D

D2

► NCAM1

D8Ertd419e

► StAR

Dachsous

► Cadherins

DAF-16

► Forkhead Box Protein O

DAG

► Dystroglycan

DAG1

► Dystroglycan

DAGK

► Diacylglycerol Kinase

DANCE

► Fibulins

Danio rerio: Zeb2b, sip1b, zfhx1b

► Zinc Finger E-Box-Binding Homeobox 2

DAP Kinase

► DAPK1

DAP Kinase-Related Apoptotic Kinase 2

► DRAK2

DAPK

► DAPK1

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DAPK1

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Synonyms

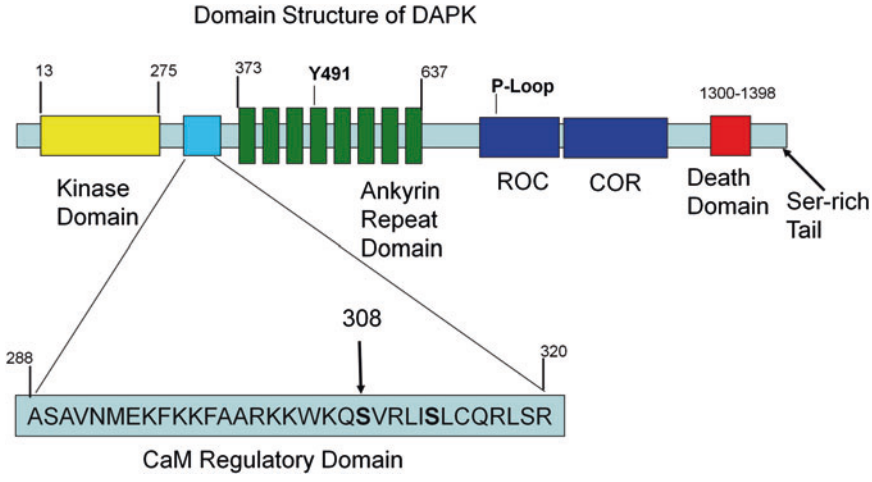
DAPK; DAP kinase; Death-associated protein kinase

Historical Background

Death-associated protein kinase (DAPK) is the inaugural member of a class of Ser/Thr protein kinases whose members exhibit homologous catalytic domains as well as share cell death-associated functions (Bialik and Kimchi 2006). DAPK was discovered by Kimchi and coworkers as a tumor suppressor gene whose expression is lost in multiple tumor types (Cohen and Kimchi 2001). This attracted the interest of several investigators resulting in an impressive body of literature concerning its function, regulation, and involvement in various diseases and conditions. For example, DAPK participates in apoptotic pathways initiated by interferon-γ, TNFα, activated Fas, and loss of attachment to the extracellular matrix (Bialik and Kimchi 2006). By activating p53 in a p19ARF-dependent fashion DAPK is an intrinsic tumor suppressor that opposes early stage oncogenic transformation (Raveh et al. 2001). However, hypermethylation of the DAPK promoter inactivates this pathway in tumorgenesis such as found in multiple myeloma (Chim et al. 2007) and other cancers (Michie et al. 2010).

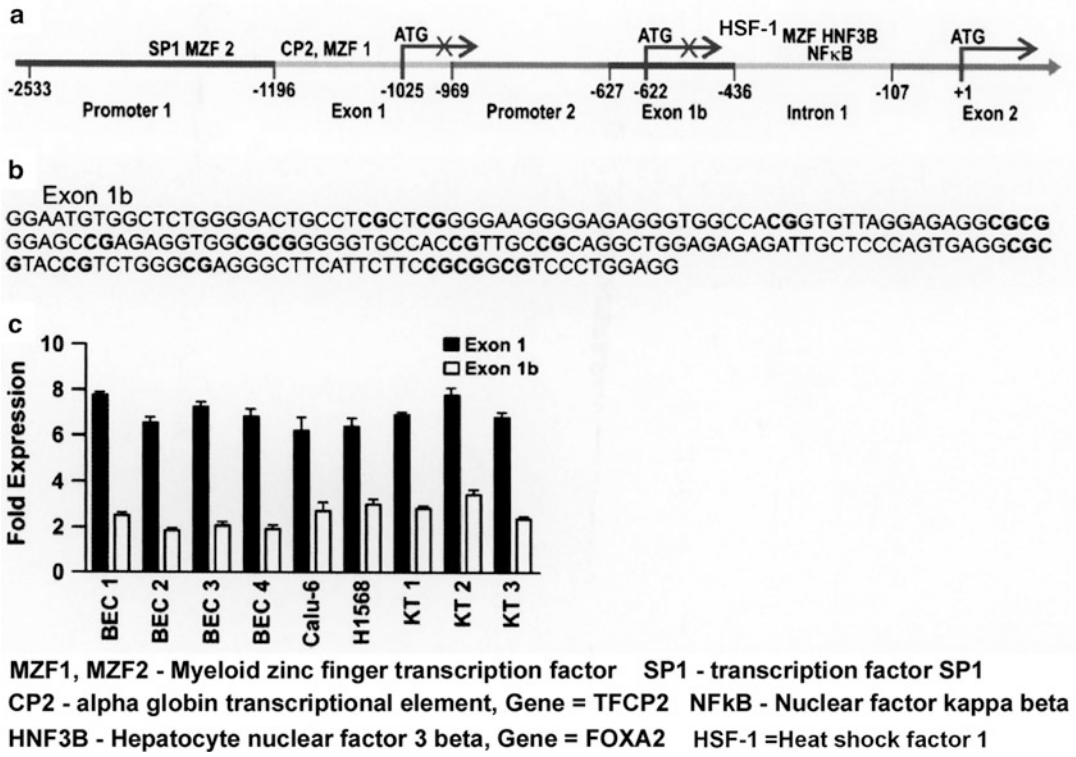
Domain Structure of DAPK and Regulation of Enzymatic Activity

DAPK has several functional domains (Fig. 1). These include a series of ankyrin-repeat domains as well as a "death" domain. Between these domains exists a tandem "ROC" and "COR" domain (Carlessi et al. 2011). These domains always occur in tandem. The ROC (Ras of complex proteins) is a GTPase domain resembling the small G-proteins such as Ras, preceding the COR (C-terminal of ROC) domain. The family of ROC-COR proteins has three members that are protein kinases. These include the Parkinson



DAPK1, Fig. 1 Domain structure of DAPK. Shown are the locations of the kinase catalytic (*yellow*), calmodulin regulatory (*light blue*), ankyrin repeats (*green*), ROC COR domain (*blue*), and death domain (*red*). The calmodulin regulatory domain is expanded to show the amino acid sequence and the location of the Ser-308 phosphosphorylation site

disease-associated kinase LRRK2 (Leucine-rich repeat kinase 2) (Drolet et al. 2011), the closely related LRRK1, and DAPK. Completing the domain structure of DAPK are the catalytic and calmodulin-regulatory domains that are similar to other calmodulin-dependent protein kinases. DAPK activation requires calmodulin (CaM), but it is further modulated by phosphorylation at sites within and outside of the CaM regulatory domain (Michie et al. 2010). Phosphorylation at two sites decrease DAPK activity; Ser-308 is within the CaM regulatory domain, and Tyr-491/492 are within one of the ankyrin repeat domains. The presence of the GTP-binding ROC domain further regulates DAPK activation. In the presence of GTP (which binds at the p-Loop (Fig. 1) DAPK activity is lower as demonstrated with a mutant that lacks the p-Loop and GTP binding (Carlessi et al. 2011). However, upon GTP hydrolysis, the dephosphorylation of Ser-308 by phosphatase PP2a is facilitated (Carlessi et al. 2011), allowing calmodulin activation. Phosphorylation of Ser-735 by activation of the extracellular-regulated kinases ERK1 and 2 results in an increase in DAPK activity (Michie et al. 2010). It is thought that this tight control over DAPK activation is one of the checkpoints for committing a cell to apoptotic/necrotic death.



DAPK1, Fig. 2 DAPK genomic structure, exon 1b sequence, and expression of exon 1 and 1b transcripts. (**a**) Schematic diagram depicting the location of promoter 1, exon 1 and 1b, promoter 2, and intron 1 in relation to the translational start site within exon 2 is shown. *Shaded areas* and *numbers* depict the boundaries and location of each region. The location of the ATGs in exon 1 and 1b that are not translational start sites due to stop codons in these exons are shown as are the location of transcription factor-binding sites that affect reporter activity. (**b**) Sequence of the 186 bp region designated exon 1b with the 17 CpG dinucleotides bolded is shown. (**c**) Quantitative expression of DAPK exon 1 and 1b transcripts in H1568, Calu-6, BEC, and keratinocyte (KT) cell lines. Fold expression between exon 1 and 1b transcripts is compared (Reproduced from Pulling et al. (2009) with permission. Transcription factor descriptions were added)

Regulation of DAPK Expression

The upstream genetic sequences that contain the promoter(s) for the human DAPK gene are diagrammed in Fig. 2a. There are two transcriptional start sites that are controlled by independent promoters. Two alternative translational start sites at -1025 and -622 are followed by termination codons within exon 1 and 1b, respectively, rendering them nonfunctional. These sites precede the active start site in exon 2. There is no TATA box in either promoter, but there are several transcription factor binding sites including AP2, E-box, CAAT box, AP1, and nuclear factor kappa B (NF-kB) (Pulling et al. 2009). A CpG island of 590 bp containing 46 CpG dinucleotides is directly upstream of the translational start site. An additional 100 CpGs are located 1000 bp upstream of this region. Exon 1b contains 17 CpG dinucleotides that may also be methylated in cancer cells (Fig. 2b). Both promoters are active in multiple cell types. Promoter 1 (Exon 1) activity was 40–50% higher than promoter 2- Exon 1b-intron promoter using reporter constructs (Fig. 2c) (Pulling et al. 2009). Methylation analysis in 5 of 15 tumor cell lines revealed that 51–91% of the CpGs in the promoter 1-exon 1 region were methylated and associated with a complete loss of transcription from exon 1. Similarly, there was a good correlation with methylation of promoter 2-exon 1b-intron constructs and loss of expression in 9 of 15 tumor cell lines. The methylation status and location within the DAPK promoters varies greatly in cultured cells but methylation at multiple sites correlates with a decrease or loss of expression. Because of the dual promoters, DAPK expression in one cell type may be more susceptible to methylation than another cell type where the alternative promoter is used. This perhaps explains variability in DAPK promoter hypermethylation in tissue biopsy samples where a mixture of cell types (metastatic versus nonmetastatic) are analyzed. However, the association of DAPK methylation with tumor aggressiveness and disease progression (poor prognosis) is significant (Pulling et al. 2009; Chim et al. 2007). MicroRNAs have also been found to regulate DAPK expression.

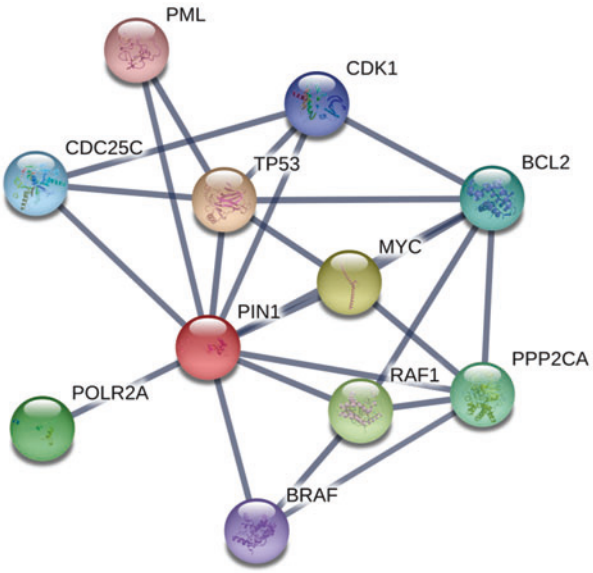
DAPK1, Table 1 Functional substrates of DAPK

Substrate	Sequence	In vivo?	Function	Reference
MLC	RPQRAT-**S**-NVF or RPQRA-**T**-SNVF	Yes	Activates myosin/ membrane blebbing	(Steinmann et al. 2015)
Zip Kinase	RRRLK-**T**-RL YTIK-**S**-H-**S**-**S**-L PNN-**S**-YADFERF-**S**-K	?	Localization and dimerization	(Steinmann et al. 2015)
Syntaxin 1A	GHMDSSI-**S**-KQA	Yes	Synaptic vesicle membrane fusion	(Steinmann et al. 2015)
CaMKK	RREERSL-**S**-APG	Yes	Inhibits activation	(Steinmann et al. 2015)
Beclin 1A	SRRLKV-**T**-GDLF	Yes	Dissociates Beclin from Bcl-xL proteins	(Steinmann et al. 2015)
MCM3	TIERRY-**S**-DLT	Yes	Unknown	(Bialik et al. 2008)
PIN1	RRP-**S**-SWRQ	Yes	Inhibits activity	(Lee et al. 2011)
NR2B (NMDA receptor	KLRRQH-**S**-YDTF	Yes	Damaging Ca2+ influx in stroke	(Tu et al. 2010)
S6	AKRRRL-**S**-SLRAS	Yes	Suppression of translation	(Schumacher et al. 2006)
P53	LSQE-**T**-F-**S**-DLWK	Yes	Stimulates transcriptional activity	(Steinmann et al. 2015)
HSF-1	YSRQF-**S**-LE	Yes	Stimulates transcriptional activity	(Benderska et al. 2014)

One, miR-103/107, promotes metastasis in colorectal cancer (Chen et al. 2012). Another, miR-191, inhibits DAPK expression in ovarian endometriosis (Tian et al. 2015). In both cases the miRNA expression is upregulated in the tumor cells resulting in a decrease in DAPK and other tumor suppressors.

Signal Transduction Pathways Involving DAPK and Its Protein Substrates

Because of its multiple protein interacting domains, DAPK is involved in several cellular processes. Two primary areas of investigative focus are apoptosis and autophagy (Bialik and Kimchi 2006) that will be discussed separately. A summary of well-characterized DAPK substrates is given in Table 1. DAPK phosphorylates regulatory myosin light chains and is involved in membrane blebbing that occurs during programmed cell death (apoptosis) (Bialik and Kimchi 2006). Calmodulin-regulated kinase kinase (CaMKK) is neuronal protein substrate of DAPK. Its phosphorylation site (Ser-511) is near the CaM recognition domain and results in an attenuation of CaM-stimulated activity (Schumacher et al. 2004). Another neuronal substrate is Syntaxin 1A where phosphorylation of Ser-188 is proposed to decrease binding of



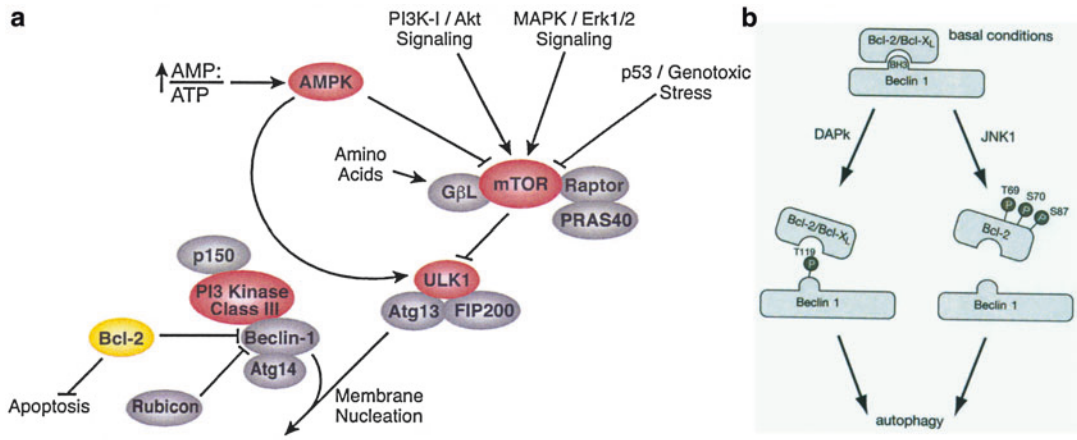
Gene	Description
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1; Serine/threonine-protein kinase
PML	promyelocytic leukemia; Phosphoprotein localizes to nuclear bodies where it functions as a transcription factor and tumor suppressor. It regulates the p53 response to oncogenic signals.
TP53	Tumor protein p53; Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis
BRAF	v-raf murine sarcoma viral oncogene homolog B;; Plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion.
CDC25C	Cell division cycle 25 homolog C; Tyrosine protein phosphatase that functions as a dosage-dependent inducer in mitotic control.
BCL2	B-cell CLL/lymphoma 2; Suppresses apoptosis in a variety of cell systems
MYC	v-myc myelocytomatosis viral oncogene homolog; Participates in the regulation of gene transcription
CDK1	Cyclin-dependent kinase 1; Plays a key role in the control of the eukaryotic cell cycle by modulating the centrosome cycle as well as mitotic onset.
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme; PP2A is the major phosphatase for microtubule-associated proteins.

DAPK1, Fig. 3 Pin1 interaction network. Illustrated is a portion of the Pin1 interactions created using String V10 and its associated database (string-db.org)

syntaxin-1A to Munc18-1, a syntaxin-binding protein that regulates a complex (known as the SNARE complex) that is necessary for synaptic vesicle docking and secretion (Tian et al. 2003). The NMDA (N-methyl-d-aspartic acid) receptor NR2B subunit is also phosphorylated by DAPK (Tu et al. 2010). The site is in the regulatory C-terminal end and enhances the Ca^{2+} conductance of the channel. Thus, if DAPK is activated in presynaptic neurons, two processes may be affected that relate to cellular homeostasis. DAPK phosphorylation of CaMKK is pro-apoptotic, because this kinase is responsible for activating survival pathways through phosphorylation of the kinase known as AMPK (Kuo et al. 2005), while phosphorylation of syntaxin-1A can lead to inhibition of neurotransmitter secretion (Bialik and Kimchi 2006). DAPK phosphorylation of Zip kinase (DAPK3) activates this kinase leading to changes in cell morphology characteristic of apoptotic cells (Shani et al. 2004). DAPK was found to phosphorylate MCM3, a protein that may be involved in the regulation of DNA replication (Bialik et al. 2008). Pin1 is a proline isomerase that specifically targets phosphor-Ser/Thr-Pro sites in proteins, and phosphorylation of Pin1 at Ser-71 by DAPK fully inactivates Pin1 activity (Lee et al. 2011). The number of proteins/processes that depend upon Pin1 activity includes many tumorogenic and cell cycle-related proteins. Figure 3 summarizes just a few of the proteins within the Pin1 network of interactions. The significance of PIN1 inactivation is that DAPK's cancer suppressing activity is extended to these processes. Finally, DAPK is one of the several protein kinases that phosphorylates the transcription factor HSF-1 (heat shock factor 1). After stimulation by TNFα, phosphorylation of Ser-230 by DAPK promotes HSF-1 translocation to the nucleus. In turn HSF-1 stimulates expression of DAPK in a positive feedback loop and results in the apoptosis of cancer cells (Benderska et al. 2014).

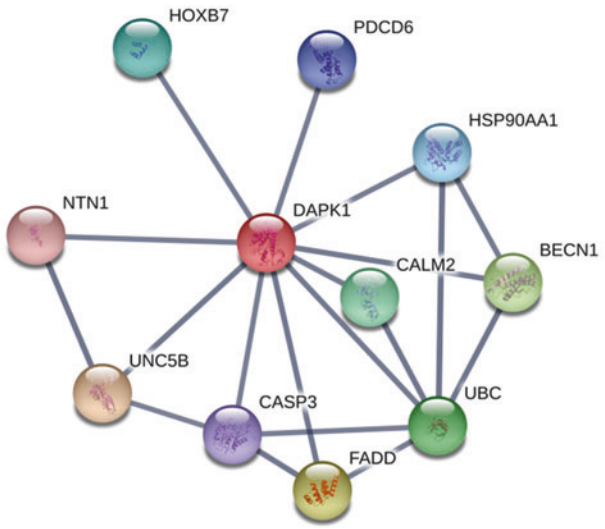
DAPK and Autophagy

Figure 4a summarizes the initial phase of autophagy and the role that DAPK and its substrate Beclin 1 play in this process. Autophagy is a highly conserved process that is characterized by the formation of membrane enclosed "autophagosomes" that function to engulf intracellular organelles and other constituents and deliver them



DAPK1, Fig. 4 (**a**) **Diagram of the early stages of autophagy that lead to membrane nucleation.** From pathways created by +Cell Signaling Technology reproduced with permission. (**b**) **A model of phosphorylation events which regulate the interaction between Beclin 1 and BCL-2/XL leading to the induction of autophagy.** DAPK-mediated phosphorylation on Beclin 1's T119 residue and JNK1-mediated phosphorylation on residues T69, S70, and S87 on Bcl-2, each reduces Beclin 1's interaction with its inhibitors leading to autophagy (Reproduced from Hu et al. (2010) by permission from the Authors)

to the lysosomes for degradation. Thus, autophagy is the cell's intrinsic "recycling" machinery that serves to provide material for cell metabolism during periods when extracellular sources of nutrients are low. Beclin 1 is an essential autophagic protein that binds BcL-2 family proteins through its BH3 domain. The phosphorylation site for DAPK (Thr-119) is located within the BH3 domain, and phosphorylation promotes dissociation of Beclin 1 from Bcl-2/XL (Fig. 4b). Dissociated Beclin 1 promotes activation of autophagic machinery by interacting with a complex centered upon phosphatidylinositol-3-kinase (PI-3 Kinase) (Fig. 4a). This multiprotein complex participates in autophagosome nucleation (Funderburk et al. 2010).



Gene	Description
DAPK1	Death-associated protein kinase 1; Calcium/calmodulin-dependent serine/threonine kinase involved in multiple cellular signaling pathways
NTN1	Netrin 1; It serves as a survival factor via its association with its UNC5 receptor which prevents the initiation of apoptosis.
HOXB7	Homeobox B7; Sequence-specific transcription factor that provides cells with specific positional identities on the anterior-posterior axis
PDCD6	Programmed cell death 6;. May mediate Ca(2+)- regulated signals along the death pathway.
FADD	Fas (TNFRSF6)-associated via death domain; Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1
CASP3	Caspase 3, apoptosis-related cysteine peptidase; Involved in the activation cascade of caspases responsible for the execution of apoptosis.
UNC5B	Unc-5 homolog B; Receptor for netrin required for axon guidance. Mediates apoptosis by activating DAPK1 in the absence of NTN1.
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1; Molecular chaperone that stabilizes DAPK and prevents its degradation by the proteasome.
BECN1	Beclin 1; Plays a central role in autophagy by initiating autophagosome membrane nucleation.
UBC	Ubiquitin C; Precursor to monomeric ubiquitin. When ubquitinated DAPK is degraded by the proteasome.

DAPK1, Fig. 5 DAPK network and apoptosis. Illustrated is a portion of the DAPK interaction network focused on apoptosis. DAPK engages several proteins in a complex that receives inputs from potential death signals (FADD) as well as survival signals (UNC5H2 –Netrin receptor). This network was created using String v10 and its associated database (string-db.org)

DAPK Protein Interactions and Apoptosis

DAPK is linked through direct binding to a number of proteins that participate in apoptosis by one or more pathways (Bialik and Kimchi 2006; Bialik and Kimchi 2014). Not all of the pathways are functional in a given cell type. The first pathway is through the binding of Fas to the death-initiator, FADD (Fig. 5). DAPK interacts with FADD by way of its death domain (Fig. 1). This interaction results in the downstream activation of caspases (CASP3) that lead to cell death (Fig. 5). The involvement of DAPK was established in multiple ways: (1) Expression of a fragment of DAPK containing the death domain protects cells from Fas-mediated cell death, (2) Expression of a DAPK mutant lacking the death domain does not promote cell death, (3) DAPK mutants that lack CaM regulation (deletion of CaM segment) resulted in massive apoptosis (Bialik and Kimchi 2006; Bialik and Kimchi 2014). Thus, both the death domain and DAPK catalytic activity are necessary for Fas-mediated cell death. A second pathway mediated by DAPK is also initiated from other signals such as UNC5H2 (Fig. 5). UNC52B (netrin 1 receptor) when bound to ligand (NTN1) blocks DAPK activation (Bialik and Kimchi 2014). However, when netrin is absent, UNC5H2 reduces the phosphorylation of DAPK at Ser-308 which induces its activation by Ca^{2+} CaM (Bialik and Kimchi 2006; Bialik and Kimchi 2014). Thus, netrin1/UNC5H2 functions as a DAPK switch. (Bialik and Kimchi 2014). In some cell types, however, depletion of DAPK by mRNA interference can promote apoptosis (Jin and Gallagher 2003). This may be mediated by a DAPK isoform that has a truncation near the C-terminus of the protein that inhibits the activity of the death domain (Bialik and Kimchi 2006). It is not clear what mediates the expression of this isoform called DAPKβ. Two examples of upregulated DAPK expression that might involve this isoform are p53 mutant cancers (Zhao et al. 2015) and human peritumoral tissues that results in phosphorylation of NR2B (Table 1) and increased exitability that is thought to lead to seizures (Gao et al. 2015).

Summary

DAPK is a fascinating kinase with respect to its involvement in multiple cellular processes that have not been fully studied. For example, although some substrates have been identified the timing and duration of DAPK activation may be critically important with respect to cellular commitment to apoptosis.

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Dardarin

▶ Receptor-Interacting Protein Kinase

Darpp32

▶ DARPP-32 (Ppp1r1b)

DARPP-32

▶ DARPP-32 (Ppp1r1b)

DARPP-32 (Ppp1r1b)

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Synonyms

DARPP-32; Darpp32; Dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein, 32 kDa; Dopamine and cAMP-regulated

phosphoprotein; Dopamine- and cAMP-regulated phosphoprotein, Mr32 kDa; Ppp1r1b; Protein phosphatase 1 DARPP-32 inhibitor protein; Protein phosphatase 1, regulatory (inhibitor) subunit 1B

Historical Background

Protein phosphorylation is one of the most important posttranslational events that regulate myriad of biological processes such as cell division, cell differentiation, metabolism and modulation of signal transduction pathways (Ubersax and Ferrell 2007).

Studies using behavioral analysis of animal models suggest that alterations in critical intracellular signaling pathways have an important role in the pathophysiology and treatment of complex neuropsychiatric disorders. The hypothesis is that if the vast majority of psychiatric medications exert their primary therapeutic actions in the first week of treatment, the therapeutic effects involve transcriptional changes initiated and maintained by critical intracellular signaling pathways.

The dopaminergic neurotransmission system has been the focus of much research throughout the last few decades, including psychiatric and neurological disorders, and drug action mechanisms. Dopamine is associated with motor behavior, pleasure and reward, cognition, among other functions. It is well known that dopamine plays a major role in the coordination and regulation of the two output pathways by acting in a bidirectional manner. Five different dopamine receptors were cloned in humans and classified into two subgroups: D1 and D2 receptors. Many electrophysiological and gene transcriptional data, obtained *in vitro* and *in vivo*, suggest that dopamine exerts stimulatory effects via D1 receptors and inhibitory effects via D2 receptors (West and Grace 2002). All of the dopaminergic receptors are metabotropic and alter cAMP signaling: D1 receptor subtypes (D1, D5) stimulate adenylyl cyclase, whereas D2 receptor subtypes (D2S, D2L, D3, D4) inhibit adenylyl cyclase (Svenningsson et al. 2004).

Initially discovered as the major target for dopamine-activated adenylyl cyclase and PKA in striatum, DARPP-32 (dopamine and cAMP-regulated phosphoprotein Mr 32,000), which is also called PPP1R1B (protein phosphatase 1 regulatory subunit 1B), is a hub for signaling by multiple receptors and its regulation has been modeled by several groups (Li et al. 2015) and has a central role in the biology of dopaminoceptive neurons in the central and peripheral nervous system (Reis et al. 2007). The phosphorylation by PKA at Thr34 converts DARPP-32 into a potent high-affinity inhibitor of the multifunctional serine/threonine protein phosphatase, PP-1 (Svenningsson et al. 2004), including several neurotransmitter receptors, ion channels, and transcription factors (Yamada et al. 2016).

DARPP-32 is also phosphorylated at Thr75 by Cdk5 converting DARPP-32 into an inhibitor of PKA. Furthermore, the state of phosphorylation of DARPP-32 at Thr34 is fine-tuned by the phosphorylation state of two serine residues, Ser102 and Ser137, which are phosphorylated by CK2 and CK1, respectively. Thus, by virtue of its unique ability to modulate the activity of both PP-1 and PKA, DARPP-32 is critically involved in regulating electrophysiological, transcriptional, and behavioral responses to physiological and pharmacological stimuli, including antidepressants, neuroleptics, and drugs of abuse (Svenningsson et al. 2004).

The serotonergic neurotransmitter system, together with the dopaminergic system, regulates emotion, mood, reward, and cognition. Detailed studies in striatal slices and whole animals have shown that serotonin causes an increase in phosphorylation of DARPP-32 at Thr34 and Ser137 and decreased phosphorylation at Thr75. The actions of serotonin in regulating phosphorylation of DARPP-32 at Thr34 and Thr75 were mediated primarily via activation of 5-HT4 and 5-HT6 receptors, whereas the regulation of phosphorylation at Ser137 was mediated primarily via 5-HT2 receptors. DARPP-32 phosphorylation is also modulated by glutamate, GABA, neuromodulators, and neuropeptides (Svenningsson et al. 2004).

In addition to the brain, DARPP-32 is expressed in the adrenal medulla, kidney, and parathyroid cells (Belkhiri et al. 2016), and beside

its main role in dopamine signaling, other recent studies also revealed wide functions of DARPP-32 such as binding to tra2-beta1 in regulating alternative splicing events (Benderska et al. 2010). For instance, it has been reported that the truncated form of DARPP-32 (t-DARPP-32), where amino acid residues encoded by exon one are missing, is highly expressed in cancers (Belkhiri et al. 2008; Vangamudi et al. 2010). Thus, deregulated or loss of DARPP-32 activities can transform normal thyroid cells to tumors. Also, overexpression of DARPP-32 enhanced interactions between EGFR and ERBB3 and promoted tumor resistance to antitumor drug gefitinib via increased phosphorylation of AKT (Ung and Teoh 2014; Zhu et al. 2011) and frequent amplification of 17q12, the locus of DARPP-32, in gastric and esophageal adenocarcinomas (El-Rifai et al. 2002).

DARPP-32 Knockout and Mutant Mice

The generation of DARPP-32 knockout (KO) mice (Fienberg et al. 1998) and mutant mice with point mutations in phosphorylation sites of DARPP-32 (Svenningsson 2003) has provided a powerful tool for the search of the DARPP-32 roles in both behavioral and neurobiological basis of several diseases. Overall, studies have shown that DARPP-32 is required for the physiological actions of dopamine. A well-accepted molecular explanation for this role is the reduced induction of gene expression after treatment with D1 receptor agonists in mice lacking DARPP-32 (Svenningsson et al. 2000a).

Studies using DARPP-32 KO mice have indicated that DARPP-32-dependent pathways are involved in the modulation of the short, and perhaps long, term actions of drugs of abuse (Svenningsson et al. 2005). In fact, addictive properties of most drugs of abuse are mediated via dopaminergic pathways, particularly through postsynaptic neurons within the striatum, which contains high levels of DARPP-32. For example, cocaine preference (Zachariou et al. 2002), ethanol reward (Risinger et al. 2001), and stimulatory effects of caffeine on motor activity (Lindskog et al. 2002) are reduced in mice lacking DARPP-32. Also, hyperlocomotor response to D-methamphetamine in mice lacking PDE1B was blocked in PDE1B-DARPP-32 double-KO mice (Ehrman et al. 2006). Similar results were reported in Thr34 and Thr75 mutant mice, which denote that behavioral and biochemical actions of cocaine depend on phosphorylation sites of DARPP-32 (Zachariou et al. 2002).

Evidence has also shown a critical role for DARPP-32 in the therapeutic drug actions. The antidepressant effects of fluoxetine, a drug that increases phosphorylation of DARPP-32 at Thr34, was strongly reduced in DARPP-32 knockout mice (Lindskog et al. 2002). For more detailed information, see Fienberg and Greengard (2000).

DARPP-32 and Human Genetics

Since the function of DARPP-32 is endogenously regulated by the action of neurotransmitters and neuromodulators, and exogenously by cocaine and therapeutic drugs, genetic variations in the DARPP-32 gene (also known as PPP1R1B; located on 17q12) have been long viewed as potential biomarkers for psychiatric disorders. The initial evidence that a chromosomal region within 17q, which includes the PPP1R1B gene, increases the risk for schizophrenia (Cardno et al. 2001) and bipolar disorder (Dick et al. 2003), has offered a useful starting point for genetic studies. However, a few studies have examined the influence of variations in PPP1R1B gene on psychiatric symptoms.

To date, genetic association studies involving the PPP1R1B gene were performed in schizophrenia, bipolar disorder, autism, addiction, neurocognitive functions, attention-deficit hyperactivity disorder (ADHD), and breast cancer. Among them, positive associations were found only for nicotine dependence, autism and a miscellaneous of neurocognitive functions. Overall, weak-to-moderate associations were reported, and the inconsistent data can be partially explained by the genetic diversity of the populations studied and polymorphism discrepancy.

In a sample of 2037 subjects, Beuten et al. (2007) found that a PPP1R1B haplotype formed by the single nucleotide polymorphisms (SNPs) rs2271309, rs907094, rs3764352, and rs3817160 was significantly associated with smoking quantity in European-Americans, but not in African-Americans (Beuten et al. 2007). These data not only suggest that nicotine addiction risk is predisposed by PPP1R1B polymorphisms, but also that it appears to be influenced by ethnic diversity. Using a translational genetics approach, Meyer-lindenberg et al. (2007a) found that the same schizophrenia-related PPP1R1B variants that impact on cognitive functions also predicted the DARPP-32 mRNA expression. Interestingly, in an independent sample of healthy Caucasian subjects, these same polymorphisms were associated with different patterns of neostriatal morphology and function (Meyer-Lindenberg et al. 2007b).

In a cohort of 112 males with autism, Hettinger et al. (2012) found an increased frequency of CC genotype for the rs1495099 SNP in affected individuals. Authors also suggested this genotype is associated with severe problems of social interaction, communication, and increased stereotypic behaviors (Hettinger et al. 2012).

The rs907094 is by far the most studied PPP1R1B SNP. This polymorphism, which is associated with striatal dopamine function, has been shown to predict some neurocognitive functions such as human reinforcement learning (Frank et al. 2007), emotional learning (ĆurČić-Blake et al. 2012), the work memory (Schuck et al. 2013; Smith et al. 2014), auditory perception (Li et al. 2013), and brain-evoked potentials (Hämmerer et al. 2013). Knowledge of how genetic variations in the PPP1R1B such as rs907094 affect dopamine-dependent behaviors may have substantial implications for several psychiatric disorders.

Psychiatric Disorders and Neurological Diseases

Schizophrenia (SCZ): SCZ is a disease that affects 1% of the worldwide population. Abnormalities in the dopamine, glutamate, and GABA neurotransmitter systems are involved in the SCZ etiology (Chiapponi et al. 2016; Rowland et al. 2013; Souza et al. 2006). The DARPP-32 signaling pathway is regulated by these neurotransmitters' signaling (Yger and Girault 2011). Several studies, using postmortem brain and lymphocytes from SCZ patients, have reported alterations in the DARPP-32 levels (see Table 1). These studies demonstrated a decrease in the protein levels, but not mRNA, in the dorsolateral prefrontal cortex of patients, a region suggested to be associated with negative symptoms and cognitive impairments of SCZ (Albert et al. 2002; Baracskay et al. 2006; Ishikawa et al. 2007; Kunii et al. 2014a). The number of DARPP-32 expressing neurons is diminished within the neurons of the layers II, III, IV, and V of SCZ dorsolateral prefrontal cortex, but there are no changes in the number of glia cells expressing DARPP-32 (Kunii et al. 2011a). On the other hand, the expression of t-DARPP-32 transcripts is increased in the dorsolateral prefrontal cortex of the patients (Kunii et al. 2014b). The decrease of DARPP-32 in this region was not affected by the age of patients (Albert et al. 2002; Ishikawa et al. 2007). It was also observed a reduction in the number of neurons and the number of DARPP-32 expressing neurons within the CA3 subregion of the hippocampus and in the layers III and IV of superior temporal gyrus cortex of SCZ (Kunii et al. 2011a; Kunii et al. 2011b). But there are no changes in the mRNA expression levels of both DARPP-32 and t-DARPP-32 within the hippocampus and caudate nucleus of patients (Kunii et al. 2014b). On the other hand, there is an increase in the number of neurons expressing DARPP-32 within the layer V of SCZ and expression of t-DARPP-32 in the striatum. Also, no alterations were demonstrated in the mRNA levels of DARPP-32 in the anterior cingulated cortex and thalamus of SCZ patients (Baracskay et al. 2006). Recently, levels of DARPP-32 were shown to be decreased in lymphocytes of SCZ patients, but not mRNA DARPP-32 levels, suggesting that DARPP-32 protein levels can be a potential biomarker for this illness (Torres et al. 2009) (see Table 1). Several studies showed that blocking D2 receptors,

DARPP-32 (Ppp1r1b), Table 1 DARPP-32 and psychiatric and neurological disorders – summary of DARPP-32 expression patterns in samples from psychiatric and neurologic disorder patients

Disorder	Sample	DARPP-32 or t-DARPP-32	Alterations
Major depression	Dorsolateral prefrontal cortex	DARPP-32	Increase: mRNA (Kunii et al. 2014a)
		t-DARPP-32	Increase: mRNA (Kunii et al. 2014b)
	Hippocampus	DARPP-32	No alterations: mRNA (Kunii et al. 2014a)
		t-DARPP-32	No Alterations: mRNA (Kunii et al. 2014b)
Schizophrenia	Dorsolateral prefrontal cortex	DARPP-32	Decrease: protein (Albert et al. 2002; Ishikawa et al. 2007; Kunii et al. 2011b); mRNA in suicidal patients (Feldcamp et al. 2008) No alterations: mRNA (Baracskay et al. 2006)
		t-DARPP-32	Increase: mRNA (Kunii et al. 2014a)
	Hippocampus	DARPP-32	Decrease: protein (Kunii et al. 2011a) No alterations: mRNA (Kunii et al. 2014b)
		t-DARPP-32	No alterations: mRNA (Kunii et al. 2014b)
	Superior temporal gyrus cortex	DARPP-32	Decrease: protein (Kunii et al. 2011a)
	Caudate nucleus	DARPP-32	No alterations: mRNA (Kunii et al. 2014a)
		t-DARPP-32	No alterations: mRNA (Kunii et al. 2014b)
	Striatum	DARPP-32	Increase: protein phosphorylation (Kunii et al. 2011b)
		t-DARPP-32	Increase: (Kunii et al. 2014b)
	Anterior cingulate cortex	DARPP-32	No alterations: mRNA (Baracskay et al. 2006)
	Thalamus	DARPP-32	No alterations: mRNA (Clinton et al. 2005)
	Lymphocytes	DARPP-32	Decrease: protein (Torres et al. 2009) No alterations: RNA (Cui et al. 2015)
Bipolar disorder	Dorsolateral prefrontal cortex	DARPP-32	Decrease: protein (Ishikawa et al. 2007) No alterations: (Kunii et al. 2014a)
		t-DARPP-32	Increase: mRNA (Kunii et al. 2014b)
	Hippocampus	DARPP-32	Increase: mRNA (Kunii et al. 2014a)
		t-DARPP-32	Increase: mRNA (Kunii et al. 2014b)
	Caudate	DARPP-32	No alterations: (Kunii et al. 2014a)
		t-DARPP-32	No alterations: (Kunii et al. 2014b)
	Lymphocytes	DARPP-32	Decrease: protein (Torres et al. 2009)
Parkinson disease	Putamen	DARPP-32	Decrease: protein (Cash et al., 1987) No alterations: protein (Girault et al., 1989)
	Substantia nigra pars reticulate	DARPP-32	Decrease: protein (Cash et al. 1987)
	Substantia nigra pars compacta	DARPP-32	Decrease: protein (Cash et al. 1987)
	Caudate nucleus	DARPP-32	No alteration: protein (Girault et al. 1989)

the main target of antipsychotics can increase the phosphorylation of DARPP-32(Thr34), affecting motor behavior (Yger and Girault 2011; Svenningsson et al. 2000b). This modulation is impaired in DARPP-32 KO mice (Fienberg and Greengard 2000; Heyser et al. 2000). However, antipsychotics do not regulate the expression of DARPP-32, suggesting that the decrease of DARRP-32 in the prefrontal cortex of SCZ patients is related to the disease and not to the pharmacological treatment (Baracskay et al. 2006; Souza et al. 2010; Feldcamp et al. 2008).

Bipolar Disorder (BD): Three studies reported alterations in DARPP-32 levels in BD patients. The levels of DARPP-32 are decreased in the dorsolateral prefrontal cortex of BD patients, indicating that DARPP-32 might be involved in the neurotransmission imbalance in the BD brain (Ishikawa et al. 2007). However, there is no alteration in the expression of DARPP-32 mRNA in the same brain region of patients (Kunii et al. 2014a). In contrast, the levels of expression of t-DARPP-32 are increased in the dorsolateral prefrontal cortex of patients. It was also reported an increase of DARPP-32 and t-DARPP-32 mRNA levels in the hippocampus, but not in caudate, of BD patients (Kunii et al. 2014b). Interestingly, chronic treatment with lithium increases the levels of DARPP-32 in prefrontal cortex of rats (Guitart and Nestler 1992). Furthermore, DARPP-32 levels are decreased in the lymphocytes of BD patients, pointing to DARPP-32 as a putative biomarker for BD (Torres et al. 2009) (see Table 1).

