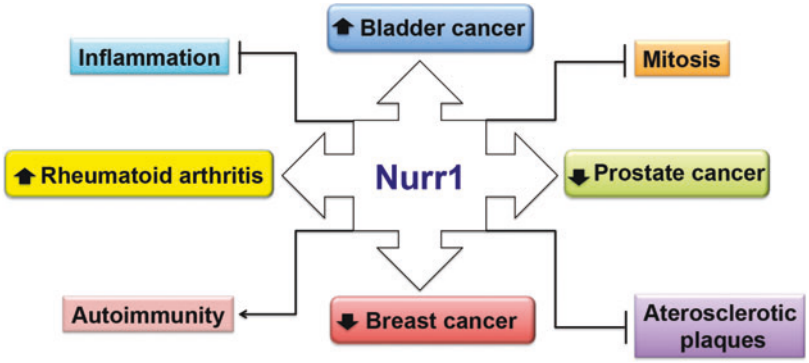
Nurr1 also influences maturation and differentiation of Th17 T-cells and plays a role in autoimmunity and in resolution of infections (Raveney et al. 2013). Thus, Nurr1, according to the context and to the pathological model, appears to play a dual role (i.e. pro- or anti-inflammatory) in the resolution of inflammatory signaling both in activated immune cells and glial cells.

Nurr1 and Cancer

In addition to the well-known role of Nurr1 in the differentiation, maturation, and maintenance of midbrain DA neurons, an involvement of Nurr1 in cancer was recently discovered. High levels of cytoplasmic Nurr1 correlate with high tumor grade, decreased survival, and increased distant metastasis in a cohort of bladder cancer patients. Immunohistochemical analyses of human prostate cancer biopsies indicated that expression of Nurr1 was significantly higher than in normal controls, suggesting a relationship between expression of Nurr1 and tumor growth. Differently, Nurr1 is upregulated in normal breast epithelium compared to breast cancer cells, suggesting an inverse correlation between breast cancer and Nurr1 expression (Safe et al. 2016). At molecular level, Nurr1 can also interact with p53 and inhibits p53-dependent apoptosis by inhibiting transactivation. p53 plays a major role in determining cell cycle progression, DNA repair, and apoptosis. In response to a stress stimulus, such as DNA damage, p53 is quickly induced. In primary breast cancer tissues Nurr1 interacts with the C-terminal domain of p53 and regulates critical p53-dependent signaling. Likewise, silencing of Nurr1 expression *in vitro* in prostate cancer cells reduced cell proliferation, invasion, and migration, indicating that Nurr1 could be a biomarker for the progression of breast and prostate cancer (Ranhotra 2015). Recent studies also show that Nurr1 expression predicts poor survival and drug resistance in colon and gastric cancer patients. Together with NR4A1 (Nur77), Nurr1 can act as tumor suppressors in hematologic neoplasms, such as acute myeloid leukemia, and a low NR4A1 and NR4A3 were described in aggressive lymphomas and associated with poor overall survival (Wenzl et al. 2015, Fig. 2).

Nurr1 and Cardiovascular Disease

Atherosclerosis is characterized by hardening of arteries with the formation of plaques that compromise normal blood flow. Atherosclerotic plaques contain cholesterol, calcium deposits, and fat and stimulate a proliferative response in smooth muscle cells within the media of arteries with further constrictions to blood flow leading to myocardial infarction, stroke, and death. The macrophages release cytokines and growth factors and aggravate the local inflammation and lead to



NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2), Fig. 2 Nurr1 protein is upregulated in bladder cancer and rheumatoid arthritis, is downregulated in prostate cancer and breast cancer, instead have anti-mitogenic and anti-inflammatory effects, its upregulation blocks the atherosclerotic plaques formation and plays a role in autoimmunity and in resolution of infections

excessive uptake of lipids and the transition of macrophages into lipid-laden foam cells that remain resident in the atherosclerotic lesion. Nurr1 seems to have an anti-mitogenic effect in smooth muscle cells antagonizing atherosclerotic plaques formation by inhibition of NFκB-dependent expression of inflammatory genes in macrophages. In addition, Nurr1 is negatively regulated by miR-145 in smooth muscle cells, and mice lacking miR-145 are resistant to the development of atherosclerotic plaques, owing to their high expression of Nurr1. Studies in human macrophages using lentiviral-mediated overexpression or silencing of Nurr1 demonstrated that Nurr1 inhibited the uptake of oxidized LDL by macrophages and reduced the expression of pro-inflammatory cytokines and chemokines supporting the role of Nurr1 in protection against cardiovascular disease (Safe et al. 2016) (Fig. 2).

Summary

Nurr1 is a member of the steroid-thyroid hormone nuclear receptor superfamily widely expressed throughout the brain. Its expression is detectable in telencephalic structures such as cortex and hippocampus, although it is well studied in midbrain dopaminergic neurons. In fact, Nurr1 regulates key genes involved in DA neurotransmission such as TH, DAT, VMAT2, Pitx3, and RET, and via the regulation of *Top IIβ* expression, it might influence the processes of axon genesis in dopaminergic neurons. Mutations in this gene have been associated with disorders related to dopaminergic dysfunction, including Parkinson's disease, schizophrenia, and manic depression. In addition to its role in DA neurotransmission, Nurr1 appears to be important in the regulation of inflammation and resolution of inflammatory signaling both in activated immune cells and glial cells. Furthermore, Nurr1 was found to be a prognostic factor for high tumor grade, decreased survival, and increased distant metastasis in bladder cancer, breast cancer, and prostatic cancer. It seems to have an anti-mitogenic effect in smooth muscle cells antagonizing atherosclerotic plaques formation.

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NR5a1

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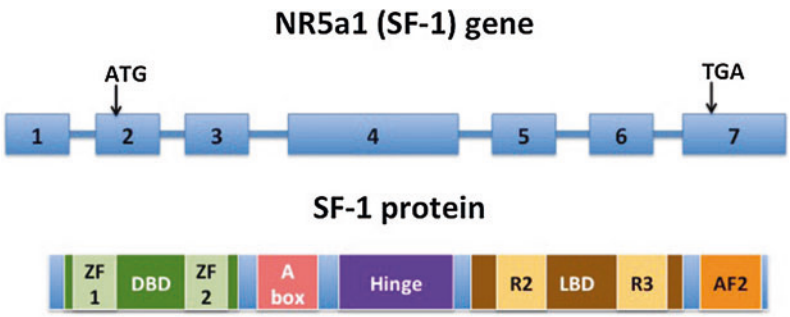
Synonyms

Adrenal 4 binding protein (Ad4BP); Steroidogenic factor 1 (SF-1)

Historical Background

Steroidogenic factor 1 (SF-1), also called Adrenal 4 binding protein (Ad4BP), was originally discovered in the early 1990s by Drs. Parker and Morohashi as a regulator of steroid hydroxylases in adrenal glands (Lala et al. 1992; Morohashi et al. 1992). It is a transcription factor, member of the nuclear receptors superfamily. Official designation of the gene encoding for SF-1 is *NR5a1*, although in the literature the name *SF-1* is still predominantly used, and therefore, we will use this designation throughout this chapter.

Numerous studies have shown wide role of SF-1 in the endocrine development and function beyond regulation of steroid hydroxylases. In



NR5a1, Fig. 1 Structure of *SF-1* (NR5a1) gene (*upper panel*) and protein (*lower panel*); ZF zinc finger, DBD DNA-binding domain, LBD ligand-binding domain, R2 and R3 highly conserved regions within ligand-binding domain, AF activational domain; proline-rich region is within hinge region).

particularly, studies with SF-1 knockout (SF-1 KO) mice have shown the importance of SF-1 for adrenal and gonadal development, development of the ventromedial nucleus of the hypothalamus (VMH), and spleen and pituitary gonadotropin-producing (gonadotrope) cells. Different studies have revealed that SF-1 does not regulate only the expression of genes encoding for steroid hydroxylases, but many other genes such as antimullerian hormone (AMH), gonadotropin-releasing hormone (GnRH) receptor, common alpha subunit of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), brain-derived neurotrophic factor (BDNF), and many others (reviewed in Schimmer and White 2010).

SF-1 Structure

SF-1 (*NR5a1*) gene resides on the chromosome 9 in humans and chromosome 2 in mice. Gene encodes for a protein that is 461 amino acids long in humans and 462 amino acids long in mice, and the gene is comprised of seven exons (Ninomiya et al. 1995). SF-1 protein is structured similarly to other nuclear hormone receptors. It has a modular domain structure with a zinc finger DNA-binding domain (DBD) at its N-terminal, a ligand-binding domain, AF-2 activation domain at its C-terminal, and an intervening proline-rich domain within hinge region. Within this proline-rich domain is also an AF-1-like region that has activational activity. Immediately proximately to the DBD lies an A box, also called fushi tarazu factor 1 box, which is believed to mediate specific binding of SF-1 protein to the hexamer target sequence of SF-1 (Schimmer and White 2010) (Fig. 1).

Regulation of SF-1 Expression

Tissue-Specific Expression

In adult tissues, SF-1 is primarily expressed in steroidogenic cells such as adrenal cortex, testicular Leydig cells, ovarian theca, and granulosa cells and at lower levels in corpus luteum. However, SF-1 is also expressed at some other, non-steroidogenic cells and tissues such as testicular Sertoli cells, VMH, pituitary gonadotrope cells, and spleen. In the fetal tissues, SF-1 is initially expressed in the adrenal and gonadal primordia and serves as an early marker for developing gonads and adrenal glands. SF-1 is also expressed in the fetal spleen and in the prosencephalon, a region of developing brain that includes the hypothalamic primordium (Morohashi et al. 1995; Majdic and Saunders 1996; Hanley et al. 2000a).

Transcriptional Regulation of Expression

Regulation of SF-1 expression is not completely understood, yet. Proximal promoter of SF-1 gene contains SOX binding site, E-box, Sp1/Sp3 site, and CCAAT box (Nomura et al. 1995; Scherrer et al. 2002; Shen and Ingraham 2002). Further upstream and downstream, there are binding sites for other transcriptional regulators such as GATA-4, WT1, and Lhx9, and studies in vitro have shown a potential role of SOX15, SOX30,

TEAD-4, and CBX2 proteins in the regulation of SF-1 expression. It is believed that variety of binding sites and transcriptional factors binding to the SF-1 promoter regions are needed to ensure tissue specificity of SF-1 expression (Schimmer and White 2010). However, in vivo studies have shown that these promoter regions are not sufficient to drive the full expression of SF-1 in all tissues that physiologically express this gene. A very large fragment of genomic DNA, flanking the *Sf-1* gene is needed to recapitulate endogenous expression of SF-1 in GFP transgenic mice. This, about 5 KB long fragment extending from exon 2 of the *Sf-1* gene into the upstream gene *NR6A1* (germ cell nuclear factor, GCNF), is able to induce gene expression in Leydig cells, theca cells, VMH, and spleen, but not in the pituitary and corpus luteum (Stallings et al. 2002). Only large rat genomic fragment extending well into the *NR6A1* and *PSMB17* genes on either side of the *Sf-1* gene duplicates patterns of Sf-1 endogenous expression (Karpova et al. 2005). Within this fragment, two conserved enhancer regions were identified that are required for the expression of *Sf-1* in the VMH (Shima et al. 2005) and pituitary (Shima et al. 2008). In addition to specific enhancers for VMH and pituitary, special enhancer has been identified that drives the expression of SF-1 in the fetal adrenal gland. This enhancer is found within the intron 4 of the *SF-1* gene and is necessary to sustain the expression of SF-1 in the fetal adrenal, but is insufficient to drive the expression of SF-1 in adult adrenal, suggesting that regulation in adult and fetal adrenal gland is differentially regulated (Zubair et al. 2006).

Epigenetic Regulation of SF-1 Expression

Several studies have shown the role of epigenetic regulation through the methylation of different sites within *SF-1* regulatory regions playing a role in the expression of SF-1. The basal promoter of *SF-1* contains 14 and 10 CpG islands in human and mouse, respectively. These CpG sites are spread across 250 bp and thus do not represent a classical CpG islands, but rather a weak CpG island, such that are frequently found in genes with high tissue specificity in their expression. Methylation pattern of *SF-1* basal promoter is in nearly perfect correlation with the levels of expression with the exception of the pituitary, where basal promoter is hypermethylated, what is in agreement with the finding that expression in the pituitary is driven from alternative promoter.

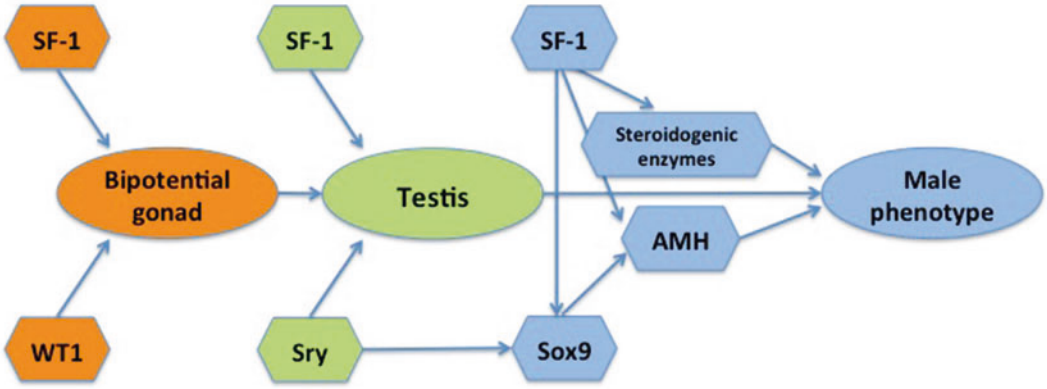
Enhancers that drive tissue-specific expression of *SF-1* in different tissues such as VMH, pituitary gonadotrope cells, and fetal adrenal all contain CpG sites, and methylation of these CpGs does follow expression patterns of *SF-1* gene. Pituitary enhancer is thus hypomethylated in the pituitary gonadotrope cells and hypermethylated in the VMH neurons and adrenal cortex. Similarly, VMH enhancer is hypomethylated in VMH neurons but hypermethylated in adrenal glands and other tissues that do not use this enhancer for tissue-specific expression of SF-1. However, fetal adrenal specific enhancer is hypomethylated in fetal adrenal cells and remains hypomethylated in adult adrenals, despite solid evidence that this enhancer is not needed for adult adrenal expression (Hoivik et al. 2013).

SF-1 Function

Fetal Development of Gonads

During development, SF-1 expression is first detected in urogenital ridges of both sexes in murine fetuses at E9.5 (Ikeda et al. 1994; Morohashi et al. 1995) and in human fetuses at 32 days post-ovulation (Hanley et al. 2000b). In mice, total ablation of *Sf-1* gene causes regression of the gonadal ridge by E11.5–12.0. The formation of the gonadal ridge is regulated also by other genes such as *Emx2, Gata4, Wt1, Lhx9*, *Cbx*, *Pod1*, *Six1*, and *Six4* most of which are believed to act upstream of the *Sf-1*. Therefore it is not surprising that promotor region of *Sf-1* gene contains binding sites for many of these genes and that impaired gonadal formation in mutant mouse fetuses for several of these genes is associated with downregulation or ectopic upregulation of the SF-1 (reviewed by Tanaka and Nishinakamura 2014).

Shift from the bipotential gonadal ridge to the testis happens in male mice fetuses around E10.5, triggered by the expression of *Sry,* which induces



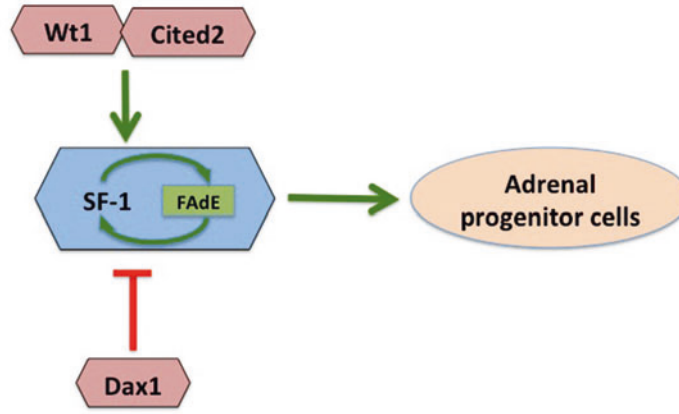
NR5a1, Fig. 2 Role of SF-1 in the development of the testis and male phenotype. Note that SF-1 is involved in all stages of male development, from bipotential gonad in the fetus to steroidogenesis in the adult testis.

differentiation of somatic cells into Sertoli cells. SF-1 is proposed to be one of the initial upstream regulators of the *Sry* gene. A few hours later the control of the differentiation process is switched from *Sry* to *Sox9*. In Sertoli cells SF-1, together with Sox9, induces activation of the gene for AMH (Arango et al. 1999) and activates Cyp26b1, an enzyme that catalyzes degradation of the retinoic acid. AMH promotes the regression of Mullerian ducts, and the absence of retinoic acid prevents male germ cells from entering meiosis prematurely. In mice fetal Leydig cells appear in the interstitium around E12.5 and persist in the testis until approximately one week after birth, when adult fetal Leydig cells develop (O'Shaughnessy et al. 2006). SF-1 is expressed in fetal Leydig cells and remains expressed in adult Leydig cells, as it is needed for the production of androgens both during fetal development and in adulthood by regulating the expression of several steroidogenic enzymes (Parker and Schimmer 1997; Ikeda et al. 2001). It is still not known to what extend SF-1 regulates Leydig cell differentiation. While loss of one *Sf-1* allele causes delayed Sertoli and Leydig cell differentiation, it does not impact the final number of adult Leydig cells (Luo et al. 1994; Park et al. 2005). However, conditional deletion of *Sf-1* in testicular somatic cells result in the absence of fetal and adult Leydig and Sertoli cells in testes, but the absence of Leydig cells in this model could also be due to the absence of Sertoli cells (Jeyasuria et al. 2004). However, an ectopic upregulation of SF-1 in $Pod1^{lacZ/lacZ}$ mice leads to remarkable increase in the number of fetal Leydig cells (Cui et al. 2004), suggesting direct involvement of SF-1 in the process of fetal Leydig cell formation.

In female mice fetuses, expression of SF-1 follows the same pattern as in males until E11.5–12.0. At E12.0 SF-1 transcripts are no longer detected in mouse developing ovaries, and its expression is switch on again just before birth (Ikeda et al. 1994; Morohashi et al. 1995; Majdic and Saunders 1996). In human ovaries down-regulation of SF-1 is less striking. At 52 days post-ovulation, SF-1 remains broadly distributed through the ovaries, with stronger localization in the germinal epithelial layers, and SF-1 expression in the human fetus persists through the entire fetal development (Hanley et al. 2000b) (Fig. 2).

Fetal Development of Adrenals

In the developing adrenal glands, SF-1 transcripts are detected in mouse fetuses at E10.5 (Ikeda et al. 1994; Morohashi et al. 1995) and in humans at 33 days post-conception (Hanley et al. 2001). Besides SF-1, several other factors including WT1, Wnt-4, activin, Pbx1, and DAX-1 regulate adrenal development. Many of these interact directly with SF-1 (reviewed by Hammer et al. 2005), for example, WT1 together with Cited2 increase SF-1 expression (Val et al. 2007), while DAX1 was shown to repress SF-1 expression in developing adrenal cells (Ito et al. 1997; Zubair



NR5a1, Fig. 3 SF-1 has a major role in the development of adrenal gland, together with WT1, Cited2, and Dax1, which are regulating SF-1 expression. The expression of SF-1 in the fetal adrenal gland is also auto regulated through the enhancer in intron 4 of SF-1 gene, called FAdE (fetal adrenal enhancer).

et al. 2006). Specific enhancer driving SF-1 expression in the fetal adrenal gland was identified in the fourth intron of the *SF-1* gene and is called fetal adrenal enhancer (FAdE). FAdE is necessary for maintenance of adrenal progenitor cells in developing adrenal primordia (Fig. 3) (Zubair et al. 2006).

Gene dosage seems to play important role in the regulation of adrenal development by SF-1. In mice, heterozygous for disrupted *Sf-1* allele (SF-1+/– mice), adrenals show a selective decrease in number of adrenal precursor cells at E10.0. Although by E13.5 cell proliferation increases, adrenals do not develop normally and are reduced in size in newborn mice (Bland et al. 2000a, 2004). While SF-1 haploinsufficiency results in the adrenal hypoplasia, overexpression of SF-1 in mice causes increased adrenal size and formation of ectopic adrenal cortical tissues in the thorax (Zubair et al. 2009).

Adult Steroidogenic Organs; Gonads and Adrenal Glands

In gonads and adrenal glands, SF-1 is a key regulator of steroid hormone biosynthesis. Control of steroid production already starts at the level of cholesterol transport. SF-1 increases the expression of the scavenger receptor B1 (SR-B1), important for cellular import of high-density lipoprotein cholesterol, and steroidogenic acute regulatory protein (StAR), which regulates transport of cholesterol from outer to inner mitochondrial membrane. SF-1 also regulates the expression of different cytochrome P450 steroid hydroxylases and other steroidogenic enzymes such as 3β-Hydroxysteroid dehydrogenase (HSD3B2 and HSD3B1) (Schimmer and White 2010). Transcription of enzymes involved in the steroid production in the adrenal cortex is primarily regulated by adrenocorticotropic hormone (ACTH) from the pituitary. After ACTH binds to its cognate receptor, the melanocortin 2 receptor (MC2-R), numerous signaling pathways are activated in adrenal cells and at least some of these pathways are believed to be regulated by SF-1 (Sewer and Waterman 2002). For example, activation of cAMP/PKA pathway, activated by ACTH, decreases the amount of sphingosine and lysosphingomyelin bound to SF-1. Both phospholipids act on SF-1 as inhibitory ligands, and reduced amount of both ligands increases the expression of P45017a -hydroxylase/17,20-lyase (CYP17A1), mediated by SF-1 (Urs et al. 2006).

In testicular Leydig cells, SF-1 regulates both basal and LH-stimulated production of testosterone through regulation of cytochrome P450 cholesterol side chain cleavage (CYP11A1), CYP17A1, HSD3B2, and also 17β-hydroxysteroid dehydrogenase (HSD17B) enzymes (Schimmer and White 2010).

In ovaries, SF-1 is expressed in theca, granulosa, and luteal cells and has a role in production of estrogens during follicular phase, as well as in production of progesterone during luteal phase (Saxena et al. 2007).

Although initial studies reported that there is no deleterious phenotype in SF-1 +/− mice, subsequent studies did show the importance of gene dosage and that both alleles of *Sf-1* gene are needed for normal development and function of adrenals (Bland et al. 2000a, b, 2004). Adrenal glands in SF-1 +/− mice are smaller, with normal zonation but marked cellular hypertrophy within the zona fasciculata and striking dilatation of the cortical vasculature. In SF-1 +/− mice stimulated plasma corticosterone levels are lower in comparison to WT mice, evening levels of ACTH are higher, and expression of StAR and MC2-R is increased, while, interestingly, expression of CYP11A1 expression is unchanged (Bland et al. 2000b).

Pituitary

SF-1 is expressed also in the anterior pituitary, specifically in gonadotrope cells (Ingraham et al. 1994; Shinoda et al. 1995). Global and pituitary-specific SF-1 KO mice have markedly diminished expression of LH, FSH, and receptor for GnRH suggesting that SF-1 regulates expression of these pituitary genes (Ingraham et al. 1994; Shinoda et al. 1995). This was confirmed by in vitro studies, demonstrating that SF-1 indeed regulates the expression of common α-subunit (αGSU) of LH and FSH, as well as β-subunits of both FSH and LH (βFSH and βLH), and GnRH receptor (Barnhart and Mellon 1994; Ingraham et al. 1994; Halvorson et al. 1996; Jacobs et al. 2003). However, it is not yet clear whether reduced expression of both LH and FSH in SF-1 KO mice is due to direct effect of missing *Sf-1* gene or perhaps secondary due to diminished expression of GnRH receptor since expression of both LH and FSH is strongly influenced by GnRH, secreted from the hypothalamus. This is supported by finding that the treatment of SF-1 KO mice with supraphysiologic doses of GnRH stimulates LH and FSH production in the pituitary in SF-1 KO mice (Ikeda et al. 1995) indicating that gonadotropin cells are capable of gonadotropin synthesis in the absence of SF-1, although this was done only qualitatively (by immunohistochemistry and levels of expression were not determined).

Ventromedial Nucleus of the Hypothalamus

In the central nervous system, the VMH is the only part where SF-1 is expressed. In the mouse its expression has been detected in the hypothalamic primordium as early as embryonic day E9.0. Initial studies showed that target disruption of the gene encoding SF-1 impaired the cytoarchitecture of the VMH (Ikeda et al. 1995; Luo et al. 1995; Shinoda et al. 1995). Subsequent studies on SF-1 KO fetuses (E15) and neonates revealed that distribution of cells is altered significantly in the region of the developing VMH, but without significantly affecting cell numbers up until birth, suggesting that disrupted neuronal migration could be the main cause of disorganized VMH in SF-1 KO mice (Davis et al. 2004). SF-1 could be also required for terminal differentiation of the VMH and for expression of BDNF since SF-1 KO neonates show complete loss of projections to the bed nucleus of stria terminalis (BNST) and amygdala, and neuronal precursors do not express BDNF (Tran et al. 2003). Similar findings were reported in CNS-specific SF-1 KO mice, which have also diminished hypothalamic expression of BDNF, CRH type 2 receptor, and cannabinoid receptor 1 (CBR1) (Davis et al. 2004; Kim et al. 2008; Zhao et al. 2008).

In adult life, SF-1 KO mice develop obesity, caused mainly by hypoactivity rather than hyperphagia, suggesting the role of VMH in the regulation of spontaneous physical activity (Majdic et al. 2002). Expression of several orexigenic and anorexigenic peptides was studied in these mice, and although changes in their expression pattern were observed, this was most likely due to disrupted neuronal migration in the VMH region and might not be functionally connected with obese phenotype (Budefeld et al. 2011). Diminished running wheel activity was observed also in the CNS-specific SF-1 KO mice, although these mice only develop obesity when on high-fat diet, probably reflecting the difference between early and later disruption of the VMH development. In addition to the obesity when on high-fat diet, CNS-specific SF-1 KO mice have impaired thermogenesis after acute exposure to high-fat diet and have decreased expression of leptin

receptors (LepR) in the VMH (Zhao et al. 2008; Kim et al. 2011).

VMH is also involved in the regulation of different social behaviors such as anxiety, aggression, defensive, maternal and female sexual behavior. CNS-specific SF-1 KO mice showed elevated anxiety (Zhao et al. 2008), impaired female sexual behavior – lordosis and subfertility with the decreased number of corpora lutea (Kim et al. 2010). Lordosis is also impaired in hormone primed agonadal SF-1 KO mice (Grgurevic et al. 2012), while in the absence of any hormones, these mice show elevated aggression toward receptive females (Grgurevic et al. 2008). In our laboratory we also noted elevated anxiety-like behavior and poorer parental behavior in global SF-1 KO mice when compared to WT mice (unpublished observations – Grgurevic), and our recent study shows that SF-1+/− females have impaired maternal behavior (Spanic et al. 2016). Study by DaSilva et al. (Silva et al. 2013) has also shown the involvement of SF-1 neurons in the dorsolateral VMH in fear response, although in this study SF-1 was only used as a marker for the VMH neurons and direct role of SF-1 protein in the regulation of fear response was not studied.

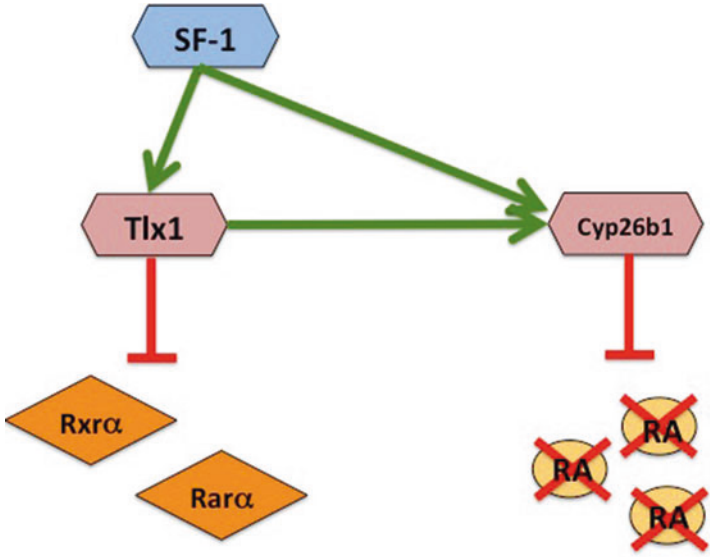
Although numerous behavioral effects have been observed in SF-1 KO mice, it is not known to what extent SF-1 directly influence these behavioral traits as many of those could arise due to impaired VMH structure, and SF-1 role might only be important during development of this nucleus. However, SF-1 might also have a direct role in the regulation of some of these behaviors as studies have shown the role of SF-1 in the regulation of expression of genes encoding for BDNF, CBR1, CRH type 2 receptor, and LepR (Schimmer and White 2010).

Spleen

In adult mice, rats, and humans, SF-1 protein was detected in the red pulp but not in the white pulp of spleen. Several immunoreactive cells were also found inside the venous sinuses, which are part of a unique vascular system of the spleen (Morohashi et al. 1999). SF-1 is required for normal spleen development in humans (Zangen et al. 2014) and in mice which after global SF-1 ablation show abnormality in spleen size and structure (Morohashi et al. 1995). A recent report described similar phenotype in a 46,XY girl with complete sex reversal and asplenia, but without primary adrenal insufficiency. Genetic analysis confirmed a homozygous *SF-1* mutation (p. R103Q) which consequently decreased SF-1 transactivation of the TLX1, a transcription factor that is essential for spleen development (Zangen et al. 2014). As shown by studies in mice, TLX1 is likely a downstream target of SF-1 through which SF-1 influence development of the spleen by modulating retinoic acid signaling pathway (Fig. 4). On the other hand, mouse Polycomb gene *M33*, another important factor for spleen development, might be an upstream regulator of SF-1 as similar splenic phenotype as in SF-1 KO mice was found in M33 KO mice, and spleens from these mice have markedly reduced expression of SF-1 (Katoh-Fukui et al. 2005; Lenti et al. 2016).

SF-1 in Human Disease

Based on in vitro and in vivo studies of SF-1, the first attempts to expose the potential roles of SF-1 in human pathology were focused on adrenal insufficiency and complete gonadal dysgenesis, a syndrome similar to SF-1 KO mice phenotype. This phenotype is very rare in humans; nevertheless, in 1999 Achermann et al. identified mutations in *SF-1* in 46,XY girl with complete gonadal agenesis, male to female sex reversal, and primary adrenal failure. In this patient, de novo mutation (p. G35E) was found in the P-box of the first zinc finger domain, affecting key amino acid in this domain, responsible for DNA binding. The second patient had similar phenotype but was found to carry homozygous recessive mutation (p. R92Q) affecting key amino acid in the A box within the Ftz-F1 box. Despite these two mutations, it turned out that mutations in *SF-1* are rare in patients with adrenal phenotype (Suntharalingham et al. 2015). In contrast, SF-1 seems to be more often involved in disorders of sexual development (DSD). Heterozygous



NR5a1, Fig. 4 SF-1 influences spleen development by regulating expression of Tlx1 and Cyp26b1 genes. These two genes regulate spleen development through the repression of the retinoic acid signaling pathway (Rxr, Rar retinoic acid receptors, RA retinoic acid).

mutations in *SF-1* in DSD patients were found to be remarkably common, present in 10–20% of all DSD patients (Suntharalingham et al. 2015). The most often observed phenotype is a chromosomally male patient (46,XY DSD) with ambiguous genitalia and with, or more often without, Mullerian structures. The testes are often well developed, and main phenotype characteristics seem to be arising due to androgen deficiency. Therefore, in some cases, the patient may be misdiagnosed for androgen insensitivity syndrome (Coutant et al. 2007). In addition to 46,XY DSDs, *SF-1* mutations have been identified in patients with hypospadias and undescended testes (Köhler et al. 2009) and bilateral anorchia (Philibert et al. 2007), and some studies suggested that mild mutations/variations in *SF-1* gene might be involved in some cases of male factor infertility (Bashamboo et al. 2010; Röpke et al. 2013; Ferlin et al. 2015).

Similarly to male patients, primary ovarian insufficiency has been reported in sisters or mothers of children with DSD due to *SF-1* mutations. The clinical phenotype in these patients is very variable, from rare reports of complete ovarian insufficiency and the absence of menarche to more common early menopause and early depletion of ovarian reserves (Janse et al. 2012; Voican et al. 2013).

Hyperactivity and/or overexpression of SF-1 has also been linked to pathologies in human patients. In high proportion of childhood adrenal carcinomas, duplication of the gene locus containing *SF-1* gene has been reported in somatic cells, and overexpression of SF-1 has been detected in many adult adrenal tumors. The degree of overexpression of SF-1 might even be an important predictor of adrenal tumor outcome, as suggested by some studies (Almeida et al. 2010; Sbiera et al. 2010).

Interestingly, although studies in mice have shown importance of SF-1 also for the development of hypothalamus, no reports about SF-1 role in obesity in humans have been reported to date. Interestingly, there is one report suggesting a possible link between *SF-1* mutations and psychiatric symptoms of anxiety and/or depression (Suwanai et al. 2013) although the direct connection between *SF-1* mutations and psychiatric symptoms has not been unequivocally established.

Summary

SF-1, first identified over 20 years ago, has been shown conclusively to be one of the most important genes involved in the regulation of gonadal and adrenal development, with important

functions in other tissues such as pituitary, hypothalamus, and spleen. Although some roles of SF-1 such as regulation of expression of steroidogenic enzymes in gonads and adrenal glands, regulation of expression of AMH and Sox9 in fetal Sertoli cells, regulation of GnRH receptor expression in the pituitary, and regulation of spleen development through Tlx1 and retinoic acid signaling have been clearly established, there are many questions still unanswered. Precise temporal and tissue-specific regulation of SF-1, functional role of the SF-1 in the hypothalamus, role of SF-1 in infertile patients, and some other human diseases are among the questions that are still not fully understood and will have to be studied in future years.

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N-Ras

▶ Ras (H-, K-, N-Ras)

Nrf2

▶ Nrf2 (NF-E2-Related Factor2)

Nrf2 (NF-E2-Related Factor2)

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Synonyms

NFE2L2; NF-E2-related factor 2; Nrf2

Historical Background

Nrf2 was found as a member of nuclear factor erythroid 2 (NF-E2) transcription factor family in 1994 (Moi et al. 1994). Nrf2 is a basic Leucine Zipper (bZIP) transcription factor that belongs to the Cap'n'Collar (CNC) family (p45-NFE2, Nrf1, Nrf2, and Nrf3) and is expressed ubiquitously in various tissues (Moi et al. 1994). Yamamoto and his colleagues showed that Nrf2 forms a heterodimer with small Mafs and induces phase-II detoxifying enzymes through antioxidant response elements (AREs) in the promoter regions of the target genes (Tong et al. 2006). A myriad of studies have identified Nrf2 as a sensor that acts against oxidative stress or electrophilic chemicals. In spite of the similarity in nucleotide sequences between Nrf2 and NF-E2, it was not involved in erythropoiesis and development in a murine model.

Oxidative stress is featured by high levels of reactive oxygen species (ROS), which exerts a harmful effect on cellular components and induces defensive responses. ROS originates from hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and peroxynitrite ($ONOO^-$) form powerful oxidants in the cell. Thus, ROS generation is the fate of aerobic organisms as a natural by-product of oxygen metabolism. In order to avoid cellular damage inflicted by oxidative perturbation, aerobic organisms have developed novel antioxidant defense systems. Among these, Nrf2 and its cytoplasmic repressor kelch-like ECH-associated protein 1 (Keap1) serve sulfhydryl-containing sensors that respond to oxidative stress (Tong et al. 2006); oxidative stress modifies reactive cysteine residues in Keap1 and/or Nrf2. Under no oxidative stimuli, Keap1 binds to the amino-terminal Nrf2-ECH homology 2 (Neh2) domain of Nrf2 and provokes its ubiquitin/proteasomal degradation (Tong et al. 2006). In cells challenged with oxidative stimuli, Keap1 dissociates from Nrf2 and thereby ubiquitin/proteasomal degradation of Nrf2 is hampered. Hence, mice deficient in Nrf2 exacerbate sensitivity to carcinogens or tumorigens, which supports the concept that Nrf2-mediated gene transcription is necessary for the prevention of chemical carcinogenesis by cytoprotective agents (Kensler and Wakabayashi 2010).

Nrf2 (NF-E2-Related Factor2), Table 1 Nrf2-activating compounds

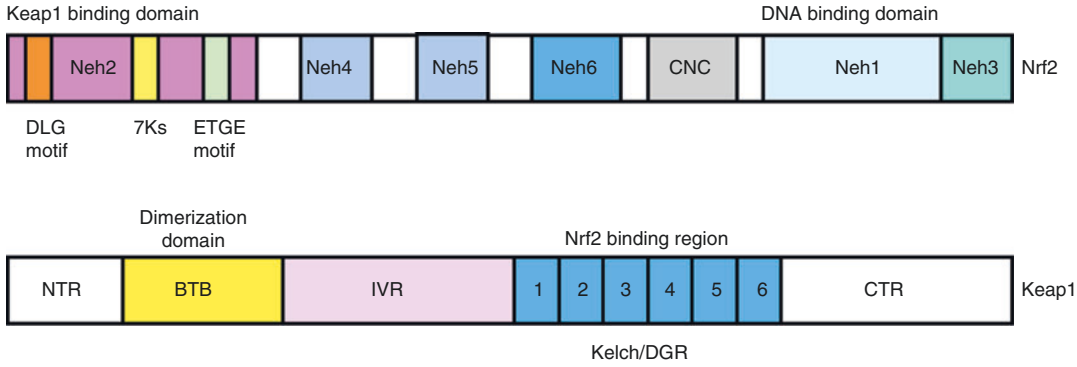
Categories	Nrf2 activators
Aromatic organic compounds	BHT (butylated hydroxytoluene)
	tBHQ(tert-butylhydroquinone)
	BHA (butylated hydroxyanisole)
Dithiolethiones	oltipraz, D3T (1,2-dithiole-3-thione)
Isothiocyantes	sulforaphane
Oleanoic triterpenoids	CDDO-Im (2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide)
Flavonoids	genistein, isoliquiritigenin
Cyclopentenone prostaglandin	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
Polyphenols	EGCG ((−)-epigallocatechin-3-gallate), resveratrol

An increasing number of studies have described a series of synthetic and phytochemical compounds that activate Nrf2 in cell or animal models (Eggler et al. 2008) (Table 1). Because most of these agents have beneficial effects in a variety of disease models, current pharmacological interventions that target the activity of Nrf2 are expected to advance into novel drug discovery for human diseases.

Regulation of Nrf2 Activity

Domain Structure of Nrf2

Nrf2-ECH homology (Neh) domains are highly conserved in mammalian cells; Nrf2 has six Neh domains (Fig. 1). The domain structure of Nrf2



Nrf2 (NF-E2-Related Factor2), Fig. 1 The domain structures of Nrf2 and Keap1

has been extensively studied by Yamamoto group. Each Neh domains has a distinct role in regulating the activity of Nrf2. First, Neh1 domain contains CNC-bZIP domain that leads to the formation of heterodimer with small Mafs lacking a transactivation domain. Using yeast two hybrid analysis, it has been shown that the Neh2 domain of Nrf2 interacts with Kelch/DGR domain of Keap1, and which induces ubiquitin/proteasomal degradation of Nrf2 via Cul3 ubiquitin ligase (Tong et al. 2006). Whereas Neh2 degron is redox-sensitive, Neh6 degron is not and is required for maximal turnover of Nrf2. Studies have shown that Neh4 and Neh5 domains cooperatively bind with either CREB-binding protein (CBP) or silencing mediator of retinoid and thyroid receptors (SMRT)(Ki et al. 2005) and enhance target gene transactivation (Li and Kong 2009). In addition, the carboxy-terminal Neh3 domain of Nrf2 contributes to Nrf2 transactivation activity.

Degradation of Nrf2 Protein

In normal cells, Nrf2 is short-lived but is rapidly stabilized by oxidative stress. In this process, Keap1 has been recognized as a key repressor molecule that causes ubiquitination/proteasomal degradation. Keap1 is a cysteine-rich protein and serves an oxidative sensor molecule in response to free radical stress. The sulfhydryl modifications of cysteine residues of Keap1 affect Nrf2 degradation (Keap1 has nine reactive cysteine residues that respond to oxidative stress) (Fig. 1). Among them, C273 and C288 are located in the intervening region (IVR) of Keap1, and thus these mutations abrogated the basal inhibitory activity of Nrf2. So, oxidative stress supposedly causes the intermolecular disulfide bond formation of C273 and C288 and, thereby, down-regulates Keap1 activity.

Keap1 has two functional domains called Kelch/DGR and broad complex, tramtrack, bric-a-brac (BTB) (Fig. 1) that are involved in Nrf2 degradation. Kelch/DGR domain interacts with Neh2 of Nrf2. BTB domain of Keap1 recruits ubiquitin-ligase complex components and also forms a homodimer of Keap1. Neh2 domain of Nrf2 contains two distinct binding motifs with Kelch/DGR domain of Keap1. One is ETGE motif (formulated as D/N-X-E-T/S-G-E) that has a high affinity ($K_a = 20 \times 10^7\ M^{-1}$) to Kelch/DGR domain of Keap1, whereas the other is DLG motif (formulated as L-X-X-Q-D-X-D-L-G) that has a low affinity ($K_a = 0.1 \times 10^7\ M^{-1}$). X-ray crystallography unraveled that Kelch/DGR domain is a shape of six-bladed β-propeller and each Kelch domain consists of four antiparallel β-strands. Kelch/DGR domain possesses arginine triad (R380, R415 and R483), and these amino acids explain why ETGE motif of Neh2 exerts higher binding affinity than that of DLG. Between these two motifs, 7 lysine residues (7Ks) are located and ubiquitinated by E3 ligase. In addition, the results of nuclear magnetic resonance analysis indicated that the binding ratio of Keap1/Neh2 domain is 2:1 (Li and Kong 2009), suggesting that Keap1 makes homodimer formation.

In addition to Keap1, the ubiquitin-proteasome system is responsible for Nrf2 degradation (Tong et al. 2006). Ubiquitin consists of 76 amino acids and is a highly conserved regulatory protein which designates certain proteins subjected to degradation. E1, an ubiquitin-activating enzyme, generates an ubiquitin-adenylate intermediate using ATP, and then transfers ubiquitin to the active cysteine residue of E1. E2, an ubiquitin-conjugating enzyme, and E3, ubiquitin ligase, cooperatively accomplish the ubiquitination of target proteins. Lysine residue of target protein and C-terminal glycine of ubiquitin forms isopeptide bond by E3 ubiquitin ligase complex. Nrf2 is ubiquitinated by Cul3-BTB^{keap1} E3 ligase which belongs to the members of RING domain E3 ligases (Tong et al. 2006). Cul3-BTB^{keap1} E3 ligase is composed of Keap1, Rbx1, Cullin3, and Ubc5 (E2 enzyme). As the member of E3 ligase complex, Kelch/DGR domain of Keap1 binds to Nrf2 and the BTB domain recruits the components of E3 ligase complex.

According to these results, Yamamoto's group proposed "hinge and latch model-two-sites binding mechanism" that describes Keap1-Nrf2 system (Tong et al. 2006). ETGE motif serves "hinge" since it forms a strong binding complex with the Kelch/DGR domain of Keap1 even under oxidative stress. In contrast, DLG motif works as a "latch" which allows Nrf2 to be disoriented under oxidative stress and impedes Nrf2 ubiquitination by Cul3-BTB^{keap1} E3 ligase. Reactive cysteines in the IVR domain of Keap1 are modified by oxidative stress. It is postulated that these sulfhydryl modifications may change the conformation of Nrf2 structure (Tong et al. 2006). "Hinge and latch model" successfully accounts for the mode of actions of various Nrf2 activators, including ROS, reactive nitrogen species (RNS), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and sulforaphane. Recently, the direct interaction between ^{154}KRR motif in p21 and DLG/ETGE motifs in Nrf2 hampers ubiquitination of Nrf2 by competitively binding with Keap1 (Chen et al. 2009), which supports the model of Yamamoto's group. Collectively, "hinge and latch model" may account for redox-sensitive Nrf2 activity regulation in association with Keap1.

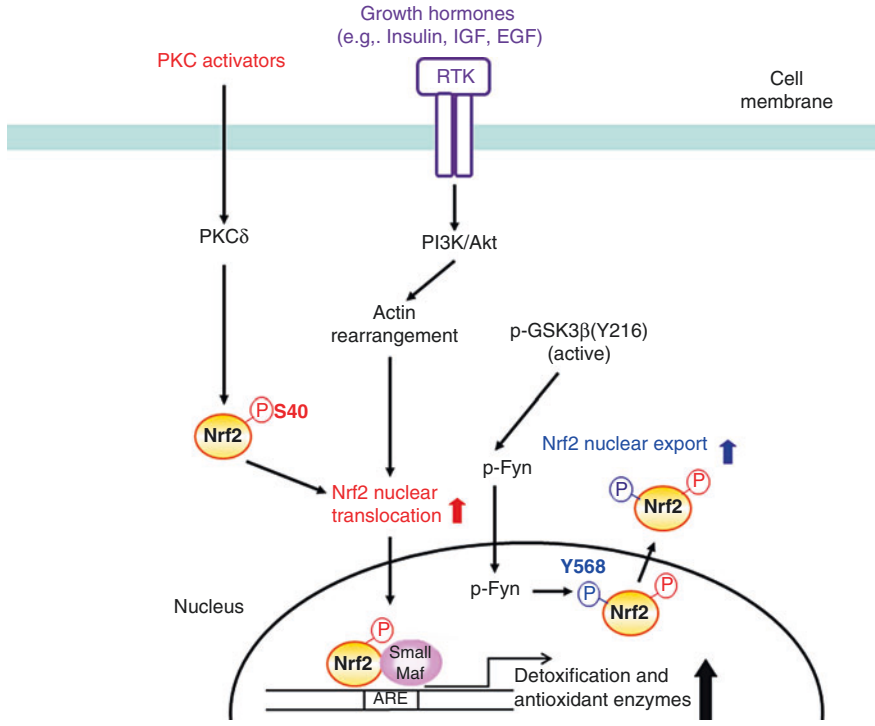
Nuclear Localization of Nrf2

Nrf2 contains three nuclear localization signal (NLS) motifs and two nuclear export signal (NES) motifs (Li et al. 2006; Li and Kong 2009). NLS_N was identified at the amino-terminus, whereas NLS_C was at the carboxy-terminus. bNLS is characterized at the basic region of Nrf2. NES_{TA} is localized at the Neh5 transactivation domain, whereas NES_{zip} is at the ZIP domain of Nrf2. Li et al. discovered NES_{TA} motif (^{175}LLSIPELQCLNI^{186}) of Neh5 domain based on the consensus leucine-rich NES motif that is formulated as $\Phi^4(X)_{2-3}\Phi^3(X)_{2-3}\Phi^2X\Phi^1$ (Φ represents hydrophobic amino acids and X represents any amino acids). EGFP-NES_{TA} chimeric protein (a truncated form of Nrf2) promoted cytoplasmic distribution of Nrf2. So, mutation of C183 of NES_{TA} abrogated oxidant-induced ARE activity. The fluorescence resonance energy transfer (FRET) assay failed to show direct interaction between NES_{TA} and Keap1, indicating that nuclear localization of Nrf2 might be modulated by NES_{TA} motif independently of Keap1. Li and Kong have proposed another Nrf2 signaling model in association with Keap1-independent Nrf2 regulation (Li and Kong 2009); in this model, the balance between NLSs and NESs determines the subcellular localization of Nrf2 in response to oxidative stress or antioxidants. NES_{TA} and bNLS motifs possess similar strong driving force for the translocation of Nrf2. Since the driving forces among the motifs are well balanced under normal condition, Nrf2 is expelled to cytoplasm. Nrf2 is translocated into the nucleus when redox-sensitive NES_{TA} is halted by oxidative stress (i.e., NES_{zip} is redox-insensitive).

The Signals of Nrf2 Regulation

Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol-4,5-trisphosphate [PtdIns(4,5)P2] into phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3], which is the secondary messenger for other kinases such as serine-threonine Akt kinase. ▶ PI3K regulates



Nrf2 (NF-E2-Related Factor2), Fig. 2 The signaling pathways for Nrf2 activity regulation

microfilaments and translocation of actin-associated proteins (Fig. 2). In response to oxidative stress, PI3K activation induces actin cytoskeleton rearrangement. Actin depolymerization promotes translocation of a complex of Nrf2 and actin into the nucleus and enables activating Nrf2 to bind to the ARE for phase II enzyme induction by oxidative stress (Kang et al. 2005).

Protein Kinase C Delta

Protein kinase C (PKC) disseminates signals into target molecules in response to extracellular stimuli. PKC pathway may be an initial triggering step for recognizing cellular redox state transition. Pickett's group revealed that Nrf2 activation requires phosphorylation at S40 by PKCδ critical for nuclear translocation of Nrf2 (Fig. 2) (Kaspar et al. 2009). Recently, both phosphorylation of Nrf2 S40 by PKCδ and antioxidant-induced modification of Keap1 C151 contribute to stabilization and nuclear translocation of Nrf2 (Niture et al. 2009).

Glycogen Synthase Kinase-3β

It has been shown that tyrosine kinase Fyn phosphorylated Y568 of Nrf2 and regulates chromosomal region maintenance 1 (Crm1/exportin 1)-mediated nuclear export of Nrf2 (Fig. 2). Glycogen synthase kinase-3β (GSK-3β) was identified as an upstream kinase of Fyn (Kaspar et al. 2009). Inhibition of GSK-3β induces nuclear accumulation of Nrf2 and transcriptionally activates the induction of Nrf2 target genes (i.e., phase-II enzymes). Hydrogen peroxide directly phosphorylates Y216 of GSK-3β, which leads to GSK-3β activation, implying that ROS affects GSK-3β-mediated Nrf2 activity regulation (Kaspar et al. 2009).

Nrf2 Target Genes and Biological Functions

The Genes That Contain ARE(s)

The induction of phase-II detoxification enzymes and phase-III efflux transporters through ARE depends on the activity of Nrf2. Major antioxidant

Nrf2 (NF-E2-Related Factor2), Table 2 Nrf2 target genes

Functions	Target genes
Phase-I enzymes	None
Phase-II enzymes	GSTA2
	NQO1
	heme oxygenase-1
	UDP-glucuronosyltransferases (UGT) 1A6
	glutamate-cysteine ligase modifier (GCLM)
	glutamate-cysteine ligase catalytic subunits (GCLC)
Phase-III enzymes	Multidrug resistance protein (MRP) 2/3/4/5/6, Organic anion transporting polypeptide (Oatp) 1a1/2b1
Iron-binding protein	Ferritin H

enzymes contain one or more functional ARE(s) in their promoter regions (Table 2). Once Nrf2 dissociates from its Keap1 binding in response to oxidative stress, the activating Nrf2 translocates into the nucleus and binds ARE comprised in the promoters of target genes. Unlike canonical bZIP proteins, Nrf2 has no ability to form homodimer (Li and Kong 2009). Instead, Nrf2 forms heterodimer with small Maf proteins such as MafF/G/K which lack canonical transactivation domain. In addition, Nrf2 is directly acetylated by p300/CREB-binding protein (CBP) under the condition of arsenite-induced stress (Sun et al. 2009). Eighteen lysine residues were identified as acetylation sites in Neh1 DNA-binding domain. Intriguingly, combined lysine-to-arginine mutations on the acetylation sites unaffected the stability of Nrf2, but compromised its DNA-binding activity (Sun et al. 2009). Nrf2 activation and target gene transcription contribute to the detoxification and excretion of detrimental xenobiotics. Phytochemicals and synthetic compounds may have cytoprotective and chemopreventive effects through Nrf2 activation (Eggler et al. 2008). Hence, a deficiency of Nrf2 abrogates the abilities of these agents to protect cells against toxic chemicals or physical stresses, as shown in the experiments using animals or cells.

As the cores of energy metabolism, mitochondria regulate the balance between constitutive and excessive levels of cellular ROS. The mitochondrial respiratory chain not only produces ROS under a basal condition, but also serves a major ROS source under pathological situations. Oxidative stress causes mitochondrial permeability transition, mitochondrial dysfunction, and apoptosis. Hence, the maintenance of mitochondrial function is crucial in protecting cells or organs from toxicants. Compared to nuclear DNA, mitochondria DNA are vulnerable to oxidative stress because of two reasons: (1) mitochondria are the organelles that produce ROS via electron transport chain, and (2) mitochondria DNA repair mechanisms are insufficient. Therefore, the induction of phase-II detoxifying enzymes by Nrf2 might be closely associated with cytoprotective effect against toxicant-induced injury, which may result from not only a decrease in cellular ROS, but protection of mitochondria (Kensler et al. 2007). Collectively, it is hypothesized that the roles of Nrf2 in apoptosis include regulation of redox-homeostasis, increase in adaptive antioxidant capacity, activation of phase-II detoxifying enzymes, and mitochondrial protection, all of which contribute to cell viability.