Major Depression (MD): Much evidence supports the involvement of dopamine in MD, for example, pharmacological treatment and dopamine metabolite levels. A recent study observed an increase of mRNA expression of both DARPP-32 and t-DARPP-32 in the dorsolateral prefrontal cortex of MD (Kunii et al. 2014b). On the other hand, there is no alteration in the DARPP-32 and t-DARP-32 mRNA levels in the MD patients' hippocampus. Acute treatment with fluoxetine increases the phosphorylation of DARPP-32 in many regions of the mouse brain (Svenningsson et al. 2000b), and chronic treatment with lithium and antidepressants increases DARPP-32 levels in the prefrontal cortex of rats (Guitart and Nestler 1992; Reis et al. 2007). Another study reported that chronic electroconvulsive stimulation, which is very useful for depression, increases the levels of DARPP-32 in rats hippocampus and striatum (Rosa et al. 2007).

Attention Deficit and Hyperactivity Disorder (ADHD): ADHD affects 3–7% of children in the world. Dopamine signaling is the main target of pharmacological treatment. It was recently reported that methylphenidate treatment regulates the expression and phosphorylation of DARPP-32. Interestingly, these regulations are dependent on drug posology and the age of the rats and mice, and it is region specific as well (Fukui et al. 2003; Souza et al. 2009).

Parkinson Disease (PD): It is well known that dopamine signaling abnormalities are involved in the PD. Two studies reported different results regarding the levels of DARPP-32 in the brains of PD patients. One reported decreased levels of DARPP-32 in the putamen, substantia nigra pars reticulate and substantia nigra pars compacta of PD patients. However, no alterations in DARPP-32 levels were found in the putamen and caudate nucleus of a different group of PD patients (Cash et al. 1987; Girault et al. 1989) (see Table 1).

DARPP-32 and Drugs of Abuse

Since its discovery three decades ago, DARPP-32 has been shown in a large body of work as a central signaling molecule activated by a diverse array of neurotransmitters such as dopamine, glutamate, serotonin, adenosine, and gamma-aminobutyric acid (GABA). In response to drugs of abuse and psychostimulants, these neurotransmitters regulate the phosphorylation state of DARPP-32, which converts it to an inhibitor of either a protein phosphatase (PP1) or a protein kinase (PKA) (Belkhiri et al. 2016; Andersson et al. 2005).

Diverse addictive stimuli share the ability to enhance dopamine signaling and modulate reward-related learning and memory; yet, the responsiveness of humans and animal models to drugs is highly dependent on a variety of genetic and environmental factors that are not entirely understood. DARPP-32 functions as a switch, reinforcing or inhibiting the action of the cAMP-dependent pathway, depending on its state of phosphorylation (Engmann et al. 2015).

Cannabis: The major psychoactive components of marihuana and hashish are cannabinoid. Cannabis and its primary psychoactive active constituent, D9-tetrahydrocannabinol (THC), can produce addiction and neuropsychiatric symptoms with

repeated use (Volkow et al. 2014). Fernández-Ruiz et al. (2010) showed that the effects of cannabinoids on dopamine transmission and dopamine-related behaviors are indirect and affected by the modulation of GABA and glutamate inputs received by dopaminergic neurons. Recent evidence suggests, however, that certain eicosanoid-derived cannabinoids may directly activate TRPV1 receptors (Fernández-Ruiz et al. 2010). These receptors have been found in some dopaminergic pathways, what allow a direct regulation of DA function by cannabinoid signaling. In the brain, cannabinoids interact with neuronal cannabinoid CB1 receptors (CB1Rs), thereby producing a marked reduction of motor activity. These receptors are coupled to Gs protein that enhances cAMP levels and, consequently, leads to the phosphorylation of DARPP-32 at Thr34. Point mutation of Thr75 does not affect the behavioral response to CP55940, a selective CB1Rs agonist. On the other hand, catalepsy induced by CP55940 is reduced in both DARPP-32 knockout mice and Thr34-Ala DARPP-32 mutant mice. Activation of CB1Rs, either by an agonist or by inhibition of reuptake of endogenous cannabinoids, stimulates phosphorylation at Thr34 (Reis et al. 2007; Andersson et al. 2005).

The stable transcription factor D FBJ murine osteosarcoma viral oncogene homolog B (DFosB) accumulates in striatal neurons during repeated administration of abused drugs, including THC (Lazenka et al. 2014). Dopamine signaling is regulated by DARPP-32, which is highly expressed in striatal medium spiny neurons (MSNs) and dopaminergic terminal fields (Greengard 2001; Nairn et al. 2004). THC-mediated phosphorylation of T34 DARPP-32 is inhibited by administration of a D1R or A2AR antagonist (Borgkvist et al. 2008), suggesting that both MSN populations contribute to cannabinoid-dopamine interactions. Lazenka et al. (2014) recently reported that repeated THC-mediated DFosB induction in the striatum was abolished in mice lacking CB1Rs (Lazenka et al. 2014). Genetic deletion of DARPP-32 enhances THC-mediated hypolocomotion as well as the development of tolerance to this response after repeated THC administration, suggesting an involvement of DARPP-32-mediated signaling in the acute and chronic motor effects of THC (Lazenka et al. 2015).

Cocaine: Acute treatment with cocaine increases the phosphorylation of DARPP-32(Thr34) and decreases the phosphorylation ofDARPP-32(Thr75). On the other hand, chronic treatment with cocaine increases phosphorylation of DARPP-32(Thr75) and decreases phosphorylation of DARPP-32(Thr34) (Svenningsson et al. 2004).

Chen et al. (2008) demonstrated that acute stimulation with cocaine activates the dopamine D1 receptors, consequently leading to DARPP-32(Thr34) phosphorylation in the striatum. Several studies have shown that DARPP-32 participates in the progressive development of behavioral sensitization to cocaine. Knock-out of DARPP-32 or DARPP-32 mutation (threonine 34 replaced by alanine) in mice attenuated the hyperlocomotor activity induced by acute cocaine treatment. Moreover, chronic treatment with cocaine decreased phosphorylation at Thr34 but increased at Thr75. This latter effect was due to enhanced Cdk5 (Chen et al. 2008).

Ro 60–0175, 5-HT2CR agonist, inhibited cocaine-induced phosphorylation of DARPP-32 at threonine residues in the nucleus accumbens (NAc) core and the selective 5-HT2CR antagonist SB 242084 reversed this effect. These findings demonstrate that 5-HT2CRs are capable of modulating mesoaccumbens dopamine pathway activity at postsynaptic level by specifically controlling dopamine signaling in the NAc core subregion (Cathala et al. 2015).

Acute and repeated cocaine exposures had been shown to induce changes in the expression of many genes in the brain (Robison and Nestler 2011). Such transcriptional modifications can be rapid and transient, or persistent and account for long-term behavioral changes following repeated exposure. It has been shown that cocaine also acts on DNA methylation involving DNA methyltransferases (DNMTs) and methyl-CpG binding domain proteins (MBDs) (Host et al. 2011). While cocaine inhibits PP1 activity through at least

dopamine D1 receptors and DARPP-32 phosphorylation, very little is known about the mechanism by which the expression of each PP1C isoform is regulated (Bodetto et al. 2013).

Opiates: Opiates act on the dopaminergic system in the brain via the μ-receptor and modulates the expression of DARPP-32, which represents an interesting nexus for drug-induced changes in neural long-term synaptic plasticity. Mahajan et al. (2009) showed that heroin significantly increased both D1 and DARPP-32 gene expression. Also, it has been demonstrated that that gene silencing DARPP-32 by siRNA in cultured normal human astrocytes cells modulated the activity of downstream effectors molecules, such as PP-1 (Mahajan et al. 2009). Opiates, such as morphine, bind to opioid receptor subtypes (mu, delta, and kappa) that are abundant in the striatum. Mu- and delta-receptors are coupled to Gi protein, which decreases phosphorylation of DARPP-32 at Thr34 and modulates both dopamine and adenosine receptor activation (Reis et al. 2007).

Ignatowski et al. (2015) investigated the role of DARPP-32-mediated signaling on withdrawal behavior in a rat model of opiate addiction, using intracerebral administration of gold nanorods (GNR) complexed to DARPP-32 siRNA to silence DARPP-32 gene expression and measure its effects on the opiate withdrawal syndrome. The results showed that opiate-addicted animals treated with GNR-DARPP-32 siRNA nanoplex showed a lack of condition place aversive behavior consequent to the downregulation of secondary effectors such as PP-1 and CREB, which modify transcriptional gene regulation and consequently neuronal plasticity. Thus, nanotechnology-based delivery systems could allow sustained knockdown of DARPP-32 gene expression, which could be developed into a therapeutic intervention for treating drug addiction by altering reward and motivational systems and interfere with conditioned responses (Ignatowski et al. 2015).

Nicotine: Nicotine is the critical component in tobacco smoke that is involved in addiction. It has been shown that nicotine modulates dopaminergic neurotransmission mainly by enhancing dopamine release in nigrostriatal and mesolimbic dopaminergic systems. Abdolahi et al. (2010) demonstrated incubation of drug seeking following widespread access to nicotine self-administration and suggested that enhanced PKA signaling in the insular cortex via phosphorylation of DARPP-32 at Thr34 is associated with this effect. At low concentrations, nicotine decreases phosphorylation of DARPP-32 at Thr34 in mouse neostriatal slices. In the other hand, high levels of nicotine increased the phosphorylation of DARPP-32 at Thr34 (Abdolahi et al. 2010). Therefore, different concentrations of nicotine lead to a different amount of dopamine release and, consequently distinct activation patterns of dopamine receptors (Reis et al. 2007).

Kuroiwa et al. (2012) investigated the function of muscarinic receptors in the striatum by monitoring DARPP-32 phosphorylation at Thr34 using mouse striatal slices and showed that muscarinic receptors, especially M5 receptors, act at presynaptic dopaminergic terminals, regulate the release of dopamine in cooperation with nicotinic receptors, and activate D1 receptor/DARPP-32 signaling in the striatonigral neurons. Muscarinic M1 receptors expressed in striatopallidal neurons interact with adenosine A2A receptors and activate DARPP-32 signaling (Kuroiwa et al. 2012).

Shin et al. (2012) studied therapeutic potential of agents affecting the dopamine system in traumatic brain injury model and showed that damage reduced pDARPP-32-T34 levels, but nicotine treatment of injured animals did not alter pDARPP-32-T34 levels, indicating that postsynaptic dopamine signaling is complex, and the restoration of dopamine release may not be sufficient for the recovery of DARPP-32 activity (Shin et al. 2012).

Ethanol: Ethanol does not have a clearly defined site of action. It can act directly as an agonist of GABAA and antagonist of NMDA receptors and indirectly as an agonist of dopamine D1 receptor. Studies with DARPP-32 knockout mice showed DARPP-32 involvement in ethanol reward induced behavior in both place preference and self-administration tests (Risinger et al. 2001). It has been demonstrated that moderate levels of ethanol increase phosphorylation of DARPP-32 at Thr34 in striatal slices. Ethanol administration was found to increase

phosphorylation of DARPP-32 (Thr34) in the nucleus accumbens (NAc) and amygdala (but not in the striatum) of wild-type and transgenic mice, with a greater effect in the amygdala of transgenic mice. It was also found to increase of DARPP-32 (Thr75) in the amygdala of the wild-type mice only and the NAc and striatum of both the transgenic and wild-type mice. The authors concluded that the effect of ethanol on the balance of DARPP-32 phosphorylation, especially in the amygdala, may contribute to differential motivational effects of ethanol (Nairn et al. 2004; Goodman 2008).

The reinforcing properties of ethanol are in part attributed to interactions between opioid and dopaminergic signaling pathways. Björk et al. (2010) report that an acute ethanol challenge induces a robust phosphorylation of DARPP-32 (Björk et al. 2010). Abrahao et al. (2014) showed that the functional hyperresponsiveness of D1 receptors in the nucleus accumbens is associated with an increased phospho-Thr34-DARPP-32 expression after D1 receptor activation (Abrahao et al. 2014).

Summary (Future Directions, Perspective, Questions, or Challenges)

The importance of DARPP-32 arises from its relationship to several different signaling systems/cascades involved in intracellular functions such as important as gene expression, cell differentiation, metabolism, and neuronal plasticity. The protein is an integrator of cellular function and as such, a putative target to fine tune those functions. Also, research should be conducted to gather information from animal models such as the *Caenorhabditis elegans*, *Drosophila melanogaster, Aplysia sp.*, and Zebrafish. An example, in *Caenorhabditis elegans*, "Area-Restricted Search" behavior is controlled by a dopaminergic response to food deprivation that modulated glutamatergic signaling. Dopaminergic pathway compounds are not entirely described, and, like humans, this behavior time course is on order of minutes, so a similar process might explain, and a DARPP-32 like protein could be part of it.

Future research will certainly shed more light on the roles of DARPP-32 in different biological processes, as well as potential new functions. Nanotechnology is having an increasing impact in the healthcare industry. The combination of diagnostic (imaging) and therapeutic capability enables the "real-time" monitoring of therapeutic progression, thus bringing "personalized medicine" closer to clinical reality. Bonoiu et al. (2009) introduced a nanotechnology approach that utilizes gold nanorod-DARPP-32 siRNA complexes that target to dopaminergic signaling pathway in the brain. Gene silencing of the nanoplexes in dopaminergic neuronal cells was evidenced by the reduction in the expression of key proteins, as DARPP-32, belonging to this pathway, with no observed cytotoxicity. Since these nanoplexes were shown to transmigrate across an *in vitro* model of the blood–brain barrier, it appears to be suited for brain-specific delivery of appropriate siRNA for therapy of drug addiction and other brain diseases. Nevertheless, the available collection of evidence suggests that DARPP-32 lies at the nexus of multiple signaling pathways that modulate critical signaling states of a given cell type, thus assuring that it will continue to be an important molecule in the quest to find new pharmacological targets.

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Dbf4

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Synonyms

ASK (activator of S phase kinase)/huDbf4 (human); Chiffon (*Drosophila*); Dbf4 (dumbbell former 4)/DNA52 (*Saccharomyces cerevisiae*); Dbf4l1; Dfp1/Him1/Rad35 (*Schizosaccharomyces pombe*); Drf1/ASKL1 (a second activator of

Cdc7 in human and *Xenopus*); nimO (*Aspergillus*); Spo6 (a second Dbf4 homologue in *S. pombe*)

Historical Background

dbf4 (dumbbell former 4) mutation was originally identified in the screening for budding yeast temperature-sensitive mutants which arrest with dumbbell-shaped phenotype at the nonpermissive temperature (Johnston and Thomas 1982). The terminal phenotypes of *dbf4*(ts) strain at a nonpermissive temperature were very similar to those of *cdc7* (ts), encoding a serine-threonine kinase known to be essential for initiation of DNA replication. Later, *dbf4* was rediscovered as a multicopy suppressor of *cdc7*(ts), suggesting physical and functional interactions between Cdc7 and Dbf4 (Kitada et al. 1992). Following this finding, Dbf4 was shown to bind to Cdc7 and stimulate its kinase activity, establishing that Dbf4 is the activation subunit for Cdc7 kinase (Jackson et al. 1993). The presence of Cdc7 homologues in species other than budding yeast was first reported in fission yeast (*hsk1*; Masai et al. 1995). Following this discovery, an ortholog of Dbf4 was reported in fission yeast (Brown and Kelly 1998, Dfp1/Him1). The orthologs of Dbf4 from vertebrates were also identified and found to form complexes with cognate Cdc7 (Kumagai et al. 1999; Jiang et al. 1999).

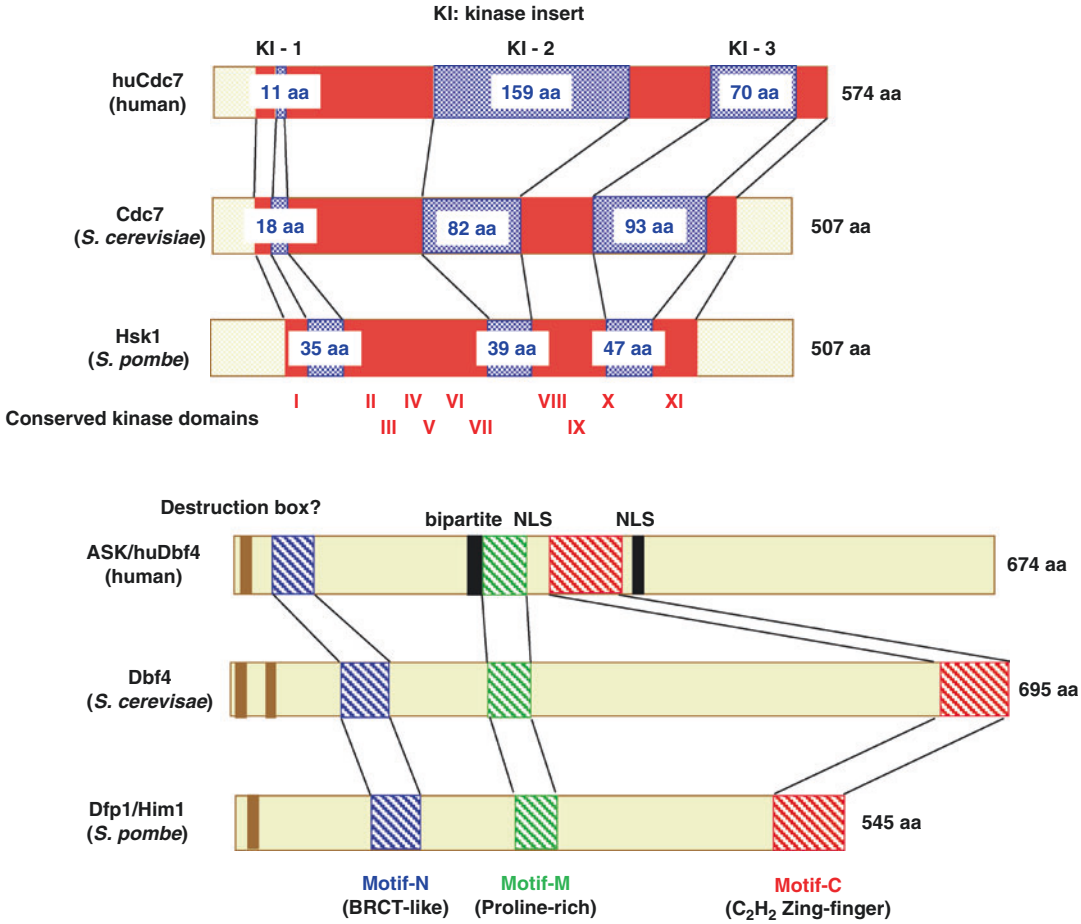
Identification of Orthologs of Dbf4 in Other Species

Purification of Hsk1 kinase from fission yeast led to identification of Dfp1, the fission yeast ortholog of Dbf4 (Brown and Kelly 1998). Two-hybrid screening with Hsk1 as a bait also led to the identification of Him1, identical to Dfp1. Interestingly, *dfp1*/ *him1* was found to be allelic to *rad35*, a radiation-sensitive mutant, implicating Dbf4 in DNA damage response pathway (Takeda et al. 1999). In *Aspergillus*, a mutant called *nimO* was isolated and was shown to encode a Dbf4 homologue (James et al. 1999). Human homologue of Dbf4, ASK (activator of S phase kinase), was isolated by two-hybrid screening using huCdc7 as a bait (Kumagai et al. 1999; Jiang et al. 1999).

A second Dbf4 subunit in human was identified and named Drf1 or ASKL1 (Montagnoli et al. 2002; Yoshizawa-Sugata et al. 2005). Drf1/ASKL1 can bind and activate huCdc7, but its role during cell cycle in human cell lines may be secondary. In fission yeast, a second set of Cdc7-Dbf4, Spo4-Spo6, is present which functions specifically during the sporulation/second meiotic division stage of meiosis (Nakamura et al. 2000). No functional homologues of Spo4-Spo6 have been identified in other species.

Activation of Cdc7 Kinase by Dbf4 Protein

Analysis of sequences of Dbf4 and its orthologs revealed the presence of three conserved motifs, motif-N, motif-M, and motif-C (Masai and Arai 2000; Fig. 1). Further analyses indicated that motif-M and motif-C can bind to Cdc7 independently. Binding of both motifs to Cdc7 is required for full activation of Cdc7 kinase. The sequences and length connecting motif-M and motif-C can be varied without affecting the kinase activity, suggesting that they serve as a linker sequence (Ogino et al. 2001; Kitamura et al. 2011). Motif-M is a proline-rich motif with no apparent similarity to known motifs. Motif-C is a C_2H_2-type zinc finger-like structure. Motif-N is composed of a single copy of a BRCT-like sequence, which could be uniquely replaced by the BRCT motif from Rev1 (Harkins et al. 2009). It was reported that zinc finger mutation exhibited slowed S-phase, DNA damage sensitivity, and hypo-mutagenic phenotype following UV irradiation. This mutant does not interact with Mcm2 and exhibits reduced phosphorylation. The same mutant was sensitive to long-term exposure to HU or MMS (Jones et al. 2010). Motif-M alone can sustain mitotic viability (Fung et al. 2002), but both motif-M and motif-C are required for viability through meiosis in fission yeast (Ogino et al. 2001). On the other hand, motif-N is not essential for viability, although it is required for interaction with Rad53 checkpoint kinase and is involved in conferring resistance to genotoxic agents and in checkpoint responses (Chen et al. 2013;



Dbf4, Fig. 1 *Comparison of the primary structures of Cdc7 and Dbf4/ASK proteins from human, budding yeast, and fission yeast.* The sequences of human, *S. cerevisiae*, or *S. pombe* Cdc7 or Dbf4/ASK were aligned, and the conserved segments are indicated. *Upper*: *red* regions are kinase-conserved domains which are similar between the three species. *Blue* regions are kinase insert sequences and are not conserved. *Lower*: motif-N, motif-M, and motif-C, shown in *blue*, *green*, and *red*, respectively, are conserved across the species

Matthews et al. 2014). In mammalian cells, motif-M and motif-C are sufficient for maximum kinase activity, but motif-N is also required for viability (Yamashita et al. 2005).

There is a long C-terminal tail segment in Dbf4/ASK from higher eukaryotes, which is not conserved except for the very C-terminal sequences rich in serines and threonines. They are major autophosphorylation sites. Truncation of the C-terminal 50 amino acids of human ASK was shown to hyperactivate the Cdc7 kinase, suggesting autoinhibition of Cdc7-ASK kinase by the C-terminal tail. LEDGF protein, a co-factor of human immunodeficiency virus DNA integration, interacts with this segment and relieves the autoinhibition (Hughes et al. 2010).

Functions of Dbf4/ASK During Cell Growth

A temperature-sensitive mutant of *dbf4* in budding yeast ceases growth at the onset of S phase, an identical position with that of *cdc7*(ts) cells. The arrested cells can reversibly enter the S phase upon return to a permissive temperature. Analyses of a temperature-sensitive mutant of *nimO*, the *Aspergillus* homologue of Dbf4, showed that NimO is required for initiation of DNA synthesis and for efficient progression through S phase as well as for DNA replication checkpoint coupling

S and M phases (James et al. 1999). *Drosophila* homologue of Dbf4, chiffon, is required for chorion gene amplification. Hypomorphic mutant alleles of the chiffon gene cause thin, fragile chorions and female sterility. Null alleles of chiffon had the additional phenotypes of rough eyes and thin thoracic bristles, phenotypes often associated with disruption of normal cell cycle progression (Landis and Tower 1999).

Dbf4 and Dfp1/Him1 are hyperphosphorylated in response to replication stress (such as treatment with hydroxyurea, which inhibits cellular nucleotide reductase and depletes cellular nucleotide pool). This phosphorylation of Dbf4 or Dfp1/Him1 depends on both Cdc7/Hsk1 and checkpoint kinase Rad53 or Cds1, respectively (Takeda et al. 2001; Brown and Kelly 1999). It has been proposed that this phosphorylation somehow inhibits the function of Dbf4, which contributes to suppression of firing of late origins. However, the nature of this inhibition is still unknown. Extensive mutagenesis of potential phosphorylation sites on Dbf4 rendered the mutant Dbf4 refractory to the checkpoint inhibition, and combination of the Dbf4 mutant with a similar phosphorylation site mutant of Sld3 abrogated the checkpoint inhibition of late origin firing, showing that Dbf4 is a critical target of DNA replication checkpoint (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). Human Dbf4/ASK and fission yeast Hsk1 may also be hyperphosphorylated by replication stress (Heffernan et al. 2007; Snaith et al. 2000). Dbf4 is generally limiting in quantity, and overexpression of Dbf4 can cause late-firing origins to fire earlier in S phase (Mantiero et al. 2011). Similar findings were made also in fission yeast (Patel et al. 2008; Wu and Nurse 2009).

In mouse ES cells, conditional knockout of Dbf4/ASK is lethal and cells undergo cell death (Yamashita et al. 2005), as was observed in ES cells in which the *Cdc7* gene was conditionally knocked down.

Functions During Meiosis

Initial characterization of budding yeast *cdc7* (ts) indicated the essential role of Cdc7 for meiotic recombination, but not for premeiotic DNA replication. In fission yeast as well, cells are arrested with one nucleus in an *hsk1*(ts) cells. In both fission yeast and budding yeast, initiation of meiotic recombination, i.e., induction of DSB (double-stranded DNA breaks), does not occur in the absence of Hsk1/Cdc7 (Ogino et al. 2006). Furthermore, Mer2, a factor essential for loading of DSB endonuclease Spo11, was identified to be a critical target of Cdc7 (Sasanuma et al. 2008; Wan et al. 2008). Premeiotic DNA replication can proceed albeit at slightly slower rate in *hsk1*(ts) mutant or *cdc7* as (a shorkat form of Cdc7 that can be chemically inactivated) in which Cdc7 can be chemically inactivated (Ogino et al. 2006; Lo et al. 2008).

On the other hand, repression of $dbf4^+$ expression regulated by the tet promoter suppressed premeiotic DNA replication in budding yeast when it was suppressed before meiosis was induced, but DNA replication was observed if $dbf4^+$ expression was suppressed later, presumably due to inadequate suppression of $dbf4^+$ expression. This result suggests that Dbf4 function is required for premeiotic DNA replication. In the latter cells, premeiotic S phase was completed, but the meiosis was still arrested before anaphase I. This arrest was relieved by *rec8* deletion, suggesting a crucial role of Cdc7 during meiotic chromosome segregation (Valentin et al. 2006). Indeed, Rec8 cleavage by separase is regulated by phosphorylation mediated by Cdc7 or casein kinase 1 (Katis et al. 2010). Cdc7 also regulates monopolar attachment of sister kinetochores by regulating the recruitment of the monopolin complex to kinetochores through phosphorylation of monopolin subunit Lrs4 (Matos et al. 2008). Thus, Dbf4, in a complex with Cdc7, may regulate multiple steps of meiotic cell cycle.

Other Dbf4-Related Molecules

Another Cdc7-Dbf4-related complex, the Spo4 (Cdc7-like)-Spo6(Dbf4-like) complex, is expressed in fission yeast during late meiosis and is specifically required for the sporulation stage (Nakamura et al. 2000). Kinase complexes related to Spo4-Spo6 have not been found in other species.

A second Dbf4/ASK-like molecule, Drf1/ASKL1, was identified in silico on the human genome and was shown to function as an

activation subunit for human Cdc7 kinase (Montagnoli et al. 2002; Yoshizawa-Sugata et al. 2005). The expression level of Drf1/ASKL1 increases during late S/G2, and inhibition of its expression resulted in accumulation of late S-G2/M populations. In contrast to Dbf4/ASK, Drf1/ASKL1 is present mostly in the nuclear soluble fractions, not in chromatin-bound fractions. Drf1/ASKL1 has been identified only in human and X*enopus*.

Association of Dbf4 with Origin Sequences in Budding Yeast

One-hybrid assays showed that Dbf4 interacts with the replication origin sequences in budding yeast (Dowell et al. 1994). Mapping of the interacting segment on Dbf4 indicated motif-N as the origin-interacting domain. Thus, Dbf4 targets Cdc7 kinase at the origin of DNA replication. At the origin, Cdc7 targets pre-replicative complex (pre-RC) which is generated on chromatin during early G1. Among the components of pre-RC, MCM is the critical and conserved substrate of Cdc7 (Lei et al. 1997; Sato et al. 1997). See the section of "Cdc7" for details on how Cdc7-mediated phosphorylation may activate initiation of DNA replication.

Interaction of Dbf4 with Other Replication Factors

Yeast two-hybrid analyses indicated the interaction of mouse Dbf4/ASK with Orc1, Orc2, Orc5, and Orc6 as well as with MCM2, MCM3, MCM4, and MCM7, consistent with the interaction of Dbf4 with pre-RC assembled at origins in budding yeast (Kneissl et al. 2003). In the same report, mouse Cdc7 was reported to interact with Orc1 and Orc6 and with MCM2, MCM4, MCM5, and MCM7 in two-hybrid assays. Two-hybrid assays also indicate that budding yeast Dbf4 interacted most strongly with Mcm2, whereas Cdc7 associated with both Mcm4 and Mcm5. They found most strong interaction between Dbf4 and N-terminal segment of Mcm2, which may serve as a second Cdc7 docking site in addition to that found in Mcm4 (Sheu and Stillman 2006).

Regulation of Expression of Dbf4 During the Cell Cycle

Expression of Dbf4 is cell cycle regulated. Regulation is generally on both transcription and protein levels. In budding yeast, transcription of Dbf4 is regulated during cell cycle, peaking during G1 (Chapman and Johnston 1989). The promoter contains a MluI cell cycle box (or MCB) and may be regulated by the MCB-binding factor (MBF). Furthermore, the budding yeast Dbf4 protein is degraded by APC during G1 phase. The protein is present during S through G2 phase, coinciding with the active Cdc7-Dbf4 kinase activity during this period. The potential degradation signal was identified in the N-terminal segment of Dbf4 (Oshiro et al. 1999; Ferreira et al. 2000). Similar regulation is likely to operate for the fission yeast Dfp1/Him1 gene, which is expressed specifically during S through G2/M phase (Brown and Kelly 1998; Takeda et al. 1999).

Expression of mammalian Dbf4/ASK is also cell cycle regulated. The transcript is repressed in the quiescent cells and induced after growth stimulation. A 63-base pair ASK promoter segment was identified, which is sufficient for mediating growth stimulation (Yamada et al. 2002). This minimal promoter segment contains an Sp1 site but no canonical E2F site but can be activated by ectopic E2F expression. Within the 63-base pair region, the Sp1 site and other elements are essential for stimulation by growth signals and by E2F, whereas an AT-rich sequence proximal to the coding region may serve as an element required for suppression in quiescence. Another report proposed the presence of MCB in the core promoter region of human Dbf4/ASK (Wu and Lee 2002). The Dbf4/ASK protein levels decrease during G1 phase, and a part of this may be attributed to cell cycle-dependent protein degradation.

Developmental Role of Dbf4

A role of Dbf4 in heart/eye development was suggested in *Xenopus* (Brott and Sokol 2005). Dbf4/ASK inhibits the canonical Wnt signaling pathway, possibly through interacting with Frodo. This role of Dbf4 does not involve its ability to activate Cdc7 kinase, since the Dbf4-motif-M,

which is known to be essential for Cdc7 kinase activation, is not required for its role in heart development. Expression of Drf1/ASKL1 and Dbf4/ASK molecules is developmentally regulated in *Xenopus*. Drf1/ASKL1 is predominantly expressed in early development but is later replaced by Dbf4/ASK in somatic cells (Takahashi and Walter 2005).

Those who are interested in learning more about eukaryotic DNA replication are recommended to read the reference (Masai et al. 2010).

Summary

Dbf4 is the activation subunit for Cdc7, a conserved kinase essential for initiation of DNA replication. Dbf4 is evolutionally conserved and carries three conserved motifs, motif-N, motif-M, and motif-C. Motif-M or motif-C interacts with Cdc7 on its own, but motif-M alone can support mitotic growth in yeast. Motif-C, the most conserved segment, is required together with motif-M for full kinase activation as well as for meiotic function of Cdc7-Dbf4. Motif-N of Dbf4 is involved in interaction with chromatin of the Cdc7-Dbf4 complex. Dbf4 may induce conformational change of Cdc7 and facilitate its binding to ATP as well as its association with the critical substrate of the Cdc7-Dbf4 kinase complex. Second Dbf4, Drf1/ASKL1, has been discovered in human and *Xenopus*. Abundance of Dbf4 is cell cycle regulated and contributes to cell cycle oscillation of Cdc7 kinase activity.

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Dbf4 (Dumbbell Former 4)/DNA52 (*Saccharomyces cerevisiae*)

▶ Dbf4

Dbf4l1

▶ Dbf4

DDIT1 (DNA-Damage Inducible Transcript 1)

▶ GADD45

DDX58

▶ RIG-I (Retinoic Acid Inducible Gene-I)

Death-Associated Protein Kinase

▶ DAPK1

Dectin-1

▶ CLEC7A

Defensin

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Synonyms

α-Defensin; β-Defensin; Human beta defensin (HBD/hBD); Human defensin (HD); Human neutrophil peptides (HNP)

Historical Background

Defensin is a cysteine-rich, cationic peptide expressed by higher organisms and plays an important role in innate immunity. Mammalian defensin is classified into alpha, beta, and theta depending on the pattern of disulfide-bridge formation between six conserved cysteine residues. In 1985, Selsted et al. first isolated a mature form of α-defensins family from human blood denoting

them as human neutrophil peptides (HNPs) in accordance to their source, property, and size. Shortly, three types of HNPs (HNP-1, HNP-2, and HNP-3) having nearly identical amino acid sequences were isolated. In 1989, a fourth type of HNPs, HNP-4, was reported after being purified to homogeneity by chromatographic methods. In 1989 and 1992, α-defensin genes were extracted from mouse and human paneth cells, respectively, and termed as HD-5 and HD-6, which are tissue-specific peptides and only restricted to human intestines. Members of human β-defensin family were soon discovered with human β defensin-1 (hBD-1) being the first to be extracted by Bensch et al. from human plasma in 1995. Subsequently, hBD-2 was identified from lesional psoriatic skin with two variants hBD-3 and hBD-4 documented in year 2001 (Wang 2014). Till now, 28 additional human and 43 mouse β-defensin genes have been identified with the help of bioinformatic studies. In the meantime, a different type of defensin called θ-defensin also was identified in neutrophils and monocytes of rhesus monkey and other nonhuman primates (Tang et al. 1999). Humans including chimpanzees and gorillas also expressed θ-defensin pseudogenes, which prevent the translation of θ-defensin precursor due to the presence of a premature stop codon (Garcia et al. 2008).

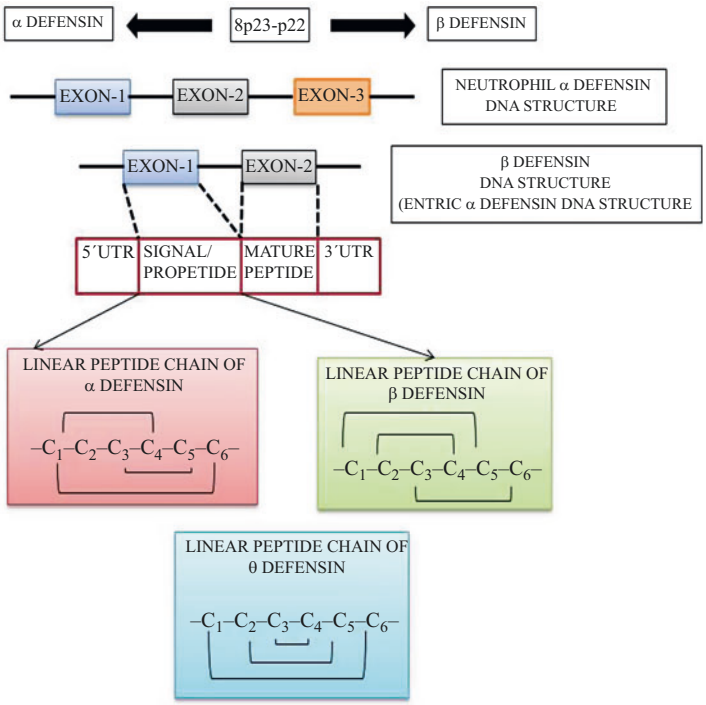
Gene Structure and Its Transcription

Human α-defensin peptides are encoded by five DEFA (Defensin-α) genes. HNP1, 2, and 3 share almost identical sequences, i.e., XC1YC2RIPA-C3IAGERRYGTC4IYQGRLWAFC5C6 with HNP 1 and 3 differing only by a single N-terminal residue ("X" is alanine in HNP1 and aspartic acid in HNP3). The proteolytic removal of X residue from N-terminal results in HNP2 explaining the absence of its individual gene. DEFA1 and DEFA3 genes are located on chromosome 8p23.1 where they show extensive copy number of polymorphism. In contrast, genes for HNP4, HD5, and HD6 do not undergo duplication with only one copy of each existing per haploid genome (Lehrer and Lu 2012). Human enteric α-defensin genes consist of two exons, whereas human neutrophils α-defensin genes consist of three exons, two of which (second and third) are homologous to enteric defensins. β-defensin gene coding consists of two exons located on human chromosome 8p23–p22. The first exon includes 5′ untranslated region and encodes the leader domain of the preproprotein, whereas the second exon encodes mature peptide with the six-cysteine domain (Meade et al. 2014).

Structure and Distribution

The human α defensins are small (3.5–4 kDa) cationic peptide, having three intramolecular cysteine bond pairing like 1–6, 2–4, and 3–5, whereas the β defensins are little larger molecule (4–6 kDa) with bonds between cysteines 1–5, 2–4, and 3–6 in their linear peptide structure. These small molecules have an intricate tertiary structure with a core of three antiparallel β sheet components resembling chemokines (Oppenheim et al. 2003). θ-Defensin has a circular architecture due to the formation of a peptide bond between its N- and C-terminal ends. It is generated by ligation of two truncated α-defensins and also stabilized by three sets of disulfide bonds (C_1–C_6, C_2–C_5, and C_3–C_5) (Lisitsyn et al. 2012; Wang 2014) (Fig. 1).

Defensin has widespread tissue distribution in epithelial and mesenchymal cells of human adult (Table 1). Apart from their constitutive expression, majority of defensin molecules could be induced by microbial signals and pro-inflammatory cytokines as observed in various experimental and clinical studies (Ebrahem 2013; Mallow et al. 1996; Wang 2014: Valore et al. 1998; Haynes et al. 2000). In addition, few researchers have demonstrated low level of defensin expression during embryological development. Schnapp et al. also isolated mRNA of hBD-1 in mesenchymal tissues of pancreas and kidney other than epithelial cells, thereby suggesting additional physiological function of host defense (Schnapp et al. 1998). In another extensive study on human enteric defensin, Mallow et al. observed limited and low expression of intestinal defensin (HD-5 and 6) as early as in



Defensin, Fig. 1 Structure of mRNA and linear peptides of α- and β-defensin encoded by 8p22–p23. The θ-defensins originate from α-defensins through a nonsense mutation in the mature peptide

Defensin, Table 1 Tissue-specific expression of important defensins in humans

Defensins	Expression in tissues	Type of expression
α-Defensins		
HNP-1 HNP-2 HNP-3	Neutrophils, macrophage Nature killer cells B&T lymphocytes	Constitutive expression
HNP-4	Neutrophils	
HD-5	Paneth cells, urogenital tracts	Constitutive and inducible expression
HD-6	Paneth cells	
β-Defensins		
HBD-1	Keratinocytes (gingiva, buccal mucosa, tongue) Salivary glands (ductal cells of parotid and submandibular gland) Kidney (epithelial layers of the loops of Henle, distal tubules, and the collecting ducts) Occular tissue (cornea, conjunctiva, lacrimal gland) Female genital tract (epithelial layers of the vagina, ectocervix, endocervix, uterus, and fallopian tubes)	Constitutive and inducible expression (induced by IFN-γ, IL-1β, IL-10, TNF-α and inhibited by IL-13 & 14) HBD-1 is unaffected by cytokines HBD-2 induced only by IL-1β
HBD-2	Gastrointestinal tract, respiratory tract, skin, salivary glands, oral cavity (stratum spinosum and granulosum)	
HBD-3	Gingiva, tongue, buccal mucosa, labial mucosa, floor of the mouth (stratum basale), dental follicle Skin (malphighian layer of epidermis, stratum corneum), trachea, pharynx, kidney, thymus, colon, stomach Placenta, uterus endometrium Submandibular gland, minor salivary gland (labial gland)	
HBD-4	Lungs, testis, stomach, neutrophils	

24 weeks of gestation putting preterm fetus at risk of development of necrotizing enterocolitis in the absence of potent local defense (Mallow et al. 1996).

Defensin in Physiology

Various researches have revealed extended cellular functions of defensin like chemoattraction and innate and adaptive immune-mediated response beyond its well-established antimicrobial role.

Role of Defensin in Immunity

Continuous expression of defensin ranging from lower to higher organisms indicates its development as an integral part of innate immunity. Moreover, its constitutive expression in protective barriers (skin and mucous membrane) makes defensin act within first line of defense in innate immunity. Newborns deficient in neutrophil defensin have shown to be at a larger risk of bacterial infections; activation of classical complement pathway while expression of beta defensin to inflammatory cytokines supports pivotal role of defensin in innate immunity. Defensin also contributes enormously in cross-linking of innate to adaptive immunity through its receptor, specific ligation ability to pathogen-associated molecular pattern (lipopolysaccharides, peptidoglycans, etc.), and activation of appropriate adaptive immune response against the pathogens. They contribute in a larger extent to adaptive immune response via various mechanisms like (i) facilitation of chemotaxis (chemotactic ability of α-defensin toward monocytes, dendritic cells, and T-lymphocytes; β-defensin for dendritic cells and memory T cells via chemokine cell surface receptors like CCR-6 also for myeloid progenitors via CCR2) and (ii) maturation of dendritic cells and monocytes (toll-like receptor-mediated activation of antigen-presenting cells and cytokine-mediated activation of monocytes) (Raj and Dentino 2002; Weinberg et al. 2012).