Cancer

Exposure to toxic external stimuli such as xenobiotics and viral infections might cause genetic defects and thus increase cancer incidence. Carcinogenesis is induced by complex mechanisms which are characterized as multiple genetic defects and uncontrolled growth. These genetically defected genes often have effects on signal transduction pathways regarding cell survival, proliferation, and trans-differentiation. In particular, excess ROS provokes DNA damage such as point mutation, deletion-insertion, and microsatellite instability. Thus, it is a reasonable prediction that antioxidants and antioxidative enzymes contribute to preventing genetic defects of cells from radical stress. Various experimental models have shown that induction of antioxidative and cytoprotective enzymes by chemicals contributes to cancer chemoprevention, and which accompanies Nrf2 activation in most cases.

Many research groups have made a huge effort to develop Nrf2 activators as chemopreventive agents. However, it is now accepted that constitutive

Nrf2 activation may also contribute to malignancy and radiation/drug resistance in cancer. The mutations in Keap1 and overwhelming expression of Nrf2 occur in the tissue of lung cancer patients (Lau et al. 2008). Mutated Keap1 possesses substantially impeded binding affinity with Nrf2, leading to augmented anti-apoptotic and antioxidative effects. Therefore, some cancer cells with Keap1 mutations acquire the capacity to survive from harsh tumor microenvironment through Nrf2 activation. Recently, Kensler and Wakabayashi proposed "U-Shaped model" that describes the modulation of cancer risk in terms of the Keap1-Nrf2 pathway (Kensler and Wakabayashi 2010). Nrf2 activation is clinically practical only between the biologically effective dose (BED) and a maximal-tolerated dose (MTD). Low level of Nrf2 expression makes cells susceptible to carcinogenesis or toxicity, whereas high level of Nrf2 expression in tumor might attribute to cancer malignancy.

Lately, epigenetic regulatory pathway of Nrf2-Keap1 has been underscored in cancer cells by several research groups. Hypermethylation of CpG islands of Keap1 was discovered in lung adenocarcinoma. In addition, CpG island methylations in the promoter region of Nrf2 gene was identified in transgenic adenocarcinoma of mouse prostate, but not in normal tissue (Yu et al. 2010). Thus, Nrf2 level and its downstream target gene expression are substantially repressed in this prostate tumor model. These results suggest that the epigenetic approach is also necessary for the understanding of Nrf2 role in cancer.

Cardiovascular Diseases

ROS is involved in the pathologic processes of cardiovascular diseases such as atherosclerosis, hypertension, and coronary heart disease. In cardiovascular diseases, ROS production (H_2O_2 and O_2^-) is increased due to NAD(P)H oxidase, peroxidase, and cyclooxygenase. In particular, vascular smooth muscle cells (VSMCs) and endothelial cells are the major sources of ROS. Nrf2 is a potential target for the intervention of cardiovascular diseases. Studies have shown that the Nrf2/heme oxygenase-1 (HO-1) pathway is associated with the inhibition of VSMC proliferation and migration, and which helps provide a condition for obtaining anti-atherosclerotic activity (Li et al. 2009). After balloon angioplasty in rabbit aorta, local adenoviral transfer of Nrf2 contributes to reducing VSMC proliferation, oxidative stress, and inflammatory responses (Li et al. 2009). However, the lack of change in neointimal hyperplasia by ectopic Nrf2 expression implies that Nrf2 may induce anti-apoptosis of VSMCs. In human aortic endothelial cells, laminar flow, but not oscillatory flow, induces Nrf2 activation and its target gene transactivation (Li et al. 2009); the degree of Nrf2 activation differs between atherosclerosis-resistant and atherosclerosis-susceptible regions of the mouse aorta. Although Nrf2 regulates antioxidant defense system, it still remains elusive what the exact molecular mechanism of Nrf2 is in cardiovascular system.

Summary

Oxidative stress is critical in homeostasis and survival of aerobic organisms. Nrf2 target gene induction plays a role in antioxidant defense systems. In cells challenged with oxidative stimuli, Keap1 is not able to degrade Nrf2 so that antioxidative and cytoprotective enzymes are activated. So, Nrf2-mediated gene induction by pharmacological agents may account for cancer chemoprevention, and amelioration of hepatic and cardiovascular diseases. However, incremental Nrf2 activation was observed in cancer tissues, implying that it may also contribute to invoking cancer malignancy and chemoresistance. The exact role and mechanism of Nrf2 regulation and its functional consequences are still elusive, and further molecular and clinical investigation is requisite.

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NRIP1

► Nuclear Receptor-Interacting Protein 1 (NRIP1)

NS1

► Tyrosine-Protein Phosphatase Nonreceptor Type 11 (PTPN11)

NSHPT

► Calcium Sensing Receptor (CASR)

NSP, Novel Serine Protease

► SARA

NTCP (Sodium Taurocholate Cotransporting Polypeptide)

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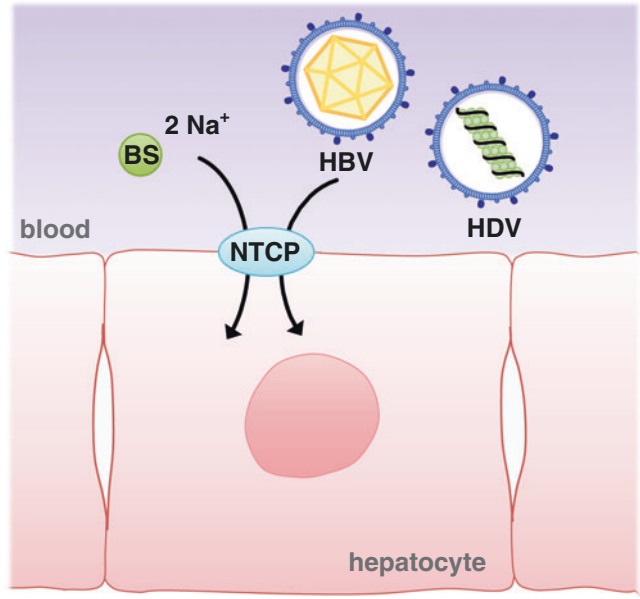
Synonyms

NTCP1; SLC10A1; Sodium/bile acid cotransporter; Solute carrier family 10 member 1

Historical Background

A sodium-dependent transport system for conjugated bile salts was extensively characterized in early studies using perfused rat liver and isolated rat and human basolateral membranes. The factor responsible for the activity of the system was first identified by Dr. Meier's group in 1990. In their seminal study, they injected rat liver poly $(A)^+$ RNA into *Xenopus laevis* oocytes and observed

These authors equally contributed to this work



NTCP (Sodium Taurocholate Cotransporting Polypeptide), Fig. 1 NTCP's functions as a transporter for bile salts (BS) and as an entry receptor for hepatitis B and D viruses (HBV, HDV). NTCP cotransports one molecule of extracellular BS together with two sodium ions to the hepatocytes. NTCP also essentially supports the entry of HBV and HDV into hepatocytes through the interaction with the preS1 region of the viral envelope protein

the functional expression of sodium-dependent taurocholate uptake (Hagenbuch et al. 1990). Meier and coworkers size-fractionated and enriched the active mRNA, and showed that the 1.5∼3 Kb subfraction supported bile salt uptake. Following expression cloning of a rat cDNA library, they succeeded in cloning rat Ntcp, which consists of 1738 nucleotides that encode an open reading frame and whose expression produces a 362 aa polypeptide (Hagenbuch et al. 1991). NTCP was subsequently cloned from many species, including human, mouse, and rabbit (Stieger 2011). In addition to its function as a transporter of bile salts, NTCP was recently identified as an entry receptor for hepatitis B and D viruses by Dr. Li's group (Yan et al. 2012).

Introduction

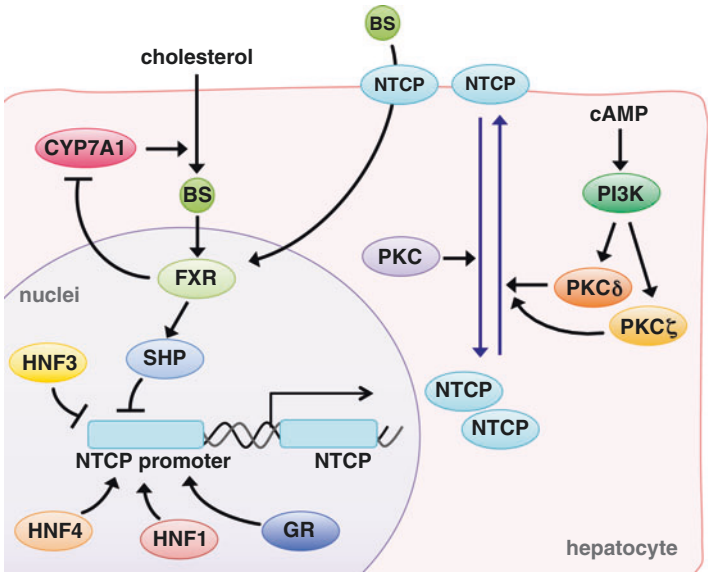
The solute carrier (SLC) 10 family comprises uptake transporters for bile salts, steroidal hormones, and various drugs. The SLC10 transporter family consists of seven members, including sodium taurocholate cotransporting polypeptide (NTCP, SLC10A1), apical sodium-dependent bile acid transporter (ASBT, SLC10A2), SLC10A3, SLC10A4, SLC10A5, sodium-dependent organic anion transporter (SOAT, SLC10A6), and SLC10A7 (Anwer and Stieger 2014). Of the family members, NTCP and ASBT have been extensively characterized as regulators for the enterohepatic circulation of bile salts. NTCP, the first identified SLC10 family member, is mainly distributed in the liver and its primary function in bile salt metabolism is to transport extracellular bile salts into hepatocytes (Fig. 1). NTCP also transports other xenobiotics, including statins, propranolol, furosemide, micafungin, and indocyanine green (Claro da Silva et al. 2013). In addition to this transporter function, NTCP was recently demonstrated to serve as an entry receptor for hepatitis B and D viruses (HBV and HDV) (Fig. 1) (Yan et al. 2012).

The human NTCP (hNTCP) gene produces a 349 aa protein with a mass of ∼56 kDa (Stieger 2011; Watashi and Wakita 2015). NTCP is expressed in not only human but also in other mammals such as chimpanzee, monkey, rabbit, rat, and mouse and in avians, reptiles, fishes, and potentially nematodes. The amino acid homologies of hNTCP to mouse (mNtcp), tupaia (tsNtcp), and crab-eating monkey Ntcp (mkNtcp) are 73.8%, 83.9%, and 96.3%, respectively. The NTCP gene product is a hydrophobic transmembrane protein. Its crystal structure remains to be

solved, but a series of mutagenesis assays suggest that this protein has putative seven to nine transmembrane domains with a predicted topology of amino-terminal extracellular and carboxy-terminal intracellular sides. NTCP is distributed mainly on the basolateral membrane of hepatocytes and functions as an uptake transporter for bile salts. Its function is strictly regulated at both the transcriptional and posttranslational levels.

Transcriptional Regulation

Transcription of NTCP is regulated differently among species, and the molecular mechanisms of rat NTCP have been extensively characterized. Expression of NTCP is regulated by multiple transcription factors, especially nuclear hormone receptors (Fig. 2). In the rat, farnesoid X receptor (FXR), a nuclear receptor of bile salts, negatively regulates the transcription of NTCP: FXR activates the expression of small heterodimer partner (SHP), and SHP then represses the transcription of NTCP mRNA by interacting with the retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer, which induces the transcription of NTCP (Dawson et al. 2009). Upon the recognition of intracellular bile salts, FXR represses not only NTCP, but also other bile salt transporters such as ASBT and organic anion transporting polypeptide (OATP) 1B1 (Claro da Silva et al. 2013). In addition, FXR upregulates bile salt efflux transporters, such as bile salt export pump (BSEP) and OATP8, to prevent intracellular accumulation of cytotoxic bile salts. Activation of FXR reduces the expression level of cholesterol 7α-hydroxylase (CYP7A1), a key enzyme involved in bile acid biosynthesis. Another nuclear hormone receptor, glucocorticoid receptor (GR), is reported to transactivate mouse and human NTCP: Hepatocytes from GR knockout mice showed a decrease in the expression of NTCP, accompanied by a reduced bile volume in the gallbladder. Hepatocyte nuclear factor (HNF) 1 and HNF4 are also reported



NTCP (Sodium Taurocholate Cotransporting Polypeptide), Fig. 2 Transcriptional regulation and posttranslational modification of NTCP. NTCP expression is regulated by several nuclear hormone receptors. Farnesoid X receptor (FXR) is activated by stimulation of BS to upregulate small heterodimer partner (SHP). SHP then represses the transcription of NTCP. FXR also negatively regulate cholesterol-7-hydrogenase (CYP7A1), a key enzyme for BS synthesis. In addition, NTCP transcription is reported to be activated by glucocorticoid receptor (GR), hepatocyte nuclear factor 1 (HNF1), and HNF4, and negatively regulated by HNF3. After NTCP protein synthesis, its subcellular localization is regulated by posttranslational modification. Cyclic AMP (cAMP) triggers the phosphoinositide 3-kinase (PI3K) signaling and activates protein kinase C (PKC)δ and PKCζ. These PKCs promote the translocation of NTCP to the plasma membrane

as positive regulators for rat NTCP transcription, while HNF3 represses the transcription of NTCP. Stimulation of cytokines, including tumor necrosis factor (TNF)α and interleukin (IL)-1β, has been reported to decrease the expression of NTCP by modulating the activity of the above transcription factors (Dawson et al. 2009; Urban et al. 2014).

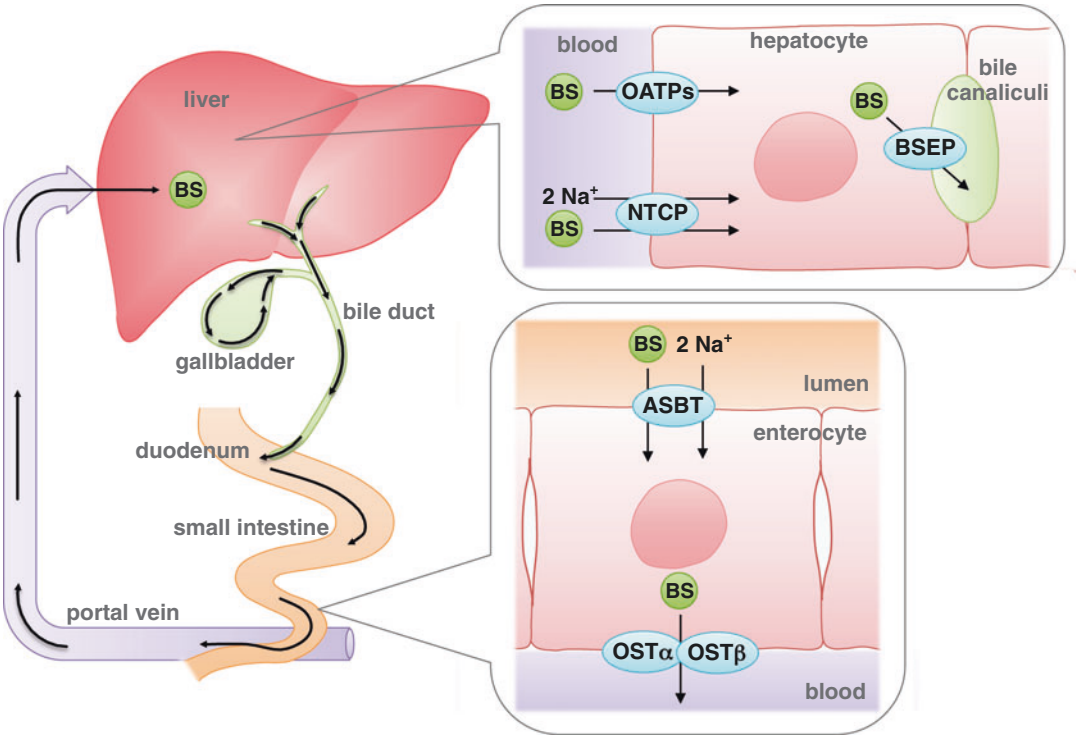
Posttranslational Modification

After transcription and translation, NTCP is destined to be localized to the plasma membrane to work as a bile salt transporter. NTCP is mainly located on the basolateral membrane of hepatocytes. This localization is regulated by several posttranslational modification events (Fig. 2) (Anwer and Stieger 2014; Claro da Silva et al. 2013). Cyclic AMP (cAMP) affects NTCP localization on the plasma membrane and transporter activity by activating phosphoinositide-3-kinase (PI3K) and protein kinase C (PKC): cAMP triggers PI3K signaling and then activates PKCδ and PKCζ. These PKCs activate Rab4, which colocalizes with NTCP, to stimulate the motility of Rab4 and NTCP-containing vesicles on the microtubules leading to the plasma membrane. PKC activation is shown to translocate NTCP from the plasma membrane to the cytosol. These findings suggest that the serine/threonine phosphorylation of NTCP is a key determinant for its localization, although the mechanism as a whole remains to be understood. It is also reported that the glycosylation of NTCP alters its localization. The R252H point mutation of NTCP reduces the complex-glycosylated NTCP, resulting in a reduction in plasma membrane NTCP, although it is not clear whether this amino acid is the glycosylation site (Vaz et al. 2015). In addition, NTCP localization on the plasma membrane is modulated by S-nitrosylation (Anwer and Stieger 2014). Treatment with nitric oxide to induce S-nitrosylation decreases NTCP membrane localization and its transport activity. NTCP is also reported to be modified by ubiquitin, and ubiquitinated NTCP mediates the degradation through the proteasome pathway. The above transcriptional and posttranslational regulation mechanisms of NTCP play a key role in regulating bile salt circulation and metabolism.

Function

Bile salts are important physiological agents for the intestinal digestion of fat and absorption of fat-soluble vitamins. They also function as intracellular signaling molecules through G protein-coupled receptors (GPCRs) and nuclear hormone receptors to regulate cellular physiological events, including lipid, glucose, energy, and metabolic homeostasis. Bile salts are synthesized mainly in the liver using cholesterol or oxysterols as starting materials via stepwise reactions involving cytochrome P450 (CYP) family enzymes. The synthesized primary bile salts can be then conjugated with either glycine or taurine to produce conjugated bile salts. These bile salts are secreted from hepatocytes into the bile and travel down the intestine, where they function to promote nutrient digestion and absorption (Fig. 3). Most of the bile salts are then carried in the portal circulation back to the liver for uptake and resecretion into bile. This cycle of bile salts is called enterohepatic circulation, and this circulation system involves several transporters, such as NTCP and the OATP family that take up bile salts from portal blood into hepatocytes, BSEP (which secretes bile salts into the bile), and ASBT and organic solute transporter alpha-beta (OSTα/β), which mediate the uptake and efflux of bile salts from enterocytes (Kosters and Karpen 2008; Stieger 2011).

The uptake of bile salts by NTCP occurs in a sodium-dependent manner (Figs. 1, 3). NTCP cotransports one bile salt molecule together with two sodium ions to the intracellular side using the energy provided by the out-to-in sodium gradient generated by Na^+/K^+-ATPase on the basolateral plasma membrane. NTCP predominantly transports taurine- or glycine-conjugated bile salts and shows higher affinity for dihydroxy bile salts (e.g., taurochenodeoxycholate and taurodeoxycholate) than for trihydroxy bile salts (e.g., taurocholate, cholate) (Kosters and Karpen 2008; Stieger 2011). A recent NTCP knockout analysis showed that about 30% of NTCP-



NTCP (Sodium Taurocholate Cotransporting Polypeptide), Fig. 3 Enterohepatic circulation of bile salts. BS are synthesized mainly in the liver and can be conjugated with either glycine or taurine. The BS are secreted from hepatocytes into the bile canaliculi through transport by bile salt export pump (BSEP) and then flow into gallbladder to travel down the small intestine. More than 90% of BS are reabsorbed into enterocytes within the terminal ileum via transport by apical sodium-dependent bile salt transporter (ASBT). BS are then secreted into the portal vein by organic solute transporter (OSTα/β) and travel back to the liver. In the liver, BS are transported into the hepatocytes by NTCP or organic anion-transporting polypeptide family (OATPs)

deficient mice remarkably accumulated conjugated bile salt in the serum (up to 500-fold compared with normal mice) and had significantly reduced body weights (Slijepcevic et al. 2015). Another NTCP knockout study reported that in addition to the elevation of serum bile salt levels, some mice developed gallbladder diseases and impaired liver function at an early age, together with altered lipid metabolism (Hou et al. 2015). These observations clearly demonstrate the critical role of NTCP in bile salt metabolism and its related physiological events. Many drugs and small molecules have been reported to interact with and impair the transporter activity of NTCP. Inhibition of the transporter activity of NTCP by these compounds may elevate serum bile salts levels and thus cause significant adverse effects. OATPs, which are other sodium-dependent bile salt transporters, can partially compensate for the inhibition of NTCP, but some of these compounds inhibit both NTCP and OATP transporters.

Polymorphism

Several single-nucleotide polymorphisms (SNPs) associate with the transporter activity of NTCP have been reported. A SNPs analysis of 90 European Americans, 90 African-Americans, 100 Chinese Americans, and 90 Hispanic Americans identified seven SNPs in the NTCP coding region, including four nonsynonymous SNPs, which resulted in the I223T, S267F, I279T, and K314E substitutions in NTCP (Ho et al. 2004). The I223T variant, seen in 5.5% of African-Americans, showed an equivalent level of total NTCP protein

expression in cells compared with the wild type, but significantly reduced NTCP expression on the cell surface: consequently, bile salt uptake was impaired. I223 is therefore likely to be important for NTCP trafficking to the cell surface. The S267F variant, seen in 7.5% of Chinese Americans, exhibited almost complete loss of both conjugated- and unconjugated-bile salt uptake, but possessed normal transport activity for another substrate, estron sulfate, suggesting that S267 is critical for bile salt binding/recognition. The I279T and K314E polymorphisms were relatively rare and were found in 0.5% of Chinese Americans and in 0.55% of Hispanic Americans, respectively. A recent paper reported other minor variants (I88T, L131 V, V200 M, L222S, M256 T, A323P, and A333T) seen in less than 0.5% of the Chinese populations. Although these variants in the NTCP gene are unlikely to cause serious diseases in individuals, a recent report analyzing a child case who showed growth retardation and motor developmental delay found the R252H polymorphism of NTCP and an extremely elevated serum bile salt level (up to mM levels) (Vaz et al. 2015). This case demonstrates the essential role of NTCP in the uptake of conjugated bile salts in human liver.

The discovery that NTCP functions as an entry receptor for HBV and HDV (see the next paragraph) prompted the analysis of SNPs that associate with HBV infection, the pathogenesis of chronic hepatitis B, and eventual hepatocellular carcinoma. In an analysis of 1899 chronic hepatitis B patients and 1828 healthy controls of Taiwanese Han Chinese individuals, the S267F NTCP variant was significantly associated with resistance to chronic hepatitis B (4.1–4.6% of the population with chronic hepatitis B vs. 10.8–11.1% of healthy individuals) and a lower incidence of acute-on-chronic liver failure (Peng et al. 2015). No other NTCP SNPs were identified in this paper showing association with hepatitis B. Another study from Taiwan on 3801 chronic hepatitis B patients and a cohort of HBV-negative individuals also consistently showed an association between the S267F variant and resistance to HBV infection and decreased risk of resultant cirrhosis and hepatocellular carcinoma (Hu et al. 2016). Although other papers showed no such association of this genetic variation with HBV infection, a relatively small number of individuals were analyzed in these studies. Cell culture analysis showed that the S267F mutation in NTCP severely impaired the function of the protein as an entry receptor for HBV and HDV (Yan et al. 2014), thus explaining the molecular basis for these epidemiological findings. However, it is interesting that some S267F individuals can still develop chronic hepatitis B in spite of the almost complete loss of receptor function observed in cell culture analysis, suggesting the existence of another entry receptor or of an HBV variant that can overcome the loss of function of NTCP (S267F). Additionally, a genetic variant located in intron 1 of NTCP (rs4646287) was shown to have association with the risk of HBV infection, although its variant effect on the NTCP expression level was not clear. Thus, NTCP polymorphisms affect susceptibility of HBV infection and HBV-related pathogenesis in addition to bile salt metabolism.

NTCP as an HBV/HDV Receptor

NTCP as a Virus Entry Receptor

Fifty years after the discovery of HBV, the cellular receptor for HBV has remained unknown despite intense efforts to analyze the viral entry process. In 2012, NTCP was revealed to function as an entry receptor for HBV and HDV (Fig. 1) (Yan et al. 2012). The liver-specific distribution of NTCP apparently accounts for some, if not all, of the hepatotropism of HBV and HDV. An HBV particle contains three types of surface envelope proteins: small (SHBs), middle (MHBs), and large surface proteins (LHBs). These proteins share a common carboxyl-terminal domain, termed the S region. SHBs comprises only the S region, while MHBs have an amino-terminal extension, referred to as the preS2 region, and LHBs carries a further amino-terminal region upstream of preS2 and S, called the preS1 region (Urban et al. 2014). A series of analyses using neutralizing antibodies and mutagenesis suggest that both the preS1 and

the S regions play pivotal roles in HBV infection, and that the 2–48 aa region of preS1 is especially essential for interaction with a cellular receptor that had remained unidentified. Recently, Yan et al. used an affinity purification method to identify NTCP as a binding protein for the 2–48 aa region of preS1. Subsequent analysis showed that knockdown of NTCP in HBV-susceptible cells impaired HBV susceptibility, and ectopic expression of NTCP rendered HBV non-susceptible human hepatocyte cell lines susceptible to HBV infection. These data demonstrate that NTCP is an HBV entry receptor. Furthermore, it was shown that NTCP is essential for the entry of HDV, which shares the same envelope proteins as HBV. This discovery represents a major breakthrough in HBV/HDV research in this decade.

Residues Responsible for Binding with Viruses, Bile Salts, and Sodium

The precise mechanisms by which NTCP mediates viral entry are currently largely unknown, but it is speculated that NTCP triggers the endocytosis of HBV/HDV through engagement of the preS1 region during the early entry process. Limited information about its molecular basis indicates which NTCP residues are required to support viral entry (Watashi and Wakita 2015; Yan et al. 2015): especially, it was shown that hNTCP and tsNtcp, but not mkNtcp, support infection of HBV and HDV. Comparison of the sequences of these three proteins suggests that 157–165 aa of hNTCP are required for viral entry. The introduction of mutations (KGIVISLVL) in the 157–165 aa region of hNTCP abolished preS1 binding, although the data could not exclude the possibility that the mutations caused a major conformational change of NTCP. Another study compared the sequence of hNTCP with that of mNtcp, which does not support HBV/HDV entry, and identified 84–87 aa (RLKN) as critical residues in hNTCP for viral entry. It is interesting that mice carrying the RLKN hNTCP mutation in the 84–87 aa region of mNtcp became susceptible to infection by HDV. Modeling analysis suggested that both the 157–165 aa and 84–87 aa regions reside on the extracellular surface or in the transmembrane region, although the precise mechanisms by which these regions support HBV/HDV entry remain to be clarified.

Most bile salts, including primary and secondary bile salts, inhibit the binding of NTCP with preS1 and subsequent infection of HBV and HDV (Watashi and Wakita 2015; Yan et al. 2015). These results suggest that the binding region of HBV/HDV and that of bile salts to NTCP are overlapped. This possibility is further supported by point mutation analyses in bile salt-binding residues, as described below. The bile salt and sodium binding sites on hNTCP have been predicted based on previous information obtained from rat Ntcp mapping and from the crystal structure of ASBT. Mutagenesis assays indicated that N262, Q293/L294, and S267 (described above) are all critical regions for bile salt binding. The introduction of mutations in any of these regions strongly impaired the ability of NTCP to bind to preS1 and thus support HBV/HDV infection. Thus, HBV/HDV interaction and bile salt binding share common molecular determinants on NTCP, at least based on the evidence available to date. Mutations in the reported or predicted sites for sodium binding, namely, Q68, S105/N106, E257 and Q261 of NTCP, also reduce viral receptor function, although to a lesser extent than mutations in the bile salt binding sites (Yan et al. 2014). Solving the NTCP protein structure is essential for fully understanding the molecular basis of NTCP function.

NTCP as a Target for Developing Anti-HBV Drugs

Approximately 240 million people worldwide are chronically infected with HBV, and this elevates their risk for developing liver cirrhosis and hepatocellular carcinoma. HDV coinfects the liver with HBV and clinically aggravates the natural history of HBV-related diseases. These viruses constitute a major public health problem worldwide and thus there is urgent need for new drugs against HBV/HDV infection. Identification of NTCP as an HBV/HDV receptor has facilitated research on developing anti-HBV/HDV agents. To date, NTCP substrates such as bile salts,

including taurocholate, tauroursodeoxycholate, and taurochenodeoxycholate, and other xenobiotic substrates, including bromosulfophthalein, have been reported to inhibit HBV/HDV infection. Furthermore, compounds known to inhibit NTCP-mediated bile acid uptake, including cyclosporin A, ezetimibe, and irbesartan, were shown to inhibit HBV entry (Yan et al. 2015). Additionally, newly identified HBV entry inhibitors such as vanitaracin A identified using cell-based chemical screening have been demonstrated to directly target NTCP and inhibit both its viral receptor function and transporter activity. These data suggest that NTCP can serve as a new target for developing anti-HBV agents (Iwamoto and Watashi 2016). In fact, myrcludex B, which is an optimized synthetic lipopeptide of the myristoylated 2–48 aa region of preS1 and is proved to bind to NTCP, strongly inhibits HBV infection in both cell culture and in in vivo mouse models. A recent report of a phase Ib/IIa clinical study demonstrated the antiviral potential of myrcludex B for anti-HBV/HDV treatment in patients (Bogomolov et al. 2016). Currently, the adverse effects of long-term administration of myrcludex B, and its inhibition of NTCP transporter and modulation of bile salt metabolism, remain unclear. Optimizing strategies for using NTCP as a drug target will require further analysis of the mechanism underlying NTCP-mediated viral entry.

Summary

NTCP transports bile salts and other xenobiotics into hepatocytes that play a critical role in the enterohepatic circulation of bile salts. Knockout mice and gene polymorphism analyses clearly demonstrate the essential role of NTCP in bile salt metabolism. In addition, NTCP was recently identified as an entry receptor for HBV/HDV, making NTCP an attractive target for the development of antiviral agents. Although the molecular mechanisms by which NTCP supports HBV/HDV infection remain largely unknown, it is likely that NTCP supports HBV/HDV entry in a manner different from that used to transport bile salts because viral particles are much larger than the pore size of NTCP. Further mechanistic analysis of NTCP-mediated HBV/HDV entry will provide new insights into NTCP function.

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NTCP1

► NTCP (Sodium Taurocholate Cotransporting Polypeptide)

Ntk

► CSK-Homologous Kinase

NTPDase

► E-NTPDase Family

NTPH

► Tryptophan Hydroxylase 2

NTR

► Neurotensin Receptor (NTSR)

NTS

► Neurotensin Receptor (NTSR)

N-Type

► Voltage-Gated Calcium Channels: Structure and Function (CACNA)

Nuclear Factor for IL-6 Expression (NF-IL6)

► CCAAT/Enhancer-Binding Protein Beta

Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells

► NF-κB Family

Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Zeta

► IkBz

Nuclear Factor RIP140

► Nuclear Receptor-Interacting Protein 1 (NRIP1)

Nuclear Factor-Kappa-B

► NF-κB Family

Nuclear Mitogen- and Stress-Activated Protein Kinase-1

▶ MSK1

Nuclear Myosin I

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Synonyms

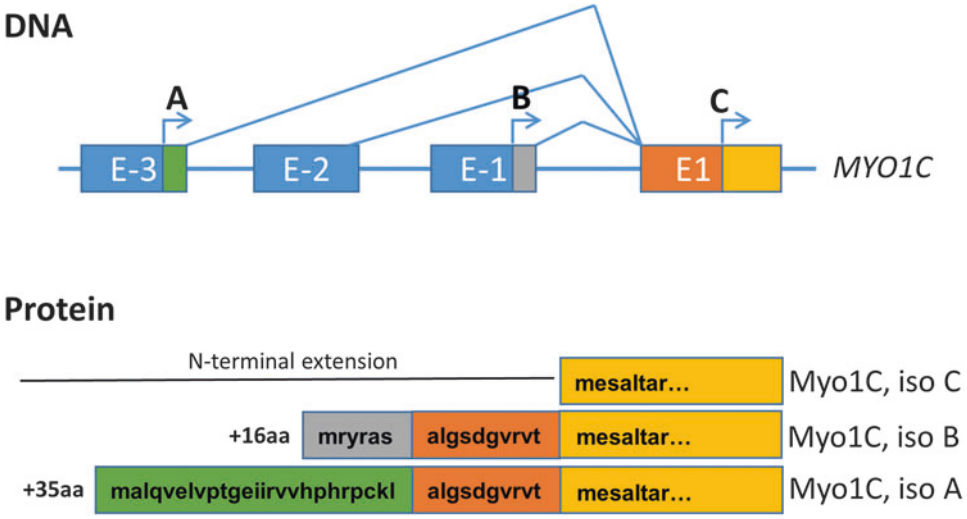
MMIb; MMI-beta; Myo1C isoform B; myr2; NM1; NMI

Historical Background

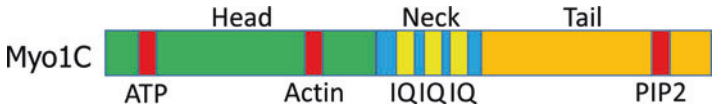
Nuclear myosin I (NM1) belongs to the group of class I myosins, which are monomeric, non-processive, slow-rate, and low-duty ratio molecular motors transforming free chemical energy stored in ATP into mechanical force. Nuclear myosin I was discovered by testing antibodies to adrenal myosin 1. The antibody was staining a 120 kDa nuclear protein with ATPase activity, and the protein was ATP-, actin-, and calmodulin-binding, which are the typical features of unconventional myosins. At that time, there were no myosins known to be present in the cell nucleus, hence the discovered protein was called nuclear myosin I (Pestic-Dragovich et al. 2000). The mass spectrometric analysis of the NM1 showed a high homology to the Myosin 1c (Myo1c) protein, the first single-headed myosin isolated from mammals, also known as mammalian myosin I, or myosin 1β. However, with the increasing numbers of myosins discovered, there was a need to unify the myosin nomenclature. Therefore, we now recognize NM1 as an isoform of Myosin 1c protein (Gillespie et al. 2001).

The human MYOIC gene encodes three isoforms. Myosin 1c, isoform C is the classic 1028 amino acid "cytoplasmic" form, usually called Myo1C. Myosin 1c, isoform B, known as nuclear myosin I (NM1), includes 16 extra N-terminal amino acids arising from an upstream exon-1. The newest isoform discovered is Myosin 1c, isoform A, which contains additional 35 amino acids on its N-terminal end in comparison to the Myo1C, isoform C (Fig. 1). This isoform is specifically expressed in some human cancer cell lines and its expression is several grades lower in comparison to the two other isoforms (Sielski et al. 2014). In this review, we will pay attention only to Myo1C and NM1.

Historically, Myo1C was considered to be purely cytoplasmic, while NM1 purely nuclear. N-terminal extension as the only difference between cytoplasmic and nuclear isoform was believed to provide a nuclear localization signal. However, there is rising evidence that these isoform cooperate together in both compartments and serve similar functions. Dzijak et al. showed that nuclear localization signal which directs these myosins to the cell nucleus is localized in the neck region of the molecule and is common for all three isoforms (Dzijak et al. 2012). Moreover, the proteomic studies of fractionated lysates showed that there is no compartment-specific enrichment of one of the isoforms. Instead, relatively small fraction of both isoforms is present in the nucleus (∼20%) in comparison to the cytoplasm (∼80%) (Venit et al. 2016). Therefore it seems plausible that rather than having separate function, the overall amount of myosin molecules together is more important in concrete cellular processes, and that they might even replace one another in these functions. This was shown in NM1 knockout mice, where the loss of NM1 was compensated by Myo1C (Venit et al. 2013). However, we cannot exclude the possibility, that these isoform has also some specific functions as the identified N-terminal extension of NM1 is highly conserved across metazoans and expression profiles of both isoforms somewhat differ in various tissues (Kahle et al. 2007).



Nuclear Myosin I, Fig. 1 Alternative splicing of human *MYO1C* gene and its protein products. On DNA, arrows mark start of the transcription of Myo1c, Iso C from exon 1 (E1), Myo1c, Iso B from exon-1 and Myo1c, IsoA from exon-3. Exon-2 is noncoding. Alternative splicing gives rise three isoforms differing in protein sequence on the N-terminus. The coloring in DNA sequence corresponds to colouring in the protein sequence



Nuclear Myosin I, Fig. 2 Schematic view of Myo1C protein structure and its binding sites

NM1 Structure and Function

The typical myosins are 1000–2000 residues long and comprise of three functional subdomains: (1) head domain which harbors an ATP-binding site and actin-binding site, (2) neck domain which binds light chains or calmodulins, and (3) tail domain often containing a cargo binding domain, such as SH3 domains, GAP domains, FERM domains, or plecstrin homology PH domains. Whereas the catalytic head domain shares a number of highly conserved elements differing only in some surface loops and the N-terminus, the tail domains of various myosin classes are highly divergent. The neck domain is relatively stable, consisting of a various number (0–6) of helical sequences termed IQ motives with consensus sequence IQXXXRGXXXR (Coluccio 1997). Identically to other class I myosins, NM1 possesses all these three main domains. In a neck domain, it contains three IQ domains responsible for CA^{2+}-dependent calmodulin binding important for nuclear translocation of the protein and specific nuclear localization signal ^{754}GRRKAAKRKWAAQ^{766}. Deletion or mutation of single basic residues does not have any effect on the transport, however mutating of all six basic residues to Alanin completely abolish nuclear import (Dzijak et al. 2012). On the C-terminal tail domain, NM1 contains PH domain responsible for binding to phosphoinositides, chiefly to phosphoinositol-4,5-bis-phosphate (PIP2) (Fig. 2).

NM1 in Transcription and Chromatin Remodeling

In the cell nucleus, NM1 associates with nuclear actin and is required for RNA polymerase I (Pol I) and RNA polymerase II (Pol II) transcription. Both NM1 and actin colocalize and coimmunoprecipitate with Pol I and Pol II complexes and depletion of NM1 inhibits transcription by both

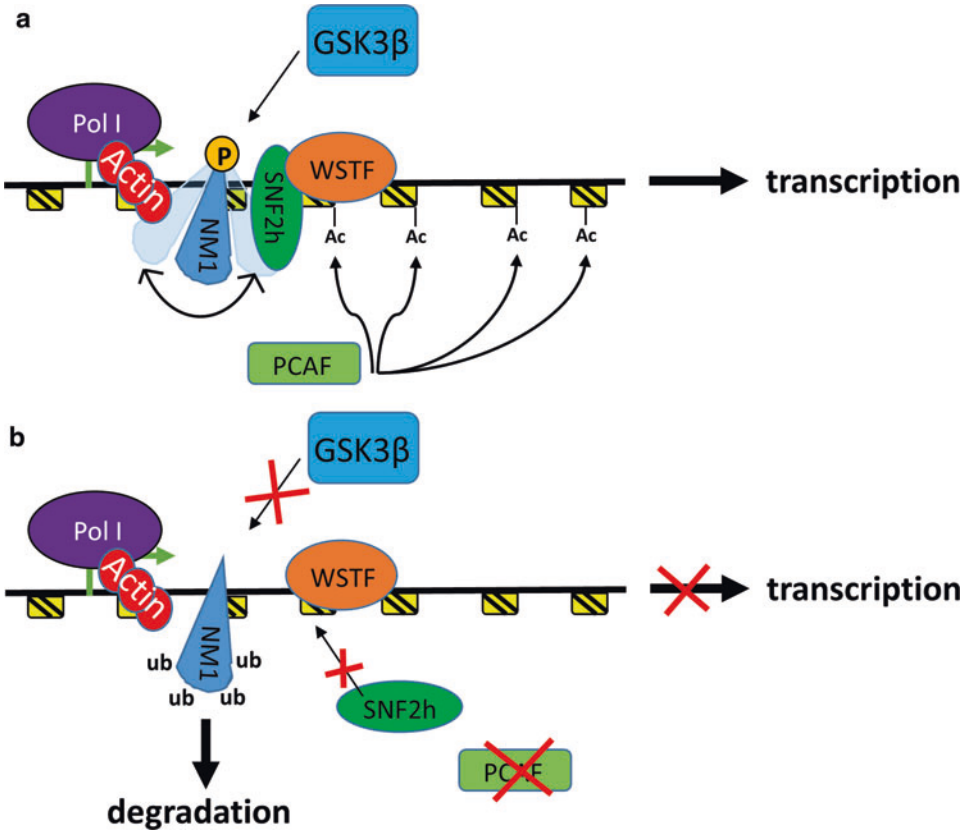
polymerases. This effect can be rescued by the addition of purified NM1, which increases the level of transcription in a dose-dependent manner (Pestic-Dragovich et al. 2000; Philimonenko et al. 2004; Grummt 2006; Ye et al. 2008). NM1 associates with initiation-competent RNA polymerase I complexes through an interaction with the basal transcription factor TIF1A (Philimonenko et al. 2004). In addition to the transcription initiation, NM1 is needed in further steps during the elongation phase where it interacts with chromatin remodeling complex WSTF-SNF2h and facilitates Pol I transcription on chromatin (Percipalle et al. 2006). It is therefore believed that NM1 bound to TIF-1A is recruited to the preinitiation complex along with Pol I and associated actin, which assemble to a functional transcription initiation complex. This is also supported by the finding that both actin polymerization and the motor function of NM1 are required for the association with the Pol I transcription machinery and transcription activation. Finally, by interacting with NM1, SNF2h promote PCAF-mediated H3K9 acetylation at the gene promoter to allow Pol I movement through the chromatin (Sarshad et al. 2013). Moreover, the interaction of NM1, with actin or SNF2h on rDNA is cell cycle dependent and these proteins associate with Pol I just after exit from mitosis. Glycogen synthase kinase 3β (GSK3β) plays principal role in this regulation as it phosphorylates NM1 in early G1 phase and protects it from proteasome-mediated degradation (Sarshad et al. 2014). Lately, ChIP seq analysis of NM1 binding chromatin revealed that, except its binding to rDNA, NM1 binds across the entire mammalian genome and this binding correlates with the occupancy of Pol II and active epigenetic marks at the gene promoters. NM1 also colocalizes with SNF2h chromatin remodeler and mediates physical recruitment of the histone acetyl transferase PCAF in similar manner as was described for Pol I transcription (Almuzzaini et al. 2015). Therefore, a speculative model has been suggested: after mitosis, NM1 is phosphorylated by GSK3β, which allows it to form a complex with actin and Pol I/Pol II in the gene promoters. Then WSTF-SNF2h complex is associated with NM1, what leads to the loading of PCAF and subsequent acetylation of H3K9 histones and allows transcription machinery to proceed (Fig. 3a). In case of GSK3β mutant, unphosphorylated NM1 is polyubiquitinated and degraded by proteasome, and B-WICH complex is not assembled on chromatin what leads to the suppression of Pol I/Pol II transcription (Almuzzaini et al. 2015) (Fig. 3b).

NM1 in Gene Movements

The cell nucleus is highly compartmentalized structure, where chromosomes occupy discreet radially organized territories. Chromosomes with more active genes are localized to the center of the nucleus while gene-poor chromosomes are localized more to the nuclear periphery. However, this organization is not static and there is always some intermingling of genes which seem to be actively moved toward the transcription machineries. In the cytoplasm, the movement of cargos is achieved by different molecular motors which move vesicles along the different kinds of filaments. The similar mechanism has been suggested to occur also in the cell nucleus. It was shown that NM1 and actin are necessary for long-range migration of chromosome site from nuclear periphery to the interior after transcription activation. Mutation in NM1 motor domain or blocking of actin polymerization blocks this movement, however the direct mechanism is still not known and results are not conclusive yet (Chuang et al. 2006; Dundr et al. 2007). Apparently, there are no long actin filaments present in the nucleoplasm, although they can form there under some specific, mostly unphysiological, circumstances (Kalendova et al. 2014). New tools, especially molecular probes, will be needed to understand actin-myosin functions in chromosome movements.

NM1 in the Cytoplasm

As it was shown that “cytoplasmic” Myo1c can compensate for the NM1 loss in the nucleus and that the two isoforms are interchangeable in the transcription, the question whether NM1 protein can function in the cytoplasm was raised up. The study by Venit and Kalendova demonstrated



Nuclear Myosin I, Fig. 3 NM1 function in Pol I transcription. (**a**) Early after cell division, GSK3β phosphorylates NM1, which subsequently binds to SNF2h and facilitates PCAF acetylation of surrounding histones allowing. (**b**) In case of mutant GSK3β, NM1 is polyubiquitinated and degraded by proteasome, B-WICH complex is not assembled and Pol I/ Pol II transcription is suppressed

that NM1 is tethered to the plasma membrane by interaction with plasma membrane-associated phosphoinositides, and NM1 and Myo1c isoforms are evenly distributed next to each other (Venit et al. 2016).

Myo1C has been shown to maintain effective cell membrane tension and regulate osmotic state of the cell by linking of plasma membrane to the underlying actin cytoskeleton. Atomic force microscopy measurements of plasma membrane of cultured fibroblasts with a deletion in NM1 showed that these cells have higher membrane elasticity, supporting the idea that NM1 regulates plasma membrane tension via its lineage to the underlying actin cytoskeleton similarly to Myo1C. This was proven also functionally, as upon deletion of NM1, the cells exhibit higher ability to tolerate strong hypotonic conditions, suggesting a deleterious effect of myosin linkage between plasma membrane and actin cytoskeleton under rapid membrane rearrangements caused by changes in osmotic conditions. Therefore, the deletion of NM1 leads to the reduction of the overall number of myosin molecules on the plasma membrane, which afterward binds to the cytoskeleton less tightly, and cells are more susceptible to swelling. On the other side, NM1 KO cells did not show any difference in adhesion, spreading, or motility, suggesting that the basic level of membrane tension is sufficiently maintained by other class I myosins. However, the regulation and dynamics between different myosin I members on the plasma membrane are not yet known. For example, the N-terminal extension of

NM1 does not affect its membrane localization since the amount of NM1 and Myo1c isoforms at the plasma membrane is the same, but as a part of the motor head domain it could affect the binding of NM1 to the actin cytoskeleton. It is also possible that multiple pathways contribute to the phenotype observed under stress conditions – because NM1 is widely spread all over the plasma membrane, not only bound to actin filaments.

Summary and Perspectives

There is an increasing evidence of specific nuclear functions of several myosin classes. However, the interplay between different myosin isoforms and classes is hardly known. In case of NM1 and Myo1C, the general idea of having one isoform in the nucleus and one in the cytoplasm is still present in recent publications. However, strictly speaking, no studies have been published directly proving isoform-specific functions of these myosins. This is especially important in the experimental design and data explanation, as most of the techniques based on the gene targeting or antibody usage affect both myosin isoforms. Therefore, one of the questions which has not been answered yet is what is the functional significance of N-terminal extension of NM1 protein? Current advances in gene knockout technologies by using of CRISP/Cas system combined with exogenous expression of single isoforms and high-throughput analysis could answer this question.

Secondly, there is an emerging evidence about different phosphoinositides in the cell nucleus. Phosphoinositol-4,5-bis-phosphate (PIP2) have been shown to be in complex with nascent RNA transcript affecting Pol I transcription by binding to UBF and fibrillarin in the nucleolus (Yildirim et al. 2013). NM1 binds to PIP2 by its PH domain in the cytoplasm and therefore similar interaction is predicted also in the cell nucleus. However, the functional significance of such interaction needs to be elucidated.

Finally, MYO1C gene is localized in a genomic locus commonly deleted in a variety of human tumors, and therefore it is one of the candidates as a novel tumor suppressor gene (Hedberg Oldfors et al. 2015). This is further supported by the increasing number of studies reporting that cancer cells show significantly higher elasticity and deformability than control cells derived from the healthy tissues. While healthy control cells display a broad distribution of the elasticity modulus, cancer cells show a significantly narrower Gaussian distribution with a significantly lower standard deviation of the Young's modulus (Suresh 2007). These data resemble elastic phenotype of the WT and NM1 KO cells, which show very similar relative patterns of the Young's modulus value distribution. Therefore, deletion, mutation, or change of the relative amounts of NM1 or other class 1 myosins might lead to an increased metastatic potential of the cells. Understanding of myosins dynamics at the plasma membrane could therefore bring new insight into a cancer research and therapy.

See Also

- ▶ Myosin I (Myo1)
- ▶ Myosins

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Nuclear Receptor Related 1

▶ NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

Nuclear Receptor Subfamily 0 Group B Member 2

▶ Nr0b2

Nuclear Receptor Subfamily 1, Group I, Member 1 (NR1I1)

▶ VDR, the Vitamin D Receptor

Nuclear Receptor Subfamily 3 Group C Member 1 (Glucocorticoid Receptor)

▶ Glucocorticoid Receptor (GR)

Nuclear Receptor Subfamily 3 Group C Member 2

▶ Mineralocorticoid Receptor

Nuclear Receptor Subfamily 4 Group A Member 2

▶ NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

Nuclear Receptor-Interacting Protein 1

► Nuclear Receptor-Interacting Protein 1 (NRIP1)

Nuclear Receptor-Interacting Protein 1 (NRIP1)

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Synonyms

NRIP1; Nuclear factor RIP140; Nuclear receptor-interacting protein 1; Receptor interacting protein 140; Receptor-interacting protein 140; RIP140

Historical Background

Determining the regulatory mechanisms of nuclear receptor action was one major focal topic of research in the 1990s. During this period, many nuclear receptor-associated proteins were identified as transcriptional coregulators, which were broadly categorized as coactivators and corepressors. Human receptor-interacting protein 140 (RIP140) was identified as a ligand-dependent interacting protein of estrogen receptor α (ERα) by far-Western blotting, and mouse RIP140 was isolated as a corepressor of orphan receptor TR2 from a yeast two-hybrid screening and later found to also interact with ► retinoic acid receptor (RAR) in a ligand-enhanced manner (Lee et al. 1998). An official gene name *nrip1* was established for RIP140 by the HUGO gene nomenclature committee. The mouse gene is located on chromosome 16, in region C3.1, whereas the human gene is located on q11.2 of chromosome 21. DNA sequence analyses conclude that this gene is conserved in all vertebrate species examined. Experimental data have validated its interaction and gene coregulatory activity for all the nuclear receptors examined, as well as several transcription factors. The list includes AR, ER, GR, RARα/β, RXRα/β, PPARα/γ/δ, PXR, LXRα/β, ► VDR, AhR, ERRα/β/γ, RORβ, HNF4α, TR2, ► TR4, c-jun, SF-1, RelA, and ► GRIP1. Most experimental data in the past have demonstrated its ligand-enhanced gene repressive activity (Wei 2004). Recent studies have begun to elucidate other functions of RIP140 beyond the nucleus. These extranuclear activities of RIP140 are needed for certain specialized signaling pathways and/or functions of fully differentiated cells such as adipocytes, neurons, and macrophages (see later cytoplasmic RIP140).

Expression of RIP140 and Its Regulation

RIP140 expression in the mouse can be detected as early as embryonic stage E12.5. In studies using promoter-driven reporters, Northern blotting, or Western blotting, RIP140 is detected in various organs and cell types (Fig. 1). Its expression level, based upon Western blot results, is higher in ovary and metabolic tissues such as adipose tissue, muscle, and liver. Regulation of its expression involves transcriptional regulation through several binding sites for hormone receptors such as ER and RAR, posttranscriptional regulation involving miR33 that targets its 3′UTR, translational regulation through the action of a microRNA miR-346 that targets its 5′UTR, and ubiquitin-mediated protein degradation (Ho et al. 2011, 2012) (Fig. 2). In hormone-sensitive lipase-null mice, its expression is altered in adipose tissues. In mice fed a short-term high-fat diet, it is upregulated in the epididymal adipose tissue. These findings suggest that the whole-body metabolic status can affect the regulatory machineries for RIP140 expression. In the brain, a novel form of RIP140 mRNA, which possesses an alternatively spliced 5′UTR, has been detected. Because miR-346 is generated from glutamate receptor ionotropic delta 1 gene that has been proposed to be involved in certain neurological

Nuclear Receptor-Interacting Protein 1 (NRIP1), Fig. 1 Expression of RIP140 in the mouse and detection methods

Major sites of RIP140 expression

Sites	Detection method
Ovary	Western blotting and reporter
Muscle	Western blotting
White Adipose tissue	Western blotting
Heart	Western blotting and Northern blotting
Macrophage	Western blotting
Testis	Northern blotting and reporter
Brain	Northern blotting and reporter
Lung	Northern blotting
Stomach	Northern blotting
Kidney	Northern blotting
Spleen	Northern blotting
Placenta	Northern blotting
Uterus	Reporter
Pituitary	Reporter

diseases, translational regulation of RIP140 by miR-346 would suggest a potential link between RIP140 and brain disorders.

Posttranslational Modifications of RIP140

Posttranslational modifications (PTMs) provide an important regulatory mechanism to control or modulate the function, location, interaction, and stability of proteins in response to extracellular and/or intracellular stimuli. Using mass spectroscopy (MS) analyses, many PTMs have been found on RIP140, and most of these PTMs appear to affect RIP140's interaction with other proteins, which ultimately affects its stability, subcellular distribution, and biological activities (Huq et al. 2008). Figure 3 shows the established PTMs of RIP140.