Antimicrobial and Antiviral Role

Both in vivo and in vitro studies support broad-spectrum antimicrobial activity of defensin. Some experimental observations showed increased defensin concentration in response to the presence of microorganisms/inflammation and synergistic interaction with other antimicrobial peptides like lactoferrin and cathelicidins, signifying its antimicrobial mechanisms. A selective detrimental effect on cell membrane is the principal mode of antibacterial action of defensin. Defensin being cationic peptide mainly interacts with negatively charged constituents of microbial cell membrane (lipopolysachharide in gram-negative bacteria and techoic acid in gram-positive bacteria). It mediates bactericidal activity through increased permeability of inner and outer cell membrane by formation of multimeric pores in gram-positive bacteria along with competitive displacement of divalent cations through its high affinity for LPS in gram-negative bacteria. Other possible antibacterial mechanisms are stimulation of autolytic enzymes and interference with bacterial DNA and/or protein synthesis. In case of fungus, a cascade of events are orchestrated by defensin as an antifungal agent like binding to the cell wall, increasing membrane permeability, triggering receptor-mediated internalization, and interacting with intracellular targets which causes the formation of reactive oxygen species and ultimately induces apoptosis (Silva et al. 2014). All six human alpha defensins and HBD 1 to 3 also play a major role in antiviral activity through mechanisms like direct neutralization or aggregations of virions.

Nonimmune Regulatory Role

Defensin is also involved in various other regulatory functions other than its much acclaimed immunological role. Functions like (i) modulation of tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells thus inhibiting fibrinolysis, (ii) interaction with ACTH, thus preventing ACTH-induced steroidogenesis, (iii) induction of histamine release from mast cells, (iv) regulation of proteoglycan-dependent catabolism of low-density lipoprotein (LDL) by vascular cells, and (v) proliferation of epithelial cell during wound healing broaden the range of biological role of defensin and establish these peptides as

multifunctional cell effectors in addition to their much acclaimed role in integration of innate and adaptive immunity.

Defensin in Pathology

As defensin is closely associated with immunity and inflammation, it has attracted many researchers to explore its role in inflammatory diseases of human. Etiopathogenesis of inflammatory diseases of human was found to be directly related to either its antimicrobial properties or immunomodulation ability. Beside its immune-related function, defensin has also extended its contribution in pathophysiology of various diseases and disorders.

In Gastrointestinal Disease

Because of its much explored antimicrobial role, defensin is thought to maintain a well-balanced microbiota, prevention of pathogenic bacterial growth and invasion, thereby ensuring normal health/function of intestinal tract. Altered levels of defensin were mainly observed in intestinal diseases like inflammatory bowel disease (IBD), Crohn's disease (CD), and Ulcerative colitis (UC). In IBD, a drastic decrease in α-defensin (HD-5&6) was noticed, suggesting its direct association with its etiopathogenesis. In CD, reduction of both α and β defensin concentration in association with mutation of an intracellular receptor nucleotide oligomerization domain (NOD) 2 indicates probable association of genetic mutation in its pathogenesis. NOD2 is normally expressed by intestinal epithelial cells (paneth cells) and acts as a recipient of bacterial cell component like muramyl diapeptide (MDP). A recent research work suggested functional mutation of CARD15 gene encoding NOD2, thus preventing NOD2/NF-κB-mediated transcription and translation of defensin. In UC, consistent observations like increase in mRNA concentration of inducible β-defensins (HBD2 and 3) and decrease in HBD-1 (constitutional defensin) clearly indicates about the regulatory role of defensin in response to inflammation (Ramasundara et al. 2009).

In Cardiovascular Diseases

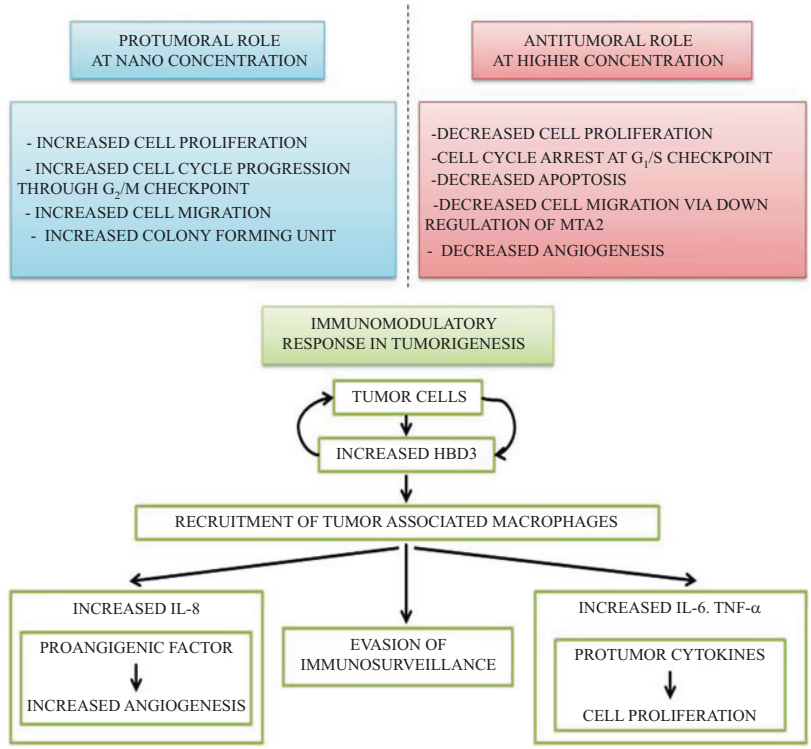
Defensin, mainly HNPs, pursue various physiological role in relation to heart such as maintaining vascular tone, mediating thrombolytic activity, and involvement in lipid metabolism. Hence, possible mechanisms for HNP-mediated cardiovascular diseases like atherosclerosis and coronary disease are: (i) promotion of accumulation of LDL by its binding to endothelial cell surface, (ii) inhibition of fibrinolytic activity, and (iii) formation of stable, multivalent complexes with LDL (Maneerat et al. 2016).

In Skin Diseases

Since its discovery, altered expression of defensin protein has been recognized in some skin diseases/lesions like psoriasis, atopic dermatitis, Kostman's syndrome, and skin injury, thus substantiating its role in determining susceptibility of patients with skin disorders to the pathogens. Conflicting observation of defensin levels in psoriasis (high) and atopic dermatitis (low) was found to be inversely proportional to their susceptibility to superadded infections. In Kostman's syndrome (severe congenital neutropenia), decreased defensin level (HNP1 to 3) suggests the importance of its proper expression for the antimicrobial function of neutrophils. In skin injury, besides protection from infections, defensin is involved in wound healing with β-defensins, i.e., hBD-2, -3, and -4, accelerating keratinocyte migration and proliferation through calcium influx and EGFR phosphorylation. Similarly in skin injury, HNP1 is said to promote extracellular matrix deposition and control its degradation by enhancing the expression of pro-α1 collagen and inhibiting the expression of matrix metalloproteinase-1 (Kenshi and Richard 2008).

In Tumorigenesis

In 1999, Abiko et al. for first time speculated about the role of human β-defensin-1 and -2 in oral carcinogenesis. Since then, many in vivo/vitro studies have publicized numerous speculations on the role of defensin in tumorigenesis. Both protumoral and antitumoral effects of defensin have been suggested, which are mainly concentration dependent through which they



Defensin,
Fig. 2 Contoversial role of defensin in tumerigenesis along with immunomodulatory expression of HBD-3. Recent reports explored the antitumoral role of beta defensin as a downregulator of metastasis-associated family protein member (MTA2)

either enhance or inhibit various cellular responses like cell proliferation, cell migration, and angiogenesis. While on one end defensin plays a vital role in innate and adaptive immunity, on other end they can modulate their protective mechanism to tumor promoting molecular/cellular responses. These observations make the whole scenario more complex which needs to be explored to capture the potentiality of this molecule, especially in the field of tumorigenesis (Mburu et al. 2011; Weinberg et al. 2012) (Fig. 2).

Summary

Defensin is well established in its roles as antimicrobial peptide, important component of innate immunity, and as an effective regulator of adaptive immunity. However, these beneficial roles need to be explored more for defensin could emerge as a novel adjuvant to amend the immunomodulatory strategies so that host responses could be enforced against the microbial challenge. Though its potential as tumor suppressor or promoter needs to be clarified, mounting evidences support defensin as a potent biomarker at least in epithelial tumors, thus necessitating an exploration in possibility of it as a candidate in targeted individualized therapy in near future.

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Deleted in Polyposis 2.5 (DP2.5)

▶ Adenomatous Polyposis Coli

Delta Glutamate Receptor (*GluD1*, *GluD2*)

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Synonyms

GluD1; GluD2; GluRδ1; GluRδ2

Historical Background

The δ1 glutamate receptor (GluRδ1 and GluD1) and the δ2 glutamate receptor (GluRδ2 and GluD2) were cloned by homology screening in 1993 at the end of the "gold rush" for cloning of ionotropic glutamate receptor (iGluR) cDNA. They were regarded as orphan receptors for a long time since their endogenous ligands were unknown. GluD1 is highly expressed in hair cells of the auditory and vestibular systems in adult mice. Indeed, deletion of a gene encoding

GluD1 (*grid1*) in mice leads to deficit in high-frequency hearing. In contrast, GluD2 is predominantly expressed in cerebellar Purkinje cells and deletion of a gene encoding GluD2 (*grid2*) results in cerebellar ataxia and characteristic phenotypes at parallel fiber (PF)–Purkinje cell synapses. Functionally, the long-term depression (LTD) of synaptic transmission, which is thought to underlie motor coordination and motor learning, is completely blunted. Morphologically, approximately 40% of dendritic spines of *grid2*-null Purkinje cells remain uninnervated by PF terminals. Although these characteristic phenotypes of *grid1*-null and *grid2*-null mice point to essential roles played by GluD1 and GluD2, mechanisms by which GluD1/2 mediate these functions remained obscure. Recent structural studies and phenotype rescue experiments have greatly advanced our understanding of mechanistic aspects of GluDs, especially GluD2. For some original references, which have been omitted in this entry for the sake of space, please refer to reviews elsewhere (Yuzaki 2008).

Genes and Expression

The size of *grid1* and *grid2* genes is much larger (~760 kb for *grid1* and ~1.4Mb for *grid2* in mice) than sizes of genes encoding other iGluRs (~200 kb). This large size and a high percentage of purine nucleotides within the locus are thought to render *grid2* susceptible to frequent spontaneous mutations in mice, called *hotfoot* (Yuzaki 2008). Human cases that carries mutations in the *GRID2* gene have not been discovered until recently (Hills et al. 2013; Utine et al. 2013; Maier et al. 2014; Van Schil et al. 2015; Coutelier et al. 2015). Their overlapping neurological phenotypes included oculomotor impairment, gross motor delay, language and intellectual deficit, and progressive cerebellar atrophy, suggesting that GluD2 also works in the cognitive processes in addition to motor control and learning.

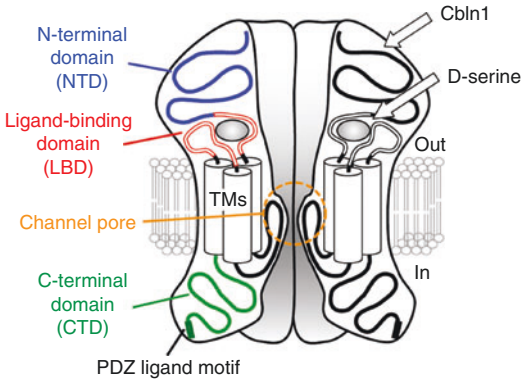
It should be noted that GluD2 expression is not confined to cerebellar Purkinje cells. It has also been reported that interneurons in the cerebellum expressed GluD2 using Purkinje cell-specific *grid2*-null mice (Yamasaki et al. 2011). Furthermore, immunohistochemical and Western blotting analyses indicated that GluD2 was widely expressed in the brain including olfactory bulb, cerebral cortex, hippocampus, thalamus, striatum, and midbrain (Yuzaki 2008, Hepp et al. 2015, Konno et al. 2014). Thus, although it is proposed that the cerebellum also contributes to cognitive and emotional function (Buckner 2013), it is still unclear which brain regions are responsible for the cognitive impairments derived from GluD2 mutations.

No spontaneous mutant mouse linked to the *grid1* locus is known so far. However, recent genome-wide association studies indicated linkage of the *GRID1* gene with bipolar disorder, major depressive disorder, and schizophrenia (Fallin et al. 2005; Guo et al. 2007; Treutlein et al. 2009). Furthermore, genome-wide copy number variation studies have implicated that *GRID1* may be associated with autism spectrum disorder (ASD; Glessner et al. 2009; Smith et al. 2009).

In addition to hair cells, GluD1 is highly expressed in molecular layer interneurons in the cerebellum (Konno et al. 2014). Furthermore, GluD1 is widely expressed at a low level in the adult mouse brain, including the cerebral cortex, striatum, hippocampus, lateral habenula, and amygdala (Konno et al. 2014). Interestingly, *grid1*-null mice showed aberrant emotional and social behaviors, other than hearing deficits (Yadav et al. 2012, Yadav et al. 2013). Although the mechanisms are still largely unknown, GluD1 is likely involved in cognitive function and its dysfunction might be associated with mental disorders including schizophrenia, mood disorders, and ASD.

Structure and Function

On the basis of sequence homology with other iGluRs, the topology of GluD1/2 in the cell membrane is predicted to be similar to that of other iGluRs, which are composed of an N-terminal domain (NTD) and a bipartite ligand-binding domain (LBD) on the extracellular side of the plasma membrane, three transmembrane (TM) domains (TM1, TM3, and TM4), an ion



Delta Glutamate Receptor (*GluD1*, *GluD2*), Fig. 1 Presumed membrane topology of GluD2. The ligand-binding domain (LBD) is separated by transmembrane (TM) domains 1 through 3. The ligand-binding pocket, to which D-serine binds, is indicated by an *arrow*. Cbln1 binds to the most N-terminal domain (NTD) outside of the LBD (indicated by an *arrow*). The channel pore is indicated by a *dotted circle*. The most C-terminal domain (CTD) of GluD2 constitutes a PDZ ligand motif, to which PDZ proteins such as PSD-93, PTPMEG, S-SCAM, n-PIST, and delphilin bind

channel-forming re-entrant loop segment (TM2 or P-loop), and a cytoplasmic C-terminal domain (CTD) (Fig. 1). GluD2 and GluD1 likely form a homomeric complex in vivo, although it is possible that a small proportion of GluD1 or GluD2 exists as a heteromer with other iGluRs, as observed in vitro (Yuzaki 2008).

Extracellular N-Terminal Domain (NTD)

The NTD of iGluRs contributes to receptor assembly and efficient surface transport of the receptor. Similarly, various small inframe deletions in the NTD of GluD2 found in many *hotfoot* mutant mice impair the homomeric oligomerization of GluD2 and its subsequent exit from the endoplasmic reticulum to reach the cell surface (Yuzaki 2008). These findings suggest that, like other iGluRs, the NTD of GluD2 is essential for receptor assembly, and that unstable oligomers may be retained in the endoplasmic reticulum by the quality control mechanism.

Virally mediated expression of wild-type GluD2 or chimeric glutamate receptor 6 (GluK2) that had the NTD of GluD2 completely rescues impaired PF synaptogenesis in *grid2*-null mice, while expression of GluD2 lacking the NTD or the chimeric GluD2 with the NTD of GluK2 does not (Table 1). Thus, the NTD of GluD2 is necessary and sufficient for synapse formation between PFs and Purkinje cells (Kakegawa et al. 2009). Interestingly, the NTD of GluD2 was shown to bind to ▶ Cbln1, which is secreted from cerebellar granule cells (Matsuda et al. 2010). ▶ Cbln1 also binds to neurexin on the presynaptic terminals (Matsuda and Yuzaki 2011). These findings indicate that the NTD of GluD2 plays a crucial role in formation and maintenance of PF synapses by forming a tripartite complex consisting of GluD2, ▶ Cbln1, and neurexin at PF-Purkinje cell synapses. Indeed, the recent structural study revealed that GluD2 NTD dimers tether Cbln1 hexamers to monomeric neurexin, indicating that neurexin-Cbln1-GluD2 trimeric complex has a stoichiometry with 2 (in monomer) : 2 (in hexamer) : 1 (in tetramer) (Fig. 2) (Elegheert et al. 2016). Since ▶ Cbln1 also bind to the NTD of GluD1 to promote synaptogenesis in cultured hippocampal neurons (Matsuda et al. 2010; Matsuda and Yuzaki 2011), the NTD of GluD1 may also play a similar role in neurons expressing GluD1.

Extracellular Ligand-Binding Domain (LBD)

An arginine (R) residue highly conserved in the LBD of iGluRs is essential for binding to amino acid ligands, including glutamate, aspartate, glutamine, glycine, lysine, serine, arginine, ornithine, and histidine. Surprisingly, the mutant GluD2 transgene ($GluD2^{R/K}$), in which the conserved arginine is replaced by lysine (K) to lose the ability of ligand binding, still rescues all the abnormal phenotypes of adult *grid2*-null mice (Table 1) (Hirai et al. 2005). Therefore, no L-glutamate analog binding is likely required for GluD2 to achieve its functions at least in adult mice.

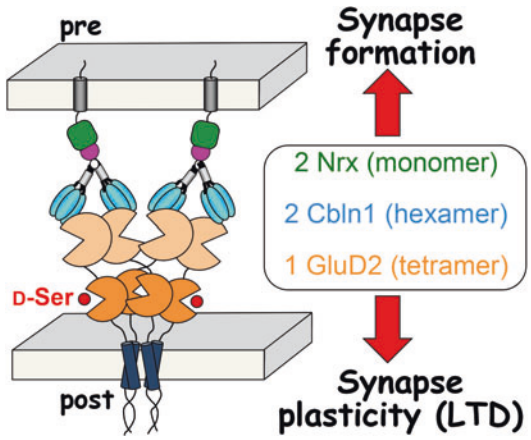
The LBD of GluD2 was crystallized and shown to bind to D-serine (Fig. 1) in a manner dependent on the arginine residue (Naur et al. 2007). Thus, D-serine fails to bind to $GluD2^{R/K}$. Interestingly, *grid2*-null mice expressing $GluD2^{R/K}$ exhibited impaired LTD and motor dyscoordination during development (Kakegawa et al. 2011). Indeed, D-serine is released from

D

Delta Glutamate Receptor (*GluD1*, *GluD2*), Table 1 Summary of *grid2*-null phenotypes rescued by various *grid2* transgenes

Mouse	Transgene	Disruptedfunction/ deleted domain	Age	Method	Ataxia	Motor learning[1]	Synapse formation	LTD
WT	None		Young/ Adult		√	√ e,r	√	√
grid2-null	None		Young/ Adult		↓	↓ e,r	↓	↓
	+GluD2^{WT}		Young/ Adult	Virus vector/TG mouse	√	√ e,r	√	√
	+GluD2$^{\Delta NT}$	N-terminal domain (NTD)	Adult	Virus vector	√*	n.e.	↓	√
	+GluD2$^{R/K}$	Ligand-binding domain (LBD)	Adult	TG mouse	√	√ r	√	√
			Young	TG mouse	√	↓ r	√	↓
	+GluD2$^{Q/R}$	Ca^{2+} permeability	Adult	TG mouse	√	n.e.	√*	√
	+GluD2$^{V/R}$	Channel function	Adult	Virus vector	√	n.e.	√	√
			Young	Virus vector	√	n.e.	n.e.	√
	+GluD2$^{\Delta CT7}$	PDZ ligand	Adult	Virus vector/ TG mouse	√	↓ e,r	√	↓

√ rescued, √* partially rescued, ↓ not rescued, *n.e.* not examined
[1]Motor learning was examined by the delayed eyeblink conditioning test (e) or the rotor-rod test (r)



Delta Glutamate Receptor (*GluD1*, *GluD2*), Fig. 2 Neurexin-Cbln1-GluD2 trimeric complex connecting pre- and postsynaptic neurons. GluD2 NTD dimers tether Cbln1 hexamers to monomeric neurexin, indicating that neurexin-Cbln1-GluD2 tripartite complex has a stoichiometry of 2 monomers: 2 hexamers: 1 tetramer. The complex dynamically regulates synapse formation and synapse plasticity in the cerebellum

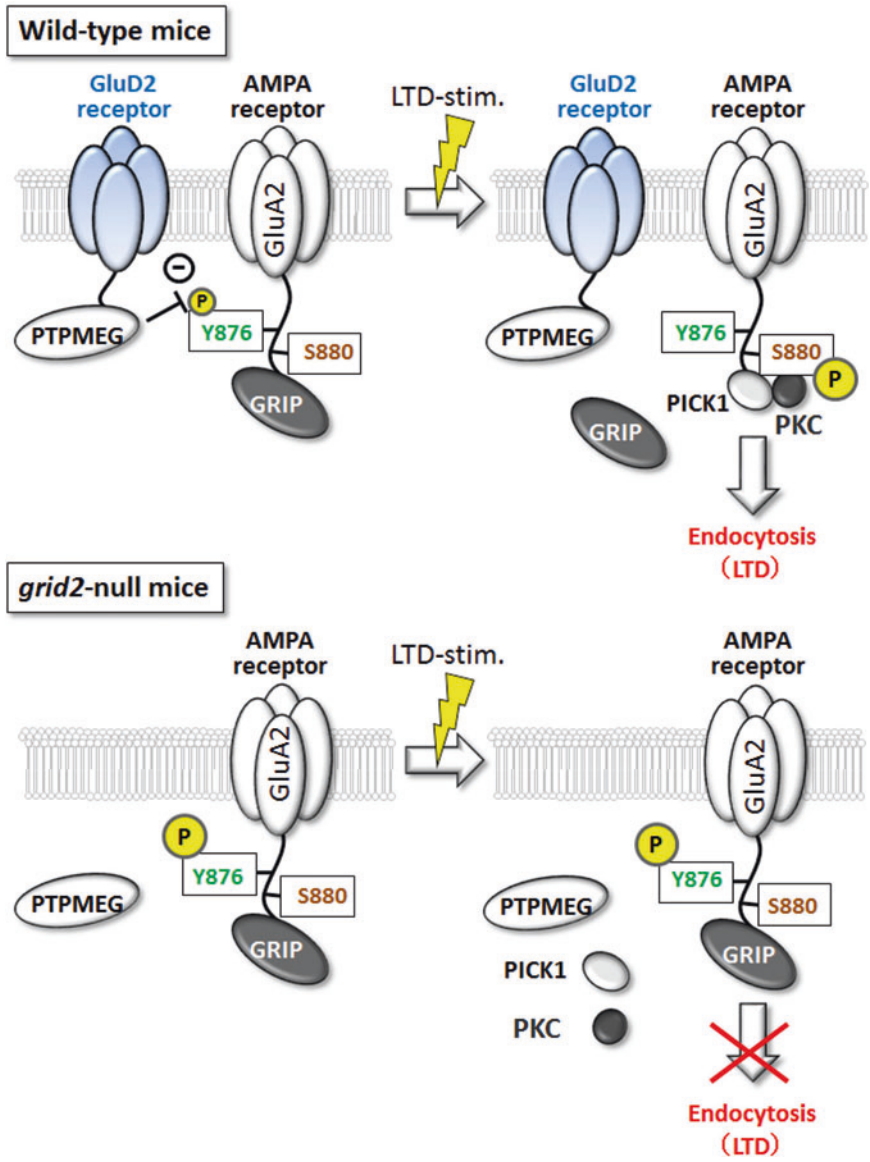
Bergmann glia after the burst stimulation of PFs in immature, but not mature, cerebellum because of developmental upregulation of D-amino acid oxidase, a D-serine-degrading enzyme. These findings indicate that D-serine serves as an endogenous ligand for GluD2 in immature cerebellum. Recently, this D-serine-GluD2-mediated LTD signaling is reportedly influenced by binding of Cbln1 to the GluD2 NTD, suggesting that potential NTD-LBD coupling is crucial for D-serine-dependent LTD (Elegheert et al. 2016). Although D-serine also binds to GluD1 (Yadav et al. 2011), its physiological significance remains to be determined.

Channel Pore-Forming Domain

A point mutation in the TM3 of GluD2 (GluD2^{Lc}) makes GluD2 channel constitutively open, causing Purkinje cell death in *lurcher* mice (Zuo et al. 1997). The Ca^{2+} permeability of GluD2^{Lc} is abolished by replacing glutamine (Q) with arginine (R) at the Q/R site (GluD2$^{Q/R}$) (Kohda et al. 2000; Kakegawa et al. 2007a). However, when a mutant GluD2$^{Q/R}$ transgene is introduced into *grid2*-null Purkinje cells, LTD and other major abnormalities of *grid2*-null mice are rescued (Table 1). These finding indicate that although cerebellar LTD depends on Ca^{2+} influx, GluD2 unlikely serves as a Ca^{2+}-permeable channel (Kakegawa et al. 2007a).

The channel activity of GluD2^{Lc} is abolished by replacing valine (V) with arginine (R) at one position upstream of the Q/R site (GluD2$^{V/R}$) (Kakegawa et al. 2007b). Surprisingly, the expression of GluD2$^{V/R}$ in *grid2*-null Purkinje cells by Sindbis virus completely restored LTD and motor coordination (Table 1), indicating that channel activity of GluD2 is not required for inducing LTD.

Together, although channel activities do not seem required to achieve major functions of



Delta Glutamate Receptor (*GluD1*, *GluD2*), Fig. 3 GluD2-CTD signaling for cerebellar LTD. (Top) In wild-type mice, GluD2 maintains low phosphorylation levels at tyrosine 876 (Y876) of the AMPA receptor's GluA2 subunit through PTPMEG, a tyrosine phosphatase binding to the most C-terminus of GluD2. An LTD-inducing stimulation (LTD-stim.) further reduces Y876 phosphorylation allowing PKC to phosphorylate serine 880 (S880) of GluA2, which is an essential step for activity-dependent endocytosis of AMPA receptors, a molecular substrate of LTD. (Bottom) In grid2-null mice, PTPMEG fails to dephosphorylate Y876 of GluA2, thereby impairing S880 phosphorylation and LTD

GluD2 at PF-Purkinje cell synapses, there still remains a possibility that GluD2 might function as an ion channel under some circumstances (Ady et al. 2014).

Cytoplasmic C-Terminal Domain (CTD)

The cytoplasmic CTD of GluD2 binds to many intracellular proteins such as Shank, PICK1, and the adaptor protein complex ▶ AP-4. In addition, the most CTD of GluD2 constitutes a PDZ ligand motif, to which PDZ proteins, such as PSD-93, PTPMEG, S-SCAM, n-PIST, and delphilin, bind (Yuzaki 2008). When the mutant GluD2 lacking the C-terminal seven amino acids ($GluD2^{\Delta CT7}$) is expressed in *grid2*-null PCs, it fails to rescue abrogated LTD and impaired delayed eyeblink conditioning, a cerebellum-dependent motor learning, in *grid2*-null mice (Table 1) (Kohda et al. 2007; Kakegawa et al. 2008). Furthermore, D-serine binding to GluD2 failed to induce LTD when the CTD is deleted (Kakegawa et al. 2011). In contrast, the mutant transgene $GluD2^{\Delta CT7}$ completely restores abnormal PF synapse formation (Kakegawa et al. 2008). These findings indicate that signaling via the CTD of GluD2 is not necessary for morphological integrity at PF synapses, but absolutely required for the induction of LTD and motor learning.

How is the CTD of GluD2 involved in the signaling for cerebellar LTD? PTPMEG, a tyrosine phosphatase, binds to the most C-terminus of GluD2 and *PTPMEG*-null mice showed impaired cerebellar LTD (Kina et al. 2007). It was found that PTPMEG directly dephosphorylated tyrosine 876 (Y876) of the AMPA receptor's GluA2 subunit in vitro and a chemical LTD stimulus mimicking the depolarization of Purkinje cells and the activation of PF inputs significantly reduced Y876 phosphorylation in wild-type mice, while it remained unchanged in *grid2*- and *PTPMEG*-null mice (Kohda et al. 2013). In addition, Y876 phosphorylation significantly attenuated subsequent phosphorylation of serine 880 of GluA2 by PKC (Kohda et al. 2013), which is an essential step for activity-dependent endocytosis of AMPA receptors, a molecular substrate of LTD (Matsuda et al. 2000). Therefore, direct interaction between GluD2 and PTPMEG likely reduces Y876 phosphorylation of GluA2, thereby enabling activity-dependent GluA2 S880 phosphorylation and LTD (Fig. 3). Although D-serine-mediated LTD also requires GluD2 CTD (Kakegawa et al. 2011), it remains unclear whether dephosphorylation of GluA2-Y876 by PTPMEG is involved in this process.

Summary

Although GluD2 was referred to an orphan receptor for a long time, it now has two unusual endogenous ligands – ▶ Cbln1 and D-serine. The extracellular NTD of GluD2 plays a crucial role in the formation and maintenance of PF synapses by forming a tripartite complex with ▶ Cbln1 and its presynaptic receptor neurexin in the cerebellum. On the other hand, the LBD of GluD2 binds to D-serine, which is released from cerebellar Bergman glia during early developmental period, to facilitate LTD and motor coordination. Although signaling via interactions of the CTD of GluD2 and PTPMEG is required for the induction of LTD and motor learning, how and whether PTPMEG activity is regulated remains unanswered. In addition, how Cbln1 binding to the NTD and D-serine binding to the LBD induce conformational changes to the whole GluD2 complex and transmit signals to the CTD and its interacting proteins remain to be determined. Finally, although ▶ Cbln1 (and Cbln2, Cbln4) and D-serine bind to GluD1, it is currently unclear whether GluD1 regulates synaptic functions similar to those exerted by GluD2 in various brain regions in vivo.

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Delta-9-Desaturase

▶ SCD (Stearoyl-CoA Desaturase)

DEP Domain Containing 2

▶ P-Rex
▶ P-Rex2

DEP.2

▶ P-Rex

Depdc2

▶ P-Rex
▶ P-Rex2

DERK (*Drosophila melanogaster*)

▶ ERK1 and ERK2

D-*Erythro*-Sphingosine-1-Phosphate

▶ Sphingosine-1-Phosphate

Desmoglein 3

▶ Desmoglein-3

Desmoglein-3

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Synonyms

130 kDa pemphigus vulgaris antigen; Cadherin family member 6; CDHF6; Desmoglein 3; DSG3; Pemphigus vulgaris antigen; PVA

Historic Background

Desmoglein-3 (Dsg3) was first identified as a pemphigus vulgaris antigen (PVA) from the human keratinocyte expression libraries in 1991 by Amagai et al. in an attempt to search for the

targets of pemphigus vulgaris autoantibodies (Amagai et al. 1991). Due to its significant homology with the cadherin family of cell adhesion molecules and most remarkably to Dsg1, Dsg3 is characterized as a member of cadherin superfamily and specifically the one in desmoglein subfamily which contains Dsg1. Pemphigus vulgaris (PV) is a potentially lethal autoimmune blistering disease that affects oral mucosa and skin with the manifestation of acantholysis characterized as the loss of adhesion between epithelial cells and structural components maintaining cell cohesion in the tissues caused by the action of autoantibodies against Dsg3 as well as Dsg1. Dsg1 is also a target antigen in another autoantibody-mediated blistering disease of the skin, pemphigus foliaceus. There are two subfamilies of desmosomal cadherins, desmoglein (Dsg) and desmocollin (Dsc), both of which are members of the cadherin superfamily of calcium-dependent adhesive proteins in desmosomes, one type of intercellular junctions between epithelial, myocardial, and other cell types that confer strong cell–cell adhesion (Thomason et al. 2010). There are four human desmoglein isoforms (Dsg1–4) and three desmocollin isoforms (Dsc1–3), which exhibit different tissue and cell type–specific expression patterns. For instance, Dsg3/Dsc3 and Dsg1/Dsc1 are more restricted to complex epithelial tissues, whereas Dsg2/Dsc2 are widely expressed in desmosomes of the basal layer of stratified epithelia, simple epithelia, and non-epithelial cells such as the cardiomyocytes and lymph node follicles. Dsg3, in particular, is found confined in the basal and immediate suprabasal layers of epidermis but uniformly distributed across the entire stratified squamous epithelia in the oral mucosa.

All the genes that encode Dsgs and Dscs are located within a tightly linked cluster in chromosome 18q12.1 (Frank et al. 2001). Although mutations in the human Dsg3 gene have not been described to date, mutations in *DSG3* underlie the balding mice (Davisson et al. 1994; Pulkkinen et al. 2002) and overt squeaky phenotype with a spectrum of severe pathology such as cyclic hair loss, obstructed airways, and severe immunodeficiency subsequent to the development of oral lesions and malnutrition (Kountikov et al. 2015). The ablation of *DSG3* gene in mice leads to fragility of the skin and oral mucous membrane, analogous to those found in PV patients, along with runting and hair loss (Koch et al. 1997, 1998). However, a recent report has revealed expression of a 31-kDa truncated protein of Dsg3 containing 282 amino acids, which corresponds to the N-terminal truncated intracellular domain of Dsg3, in differentiating keratinocytes of human epidermis (Lee et al. 2009b). This truncated Dsg3 was also found to be upregulated in psoriatic epidermis and skin tumors, including Bowen's disease and squamous cell carcinoma (SCC). In addition, recently, upregulation of the Dsg3 gene and protein (wild type) has also been reported in cancer, especially in SCC with increased levels of expression correlating with the clinical stage of malignancy, implicating its pro-cancerous role and potentiality to serve as a diagnostic and prognostic marker (Brown and Wan 2015).

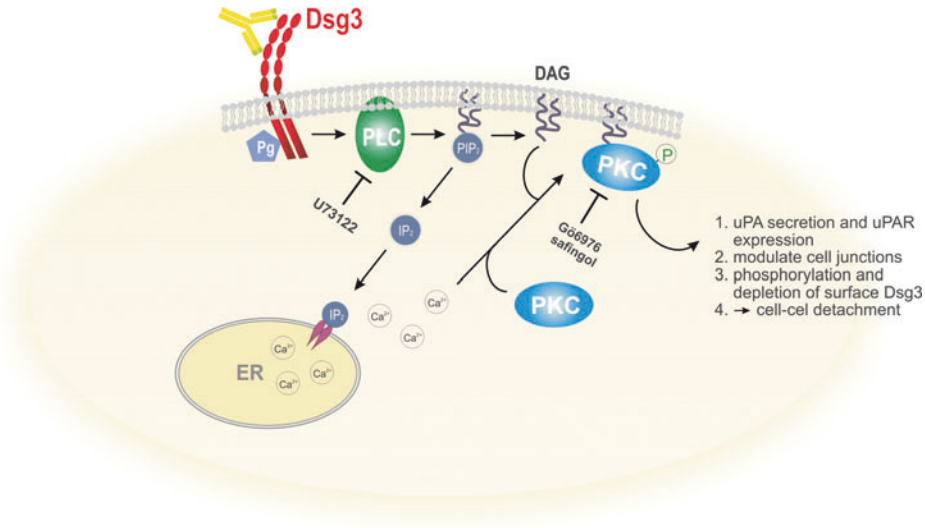
The mature form of Dsg3 (50–999 amino acids) is cleaved from a precursor protein and contains the unglycosylated peptide of 950 amino acids. The first 23 amino acids are the signal peptide. Like other cadherins, Dsg3 is a single-pass transmembrane glycoprotein consisting of the extracellular domain with five motifs of about equal size, EC1 through EC5, which, except for EC5, have homology with each other, in particular among EC1 to EC3, the most amino-terminal domains. All five extracellular motifs that contain 566 amino acids (50–615 amino acids), also show significant homology with the corresponding region in classical cadherin, such as P-cadherin (Amagai et al. 1991). Both Dsg1 and Dsg3 have a RAL site (128–130 amino acids) in EC1 that corresponds to the conserved cell adhesion recognition sequence HAV in an equivalent position in classical cadherins that is responsible for homophilic binding. Other conserved sequences in the extracellular domain are several putative Ca^{2+}-binding sites with all cadherins. The transmembrane region of Dsg3 encompasses 25 amino acids (616–640 amino acids) and the cytoplasmic domain contains 359 amino acids (641–999 amino acids) which is substantially

longer than that of typical cadherins (approximately 150 residues) but shorter than that of Dsg1 (480 residues). The cytoplasmic tail consists of several domains, such as the intracellular anchoring domain (IA), intracellular cadherin-specific domain (ICS), proline-rich linker domain (IPL), repeating unit domain (RUD), and lastly the desmoglein-specific terminal domain (DTD). The proteins that have been identified to directly bind to the cytoplasmic domain of Dsg3 includes p120 that binds to IA, plakoglobin (Pg) and caveolin-1 (Cav-1) that bind to ICS, and actin with the specific domain not yet defined (Andl and Stanley 2001; Brown et al. 2014; Wan et al. 2016; Kanno et al. 2008). The evidence for the interaction with actin proteins came from the mass spectrometry analysis of the Halo immunoprecipitates from A431 cells transfected with plasmid containing the entire Dsg3 cytoplasmic tail tagged with Halo at the N-terminus (Brown et al. 2014). Dsg3 has also been found to form complexes with many other proteins, in particular the signal molecules that are described below. Although Dsg3 belongs to the desmoglein subfamily, it has been found to be uniformly distributed on keratinocyte cell surface of stratified squamous epithelia, suggesting that Dsg3 does not solely function in desmosome adhesion but rather plays an extrajunctional role associating with other cellular processes than cell–cell adhesion.

The evidence that suggests Dsg3 acting as a signaling molecule comes from plethora studies in pemphigus research over the past two decades that demonstrates that PV-IgG binding to Dsg3 on keratinocyte surface triggers a cascade of intracellular events, such as Dsg3 phosphorylation, activation of phospholipase C (PLC) signal pathway including production of inositol 1,4,5-trisphosphate (IP3) and induction of a Ca^{2+}/PKC, signaling through p38MAPK/heat shock protein 27 (HSP27), Src, epidermal growth factor receptor (EGFR), apoptosis, c-Myc, adaptor protein plakoglobin/plakophinlin-3, as well as Rho GTPases and rearrangement of actin cytoskeleton, etc. All these observations have implicated a key role of Dsg3 in mediating outside-in signaling that leads to desmosome remodeling, cell proliferation and differentiation, or apoptosis. Deregulation of these signal pathways causes depletion of Dsg3 from the desmosomes and keratinocyte surface, resulting in cell dissociation and blister formation as observed in pemphigus disease. For convenience, the discussion below regarding the PV-IgG induced Dsg3 signaling is referred to as "PV-IgG signaling" in this review, unless otherwise specified.

Phospholipase C (PLC) and Protein Kinase C (PKC) Signaling

In 1995, Kitagima and his colleagues had made the first discovery that pemphigus IgGs, but not IgGs from bullous pemphigoid or normal sera, caused an induction of rapid and transient increase of Ca^{2+} and inositol triphosphate (IP3) in cultured human keratinocytes, and this induction was correlated with secretion of plasminogen activator (PA) and disruption of cell–cell adhesions (Seishima et al. 1995). It was later demonstrated by the same group that PLC is actually involved in these events since preincubation of keratinocytes with a specific PLC inhibitor U73122 dramatically reduced the pemphigus IgG-induced increase in Ca^{2+} and IP3 as well as the PA activity and cell–cell detachment (Esaki et al. 1995). PLC mediates catalytic hydrolysis of phosphatidylinositol bisphosphate, generating IP3 and diacylglycerol (DAG), with the former rendering an increase of intracellular Ca^{2+} and the latter activating protein kinase C (PKC), a family of Ca^{2+} and phospholipid-dependent serine/threonine kinases (Fig. 1). Indeed, in a time-course study on the involvement of PKCs in PV-IgG induced cell–cell detachment, the distribution of PKC isozymes, including conventional isoform PKC-alpha, novel PKC-delta and PKC-eta, and atypical PKC-zeta which are known to be expressed in human keratinocytes, was shown to be modulated in a time-dependent manner and all the PKC isozymes analyzed were translocated from the cytosol to the cytoskeletal fractions within 30 min after PV-IgG stimulation



Desmoglein-3, Fig. 1 PLC and PKC signaling. Autoantibodies targeting Dsg3 on keratinocyte surface activate PLC and its downstream PKC (phosphorylation), leading to uPA secretion, Dsg3 phosphorylation, and ultimately cell–cell detachment

(Osada et al. 1997). Both PKC-alpha and PKC-delta were immediately translocated to the cytoskeletal-associated fractions within seconds, with a peak at 1 min and 5 min, respectively, and this increase gradually declined after 30 min. PKC-eta's translocation, however, was induced slowly, taking more than 5 min, and was decreased to approximately half-maximum at 30 min. The PKC-zeta's translocation reached a maximum at 30 s rapidly and returned to baseline after 5 min followed by PV-IgG stimulation. It was also shown that the activity of total PKCs in cytoskeletal fraction was increased too after PV-IgG exposure, with a peak at 1 min, and was sustained for at least 30 min. It was thus suggested that enhanced PKC activity in PV-IgG-treated keratinocytes may play a role in modulating desmosome turnover and dysfunction of hemidesmosomes through the mechanisms of serine phosphorylation of junctional proteins as well as secretion of urokinase-type PA and expression of its receptor uPAR (Kitajima et al. 1999). Furthermore, serine phosphorylation of Dsg3 was detected in cells treated with PV-IgG and this was accompanied by its dissociation from plakoglobin (Aoyama et al. 1999). Importantly, inhibition of PKC by either Gö6976 or safingo blocked PV-IgG-mediated depletion of Dsg3 in cultured human epidermis and blister formation in a neonatal mouse (Spindler et al. 2011). Together, these findings suggest that PV-IgG binding to Dsg3 stimulates PLC-signaling pathway and its downstream events, such as calcium release, PKC activation, uPA secretion, and its receptor expression, that collectively lead to the depletion of Dsg3 from the cell surface and the desmosomes followed by the disruption of cell cohesion and blister formation in keratinocytes both in vitro and in vivo (Fig. 1).