Phosphorylation

RIP140 can be phosphorylated at nine serine and two threonine residues. Phosphorylation at Thr202 and Thr207 by ERK2 facilitates its recruiting p300 which in turn acetylates RIP140 at Lys158 and Lys287. These sequential PTMs enhance RIP140's gene repressive activity by enhancing the recruitment of HDAC3. Phosphorylation at Ser102 and Ser1003 is catalyzed by protein kinase C epsilon (PKCε), which promotes PRMT1-mediated methylation at Arg240, Arg650, and Arg948. These sequential PTMs lead to the recruitment of exportin 1, which would facilitate its nuclear export (see following). Interestingly, a high-fat diet can initiate this nuclear export pathway in adipocytes by activating nuclear PKCε activity.

Acetylation

Nine lysine residues on RIP140 can be modified by acetylation. Acetylation at Lys482, Lys 529, and Lys607 in repressive domain 2 (RD2)/RD3 region promotes nuclear export and reduces its gene repressive activity, whereas acetylation at Lys158 and Lys287 in RD1 region leads to a stronger gene repressive activity and nuclear retention. Acetylation at Lys158 and Lys287 is catalyzed by p300 following ERK2-mediated phosphorylation (see above). Interestingly, this is elevated in the adipocyte differentiation process of the 3T3-L1 model indicating that RIP140's

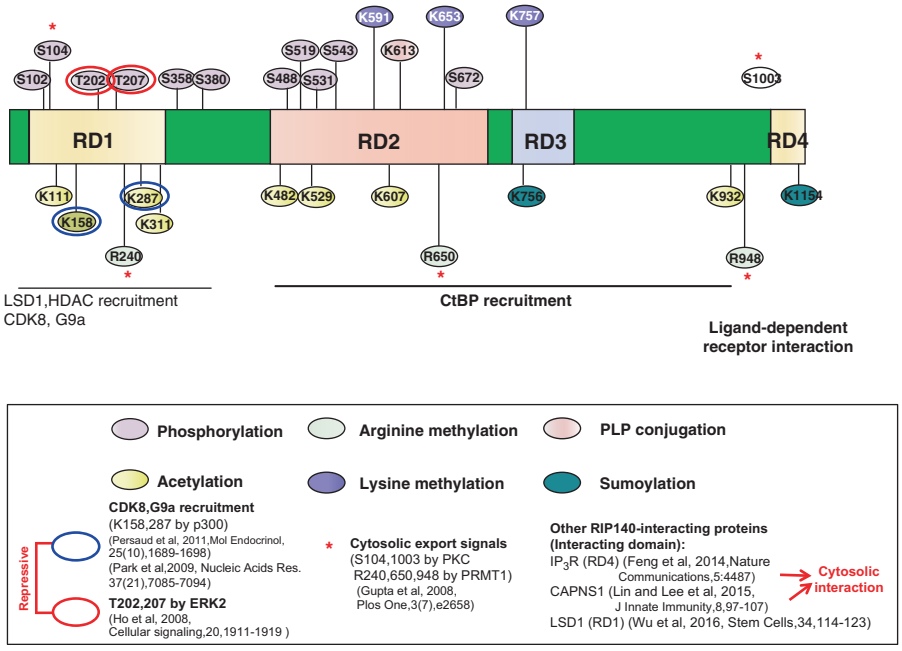
Up-regulation of RIP140

Stimuli	Mechanism	Cell type
Androgen	Not clear	LNCaP
Estrogen	Transcriptional	MCF-7
Vitamin D	Not clear	SCC25
Retinoid	Transcriptional	MCF-7 and NT2/D1
pregnant mare serum gonadotrophin (PMSG)	Transcriptional?	Rat granulosa cell
Dioxin	Transcriptional	MCF-7
miR-346	Translational	P19

Down-regulation of RIP140

Stimuli		
Progestin	Not clear	T-47D
human chorionic gonadotropin (hCG)	Transcriptional?	Rat granulosa cell
HSL-null mice	Not clear	White adipocytes
Lipid accumulation	miR-33	adipocytes
Cold exposure / β-adrenergic receptor activator	miR-30b/c	Brown adipocytes
Lipopolysaccharide (LPS)	Post-transcriptional (ubiquitin-mediated)	macrophages

Nuclear Receptor-Interacting Protein 1 (NRIP1), Fig. 2 Regulatory mechanisms of RIP140 expression, including the stimuli, underlying mechanisms, and cells/tissues in which stimuli are identified



Nuclear Receptor-Interacting Protein 1 (NRIP1), Fig. 3 Posttranslational modifications (PTMs) of RIP140. The boxes show the full-length protein with four repressive domains (RDs 1–4)

gene repressive activity is increasingly needed in later stages of adipocyte differentiation.

Methylation

Methylation can occur at either arginine or lysine residues of RIP140. Arginine methylation at Arg240, Arg650, and Arg946 is mediated by PRMT1, which promotes the interaction of RIP140 with the exportin 1 subunit CRM1, thereby stimulating RIP140's nuclear export and reducing its nuclear activity in transcriptional repression. On the other hand, lysine methylation at Lys591, Lys653, and Lys757 elevates its gene repressive activity by unknown mechanisms. Interestingly, demethylation at these three lysine residues of RIP140 is required for its methylation at the three arginine residues. This suggests a signal cross talk among various protein methylation enzyme machineries in the nucleus in order to coordinate RIP140's PTMs and, as a result, its interacting partners, subcellular localization, and biological activity.

PLP Conjugation

Pyridoxal 5′-phosphate (PLP) conjugation is found at Lys613 of RIP140, which elevates its gene repressive activity. This modification is regulated by the cellular status of PLP level, the active form of vitamin B6. This finding suggests that RIP140 may sense PLP or other nutritional factors through PTMs, which then modulates its property and biological activity.

Sumoylation

Sumo-1 conjugation is found for the human protein at Lys 756 and Lys 1154 (Lys757 and Lys 1157 for the mouse RIP140), which may regulate its gene repressive activity and nuclear distribution. The sumoylation enzyme for RIP140 has not been identified. Interestingly, Lys757 on the mouse protein can be methylated, but its relationship with potential Lys757 sumoylation is unclear.

Functional Roles of RIP140

The ubiquitous expression profile of RIP140 suggests its role in many biological processes. Based on the phenotype of RIP140-null mice, this gene is essential for normal ovulation in female animals, and metabolism in general. At the molecular level, RIP140 is known, mostly, for its corepressive activity in gene transcription through its four RDs which mediate its interaction with HDAC, CtBP, and other chromatin remodeling proteins (Wei 2004). Recent studies indicate that RIP140 can also function as a coactivator for transcription of certain genes, including fatty acid synthase (FAS) in hepatocytes and several proinflammatory genes in macrophages. The demonstrated opposing activities, with regard to gene transcriptional control, of RIP140 would indicate that the coregulatory function of RIP140 in transcription might be gene, transcription factor, and/or cell context specific. A hypothesis for its diverse functions was first proposed based upon its extensive PTMs. This hypothesis has been validated in several studies which examined the functional significance of specific PTMs of RIP140 as detailed above (Huq et al. 2008). In addition, its tightly regulated nuclear export and cytoplasmic distribution strongly suggest other functional roles for RIP140 outside the nucleus. This has also begun to be established in more recent studies, including its activity in modulating insulin-stimulated glucose uptake in adipocytes and adipokine secretion (Ho et al. 2009; Ho and Wei 2012), interacting with IP3R to modulate calcium signaling in modulating neuronal stress response (Feng et al. 2014), and interacting with CAPNS1 to activate calpain for suppressing STAT6 activation in macrophage M2 polarization (Lin et al. 2015a).

It is clear that the biological activity of RIP140 is tightly regulated by its PTMs in response to certain extracellular/intracellular cues or stimuli. The functional roles of RIP140 in animals have been demonstrated using, primarily, genetically manipulated mice. It is important to note that phenotypes of whole-body gene manipulation in animals, either gene knockout or overexpression, are the manifestation of multiple defects in various tissues/organs and therefore should not be simply interpreted as the direct consequence of a single gene defect. Moreover, cytoplasmic functions of RIP140 will complicate the phenotypes of

these genetically altered animals. Therefore, in interpreting these animal data, it is critically important to take into consideration the physiological or pathological context where RIP140 can possibly function in the nucleus and/or cytoplasm and how RIP140 protein may be modified. In the following, its functional roles deduced from studying whole animals or cell cultures are summarized.

Role in Ovulation

Depletion of RIP140 in mouse revealed its essential role in female fertility, particularly for the control of ovulation. The absence of RIP140 impacts many steps including follicular rupture, cumulus cell-oocyte complex expansion, and oocyte release (White et al. 2000). Further studies indicate that RIP140 is also critical for the expression of EGF-like factors which are essential in cumulus expansion and, possibly, follicular rupture.

Role in Adipocyte

RIP140-null mice exhibit a lower fat content and are resistant to diet-induced metabolic disorders. In the 3T3-L1 adipocyte differentiation model, RIP140 suppresses hormonal (such as thyroid hormones and retinoic acid) responses by acting as hormone-dependent corepressor (Wei 2004; Park et al. 2009; Persaud et al. 2011). In this model, depletion of RIP140 can substantially change lipid accumulation, fatty acid oxidation, glycolysis, glucose uptake, and mitochondria biogenesis in these cells. These results demonstrate RIP140's activity in modulating metabolism in adipocytes. Among potential target genes of RIP140 in adipocytes, UCP-1 is important for energy metabolism. In brown adipocytes that express a higher level of coactivator PGC-1, UCP-1 is upregulated to enhance thermogenesis. In white adipocytes where RIP140 is detected at a higher level, UCP-1 is downregulated to reduce thermogenesis. Importantly, in RIP140-null animals, white adipose tissue gains certain brown adipocyte features, indicating potential antagonism between RIP140 and PGC-1 in adipocyte metabolism. RIP140 is also found to have specific functions in the cytoplasm of adipocytes (see later section). RIP140 has been reported to repress the "brown-in-white" program in white adipocytes by preventing the expression of brown fat genes and inhibiting triacylglycerol breakdown/resynthesis (Kiskinis et al.) and regulate UCP1 and Cidea in brown adipocytes (see citations in Fig. 4).

Role in Hepatocytes

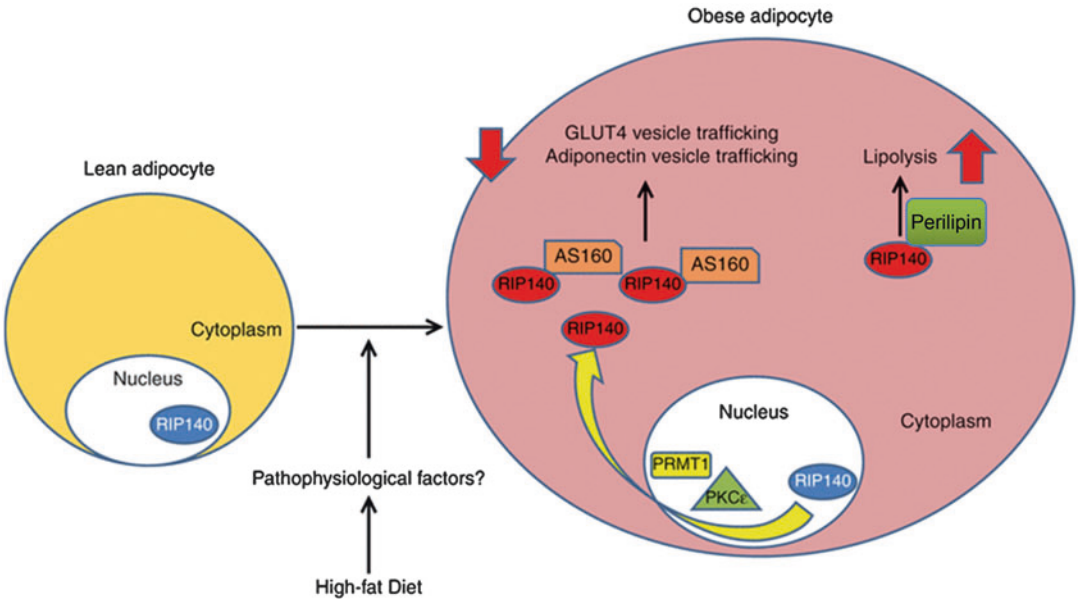
In liver cells, RIP140 can function as a corepressor or coactivator for LXR. As a corepressor of LXR, RIP140 suppresses PEPCK expression to reduce gluconeogenesis. As a coactivator of LXR, RIP140 enhances SREBP-1c and FAS expression to increase lipogenesis. Interestingly, RIP140-null mice are resistant to the development of hepatic steatosis under a high-fat diet. In studying these animals, RIP140 has also been identified as an important regulator for triglyceride storage in sepsis, starvation, and cancer cachexia. These results indicate a potential role for RIP140 in liver lipogenesis and triglyceride storage.

Role in Muscle Cells

RIP140 is differentially expressed in muscle cells: glycolytic fibers express a higher level, but oxidative fibers express a lower level, of RIP140. Microarray and metabolic analyses of muscle cells reveal that depleting RIP140 enhances the expression of genes involved in oxidative phosphorylation, fatty acid oxidation, and mitochondria biogenesis. This study demonstrates the role of RIP140 in controlling muscle metabolism and provides a clue for the defect of cardiac function in RIP140 overexpressed transgenic mice (see later sections). RIP140 regulates fatty acid oxidation via the control of Akt signaling in skeletal muscle cells. RIP140 also negatively regulates GLUT4 trafficking and glucose uptake in oxidative skeletal muscle through UCP1-associated AMPK activation. These studies indicate RIP140 as an important player in skeletal muscle where it can modulate mitochondrial function (Constantinescu et al. and Fritah et al. See citations in Fig. 4).

Role in Macrophages

For the monocyte-macrophage lineage, nuclear RIP140 functions as a coregulator for ▶ NF-κB/



Nuclear Receptor-Interacting Protein 1 (NRIP1), Fig. 4 The activities of cytoplasmic RIP140 related to adipocyte dysfunctions. In a normal state, adipocytes are lean, and RIP140 is mainly localized within nuclei. After a short-term high-fat diet, endothelin-1(ET-1) promotes cytoplasmic accumulation of RIP140 by activating ET_A receptor PLCβ-PKCε pathway (Ho et al. 2012, Molecular Cellular Endocrinology 351, 176–83). Cytoplasmic RIP140 interacts with AS160 to reduce GLUT4 (Ho et al. 2009) and adiponectin vesicle trafficking (Ho et al. 2012, Cellular Signaling 24, 71–6) and interacts with perilipin to promote (Ho et al. 2011, Cellular Signaling)

CBP complex in classic (M1) macrophage activation. This is important for LPS-stimulated transcription of several inflammatory genes. Depleting RIP140 in macrophages impairs LPS-stimulated proinflammatory cytokine production, because NF-κB transcriptional activity is reduced (Zschiedrich et al.). These studies reveal that RIP140 is a principal regulator of inflammation and/or inflammation-related diseases. Additionally, LPS-activated Syk-mediated RIP140 protein phosphorylation and then ubiquitination/degradation contribute to resolution of inflammatory responses and endotoxin tolerance (Ho et al. 2012). Related to cholesterol metabolism and inflammation, RIP140 mRNA and protein levels can be regulated by cholesterol via miRNA-33 that targets 3′UTR of RIP140 mRNA (Ho et al. 2011). RIP140 also coregulates LXR to modulate cholesterol levels in foam cells derived from macrophages (Lin et al. 2015b). Lowering RIP140 expression not only prevents M1 activation but also activates M2 activation through its cytosolic function that suppresses STAT6 (master regulator of M2 polarization), indicating that RIP140 plays a critical role in M1-M2 phenotype switch (Lin et al. 2015a). The relevance to metabolism is validated in a macrophage-specific RIP140 knockdown (mϕRIP140KD) mouse model which showed improved systemic insulin sensitivity and white adipose tissue browning under a high-fat diet challenge (Liu et al. 2014). As a proof of concept, Liu et al. successfully developed a therapeutic strategy, using designer macrophages where RIP140 level was greatly reduced, to manage (both prevent and treat) diet-induced metabolic diseases (Liu et al. 2015b, 2015a). When bred to atherosclerosis-prone ApoE mice, mϕRIP140KD also reduced foam cell formation because of reduced RIP140-mediated suppression of cholesterol transport genes ABCA1 and ABCG1, significantly alleviating western diet-induced plaque burden and atherosclerosis (Lin et al. 2015b). Recently, RIP140 was shown to act as a

corepressor of orphan receptor TR4 which suppressed osteoclastogenesis. This pathway was also validated in the mϕRIP140KD mouse model, which indeed exhibited an osteopenia phenotype (in prep). These recent studies demonstrate RIP140's pleiotropic activities in the monocyte-macrophage lineage, all are critically relevant to various pathophysiological conditions including septic shock, wound healing, diabetes, atherosclerosis, and osteopenia. However, the exact mechanism that regulates RIP140's intracellular localization in macrophages and cells derived from this lineage remains to be examined.

Role in Cardiomyocytes

In studying whole-body RIP140-overexpressing transgenic mice, a role for RIP140 in cardiac hypertrophy and functions was identified. This finding suggests a need to control the expression level of RIP140 in cardiomyocytes. However, it remains unclear if the cardiac defects are results directly from the expression of RIP140 in cardiomyocytes or that may be caused by systemic changes in whole-body metabolism. To this end, RIP140 overexpression induces NF-kB-mediated inflammatory responses and metabolic dysfunction in neonatal rat cardiomyocytes, also supporting RIP140's role as a coactivator of NF-kB in certain types of cells (Zhang et al.; see citation in Fig. 4).

Role in Brain Cells

RIP140 has been implicated in animal behaviors related with memory, stress, and emotional regulation. RIP140-deficient mice showed long-term learning and memory deficits and stress response indicating its role in the neurophysiological development (see citations in Fig. 4). Feng et al. uncovered RIP140's function in neurons, in response to neuronal damage/stress, by translocating to the cytoplasm to bind IP3R, thereby modulating calcium release to protect neurons from apoptotic death (Feng et al. 2014). RIP140's role in microglia is demonstrated in mϕRIPKD mice which showed reduced RIP140 expression in the ventromedial hypothalamus. These mice exhibited increased depressive and anxiety-like behavior. Mechanistically, this study reported that RIP140 depletion in macrophages reduced NPY production in astrocytes (Flaisher-Grinberg et al. 2014). Importantly, RIP140 expression can be dampened by behavioral stress, which is also associated with an elevated brain cholesterol level, indicting RIP140's additional role in brain cholesterol homeostasis (Feng et al. 2015). In embryonic stem cell differentiation, RIP140 is a potent repressor of genes in retinoic acid-induced early differentiation such as *Oct4* (Wu et al. 2014), and it further suppresses neuronal differentiation by forming a complex with LSD1 to specifically regulate certain retinoic acid target genes, such as *Pax6* (Wu et al. 2016). These studies suggest that RIP140 plays important roles in regulating different brain cells, including neuron stem cells, microglia, astrocytes, and neurons, to affect brain functions as reflected in various types of neurological disorders such as memory, stress response, and emotional disorder.

Role in Cancer Cells

RIP140 was first identified in human breast cancer cells and has been suggested to play important roles in oncogenic signaling pathways in various tumors including breast, ovary, colon, and liver tumors. Docquier et al. reported that RIP140 enhances ERβ's suppressive effect on 17 β-estradiol-induced transactivation and cell proliferation in ovarian cancer cells. In colorectal cancer, RIP140 was found to repress proliferation of intestinal epithelial cells and human colon cancer cells through the APC/β-catenin signaling pathway, supporting a tumor suppressor role of RIP140. RIP140 is also shown to inhibit hepatocellular carcinoma cell proliferation and migration via inactivating β-catenin/TCF signaling. Clinically, RIP140 was first identified as a chronic lymphocytic leukemia (CLL) prognostic factor, and low RIP140 expression was associated with poor prognosis for overall survival, but the molecular mechanism of RIP140's action in CLL has not been studied. These results suggest that RIP140 acts as

a tumor suppressor and can be a potential biomarker for specific tumors (see citations in Fig. 4).

Cytoplasmic RIP140: Player in Pathophysiology of Metabolic Diseases, Inflammation, and Neurological Disorders

The finding that PTMs dramatically alter RIP140's property and subcellular distribution (nuclear export) would suggest certain functions for RIP140 in the cytoplasm. Studies have shown that a short-term high-fat diet can promote cytoplasmic accumulation of RIP140 in epididymal adipose tissue, accompanied by the upregulation of nuclear PKCε activity which provides the initial trigger for RIP140's nuclear export (Ho et al. 2009). Detailed molecular studies show that RIP140 interacts with AS160, which blocks AS160 inactivation by Akt/PKB and retards GLUT4 vesicle trafficking (Fig. 5). These studies establish the first cytoplasmic function of RIP140. In addition to this finding, Ho et al. also reported that endothelin-1 promotes cytoplasmic RIP140 accumulation by activating ET_A receptor PLC-β-PKCε. Cytoplasmic RIP140 can also retard adiponectin secretion through its action on AS160 and enhance lipolysis by interacting with perilipin (Fig. 5, see citations in the legend). These adipocyte defects could have severe pathological consequences. For instance, increased lipolysis in adipocytes causes high circulating fatty acid levels, and these fatty acids can accumulate in muscle cells, hepatocytes, and cardiomyocytes to trigger apoptosis. Adiponectin is the most abundant and protective adipokine that is known to modulate systemic glucose homeostasis and lipid metabolism. Reduction in adiponectin secretion is an important feature in diabetic mice and human patients.

The findings that cytoplasmic accumulation of RIP140 in adipocytes may contribute to their dysfunctions such as impaired glucose uptake, adipokine secretion, and lipolysis would strongly support its functional role in regulating systemic metabolism as demonstrated in whole-body knockout mice, which, in part, is attributable to the cytoplasmic RIP140. In animals, a high-fat diet promotes RIP140's nuclear export in adipocytes. From a clinical point of view, targeting cytoplasmic RIP140, or blocking signaling pathways that promote RIP140's cytoplasmic accumulation, may provide a more specific, beneficial/protective effect in the management of metabolic disorders (Ho and Wei 2012).

Cytoplasmic function of RIP140 has also been found in IL-4-stimulated macrophage in M2 polarization. Upon IL-4 stimulation, RIP140 is translocated to the cytoplasm to interact with CAPNS1 to activate calpain1/2, which cleaves PTP1B, a negative regulator of STAT6 (Lin et al. 2015a). Through this pathway, cytoplasmic RIP140 ultimately suppresses STAT6 activity in M2 polarization. Of a physiological relevance, it was found that wound healing indeed was more efficient in mϕRIP140KD mice, which have significantly reduced M1 proinflammatory macrophages and enhanced M2 anti-inflammatory macrophages. Therefore, RIP140 not only acts as a positive regulator of M1 activation but also acts as a negative regulator of M2 activation, indicating a pivotal functional role for RIP140 in maintaining the balance between M1 and M2 polarization in activated macrophages. These studies further support the notion that RIP140 can be a potential therapeutic target for metabolic and inflammatory pathological conditions (Liu et al. 2015b).

The third type of cell where RIP140 also plays a significant role in the cytoplasm is neuron. In stressed neurons, unfolded protein response induces RIP140's translocation to the endoplasmic reticulum (ER) to form complex with IP_3R. Through this interaction, increasingly ER localized RIP140 attenuates IP_3R-mediated Ca^{2+} release, thereby protecting stressed neurons from apoptotic death (Feng et al. 2014). This study uncovers a new function of RIP140 in neurons and sheds lights into an important physiological/pathological context where cytoplasmic RIP140 can modulate various neurological diseases.

Currently found RIP140-associated diseases		
Symptoms/Diseases	Mediating tissues/cells	References
Acute lung injury	macrophage	C. Lei et al, 2016, Pulm Pharmacol Ther. 37,57-64
Heart disease	cardiomyocytes	L. Zhang et al, 2014, Arch Biochem Biophys. 554,22-27
Atherosclerosis	macrophage	Y.-W. Lin et al, 2015, J Molecular Cellular Cardiology, 79,287-294
Wound healing	macrophage	Y.-W. Lin & B. Lee et al, 2015, J Innate Immunity, 8,97-107
Type2 diabetes, obesity	muscle	A. Fritah et al, 2012, Plos One 7(2), e32520
	skeletal muscle	S. Constantinescu et al, 2013, Exp Physiol. 98.2,514-525
	brown adipocyte	F. Hu et al, 2015, Diabetes, 64,2056-2068
	white adipose tissue	E. Kiskinis et al, 2014, Mol Endocrinol. 28,344-356
	macrophage, adipocyte	P.S. Liu et al, 2014, Diabetes, 63,4021-4031
	adipocyte	P.-C. Ho et al, 2012, Molecular and cellular Endocrinology, 351, 176-183
Type 2 diabetes and bone disease	bone	P.M. Miotto et al, 2016, Mol Nutr Food Res. 00,1-14
Osteoporosis, osteopenia	osteoclast	H. Piao et al, 2016, J Physiol Sci. (Epub, PMID:27016936)
	macrophage, osteoclast	B. Lee et al, In preparation
Cognitive impairments	brain	F. Duclot et al, 2012, Genes Brain Behav. 11,69-78
Down syndrome	fibroblasts	A. Izzo et al, 2014, Hum Mol Genet. 23(16),4406-4419
	neuron	X. Feng et al, 2015, Chin Med J. 128(1),119-124
Stress-mediated neurological disorder	astrocyte, neuron	X. Feng et al, 2014, Nature Communications, 5:4487 X. Feng et al, 2015, Brain Behav Immun. 46,270-279
Emotional regulation disorders	microglia, astrocyte in ventromedial hypothalamus	S.F-Grinberg et al, 2014, Brain Behav Immun. 40,226-234
Cancer	liver, hepatocytes	D. Zhang et al, 2015, Tumor Biol. 36,2077-2085
	colon	M. Lapierre et al, 2014, J Clin Invest. 124(5),1899-1913
	ovary	A. Docquier et al, 2013, Mol Endocrinol. 27,1429-1441
	lymphocyte	M. Lapierre et al, 2015, J Hematol Oncol. 8:20

Nuclear Receptor-Interacting Protein 1 (NRIP1), Fig. 5 RIP140 (Nrip1)-associated diseases. This table shows identified diseases or symptoms that are associated with RIP140's expression or functions in relevant tissues and cell types

Summary

RIP140 was first identified as a universal gene transcriptional corepressor that acts, primarily, to regulate the activities of nuclear receptors in a ligand-enhanced manner. Its gene repressive activity is mediated by its four RDs that recruit various corepressive factors, histone-modifying enzymes, and chromatin remodelers. This was complicated by the fact that RIP140 can be extensively modified by PTMs, which drastically affect its ability to recruit interacting partners and alter its subcellular distribution, stability, and functions. Studies of whole-body gene knockout, overexpression of transgenic mice, and lineage-specific KD transgenic mice provide insights into its multiple physiological roles in regulating metabolism, inflammation, reproduction, and behavior in animals. While most of these activities have been associated with its nuclear functions, it

has become clear that some might have been affected by its extranuclear activities.

In the nucleus, the canonical activity of RIP140 is to suppress hormone-activated transcription activation, such as by antagonizing coactivators PGC-1, PCAF, and p300 in a hormone-dependent manner. However, nuclear RIP140 can also function as a coactivator for those without hormone ligands, such as NF-κB in macrophages. It is likely that nuclear RIP140 provides the principal gatekeeper to dampen hormonal activation of most nuclear receptors in hormone-targeted cells, which would be representative of the canonical activity of nuclear RIP140. For certain transcription factors, such as NF-κB, nuclear RIP140 can function as a coactivator, which is likely gene or context dependent. In the context of system biology, RIP140 seems to provide, primarily, a counteracting force to maintain homeostasis in gene expression and cellular responses in the face of challenges such as hormones, cytokines, and stress. Most of these activities are manifested in a cell autonomous fashion and as a negative feedback control both in the nucleus and the cytoplasm (see the following).

Studies of RIP140's PTMs reveal its cytoplasmic distribution and novel functions in multiple specialized cell types, including adipocytes, neurons, and macrophages. In adipocytes, cytoplasmic RIP140 interacts with AS160 and perilipin to modulate glucose homeostasis, lipid metabolism, and vesicle trafficking. In macrophages, cytoplasmic RIP140 interacts with CAPNS1 to dampen STAT6 activation (and M2 polarization). In neurons, cytoplasmic RIP140 is primarily localized on the ER to form a complex with IP_3R (ER stress-induced neurons), which attenuates calcium release thereby protecting cells from apoptosis.

Because of its highly specialized function and modification in response to stimuli/inputs presented to certain specific cell types, RIP140 can be a potential therapeutic target. Therapeutics targeting RIP140, either the nuclear or the cytoplasmic form, can be developed for numerous diseases including metabolic disorders, inflammation, cancers, and neurological disorders. As a proof of concept, targeting RIP140 in specialized cell types to manage diseased conditions has been demonstrated in several recent studies of mouse models. Future challenges reside at the identification of specific agents that can specifically augment RIP140's expression and/or PTM in order to target specialized pathways/cells that are most specific to disease progression. This would drastically reduce side effects and toxicity as seen in most therapeutics developed to target molecules/pathways common to multiple organs/tissues/cell types. This is particularly important because many of these medical conditions are chronic in nature, and therefore toxicity of therapeutics is of most concern.

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Nucleoside Triphosphate Diphosphohydrolase

► E-NTPDase Family

Nucleotide Receptor P2x

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Historical Background

The first evidence of purinergic signaling was described in 1929, when purines were found to underlie physiological responses in the circulatory and digestive system. After 50 years and a wealth of data supporting purine mediated effects in different systems, Burnstock presented the first direct evidence that ATP acts as a transmitter and introduced the concept of purinergic neurotransmission (Burnstock et al. 2010). Thus, ATP was recognized as both an intracellular energy source and an extracellular signaling molecule. Extracellular ATP has been implicated in intercellular communication in a wide variety of cells from different organisms and associated with a diverse array of biological effects. ATP is an ideal molecule for extracellular signaling, it is small, rapidly diffusing, highly unstable due to the presence of extracellular degrading enzymes and not abundant in the extracellular environment at resting conditions (Soto et al. 1997). ATP exerts it actions by binding to cell surface receptors called P2 receptors. P2 receptors are subsequently divided into two different families: P2Y receptors are metabotropic G-protein-coupled receptors while P2X receptors are ligand-gated ion channels (North 2002). Binding of ATP to P2X receptors opens within milliseconds an integral ion channel. While P2X receptors are functional homologues to the Cys-loop and glutamate receptor families of ligand-gated ion channels, they form a structurally distinct group of membrane receptors. Purinergic signaling via P2X receptors has a remarkably wide range of action, influencing epithelia and endocrine cell secretion, immune and

inflammatory processes, cardiovascular performance, skeletal and smooth muscle contraction, and glial and neuronal function (Surprenant and North 2009). This chapter is devoted to the molecular and functional properties of P2X receptors and their involvement in physiological and pathological processes.

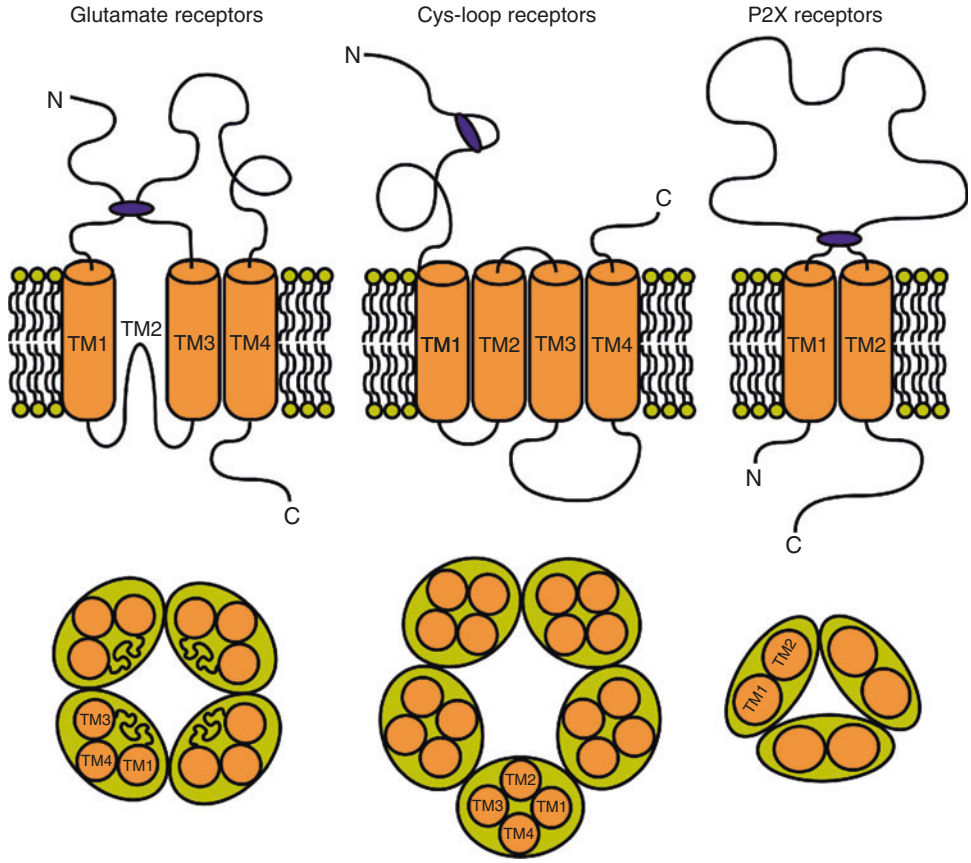
Molecular Properties of P2X Subunits

The first two P2X subunits (P2X1 and P2X2) were isolated from rat vas deferens smooth muscle and from PC12 cells by expression cloning. Based on sequence similarity, five additional subunits were identified in the rat and shortly after in human and mouse tissues (North 2002). The sequence identity between subunits (approx. 30–50%) and the lack of similarity to other cloned ligand-gated ion channels indicated they constitute a new family of membrane receptors. P2X subunits have been isolated and characterized from additional vertebrate classes (e.g., aves) and are present in all vertebrate species. They have also been found in fish, protozoa, trematode, fungi, and algae. In contrast, no P2X subunits have been identified in the genomes of the nematode worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), or in prokaryotes (Fountain and Burnstock 2009). Mammalian P2X receptor subunits are 379–595 amino acids long. Multiple splice variants of the originally cloned P2X subunits have been described, showing different amino acid lengths and properties. It was predicted using hydrophobicity plots that each subunit has two transmembrane domains linked by an extracellular loop comprising between 50% and 70% of the total protein length (Fig. 1). This extracellular loop contains 10 cysteine residues conserved in all cloned vertebrate P2X subunits. Both N- and C- terminus were suggested to be intracellular, with the length of the C-terminal domain being the main source of structure variation between the different subunits. The predicted membrane topology has been confirmed using multiple approaches, including mutating the extracellular domain, use of extracellular antibodies, concatemers and chimeric constructs (North 2002). The proposed membrane topology differs from that of the members of Cys-loop receptors superfamily and glutamate ionotropic receptors family but closely resembles that of the degenerins/ENaC/ASIC family (Fig. 1) (North 2006). P2X receptors are trimeric combinations of P2X subunits. Questions about the membrane topology of P2X subunits as well as the quaternary structure of P2X receptors were recently and definitely answered by X-ray crystallography (Kawate et al. 2009). The authors solved the crystal structure of zebrafish P2X4 receptor in its closed stated, as a symmetrical assembly of three P2X subunits surrounding a central ionic channel pore. This study confirms the proposed membrane structure and will further the understanding about the protein domains involved in P2X receptor function.

Genomic Organization and Splicing

The chromosomal localization (obtained from the Ensembl database, www.ensembl.org) of the seven P2X subunit genes is summarized in Table 1. Several P2X genes are localized in the same human chromosome. Thus, P2X4 and P2X7 genes are located in the long arm of chromosome 12. Similarly, P2X1 and P2X5 genes are located within 1 Mb in the short arm of chromosome 17. A co-localization of paralog genes in chromosomes could arise from tandem duplication. However, it might also indicate the formation of a gene cluster, in which the expression of functionally related genes is co-regulated (Makino and McLysaght 2008). This could be the case for P2X subunits since co-expression of P2X4 and P2X7 has been detected in many different tissues and cell types, including microglia, vascular endothelium, ciliated epithelium, and the immune system. Moreover, P2X1 and P2X5 subunits co-express and heteromerize to form the P2X receptor in astrocytes (Surprenant and North 2009).

The number of exons comprising the sequence of P2X subunits varies between 11 for P2X2 and 15 for P2X7 (Cheewatrakoolpong et al. 2005, Nicke et al. 2009), while the remaining P2X genes contain 12 identified exons. Most exon–intron borders are conserved between the



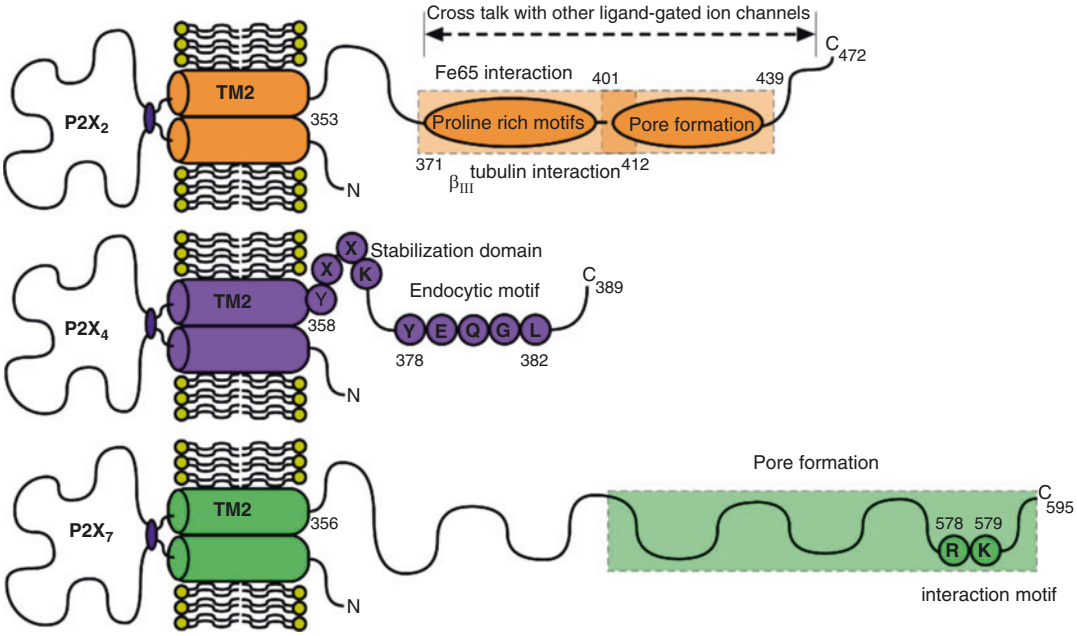
Nucleotide Receptor P2x, Fig. 1 *Ligand-gated receptor membrane topology and stoichiometry.* The membrane topology of a single subunit belonging to the three main families of ligand-gated ion channels is shown. The place of agonist interaction for the three types of receptors is shown in *purple*. In the *lower* part of the figure, the arrangement of subunits around the channel pore to form a functional receptor is depicted. *TM* transmembrane domain. The membrane topology and stoichoimetry is shared between the P2X receptor family and the degenerins/ENaC/ASIC family

Nucleotide Receptor P2x, Table 1 Chromosomal location and length of human P2X subunit genes

Gene	Chromosomal location	Gene length (Kb)
P2RX1	17p13.2	20.1
P2RX2	12q24.3	3.7
P2RX3	11q12.1	31.6
P2XR4	12q24.3	24.2
P2XR5	17p13.2	23.9
P2XR6	22q11.2	13.8
P2XR7	12q24.3	53.2

Data obtained from the Emsembl database at http://uswest.ensembl.org/index.html

different genes, indicating, as expected, a common evolutionary origin. In contrast, intronic length varies largely between genes, as reflected by the differences in gene length listed in Table 1. Splice variants have been identified for all P2X subunits. Many of the isolated splice variants lack transmembrane domains or part of the extracellular domain giving rise to truncated P2X subunits that are not able to assemble in functional receptors. In addition, several subunits present a shorter version of their C-terminal domain due to the use



Nucleotide Receptor P2x, Fig. 2 *Carboxy terminus of P2X subunits.* Representation of the functional and interacting domains present in the C-termini of P2X2, P2X4, and P2X7 subunits. The surface stabilization sequence YXXK present in all P2X subunits C-terminus proximal domain is represented only for P2X4

of cryptic splice sites inside exon sequences or of inclusion of intron sequences in the corresponding DNA (Cheewatrakoolpong et al. 2005, Koshimizu and Tsujimoto 2006). The importance of this splicing in the receptor function will be described in the next section in more detail.

Regulation of P2X Receptors via Their C-terminal Domain

The C-terminal domain of P2X subunits is the least conserved part of the protein both in length and amino acid composition (North 2002), indicating that it might confer subunit specific properties. Moreover, the intracellular location of the domain makes it a candidate for interaction with intracellular adaptor and cytoskeletal proteins. Indeed, it has been shown that the P2X2 C-terminus interacts with the adaptor protein Fe65, a cytosolic protein containing several protein-binding domains (Fig. 2). P2X subunits and Fe65 colocalize in brain synapses and Fe65 has been shown to modify the functional properties of P2X2 receptors upon co-expression in heterologous systems (Masin et al. 2006). A splice variant of P2X2 with a 69 amino acid deletion in the C-terminal domain, assemble into a membrane receptor with different kinetic properties, and it does not interact with Fe65 (Masin et al. 2006).

A role for the cytosolic C-terminal domain of the P2X4 subunit in trafficking of the receptor in and out of the plasma membrane has been described. Thus, a tyrosine-based non-canonical sorting signal in the C-terminal tail of P2X4 subunits (YXXGL, Fig. 2) that directly binds to the AP2 adaptor protein has been identified. When the interaction was disrupted by mutations in the sorting signal, an increase in the surface expression of $P2X_4$ receptors in transfected neurons was observed (Murrell-Lagnado and Qureshi 2008). In addition, another tyrosine-containing domain (YXXXK) present in the proximal C-terminus of all P2X subunits was shown to be involved in

membrane stabilization of P2X receptors (Murrell-Lagnado and Qureshi 2008).

Arguably, the best studied C-terminal domain is that of P2X7 receptors. In the immune system, P2X7 receptors mediate ATP-induced inflammatory responses by activating caspase-1 and the subsequent release of interleukins. Macrophages of a mouse line in which the C-terminal domain of P2X7 has been deleted by knock-in methods lack the immune response elicited by ATP. Moreover, efficient caspase-1 activation by ATP requires priming by bacterial membrane lipopolysaccharides (LPS) and an LPS binding domain has been identified in the distal C-terminal sequence (Surprenant and North 2009) (Fig. 2). P2X7 receptors have been shown to interact with many intracellular proteins, including pannexin-1, protein tyrosin-phosphatase β, heat shock, and epithelial membrane proteins (Surprenant and North 2009); however, the domains mediating the interaction are not known. In addition to playing a role in protein interaction and intracellular signaling, the C-terminal domain is modulating the channel properties of P2X7 receptors. Thus, the increases in permeability to big cations or fluorescent dyes of P2X7 receptors upon repetitive application of ATP also depend on the C-terminal domain (North 2002).

Pharmacology, Tissue Expression, and Physiological Roles of P2X Receptors

In considering the pharmacology and P2X receptors (P2XR), a number of considerations are needed. First, P2X receptors are ligand-gated ion channels, and as such serve as both cell-surface receptors and ion channels. The natural agonist is extracellular ATP or adenine nucleotides. A number of nucleotide and non-nucleotide antagonists have been developed with selectivity at each of the seven P2X receptors. Most of the agonists are relatively nonselective while the antagonists with a high degree of selectivity have been developed. Second, the endogenous or native P2XR may be homomeric or heteromeric. Each P2X channel is a subunit of the trimeric channel. Each P2X receptor is capable of complexing with itself to form a homotrimeric channel or with another P2X receptor as a heterotrimer. A total of seven heterotrimers have been demonstrated. P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6, P2X4/7. Pharmacology of heterologously expressed P2XR subtype in oocytes or HEK293 cells is often different from that of P2XR in native tissues. Third, agonist and antagonist selectivity is different at homomeric vs. heteromeric receptors. Individual P2X subunit of a heterotrimeric P2XR retains some of its selective characteristic response to agonist, antagonist, extracellular zinc, pH, or desensitization kinetics. Fourth, heteromers can exhibit unique pharmacology of agonist and/or antagonist actions or other properties that are different from those for homomeric receptors. In some heteromers, the individual characteristic properties specific to one of its subunit can become dominant and make the heteromer more like the homomeric assembly of the "dominant" subunit. Table 2 summarizes the EC50s of known agonists and IC50 of antagonists at both homomeric and heteromeric receptors (Ralevic and burnstock 1998; Gever et al. 2006; North 2002; Jarvis and Khakh 2009; Roberts et al. 2006; Burnstock and Knight 2004). Table 3 summarizes the tissue expression pattern of both homomeric and heteromeric P2X receptors (Ralevic and burnstock 1998; Gever et al. 2006; North 2002; Burnstock and Knight 2004; Surprenant and North 2009). Each homomeric or heteromeric receptor will be discussed in the following sections.

Homomeric P2X1R

P2X1 mRNA and protein are detected in a fairly broad range of tissues, such as urinary bladder, smooth muscle of small arteries and vas deferens, brain, spinal cord and several neuron ganglions, and platelets. Low levels of P2X1 are also found in lungs, spleen, and heart. Notably, P2X1R is the significant P2X subtypes in smooth muscle of blood vessels and other hollow organs including bladder, intestine, and vas deferens. Studies demonstrated that ATP or α,β-meATP could elicite an inward current and membrane depolarization, induce the contraction in a variety of smooth muscle tissues. These effects of that ATP or α,-β-meATP in smooth muscle are eliminated or reduced in P2X1R knockout (KO) mice, confirming the significant role of P2X1R in the

Nucleotide Receptor P2x, Table 2 Agonists and antagonists at P2X receptors

Agonists	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7	P2X1/2	P2X1/4	P2X1/5	P2X2/3	P2X2/6	P2X4/6	P2X4/7
ATP	≤1	2–10	≤1	7–10	5–10	10	≥100	0.5–0.6	10	1–5	0.7–2	30	6	>300
2-meSATP	≤1	≤2	≤1	10	10	9	100	0.07		1	1	35	7	
α, β-meSATP	≤1	>300	≤1	>300	>300	>100	>300	0.1	10	3	5	>100	12	
BZ-ATP	0.003	0.8	0.08	7	>500		20	0.003			0.8			9
Antagonists														
Suramin	1–5	1–10	3–5	>500, 180[a]	4	>100	>300				10			
PPADS	1	1–2	1	>100, 28[a]	3	>100	3–4							
TNP-ATP	0.001–0.006	≥1	0.001	15	>10		>30		0.5	0.4	0.007			1
NF 449	0.0003, 0.0005[a]	>50	2	>100			>100, 40[a]			0.0007	0.12			
NF 023	0.2	>50	30	>100										
NF 279	0.02, 0.05[a]	0.8–1	2	>300			10–20, 3[a]							
RO 0437626	3	>100	>100								>100			
NF 110	0.08	4	0.01	>300										
RO −3	>10	>10	0.01–0.1	>10	>10		>10				1–2	>10		
A 317491	>10	>100	0.1	>100		>100	>100				0.1			
5 -BDBD				0.5										
A 740003	>100	>100	>100	>100		>100	0.02–0.05							
A 438079	>100	>100	>100	>100		>100	0.06–0.07							
A 804598	>100	>100	>100	>100		>100	0.01							
GSK314181A	>10	>10	>10	>10			0.1							
AZ11645373	>10	>10	>10	>10	>10		0.1							
IP_5I	0.003	>300	3–7	Potentiation							3			
KN −62							0.3							

Value of EC50 (agonists) or EC50 (antagonists) are μM. Most of the data are taken from Antonio et al. (2009), Aschrafi et al. (2004), Ase et al. (2010), Burnstock and Knight (2004), Burnstock et al. (2010). Additional data are taken from Donnelly-Roberts et al. (2008), Evans et al. (1995), Rettinger et al. (2000), Klapperstück et al. (2000), Rettinger et al. (2005), Hechler et al. (2005), Hülsmann et al. (2003), Ford et al. (2006), Honore et al. (2006), Donnelly-Roberts et al. (2009), King (2007), Sneddon et al. (2000), Soto et al. (1999), Hausmann et al. (2006), Kassack et al. (2004), King et al. (2004), Jaime-Figueroa et al. (2005)

ATP adenosine 5′-triphosphate, *2meSATP* 2-methythioadenosine 5′-triphosphate, *α,β-meATP* 2-methythioadenosine 50-triphosphate, *BzATP* 2,3-O-(4-benzoylbenzoyl)-ATP, *PPADS* pyridoxal-5′-phosphate-6-azophenyl-20 0,40 0-disulphonic acid, *TNP-ATP* 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate, *IP5I* diinosine pentaphosphate, *KN-62* 1-[N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, *A-317491* 5-({(3-phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetricarboxylic acid, *RO-3* 5-(2-isopropyl-4,5-dimethoxybenzyl)pyrimidine-2,4-diamine, *A-740003* N-[1-(N0 0-cyano-N′-quinolin-5-ylcarbamimidamido)-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl)acetamide, *A-438079* 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine, *A-804598* 2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine, *MRS2179* 20 0-deoxy-N^6-methyl adenosine 3′ 0,5′ 0diphosphate, *NF279* 8,80-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(naphthalene)-1,3,5-trisulfonic acid, *NF449* 4-[({3-[({3,5-bis[(2,4-disulfophenyl)carbamoyl]phenyl}carbamoyl)amino]-5-[(2,4-disulfophenyl)carbamoyl]phenyl}carbonyl)amino]benzene-1,3-disulfonic acid, *GSK314181A* 5-{[(3R)-3-aminopyrrolidin-1-yl]methyl}-2-chloro-N-(tricyclo[3.3.1.13,7]dec-1-ylmethyl) benzamide, *AZ11645373* 3-(1-(30-nitrobiphenyl-4-yloxy)-4-(pyridine-4-yl)butan-2-yl)thiazolidine-2,4-dione, *Cibacron Blue* 1-amino-4-(4-(4-chloro-6-(2-sulfophenylamino)-1,3,5-triazin-2-ylamino)-3-sulfophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid.

[a]Human P2XR

Nucleotide Receptor P2x, Table 3 Tissue distribution

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$	$P2X_{1/2}$	$P2X_{1/4}$	$P2X_{1/5}$	$P2X_{2/3}$	$P2X_{2/4}$	$P2X_{2/6}$	$P2X_{4/7}$
Skeletal muscle	+	–			+	+	+							
Cardical muscle	+	–	+	+	+	+								
Smooth muscle														
Urinary bladder	+	+		+	+	+	+							
Gut	+	+					+							
Blood vessels	+	+	+	+	+		+		+					
vas Deferens	+	+		+			+							
Ureter	+	+			+	+								
Overy		+												
Epithelial cell														
Nasal mucosa		+			+									
Gut		+			+		+							
Bladder			+		+	+	+							
Ureter				+	+	+	+							
Skin					+		+							
Bronchial						+								
Salivary gland				+										
Overy	+	+				+								
Thymus		+	+			+	+							
Blood vessels	+	+	+	+	+		+							
Organs														
Lung	+	–		+	+									
Trachea				+			+							
Spleen	+	–												
Liver		–		+										
Kidney	+	+	+	+	+	+	+							

Thymus	+			+		+	+							
Testis (Leydig)		+	+	+			+	+				+		
Salivary gland				+			+							
Adrenal gland		+	+	+	+	+	+							
Pancrease				+										
Uterus						+								
CNS														
Brain	+	+	+	+	+	+	+			+				
Spinal cord	+	+	+	+		+	+							
Cortical astrocytes	+				+					+				
Neuron ganglion														
Sensory	+	+	+	+		+	+				+	+		
Dorsal root	+	+	+	+		+						+		
Trigeminal	+	+	+	+	+	+								
Coeliac	+	+	+	+		+	+							
Autonomic	+	+	–	+	+	+	+	+			+			
Esophagus myenteric														
Enteric sensory neurones													+	
Otic neurones		+	+								+			
Blood cells														
Plateletes	+													
Lymphocytes	+	+		+			+							
Granulocytes							+							
Monocytes							+							
Macrophage							+							+
Neutrophils	+			+	+		+							

+ Detected
– Not present

regulation of native smooth muscle contractility (Ralevic and burnstock 1998; Gever et al. 2006).

P2X1R can also regulate platelet functions as the main ATP-gated ion channel in platelets and megakaryocytes. The endogenous platelet P2XR has a similar pharmacology as that of the heterologously expressed recombinant P2X1R. In P2X1R KO mice, functions of platelets, such as aggregation, secretion, adhesion, and thrombus formation, are impaired. Vascular disease superimposed on P2X1R KO mice showed reduced mortality in the presence of systemic thromoembolism and laser-induced vessel injury. On the other hand, hypersensitive ex vivo platelet response and increased mortality in vivo secondary to increased thromboembolism were observed in transgenic mice overexpressing human P2X1R in megakaryocytic cell line (Gever et al. 2006).