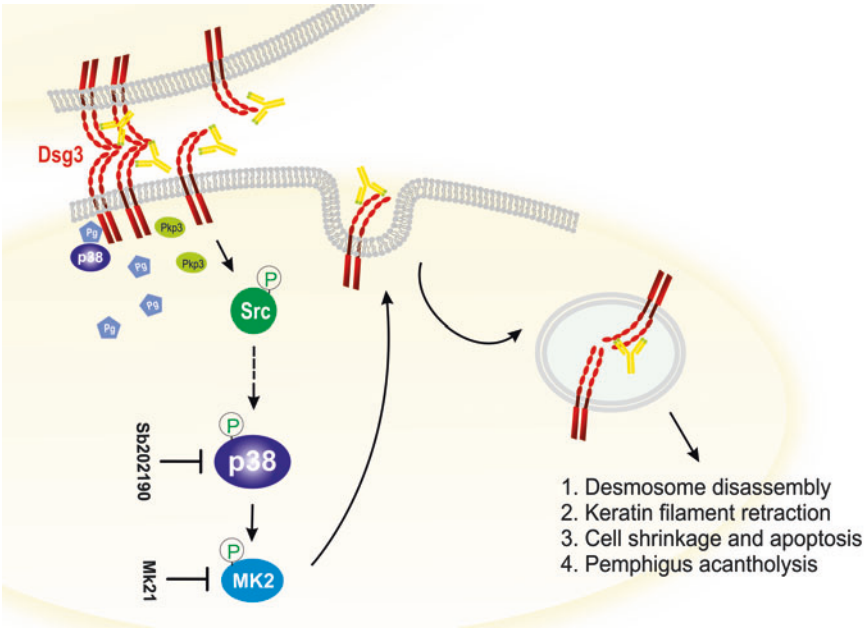
p38 MAPK Signaling

Strong evidence suggests that Dsg3 acts as an upstream regulator of p38 mitogen-activated protein kinase (MAPK), and this signaling pathway plays a crucial role in PV-IgG signaling that causes onset of pemphigus disease (Vielmuth et al. 2015; Kawasaki et al. 2006; Berkowitz et al. 2005, Berkowitz et al. 2006; Kitajima. 2014; Mao et al. 2011). This finding again originates from a plethora of pemphigus research that

show autoantibodies from patients with PV and pemphigus foliaceus (PF) target Dsg3/Dsg1 and trigger the activation of p38 MAPK and its downstream HSP27 (Berkowitz et al. 2005, 2006, 2008a, b). When human keratinocytes were treated with pathogenic anti-Dsg3 autoantibodies, p38 MAPK and HSP27 were rapidly phosphorylated, in response to PV-IgG that can be abrogated by the pharmacological inhibition of p38 MAPK activity, and this finding was demonstrated in both in vitro and in vivo experiments. It is worth noting that this was further evaluated by an independent study using the characterized potent pathogenic monoclonal antibody against Dsg3 (AK23 mAb), and the treatment of human keratinocytes DJM-1 cells with AK23 caused augmented levels of p38 MAPK activity and concomitantly increased serine phosphorylation of Dsg3 (Kawasaki et al. 2006). The time-course study is critical by providing the insight of activation cascades stimulated by PV-IgG, and it was revealed that the activation of Src peaked at 30 min, EGF receptor kinase at 60 min, and p38 MAPK at 240 min after exposure of PV-IgG in keratinocytes (Chernyavsky et al. 2007). Inhibition of Src by its specific inhibitor PP1 showed only partial abrogation of the activity of EGFR kinase and p38 by approximately 45 and 30%, respectively, suggesting that Src is part of signaling events induced by PV-IgG signaling. In parallel to the p38 MAPK activation at approximately same time, cell surface Dsg3 was found to be internalized and translocated into endosomes followed by degradation in lysosome. Such a depletion of Dsg3 can be sufficiently blocked by p38 MAPK specific inhibitor SB202190 (Jolly et al. 2010; Vielmuth et al. 2015), suggesting that p38 activation is directly responsible for the endocytosis and degradation of Dsg3 followed by PV-IgG exposure. In line with this finding, another study provided the evidence that p38 MAPK is activated downstream to the loss of cell–cell adhesion (the secondary event) as both knockdown of p38 in cultured cells and p38-alpha ablation in animal model showed the surface Dsg3 retained in acantholysis cells treated with PV-IgG albeit there was blister formation (Mao et al. 2011). Correspondingly, exogenous expression of p38-accelerated Dsg3 internalization and degradation alongside other desmosomal proteins, desmocollin 3 and desmoplakin, indicating that p38 activation is responsible for desmosome disassembly and blister formation. Interestingly, another study indicates that such p38 MAPK-mediated endocytosis of surface Dsg3 is caused only by pathogenic polyclonal PV-IgG but not by pathogenic monoclonal antibodies that are capable of inducing compromised cell adhesion strength but unable to cause Dsg3 clustering and endocytosis (Saito et al. 2012). Furthermore, it has been identified that (mitogen-activated protein kinase-activated protein) kinase 2 (MK2) acts as a key downstream effecter of p38 signaling in PV (Mao et al. 2014). Altogether, these findings underscore that polyclonal antibodies targeting Dsg3 trigger a cascade of intracellular signaling involving p38 MAPK activation that plays a central part in the PV-IgG induced Dsg3 depletion, desmosome dissolution, and pemphigus acantholysis, as illustrated in Fig. 2. The consequence of such signaling events is the keratinocyte cell shrinkage and apoptosis leading to accelerated acantholysis and blistering presented in pemphigus disease (Lee et al. 2009a) (discussed below).

EGF Receptor and Apoptosis Pathways

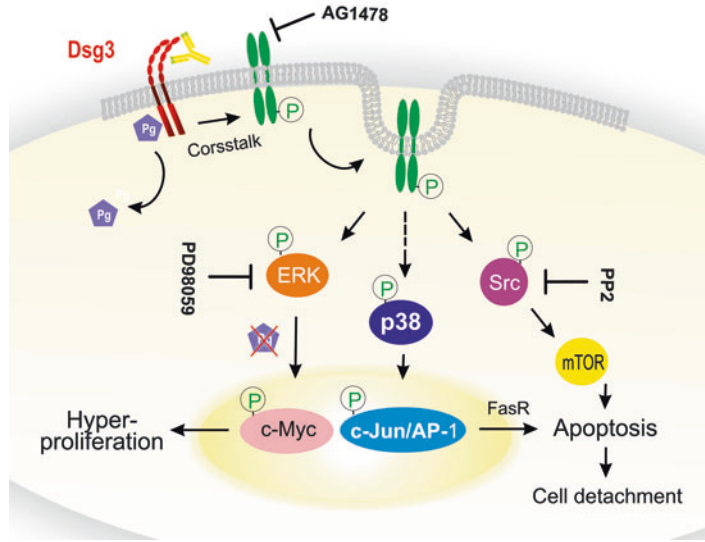
It has been shown that p38 MAPK activation not only stimulates but also enhances cell apoptosis in PV-IgG-treated keratinocytes, in PV patients, and also in passive transfer model, suggesting that apoptosis could be a major causal factor of the acantholytic phenomenon (Lee et al. 2009a; Pelacho et al. 2004; Wang et al. 2004; Grando et al. 2009). The observations of apoptosis in PV includes (1) secretion of soluble FasL; (2) elevated cellular amounts of FasR, FasL, Bax, and p53 proteins; (3) reduction in the levels of cellular Bcl-2; (4) enrichment in caspase 8 and activation of caspases 1 and 3; and (5) coaggregation of FasL and FasR with caspase 8 in death-inducing signaling complex (Wang et al. 2004).



Desmoglein-3, Fig. 2 PV-IgG targeting Dsg3 on keratinocytes induces Src phosphorylation and activation of p38 pathway that in turn causes desmosome disassembly and cell apoptosis that lead to blistering and pemphigus acantholysis in skin and oral mucosa. Dissociation of Pg from Dsg3 is also a concurrent event in PV-IgG signaling

Furthermore, in skin organ cultures and in in vitro cultured keratinocytes, PV-IgG is shown to be able to induce the caspase activation and DNA fragmentation, and the caspase inhibitors can prevent the formation of PV-IgG-induced epidermal lesions. In support, another independent report showed caspase-3 activation, Bcl-2 depletion, and Bax expression, and the p38 MAPK inhibitors were able to block the activation of caspase-3, the proapoptotic proteinase, suggesting that initiation of apoptosis is downstream to, and a consequence of, p38MAPK activation (Lee et al. 2009a). Hence, the Fas-mediated cell death seems to be involved in PV-IgG signaling and ultimately the pemphigus acantholysis.

Later studies suggest that the activation of EGFR, followed by its internalization, is instrumental in PV-IgG induced p38 MAPK signaling and apoptotic pathway (Bektas et al. 2013; Frusic-Zlotkin et al. 2006; Grando et al. 2009) (Fig. 3). Keratinocytes stimulated with PV-IgG exhibited activation and autophosphorylation of EGFR and its downstream signaling via ERK/c-Jun. Inhibition with the specific tyrosine kinase inhibitor AG1478 abrogated the EGFR autophosphorylation, cell death, FasL appearance, and acantholysis (Frusic-Zlotkin et al. 2006). Concomitantly, EGFR inhibition also prevented PV-IgG-induced Dsg3 internalization and keratin intermediate filament retraction (Bektas et al. 2013). Importantly, this finding has been verified in both neonatal and adult mouse models by independent groups. In the passive transfer neonatal model of pemphigus, EGFR inhibition showed prevention of PV-IgG-induced blister formation and skin lesion (Bektas et al. 2013). In an adult mouse model with passive transfer of pathogenic AK23 that specifically targets Dsg3, Müller and colleagues demonstrated that 2 h after AK23 transfer, detachment of desmosomes and activation of EGFR occurred, and this was followed by several events such as the increased c-Myc expression, epidermal hyper-proliferation, Dsg3 depletion, and blister formation in oral mucous membrane and hair follicles (Schulze et al.



Desmoglein-3,
Fig. 3 Dsg3 cross talks with EGFR. PV-IgG binding to Dsg3 triggers the EGFR signaling in keratinocytes that involves p38, Src, and ERK pathways and ultimately elicit activation of the transcription factors c-Myc and c-Jun/AP-1, leading to enhanced cell proliferation, apoptosis, and cell–cell detachment

2012). Furthermore, an imbalance in Akt/mTOR (with almost lack of activated Akt and high levels of activated mTOR) was detected in basal epidermal keratinocytes in passive transfer model, suggesting the imbalance in Akt/mTOR could be involved in the development of apoptosis and acantholysis downstream of EGFR (Pretel et al. 2009). In line with these findings, another report indicated enhanced FAK phosphorylation on Tyr397/925 in the basal layer of epidermis in passive transfer model and inhibition of FAK reduced the expression of phosphorylated Src and mTOR in the epidermis (Gil et al. 2012). When mouse was pretreated with the specific FAK inhibitor, the acantholysis was disappeared. What is more, when inhibitors for HER isoforms, Src, mTOR, and pan-caspases were employed together before PV-IgG administration, the phosphorylated FAK (Y397/925) was decreased, suggesting that likely there is a positive feedback loop in PV-IgG-induced signaling. Besides, pretreatment with the FAK inhibitor also showed to prevent alterations in the Bax and Bcl-2 expression and caspase-9/caspase-3 activities induced by PV-IgG.

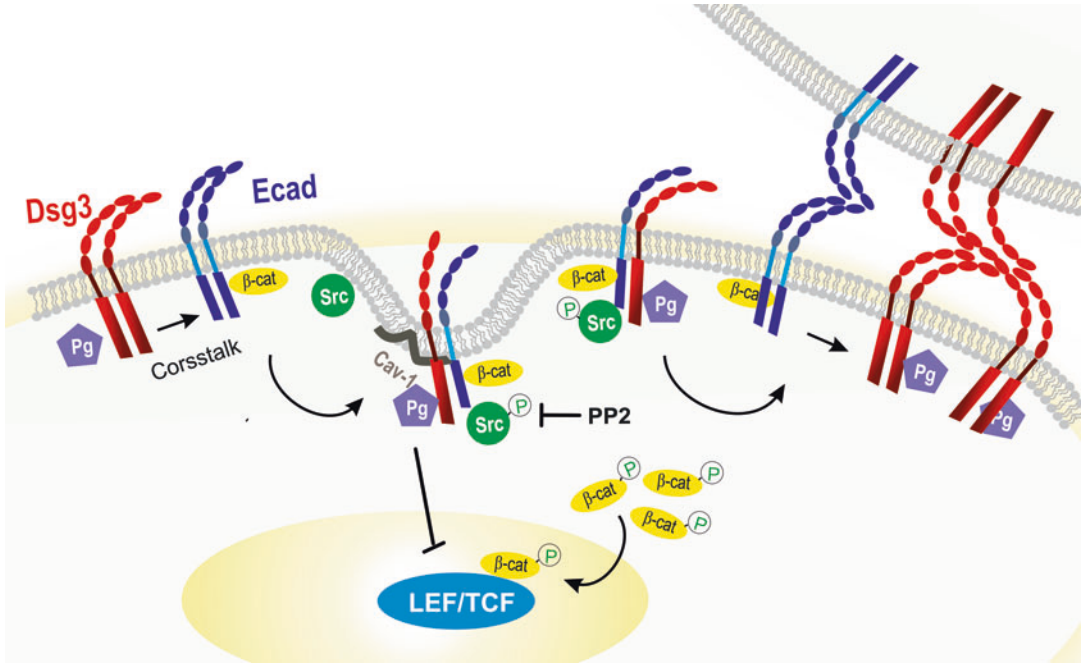
Plakoglobin (Pg), a binding partner of Dsg3 and with dual functions in both desmosomes and nucleus, plays a role in suppression of transcription factor c-Myc and is described as the principle effecter of PV-IgG-mediated signals downstream of c-Myc (Williamson et al. 2006; Williamson et al. 2007; de Bruin et al. 2007). When keratinocytes treated by PV-IgG, the non-junctional pool of Dsg3 along with Pg undergoes a process of enhanced protein turnover leading to depletion of nuclear Pg and attenuation of its suppression of c-Myc (Fig. 3). As a consequence, a marked increase in c-Myc accumulation and proliferating Ki67-positive cells was presented in PV of both human and animal patients. In line with this finding, Pg-knockout mouse exhibited high levels of c-Myc in epidermal keratinocytes, further confirming a suppressive role of Pg in c-Myc activity. What is more, another desmosomal adaptor protein plakophilins 3 (Pkp3) is implicated as an effecter of Src signaling, and upon PV-IgG binding to keratinocytes and activation of Src, tyrosine phosphorylation of Pkp3 was induced leading to the detachment of Pkp3 from Dsg3 and cytoplasmic accumulation accompanied with cell dissociation (Cirillo et al. 2014). Taken together, all these data support the notion that Dsg3 cross talks with EGFR and regulates its signaling pathway, including the downstream effecters Src, ERK, p38 MAPK, and c-Jun as well as the desmosomal adaptor proteins Pg and

Pkp3 that collectively result in gross effects on cell proliferation, apoptosis, as well as cell–cell detachment.

Although strong evidence suggests activation of EGFR is necessary for PV-IgG-induced apoptosis and cell–cell detachment, there is also reports, however, arguing against this theory with the findings implying that PV-IgG-mediated signaling does not require EGFR and its internalization, especially the phosphorylation at Tyrosine (Tyr)-1173 (the canonical activation of EGFR) and the c-Src-dependent site Tyr-845, nor apoptosis as absence of apoptosis was observed in the early lesions of PV acantholysis in vivo and also in the keratinocyte cultures treated with PV-IgG in vitro (Heupel et al. 2009; Schmidt and Waschke 2009; Schmidt et al. 2009). Even if both PV-IgG and EGF elicit cell discohesion and cytokeratin retraction, only the effects of EGF can be blocked by inhibition of EGFR and c-Src. In support, laser tweezer experiments showed that impaired bead binding of Dsg3 and Dsg1 in response to PV-IgG was not affected by inhibition of either EGFR or c-Src. These findings implicate that neither EGFR nor Src attributes to the loss of Dsg-mediated adhesion, cytokeratin retraction, and keratinocyte dissociation and suggest that apoptosis detected in PV may occur secondary to acantholysis event.

Signaling to E-Cadherin/Src and the Wnt/Beta-Catenin Pathways

Src family of nonreceptor tyrosine kinases plays a positive role in control of junction assembly and cell adhesion through a mechanism of regulating tyrosine phosphorylation of adherens junction and desmosomal proteins, including E-cadherin and Dsg3, in differentiating keratinocytes (Calautti et al. 1998; Rotzer et al. 2015; Tsang et al. 2010; Tsang et al. 2012b). Accompanied with protein phosphorylation, the formation of E-cadherin complex and its mediated cell cohesion is enhanced. Inhibition of tyrosine kinase not only prevents increased association of catenins with E-cadherin but also has a negative impact on the cell adhesive structures resulting in a significant reduction in the adhesive strength of keratinocytes. As described above, activation of Src is found in keratinocytes treated with PV-IgG both in vitro and in vivo, and inhibition of tyrosine kinases has proved to be successful in preventing Dsg3 internalization and PV-IgG-induced disruption of desmosomes and epidermal acantholysis (Delva et al. 2008; Tsang et al. 2012b). Besides, recent studies have revealed that Dsg3 cross talks with E-cadherin and acts as an upstream regulator of E-cadherin/Src signaling (Tsang et al. 2010; Tsang et al. 2012b) (Fig. 4). A complex formation containing Dsg3, E-cadherin, and Src has been demonstrated in a calcium-dependent manner, and overexpression of Dsg3 in epithelial cells elicits an increase of Src and its activity within the E-cadherin complex and knockdown of Dsg3 resulted in an inverse effect on the Src expression levels in the complex. As a consequence, Dsg3 depletion affects not only the E-cadherin junction assembly but also the expression of desmosomal proteins and proper formation of the desmosome junctions, resulting in compromised cell–cell adhesion as observed in pemphigus disease. It is worth noting that overexpression of Dsg3 in cancer cell lines does not necessarily enhance cell cohesion but rather caused accelerated cell migration and invasion due to aberrant activation or hijack of several signaling pathways including Src and its downstream effecters such as tyrosine phosphorylation of adherens junction proteins, leading to lysosomal degradation of proteins including E-cadherin (Tsang et al. 2010). In line with this finding, recently, another independent study has also demonstrated the existence of such a protein complex containing Dsg3, E-cadherin, and Src in keratinocytes and indicates that Src is required for the stability of the complex as well as the association of Dsg3 with cytoskeleton that is required for desmosome assembly (Rotzer et al. 2015). Inhibition of Src by PP1, a potent Src inhibitor, caused a reduction of tyrosine phosphorylation of Dsg3 and E-cadherin and their association with Src in the cytoskeletal fraction. Furthermore, it has been proposed that the Dsg3-mediated Src activation likely involves Cav-1, a scaffolding protein in a special type of lipid raft known as caveolae (Fig. 4), and the



Desmoglein-3, Fig. 4 Dsg3 forms a complex with and regulates E-cadherin/Src signaling. A nonjunctional pool of Dsg3 that forms a complex with E-cadherin is also found to bind to caveolin-1 (Cav-1). Such an interaction may enable Dsg3 to compete with inactive Src for binding to Cav-1 and thus causes release of Src from its interaction with Cav-1 followed by its autoactivation (Wan et al. 2016). The complex of Dsg3, E-cadherin, and Src, as well as the Src-mediated phosphorylation of cadherins, seems to be required for E-cadherin and desmosome junction formation (Rotzer et al. 2015). Binding and recruitment of Pg by Dsg3 is also necessary for enhancing β-catenin-LEF/TCF interaction and activation (Chen et al. 2013)

overexpression of Dsg3 leads to its competition with the inactive form of Src for binding to Cav-1, thus causing the release of Src followed by its autoactivation (Wan et al. 2016). Cav-1 is known to negatively regulate the Src activity through an inhibitory interaction which prevents its autophosphorylation (Li et al. 1996; Okamoto et al. 1998). Caveolae is thought to modulate signal transduction through the compartmentalization of specific signaling molecules and regulation of their activity (Lisanti et al. 1994). Emerging evidence suggests that both Src and Cav-1 associate with Dsg3 and a modulation of Dsg3 levels (overexpression or knockdown) causes an inverse effect on the amount of Src molecules bound to Cav-1 (Wan et al. 2016). In support, a potential binding site for the scaffolding domain of Cav-1 is identified within the C-terminus of human Dsg3 at amino acids 788–798 that contain four aromatic amino acid residues (Wan et al. 2016), and this region shares some common feature with the characterized amino acid sequence that binds to the scaffolding domain of Cav-1 (Couet et al. 1997). It is worth noting that this region is highly conserved within the desmoglein subfamily members (Dsg1–4) as well as across most of 18 species (Wan et al. 2016).

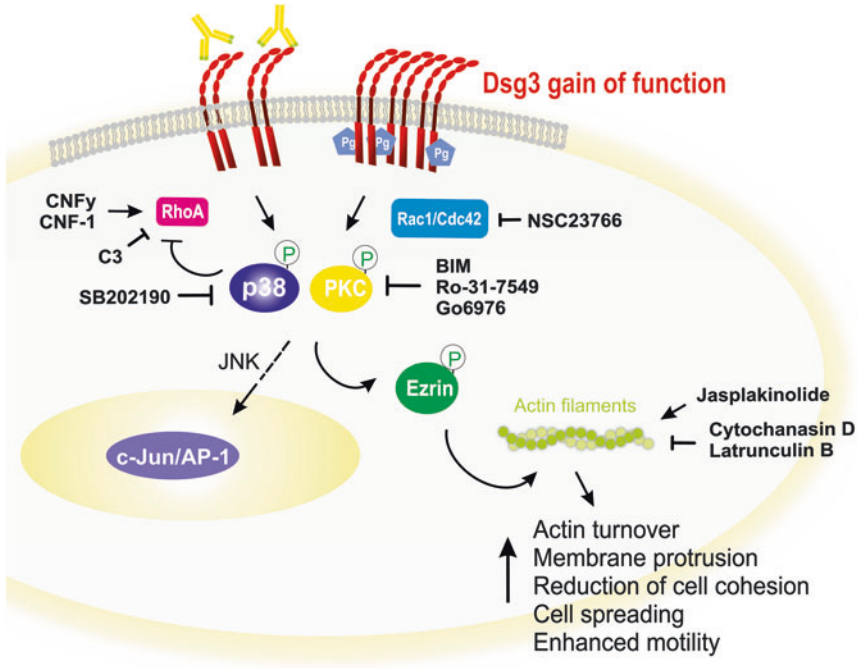
Dsg3 is also implicated to play a part in influencing Wnt/beta-catenin signal pathway (Fig. 4). In addition to a cell adhesive role in adherens junctions, beta-catenin is known to act as a transcriptional coregulator in Wnt-signal pathway (Novak and Dedhar 1999; Kikuchi et al. 2006). The conserved Wnt/beta-catenin signaling plays an important role in regulating development, cell proliferation, migration, and cell-fate decision. Activation of this signal pathway stabilizes beta-catenin in the cytoplasm, leading its

translocation to the nucleus via Rac1 and other factors, where it binds to LEF/TCF transcription factors and increases the expression of Wnt target genes, such as Myc, cyclin D1, TCF-1, PPAR-δ, MMP-7, Axin-2, and CD44, etc., in order to facilitate cell migration. In the absence of Wnt-signal, beta-catenin is targeted to ubiquitination and proteasomal degradation. In vitro study showed that overexpression of Dsg3 in A431 cancer cell line activates beta-catenin and its transcriptional activity to some degree (unpublished data). In line with this, a recent report suggested that Dsg3 regulates Wnt/β-catenin signaling indirectly, in a Pg-dependent manor, by a mechanism of sequestering Pg and preventing its nuclear translocation and suppression of LEF/TCF transcriptional activity (Chen et al. 2013). Dsg3 deletion caused an inverse effect with increased nucleus translocation where Pg interacts with and inhibits TCF/LEF transcription activity, resulting in suppression of tumor growth and invasion. Correspondingly, the increased levels of Dsg3 were shown to be correlated with reduced nuclear Pg accompanied with the elevated expression of the LEF/TCF transcriptional targets, cyclin D1, c-Myc, and MMP7, in both the head and neck cancer tissues and the cultured oral cancer cell lines (Chen et al. 2013). Thus, upregulation of Dsg3 in squamous cancer could potentially tip the balance in favor of the β-catenin-LEF/TCF interaction and activation via the suppression of the Pg nuclear translocation.

Dsg3 Regulates Rho GTPase, Ezrin, c-Jun/AP-1, and Organization of Actin Cytoskeleton

Since pemphigus is a cell-adhesion disease, it is not surprising that the activity of Rho GTPase and actin cytoskeleton are implicated in PV-IgG signaling and acantholysis. Waschke et al. first reported that the interference of RhoA signaling occurs in both PV- and PF-IgG induce skin blistering as the treatment with bacterial toxins for Rho GTPases (*Escherichia coli* cytotoxic necrotizing factor 1 (CNF-1) that activates RhoA, Rac1, and Cdc42, and CNFy from *Yersinia pseudotuberculosis* that selectively activates RhoA) showed to prevent epidermal splitting after 24 h of incubation along with pemphigus-IgG (Waschke et al. 2006). In vitro study based on HaCaT keratinocytes showed a reduction of RhoA activity accompanied with pemphigus-IgG-induced disruption of cell adhesion as well as in the loss of Dsg1-mediated binding probed by laser tweezers, the events found to be dependent on p38 MAPK (Figs. 2, 3, 5). In support, profound alterations of the F-actin distribution, along with the depletion of Dsg3 from the cytoskeleton pool in PV-IgG treated cells, were abolished by treating the cells with CNFy, suggesting that the activity of RhoA is required for desmosomal adhesion, stability, and homeostasis. In line with this study, the same group has demonstrated that PV-IgG has an influence on actin dynamics (Gliem et al. 2010). Using various pharmacological agents, they showed that manipulation of actin polymerization is able to modulate the pathogenic effects of PV-IgG. For instance, jasplakinolide that stabilizes actin filaments significantly blocked cell discohesion, whereas cytochalasin D that disrupts the polymerization of actin filaments caused augmented pathogenic effects of PV-IgG. It was also shown that the CNF-1-mediated activation of Rho-GTPases enhanced the cortical actin belt and blunted PV-IgG-induced cell dissociation. However, when actin polymerization was blocked under these conditions via addition of latrunculin B, the protective effects of CNF-1 were abolished. Taken together, all these findings underscore that reorganization of cortical actin is involved in PV-IgG-induced keratinocyte dissociation and epidermal acantholysis.

In vitro study using gain-of-function approach showed that overexpression of full-length human Dsg3 in epithelial cells causes remarkable induction of Rac1/Cdc42 and to a lesser extent, RhoA (Tsang et al. 2012a). Such an activation of Rho GTPases is accompanied with significant increase of actin turnover and pronounced membrane protrusions and dynamics in various epithelial cell lines, including MDCK and A431 epidermoid cells (Tsang et al. 2012a). As a consequence, enhanced cell migration and invasion is consistently observed in these cells when compared to



Desmoglein-3, Fig. 5 The PV-IgG targeted activation of p38 MAPK inhibits the activity of RhoA. On the other hand, overexpression of Dsg3 (gain-of-function) activates Rac1/Cdc42 GTPases, leading to the activation of PKC/Ezrin pathway and also c-Jun/AP-1 in PKC-dependent manner, that in turn regulates actin organization and dynamics with a gross effect on cell adhesion, morphology, and motility.

the respective control cells with relatively low levels of endogenous Dsg3 expression. Knockdown of Dsg3 results in perturbation in junction formation and accompanied cell polarization that require cortical actin assembly. Using various techniques, such as immunofluorescence, biochemical methods, as well as mass spectrometry, it was shown that Dsg3 not only colocalizes with but also physically interacts with actin proteins although the actual nature of their association remains undefined (Tsang et al. 2012a; Brown et al. 2014). In line with this finding, it is not surprising that Dsg3 is found to interact with an actin binding protein Ezrin, a member of the ERM family. Partial colocalization between Dsg3 and Ezrin is displayed at the plasma membrane, especially in the membrane projections of A431 cells. Furthermore, this interaction is proved by coimmunoprecipitation analysis with the rabbit anti-Ezrin antibody in Dsg3-dosage-dependent manner (Brown et al. 2014). In addition, Dsg3 is found to be capable of regulating the Ezrin activation by enhancing phosphorylation at Thre567 residual which is known to attribute to the accelerated motility of cancer cells. The functional importance of such an interaction was further validated by colocalization analysis of Ezrin/F-actin or CD44/F-actin at the plasma membrane that suggests that Dsg3 is required for the proper function of Ezrin at the plasma membrane since Dsg3 knockdown impaired the associations between these proteins (Brown et al. 2014). The increased expression and activation of Ezrin is known to have positive correlation with cancer development, progression, and metastasis (Kong et al. 2013; Clucas and Valderrama 2014). The increased Ezrin phosphorylation in Dsg3-overexpressing cells could be abrogated substantially by inhibition of PKC, p38 MAPK, Rho kinases that are known to be involved in Ezrin

activation (Brown et al. 2014) (Fig. 5). This finding indicates that Ezrin is likely a downstream effecter of Dsg3 signaling that regulates cortical actin organization as well as junction assembly. What is more, activation of c-Jun/AP-1 has also been identified in Dsg3-overexpressing cells by phospho-kinase array analysis and the luciferase activity assay in various cell lines with a marked increase in c-Jun S63 phosphorylation (Brown et al. 2014) (unpublished data) (Fig. 5). Knockdown of Dsg3 in A431 cell line showed more than twofold reduction of AP-1 activity relative to the matched control cells, assessed by the luciferase activity assay. Altogether, these additional data place Dsg3 as a key surface regulator for PKC-dependent Ezrin phosphorylation (activation) as well as the c-Jun/AP-1 activation that likely contributes to its procancerous role in Dsg3 associated cancer progression and metastasis.

Summary

Although Dsg3 was originally described as a desmosomal adhesion protein, accumulating evidence suggests that it is a versatile molecule with its role more related to cell signaling in epithelial cells. It is worth noting that Dsg3 is not only found in the core domain of desmosomes but also recognized to be distributed on the entire surface at the plasma membrane, and thus its signaling roles as described above are likely associated with its nonjunctional pool beyond the desmosome adhesion. In fact, the protein complex containing Dsg3 and other signal molecules such as Src and Ezrin is detectable in nonionic detergent soluble fraction that is disassociated with the cytoskeleton. Of interest, when expressing chimeric construct that encodes the extracellular domain of Dsg3 and the cytoplasmic domain of E-cadherin in fibroblast L-cells, they only displayed slight aggregation in contrast to that transfected with the wild type of E-cadherin (Amagai et al. 1994). In line with this finding, overexpression of wild-type Dsg3 in epithelial cell lines failed to render enhanced cell–cell adhesion but instead showed promoted cell migration and invasion accompanied with the downregulation of E-cadherin adhesion due to the activation of Src and other signaling pathways (Brown et al. 2014; Tsang et al. 2010).

Dsg3 is found to be associated with two human diseases, PV and squamous cell carcinoma. As a major target of PV-IgGs, Dsg3 is able to trigger a cascade of intracellular events, including the phosphorylation of itself and its depletion from the desmosomes and cell surface and concomitant activation of various signal molecules, such as PLC, PKC, EGFR, Src, p38 MAPK, and c-Myc. As a consequence, cell–cell detachment occurs, leading to blistering and pemphigus acantholysis in Dsg3 baring tissues such as skin and oral mucosa. Disruption of cell cohesion is also accompanied with changes in cell proliferation and induction of apoptosis due to the activation of c-Myc and p38 MAPK pathways, respectively, that attribute to pemphigus acantholysis. On the other hand, upregulation of Dsg3 is found in squamous cell carcinoma in various organs and shows a positive correlation with clinical grade and poor differentiation of cancers. In support, in vitro studies have demonstrated that overexpression of Dsg3 in cancer cell lines (gain-of-function) elicits activation of signal pathways, including Src, Rac1/Cdc42 GTPases, Ezrin, as well as transcription factors c-Jun/AP-1, all of which are known to be responsible for cancer invasion and metastasis. Thus, deregulation of Dsg3 signaling may play a key part in cancer development and progression. However, our current understanding of the biological function of Dsg3 and its related cellular and molecular mechanisms remains limited, and there are still many questions unanswered. For instance, we still do not understand why the distinct expression patterns of Dsg3 exist in epidermis and oral mucous membrane, both of which are the stratified squamous epithelia, with more restricted basal distribution in the former and broad uniform expression in the latter. Is there any specific function for Dsg3 that is correlated to such a distinct tissue distribution? What is the actual ligand or environmental cue that triggers the Dsg3 signaling? Does it exert a function in response to tissue mechanics as many of the pathways regulated by Dsg3 are

known to be involved in mechanotransduction? Can Dsg3 serve as a drug target in cancer therapy? Of course, it is challenging to address all these questions. Future investigation will advance our knowledge and shed light on the additional role of Dsg3 in cell biology.

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Destruction-Box, D-Box

▶ Monopolar Spindle 1 (Mps1)

Deubiquitinating Enzyme CYLD

▶ Ubiquitin Carboxyl-Terminal Hydrolase CYLD

DExD/H-Box Helicase 58

▶ RIG-I (Retinoic Acid Inducible Gene-I)

DEXRAS1, Dexamethasone-Inducible RAS Protein 1

▶ RASD1

DF3 Antigen

▶ Mucins (MUCs)

DFNB15

▶ GIPC

DFNB39

▶ HGF (Hepatocyte Growth Factor)

DFNB72

▶ GIPC

DFNB95

▶ GIPC

DFNB97

▶ Hepatocyte Growth Factor Receptor

dFoxO

▶ Forkhead Box Protein O

Dfp1/Him1/Rad35 (*Schizosaccharomyces pombe*)

▶ Dbf4

Dfz1

▶ FZD (Frizzled)

Dfz2

▶ FZD (Frizzled)

DG Kinase

▶ Diacylglycerol Kinase

DGK

▶ Diacylglycerol Kinase

DHHC Proteins

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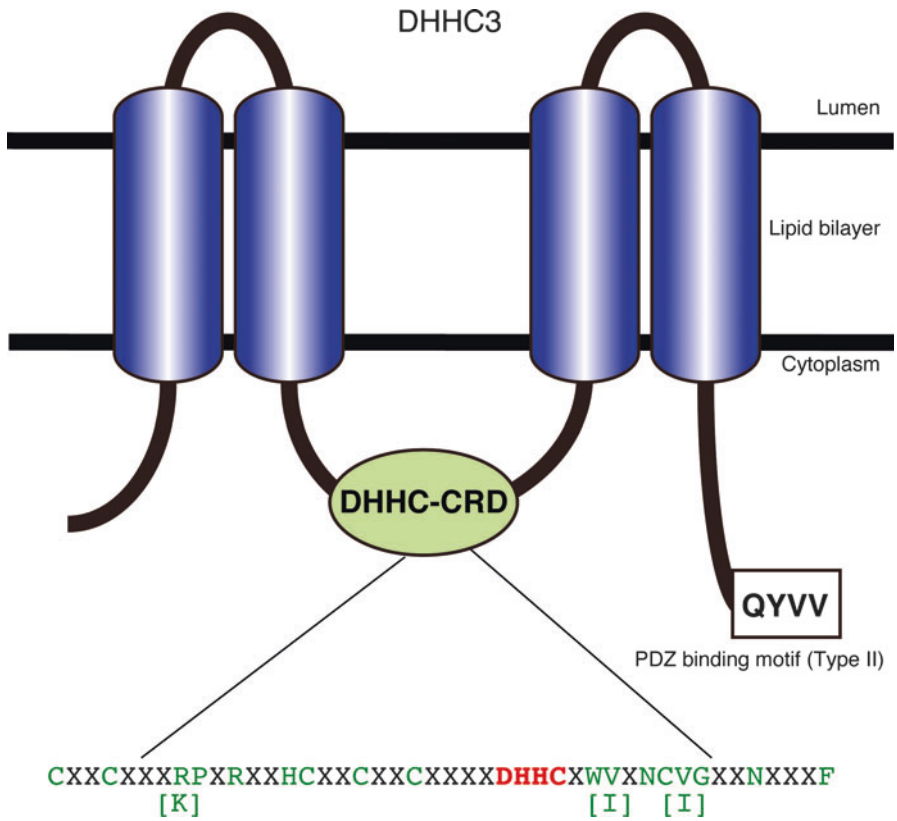
Synonyms

Palmitoyl acyl transferase (PAT); Palmitoylating enzyme; zDHHC proteins

Historical Background

Protein palmitoylation is the first discovered and the most common lipid modification. This posttranslational change involves addition of the saturated 16-carbon palmitate to specific cysteine residues by a labile thioester linkage (Linder and Deschenes 2007). Although reversible palmitoylation was

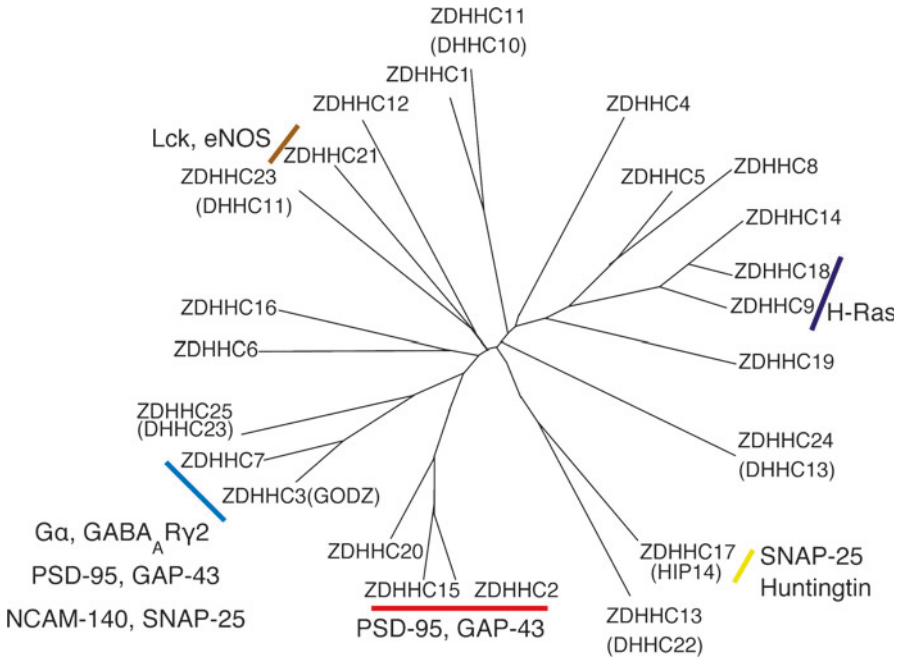


DHHC Proteins, Fig. 1 The domain structure of DHHC3, a prototype of mammalian DHHC proteins. DHHC proteins contain four or six transmembrane domains and a conserved DHHC (Asp-His-His-Cys) motif in the cytoplasmic cysteine-rich domain (*CRD*). Some members of DHHC proteins have unique motif/domain, such as PDZ-binding motif (in DHHC3). The consensus sequence of DHHC-CRD is indicated (*green* and *red*). X, a variety of amino acids

discovered over 30 years ago, the enzymes that add palmitate to proteins (palmitoyl acyl transferases, PATs) and those that cleave the thioester bond (palmitoyl protein thioesterases, PPTs) had been elusive. In 2002, genetic screening in yeast identified proteins that mediate PAT activity. Erf2/4 (Lobo et al. 2002) and Akr1 (Roth et al. 2002) were identified as PATs for yeast Ras2 and Yck2, respectively. Erf2 and Akr1 share a conserved DHHC (Asp-His-His-Cys) cysteine-rich domain (CRD) (Fig. 1) and have four or six transmembrane domains. The DHHC sequence and its surrounding CRD sequence are essential for their enzymatic activity. In 2004, 23 kinds of mammalian DHHC proteins were systematically isolated and some of them were characterized as PATs (Fukata et al. 2004). DHHC proteins have now emerged as evolutionally conserved PATs from plants, yeast (7 genes), *Caenorhabditis elegans* (15 genes), *Drosophila melanogaster* (22 genes) to mammals (24 genes).

Subfamily Classification of Mammalian DHHC Proteins

The large family of DHHC proteins can be classified into several subfamilies based on the homology of the DHHC-CRD core catalytic domains. DHHC2 and DHHC15 belong to one subfamily, while DHHC3 and DHHC7 form another subfamily. Importantly, DHHC proteins



DHHC Proteins, Fig. 2 Phylogenetic tree of mammalian DHHC protein family members. 23 DHHC proteins can be categorized into several subfamilies based on the homology of the catalytic DHHC domains. For example, DHHC2 and DHHC15 belong to one subfamily (in *red*), while DHHC3 and DHHC7 form another subfamily (in *blue*). The discovery of this mammalian DHHC protein family and the establishment of simple screening system using DHHC protein library have facilitated identification of palmitoyl substrate-enzyme pairs. Importantly, the same subfamily of DHHC proteins often shares substrates, for example, DHHC2/15 subfamily specifically palmitoylates PSD-95 and GAP-43. DHHC3/7 subfamily palmitoylates most of palmitoyl-proteins, such as PSD-95, GAP-43, Gα, GABA$_A$Rγ2, and SNAP-25. DHHC9/18 subfamily (in *purple*) palmitoylates H-Ras, DHHC21 (in *brown*) palmitoylates Lck and eNOS, and DHHC17 (in *yellow*) palmitoylates SNAP-25 and huntingtin. Note that some DHHC clone numbers initially collected by Fukata et al. (2004) are different from a current standard nomenclature (ZDHHC clone numbers) (Fukata and Fukata 2010). At present, 24 zDHHC genes have been annotated in the mouse genome (ZDHHC22 is recently annotated)

in the same subfamily often share their substrates (Fig. 2) (Fukata and Fukata 2010; Greaves and Chamberlain 2011). DHHC3/7 subfamily palmitoylates most of palmitoyl-proteins, such as PSD-95, GAP43, Gα, SNAP-25, NCAM-140, and GABA$_A$ receptor γ subunit. In contrast, DHHC2/15 subfamily more specifically palmitoylates PSD-95 and GAP43 (Fukata et al. 2004), but not Gα, NCAM-140 nor GABA$_A$ receptor γ subunit (Fig. 2). H/N-Ras is the only well-established substrate for DHHC9/18 subfamily. Because the number of identified substrate-enzyme pairs is limited, enzymatic activities of some DHHC proteins, such as DHHC1/10 subfamily, still remain undocumented.