Homomeric P2X2R

P2X2R is expressed throughout the central and peripheral nervous (CNS) systems as well as other non-neuron cell types, such as in bladder, adrenal medulla, endothelial and epithelial cells, skeletal, cardiac and smooth muscles, lymphocytes, intestine, and vas deferens (see Table 3). In the heart, P2X2R mRNA was detected in smooth muscle cells of coronary artery and only in atrium myocardium. Homemeric P2X2R appears to play a significant role in ATP-mediated fast synaptic transmission at both nerve terminals and interneuronal synapses. Thus, P2X2R is likely involved in memory, learning, motor function, autonomic coordination, and sensory integration in CNS as well as afferent and efferent signal pathway in peripheral nervous system (PNS). P2X2R is also expressed in many other non-neuron tissues in which its function is still not clear, although it may have a role in autocrine/paracrine hormone release, exocytosis/endocytosis, smoother muscle contractility, and pacemaker activity.

Heteromeric P2X1/2R

Phenotypically, heteromeric P2X1/2R is identical to homomeric P2X1R except the different sensitivity to pH changes. Furthermore, Aschrafi et al. found that assembly of heteromeric P2X1//P2X2R is favored over the respective homomeric P2X1R. It suggested that ATP-stimulated currents originally attributed to homomeric P2X1R in native tissue may actually be mediated by heteromeric P2X1/2R (Aschrafi et al. 2004). Using pharmacology profile and specific P2X subunit gene knockout mice, Calvert JA et al. confirmed existence of functional heteromeric P2X1/2R in sympathetic neurons from the superior cervical ganglion and it is implied that heteromeric P2X1/2R may have a broad role in regulation of the neuron function. Although the dominant phenotype of endogenous P2XR in sympathetic neurons is P2X2-like, a subpopulation of neurons showed $P2X_1$ property such as α,β-meATP responsiveness that was reduced in P2X1R KO mice. P2X2-like means slow desensitization, sensitivity to blockade by antagonists, potentiation by acidic pH (this property is unique to P2X2 receptor) and extracellular zinc, and partial inhibition by high extracellular calcium. It is of interest that the α,β-meATP responsiveness was abrogated by high extracellular calcium and alkaline pH; the latter two properties are more characteristic of P2X2R. Thus, a presumed heteromeric P2X1/2 native receptor includes properties of both subunits (Calvert and Evans 2004).

Homomeric P2X3R

Homomeric P2X3R has restricted distribution and is only expressed in peripheral terminals of unmyelinated C-fiber and thinly myelinated afferent neurons, such as trigeminal and dorsal root ganglions. These receptors are mainly expressed on nociceptive sensory neurons. P2X3R can be activated by both α,β-meATP and 2-methylthioATP and are sensitive to blockade by suramin, PPADS, and TNP-ATP and is selectively antagonized by NF023. The functional role of homomeric P2X3R in these neurons is to mediate the sensory neurotransmission.

Heteromeric P2X2/3R

These heteromeric receptors have a mixed property that incorporates that of both P2X2 and P2X3 receptors. Thus, they can be activated by α,β-meATP and high sensitivity by TNP-ATP (P2X3-like), and show slow desensitization and

potentiation by acidic pH (P2X2-like). A feature unique to this heteromer is that diinosine pentaphosphate is a much more potent blocker at homomeric P2X3 than at heteromeric P2X2/3R. This feature is useful in characterizing whether any of the native tissue P2XR is a P2X2/3R heteromer. These heteromers are expressed in subpopulations of sensory neurons, sympathetic ganglion cells, and brain neurons. They are thought to be important in initiating sensory signaling in pathways for taste, chemocreception, visceral distension, and neuropathic pain (Ralevic and Burnstock 1998). Heteromeric P2X2/3R is the first heteromeric channel to be studied following gene knockout confirmed the role of heteromeric P2X2/3R in several sensory signaling (Roberts et al. 2006).

Homomeric P2X4R

Homomeric P2X4R is not activated by α,β-meATP but is responsive to ATP and 2-methylthioATP. Of all the P2X receptors, it is the only receptor that can be potentiated by ivermectin via allosteric enhancement. Another unique feature is its lack of sensitivity to blockade by suramin or PPADS (North 2002). It has a desensitization kinetics intermediate between that for P2X1 and P2X2 receptors. It may be the most widely distributed of all the P2X receptors with expression in brain and spinal cord, automatic and sensory ganglions, arterial smooth muscle, osteoclasts, parotid acinar cells, kidney, lung, heart, bladder, thymus, colon, pancreas, and B lymphocytes (Gever et al. 2006; Burnstock and Knight 2004). However, the functional roles in some of these tissues are not clearly defined yet. However, recent studies showed that P2X4R are expressed in microglial cells and their activation can mediate neuropathic pain. P2X4R are also expressed in the endothelium in which activation can mediate nitric oxide-mediated vasorelaxation/vasodilatation. Global P2X4R KO mice showed hypertension and smaller arteries. Recent studies have also implicated P2X4 receptor as an important subunit of the endogenous cardiac myocyte P2X receptors. Increased expression of cardiac P2X4R by cardiac myocyte-specific transgenic overexpression or by stimulation with hydrolysis-resistant P2X agonist can confer a protected phenotype in models of both ischemic and non-ischemic heart failure (Zhou et al. 2010). Expression of a P2X4-like contractile phenotype in human atrial myocardium was recently described. P2X4R may also interact with P2X7 receptors in inflammatory responses such as pain signaling (see P2X7 receptors).

Heteromeric P2X1/4R

Both the P2X1R and P2X4R are expressed in the smooth muscle in renal resistance arteries. α,β-meATP evoked a spike-like membrane depolarization followed by a sustained depolarization which could be partially blocked by nanomolar P2X1 selective antagonist, NF279. The residual current could further be blocked by millimolar NF279, consistent with the existence of heteromeric P2X1/4R. Thus, heteromeric P2X1/4R showed both agonist and antagonist pharmacology that are more P2X1-like. TNP-ATP can block this heteromer with affinity that is higher than but closer to that for the homomeric P2X1 receptor. The P2X1/4R heteromer participates in the sympathetic control and paracrine regulation of renal blood flow (Harhun et al. 2010).

Homomeric P2X5R

Homomeric P2X5R is expressed in brain, spinal cord, heart, and eye. These homomeric receptors have pharmacology and desensitization kinetics similar to those of P2X2 receptors. The current mediated by rat P2X5R has a smaller amplitude than that induced by P2X1, P2X2, P2X3, or P2X4 receptors. P2X5 channels have a uniquely high chloride conductance. Recently, high levels of homomeric P2X5R are found in differentiating tissues, such as skeletal muscle, epithelial cells of nasal mucosa, gut, bladder, utter, and skin. Activation of homomeric P2X5R inhibits proliferation while increases the differentiation of rat skeletal muscle satellite cells. Homomeric P2X5R may be also involved in the regulation of proliferation and differentiation of certain type of cancer cells in skin and prostate.

Heteromeric P2X1/5R

P2X1R and P2X5R can assemble into a heteromeric P2X1/5R. A defining property of this heteromer is its activation by α,β-meATP, which

cannot activate P2X5R. Other characteristic properties of this heteromer is a greater sensitivity to activation by ATP, a biphasic response to ATP with a transient peak current followed by a sustained plateau current, and a sensitivity to TNP-ATP intermediate between the sensitive homomeric P2X1 and the insensitive homomeric P2X5 receptors. Further, this heteromer does not appear to dilate to a larger pore on prolonged ATP exposure. Although the physiological role of heteromeric P2X1/5R is not defined, it has been postulated that it may mediate excitatory junction potentials at arterial neuroeffector junctions in guinea pig (Gever et al. 2006; North 2002). Recently, Ase et al. identified the heteromeric P2X1/5R in astrocytes from mouse brain and implied that it might participate in the astroglial Ca^{2+} signaling and excitability (Ase et al. 2010).

Homomeric P2X6R

P2X6R is expressed mainly in CNS. However, P2X6R is the only P2X receptor that usually does not form the functional homomeric P2X6R without extensive glycosylation. When such functional homomeric P2X6R are formed (after glycosylation), the heteromer has significantly higher responsiveness to α,β-meATP. Since P2X4R and P2X2R are the other two P2X subunits that usually coexist with P2X6R, the physiological role of the P2X6R is mediated either by the heteromeric P2X2/6R or heteromeric P2X4/6R (Gever et al. 2006; North 2002).

Heteromeric P2X2/6R

The P2X2R and P2X6R can be co-immunoprecipitated after co-expression in HEK293 cells or oocytes. The P2X2/6R heteromer showed a hybrid sensitivity to blockade by suramin at pH 6.5. There was a bi-phasic inhibition with a high sensitivity P2X2-like component and a lower sensitivity portion that is more P2X6-like. In general, the pharmacology of P2X2/6R heteromer is similar to that of P2X2 receptors (much less like that of homomeric P2X6 receptors). A subtle difference is that the P2X2/6R heteromer has a greater sensitivity to pH and to α,β-meATP. This heteromer is expressed by respiratory neurons in the brain stem (Gever et al. 2006; North 2002).

Heteromeric P2X4/6R

P2X4 receptors can also form a heteromer with P2X6 receptors. The pharmacology of this heteromer is similar to that of the homomeric P2X4 receptor. The heteromer, like the homomeric P2X4 receptor, is relatively insensitive to blockade by PPADS or suramin or reactive blue-2. The heteromeric P2X4/6R is similar to homomeric P2X4 receptors in its potentiation by the P2X4-specific allosteric enhancer and by zinc. In fact, the P2X4/6R heteromer appears more sensitive to the potentiation effect of ivermectin. The incorporation of P2X6 receptor in this heteromer also modestly increases the sensitivity to 2-meSATP and α,β-meATP(Gever et al. 2006; North 2002). On tissue expression and function of P2X2/6R and P2X4/6R heteromers, Antonio et al. confirmed the presence of the heteromeric P2X2/6R and P2X4/6R in mouse Leydig cells using immunofluorescence and pharmacologic profiles. Both heteromers are involved in regulating testosterone secretion (Antonio et al. 2009). Heteromeric P2X2/6R and P2X4/6R are also found in rat dorsal root ganglion neuron where they contribute to the transmission of nociceptive message, especially under inflammatory condition (De Roo et al. 2003).

Homomeric P2X7R

Homomeric P2X7R is mainly localized on glia and immune cells such as mast cells, macrophages lymphocytes, erythrocytes, and erythroleumemia. With prolonged exposure to high extracellular ATP concentrations, the homomeric P2X7R becomes open to larger size molecules such as ethidium and YO-PRO-1, leading to cell death. At low ATP concentration, the receptor is a cation channel like the other P2X receptor. Activation of homomeric P2X7R has been associated with the processing and releasing of active interleukin-1β and interleukin-18 from immune cells and glia. Selective P2X7R antagonist KN-62 (known to be selective at human P2X7R) could block the

release of interleukin-1 β in macrophages and microglia. This function of P2X7R is further confirmed in experiment in P2X7R KO mice, which fail to release interleukin-1 β when challenged by ATP or BzATP. P2X7R also has a role in mediating the release of cytokine, reactive oxygen species, and neurotransmitter in microglia and astrocytes. Other roles implicated include apoptosis around β-amyloid plaques in Alzheimer's disease model and neurodegeneration and cell death in models of spine cord injury or cerebral ischemia (Gever et al. 2006; North 2002).

Heteromeric P2X4/7R

Homomeric P2X7R is structurally similar to other P2XR except its significantly longer intracellular C-terminal. Originally, P2X7R was thought not able to heteropolymorize with any other P2XR. However, Guo et al. reported a functional heteromeric P2X4/7R in macrophages, representing the latest example of heteromeric P2X receptors (Guo et al. 2007). The two P2X receptors can be co-immunoprecipitated in detergent extracts from co-transfected HEK293 cells and from murine macrophages that express endogenous P2X4 and $P2X_7$ receptors. A mutant P2X4R lacks ATP-gated channel activity but is capable of trafficking to the plasma membrane. When this mutant P2X4R (Named S341W) was co-expressed with P2X7R, ivermectin was able to potentiate the ATP-induced current in cells co-expressing these receptors. Since ivermectin could not potentiate homomeric P2X7R -mediated current, the potentiation by ivermectin of the co-expressed S341W P2X4R and P2X7R is most likely due to allosteric enhancement of the heteromer via the S341W P2X4 receptor. The overlapping expression of P2X4R and P2X7R have been identified in a number of tissues including some non-excitatory cells such as epithelial cells from salivary glands, exocrine pancreas, airway, leukocytes, microglial cells, and osteoclasts. The possible presence of heteromeric P2X4/7R in these tissues or cells raises the question that heteromeric P2X4/7R may mediate important physiological function(s) in these tissues (Dubyak 2007).

Future Directions

Further systematic examination of the effect of each agonist and antagonist will be needed at heteromeric P2X receptors. This can be performed in cells co-expressing individual P2X subunits using heterologous systems such as HEK293 cells or oocytes. Features including pharmacology, desensitization kinetics, and sensitivity to pH, extracellular calcium, or zinc will all need to be determined. Once characterized, these features should be compared to those obtained in the native tissues to ascertain whether they are similar. Similarity of features would support the particular heteromer as the native tissue receptor. Another possibility is that a heteromer may comprise three different P2X receptor subtypes. If true, this possibility will add further complexity.

Summary

P2X receptors are a family of ligand-gated ion channels. The natural agonist ligands are ATP. There are seven P2X receptors (P2X1–7) and each receptor is a subunit of the trimeric channel. Each subunit can complex with itself to form a homotrimeric channel or with other subunits to form a heterotrimeric channel. Evidence points to existence of both homo- and hetero-trimeric channels as endogenous channels in the tissue. In the heterotrimeric channel, molecular and pharmacological properties of the constituent subunit can both dominate or become masked. Evidence is accumulating to indicate potentially important biological and pathophysiolgical roles of these receptor channels. It is expected that many of these homo- or heterotrimeric channels will become novel therapeutic targets for various diseases. Much more work is needed.

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Nucleotide Receptor P2Y

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Synonyms

Nucleotide receptors; Purinergic receptors

Historical Background

Signaling by extracellular ATP was first reported in 1929 (Drury and Szent-Györgyi 1929). Subdivision of purinergic receptors between P1 (adenosine) and P2 (ATP, ADP) was proposed in 1978 (Burnstock 1978), and further subdivision of P2 receptors between P2Y and P2X was made in 1985 (Burnstock and Kennedy 1985). The $P2Y_1$ and $P2Y_2$ receptors were the first P2Y receptors to be cloned in 1993 (Webb et al. 1993; Lustig et al. 1993).

Release of Nucleotides in the Extracellular Fluids

Although nucleotides, such as ATP and UTP, are mainly intracellular, they are released in the extracellular fluids by various mechanisms. One of them is cell damage: both necrotic and apoptotic cells release ATP and other nucleotides that thus constitute "danger signals" or DAMP (damage-associated molecular pattern) (Elliott et al. 2009). But they can also be released without cell lysis by specific mechanisms: exocytosis of secretory

granules, vesicular transport, and membrane channels, such as ABC transporters, pannexins, and connexins (Abbracchio et al. 2006). Nucleotides are released by exocytosis during platelet aggregation and synaptic transmission. They are also released in response to various types of stress: mechanical stimulation (stretch, shear stress), hypoxia, or pathogen invasion.

Extracellular nucleotides are rapidly degraded by a variety of ectonucleotidases such as the ENTDPases that degrade ATP into ADP and ADP into AMP, and 5′-nucleotidase that converts AMP into adenosine (Abbracchio et al. 2006).

Signaling by extracellular nucleotides is mediated by two families of receptors: metabotropic G protein–coupled P2Y receptors and ionotropic P2X receptors.

Structure and Signaling Properties of P2Y Receptors

The P2Y family is composed of eight members encoded by distinct genes that can be subdivided into two groups based on their coupling to specific G proteins, as well as structural features (Abbracchio et al. 2006).

Whereas the P2X receptors are all receptors for ATP, the various P2Y receptors differ by their selectivity for distinct nucleotides (Table 1). $P2Y_{11}$ is primarily an ATP receptor, whereas $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ are ADP receptors. $P2Y_4$ and $P2Y_6$ are pyrimidinergic receptors activated by UTP and UDP, respectively. $P2Y_2$ is a dual ATP and UTP receptor. $P2Y_{14}$ is a receptor for UDP-glucose and other nucleotide sugars as well as for UDP itself.

Comparisons of the structural characteristics and functionally important amino acid residues within the family have been performed using mutagenesis and modeling (Abbracchio et al. 2006). Conserved cationic residues that interact with the negatively charged phosphate groups have been identified in transmembrane domains 3, 6, and 7. The 8 P2Y receptors have a H-X-X-R/K motif in TM6. The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors share a Y-Q/K-X-X-R motif in TM7, whereas another motif, K-E-X-X-L., is found in $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. This last motif is not specific for P2Y receptors since it is also found in GPR87, a lysophosphatidic acid receptor.

The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors couple mainly to G_q and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple to G_i (Table 1). This coupling has been demonstrated directly in reconstitution experiments: ADP-activated GTP hydrolysis in vesicles containing either $P2Y_1$ and $G\alpha_q$ or $P2Y_{12}$ and $G\alpha_{i2}$ (Waldo and Harden 2004). However, the dichotomy between G_q- and G_i-coupled P2Y receptors is an oversimplification. Indeed, the $P2Y_{11}$ receptor has the unique property to couple through both G_q and G_s. It is also unique by its late appearance during evolution since no $P2Y_{11}$ gene can be identified in the genome of rodents (Communi et al. 2001a). Furthermore, the $P2Y_2$ and $P2Y_4$ receptors are also coupled to G_i, as shown interalia by a sensitivity of the responses mediated by those receptors to inhibition by pertussis toxin.

Nucleotide Receptor P2Y, Table 1 Properties of P2Y receptors

Group	Receptor	Chromosome (human)	Agonist (human)	G protein
A	$P2Y_1$	3q24–25	ADP	G_q
	$P2Y_2$	11q13.5	ATP = UTP	G_q (+ G_i)
	$P2Y_4$	Xq13	UTP	G_q (+ G_i)
	$P2Y_6$	11q13.5	UDP	G_q
	$P2Y_{11}$	19p31	ATP	G_q + G_s
B	$P2Y_{12}$	3q21–25	ADP	G_i
	$P2Y_{13}$	3q24–25	ADP	G_i
	$P2Y_{14}$	3q24–25	UDP-glucose	G_i
			UDP	

Nucleotide Receptor P2Y, Table 2 Effects of P2Y gene silencing

Receptor	Gene silencing method	Consequence of gene silencing
$P2Y_1$	Knockout mice	Inhibition of platelet aggregation and resistance to thromboembolism
		Smaller atherosclerotic lesions
$P2Y_2$	Knockout mice	Decreased neutrophil and monocyte/macrophage chemotaxis
		Decreased infiltration of eosinophils in asthmatic airways
		Abolition of ATP-induced Cl^- secretion in airways and intestinal tract
$P2Y_4$	Knockout mice	Microcardia phenotype related to defective cardiac angiogenesis
		Protection against cardiac ischemia
		Abolition of ATP-induced Cl^- secretion in the gut
$P2Y_6$	Knockout mice Antisense	Cardiac hypertrophy
		Reduced vascular inflammation
		Decreased microglial phagocytosis
	siRNA	Decreased pressure overload-induced cardiac fibrosis
$P2Y_{12}$	Knockout mice	Inhibition of platelet aggregation and resistance to thromboembolism
		Decreased microglial migration
$P2Y_{13}$	Knockout mice	Decreased differentiation of bone marrow stromal cells into osteoblasts
		Reduced bone turnover
		Decreased reverse cholesterol transport

There is limited evidence for non-G-protein-mediated signaling by P2Y receptors. In particular, the $P2Y_2$ receptor has been shown to transactivate the VEGF receptor-2, by a mechanism involving the binding of ▶ Src tyrosine kinase to SH3 binding sites in the C-terminal domain of $P2Y_2$ (Seye et al. 2004).

The pharmacology of some P2Y receptors exhibits species differences: while the human $P2Y_4$ is a UTP receptor, the rat and mouse $P2Y_4$ receptors are activated equipotently by ATP and UTP.

The missing numbers in the classification represent either nonmammalian orthologs or receptors having some sequence homology to P2Y receptors, but for which there is no functional evidence of responsiveness to nucleotides.

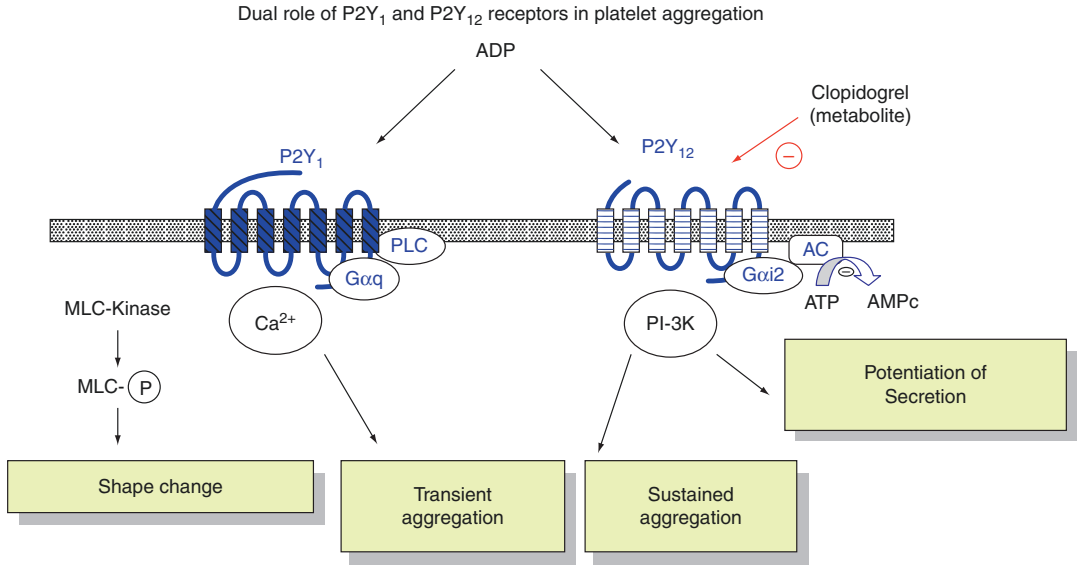
Primary references are as follows: $P2Y_1$ (Webb et al. 1993), $P2Y_2$ (Lustig et al. 1993), $P2Y_4$ (Communi et al. 1995), $P2Y_6$ (Communi et al. 1996), $P2Y_{11}$ (Communi et al. 1997), $P2Y_{12}$ (Hollopeter et al. 2001), $P2Y_{13}$ (Communi et al. 2001b), and $P2Y_{14}$ (Chambers et al. 2000).

Functions of P2Y Receptors

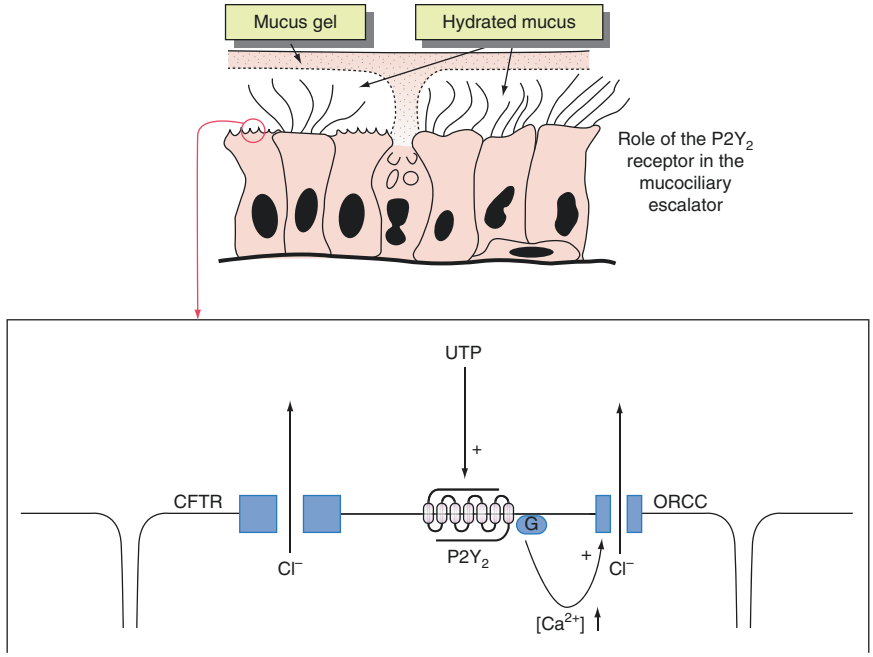
Gene silencing techniques have been instrumental in establishing the function of P2Y receptors (Table 2). ADP released from platelet dense granules amplifies platelet aggregation. This action requires the cooperation between two P2Y receptors: $P2Y_1$ and $P2Y_{12}$ (Fig. 1). $P2Y_1$ is involved in the initial platelet shape change and transient aggregation, while $P2Y_{12}$ is responsible for sustained aggregation and secretion. Both $P2Y_1{}^{-/-}$ and $P2Y_{12}{}^{-/-}$ mice show defective platelet aggregation ex vivo, increased bleeding time, and resistance to thrombosis (Leon et al. 1999; André et al. 2003). The only P2Y receptor ligands currently used as medicinal products are the thienopyridine antagonists of the $P2Y_{12}$ receptor, ticlopidine, clopidogrel, and prasugrel, which are used as antithrombotic agents.

ATP and UTP stimulate the secretion of chloride by epithelial cells through a channel distinct from Cystic Fibrosis Transmembrane Regulator (CFTR) (Fig. 2). Studies of knockout mice have demonstrated that this action is mediated by the $P2Y_2$ receptor in the airways (Cressman et al. 1999) and by $P2Y_4$ in the gut (Robaye et al. 2003). The $P2Y_2$ agonist denufosol was developed for the treatment of cystic fibrosis, but Phase 3 trials ended up in failure (Ratjen et al. 2012)

Multiple P2Y receptors are expressed in the heart: $P2Y_2$ and $P2Y_6$ receptors on cardiomyocytes



Nucleotide Receptor P2Y, Fig. 1 Cooperation between $P2Y_1$ and $P2Y_{12}$ receptors in platelet aggregation. Activation of the $P2Y_1$ receptor by ADP induces a shape change of platelets and their transient aggregation, while its stimulatory effect on $P2Y_{12}$ induces a stable aggregation and potentiates the secretion of dense granules content



Nucleotide Receptor P2Y, Fig. 2 Role of the $P2Y_2$ receptor in the regulation of the airways mucociliary escalator. Activation of the $P2Y_2$ receptor by ATP stimulates the three components of this escalator: mucus secretion, mucus hydration, and mucus mobilization by ciliary activity. Mucus hydration results from Cl^- secretion which is mediated either by Cystic Fibrosis Transmembrane Regulator (CFTR) or Outwardly rectifying chloride channels (ORCC) that are opened by an increase in $[Ca^{2+}]_i$ in response to ATP

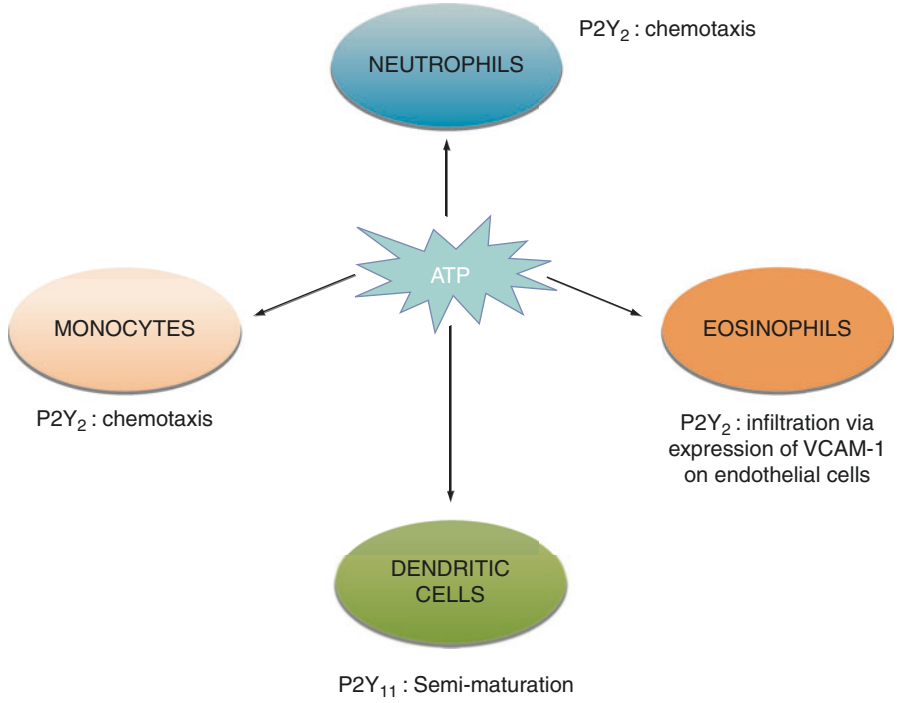
and $P2Y_4$ on microvascular endothelial cells. Nucleotides are released from cardiomyocytes in response to mechanical stretch or ischemia. Both $P2Y_2$ and $P2Y_4$ nucleotide receptors are involved in cardioprotection through distinct mechanisms. Administration of UTP to rats reduces infarct size through activation of $P2Y_2$ receptors and protects rat cardiomyocytes against hypoxic stress (Cohen et al. 2011). Loss of mouse $P2Y_4$ receptor is associated with a protection against infarction and reduction of cardiac inflammation, permeability, and fibrosis (Horckmans et al. 2015). The $P2Y_4$ receptor is also involved in postnatal cardiac development (Horckmans et al. 2012a) and exercise tolerance (Horckmans et al. 2012b). The use of siRNA revealed that the $P2Y_6$ receptor plays a role in cardiac fibrosis resulting from pressure overload (Nishida et al. 2008). Recently, loss of $P2Y_6$ was associated with a macrocardia phenotype and amplified pathological cardiac hypertrophy induced after isoproterenol injection (Clouet et al. 2016).

Interestingly, $P2Y_4$ receptor was reported as a potential pivotal regulator in osteogenic and adipogenic differentiation of human mesenchymal stem cells (Ciciarello et al. 2013). $P2Y_{13}$ plays also a role in the balance of osteoblast and adipogenic differentiation of bone marrow progenitors (Biver et al. 2013), which may explain the reduced bone turn-over in $P2Y_{13}^{-/-}$ mice (Wang et al. 2012).

Multiple P2Y receptors might play a role in the development of atherosclerotic lesions, independently from their role in platelet activation. Aortic lesions were smaller in double ApoE/$P2Y_1$ knockout mice than in $ApoE^{-/-}$ mice (Hechler et al. 2008). This difference was unrelated to the role of $P2Y_1$ in platelet activation since it was unaffected by bone marrow transplantation from $P2Y_1$ wild-type mice, indicating the role of $P2Y_1$ in nonhematopoietic-derived cells, most likely endothelial cells. On the other hand, the $P2Y_{13}$ receptor plays a role in the reverse cholesterol transport, at the level of hepatocytes. It has indeed been shown that HDL Apo A-I activates an ecto-ATPase that generates ADP from ATP on the surface of hepatocytes. ADP then stimulates the endocytosis of HDL particles via the activation of $P2Y_{13}$ receptors, as demonstrated by the use of siRNA (Jacquet et al. 2005). Endocytosis of HDL and biliary lipid secretion are decreased in $P2Y_{13}^{-/-}$ mice (Fabre et al. 2010).

P2Y receptors are involved at various steps in the inflammatory process (Fig. 3). ATP released from neutrophils amplifies their attraction by chemotactic signals, and its release from apoptotic cells constitutes a "find-me signal" for monocytes/macrophages (Chen et al. 2006; Elliott et al. 2009). In a murine model of asthma, the infiltration of esosinophils in the airways involves the $P2Y_2$-mediated expression of Vascular Cell Adhesion Molecule-1 (VCAM-1) on lung endothelial cells (Vanderstocken et al. 2010). Expression of the $P2Y_6$ receptor on endothelial cells is increased during vascular inflammation that was reduced in $P2Y_6^{-/-}$ mice (Riegel et al. 2011). P2Y receptors are also involved in adaptive immunity. In particular, ATP induces via the $P2Y_{11}$ receptor the semimaturation of human monocyte-derived dendritic cells, characterized by the upregulation of co-stimulatory molecules and the inhibition of IL-12 secretion, resulting in an enhanced ability to induce Th2 differentiation of T lymphocytes (Wilkin et al. 2001). Moreover, ATP confers tolerogenic and tumorigenic properties to dendritic cells (Marteau et al. 2005; Bles et al. 2010).

Microglia from $P2Y_{12}^{-/-}$ mice are unable to polarize, migrate, or extend processes toward ADP, and in vivo they showed decreased directional branch extension toward sites of laser-induced cortical damage (Haynes et al. 2006). Independently from this chemotactic action of ADP, UDP stimulates the uptake of microspheres by rat microglia, and this action was blocked by an antisense oligonucleotide targeting the $P2Y_6$ receptor (Koizumi et al. 2007). These complementary actions of ADP, a find-me signal, and UDP, an eat-me signal, involving a cooperation between $P2Y_{12}$ and $P2Y_6$, might be beneficial in neurodegenerative conditions such as Alzheimer's disease, via an increased clearance of amyloid-β deposits.



Nucleotide Receptor P2Y, Fig. 3 Role of P2Y receptors in various immune cells. The $P2Y_2$ receptor plays a role in the tissue infiltration of eosinophils and the chemotaxis of neutrophils and monocytes. The $P2Y_{11}$ receptor mediates the semimaturation of dendritic cells that favors Th2 differentiation or tolerance

Summary

Nucleotides are released in the extracellular fluids following cell damage (necrosis or apoptosis), mechanical stimulation, or by exocytosis. They act on G protein–coupled P2Y or ionotropic P2X receptors. There are eight P2Y receptors encoded by distinct genes. They can be divided in two groups according to structural features and coupling to specific G proteins. The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors couple mainly to G_q, and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple to G_i. They exhibit a selectivity for distinct nucleotides: ATP, ADP, UTP, UDP, and UDP-glucose. The study of knockout mice and other methods of gene silencing have demonstrated their involvement in multiple biological processes: platelet aggregation, epithelial surface lubrication, cardiac development and ischemia, stem cell differentiation, migration of neutrophils, monocytes and microglia, microglial phagocytosis, etc.

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Nucleotide Receptors

▶ Nucleotide Receptor P2Y

Nurr1

▶ NR4A2 (Nuclear Receptor Subfamily 4, Group A, Member 2)

N-WASP

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Synonyms

Neural Wiskott–Aldrich Syndrome Protein; Wiskott–Aldrich Syndrome-Like (WASL)

Historical Background

Wiskott–Aldrich syndrome (WAS), a pediatric disorder, was first described in 1937 by Alfred Wiskott as a "hereditary thrombopathy" in males, presenting with thrombocytopenia, eczema, bloody diarrhea, episodes of fever, and recurrent bacterial infections. Robert A. Aldrich would later demonstrate an X-linked mode of inheritance of this disease. Other features of WAS were later recognized, including immunodeficiency involving both humoral and cellular immunity, high rate of autoimmunity and malignancies, abnormal apoptosis, and defective cell motility. The mutated gene giving rise to this disease was identified in 1994 by positional cloning and referred to as *WAS*, and mutations of the WAS protein (WASP) were demonstrated not only in patients with WAS, but also in those with X-linked thrombocytopenia (XLT), a disease showing milder clinical phenotype with a more favorable prognosis (Notarangelo et al. 2008). Northern blot analysis indicated that WASP mRNA is expressed exclusively in hematopoietic cells. Two years later, a novel protein with ~50% amino acid identity to the *WAS* gene product was reported as a binding partner for the Grb2/Ash adapter protein. In contrast to WASP, this protein was expressed ubiquitously, but strongest expression was observed in neuronal cells and was thus named Neural-WASP (▶ N-WASP) (Miki and Takenawa 2003).

► N-WASP and WASP are actin-nucleating promoting factors (NPF) and are the founding members of the WASP-family of NPFs that contain tandem V (Verprolin homology, also known as WH2; WASP homology 2), C (Central or Connecting), and A (Acidic) regions, referred to as the VCA domain. Other members of this family are the WAVE/Scar, WASH, and WHAMM/JMY subfamilies. Evolutionary analyses indicate that these VCA domain-containing proteins are widely expressed among eukaryotes and evolutionarily ancient (Veltman and Insall 2010).

Structure of N-WASP

► N-WASP and WASP have a conserved domain organization that allows interaction with multiple distinct binding proteins (Table 1). They have a WASP homology 1 (WH1; also known as EVH1) domain that binds primarily WASP interacting protein (WIP)-family members, a basic sequence that binds phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), a GTPase-binding domain (GBD), containing a Cdc42 and Rac interactive binding (CRIB) motif that binds Cdc42-GTP, and a proline-rich region that binds SH3 proteins. The C-terminus of both proteins contains a VCA domain that binds actin via the V region and the Arp2/3 complex via the CA region but ► N-WASP has an additional V region (Takenawa and Suetsugu 2007) (Fig. 1). In vitro data using the purified VVCA domain of ► N-WASP showed that this domain alone was sufficient for actin polymerization and its activity was higher than the isolated VCA domain of WASP (Zalevsky et al. 2001).

Regulation of N-WASP

Autoinhibition and Activation by Cdc42

► N-WASP is regulated by diverse signals including Rho family GTPases, phospholipids, kinases, many SH3 domain-containing proteins, and both bacterial and viral pathogen proteins (Takenawa and Suetsugu 2007) (Table 1). In the resting state, ► N-WASP and WASP are autoinhibited through intramolecular interactions between the C-terminal VCA region and a hydrophobic pocket in the GBD but other regions, such as from the WH1 domain, may also participate in autoinhibition. In addition, it is generally considered that favorable electrostatic interactions between the basic region and the A region further stabilize the GBD–VCA interface. The Rho family GTPase Cdc42 was the first protein shown to bind ► N-WASP/WASP and is an important regulator of both proteins. The active, GTP bound form of Cdc42 disrupts the hydrophobic core of the GBD and releases the VCA domain, which can now bind G actin and the Arp2/3 complex. The Arp2/3 complex, which is composed of seven polypeptides, nucleates actin polymerization and contributes to branched filamentous actin (Takenawa and Suetsugu 2007). Another Rho family GTPase, Rac1, can activate ► N-WASP in vitro, but is probably not involved in WASP activation (Tomasevic et al. 2007). Evidence also suggests that Cdc42 may require an additional intermediate for the activation of ► N-WASP, such as F-BAR proteins of the CIP4 subfamily, which include Toca-1, CIP4, and FBP17. These proteins bind directly to Cdc42 and also contain a SH3 domain that binds to the polyproline region of ► N-WASP, and is required for ► N-WASP functions, such as endocytosis.

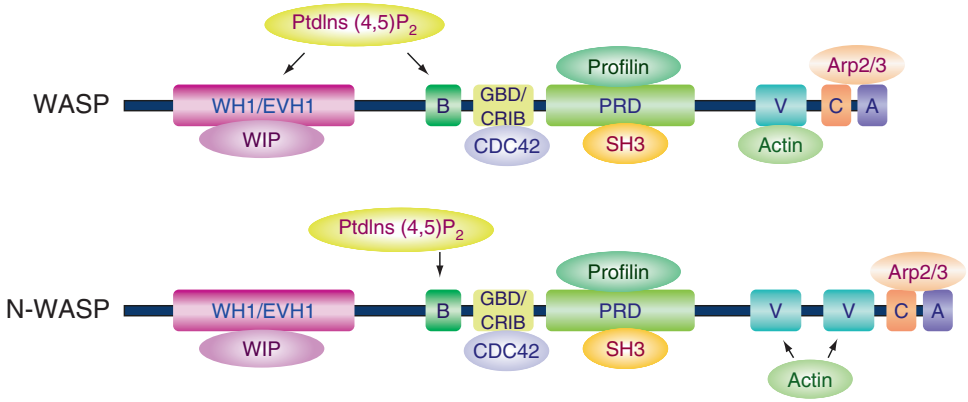
Regulation by Phosphorylation

Although it is generally accepted that the binding of Cdc42-GTP to WASP is important for allosteric release from autoinhibition, other signaling events can also modulate ► N-WASP function. One such event is phosphorylation of a conserved tyrosine residue (Y256 in human ► N-WASP and Y291 in human WASP) by non-receptor tyrosine kinases, e.g., ► Src, and is required for several cellular functions of ► N-WASP, such as neurite extension. Remarkably, this residue is located in the C-terminus of the GBD, and its phosphorylation is thought to alter the charge and therefore stability of the autoinhibited form of both proteins shifting the proteins toward the open, active conformation (Thrasher and Burns 2010). Additionally, tyrosine phosphorylation may prime the molecule for activation by proteins that contain

N-WASP, Table 1 ▶ N-WASP/WASP binding proteins

Binding protein	Binding domain	N-WASP/WASP binding region	Effect on (N)WASP/function
WIP, CR16, WICH/WIRE	Proline-rich	WH1	Stabilization, targeting, inhibition of activity
CIB		WH1	Coupling to integrin $\alpha_{IIb}\beta_3$
Cdc42/Chp		CRIB	Activation
TC10/RhoT		CRIB	Activation
mDab1	PTB	CRIB	
PtdIns(4,5)P_2		Basic	Localization, synergistic activation with Cdc42 and Nck
Hsp90		Basic	Stabilization
FBP11	WW	Proline-rich	
Src-family kinases (Src, Hck, Lck, Lyn, Fyn, Fgr)	SH3 (SH2?)	Proline-rich	Phosphorylation, activation, protein degradation
Grb2	SH3	Proline-rich	Activation, localization
Nck	SH3	Proline-rich	Activation, localization
Crk adaptors (CrkII, CrkL)	SH3	Proline-rich	Activation, localization
Vinexin beta	SH3	Proline-rich	
Cortactin, HS1	SH3	Proline-rich	Targeting, activation, invadopodium/ podosome formation
Abi1	SH3	Proline-rich	Activation, endocytosis
Endophilin	SH3	Proline-rich	Activation, endocytosis
Amphiphysin	SH3	Proline-rich	Endocytosis
Tuba	SH3	Proline-rich	Activation via Cdc42
CIP4, Toca-1, FBP17	SH3	Proline-rich	Cdc42-mediated activation
Tec family kinases (Tec, Btk, Itk)	SH3	Proline-rich	Phosphorylation, activation
WISH/DIP	SH3	Proline-rich	Activation
PSTPIP1	SH3	Proline-rich	Activation, localization
Syndapin	SH3	Proline-rich	Activation, endocytosis
Nostrin	SH3	Proline-rich	Endocytosis
srGAP	SH3	Proline-rich	
Profilin	SH3	Proline-rich	Actin polymerization
IRSp53	SH3	Proline-rich	Activation, localization
PTP-PEST		Proline-rich	Dephosphorylation, inactivation
Sorting nexin (SNX9, SNX18)	SH3	Proline-rich	Activation, endocytosis
Arg	SH3	Proline-rich	Phosphorylation, activation
Intersectin-1, -2	SH3	Proline-rich	Localization, activation
Abp1	SH3	Proline-rich	Activation, endocytosis
VASP		Proline-rich	Localization, actin polymerization
Casein kinase	?	?	VCA phosphorylation
Arp2/3		CA	Actin polymerization
G-actin		V	Actin polymerization
F-actin		Basic	Branching
Merlin, ERM (ezrin/radixin/ moesin)	FERM domain	WH1	Inhibition of actin polymerization
CD44			Stabilization, localization
IQGAP1		Basic-CRIB	Activation

? unknown



N-WASP, Fig. 1 *Domain organization and molecular interactions of* ▶ *N-WASP and WASP.* ▶ N-WASP and WASP have similar overall domain organization, with an additional V region present in ▶ N-WASP. The WH1 domain interacts with WIP and its related proteins. The basic domain mediates interactions with PtdIns(4,5)P_2 on membranes. The CRIB motif inside the GBD region binds to Cdc42-GTP and is instrumental in ▶ N-WASP activation. The polyproline stretch is a site for docking of SH3 domains, while distinct regions mediate binding to profilin. Finally, the VCA domain binds G-actin and the Arp2/3 complex and nucleates actin polymerization

SH2 domains contributing to an additional regulatory input. However, tyrosine phosphorylation may also target both WASP and ▶ N-WASP to various protein degradation pathways, thus resulting in signal termination (Dovas and Cox 2010).

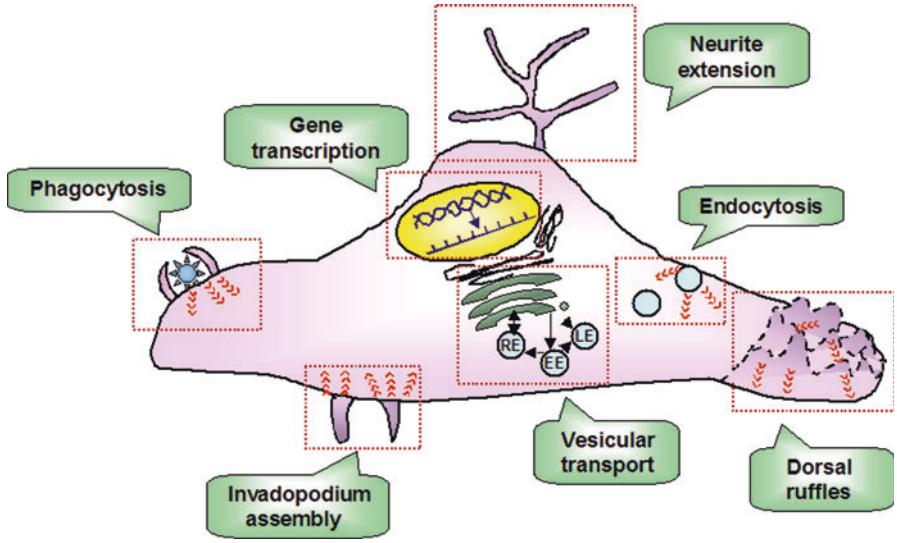
PtdIns(4,5)P_2, SH3 Domains, and EspFu

Additional levels of control of ▶ N-WASP activity following allosteric activation also exist, facilitating the integration of multiple signals in the efficient control of ▶ N-WASP activity. These are generally thought of as influencing the activity of ▶ N-WASP by correctly localizing the molecule and/or by influencing its dimerization status (Padrick and Rosen 2010). The latter is an important means of control of ▶ N-WASP-dependent actin polymerization since VCA domain dimers bind the Arp2/3 complex with higher affinity than monomers, thus contributing to enhanced actin polymerization. PtdIns(4,5)P_2 is an important regulator of actin organization mediated by both ▶ N-WASP and WASP. PtdIns(4,5)P_2 synergizes with Cdc42 in the activation of ▶ N-WASP. Increased PtdIns(4,5)P_2 density hyperactivates ▶ N-WASP in vitro, and is required for actin comet tail formation in vivo, pointing to both membrane targeting and dimerization as means by which PtdIns(4,5)P_2 regulates ▶ N-WASP.

Adaptor proteins that contain multiple SH3 domains participate in an additional level of control of ▶ N-WASP activity via dimerization. One prominent example is Nck1, which contains five SH3 domains and is able to hyperactivate ▶ N-WASP in vitro, while dimeric BAR domain proteins that contain SH3 domains may also act by inducing ▶ N-WASP dimers. Importantly, the enhanced activity of ▶ N-WASP upon dimerization is exploited by enterohemorrhagic *Escherichia coli*, to induce its spread. The bacterial protein EspFu, which is injected into the host cell cytoplasm, contains multiple repeats of a segment that can bind the GBD of ▶ N-WASP. Therefore, EspFu activates ▶ N-WASP allosterically by competing for GBD binding with the VCA domain while at the same time clustering multiple ▶ N-WASP molecules to stimulate Arp2/3-dependent actin polymerization and pedestal formation. Therefore, these data suggest that the proline-rich region may receive activation signals as well as localization signals from proteins containing SH3 domains, and that similar mechanisms are exploited by pathogens to induce their spread.

WIP: A Major Binding Partner

WIP interacts with the WH1 domain of ▶ N-WASP and WASP and performs critical



N-WASP, Fig. 2 *Cellular functions of* ▶ *N-WASP.* ▶ N-WASP plays important functions inside cells. It mediates endocytosis and vesicle motility; participates in plasma membrane extensions, such as in the outgrowth of neurites, during phagocytosis, dorsal ruffle formation and invadopodium formation, and regulates gene expression by shuttling in and out of the nucleus

regulatory functions (Ramesh and Geha 2009). Primarily, it is required for the stability of WASP protein levels in vivo. This is highlighted by the fact that the most frequent WAS mutations are clustered at the WH1 domain and affect amino acids important for the interaction with WIP. WIP also targets WASP to sites of activity. For example, WIP targets WASP to sites of T cell receptor clustering and also recruits ▶ N-WASP to vaccinia virus particles. However, WIP also inhibits the ability of ▶ N-WASP to stimulate actin polymerization, at least in vitro. It may be possible that WIP does not dissociate from ▶ N-WASP but may undergo conformational changes that relieve inhibitory interactions and allow ▶ N-WASP activation in vivo (Ramesh and Geha 2009).

The Role of N-WASP

▶ N-WASP exerts its functions primarily via the regulation of Arp2/3-mediated actin polymerization. As such, it participates in a plethora of actin-dependent functions including endocytosis, phagocytosis, invadopodium assembly, neurite extension, vesicular transport, pathogen infection, and dorsal ruffle formation (Fig. 2). ▶ N-WASP is also found in the nucleus where it participates in gene transcription through association with a nuclear complex that contains RNA polymerase II. This role of ▶ N-WASP also relies on its ability to polymerize nuclear actin (Wu et al. 2006). Similar nuclear functions have been described for WASP, where it is important in the transcription of key genes required for the differentiation of $CD4^+$ T_H1 cells (Taylor et al. 2010). ▶ N-WASP also regulates sarcomeric actin assembly in skeletal muscle, though independently of the Arp2/3 complex (Takano et al. 2010).

▶ N-WASP has not been associated with human disease and appears to be essential in mammals as ▶ N-WASP deficiency in mice results in embryonic death at day E12. Tissue-specific ablation of ▶ N-WASP, however, has revealed roles for ▶ N-WASP in T cell development (Cotta-de-Almeida et al. 2007), hair follicle cycling (Lefever et al. 2010), and myelin sheath formation by Schwann cells (Novak et al. 2011). The importance of tight regulation of ▶ N-WASP activity is suggested by the hematopoetic-restricted WASP in which activating mutations have been shown to induce a separate syndrome,

X-linked neutropenia (XLN). Therefore, both inactivating and activating mutations in WASP contribute to human disease.

Common and Distinct Functions of N-WASP and WASP

Although there is a high degree of homology in the functional domains of ▶ N-WASP and WASP, they may not be able to completely substitute for one another. WASP and ▶ N-WASP may serve both redundant and nonredundant functions depending on the cellular context. For example, only ▶ N-WASP, and not WASP, can support *Shigella* motility in cells (Snapper et al. 2001). Also, while WASP is expressed exclusively in hematopoietic cells, ▶ N-WASP is also expressed in these cells, albeit at low levels (Isaac et al. 2010). Recently, unique functions for ▶ N-WASP were demonstrated in macrophages. A striking feature of WASP-deficient macrophages is the lack of podosomes. Podosomes mediate adhesion to the extracellular matrix and perform matrix degradation. WASP localizes to the F-actin-rich core along with other actin-regulatory proteins, such as cortactin/HS1 and Arp2/3. Interestingly, certain aggressive cancer cells and ▶ Src-transformed cells possess podosome-like structures called invadopodia that appear to be directly responsible for extracellular matrix degradation. Invadopodia have similar organization and actin regulatory machinery localization compared to podosomes. However, invadopodia are regulated by ▶ N-WASP given the absence of WASP expression in these cells. When ▶ N-WASP was reduced in macrophage cells, podosomes still formed, but they were unable to perform matrix degradation (Nusblat et al. 2011). This defect was rescued by re-expression of ▶ N-WASP, but not by overexpression of WASP. Additionally, reducing ▶ N-WASP levels mistargets the matrix-degrading enzyme MT1-MMP and it no longer localizes to podosomes. Additionally, ▶ N-WASP only co-localizes with MT1-MMP positive vesicles at podosomes, suggesting that ▶ N-WASP may play a role on the targeting or fusion of MMP-containing vesicles to podosomes in macrophage cells (Nusblat et al. 2011). A unique role for ▶ N-WASP was also found for phagocytosis by macrophages and a study indicated that ▶ N-WASP may play a role in membrane delivery to the growing phagocytic cup while WASP may be required for the actin polymerization during phagocytosis (Park and Cox 2009). ▶ N-WASP and WASP have both overlapping and unique functions when expressed in the same cell.

Summary

Both ▶ N-WASP and WASP proteins play critical roles in rapid reorganization of actin filaments induced in response to diverse extracellular stimuli. Although they have high homology in their functional domains, ▶ N-WASP and WASP also have different requirements for activation and participate in distinct cellular processes. Studies in leukocytes, which express both proteins, may reveal unique functions and distinct aspects of regulation and can contribute to a more profound understanding of their cellular activities and further delineate the immunological abnormalities in WAS. Identification of different binding partners between ▶ N-WASP and WASP will provide important answers to the intriguing questions about the differential activation and unique roles of these proteins. Tissue-specific ablation of ▶ N-WASP will also help reveal its functions in vivo. Novel findings on the nuclear or Arp2/3-independent functions of ▶ N-WASP suggest that many aspects of this molecule remain unknown and promise exciting new avenues for research.

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NY-REN-37

► ZC3H14