Enzymatic and Regulatory Mechanisms for DHHC Proteins

DHHC-CRD region is the catalytic core site as mutation of the cysteine in the DHHC motif makes DHHC proteins catalytically inactive. Cysteines in this DHHC-CRD region are often autopalmitoylated (Roth et al. 2002; Fukata et al. 2004). Two mechanisms for palmitate transfer to a substrate have been proposed: (1) DHHC protein directly transfers palmitate from palmitoyl-CoA to the substrate and (2) DHHC protein first forms a thioester intermediate with palmitate (i.e., autopalmitoylation of DHHC proteins), followed by the transfer of the palmitate to the substrate.

Recent mutational analysis in yeast revealed that palmitoylation reaction by DHHC proteins, at least yeast Erf2, is mediated by the latter two-step reaction (Mitchell et al. 2010). In addition to the catalytically critical DHHC-CRD domain, some DHHC proteins have regulatory regions such as SH3 domain and ankyrin repeats at the C-terminal or N-terminal cytoplasmic regions. These domains may recruit specific substrate proteins to the DHHC enzyme. In fact, ankyrin repeats of DHHC17/HIP14 contribute to determining the substrate specificity (Huang et al. 2009).

DHHC3/7, 17, and 2/15 subfamily members do not require additional proteins (cofactors) for their PAT activity. In contrast, yeast Erf2 and its mammalian ortholog DHHC9 are inactive PATs in the absence of accessory proteins, Erf4 (for Erf2) or GCP16 (for DHHC9; Golgi complex protein of 16 kDa) (Swarthout et al. 2005). So far, the regulatory mechanism that directly activates or inactivates DHHC PAT activity is not known.

Akin to protein phosphorylation, protein palmitoylation is a reversible reaction that can be regulated by specific extracellular signals, for example, β-adrenergic receptor activation accelerates depalmitoylation and palmitoylation levels of $G\alpha_s$ (Degtyarev et al. 1993; Wedegaertner and Bourne 1994). Palmitoylation of ▶ LAT, linker for activation of T-cells, is reduced by T-cell receptor activation by ionomycin (Hundt et al. 2006). Also, glutamate-induced synaptic activity induces depalmitoylation of PSD-95 scaffolding protein (El-Husseini et al. 2002) and Cdc42 small GTPase (Kang et al. 2008). It is conceivable that unidentified PPTs, rather than DHHC-PATs, might play dominant roles in these processes. In contrast, blockade of neuronal activity rapidly increases PSD-95 palmitoylation. This dynamic palmitoylation is mediated by synaptic translocation of a PSD-95 PAT, DHHC2. This contrasts with the constitutive PSD-95 palmitoylation mediated by Golgi-resident DHHC3. Thus, the large family of DHHC palmitoylating enzymes are differentially localized and regulated in polarized cells (Noritake et al. 2009).

Inhibitors of DHHC Proteins

Several lipid-based compounds including 2-bromopalmitate (2BP), tunicamycin, and cerulenin have been used to inhibit protein palmitoylation (Ducker et al. 2006). Among them, the palmitate analog 2BP has been the most widely used. 2BP irreversibly inhibits DHHC-mediated palmitoylation directly acting on DHHC proteins in vitro (Fukata et al. 2004; Jennings et al. 2009), although 2BP also inhibits fatty acyl-CoA ligase and other enzymes involved in lipid metabolism in cells. In addition, 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one (Compound V) reversibly inhibits DHHC-mediated palmitoylation (Jennings et al. 2009). Because these inhibitors are not specific for individual DHHC-PATs, development of DHHC member-specific inhibitors is awaited.

Pathophysiological Significance of DHHC Proteins

DHHC protein members are linked to several human diseases (Table 1). DHHC8 is one of susceptibility genes for schizophrenia. Originally, microdeletions of human chromosome 22q11.2 locus, containing the DHHC8 gene, were reported to cause cognitive deficits and be associated with a high risk of developing schizophrenia. DHHC8 knockout mice showed behavioral phenotypes, a deficiency in prepulse inhibition and decreased exploratory activity in a new environment, and

DHHC Proteins, Table 1 Human disorders associated with DHHC proteins

DHHC protein	Disorders associated with DHHC-PATs
DHHC2	Colorectal cancer
DHHC8	Schizophrenia
DHHC9	X-linked mental retardation
	Colorectal cancer
DHHC11	Bladder cancer
DHHC15	X-linked mental retardation
DHHC17/HIP14	Huntington's disease

abnormalities in dendritic spines of neurons (Mukai et al. 2004, 2008). DHHC17/HIP14 is an associated protein with huntingtin protein, which is a causal gene for Huntington's disease. DHHC17 palmitoylates huntingtin and regulates its trafficking and function (Yanai et al. 2006). Polyglutamine expansion of huntingtin gene reduces interaction of huntingtin protein with DHHC17, resulting in decreased huntingtin palmitoylation. Knockout mice of DHHC17/HIP14 display behavioral, biochemical, and neuropathological defects that are reminiscent of Huntington's disease (Singaraja et al. 2011). Finally, mutations of DHHC9 and DHHC15 have been reported to be associated with X-linked mental retardation.

Summary

DHHC proteins are evolutionally conserved palmitoylating enzymes and add palmitates to proteins at specific cysteine residues by a reversible thioester linkage. DHHC proteins are integral membrane proteins with DHHC-CRD domain as a catalytic domain. Enzymatic activity and subcellular distribution of DHHC proteins can be dynamically regulated by extracellular stimulations. However, signal transduction pathways from receptors on the plasma membrane to DHHC proteins are poorly understood. Because mutations of DHHC proteins are closely associated with human diseases like schizophrenia and cancers, it would be beneficial to develop drugs acting on a specific DHHC protein for human health.

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Diacylglycerol Kinase

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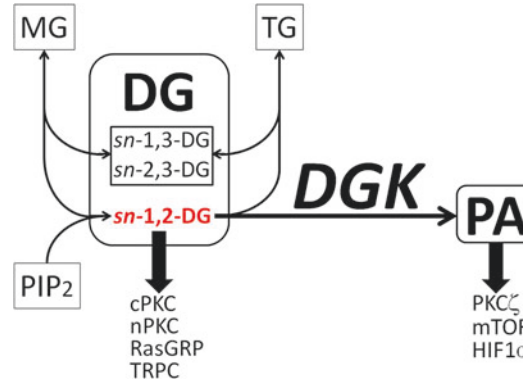
Synonyms

DAGK; DG kinase; DGK

Historical Background

Diacylglycerol kinase (DGK) is the enzyme which phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA) (Goto et al. 2007). Its enzymatic activity was first described in cabbage a half century ago by Hokin and Hokin (1959). Since their report, DGK activity has been found widely in many animal species. The substrate of DGK, DG, is a lipid derived from various sources, including phosphatidylinositol 4, 5-bisphosphate by the action of phospholipase C (PLC), phosphatidylcholine by phospholipase D (PLD), monoacylglycerol (MG) by acyltransferase, and triglyceride (TG) by TG lipase. DGs from different sources have distinct acyl chain composition. At least 50 distinct molecular species of DG have been identified. In the mid-1980s, the biological



Diacylglycerol Kinase, Fig. 1 DG pool and DGK. DG pool contains a couple of DGs with different acyl chain positions, which are produced from various sources including phosphatidylinositol 4, 5-bisphosphate (PIP_2), monoacylglycerol (MG), and triglyceride (TG). Of these, *sn*-1,2-DG activates cPKC, nPKC, RasGRP, and TRPCs. DGK phosphorylates *sn*-1,2-DG into phosphatidic acid (PA). PA activates PKCζ, mTOR, and HIF1α

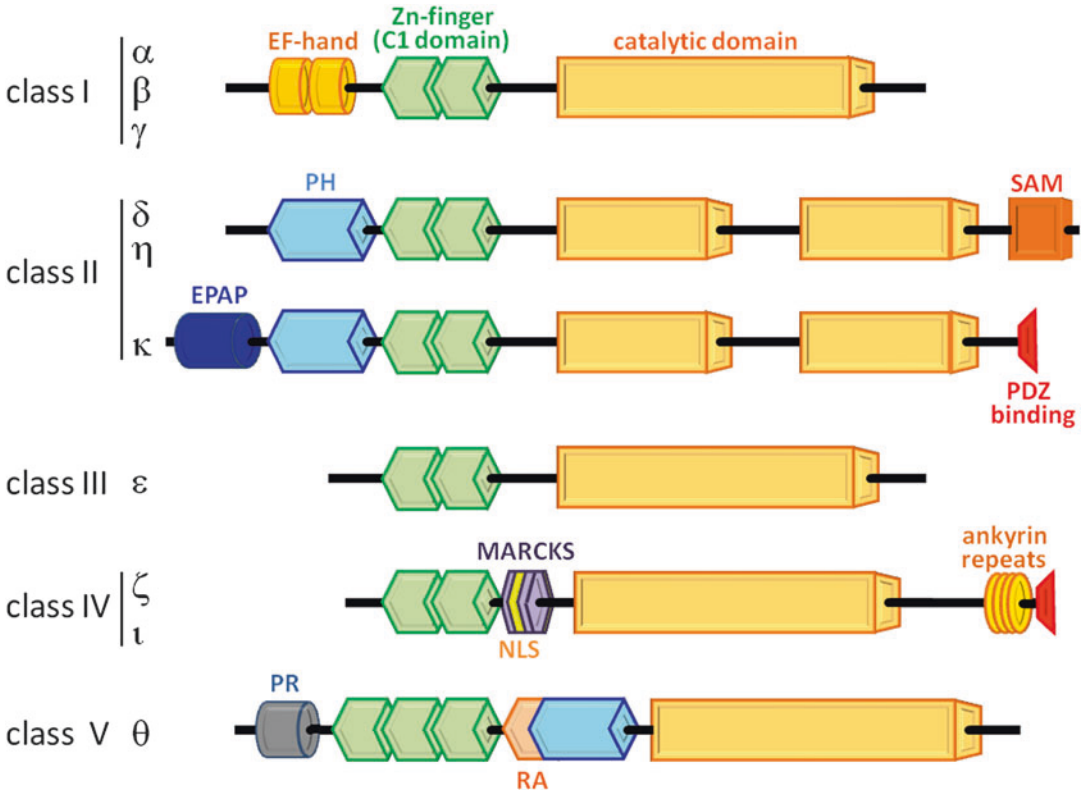
significance of *sn*-1,2-DG was highlighted: It is an allosteric activator of protein kinase C (PKC). The PKC family comprises of three classes (conventional, novel, and atypical). Of those, the activity of novel PKC is dependent solely on DG whereas that of conventional PKC depends upon both DG and Ca^{2+}. Along with accumulating reports of widely various PKC functions such as proliferation and differentiation, DGK has attracted much attention as a physiological regulator of PKC activity (Fig. 1). Because of technical difficulties, however, the purification of DGK had to wait until 1983, followed by molecular cloning of 80 kDa DGK from porcine brain in 1990. The significance of DGK in various cellular functions has been inferred because RasGRP, a canonical transient receptor potential channels, Unc-13, and protein kinase D were found to be activated by DG. Furthermore, PA, the product of DGK, is shown to work as a second messenger to activate signaling molecules such as mammalian target of Rapamycin (mTOR), hypoxia-inducible factor 1α (HIF1α), and PKCζ. To date, DGK cDNAs have been cloned in widely various species including *Homo sapiens, Mus musculus, Rattus norvegicus, Escherichia coli*, *Drosophila melanogaster*, *Dictyostelium discoideum, Streptococcus mutans,* and *Arabidopsis thaliana*.

Distinct Molecular Structure of DGK Isozyme

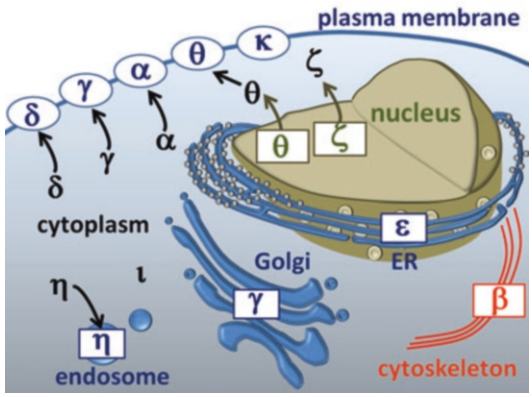
Ten mammalian DGK isozymes have been reported. They are classified into classes I–V, based upon their distinct structures (Fig. 2). DGKα, β, γ are grouped as class I; δ, η, and κ as class II; ε as class III; ζ and ι as class IV; and θ as class V. All isozymes share a conserved catalytic domain in the C-terminal region and at least two zinc fingers (C1 domains). In addition to these domains, class I isozymes have two EF-hand motifs that bind Ca^{2+}, whereas class II isozymes contain a pleckstrin homology (PH) and separated catalytic domains. DGKδ and η are characterized by a sterile α-motif (SAM) domain for oligomer formation at the C-terminus. DGKκ has a postsynaptic density protein-95/discs large/zona occludens-1 (PDZ)-binding domain instead of the SAM domain. This isozyme contains a Glu-Pro-Ala-Pro (EPAP) repeat at the N-terminus. DGKε solely constitutes class III DGK, which is the simplest, but uniquely has substrate specificity towards 2-arachidonoyl-DG. Class IV DGKζ and ι contain ankyrin-like repeat domain at the C-terminus and nuclear localization signal (NLS) overlapped with myristoylated alanine-rich C kinase substrate (MARCKS). Class V DGKθ has three zinc fingers, proline/glycine rich (PR) and Ras-associating (RA) domains.

Subcellular Localization and Functional Significance

DGK isozymes catalyze DG species irrespective of acyl chain composition except arachidonoyl-specific DGKε. What is the significance of the structural diversity of the DGK family? DG is a



Diacylglycerol Kinase, Fig. 2 Schematic representation of mammalian DGK isozymes. Ten DGK isozymes are grouped into classes I–V based on distinct molecular structures. *PH* pleckstrin homology domain, *SAM* sterile α-motif, *EPAP* Glu-Pro-Ala-Pro repeat, *PDZ binding* postsynaptic density protein-95/discs large/zona occludens-1 binding domain, *MARCKS* myristoylated alanine-rich C kinase substrate, *NLS* nuclear localization signal, *PR* proline/glycine rich domain, *RA* Ras-associating domain



Diacylglycerol Kinase, Fig. 3 Subcellular localization and translocation of DGK isozymes. Each isozyme shows unique subcellular localization and distinct translocation pattern including nucleus-cytoplasm, cytoplasm-plasma membrane, and cytoplasm-endosome. DGKκ and ι stably localize to the plasma membrane and cytoplasm, respectively

minor component of the plasma membrane, but it is also distributed to other intracellular compartments including the nucleus, endoplasmic reticulum (ER), and Golgi apparatus. It is apparently reasonable that DGK isozymes are targeted to distinct subcellular regions to handle DG in a unique manner. Transfection of cDNA and immunocytochemistry using specific antibodies have revealed detailed subcellular localization and the site-specific function of DGK isozymes (Fig. 3).

Cytosolic DGKα Translocates to the Plasma Membrane in Response to Extracellular Stimuli

Cytoplasmic localization of DGKα has been reported in various cell types including T cells, canine kidney cell line MDCK cells, melanoma cell lines, rat aortic smooth muscle cells, rat hepatocytes, and rat adrenal medullary chromaffin cells. The functional significance of DGKα has been investigated intensely in T cells, in which DGKα is expressed abundantly. DGKα is reported to regulate interleukin-2 (IL-2)-induced cell proliferation positively through PA production. In this case, DGKα translocates to the plasma membrane in response to the stimuli. T cell receptor (TCR) stimulation also moves DGKα to the plasma membrane. However, DGKα negatively regulates TCR stimulation by inhibiting RasGRP activity. This seems to be the opposite effect to that of IL-2-induced cell proliferation. However, these contradictory actions may be explained by the fact that distinct downstream signaling cascades of TCR are differently regulated (Sakane et al. 2007).

DGKβ, Associated with Actin Cytoskeleton, Facilitates Its Remodeling in a Kinase-Dependent Manner

DGKβ is colocalized with actin cytoskeleton in transfected COS7 cells. DGKβ transfection alters actin stress fiber arrangement, whereas kinase-dead mutant abolishes the association with stress fibers (Kobayashi et al. 2007). Furthermore, detailed immunoelectron microscopy reveals that DGKβ localizes to the perisynaptic membrane on dendritic spines of neurons (Hozumi et al. 2008). Overexpression of DGKβ into hippocampal neurons induces dendritic outgrowth and spine maturation depending on its enzymatic activity. In microglia, transfected DGKβ localizes to the plasma membrane before stimulation and moves to the phagocytic cup to engulf IgG-opsonized glass beads (Ueyama et al. 2004). Since both spine formation and phagocytic processes are regulated by actin rearrangement, DGKβ may play a role in actin cytoskeletal reorganization.

Cell Type-Dependent Expression Pattern and Function of DGKγ

Cell type-dependent subcellular localization and function are reported on DGKγ. DGKγ localizes to the Golgi apparatus in transfected COS7 cells (Kobayashi et al. 2007) and immunostained rat aortic endothelial cells (Nakano 2015). The Golgi apparatus is the organelle for posttranslational modification and sorting of proteins synthesized in the ER, suggesting involvement of DGKγ in these functions. However, some reports have described that DGKγ may play a role in cytoskeletal reorganization. In the process of differentiation of leukemic cell lines (HL-60 and U937 cells) into macrophages in response to phorbol ester (DG analogue serving as a strong PKC activator), DGKγ translocates from the cytoplasm to the F-actin-labeled cell margin. Upon stimulation with growth factors, DGKγ is shown to regulate

cytoskeletal organization through Rac1 activity. In this regard, DGKγ interacts with β2-chimerin, a Rac-specific GAP. DGKγ may negatively regulate macrophage differentiation via its catalytic activity (Sakane et al. 2007).

Cytoplasmic DGKδ Translocates to the Plasma Membrane in Response to Glucose Stimulation

Class II DGKs, DGKδ and η, localize to the cytoplasm but they behave differently after stimulation. Splicing variants of DGKδ (DGKδ1 and δ2) and DGKη reside in the cytoplasm of HEK293 and C2C12 myoblast cells (Takeuchi et al. 2012). Of those, only DGKδ1 translocates to the plasma membrane upon stimulation with high glucose. In this process, PH and C1 domains are responsible for this action. DGKδ2 and DGKη remain cytoplasmic after high-glucose stimulation, although DGKη translocates from the cytoplasm to endosomes by osmotic shock in transfected COS7 cells (Murakami et al. 2003).

DGKκ Stably Localizes to the Plasma Membrane

DGKκ stably localizes to the plasma membrane in HEK293 cells under basal and stimulated conditions (Imai et al. 2005), which contrasts sharply to other type II DGKs (δ and η). Functional significance of this isozyme remains unknown.

DGKε Resides in the ER and Is Implicated in Arachidonic Acid Metabolism

DGKε, which localizes to the ER in transfected COS7 and HeLa cells (Kobayashi et al. 2007), contains no specific ER retention signal such as the KDEL sequence, but the hydrophobic region in the N-terminus determines its ER localization (Matsui et al. 2014). The ER plays a pivotal role in protein and lipid synthesis and Ca^{2+} homeostasis. As described above, DGKε is a unique isozyme with respect to substrate specificity towards arachidonoyl-DG. Arachidonate is a component of phospholipids, especially in phosphoinositide (PI), of the plasma and ER membranes. Cyclooxygenase (COX), which plays a central role in arachidonate metabolism, also localizes to the ER. Under recurrent epileptic seizures, it is noteworthy that the COX-2 level is higher in the hippocampus of DGKε-knockout (KO) mice than the wild-type mice (Lukiw et al. 2005). DGKε may be intimately involved in the metabolism of PI together with COX-2.

DGKζ Shuttles Between the Nucleus and Cytoplasm

DGKζ, which is characterized by NLS, is well known as "nuclear DGK." In both cDNA transfection and immunocytochemistry, DGKζ is detected in the nuclei of neurons, alveolar epithelial cells, macrophages, and aortic smooth muscle cells. Under conditions of transient ischemia and kainate-induced seizures, DGKζ translocates from the nucleus to cytoplasm. It never relocates to the nucleus in hippocampal neurons. In addition to NLS, the nuclear export signal (NES) is identified in DGKζ. Subcellular localization of DGKζ may be determined by the balance between the NLS/importin and NES/exportin systems (Goto et al. 2014).

DGKι Localizes to the Cytoplasm Irrespective of NLS

DGKι belongs to class IV DGK. This isozyme contains the NLS similar to DGKζ but localizes to the cytoplasm (Ito et al. 2004). Deletion of DGKι is shown to reduce Ras signaling through Rap1 activity (Regier et al. 2005), suggesting involvement in Ras signaling.

DGKθ Resides in the Nucleus and Cytoplasm and Translocates to the Plasma Membrane

DGKθ localizes mainly to the nucleus of MDA-MB-453, MCF-7, PC12, HeLa, and IIC9 cells. In H295 human adrenocortical cells, DGKθ is activated transcriptionally by a complex of nuclear steroidogenic factor-1 (SF-1) and sterol regulatory element binding protein 1 (SREBP1) in response to cAMP stimulation, thereby leading to glucocorticoid production (Cai and Sewer 2013). DGKθ responds to cAMP both in the cytoplasm and the nucleus, but nuclear DGKθ is more responsive to cAMP. In A431 and HEK293 cells, DGKθ translocates from the cytoplasm to plasma membrane in a PKC-dependent manner and regulates epidermal growth factor receptor (EGFR) signaling (van Baal et al. 2012).

DGKs in Health and Diseases

Numerous reports of investigations of human patients, animal models, and population studies suggest that DGKs are closely associated with disease pathogenesis (Nakano 2015).

Cancer

Cancer cells reach an abnormally hyperproliferative state. The primary function of DGK is an attenuator of PKC activity via DG metabolism. Therefore, DGK downregulation theoretically engenders prolonged activation of PKC, thereby stimulating cell proliferation, a characteristic feature of cancer pathogenesis. However, downregulation of DGKδ engenders activation of PKCα and subsequent PH domain leucine-rich repeat protein phosphatase 2 (PHLPP2), resulting in Akt dephosphorylation. DGKη can modulate EGFR signaling. Its downregulation reduces cellular proliferation in HeLa cells. In addition, DGKη downregulation reduces the proliferation of human lung cancer cells. DGK inhibitor R59949 strongly inhibits hepatocyte growth factor (HGF)-induced cellular proliferation in Kaposi's sarcoma. Taken together, under certain conditions, DGK downregulation exerts an inhibitory, rather than stimulatory, effect on cellular proliferation. These findings suggest that DGK regulates not only DG-PKC pathway but also other signaling pathways.

Ischemia

In brain ischemia, DGKζ is deeply involved in the pathogenesis in both neurons and nonneuronal cells. Neurons are susceptible to cell damage by hypoxia because of their high metabolic rate. Transient loss of blood supply easily engenders ischemic injury. In rat forebrain ischemia, DGKζ disappears rapidly from the nucleus and translocates to the cytoplasm of hippocampal CA1 neurons. This phenomenon is recapitulated in an acute hippocampal slice culture model under oxygen-glucose deprivation (Goto et al. 2014). In an infarction model of rat brain, DGK-ζ-immunoreactivity disappears rapidly in cortical neurons. It is particularly interesting that the immunoreactivity reappears in endothelial cells and activated microglia.

Diabetes

Reduced expression of DGKδ in skeletal muscles of type II diabetes patients and diabetic rats has been reported. Skeletal muscles of DGKδ-KO mice are characterized by defects in glucose usage, together with reduced tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1), suggesting insulin-resistant states in DGKδ-deficient skeletal muscles. Decreased levels of DGKδ engender PKCδ activation, which results in serine phosphorylation of IRS-1 and subsequent insulin resistance. Another study reveals that DGKδ deficiency causes decreased activity of AMP-activated protein kinase (AMPK), which is known to be an energy sensor in skeletal muscles (Jiang et al. 2016). Consequently, modulating DG levels by DGK seems to play an important role in the regulation of glucose homeostasis. A single nucleotide polymorphism (SNP) study of human population has shown that DGKε polymorphism is associated with higher plasma TG levels. Taken together, these studies suggest that DGKs are deeply involved in both glucose and lipid metabolism. Further investigations are warranted to unveil precise mechanisms of DGK in the pathogenesis of metabolic syndromes such as diabetes and obesity.

Kidney Disease

DGKε is implicated in the field of kidney diseases such as hemolytic uremic syndrome. Mutations in DGKε gene cause thrombotic microangiopathy, characterized by endothelial injury of small vessels (Lemaire et al. 2013). In the pathogenesis of DGKε-KO mice, reduced prostaglandin production induces cell adhesion, causing endothelial cell injury in kidney glomeruli (Zhu et al. 2016).

Summary

DGK phosphorylates DG to produce PA. DG serves as an activator of conventional and novel classes of PKC, RasGRP, TRPCs, etc. PA also works as an activator of PKCζ, HIF1α, etc. These facts suggest that DGK acts as a hub between DG-mediated and PA-mediated

signalings. To date, ten mammalian isozymes have been reported. These isozymes constitute the enzyme family, which comprises five subgroups (class I, α, β, γ; class II, δ, η, κ; class III, ε; class IV, ζ, ι; and class V, θ) depending upon their distinct molecular structures. Each DGK isozyme exhibits diverse subcellular localization and translocation patterns. DGKα localizes to the cytoplasm and translocates to the plasma membrane in response to extracellular stimuli. DGKβ is associated with actin cytoskeleton. DGKγ localizes to the Golgi apparatus and may be associated with cytoskeleton. DGKδ/η localizes mainly to the cytoplasm and translocates to the plasma membrane. DGKκ stably localizes to the plasma membrane. DGKε resides in the ER. DGKζ and θ are nuclear isozymes, but they translocate to the cytoplasm or the plasma membrane. DGKι is in the cytoplasm irrespective of the NLS. DG includes at least 50 molecular species, but only DGKε shows substrate specificity towards arachidonoyl-DG. DGKs are implicated in widely various diseases including cancer, diabetes, ischemia, and kidney diseases. The original idea on DGK function was based upon the regulation of PKC activity, the so-called DG-PKC pathway, but diverse effects on the other signaling pathways are emerging now.

See Also

- ▶ mTOR
- ▶ Protein Kinase CK2
- ▶ RasGRP
- ▶ TRP

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Dickkopf 3

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Synonyms

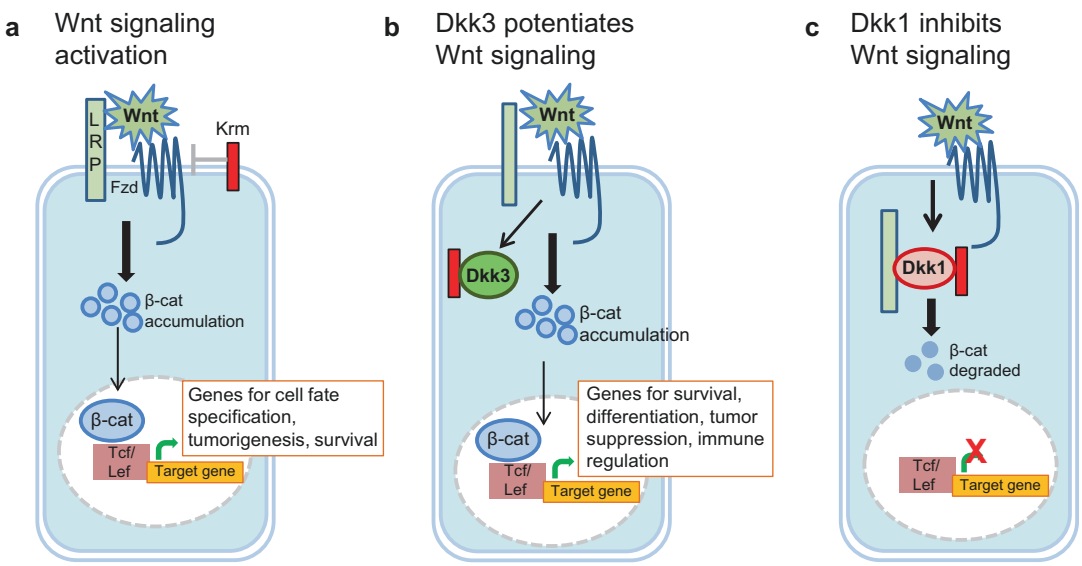
Dkk3; REIC

Historical Background

Dickkopf 3 (Dkk3) belongs to the Dkk family of proteins, which are secreted glycoproteins that regulate the canonical Wnt/β-catenin ("Wnt") signaling pathway. Wnt signaling is an essential cellular communication pathway that mediates a diverse array of cellular and molecular activities in embryonic development, tissue homeostasis, and disease pathogenesis. The prototypic Dkk family member, Dkk1, has been well characterized as an essential modifier of Wnt signaling. In contrast, the function of Dkk3 was initially unknown because it was a weak regulator of Wnt signaling, and mice deficient in Dkk3 had only mild phenotypes (see below), despite its sequence similarity to the other Dkk genes. However, in recent years, new functions for Dkk3 have been identified in immune regulatory pathways, malignancies, and neurogenesis, raising the exciting possibility that Dkk3 is a critical regulator of these processes and may be an important novel therapeutic target for several types of diseases.

The Dkk family includes five different proteins: Dkk1, Dkk2, Dkk3, Dkk4, and Soggy (Sgy). Dkk1–4 share 37–50% protein identity and contain two highly conserved cysteine-rich domains (CRD; cys-1 and cys-2) separated by a variable sequence linker region and also contain a signal peptide sequence, colipase region, and putative sites for N-linked glycosylation. The cysteine-rich CRD domains contain ten conserved cysteine residues in Dkk1–4, although the CRD of Dkk3 has lower sequence similarity to the other Dkk family members and has a relatively shorter linker region between the two CRDs (12 amino acids in Dkk3 and 50–55 amino acids in the Dkk1, Dkk2, and Dkk4 genes). Dkk3 also has longer N- and C-terminal regions compared to the other Dkks. The fifth family member, the Sgy gene, has sequence homology only to Dkk3 and lacks the CRD domains, indicating that it is most likely evolutionarily derived from Dkk3. Comparative sequence analysis of the Dkk family members across the evolutionary tree indicated that Dkk3 separated from the other Dkk family members prior to the divergence of cnidarians and bilaterians, suggesting an ancient phylogenetic separation, and raising the possibility that Dkk3 may have unique physiological roles that are distinct from the other Dkk family members.

Dkk3 was initially identified in mouse tissues by Glinka et al., in a study using EST database mining to search for sequences similar to the embryogenesis regulator Dkk1 (Glinka et al. 1998). Subsequently, a Dkk3 cDNA containing the complete open reading frame was cloned from a newborn mouse library by PCR amplification (Monaghan et al. 1999). Shortly after its initial isolation, Dkk3 was also cloned and characterized in an analysis of genes that were decreased in human tumor-derived cell lines; this Dkk3 clone was called REIC, which stood for reduced expression in immortalized cells (Tsuji et al. 2000). Therefore, Dkk3 is often referred to as REIC/Dkk3 to denote both its association with tumor cells and its membership in the Dkk family.



Dickkopf 3, Fig. 1 Regulation of Wnt signaling by Dkk1 and Dkk3. **a** Wnt signaling is induced by Wnt ligands forming a complex with the Frizzled (Fzd) receptor and LRP5/6 receptor (LRP), which leads to a series of molecular events that lead to stabilization of β-catenin in the cytosol. When levels of β-catenin accumulate, it is translocated into the nucleus where it binds to Tcf/Lef transcription factors and induces expression of Wnt target genes. Expression of Krm has been reported to inhibit Wnt signaling, presumably through endocytosis of LRP receptors. **b** Proposed model of Dkk3 and Krm in regulating Wnt signaling, based on Nakamura and Hackam (2010). Dkk3 forms a complex with Krm within intracellular membranes, which prevents Krm from inhibiting the interactions between Wnt and its receptors. Therefore, reduced levels of Krm at the surface provide a favorable environment for Wnt ligands to activate Wnt signaling and induce gene expression. **c** Unlike Dkk3, Dkk1 binds to the LRP receptor and induces endocytosis of LRP and Krm. The reduced surface expression of LRP blocks the ability of Wnt ligands to bind to form the Wnt- Fzd-LRP complex that is needed to stimulate the pathway. Krm and Dkk1 are Wnt inhibitors (labeled in *red*). LRP, Fzd, and Dkk3 are Wnt activators (labeled in *green*)

Dkk1, Dkk2, and Dkk4 inhibit Wnt signaling by binding to the Wnt receptor low-density lipoprotein receptor-related protein (LRP) 5/6 and the transmembrane protein Kremen (Krm) 1/2 via the cys-2 domain, which results in LRP5/6 internalization by endocytosis and prevents Wnt ligands from forming an active complex with Frizzled receptors and LRP5/6 (Fig. 1). In contrast, Dkk3 activates Wnt signaling in human embryonic kidney (HEK) 293 and Muller glia MIO-M1 cell lines by forming an intracellular protein complex with Krm1/2. Dkk3 also activates Wnt signaling in cardiac tissue (Lu et al. 2016). However, Dkk3 inhibits or has no effect on Wnt signaling in other cell lines, such as ARPE-19, RGC5, and Cos-7, and in various tissues (Nakamura et al. 2007). Although Dkk3 interacts with Krm1 and Krm2 (Nakamura and Hackam 2010), it does not bind to LRP6 due to a seven amino acid insertion in cys-2, indicating that Dkk3 likely has a different mechanism of action and distinct functions from Dkk1, Dkk2, and Dkk4 (Fig. 1).

Dkk3 is expressed during embryonic development in many organs, including the neural epithelium, limb bud, bone, and heart, particularly in regions of epithelial-mesenchymal transformation (EMT). Expression of Dkk3 in fetal liver may be important for immune cell development, especially B cells. Dkk3 is also widely expressed in adult tissues, with the highest levels found in the heart, retina, adrenal cortex, and brain. In adult mouse, Dkk3 is expressed in neurons in distinct areas of the brain, including the hippocampus, medulla, and the visual area of the cerebral cortex, with weaker expression in the somatomotor area of the cerebral cortex and substantia nigra of the midbrain. Dkk3 is not expressed in GFAP-positive astroglia in the brain (Meister

et al. 2015), although it is expressed in the GFAP-expressing Muller glia in the retina (Nakamura et al. 2007), implying a unique Dkk3 activity in retinal glia that is not observed in the brain. Despite the widespread expression distribution of Dkk3, Dkk3 knockout mice (*Dkk3*$^{-/-}$) develop normally, are fertile, and have a mild phenotype that includes hyperactivity, increased immunological and hematological markers, and a slight decrease in lung ventilation (Barrantes Idel et al. 2006). The absence of severe phenotypes in *Dkk3*$^{-/-}$ mice may be due to compensation from the Dkk3 homolog *Soggy* (Barrantes Idel et al. 2006). Alternatively, physiological stress or injury may be required for the appearance of a Dkk3-dependent phenotype, as demonstrated in a study on cardiac stress, described below (Lu et al. 2016).

The Role of Dkk3 in the Heart

Cardiac hypertrophy is an important cause of congestive heart failure and sudden death. Hypertrophy is often caused by hemodynamic overload, which leads to structural remodeling and dysfunction of the heart muscle. Recent evidence demonstrated that Dkk3 expression in the heart is reduced in patients with end-stage heart failure and in mice with pressure-overloaded cardiac hypertrophy (Zhang et al. 2014). Furthermore, overexpression of Dkk3 in rodent cardiac myocyte cultures led to reduced hypertrophic responses, and siRNA-mediated knockdown of Dkk3 led to increased hypertrophic responses. These findings were confirmed in vivo, in which cardiac hypertrophy was enhanced in mice with genetic loss of Dkk3, and the phenotype was reversed by transgenic overexpression of Dkk3. Therefore, these data provided evidence for a role of Dkk3 in cardiac protection. The authors also demonstrated in an elegant series of experiments that Dkk3 reduces cardiac hypertrophy by blocking activation of apoptosis signal-regulating kinase 1 (Ask1) signaling (Zhang et al. 2014). Therefore, Dkk3 has an important role in protecting the heart from pathological hypertrophy and acts as a negative modulator of cardiac hypertrophy in response to pressure overload. Whether Dkk3 functions as a cardiac protector through regulating Wnt signaling and Krm1/2 is an important mechanistic question that was not addressed in the study. Additional evidence supporting a role for Dkk3 as a cardioprotector came from a study by Lu et al., who demonstrated that Dkk3 reduced cardiac pathologic changes in a mouse model of familial dilated cardiomyopathy (FDCM), and the mechanism of protection involved activation of the canonical Wnt pathway and inhibition of the noncanonical Wnt pathway (Lu et al. 2016). In the *Dkk3*$^{-/-}$ mice, loss of Dkk3 caused enhanced cardiac pathology and dysfunction and led to decreased survival compared with wild-type mice, whereas overexpression of Dkk3 in a mouse model of FDCM improved cardiac morphology and echocardiographic parameters and led to increased survival (Lu et al. 2016). Together, these studies demonstrated that Dkk3 is important for protecting cardiac tissue from physiological damage.

The Role of Dkk3 in Immune Regulation

An important route by which Dkk3 could influence tissue homeostasis throughout the body is by regulating immune cell maturation and activation processes. Initial analyses of the *Dkk3*$^{-/-}$ mice suggested a regulatory role for Dkk3 in the immune system because the knockout mice had higher levels of IgM and increased natural killer cells compared with wild-type controls (Barrantes Idel et al. 2006). Indeed, subsequent studies demonstrated that Dkk3 is an important modulator of several immune cell types, including B cell and $CD4^+$ and $CD8^+$ T cells. In a recent study by Ludwig et al., Dkk3 was shown to regulate B-cell development, survival, proliferation, and autoreactivity (Ludwig et al. 2015). Loss of Dkk3 led to changes in B-cell-mediated immune responses, including altered antibody production and cytokine release. Notably, the role of Dkk3 in influencing B-cell fate was shown to be important to autoimmune disease because blocking Dkk3 with a neutralizing antibody increased the severity of disease in a mouse model of systemic lupus

erythematosus (SLE, lupus) (Ludwig et al. 2015). Furthermore, Dkk3 is expressed in mouse tolerant to $CD8^+$ T cells, and Dkk3 controls peripheral $CD8^+$ T-cell tolerance to self-antigens, which is critical to preventing autoimmunity. Adoptive T-cell transfer experiments and blocking Dkk3 function demonstrated that Dkk3 is essential for inhibiting T-cell proliferation and IL-2 production. Inhibiting Dkk3 reversed $CD8^+$ T-cell tolerance, leading to rejection of tumors and autologous skin grafts in mouse models, whereas soluble Dkk3 inhibited $CD8^+$ T-cell responses (Papatriantafyllou et al. 2012). Similarly, Dkk3 was also shown to function within the local microenvironment to control $CD4^+$ and $CD8^+$ T-cell responses by suppressing activation and differentiation of T cells in the periphery, which is consistent with its high expression in immune-privileged sites, such as the eye, placenta, and brain (Meister et al. 2015). Inhibiting Dkk3 led to enhanced T-cell responses and increased disease severity in a mouse model of experimental autoimmune encephalomyelitis, and Dkk3 was shown to act locally during the T-cell effector phase, rather than systemically in the spleen and lymph nodes. Therefore, these findings demonstrate that Dkk3 is a novel regulator of immune responses in mice and suggest that Dkk3 may be a target for controlling immunosuppression in inflammatory diseases and transplantation (Meister et al. 2015). Although Wnt signaling has been implicated in various aspects of immune system regulation, it is not known yet whether Dkk3 regulates B- and T-cell fates by modifying the Wnt signaling pathway, or whether it acts independently from Wnt signaling.

The Role of Dkk3 in the Central Nervous System (CNS)

Dkk3 is highly expressed in developing and adult neurons in multiple regions in the brain. As expected from its widespread distribution in the CNS, Dkk3 has been implicated in essential neuronal processes, including survival, neurogenesis, and differentiation. For example, Dkk3 increases survival of mutant dopaminergic neurons (Zhang et al. 2015) and differentiating ventral midbrain neurons (Fukusumi et al. 2015), which is consistent with its anti-apoptotic activity in cell lines (Nakamura et al. 2007). Also, a recent paper by Fukusumi et al. demonstrated that Dkk3 is necessary and sufficient for differentiation and survival of a neuronal precursor subset into rostrolateral mesodiencephalic dopaminergic neurons (mdDA) in the developing mouse ventral midbrain. Loss of Dkk3 impeded mdDA differentiation, and Dkk3 was required for expression of the transcription factors LMX1A and PITX3 in neuronal precursors. Furthermore, treating pluripotent stem cells with Dkk3 and Wnt1 ligand in vitro increased the proportion of differentiated neurons of the SNcDA type (Fukusumi et al. 2015). Because dopaminergic neurons are lost during Parkinson's disease (PD), these studies suggest that Dkk3 may be developed as a therapeutic strategy for replacing neurons in PD patients. The demonstration of Dkk3 expression in many other regions of the CNS suggests that Dkk3 has additional roles in the developing and/or adult brain and retina. Indeed, the hyperactivity phenotype in *Dkk3*$^{-/-}$ mice suggests that there are novel functions of Dkk3 in the brain that are yet to be discovered.

Dkk3 and Cancer

Numerous studies have reported that loss of Dkk3 is associated with various cancers, leading to the conclusion that Dkk3 functions as a tumor suppressor. It is notable that the original description of Dkk3 function was as a molecular marker with reduced expression in immortalized cells (*REIC*) compared to expression in normal cells (Tsuji et al. 2000). Reduced Dkk3 expression is implicated in oncogenesis in many cancer types, including the prostate, testes, ovarian, breast, gastrointestinal, malignant glioma, bone, renal, liver, non-small cell lung cancer, and many others (for a complete review, please see (Veeck and Dahl 2012)). Dkk3 expression levels are decreased in tumor cells due to epigenetic changes (hypermethylation) of its promoter region, as well as by direct reduction of Dkk3 transcripts via transcriptional inhibition by the oncogene MycN. Thus, loss or reduction of Dkk3 protein

levels would relieve cancer cells of the tumor-suppressing activity of Dkk3 and permit aberrant proliferation and survival of the cancer.

Because of the association of Dkk3 expression with cancer, efforts have been made by several research groups to utilize Dkk3 levels as a potential biomarker for tumor progression and disease prognosis. For example, methylation levels in the Dkk3 promoter were quantified and associated with survival prediction in cervical cancer, and downregulated Dkk3 expression was a marker of poor prognosis in endometrial cancer (Dellinger et al. 2012). Additionally, Dkk3 expression in serum was shown to have diagnostic application for detecting early stage disease in colorectal cancer patients before symptoms are evident, when used in a biomarker panel along with two other gene markers (Fung et al. 2015).

Targeted overexpression of Dkk3 to tumors has shown promising results in experimental gene therapy studies in rodent models of several cancers. Dkk3 was incorporated into an adenovirus for sustained gene expression, and it was shown to inhibit tumor growth, induce cancer cell-specific apoptosis, and reduce EMT in cancers of the pancreas, prostate, and breast. The mechanisms of action for antitumor activity of Dkk3 include systemic immunity effects on the tumor, maintenance of tumor cells in a dedifferentiated state (Zenzmaier et al. 2012), enhanced anticancer immune activity of splenocytes, reduced Wnt signaling, and localized apoptotic and ER-stress-mediated JNK activity in the tumor. Based on these preclinical results for adenoviral Dkk3 in animal models, a clinical trial is currently in phase 1/2a to test Dkk3 therapy for localized prostate cancer (ClinicalTrials.gov identifier NCT01931046).

In contrast, there are several reports that showed that Dkk3 acts as a tumor-promoting gene. Increased Dkk3 expression was observed in esophageal adenocarcinoma, and Dkk3 treatment of adenocarcinoma cells increased proliferation and invasion in cultured cells and in NOD/SCIDγ mice by modulating the TGFβ signaling pathway (Wang et al. 2015). Dkk3 also promoted tumor invasion and neoangiogenesis via TGFβ in esophageal cancer and was oncogenic in oral squamous cell carcinoma independent of Wnt signaling. Because TGFβ can act as a tumor suppressor early in oncogenesis yet promotes metastasis in advanced cancer, the authors concluded that Dkk3 activities in cancer, similar to TGFβ, depend on tumor type and stage (Wang et al. 2015).

Summary

Dickkopf 3 (Dkk3), also known as *REIC*, is the least conserved member of the Dkk family of proteins, which are secreted glycoproteins that regulate the canonical Wnt/β-catenin signaling pathway. The prototypic member Dkk1 is a powerful inhibitor of Wnt signaling, but Dkk3 binds atypically to Dkk1 receptors, showing an interaction with Krm1/2 but not with LRP5/6, and induces Wnt signaling activation, inhibition, or even has no effect, depending on the cell type and tissue. Dkk3 is expressed in numerous cell types in developing and adult tissues, but the $Dkk3^{-/-}$ mouse had a very mild phenotype, which initially made the physiological role for Dkk3 unclear. However, in recent years, a flurry of publications has described new roles for Dkk3 in cardiac protection, immune regulation, and neurogenesis. Furthermore, numerous studies have demonstrated that Dkk3 acts as a tumor suppressor in a large number of cancer types and induces tumor cell-specific apoptosis, and Dkk3 expression levels have been proposed as a biomarker for cancer prognosis. Additionally, a clinical trial is currently underway that uses adenoviral vectors to overexpress Dkk3 in tumors. Because of the role of Dkk3 in regulating key homeostatic events, such as B- and T-cell maturation and neuronal survival, there has been increasing interest in Dkk3 action and its potential as a therapeutic target. An unexplored area of research is the precise role of Dkk3 in the CNS and whether it acts by regulating the Wnt pathway or independent of Wnt signaling. Future studies will further characterize mechanisms of action of Dkk3 and identify interacting proteins, all of which will lead to improved understanding of this interesting molecule.

See Also

- ▶ Beta-Catenin
- ▶ WNT

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D

Dipeptidyl Peptidase 4

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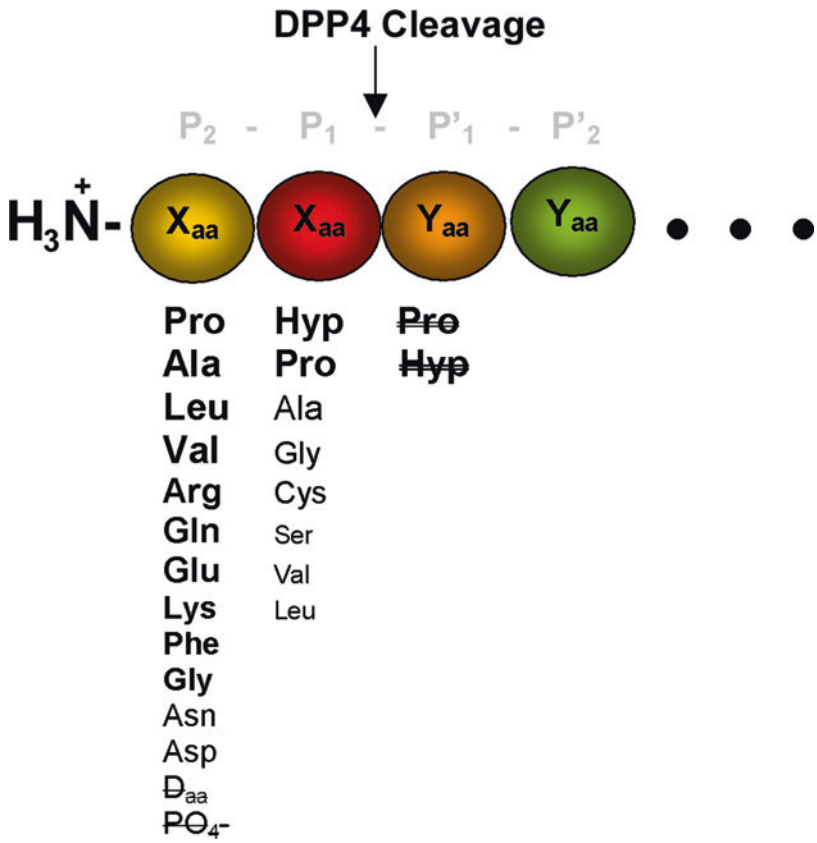
Synonyms

ADABP; CD26; DPP4; DPPIV

Historical Background

Nature has evolved a number of regulatory, neuronal, and immune peptides with a proline residue at the penultimate position determining their structural conformation and biological activity. Generally, the proline peptide bonds are resistant to proteolytic cleavage, yet an exclusive number of postproline-specific peptidases have emerged to regulate these peptides. The best-characterized one is dipeptidyl peptidase 4 (DPP4), though additional functional homologues of DPP4-like



Dipeptidyl Peptidase 4, Fig. 1 Substrate specificity of dipeptidyl peptidase 4 (DPP4). Substrate specificity of DPP4, indicating preferences of amino acids (aa) at P2, P1, and P′1 position as well as requirements for substrate hydrolysis

enzymes have been discovered, some structurally related, others without any structural homology. Since DPP4 is involved in glucose homeostasis and immune response, it is of medical and pharmaceutical interest to distinguish between these enzymes (Klemann et al. 2016; Lambeir et al. 2003; Wagner et al. 2016b).

DPP4 [EC 3.4.14.5] was first discovered in 1966 by Hopsu-Havu and Glenner and denoted with glycyl-prolyl-β-naphtylamidase. Other names include postproline dipeptidyl aminopeptidase IV, glycoprotein gp110, peptide Hep105, glycoprotein gp108, adenosine deaminase binding protein ADAbp, and T cell activation antigen CD26. The enzyme is a serine protease that hydrolyses proline, hydroxyproline, dehydroproline, to a lesser extent alanine, and at slow rates serine, glycine, threonine, valine, and leucine at the penultimate position. To allow proteolysis, the N-terminus must be protonated, P1 and P2 in trans configuration, amino acids at P2 neither be phosphorylated nor in D-configuration, whereas P′1 position must not be occupied by proline or hydroxyproline (Fig. 1) (Klemann et al. 2016; Lambeir et al. 2003).

Structure, Expression, and Distribution

DPP4 [EC 3.4.14.5] belongs to the serine peptidase clan SC, subfamily 9B referred to as the DPP4-gene family, that includes dipeptidyl peptidase 4 (DPP4), fibroblast activation protein alpha (FAP), dipeptidyl peptidase 8 (DPP8), and dipeptidyl peptidase 9 (Klemann et al. 2016; Yu et al. 2010).

DPP4 as representative member of the DPP4 gene family is the best-characterized postproline-dipeptidyl peptidase with most known in vivo substrates (Table 1) (Klemann et al. 2016; Lambeir et al. 2003). The human gene of DPP4 is localized at 2q24.2, encompassing 81.8 kb, spanning 26 exons, that code for two mRNAs of 2.8 kb and 4.2 kb, respectively. Interestingly, the nucleotides coding for the residues of the catalytic triad are found on three different exons. The resulting protein has 766 amino acids and the primary structure consists of a short six amino acid cytoplasmic tail, a 22 amino acid transmembrane, a 738 amino acid extracellular portion comprised of a flexible stalk, glycosylation-rich region, cysteine-rich region, and catalytic region with the catalytic triad. Although DPP4 is a type II trans-membrane glycoprotein, a soluble shedded form is also found in the blood circulation. The human crystal structure of DPP4 reveals two domains, an eight bladed β-propeller and a catalytic α/β-hydrolase domain. The active site is composed of the catalytic triad Ser630, Asp708, and His740, two anchoring residues Glu204 and Glu205, as well as substrate stabilizing residues Arg125, Asn710, and Tyr457. The β-propeller is open and consists of two subdomains made up of blades II–V and VI–VIII, I, and represents the glycosylation- and cysteine-rich regions, respectively. Each blade has four antiparallel β-sheets, except for blade IV that has an additional α-helix and two β-sheets forming an extended arm for dimerization. Almost all binding partners of DPP4 and monoclonal antibodies bind to the β-propeller domain, with adenosine deaminase and Caveolin-1 (Cav-1) binding to the glycolsylation-rich region and HIV-gp120, collagen, fibronectin (FN), streptokinase (SK), and plasminogen receptor (PgR) to the cysteine-rich region. Cav-1 and HIV-TAT also bind to the active site (Klemann et al. 2016; Weihofen et al. 2004; Weihofen et al. 2005). There are two openings, a side opening and a propeller tunnel (Rasmussen et al. 2003). The substrate NPY is suggested to enter via the side opening (Aertgeerts et al. 2004). Tetramerization has also been described in porcine DPP4 (Engel et al. 2003) (Fig. 2).

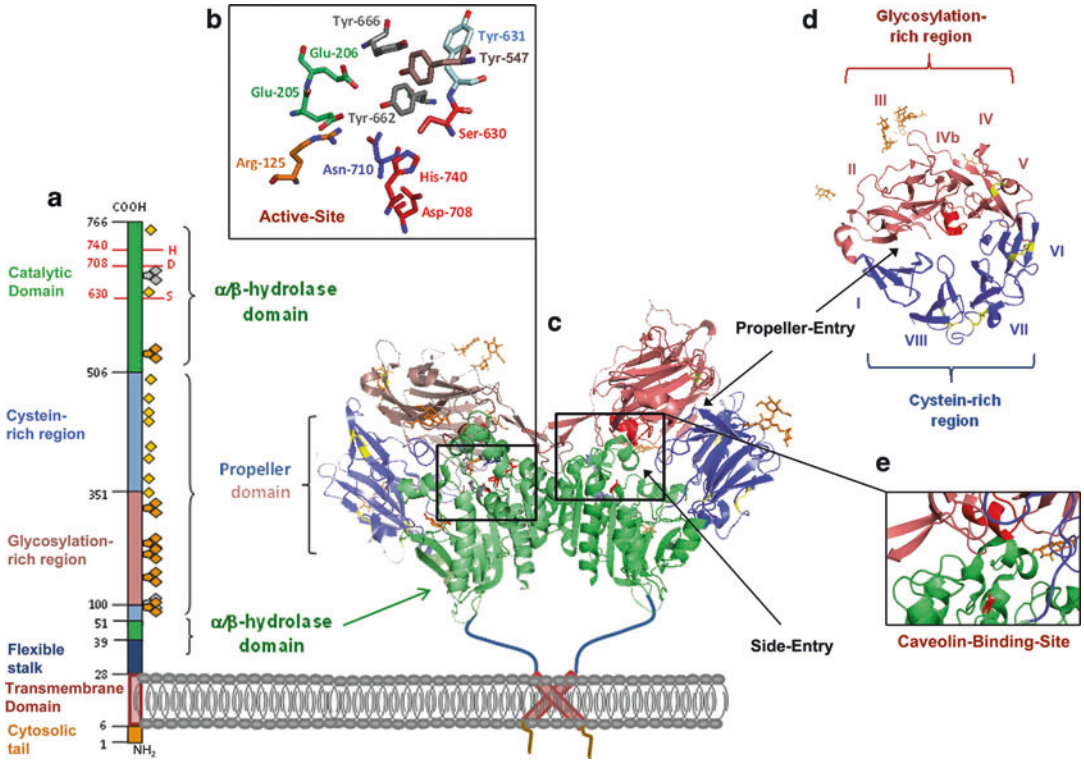
In addition, DPP4 is reported to be a homodimer with glycosylation contributing to 23% of the molecular weight of 110 kDa per subunit (Klemann et al. 2016). Post-translational modifications include nine N-terminal glycolsylation sites and five disulfide bonds, though O-glycosylation and phosphorylation have also been reported (Klemann et al. 2016).

DPP4 is ubiquitously distributed with the highest expression in kidney, lung, liver, and small intestine, whereas low expression is found in brain, heart, and skeletal muscle. It is predominantly found on endothelial and epithelial cells throughout the body, but also found on immune cells like T cells, activated B-, activated natural killer (NK) cells, and myeloid cells (Klemann et al. 2016; Lambeir et al. 2003; Wagner et al. 2016b; Waumans et al. 2015). DPP4 contains neither TATAA nor CCAAT box as a promoter, but has a C- and G-rich region, containing several consensus binding sites for transcriptional factors like NFκB, SP-1, EGFR, and AP-1 factor NF-1 (Bohm et al. 1995; Lambeir et al. 2003; Rohrborn et al. 2015). Expression is regulated at RNA level and is organ specific. Within an organ, it is dependent on cell type, differentiation state, and activation state. Several cytokines such as IL-12 are known to regulate DPP4 expression in a cell-type-specific manner. In some pathogenic tissues, the binding of the transcription factors is enhanced by certain cytokines like STAT1α by interferons, α, β, and γ in lymphocytic leukemia cells (Frerker et al. 2007; Klemann et al. 2016; Lambeir et al. 2003; Rohrborn et al. 2015). Furthermore, expression of DPP4/CD26 is modulated on malignant hematologic or solid tumor cells and proposed as potential diagnostic biomarker (Mortier et al. 2016; Wagner et al. 2016b; Yu et al. 2010).

In addition, CD26/ DPP4 exists in a soluble active form, shed from the membrane into plasma by MMPs (MMP1, MMP2, MMP9, MMP14) (Rohrborn et al. 2014). Recently, the bone marrow – but not the kidney – could be determined as one of the sources of soluble serum CD26/DPP4 in rats (Wang et al. 2014). Alterations of DPP4 activity and CD26/DPP4 concentrations in human serum have been reported in numerous diseases (Klemann et al. 2016). High levels of soluble DPP4 are also

Dipeptidyl Peptidase 4, Table 1 Summary of DPP4-substrates, receptor-binding after DPP4-truncation and the subsequent response (De Meester et al. 2002; Harmar 2001; Klemann et al. 2016; Lambeir et al. 2003; Mortier et al. 2016; Sah et al. 2007; Sherwood et al. 2000; Wagner et al. 2016a). ⇑, increased receptor binding; ⇓, decreased receptor binding; ≈, receptor binding unchanged; neuropeptide Y, NPY; peptide YY, PYY; GALP, galanin-like-peptide; substance P, SP; vasoactive intestinal peptide, VIP; Endomorphin- 1/2, EM-1/2; Tyr-Melanocyte-inhibiting factor-1, Tyr-MIF-1; glucagon-like peptide 1/2, GLP-1/2; glucose-dependent insulinotropic peptide, GIP; pituitary adenylate cyclase activating peptide, PACAP; growth hormone releasing hormone, GHRH; insulin-like growth factor-1, IGF-1; gastrin releasing peptide, GRP; Brain natriuretic peptide, BNP; CXCL2, GRO-β; CXCL6, GCP-2; CXCL9, Mig; CXCL10, IP-10; CXCL11, I-TAC; CXCL12α; SDF-1α; CXCL12β, SDF-1β; CCL3L1, LD78β; CCL4, MIP-1α; CCL5, RANTES; CCL11, Eotaxin; CCL14, HCC-1; CCL22, MDC

DPP4-substrate	Receptors binding after DPP4-truncation	Physiological/pathophysiological output
Neuropeptides		
NPY	≈ Y_2-R, ≈Y_5-R, ⇓Y_1-R, ⇑ GIR	⇑ anxiety, ⇑ depression, ⇑ psychosis, ⇓ food intake, ⇑ angiogenesis, ⇓ vasoconstriction/vascular stress
PYY	≈ Y_2-R, ≈Y_5-R, ⇓Y_1-R	⇓ food intake, ⇑ energy expenditure
$GALP_{32}$	≈ Gal_1-R, ≈Gal_2-R, ⇑ Gal_3-R	⇑ anxiety, ⇑ depression, ⇑ stress, ⇑ alcohol consumption
SP	≈ NK-R1, ⇑ NK-R2, ⇑ NK-R3	⇑ nociception, ⇑ anxiety, ⇑ depression, ⇑ psychosis, ⇑ vasodilation
VIP	⇓ $VPAC_1$-R ⇓ $VPAC_2$-R	⇑ sleep, ⇓ vasorelexant of vascular/non-vascular smooth muscles; ⇓ insulin/glucagon secretion depending on plasma glucose level
EM-1	⇓ μ-opioid-R.	⇑ nociception
EM-2	⇓ μ-opioid-R	⇑ nociception
Tyr-MIF-1	⇓ μ-opioid-R	⇑ nociception
Spinorphin	?	⇑ nociception, ⇓ ACE-, APN-, NEP-, DPPIII-, DPP4-inhibition
Peptide Hormones		
GLP-1	⇓ GLP1-R	⇓ insulin secretion
GIP	⇓ GIP-R	⇓ insulin secretion
Glucagon	⇓ GCG-R	⇓ glucagon secretion
PACAP	⇓ PAC_1-R, ⇓ $VPAC_1$-R, ⇓ $VPAC_2$-R	⇓ insulin/glucose secretion in glucose dependent manner, ⇓sleep, ⇓ learning/memory, ⇑ anxiety, ⇓ aggression
GLP-2	⇓ GLP2-R	⇓ intestinal adaptation, ⇓ intestinal growth
Sectretin	⇓ SCTR	⇓secretion of HCO_3^-, ⇓ enzymes
GHRH	⇓ GHRH-R	⇓ growth hormone secretion
IGF-1	⇓ IGF1R	⇓ cell proliferation
GRP	⇓ GRP-R	⇓ secretion of PACAP/VIP, ⇓ secretion of gastric hormones
BNP	⇓ NPRA, ⇓ NPRB	⇓ vasodilation, ⇓ natriuresis,
Chemokines		
CXCL2	? (CXCR2)	? polymorphonuclear leukocytes, hematopoietic stem cells
CXCL6	? (CXCR1, CXCR2)	≈ Neutrophil trafficking
CXCL9	⇓ CXCR3	⇓ Th1 response, ⇓ Th1, CD8 + NK trafficking
CXCL10	⇓ CXCR3	⇓ Th1 response, ⇓ Th1, CD8 + NK trafficking
CXCL11	⇓ CXCR3	⇓ Th1 response, ⇓ Th1, CD8 + NK trafficking
CXCL12α	⇓ CXCR4	⇓ Bone marrow homing
CXCL12β	⇓ CXCR4	⇓ Bone marrow homing
CCL3L1	⇑ CCR1, ⇓ CCR3, ⇑ CCR5	⇑ Macrophage-NK migration; ⇑ T cell/DC interaction; ⇓ eosinophiles
CCL4	⇑ CCR1, ⇑ CCR2, ≈ CCR5	⇑ Monocytes, ⇑ peripheral lymphocytes, ≈ T cell/DC interaction, ⇓ hematopoietic progenitor cells proliferation
CCL5	⇓CCR1, ⇓ CCR3, ⇑ CCR5	⇑ Macrophage-NK migration; ⇑ T cell/DC interaction; ⇓ eosinophiles
CCL11	⇓ CCR3	⇓ Eosinophil and basophil migration
CCL14	⇓CCR1, ⇓ CCR3, ⇓ CCR5	⇓ Monocyte activation
CCL22	⇓ CCR4	⇓Th2 response, ⇓ Th2 cell migration, ⇓ T reg migration

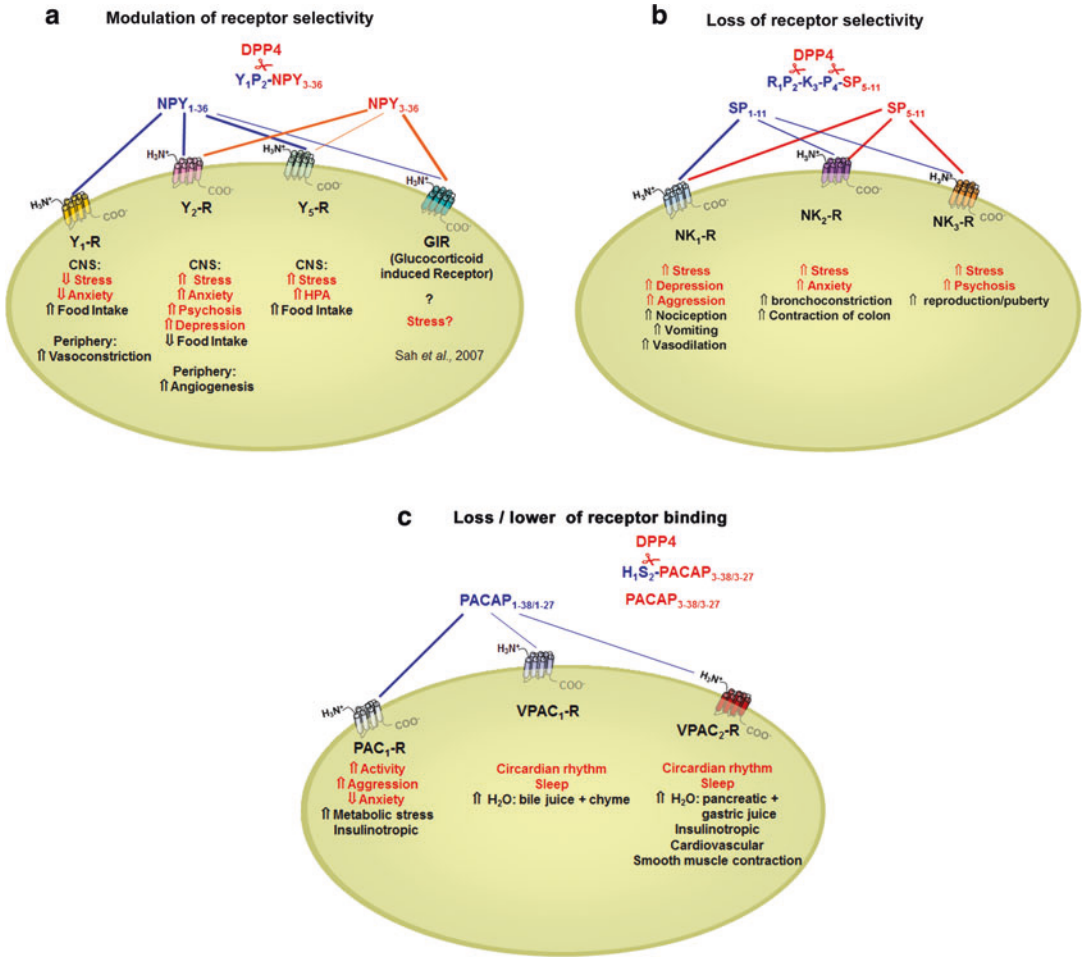


Dipeptidyl Peptidase 4, Fig. 2 Primary and quaternary structure of human dipeptidyl peptidase 4 (DPP4), based on Protein Data Bank: 1N1M. (**a**) Primary structure of DPP4 subunit, consisting of an intracellular tail (aa 1–6), transmembrane region (aa 7–28), flexible stalk (aa 29–39), glycosylated region (aa 101–350), cysteine-rich region (aa 55–100, 351–497), and catalytic region (aa 506–766); *orange symbols*, N-glycosylation; *grey symbols*, potential unoccupied N-glycosylation; *yellow diamonds*, cysteine residues involved in S-bridges; *red numbers* and *letters* indicate the catalytic triad. (**b**) Active site zoomed in, depicting the residues involved in catalysis, catalytic triad Ser630, Asp708, His740 are shown in *red*, Tyr547 responsible for oxyanion hole in *brown*, Tyr662 and Tyr666 forming the hydrophobic pocket in *grey*, Arg125 and Asn710, contributing to an electrostatic sink in *orange* and *blue*, respectively, and Glu205 and Glu206 ensuring N-terminal anchoring in *pale green*. (**c**) Quaternary structure of homodimeric human recombinant DPP4 as determined by Rasmussen et al. 2003, showing the α/β-hydrolase domain (aa 39–51 and 506–766) in *green* and β-propeller domain (aa 55–497) with the glycosylation-rich subdomain (*salmon*) and the cystein-rich subdomain (*blue*). (**d**) Propeller domain viewed from the top, illustrating the eight propeller blades designated with roman numbers and two subdomains. S–S bridges are illustrated in *yellow* and carbohydrates in *orange*. (**e**) Caveolin-1 binding site at aa 201–210 and Ser630 in *red*. Structures were drawn with PyMOLTM 2008 DeLano Scientific LLC, using Protein Data Base: 1N1M

found in seminal fluids, whereas only small levels are detected in CSF and urine (Lambeir et al. 2003).

DPP4 Substrates and Binding-Partners

DPP4 is known to cleave neuropeptides such as neuropeptide Y (NPY), substance P (SP), vasoactive intestinal peptide (VIP), galanin-like-peptide (GALP), endomorphins 1/2; peptide hormones like glucagon-like peptides 1/2 (GLP-1/ GLP-2), glucose-dependent insulinotropic peptide (GIP), glucagon, pituitary adenylate cyclase activating peptide (PACAP), growth-hormone-releasing hormone (GHRH), insulin-like growth factor-1 (IGF-1), gastrin releasing peptide (GRP), and the chemokines CXCL2, CXCL6, CXCL9, CXCL10, CXCL11, CXCL12, CCL3L1, CCL4, CCL5, CCL11, CCL14, and CCL22 as summarized in Table 1 (Klemann et al. 2016; Lambeir et al. 2003; Mortier et al. 2016). Truncation of substrates



Dipeptidyl Peptidase 4, Fig. 3 Influence of substrate-truncation by DPP4 on receptor binding. (**a**) N-terminal truncation of NPY by DPP4 results in modulation of Y-receptor selectivity. (**b**) subsequent cleavage of substance P by DPP4 abolished receptor selectivity. (**c**) N-terminal truncation of PACAP by DPP4 results in loss of receptor binding (De Meester et al. 1999; De Meester et al. 2002; Klemann et al. 2016; Lambeir et al. 2003; Sah et al. 2007). *NPY* neuropeptide Y, *SP* substance P, *PACAP* pituitary adenylate-cyclase peptide, *GIR* gluco-corticoid-induced receptor, *R* receptor, *red scissors* DPP4-cleavage site

by DPP4 results either in change of receptor selectivity (NPY, PYY CCL3L1, CCL4, and CCL5), loss of selectivity (SP), lower/lack of binding with no response (PACAP, VIP, GLP1, GLP-2, GIP, GRP, and IGF-1), or receptor activation (GALP) as depicted in Fig. 3. Hence, based on its substrates, DPP4 is involved in glucose metabolism, cardiovascular system, nutrition, neuroendocrine system, nociception, and chemotaxis (Table 1) (De Meester et al. 2002; Klemann et al. 2016; Lambeir et al. 2003; Mortier et al. 2016). Finally, most DPP4 substrates are further proteolytically degraded after N-terminal removal of the dipeptide by DPP4. Thus, GLP-1 is further degraded by NEP in the kidney and SP by ACE and NEP, respectively (Klemann et al. 2016).

Furthermore, CD26/DPP4 is implicated in various immune responses via its interaction with ADA, M6P/IGFRII, CD45, caveolin-1, CARMA1, or CxCR4 and also acts as a marker for activated T-cells (De Meester et al. 1999; Klemann et al. 2016; Ohnuma et al. 2008a) as summarized in Table 2 and shown in Figs. 1 and 4, respectively. In addition, it functions as an

Dipeptidyl Peptidase 4, Table 2 Summary of binding partners of DPP4

Function	Binding partner	Binding site
Immunology	ADA	Glyco-rich region[a]
	CD45	?
	Caveolin-1	Glyco-rich + Ser_{630}
	CARMA1	Cytoplasmic tail
	MP6	Carbo-M[b]
	M6P/IGFRII	M6P + Carbo-M[b]
	CXCR4	?
	Tromboxane A_2-R	?
	HIV-TAT	Sia-M[c] + active site
	HIV-gp120	Cys-rich region[d]
Cell adhesion/ cell to cell communication	Collagen	Cys-rich region
	Fibronectin	Cys-rich region
	PgR – Pg/Pl	Cys-rich region
	Streptokinase	Cys-rich region
	Vitronectin	Sia-M[c]
	Glypican-3	?
	FAP	?
	Tetramerization	Blade IV
Peptide transport	Na1/H1 exchanger isoform NH3	?

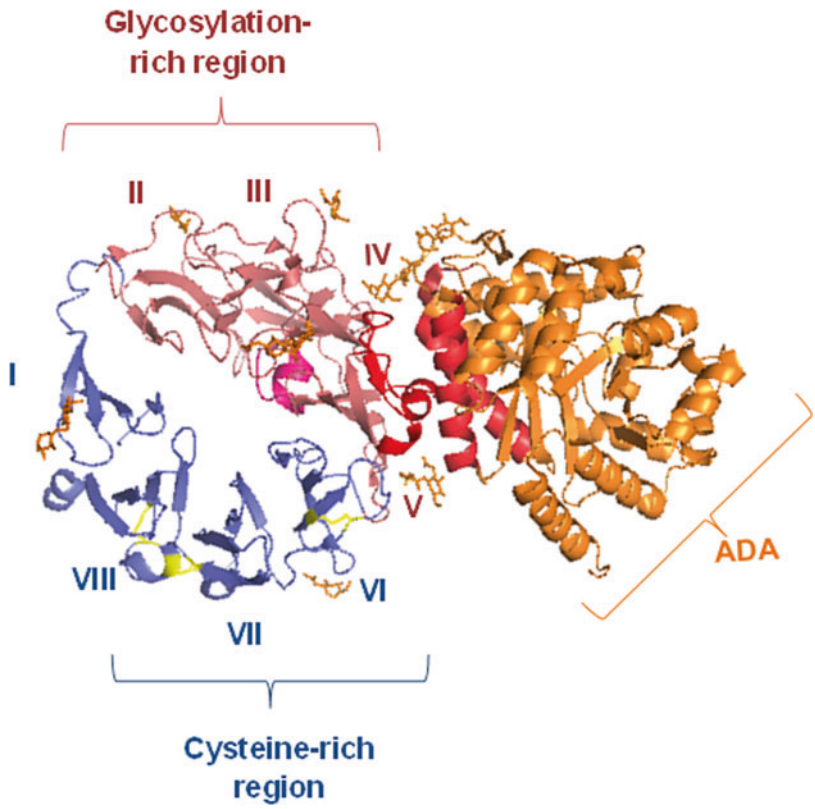
[a]gyco-rich region, glycosylation-rich region
[b]Carbo-M, carbohydrate-moiety
[c]Sia-M, sialic acid moiety
[d]Cys-rich region, cysteine-rich region
ADA, adenosine deaminase, *PgR* plasminogen-receptor, *Pg* plasminogen, *Pl* plasmin

extra-cellular adhesion molecule by binding to collagen, fibronectin, plasminogen receptor, glypican-3, FAP, and DPP4 via tetramerization (Fig. 5) (Engel et al. 2003; Klemann et al. 2016). Association of DPP4 with Na1/H1 exchanger isoform NH3 in kidney and intestine serves as peptide transporter for the assimilation of proline-containing dipeptides.

DPP4 Regulates Glucose-Homeostasis

DPP4 has been identified as a therapeutic target for type-2 diabetes mellitus due to its ability to cleave and inactivate insulinotrophic incretins such as GIP and GLP-1 as well as PACAP, VIP, GHRH, and GRP as illustrated in Fig. 6. The incretins are released into the blood circulation from the intestinal jejunum upon glucose intake and enhance the insulin secretion in β-cells from the Langerhans' islets. Their half-life of a few minutes is strictly dependent upon DPP4-like enzymatic activity. Furthermore, incretins exhibit positive effects on pancreatic β cells in the islets, including upregulation of insulin expression by stimulation its gene transcription. Once released into the blood circulation, GIP and GLP are degraded rapidly by DPP4 and thus DPP4-inhibition prolongs GIP/GLP half-life and has insulinotrophic effect (Klemann et al. 2016; Rohrborn et al. 2015). Currently, there are nine DPP4 inhibitors commercially available on the market, with sitagliptin Januvia® (Merck & Co., Inc., Kenilworth, NJ, USA), saxagliptin Onglyza® (Bristol Myers Squibb, New York, NY, USA), and linagliptin TradjentaTM (Böhringer Ingelheim, Ingelheim, Germany) being approved by the FDA and EMA. Vildagliptin Galvus® (Norvatis, Basel, Switzerland) is only approved by the EMA, whereas alogliptin Nesina® (Takeda Pharmaceuticals, London, UK) only by the FDA. Anagliptin Suiny® (Sanwa Kagaku Kenkyusho Company Ltd. and Kowa Company Ltd., Nagoya, Japan), teneligliptin Tenelia® (Mitsubishi Tanabe Pharma and Daiichi Sankyo, Dusseldorf, Germany), trelagliptin Zafatek® (Takeda Pharmaceuticals), and omarigliptin Marizev® (Merck & Co., Inc.) being approved in Japan. All of them are administered orally and taken daily, except for omarigliptin, which has weekly doses.

Generally, DPP4 inhibitors reduce DPP4 activity at approximately 70–90% of baseline and also lower the hemoglobin A1c (HbA1c) 0.74%. All DPP4 inhibitors are excreted via the renal route except for linagliptin, which is eliminated via the biliary route. DPP4-inhibitors are weight neutral and have no hypoglycemic effect as they are glucose-dependent and are overall well tolerated, with mild side effects (Klemann et al. 2016; Rohrborn et al. 2015). However, DPP4-dependent glucose-homeostasis is rather complex as insulin secretion is regulated by many DPP4-substrates, involved in the endocrine, paracrine, and neuronal systems (Fig. 6) (Lambeir et al. 2003).



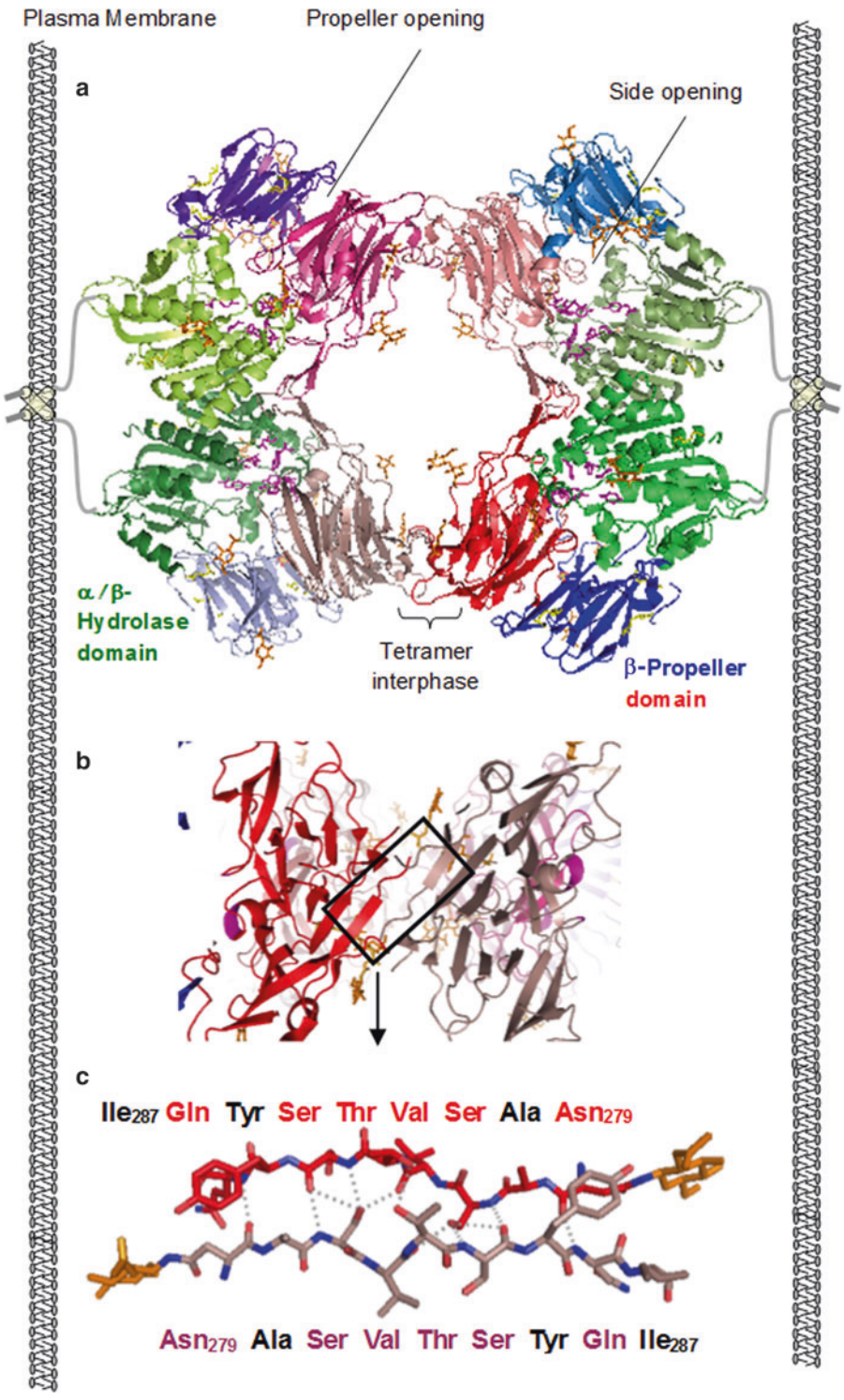
Dipeptidyl Peptidase 4, Fig. 4 Crystal structure of human dipeptidyl peptidase 4 (DPP4) and bovine adenosine deaminase (ADA) obtained from Protein Data Bank: 1W1I. Top view of DPP4-propeller domain, showing ADA binding site at blade IV of carbohydrate-rich region as well as ADA interactions with carbohydrates at N229 of DPP4 (Weihofen et al. 2004)

DPP4 in the Neuroendocrine System

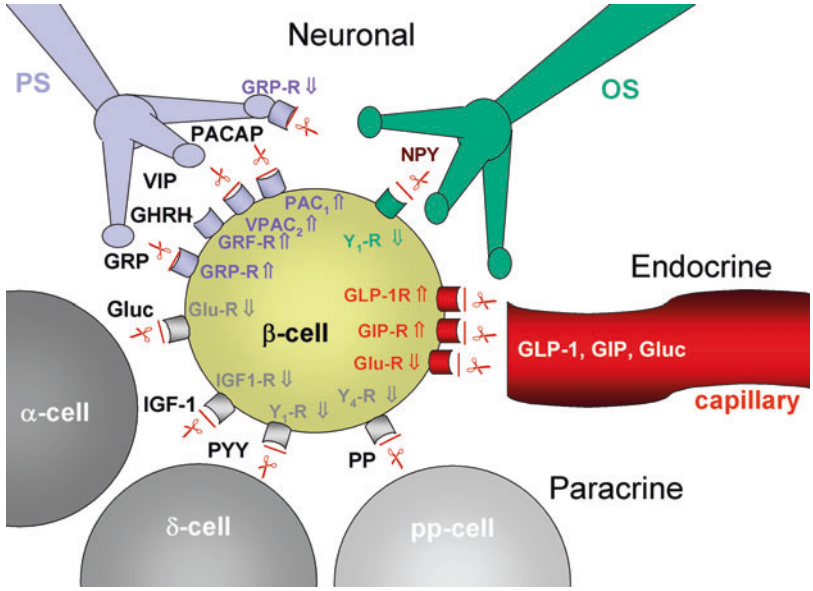
Although low levels of DPP4 are found in brain parenchyma, elevated activity and expression of DPP4 could be detected in the meninges, brain capillaries, choroid plexus, and circumventricular organs (CVOs) (Cynis et al. 2013; Wagner et al. 2015). These results imply that DPP4 is at the interphase between the CNS and the periphery via the blood circulation and CSF, respectively, thereby modulating and inactivating neuropeptides and neurotrophic growth factors (Table 1). Expression and activity of DPP4 in the CVO's median eminence (ME) and area postrema (AP) explain the involvement of DPP4 in social and stress-related hypothalamic-pituitary-adrenal axis (HPA) and neuro-sympathico axis (NSA), resulting in the release of stress hormones (norepinephrine, epinephrine, and cortisol), neuropeptides (NPY, SP), and altered cytokines (IL-1β, IL-6, TNF-α, MCP-1) as illustrated in Fig. 7 (Elenkov et al. 2000; Frerker et al. 2009; Wagner et al. 2015, 2016a).

Immunological Functions of DPP4

CD26/DPP4 is expressed on only a fraction of resting CD4+CD45RO+ memory T cells, but is strongly upregulated upon T cell activation. Also, CD26/DPP4 has been described as a negative selection marker for human regulatory T cells (Tregs), whereas human T helper type 17 (Th17) cells showed high expression of CD26/DPP4. Recently,



Dipeptidyl Peptidase 4, Fig. 5 **Schematic presentation of crystal structure of native porcine DPP4, depicting the residues important for tetramerization (protein data bank: 1ORV).** (**a**) Soluble DPP4 forms a symmetric assembly as a dimer of dimers. *Arrows* indicate respective propeller and side opening. Each subunit



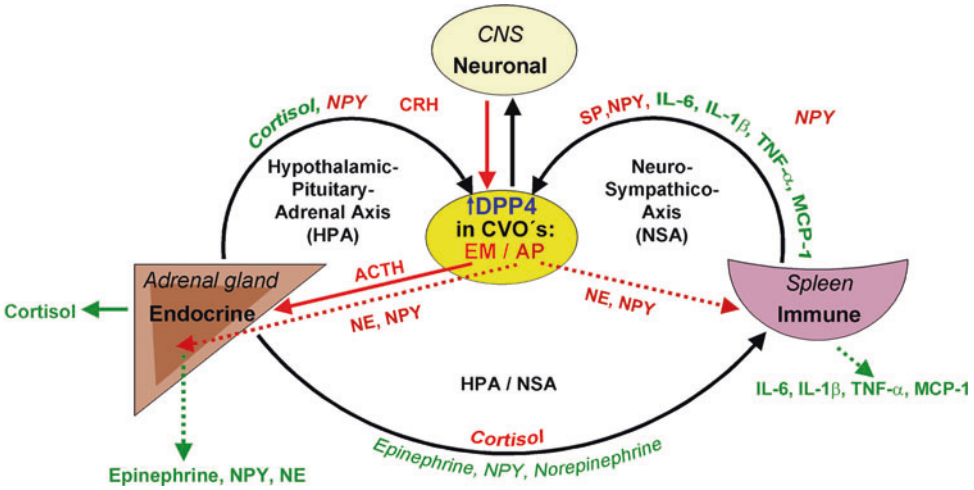
Dipeptidyl Peptidase 4, Fig. 6 Influence of DPP4 on neuronal, endocrinal, and paracrinal regulation of insulin secretion. *Red scissors*, inactivation of peptide/neuropeptide by DPP4; ⇑, enhanced insulin secretion via stimulation of peptide/neuropeptide receptor; ⇓, binding of neuropeptide/peptide to its receptor results in decreased insulin secretion (Lambeir et al. 2003)

mucosal-associated invariant T cells (MAITs) have also been shown to express high levels of CD26/DPP4 in humans (Klemann et al. 2016).

DPP4 is known to stimulate T-cell activation as CD26, and it could recently be shown to trigger T-cell activation and proliferation directly via CARMA1-mediated nuclear factor (NF)-κB. Additionally, CD26/DPP4 on T-cells interacts directly with antigen-presenting cells (APCs) via caveolin-1 that binds to the glycosylation-rich region of the β-propeller as well as the active Ser630 of DPP4. Upon linkage, Tollip and interleukin-1 receptor-associated kinase 1 (IRAK-1) disengage from caveolin-1 leading to subsequent IRAK-1 phosphorylation. As illustrated in Fig. 8, this results in an upregulation of the co-stimulatory molecule CD86, which enhances the bond of the immunological synapse (Klemann et al. 2016; Ohnuma et al. 2008a, b).

CD26 binds at the intracellular PTP2 domain of CD45RO upon internalization, thereby causing recruitment of both enzymes to the lipid rafts. Association of CD26 and CD45RO as well as their compartmentation to lipid rafts is IL-12 dependent and results in signal transduction, by inducing tyrosine phosphorylation of Erk1/2, TCRζ, ZAP70, and p56lck (Ishii et al. 2001; Salgado et al. 2003).

Dipeptidyl Peptidase 4, Fig. 5 (continued) consists of a catalytic α/β-hydrolase (*green shades*) and β-propeller domain as indicated. The β-propeller is again subdivided into a carbohydrate rich subdomain (*salmon shades*) and cysteine-rich subdomain (*blue shades*). Carbohydrates and disulfite bonds at the β-propeller tunnel are indicated in *orange* and *yellow*, respectively. (**b**) Tetramerization interphase of β-propeller domains of subunit A and subunit C as viewed from the bottom, illustrating the involvement of the strands Asn279-Gln286 to form an antiparallel sheet. (**c**) residues of strands Asn279-Gln286 of subunits A and C forming an antiparallel sheet, responsible for tetramerization and stabilized by H-bonds (*gray dots*). The transmembrane helices and their orientation to the membrane were drawn in to illustrate how tetramerization of DPP4 may mediate cell-cell contacts (Chung et al. 2010) (Figures were prepared with Pymol, using pdb: 1ORV (Engel et al. 2003))



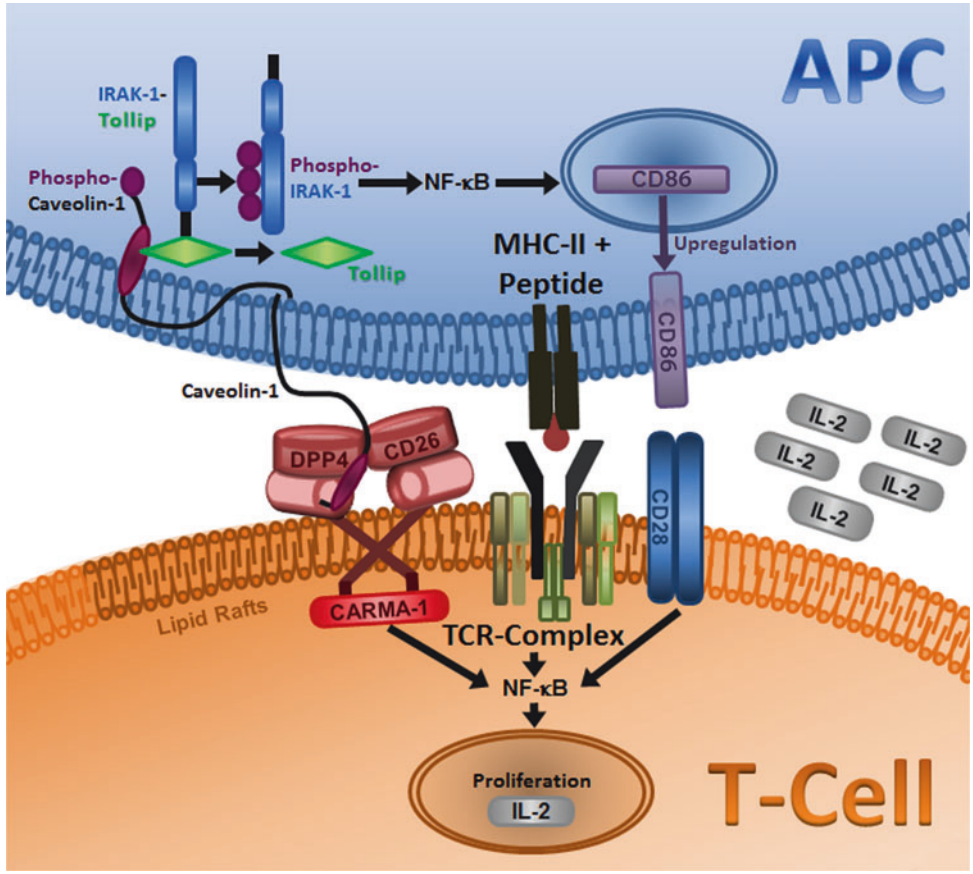
Dipeptidyl Peptidase 4, Fig. 7 Psycho-neuro-endocrino-immunological role of DPP4 at the CVO's median eminence (EM) and area postrema (AP). EM and AP form the interphase between the neuronal, endocrinal and immunological systems, involving the two stress axis hypothalamic-pituitary-adrenal (HPA) and the neuro-sympathico-axis (NSA). Activation of HPA and NSA results in the release of stress hormones cortisol and epinephrine, epinephrine, norepinephrine, cytokines, and NPY, which in turn interact with the brain via EM and AP and the lymph organs such as the spleen (Cynis et al. 2013; Elenkov et al. 2000a; Frerker et al. 2009; Wagner et al. 2015, 2016a). *NE* noradrenalin, *NPY* neuropeptide Y, *SP* substance P, *CVO* circumventricular organ, *CRH* cortico-releasing hormone, *ACTH* Adreno-corticotropic hormone, *DPP4* dipeptidyl peptidase 4, *solid red arrow* stimulating HPA-axis, *dotted red arrows* stimulating NSA, *green arrows* secretion by HPA (*solid line*) and NSA (*dotted line*), *black arrow* feedback by neuropeptides, stress hormones, cytokines or chemokine resulting in supression (*dark red*) or stimulation (*green*)

Although CD26/DPP4 is mainly upregulated in T helper type 1 (Th1) cells, T-cell activation in T helper type 2 (Th2) cells is mediated by binding of mannose-6 phosphate and insulin-like growth factor II receptor (M6P/IGFRII) to CD26, followed by subsequent internalization (Ikushima et al. 2000).

Binding of extracellular ADA to AB2 receptor on dentritic APC cells and CD26 on T cells to form a ternary complex results in co-stimulation of T cells, T cell proliferation, and T cell protection (Pacheco et al. 2005).

CD26/DPP4 is only found on activated B-cells and animal models deficient of CD26/DPP4 point to impaired cytokine production and B-cell numbers (Klemann et al. 2016). NK cells usually express only low amounts of CD26/DPP4, but surface expression increases significantly up to 30% after IL-2, IL-12, or IL-15 stimulation. Upregulation CD26/DPP4 on NK cells appears to correlate with increased CD16-dependent lysis. This may be caused by the mediation of protein tyrosine phosphorylation and an involvement of CD26/DPP4 in the production of cytokines by NK cells (Klemann et al. 2016; Wagner et al. 2016b). Additionally, the capacity of single NK cells to lyse tumor target cells is reduced in a congenic rat model, suggesting that CD26/DPP4 enzymatic activity sustains NK cytotoxicity. NK cells exert their cytotoxicity via secretory lysosomes, and CD26/DPP4 was identified on the membrane of secretory lysosomes in NK cells by proteomic analysis. Concerning the NK cell maturation, the percentage of NK cells in DPP4-deficient animals was increased significantly, while total leucocyte numbers were decreased in a congenic DPP4-deficient rat model, as well as in knock-out mice (Frerker et al. 2009; Klemann et al. 2016; Waumans et al. 2015).

CD26/DPP4 was shown to be chemorepellent for human and murine neutrophils, whereas DPP4 truncation affected recruitment of eosinophils via its substrate eotaxin (CCL11). Expression of CD26/DPP4 is increased upon activation on



Dipeptidyl Peptidase 4, Fig. 8 Scheme of CD26 interacting with caveolin-1 resulting in T cell costimulation and activation as proposed by (Ohnuma et al. 2008a): After antigen uptake via caveolae by antigen-presenting cells (APCs), caveolin-1 is exposed on the cell surface and aggregates in the immunological synaps in lipid rafts. Consequently, caveolin-1 binds to the β-propeller and active center of CD26 and its phosphorylation results in dissociation of interleukin (IL)-21 receptor-associated kinase 1 (IRAK-1) and Tollip. This leads to activation of nuclear factor (NF)-κB and upregulation of CD86, supporting the immunological synapse and thus T cell co-stimulation. In T cells, after caveolin-1 to CD26 binding, CARMA1 is recruited to the cytosolic portion of CD26. Activation of NF-κB, in turn, leads T cell proliferation and IL-2 production

dendritic cells and monocytes/macrophages as well as in Kupffer- and microglia cells, where it is localized in lysosomes (Klemann et al. 2016; Waumans et al. 2015, 2016).

However, enzymatic activity of DPP4/CD26 also influence immune response, and therefore, it has not been surprising that one of the side effects of DPP4-inhibitors includes increase of infection such as SP in rhinosinusitis and angioedema, bioactive SDF-α in arthritis and NPY/PYY, as well as SP in blood pressure. Furthermore, DPP4/CD26 was also shown to regulate HIV infection by truncating CCL5 (RANTES) and CXCL12α (SDF-1α), respectively. Yet, while truncation of CCL5 (RANTES) by DPP4 results in protection of the M-tropic virus via CCR5, truncation of CXCL12α by DPP4 enhances entry of the T-trophic virus via CXCR4 (De Meester et al. 1999; Mortier et al. 2016). Apart from type-2-diabetes mellitus and HIV-infection, DPP4/CD26 is also involved in arthritis, atherosclerosis, inflammatory bowel disease, multiple sclerosis, stress-related disease, asthma, atopic dermatitis, and cancer as recently reviewed (Klemann et al. 2016; Ohnuma et al. 2011; Wagner et al. 2016b; Waumans et al. 2015; Yu et al. 2010).

Summary

DPP4/CD26 is a multifunctional protein that is involved in signal transduction by means of its enzymatic activity and binding partners. As a postproline dipeptidyl peptidase, it regulates the bioactivity of its substrates, resulting either in modulation of receptor selectivity such as in case of NPY, PYY, CCL3L1, CCL4, CCL5, loss of receptor selectivity like SP or lower/loss of receptor binding such as GLP-1, GIP, and PACAP. Association of CD26 on T-cells with caveolin-1 on antigen-presenting cells results in T-cell activation mediated by NF-κB and subsequent upregulation of CD86. Binding of CARMA1 to cytoplasmic tail of CD26 causes T-cell proliferation mediated by NF-κB and secretion of IL-2. Binding of CD26 to ADA, CD45RO, and M6P/IGFRII also leads to T-cell activation, T-cell proliferation, and T-cell protection. Thus, based on its multifunctional roles, DPP4/CD26 is involved in many physiological and pathological conditions including glucose-homeostasis and neuroendocrine-, cardiovascular- and immune-systems.

See Also

- ▶ CARMA1
- ▶ Caveolin-1
- ▶ Dipeptidyl peptidase 8
- ▶ Dipeptidyl peptidase 9

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Dipeptidyl Peptidase 8

► DPP8

Dipeptidyl Peptidase 9

► DPP9

Dipeptidyl Peptidase-Related Protein 1

► DPP8

Dipeptidyl Peptidase-Related Protein 2

► DPP9

Diphtheria Toxin Receptor

► HB-EGF (Heparin-Binding EGF-Like Growth Factor)

dJ1187J4.5

► Alpha-1-Syntrophin

DJ4

► DNAJB6

Dkk3

► Dickkopf 3

DLG4 (Discs Large Homolog 4)

► PSD-95 (Postsynaptic Density Protein-95)

DLK

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

DLK (Dual Leucine Zipper-Bearing Kinase)

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Synonyms

DLK; Dual leucine zipper bearing kinase; Dual leucine zipper-bearing kinase; Dual leucine zipper kinase; MAP kinase upstream kinase; Map3k12; Mitogen-activated protein kinase kinase kinase 12; MUK; Zipper protein kinase; ZPK

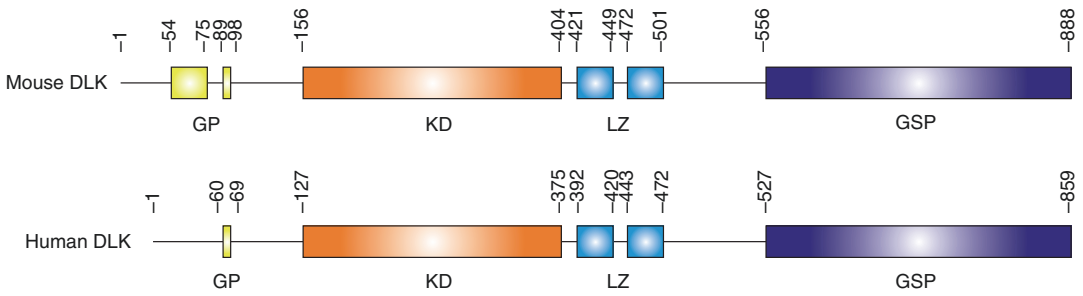
Historical Background

DLK is a serine/threonine kinase that belongs to the mixed-lineage kinase (MLK) family of mitogen-activated protein kinase kinase kinases (MAPKKKs) (Gallo and Johnson 2002). It was discovered in 1994 as a protein differentially expressed during the retinoic acid-induced neuronal differentiation of human NT2 teratocarcinoma cells and originally denoted zipper protein kinase (ZPK) (Reddy and Pleasure 1994). Parallel and subsequent studies led to the identification and cloning of the mouse and rat homologs of ZPK, respectively, termed DLK (Holzman et al. 1994) and MAP kinase upstream kinase (MUK) (Hirai et al. 1996).

Structure, Expression, and Subcellular Localization

DLK is a protein of about 120 kDa that shares with other MLKs structural characteristics unique among the protein kinase family, namely a catalytic domain hybrid between those found in serine/threonine and tyrosine kinases, and two leucine zipper motifs involved in protein dimerization and activation (Gallo and Johnson 2002). The kinase domain of mouse DLK contains a functional nuclear localization signal at amino acids 185–200 that is conserved in human, rat, and *Drosophila* (Wallbach et al. 2016). DLK also possesses glycine-, serine-, and proline-rich sequences located upstream and downstream of the catalytic domain that are presumably important for mediating protein interactions and/or for controlling subcellular localization (Fig. 1).

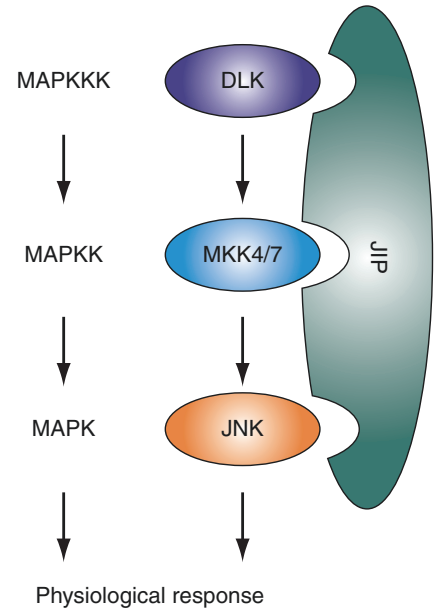
Northern blot analysis of human and mouse tissues revealed that the highest levels of DLK mRNA are observed in brain and kidney (Reddy



DLK (Dual Leucine Zipper-Bearing Kinase), Fig. 1 Primary structure of mouse and human DLK. Both proteins share 99% identity within their catalytic domains and 95% identity throughout their overall primary structure. The most significant difference between mouse and human DLK resides in the amino (N)-terminal extracatalytic region, where an additional stretch of 33 amino acids is found in the murine sequence. Numbers indicate positions relative to the first amino acid. *GP* Gly, Pro-rich domain; *KD* Kinase domain; *LZ* Leucine zipper motif; *GSP* Gly, Ser, Pro-rich domain

and Pleasure 1994; Holzman et al. 1994). DLK mRNA was also detected by in situ hybridization in mouse skin, stomach, small intestine, liver, pancreas, and testis (Nadeau et al. 1997). In all these tissues, the expression of DLK mRNA increases with development and correlates with areas occupied by differentiated rather than proliferating cells (Nadeau et al. 1997). For example, in developing mouse skin, DLK mRNA expression was detected in the suprabasal cell layers of the epidermis but not in the innermost basal layer, which contains mitotically active cells.

Consistent with the expression results described above, immunostaining experiments on mouse embryos revealed that the DLK protein is predominantly detected in neural tissues, including brain, spinal cord, and dorsal root ganglion (Hirai et al. 2002). In the developing brain, DLK is most abundant in cells of the subventricular and intermediate zones, which are comprised primarily of immature neurons (Hirai et al. 2002). Examination of the subcellular localization of DLK in cultured embryonic neurons indicated that it is preferentially associated with the microtubules and the Golgi apparatus (Hirai et al. 2002). In pancreatic β-cells, DLK is also known to localize in the cytoplasm and translocate into the nucleus upon exposure to inflammatory cytokines (Wallbach et al. 2016).



DLK (Dual Leucine Zipper-Bearing Kinase), Fig. 2 Schematic representation of the DLK-JNK signaling pathway. DLK mediates signals to JNK through phosphorylation and activation of MKK4 and/or MKK7. The scaffold protein JIP-1 negatively regulates DLK by preventing its oligomerization and activation

Signaling Properties and Regulation

DLK is a MAPKKK that serves as a pivotal component of the mitogen-activated protein kinase (MAPK) pathways, of which the best characterized in mammals are: extracellular signal-regulated kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs) (Gallo and Johnson 2002). The MAPKs are essential for transducing extracellular signals that regulate different cellular responses such as growth, differentiation, migration, survival, death, and metabolism (Gallo and Johnson 2002). Similar to other MLKs, DLK preferentially activates JNK, potentially by phosphorylating the JNK direct upstream activators MAPK kinase (MKK) 4 and/or 7 (Fig. 2) (Gallo and Johnson 2002), but a role for DLK in activation of ERK and p38 MAPK has also been proposed (Fan et al. 1996; Daviau et al. 2009).

To date, much remains to be established about the molecular mechanisms regulating DLK activation and signal suppression. Some evidence suggests, however, that dimerization or oligomerization of DLK mediated by the leucine zipper motifs is a prerequisite for autophosphorylation, activation, and stimulation of the JNK pathway (Gallo and Johnson 2002). Work from a number of laboratories has also indicated that the regulation of DLK is achieved by heterologous interactions with various cellular proteins. The binding of DLK to these proteins, in particular the scaffold JNK-interacting protein (JIP)-1 and MUK-binding inhibitory protein (MBIP), plays an important role in DLK regulation by preventing its dimerization and activation (Fukuyama et al. 2000; Gallo and Johnson 2002). Another important mechanism of DLK regulation is phosphorylation, a process that modulates the stability and enzymatic activity of DLK. In this regard, it was

shown that DLK undergoes JNK-dependent phosphorylation and stabilization in response to neuronal stress (Huntwork-Rodriguez et al. 2013). This phosphorylation at critical residues outside of the catalytic domain upregulates DLK protein abundance via reduction of DLK ubiquitination, which is mediated at least in part by the E3 ubiquitin ligase Phr1. More recently, DLK was also shown to have increased stability in cultured embryonic rat cortical neurons exposed to forskolin, an activator of adenylyl cyclase and protein kinase A (PKA). This effect of forskolin was blocked by pretreatment with the PKA inhibitor H89, indicating that the increase in DLK protein levels is PKA-dependent. In support of this, it was found that overexpression of the catalytic subunit of PKA alone could activate DLK and promote its stabilization, thus reinforcing the importance of phosphorylation in DLK regulation (Hao et al. 2016). Besides its stimulatory effect, phosphorylation is also implicated in the negative regulation of DLK activity and function. Indeed, it has been demonstrated that the kinase activity of DLK can be inhibited in mouse embryonic stem cells by Akt-mediated phosphorylation at two distinct sites (Wu et al. 2015). Such an inhibition was found to be required for maintaining the self-renewal capacity of these cells. Finally, in addition to phosphorylation, palmitoylation is another modification involved in DLK regulation. A recent study has reported that DLK is palmitoylated on a conserved cysteine residue adjacent to the kinase domain and that palmitoylation is required for DLK-dependent retrograde signaling in axons (Holland et al. 2016). Mechanistically, this was associated with the ability of palmitoylation to modulate DLK attachment to trafficking vesicles, interactions with protein partners and catalytic activity.

Wallenda and DLK-1, the respective *Drosophila* and *C. elegans* orthologues of DLK, are also known to be regulated by a ubiquitin-proteasomal degradation mechanism involving the E3 ligase Highwire/RPM-1 (Nakata et al. 2005; Collins et al. 2006). In the worm, this degradation is facilitated by PPM-2, a protein phosphatase that dephosphorylates DLK-1 at a serine residue critical for the binding of a shorter DLK-1 isoform (Baker et al. 2014). The primary function of this isoform is to inhibit DLK-1 function because its Ca^{2+}-mediated dissociation switches DLK-1 activity from off to on state in neurons (Yan and Jin 2012). Furthermore, Wallenda and DLK-1 are both activated by conditions that affect cytoskeletal stability, such as mutations in the actin-microtubule cross-linking protein Short stop (Valakh et al. 2013) or tubulin (Chen et al. 2014). Consistent with these data are studies in mammalian sensory neurons showing that genetic ablation of DLK abolished almost completely phosphorylation of the JNK substrate c-Jun in response to cytoskeleton-disrupting drugs (Valakh et al. 2015). Taken together, these findings point to a role for DLK signaling in the detection of cytoskeletal perturbations.

Biological Functions

In vitro studies with different types of cells have identified a role for DLK in the regulation of many physiological processes. For example, in rat pheochromocytoma PC12 cells and sympathetic neurons, ectopic expression of DLK induces apoptosis, whereas kinase-deficient DLK severely inhibits death caused by nerve growth factor deprivation (Xu et al. 2001). Consistent with a role in cell death, downregulation of DLK by RNA interference in mouse NIH 3 T3 fibroblasts and human MDA-MB-231 breast cancer epithelial cells blocked the apoptotic response induced by calphostin C (Robitaille et al. 2008). Based on its distribution during development, a role for DLK in cell differentiation has also been proposed (Nadeau et al. 1997). Accordingly, it was reported that DLK overexpression in poorly differentiated normal keratinocytes is sufficient to induce phenotypic changes associated with keratinocyte terminal differentiation, including upregulation of filaggrin expression, DNA fragmentation, activation of transglutaminases, and formation of corneocytes (Robitaille et al. 2005). Other evidence suggests that DLK may also play a role in adipocyte cell differentiation. Hence, in 3T3-L1 cells induced to undergo adipocyte differentiation, DLK expression is upregulated, and its

knockdown by RNA interference completely blocks the accumulation of lipid droplets as well as the expression of the adipogenic markers adiponectin and fatty acid synthase. In agreement with this, cells lacking DLK show significantly less expression of the master regulators of adipogenesis, peroxisome proliferator-activated receptor (PPAR)-γ2, and the CCAAT enhancer-binding protein (C/EBP)α (Couture et al. 2009). Interestingly, DLK was also identified as a key regulator of axon growth in mammals. This is supported by the findings that DLK knockdown prevents neurite extension from cultured PC12 cells and mouse embryonic cortical neurons (Eto et al. 2010; Hirai et al. 2011). Finally, a recent work by Stahnke et al. (2014) has revealed a new role for DLK in regulating insulin gene expression through modulation of the transcriptional activity of MafA in islet beta cells. Collectively, these data indicate that DLK may fulfill different functions, depending on the stimuli and the cellular context.

Considerable progress has also been made within the last few years in understanding the in vivo biological functions of DLK. In mice, knockout of the *DLK* gene results in a lethal phenotype around birth (Hirai et al. 2006), suggesting that it might have a crucial role during embryogenesis and organogenesis. Embryos lacking DLK display abnormal brain development, characterized by defects in axon growth, neuron migration, apoptosis, and axon degeneration (Hirai et al. 2006, 2011; Bloom et al. 2007; Ghosh et al. 2011; Itoh et al. 2011). In addition, these mice have reduced JNK activity and reduced phosphorylation of JNK substrates, such as the microtubule-stabilizing proteins Doublecortin and MAP2c (Hirai et al. 2006). Besides its role during development, it has been reported that DLK plays a crucial role in both the degeneration and regeneration of mature neurons in response to injury. Support for this idea is provided by the observations that conditional deletion of DLK significantly attenuates the neuronal and axonal degeneration caused by mechanical injury and glutamate-induced excitotoxicity (Miller et al. 2009; Pozniak et al. 2013; Watkins et al. 2013; Welsbie et al. 2013). Additionally, the absence of DLK in mouse has been shown to impair axonal growth in optic and sciatic nerve crush injury models (Shin et al. 2012; Watkins et al. 2013). The exact mechanism of this response remains unclear, but these studies showed the involvement of DLK in retrograde axonal transport of injury signals as well as in transcriptional regulation of both pro-apoptotic and regeneration-associated genes. Interestingly, this dual function of DLK seems to be conserved throughout evolution, since a dramatic defect in axon degeneration or regeneration after injury has been noticed in *Drosophila* and *C. elegans* mutants defective in Wallenda and DLK-1 (Miller et al. 2009; Hammarlund et al. 2009). Thus, these findings demonstrate a key role for DLK in controlling neuronal development as well as degenerative and regenerative responses to axonal injury.

Summary

DLK is a serine/threonine kinase that functions as an upstream activator of the MAPK pathways. It is expressed in a tissue-specific manner and regulated by mechanisms that involve phosphorylation, palmitoylation, interactions with different protein partners, and ubiquitin-mediated degradation. The functions of DLK are diverse and include regulation of development, cell differentiation, and apoptosis. Studies of mouse, fly, and worm mutants defective in DLK show that this interesting kinase may also be required for axonal degeneration and regeneration in response to injury. Thus, DLK appears to be a pivotal signaling component for regulation of various fundamental biological processes, although the precise molecular mechanisms by which it is activated and by which it mediates such effects are still elusive. Further work is therefore required to address these issues and expand our understanding of DLK's action.

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Dmel

▶ Rab8

dMiro

▶ Miro (Mitochondrial Rho)

DNA Damage Inducible Transcript 3

▶ Chop/GADD153

DNA Polymerase Delta Auxiliary Protein

▶ Proliferating Cell Nuclear Antigen

dNab2 (*D. melanogaster*)

▶ ZC3H14

DnaJ

▶ DNAJB6

DNAJ: HSP40

▶ Heat Shock Protein (HSP)

DNAJB6

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Synonyms

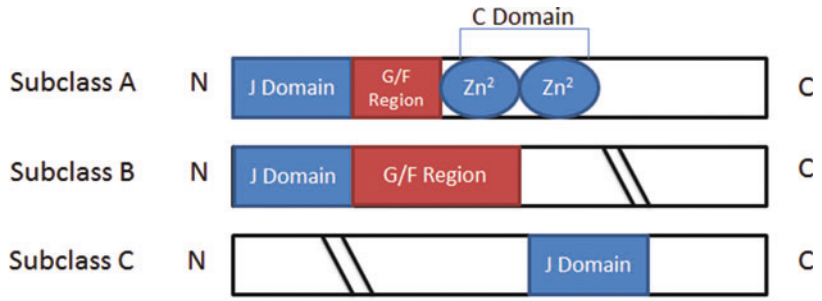
DJ4; DnaJ; HHDJ1; HSJ-2; HSJ2; LGMD1D; LGMD1E; MRJ; MSJ-1

Historical Background

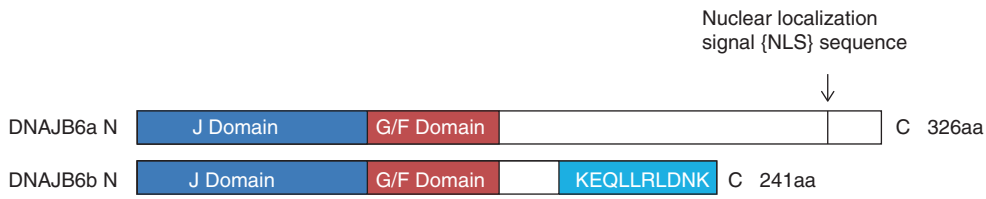
Heat shock proteins were first identified in the late 1970s by Dr. Alfred Tissieres laboratory who noticed that when *Drosophila* cells in culture were exposed to 37 °C a new set of proteins were synthesized (Arrigo et al. 1980). These proteins have been identified as heat shock proteins (HSP) and play a role in a wide variety of biological processes. Heat shock proteins act as chaperones helping move various client proteins to different cellular compartments. They also ensure the client proteins are folded correctly, as well as aid in degradation of misfolded or damaged proteins (Mitra et al. 2009). Since then, many heat shock proteins have been identified, including DNAJB6. It was initially identified for its role in the development of the embryo and placenta (Hunter et al. 1999). Recently, however, its role in cancer and many other diseases has been investigated.

Structure and Isoforms of DNAJB6

Heat shock proteins are typically classified by their molecular weight: Hsp60, Hsp70, Hsp40/DNAJ, etc. These HSPs are then further divided into subgroups. For example, Hsp40, or DNAJ, is further divided into three subclasses: A, B, and C, which are determined based upon the similarity of their domain to the *E. coli* DNAJ. These proteins bind to misfolded proteins and transport them to HSP70 proteins for refolding or destruction. There are three distinct domains associated with DNAJ proteins. The first is the J domain which is highly conserved and located near the amino terminus. It has been shown to stimulate ATPase activity of HSP70. The second domain is a glycine and phenylalanine rich region, and the third domain is a cysteine-rich region that contains four zinc finger-like regions known as the C domain (Ohtsuka and Hata 2000). In DNAJ proteins, the various subclasses are named based on which of these three domains they possess. DNAJ subclass A members contain all three of the aforementioned motifs. DNAJ subclass B contains the J domain and the G/F-rich region but lacks the C domain. Finally, DNAJ subclass C possesses only the J domain (Cheetham and Caplan 1998). See Fig. 1 for illustration. As its name implies, DNAJB6 contains both the J domain as well as the G/F-rich region but lacks the C domain.



DNAJB6, Fig. 1 Schematic representation of DNAJ family subclasses



DNAJB6, Fig. 2 A graphic representation of DNAJB6 gene, variant I and II, and DNAJB6 isoforms

DNAJB6 is located on the distal end of the q arm of chromosome 7 (Hanai and Mashima 2003). It has several isoforms as a result of alternative splicing; isoform a (2.5 kb transcript variant I) and isoform b, which is shorter, are the most well characterized to date (1.6 kb transcript variant II). Additionally, DNAJB6b has a different 3′ coding region and a distinct 3′ untranslated region (UTR) (Bock et al. 2010). The longer isoform message (DNAJB6) has 10 exons and is 326 amino acids long, while the short isoform message (DNAJB6b) has only 8 exons and is only 241 amino acids long. Isoforms share an almost identical sequence and structure; however, DNAJB6b lack the last 95 amino acids of the carboxyl terminal of DNAJB6a. Instead, DNAJB6b contains an additional ten amino acids (KEQLLRLDNK) on the carboxyl terminal that are unique to the short isoform (Hanai and Mashima 2003). See Fig. 2 for illustration.

Despite the fact that DNAJB6a and DNAJB6b are splice variants of the same gene, they still have unique physiological functions. DNAJB6a contains a nuclear localization signal sequence and therefore localizes primarily in the nucleus, while DNAJB6b is found ubiquitously throughout the cell, primarily in the cytosol (Hanai and Mashima 2003). Additionally, they both play a very distinct role in various pathologies and diseases.

The Role of DNAJB6 in Pathology and Disease

DNAJB6 is involved in a wide variety of pathological states and diseases. DNAJB6 and its cochaperones, members of the HSP70 family, play a crucial role in preventing the aggregation of misfolded proteins, many of which play roles in degenerative nervous system disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. Mutations in DNAJB6 have also been shown to cause dominantly inherited myopathies such as limb-girdle muscular dystrophy.

Alzheimer's Disease: Beta amyloid is a major component of Alzheimer's disease (AD) pathology. It is the most common neurodegenerative disease to date. Clinically, AD is characterized by cognitive decline and memory loss. Pathologically it is characterized by the extracellular accumulation of amyloid β 1-42 (Aβ42) plaques that lead to the production of reactive oxygen species as well as intracellular hyper-phosphorylated tau aggregates (Varadarajan et al. 2000). DNAJB6 has been shown to prevent the fibrillation of Aβ42 in vitro. Recent studies have also shown that DNAJB6 plays a role in preventing the aggregation of Aβ42 intracellularly in cell lines but was unable to clear extracellular Aβ42 that had already formed (Hussein et al. 2015). DNAJB6 has been found to prevent the formation of Aβ42 fibrils by inhibiting the nucleation and growth of the fibrils by binding with high affinity to the growing fibrils. It has been proposed that the high-binding affinity of DNAJB6 to Aβ42 is due to interactions of the exposed hydrophobic residues on Aβ42 and DNAJB6 which prevent nucleation of Aβ42 from occurring (Masson et al. 2014).

Parkinson's Disease: Parkinson's disease (PD) is the second most common neurodegenerative disorder, second only to Alzheimer's disease. It is characterized by bradykinesia, rigidity, and tremors. The pathological features associated with PD include deterioration of dopaminergic neurons in the substantia nigra as well as the formation of protein aggregates called Lewy bodies (Rose et al. 2011). DNAJB6b has been found in the core of

these Lewy bodies in dopaminergic nerve cells as well as in astrocytes in PD patients. Alpha-synuclein is also a major contributor to the formation of these Lewy bodies and the presence of these aggregates can lead to an upregulation of heat shock proteins that can suppress the aggregation of alpha-synuclein. It is likely, based on its localization in Lewy bodies, that DNAJB6b is involved in the early formation of Lewy bodies; however, it is still unclear whether the upregulation of DNAJB6 is a cause or a result of the disease (Durrenberger et al. 2009).

Huntington's Disease: Protein aggregations are also a critical part of Huntington's disease pathology, which is characterized by a CAG repeat expansion in the huntingtin gene, that is causing additional polyglutamine (polyQ) repeats in the mutant huntingtin protein (The Huntington's Disease Collaborative Research Group et al. 1993). DNAJB6 has been shown to decrease the amount of polyQ aggregates; when DNAJB6 was removed from the system, the aggregation of polyQ increased (Gillis et al. 2013). The shorter isoform (DNAJB6b) was found to be more effective over all at decreasing buildup of polyQ plaques because it is present in both the nucleus and the cytosol during cellular stress. DJANB6a is effective at preventing the aggregation of polyQ plaques located in the nucleus but is not present in the cytosol and therefore cannot prevent the aggregation of plaques in the cytosol. This indicates that DNAJB6 proteins need to be able to freely diffuse in order to prevent the formation of the polyQ plaques associated with Huntington's disease (Hageman et al. 2010).

Limb-Girdle Muscular Dystrophy: The fact that DNAJB6 plays a protective role in many neurodegenerative diseases makes it even more intriguing that mutations in DNAJB6 present as diseases of the skeletal muscle. The most common disease caused by a DNAJB6 mutation is limb-girdle muscular dystrophy 1D and is caused by mutations in Phe89 or Phe93. Additionally, mutations of the proximal end of the G/F domain have been shown to cause proximal limb-girdle myopathy while mutations in the distal end of the G/F domain cause distal limb-girdle myopathy (Ruggieri et al. 2015). These mutations have been shown to increase the half-life of DNAJB6, which increases the toxic effect of the mutated DNAJB6 and reduces its protective antiaggregation effects. Although both isoforms have these detrimental mutations, only the small isoform, DNAJB6b, is pathological (Sarparanta et al. 2012).

Cardiac Myopathy: In addition to its involvement in many different neurological diseases, DNAJB6 has also been implicated in playing a role in reducing cardiomyocyte hypertrophy. The calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway has been shown to be a prominent player in pathological cardiac hypertrophy. When activated, calcineurin dephosphorylates members of NFAT transcription factor family in the cytoplasm causing them to translocate into the nucleus where they upregulate the expression of several immune response genes (Molkentin 2004). The upregulation of the immune response genes includes TNFα, and increased TNFαexpression is associated with a variety of cardiac pathologies including cardiac hypertrophy. DNAJB6 has been found to form complexes with histone deacetylase4 (HADC4) which represses TNFα activity in the nucleus thus leading to a decrease in cardiac hypertrophy (Dai et al. 2005). A complete understanding of the functions and roles of DNAJB6 in cell biology is evolving. The section below summarizes the reported roles of DNAJB6 in mechanisms and signaling related to cancer biology.

The Role of DNAJB6a in Cancer

DNAJB6a has been shown to play an important role in several different kinds of cancers including breast cancer. The level of DNAJB6 found in normal tissue varies considerably depending on the type of tissue; however, the level of DNAJB6 found in each of the tissues changes when the organ is affected by cancer. No consistent pattern of up- or downregulation is evident across the various tissues, though all tissues do exhibit some level of change in the level of DNAJB6 expression when affected by cancer in comparison to normal tissue. See Table 1. This table does

DNAJB6, Table 1 Expression levels of DNAJB6 in normal and cancerous organs

Organs expressing DNAJB6	Level of DNAJB6 expression	
	Normal	Cancer
Breast	Undetected	Low, medium
Cervical	Low, medium	Undetected, low, medium
Colon/rectum	Medium	Low, medium
Endometrial	Medium	Undetected, low, medium
Head and neck	Low, medium	Medium
Liver	Undetected	Undetected, low, medium
Lung	Medium	Undetected, medium
Lymphoma	Undetected, low	Undetected, low, medium
Ovarian	Undetermined	Undetected, low, medium
Pancreatic	Undetected	Low, medium
Prostate	Low	Undetected, low
Renal	Medium	Undetected, low, medium
Skin	Undetected	Low, medium
Stomach	Low, medium	Low, medium, high
Testis	Medium	Medium, high
Thyroid	Medium	Undetected, low
Urinary bladder	Medium	Low, medium, high

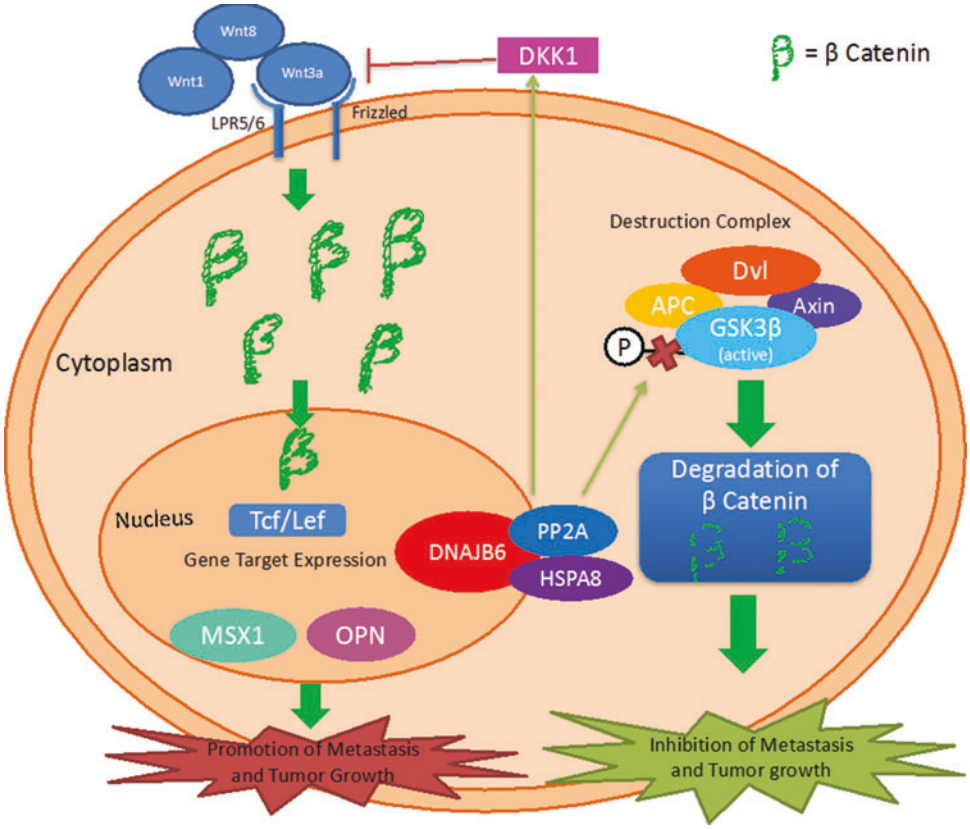
Modified from the data provided by proteinatlas.org. (http://www.proteinatlas.org/ENSG00000105993-DNAJB6/cancer)

not account for the different isoforms of DNAJB6, only the total protein level. It has also been suggested that not only the level of DNJAB6 expression is an important component of cancer pathology but the ratio of the long isoform (DNAJB6a) to the short isoform (DNAJB6b) that plays a key role in the development and progression of cancer in various types of cancer.

Metastasis: DNAJB6a has been found to be significantly reduced in aggressive breast cancer, as well as in advanced grade infiltrating ductal carcinoma and other metastatic cancer types. Conversely, cells expressing DNAJB6a are more capable of suppressing metastasis and tumorigenicity. This is achieved by downregulating the production of tumor-promoting proteins such as osteopontin and upregulating breast and melanoma metastasis suppressor proteins (Mitra et al. 2008).

Unlike in breast cancer, the overexpression of DNAJB6 has been found to promote the invasion of colorectal cancer cells. Both isoforms of DNAJB6 were found to be overexpressed in 39% of colorectal tissue and this overexpression correlated directly with the depth of tumor invasion both in vitro and in vivo. Based on these findings, it is possible that expression levels DNAJB6 could be used as a prognostic marker for colorectal cancer (Zhang et al. 2015).

Epithelial to Mesenchymal Transition (EMT): DNAJB6a also plays a crucial role in maintaining the epithelial-like phenotype in cancer cells. Loss of DNAJB6a causes the cells to shift to a more mesenchymal phenotype and become more aggressive. DNAJB6a also upregulates dickopf 1 (DKK1), a known inhibitor of the Wnt signaling pathway that binds to the Wnt coreceptor LRP5/6. DNAJB6, therefore, reverses the epithelial to mesenchymal transition (EMT) by upregulating the production of DKK1 to inhibit the Wnt/β-catenin pathway (Mitra et al. 2010). Additionally, DNAJB6a chaperones for protein complex consisting of DNAJB6a-HSPA8(HSP70)-PP2-A. The J domain of DNAJB6a binds to HSPA8 which recruits protein phosphatase, PP2A which dephosphorylates GSK3β leaving it in its active state. The activated GSK3 β works in concert with the degradation complex to mediate β-catenin degradation. The degradation of β catenin results in lower levels of β catenin in the cytosol and therefore less β catenin to import into the nucleus, ultimately resulting in a downregulation of Tcf/Lef-dependent transcription and reduced levels of MSX1. Reduction of MSX1 levels leads to an upregulation of DKK1 which interacts with LRP5/6 and prevents Wnt signals from binding (Menezes et al. 2012). The destabilization of β catenin also leads to a decrease in the level of OPN which is required for the expression of EMT markers (Mitra et al. 2012; Bhattacharya et al. 2012). DNAJB6a is also significantly decreased in patient-derived specimens of invasive and metastatic breast cancer as well as melanoma.



DNAJB6, Fig. 3 A simplified diagram of the role of DNAJB6 in the Wnt/β-catenin pathway

Additionally, nude mice injected with DNAJB6a showed reduced tumor growth rates as well as fewer lung metastases than their untreated counterparts (Mitra et al. 2008). See Fig. 3 for an illustration of Wnt/β-catenin pathway and DNAJB6a's role in it.

Regulation: Although little is currently known about how DNAJB6 expression levels are regulated, a knowledge-based screen of miRNAs identifies hsa-miR-632 (miR-632) that was found to target the coding region of DNAJB6. Exogenous miR-632 expression was found to downregulate levels of DNAJB6 causing significantly increased invasive ability in cell populations. Additionally, silencing of miR-623 was found to reverse the invasive ability of the cells and promote an epithelial like phenotype (Mitra et al. 2012). This could potentially be developed as a therapeutic target for the treatment of breast cancer.

The Role of DNAJB6b in Cancer

Despite the fact that DNAJB6a and DNAJB6b are two splice variants from the same gene, they have remarkably different roles in cancer. DNAJB6b is predominantly found in the cytosol unlike DNAJB6a which is located primarily in the nucleus. Additionally, DNAJB6b lacks the nuclear localization sequence that is found on DNAJB6a; however, during times of cellular stress, such as hypoxia, DNAJB6b does translocate into the nucleus. Currently, little is known about the role of DNAJB6b in cancer. While DNAJB6a suppresses metastasis and tumor growth, DNAJB6b has been shown to promote an increase in contact-dependent growth and accelerated proliferation rates as well as a shift to a more invasive morphology when localized to the nucleus as a result of heat shock or hypoxia

(Andrews et al. 2012). One possible mechanism for this translocation is that a pathological increase in cell number causes the formation of a tumor that will result in increased oxygen consumption. The vessels stimulated by angiogenesis are functionally abnormal and therefore unable to supply an adequate amount of oxygen to the tumor cells, which creates a hypoxic environment and leads to the import of DNAJB6b into the nucleus where it could promote further tumor growth (Krishnamachary et al. 2003). This hypothesis seems logical when DNAJB6b localization to the nucleus is looked at in the context of hypoxia, which promotes tumor growth and metastasis as well as drug resistance (Comerford et al. 2002). More investigation is needed in order to gain a more complete understanding of the role of DJANB6b in cancer.

Summary

DNAJB6 is a HSP40 this has two splice variants that play very individual roles in a variety of different diseases including cancer. It has been shown to be beneficial in preventing and/or clearing protein aggregates that are common in many neurodegenerative pathologies. Mutations in DNAJB6 lead to limb-girdle muscular dystrophy 1D as well as proximal muscular dystrophy. The long isoform DNAHB6a is primarily localized in the nucleus and is absent in aggressive or late stage cancers and has been shown to inhibit tumor growth and metastasis through its involvement in the Wnt/β-catenin pathway. Inversely, DNAJB6b, which is normally found in the cytosol, has been found to promote metastasis, invasiveness, and tumor growth when it localizes to the nucleus. It is crucial to gain a deeper understanding of DNAJB6 and the role of both its splice variant in diseases such as cancer. This knowledge could lead to a better understanding of signaling pathways underlying tumor metastasis or possibly uncover a novel therapeutic target to better treat malignant diseases.

Acknowledgments NIH R01CA194048 grant to R.S.S.

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DNA-PK Catalytic Subunit

► PRKDC

DNA-PKcs

► PRKDC

DNPK1

► PRKDC

DOCK2; Dedicator of Cytokinesis 2

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Synonyms

FLJ46592; KIAA0209

Historical Background

DOCK2 was initially designated by Nishihara et al. in 1999 (Nishihara et al. 1999) as a hematopoietic cell-specific homologue of the CDM (*c*ed-5 of *Caenorhabditis elegans*, *D*OCK180 of humans, and *m*yoblast city of *Drosophila melanogaster*) family proteins. The name "DOCK" was originally designated as "*D*ownstream *o*f *C*R*K*" for DOCK180, an archetype of CDM family proteins in 1996 (Hasegawa et al. 1996).

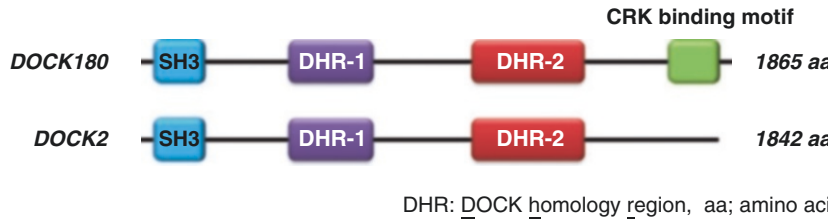
Molecular Mechanism of DOCK2 as a Rac-Specific GEF (Guanine Nucleotide Exchange Factor)

In mammals, 11 DOCK180-related proteins have been identified, and the family members can be subcategorized into four groups denoted DOCK-A, -B, -C, and -D, and DOCK2 belongs to DOCK-A as well as DOCK180 (Fig. 1a) (Cote and Vuori 2007). The structural analysis revealed that DOCK2 consists of an SH3 domain in the N-terminus and DHR (DOCK homology region)-1/2 in the middle to C-terminus (Fig. 1b)

a

Subgroups	***DOCK-A***	DOCK-B	DOCK-C	DOCK-D
Human DOCK -related proteins	DOCK180 ***DOCK2*** DOCK5	DOCK3 DOCK4	DOCK6 DOCK7 DOCK8	DOCK9 DOCK10 DOCK11
Target GTPase	Rac	Rac	Rac Cdc42	Cdc42

b



DHR: DOCK homology region, aa; amino acids.

DOCK2; Dedicator of Cytokinesis 2, Fig. 1 (**a**) The four subfamilies of human DOCK180-related proteins (DOCK-A, -B, -C, and -D) and the target small GTPases are indicated. DOCK2 belongs to DOCK-A and exclusively activates Rac, but not Cdc42. (**b**) Schematic diagram of the structure of the human DOCK180 and DOCK2. The DHR-1 domain is a unique evolutionarily conserved domain in all DOCK180-related proteins, and in the case of DOCK180, the DHR-1 domain was revealed to mediate a specific interaction with PtdIns (3, 5)P_2 and PtdIns (3, 4, 5)P_3 signaling lipids. The DHR-2 domains have been shown to interact with the GTPases of the Rho family including Rac1, 2 and Cdc42 leading to the exchange of GDP for GTP. Inactivation of the DHR-2 domain in DOCK180 has been shown to abrogate Rac activation, cell migration, and phagocytosis, highlighting the significance of this domain in the biological function of the DOCK180-related proteins. DOCK2 lacks the Crk-binding motif in the C-terminus which is an indispensable region in DOCK180 for binding to CRK, while CrkL was identified as a binding partner to DOCK2 through its SH3 domain in the N-terminus

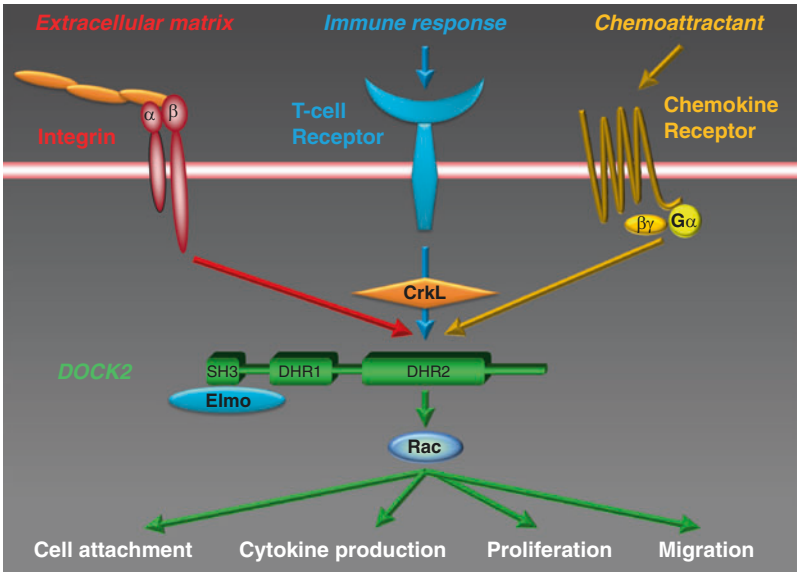
(Cote and Vuori 2002). DHR-2 is highly conserved throughout DOCK180-related proteins and identified as a Rac-specific GEF in DOCK2, although DHR-2 in other family proteins, such as DOCK3, may activate Cdc-42. DOCK2 lacks the PXXP motif in the C-terminus which is an indispensable region in DOCK180 for binding to CRK (Hasegawa et al. 1996), while CrkL was identified as a binding partner to DOCK2 through its SH3 domain in the N-terminus (Fig. 2) (Nishihara et al. 2002a). Similar to DOCK180, the interaction of DOCK2 with ELMO1, which was shown to increase the catalytic activity of DOCK180 toward Rac (Lu et al. 2004), was reported (Janardhan et al. 2004; Sanui et al. 2003b).

DOCK2 in Cytoskeletal Regulation and Tumorigenesis

DOCK2 regulates the motility of lymphocytes through actin-cytoskeletal reorganization and also cell proliferation in several types of B lymphocytes through the activation of Rac (Nishihara et al. 2002a; Sanui et al. 2003b; Wang et al. 2010), although DOCK2 itself, and even Rac, seems to lack the oncogenic ability. DOCK2-knockdown of the B-cell lymphoma cell lines using shRNA represented abrogated tumor formation in nude mice, suggesting the prominent role of DOCK2 in the progression of hematopoietic malignancy through DOCK2-Rac-ERK pathway (Wang et al. 2010). In fact, the high-level expression of DOCK2 mRNA in the leukemia/lymphoma cells obtained from the patients' blood samples has been ascertained (unpublished data).

The Immunomodulatory Role of DOCK2 in Lymphocytes

Because of the exclusive expression of DOCK2 in hematopoietic cells (Nishihara et al. 1999), a



DOCK2; Dedicator of Cytokinesis 2, Fig. 2 The signal transduction through DOCK2. DOCK2 transmits the signals from the several types of the integrins and receptors to small GTPase Rac, and regulates the cell motility, proliferation, and immune reaction of the hematopoietic cells. The protein interaction with CrkL and Elmo has been noted in the regulation of these cellular functions

hematopoietic cell-specific function such as regulation of immunity had been estimated. The analysis using DOCK2 knockout mice revealed the failure of T- and B-lymphocyte migration toward cytokines in vitro and the lack of their homing into the lymph nodes and the spleen in vivo (Fukui et al. 2001). The activation of DOCK2-Rac pathway is indispensable for CXCL12 (SDF-1)-stimulated human T-lymphocyte adhesion which is mediated by alpha4beta1 integrin (Gollmer et al. 2009), and also CCL21-mediated costimulation in CD4 (+) T cells (Garcia-Bernal et al. 2006). In addition, the role of DOCK2 in T-cell receptor (TCR) has been clarified: The in vitro analysis using the dominant negative form of DOCK2 confirmed that DOCK2 mediates TCR-dependent activation of Rac2 leading to the regulation of IL-2 (interleukin-2) promoter activity (Nishihara et al. 2002b), and in DOCK2$^{-/-}$ T cells, antigen-induced translocation of TCR and lipid rafts was significantly impaired, resulting in a marked reduction of antigen-specific T-cell proliferation (Sanui et al. 2003a). Furthermore, DOCK2 seems to be required in T-cell precursors for development into natural killer T cells (Kunisaki et al. 2006b). Taken together, these results indicate that DOCK2 is a key regulator of immunity, and explain the fact that cardiac allografts in DOCK2 knockout mice across a complete mismatch of the major histocompatibility complex molecules were not rejected by preventing potentially alloreactive T cells from recruiting into secondary lymphoid organs (Jiang et al. 2005).

DOCK2 in Myeloid Cell Lineage

Dendritic cells (DCs), macrophages, and neutrophils are also key players in immune response. The equivalent expression of DOCK2 during the maturation from CD34 (+)-myeloid precursor cells to these cells has been observed (unpublished data); therefore, the ubiquitous cellular functions of DOCK2 in this cell lineage are anticipated. In fact, the migration of neutrophil and plasmacytoid DCs was significantly abrogated in DOCK2$^{-/-}$ mice, although myeloid DCs did not show any defects in migration, suggesting the presence of alternative molecules to activate Rac during chemotaxis in myeloid DCs (Gotoh et al. 2008; Kunisaki et al. 2006a). Furthermore, DOCK2 is essential for toll-like receptor (TLR) 7- and 9- mediated interferon-alpha induction in plasmacytoid DCs, which play a key role in antiviral immunity (Gotoh et al. 2010).

Summary

As shown above, DOCK2, i.e., activation of Rac by DOCK2, plays indispensable roles in the regulation of immune response and also in the development of hematopoietic malignancy (Fig. 2). The activation of Rac is modulated by DHR-2, and several effectors for activated Rac have been identified, although the molecular mechanism of the activation of DOCK2 is still unclear. In DOCK180, autoregulation of GEF activity by its SH3 domain, ubiquitylation, and phosphorylation associated with Elmo are regarded, although the details including the upstream molecules remain undefined (Cote and Vuori 2007).

Because deficiency of DOCK2 resulted in impairment of the immune system, pharmacological inhibition of DOCK2 could be beneficial in the treatment for autoimmune diseases (Gotoh et al. 2010) and in preventing graft rejection (Jiang et al. 2005), as well as in the regulation of the progression of hematopoietic malignancy including malignant lymphoma and leukemia (Wang et al. 2010). In addition, DOCK2+ microglia which are associated with human Alzheimer's disease have been identified in a recent report (Cimino et al. 2009), suggesting that DOCK2 could be a possible therapeutic target for neurodegenerative disorders.

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Domain Protein

► ARD1/TRIM23

Dopamine and Adenosine 3′,5′-Monophosphate-Regulated Phosphoprotein, 32 kDa

► DARPP-32 (Ppp1r1b)

Dopamine and cAMP-Regulated Phosphoprotein

► DARPP-32 (Ppp1r1b)

Dopamine- and cAMP-Regulated Phosphoprotein, Mr32 kDa

► DARPP-32 (Ppp1r1b)

Double Minute 2 Protein

► MDM2 (Murine Double Minute 2)

Double Minute 4 Protein

► MDM4 (Murine Double Minute 4)

Double-Stranded RNA-Activated Protein Kinase

► PKR

Down Syndrome Candidate Region 1 (DSCR1)

► Regulator of Calcineurin 1 (RCAN1)

Down Syndrome Critical Region 1 (DSCR1)

► Regulator of Calcineurin 1 (RCAN1)

Downstream Regulatory Element Antagonist Modulator

► DREAM (Downstream Regulatory Element Antagonist Modulator)

DP8

► DPP8

DP9

▶ DPP9

DPP4

▶ Dipeptidyl Peptidase 4

DPP8

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Synonyms

Dipeptidyl peptidase 8; Dipeptidyl peptidase-related protein 1; DP8; DPRP1

Historical Background

Dipeptidyl peptidase (DPP) 8 is a member of the DPP4/DPP-IV gene and enzyme family, which belongs to clan SC of serine proteases, family S9, subfamily S9B. S9B proteases have a unique ability to remove Xaa-Pro dipeptides from the N-terminus of substrates. Fibroblast activation protein (FAP), DPP4, DPP8, and DPP9 are the S9B peptidases. Several extensive reviews provide detail (Zhang et al. 2013; Waumans et al. 2015; Klemann et al. 2016; Wilson et al. 2016).

DPP8 has been localized to human chromosome 15q22.32 (Abbott et al. 2000) and 9:65032458 in the mouse. The human DPP8 gene spans 71 kb and comprises 20 exons (Abbott et al. 2000), which is shorter and with fewer exons than DPP4 but encodes more amino acids (882 versus 766 residues). In DPP8 and DPP9, the gene sequence encoding the catalytic serine and its nearby highly conserved amino acids is in a single exon, whereas in DPP4 and FAP this region is split into two exons. Therefore, perhaps the DPP4 and FAP genes were derived from a DPP8- or DPP9-like gene. DPP8 (AF221634; encoding 882 amino acids) is ubiquitous across mammalian tissues and is 27% identical to DPP4, but 35% identical to DPP4 in the enzymatic region. DPP8 has no N-linked or O-linked glycosylation (Abbott et al. 2000). The dominant form of DPP8 mRNA in adult testis is a longer form encoded by 22 exons.

The crystal structure of DPP8 has not been solved, but protein homology modeling suggests that DPP8 is very similar to DPP9 and, apart from the N-terminal extension, shares a similar tertiary structure with DPP4 and FAP (Park et al. 2008). DPP8 is dimeric, with each monomer very likely consisting of an α/β-hydrolase domain and an eight blade β-propeller domain. The active site of the peptidase lies in the interface of these two domains, consisting of Ser^{739}, Asp^{817}, and His^{849} (Abbott et al. 2000; Klemann et al. 2016). Near this catalytic pocket lie a pair of glutamates in the β-propeller domain that are essential for catalytic activity in all members of the DPP4 enzyme family. Endogenous natural DPP8 protein has not been isolated. Human recombinant DPP8 has been produced in various expression systems and is consistently enzymatically active (Abbott et al. 2000; Ajami et al. 2008; Park et al. 2008; Geiss-Friedlander et al. 2009; Yao et al. 2011; Wilson et al. 2013; Justa-Schuch et al. 2014; Zhang et al. 2015a).

Expression and Localization In Vivo

Proteins must be localized to their appropriate subcellular compartments to fully function. Unlike DPP4 and FAP, which are cell surface expressed type II membrane glycoproteins with a very small cytoplasmic tail, DPP8 lacks a transmembrane domain and is an intracellular protein (Abbott et al. 2000). DPP8 has not been seen on the plasma membrane. The intracellular distribution of DPP8 as a chimeric fluorescent protein in human 293 T cells and hepatoma Huh7 cells is diffuse, associated with endoplasmic reticulum

(Abbott et al. 2000), where all proteins are manufactured. DPP9 has a long form, DPP9-L, that can enter the nucleus and contains a nuclear localization sequence near its N-terminus (Justa-Schuch et al. 2014). Like DPP9, DPP8 has been detected in the nucleus, but lacks a nuclear localization sequence (Justa-Schuch et al. 2014).

DPP8 is ubiquitously expressed in normal tissues from mouse, human, baboon, cynomolgus monkey, and Sprague Dawley rat (Yu et al. 2009; Zhang et al. 2013). Lymphocytes and epithelial cells from many organs, including lymph node, thymus, spleen, liver, lung, intestine, pancreas, muscle, and brain, express DPP8 (Yu et al. 2009). The expression of DPP8 is altered in many disease conditions such as liver disease, inflammatory bowel disease, and cancers (Zhang et al. 2013; Waumans et al. 2015).

Regulation of DPP8 Activity

DPP8 activity reversibly increases in reducing conditions (Park et al. 2008). DPP8 associates with Hras, but not as tightly as does DPP9 (Yao et al. 2011). DPP8 and DPP9 can be acetylated but whether that alters activity is unknown (Zhang et al. 2013).

Proteases regulate many physiological processes by cleaving peptide and protein substrates. Synthetic substrates with a proline at position P1 have been widely used in the discovery of DPP8 inhibitors and in the investigations of DPP8 biochemical and biological functions. DPP8 is most active on Ala-Pro-, Arg-Pro-, and Gly-Pro-containing chromogenic (p-nitroaniline; pNA) or fluorogeneic substrates, such as 7-Amino-4-methylcoumarin (AMC) or 7-Amino-4-trifluoromethylcoumarin (AFC) (Abbott et al. 2000).

The cleavage of the peptide hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) by DPP4 makes DPP4 inhibitors an effective treatment for type II diabetes (Klemann et al. 2016). Therefore the identification of DPP8 natural substrates can produce insights into biological function. The first natural substrates identified for DPP8 were hormones and chemokines that are also cleaved by DPP9 and DPP4 (Ajami et al. 2008). DPP4 has many other natural substrates and binding partners (Klemann et al. 2016) that have not been investigated as potential DPP8 substrates and binding partners.

Mass spectrometry–based proteomic strategies have been successfully applied to identify substrates of DPP8. Using N-terminal amine isotopic labeling of substrates (TAILS), proteomic analysis of cytosolic proteins has revealed many putative DPP8 substrates in cells stably expressing either enzymatically active or inactive DPP8 on a background of endogenous DPP8 (Wilson et al. 2013). In addition, using 2D DIGE in a cell line expressing enzyme inactive DPP9, proteins were confirmed as DPP9 substrates by MALDI-TOF or immunoblotting and all of these substrates are also DPP8 substrates (Zhang et al. 2015b; Wilson et al. 2016).

A compound that selectively inhibits DPP8 would be a useful biochemical tool to identify DPP8 substrates and to study the functions of DPP8. However, all the synthetic compounds that inhibit DPP8 also inhibit DPP9 to significant extents, with 1G244 being the most potent and selective (IC_{50} values of 14 nM and 53 nM against DPP8 and DPP9, respectively) (Wu et al. 2012). Thus, although 1G244 has low activity on DPP4 and FAP, a limitation for studies using 1G244 is that DPP8 and DPP9 cannot be discriminated. Therefore, the development of a selective DPP8 inhibitor is very important for progressing DPP8 research. The identification of dipeptide Val-Ala as a site for DPP9 cleavage not recognized by DPP8 (Zhang et al. 2015b) may facilitate the development of selective inhibitors.

DPP8 in Metabolism

The TAILS proteomic analysis of DPP8 substrates included several mitochondrial proteins. Most notably, the mitochondrial enzyme adenylate kinase 2 (AK2) is a confirmed substrate of both DPP8 and DPP9 (Wilson et al. 2013). Therefore, DPP8 probably has a role in energy metabolism. Adipogenesis in preadipocytes can be blocked by the DPP8/DPP9 inhibitor 1G244

(Han et al. 2015). In addition, the knockdown of DPP8 or DPP9 significantly impairs adipocyte differentiation from preadipocytes in vitro. Blocking the expression or activities of DPP8 and DPP9 attenuates PPARγ2 induction during preadipocyte differentiation. Addition of PPARγ agonist or ectopic expression of PPARγ2 is able to rescue the adipogenic defect caused by DPP8/DPP9 inhibition in preadipocytes (Han et al. 2015). These data indicate a possible role of DPP8 in adipogenesis.

DPP8 in Cellular Functions and Implications for Cancer

Emerging evidence points to the role of DPP8 in a number of cellular processes, including extracellular matrix interactions and epithelial growth factor–driven proliferation (Yao et al. 2011; Zhang et al. 2015a). DPP8 is ubiquitously expressed in tumor cell lines and has so far been investigated in several types of tumors, including Ewing sarcoma, testicular, liver, breast and ovarian cancer, and leukemia and lymphoma (Yu et al. 2009; Yao et al. 2011; Spagnuolo et al. 2013; Zhang et al. 2015a). HEK293T cells overexpressing DPP8 have impaired cell adhesion and monolayer wound healing, suggesting a role for DPP8 in cell adhesion and migration, but the underlying mechanisms remain elusive (Yao et al. 2011; Zhang et al. 2015a). In Ewing Sarcoma, cell death can be enhanced by blocking DPP8 and DPP9 with either enzyme inhibition or siRNA knockdown and DPP8/DPP9 inhibition enhances parthenolide's tumor cell cytotoxicity (Spagnuolo et al. 2013). Similarly, the inhibition of DPP8 and/or DPP9 probably contributes to the tumor regression induced by the compound Val-boro-Pro (Talabostat) (Walsh et al. 2013). These findings may thus implicate DPP8 and/or DPP9 in tissue and tumor growth and metastasis. Possibly, the regulation of DPP8 in cell proliferation is mediated by inhibiting Akt activation involving the epidermal growth factor signaling pathway (Yao et al. 2011). However, selective inhibitors targeting DPP8 and conditional DPP8 knockout mice are needed to differentiate DPP8 from DPP9 for future investigations.

DPP8 in Immunology

The expression patterns of DPP8 and DPP9 in immune organs are similar (Yu et al. 2009). DPP8 may have a role in the immune system and the inflammatory response, but where it has been compared with DPP9 the latter enzyme has had a much more significant role (Geiss-Friedlander et al. 2009). DPP8 and DPP9 have been implicated in lymphocyte proliferation and activation and are expressed in ill-defined subsets of leucocytes, including lymphocytes and macrophages. DPP8/DPP9 inhibition attenuates macrophage activation, possibly due to the reduced interleukin-6 secretion (Waumans et al. 2016).

The identified substrates of DPP8 also suggest potential immune roles of DPP8. Two important immune regulators, CXCL10 and IL1RA, have been shown to be DPP8 substrates (Ajami et al. 2008; Zhang et al. 2015b). Cells and molecules of the immune system are a fundamental component of the tumor microenvironment. The chemokine CXCL10 is converted to its antagonist form by DPP4 in certain tumors (Rainczuk et al. 2014) in which the active form of CXCL10 attracts lymphocytes that can attack tumor cells. CXCL10 can be cleaved by DPP8 (Ajami et al. 2008), so DPP8 potentially also inhibits CXCL10-mediated processes. Moreover, the putative DPP8 and DPP9 substrates S100-A10, SET, and NUCB1 are mediators of immunity and/or inflammatory response (Zhang et al. 2015b). However, a functional or physiological outcome of DPP8 mediated cleavage has not been investigated.

Summary

DPP8 is a large, dimeric nonglycosylated intracellular polypeptide with a narrow specificity post-proline proteolytic activity. This protease is ubiquitous and abundant, and yet the understanding of DPP8 in biology is rudimentary. DPP8

might have a role in nuclear events, in energy metabolism, in the brain, and in cancer and immunology. Further investigations are essential.

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DPP9

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Synonyms

Dipeptidyl peptidase 9; Dipeptidyl peptidase-related protein 2; DP9; DRP2

Historical Background

Dipeptidyl peptidase (DPP) 9 is a member of the DPP4 (DPP-IV) family, which are all members of clan SC of serine proteases, family S9, subfamily S9B. S9B proteases have a unique ability to remove Xaa-Pro dipeptides from the N-terminus of substrates. Fibroblast activation protein (FAP) and DPP8 are the other S9B peptidases.

DPP9 has been localized to human chromosome 19p13.3 (Olsen and Wagtmann 2002). The human DPP9 gene spans 48.6 kb and comprises 22 exons that are 53 bp to 1431 bp in length (Ajami et al. 2004). A predominant DPP9 mRNA transcript of 4.4 kb (AY374518; encoding 863 amino acids, the short form) is ubiquitous, with the highest levels in liver, heart, and skeletal muscle (Olsen and Wagtmann 2002; Ajami et al. 2004). A less abundant 5 kb transcript (AF542510; encoding 971 amino acids) that is abundant in skeletal muscle contains a second ATG translation start site that encodes a 892 amino acid protein (NM_139159.4), which is called the long form of DPP9 (DPP9-L) (Ajami et al. 2004).

Given that the crystal structures of DPP9 have not yet been solved, protein homology modeling has shown that DPP9 likely shares a similar tertiary structure with DPP4 and FAP. DPP9 is dimeric, with each monomer likely consisting of an α/β-hydrolase domain and an eight-blade β-propeller domain. The active site of the peptidase locates at the interface of these two domains, consisting of Ser^{730}, Asp^{808}, and His^{840} (Olsen and Wagtmann 2002; Ajami et al. 2004).

Until now, natural DPP9 has been purified only from bovine testes and was identified as the short form. Endogenous natural DPP9-L protein has not been isolated. Human recombinant DPP9, both the short and long forms, have been produced in various expression systems for research investigations and both forms are enzymatically active (Justa-Schuch et al. 2014; Zhang et al. 2015a, b).

Expression and Distribution

Proteins must be localized to their appropriate subcellular compartments to fully function. Different from DPP4 and FAP, which are cell surface expressed type II membrane glycoproteins, DPP9 lacks a transmembrane domain and is an intracellular protein. Inside the cell, the short and long forms of DPP9 localize differently. The intracellular distribution of the short form (DPP9-S) as a chimeric fluorescent protein in human hepatoma Huh7 cells and HeLa cells is diffuse, with some DPP9-S associated with mitochondria and with microtubules (Zhang et al. 2015a). Under steady-state conditions, DPP9-S was not seen at the plasma membrane, but upon stimulation with either phorbol 12-myristate 13-acetate or epidermal growth factor, some DPP9-S redistributes towards the inside of the ruffling membrane at the leading edge of moving cells (Zhang et al. 2015a). DPP9-L contains a nuclear localization sequence (NLS) near its N-terminus and is located in the cytoplasm and nucleus (Justa-Schuch et al. 2014). Overexpressed DPP9-L was shown not to be colocalized with a nuclear-rim protein RanBP2 and is wholly within the nucleus, not associated with the nuclear rim (Justa-Schuch et al. 2014).

DPP9 is ubiquitously expressed in normal tissues from mouse, human, baboon, cynomolgus monkey, and Sprague-Dawley rat (Zhang et al. 2013). Lymphocytes and epithelial cells from many organs, including lymph node, thymus, spleen, liver, lung, intestine, pancreas, muscle, and brain, express DPP9. The expression of DPP9 is altered in many disease conditions such as in liver

disease, inflammatory bowel disease, and chronic lymphocytic leukemia (Zhang et al. 2013).

Regulation of DPP9 Activity

DPP9 is associated with H-Ras (Yao et al. 2011), and DPP9 activity reversibly increases in reducing conditions. DPP9 also interacts with small ubiquitin-related modifier 1 (SUMO1) and is allosterically regulated by SUMO1 binding (Pilla et al. 2013).

Proteases regulate many physiological processes by cleaving substrates. Synthetic substrates with a proline at P1 position have been widely used in the discovery of DPP9 inhibitors and in the investigations of DPP9 biochemical and biological functions. DPP9 is most active on Ala-Pro-, Arg-Pro-, and Gly-Pro- containing chromogenic (p-nitroaniline; pNA) or fluorogeneic substrates, such as 7-Amino-4-methylcoumarin (AMC) or 7-Amino-4-trifluoromethylcoumarin (AFC).

The cleavage of peptide hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) by DPP4 makes DPP4 inhibitors an effective treatment for type II diabetes. Therefore, the identification of DPP9 natural substrates can produce insights into biological function. The first natural substrate identified for DPP9 is the antigenic RU131–42 peptide which has a role in antigen presentation (Geiss-Friedlander et al. 2009). Mass spectrometry-based proteomic strategies have been successfully applied to identify substrates of DPP9 (Wilson et al. 2016). Using N-terminal amine isotopic labeling of substrates (TAILS), proteomic analysis of cytosolic proteins has revealed ten DPP9 substrates in cells stably expressing either enzymatically active or inactive DPP9 on a background of endogenous DPP9. Of these, nine were confirmed as having in vitro cleavable N-terminal peptides (Wilson et al. 2013), including several mitochondrial proteins. Thus, there are potential roles for DPP9 in cellular metabolism. Using 2D DIGE in a cell line expressing enzyme-inactive DPP9, nine proteins/peptides were confirmed as DPP9 substrates by MALDI-TOF or immunoblotting (Zhang et al. 2015b). Moreover, dipeptide Val-Ala was identified as a consensus site for DPP9 cleavage that was not recognized by DPP8, suggesting different in vivo roles for these closely related enzymes (Zhang et al. 2015b).

A DPP9 specific inhibitor would be a useful biochemical tool to identify DPP9 substrates and to study the functions of DPP9. However, there is none reported. Almost all the synthesized compounds targeting DPP9 also inhibit DPP8 to some extent, with 1G244 being the most potent one (IC_{50} values of 14 nM and 53 nM against DPP8 and DPP9, respectively) (Wu et al. 2009). A limitation for studies using 1G244 is that despite not inhibiting DPP4, DPP8 and DPP9 cannot be discriminated. Therefore, the development of a specific DPP9 inhibitor is of great urgency in the field of DPP9 research.

DPP9 Mouse Model and DPP9 in Metabolism

The only published DPP9 mouse line is a gene knock-in (gki) mouse line with a serine to alanine point mutation at the DPP9 active site (Ser729Ala). The nonsurvival of homozygous DPP9 gki mice shows an essential role of DPP9 enzyme activity in neonatal development. Pathology and histochemistry studies of embryos and neonates have not yet revealed a cause of death. The death of DPP9 gki neonates within hours of birth is intriguing. These DPP9 gki neonatal mice exhibit differential expression of genes involved in cell growth, innate immunity, and metabolic pathways in the DPP9 gki mice compared to wild-type littermates (Chen et al. 2016), suggesting that DPP9 enzyme activity is involved in gluconeogenesis and lipid metabolism in neonatal liver and gut.

A mitochondrial enzyme, adenylate kinase 2 (AK2), has been identified as a substrate for DPP9 and shown to colocalize with DPP9 (Wilson et al. 2013). DPP9 shows some colocalization with mitochondria (Zhang et al. 2015a) and potential substrates identified by 2D DIGE include several mitochondrial proteins (Zhang et al. 2015b), providing further evidence that DPP9 has access to mitochondrial proteins

that may be substrates. Therefore, DPP9 probably has a role in energy metabolism.

Adipogenesis in preadipocytes can be blocked by a DPP8/DPP9 selective inhibitor 1G244 (Han et al. 2015). In addition, knockdown of DPP8 or DPP9 significantly impairs adipocyte differentiation from preadipocytes. Blocking the expression or activities of DPP8 and DPP9 attenuates PPARγ2 induction during preadipocyte differentiation. Addition of PPARγ agonist or ectopic expression of PPARγ2 is able to rescue the adipogenic defect caused by DPP8/DPP9 inhibition in preadipocytes (Han et al. 2015). Although the contribution of DPP9 compared to DPP8 cannot be elucidated due to the lack of a selective DPP9 inhibitor, this piece of data is indicative of the possible role of DPP9 in adipogenesis.

DPP9 in Cellular Functions and Implications for Cancer

Emerging evidence points to the role of DPP9 in a number of cellular processes, including extracellular matrix interactions, proliferation, and apoptosis (Yu et al. 2006, 2009; Yao et al. 2011; Zhang et al. 2015a). DPP9 is ubiquitously expressed in tumor cell lines and has so far been investigated in several types of tumors, including Ewing sarcoma, testicular, breast, and ovarian cancer, and leukemia and lymphoma (Ajami et al. 2004; Yu et al. 2009; Spagnuolo et al. 2013; Waumans et al. 2015). HEK293T cells overexpressing DPP9-S have impaired cell adhesion and monolayer wound healing, suggesting a role for DPP9 in cell adhesion and migration, but the underlying mechanisms remain elusive (Yao et al. 2011; Zhang et al. 2015a). Overexpression of either DPP9-S or DPP9-L causes increased apoptosis in human hepatoma Huh7 cells, suggesting overexpression provides limited and perhaps nonphysiological information on DPP9 function. Downregulating DPP9 by siRNA knockdown or enzyme inhibition of DPP9 by 1G244 in Huh7 cells has been found not to cause apoptosis, and these approaches target all endogenous DPP9, including both the short and long forms. DPP9-gene silencing or enzyme inhibition reduced cell adhesion and migration and expression of integrin-β1 and talin in Huh7 cells (Zhang et al. 2015a). There is a concomitant decrease in the phosphorylation of focal adhesion kinase and paxillin, indicating that reduced DPP9 suppressed the associated adhesion signaling pathway, thereby slowing cell movement (Zhang et al. 2015a). In Ewing Sarcoma, cell death can be enhanced by blocking DPP9 with either selective enzyme inhibition or siRNA knockdown, and DPP8/DPP9 inhibition enhances parthenolide's tumor cell cytotoxicity (Spagnuolo et al. 2013). Similarly, inhibition of DPP8 and DPP9 probably contributes to the tumor regression induced by the compound Val-boro-Pro (Walsh et al. 2013). These findings may thus implicate DPP9 in tissue and tumor growth and metastasis. Possibly, the regulation of DPP9 in cell survival and proliferation is mediated by inhibiting Akt activation involving epidermal growth factor signaling pathway (Yao et al. 2011; Zhang et al. 2015a). However, selective inhibitors targeting DPP9 and conditional DPP9 knockout mice are needed to differentiate DPP9 from DPP8 for future investigations.

DPP9 in Immunology

The involvement of DPP9 in the immune system and the inflammatory response is an emerging interest. DPP9 has been implicated in lymphocyte proliferation and activation (Zhang et al. 2013; Waumans et al. 2015). DPP9 is expressed in lymphocytes and macrophages from immune organs and has been implicated in inflammatory diseases including atherosclerosis (Waumans et al. 2015). Particularly, DPP9 is expressed in macrophage-rich regions of atherosclerotic plaques, and DPP8/DPP9 inhibition attenuates macrophage activation, possibly due to the reduced IL-6 (interleukin-6) secretion (Waumans et al. 2016).

The identified substrates of DPP9 also suggest potential roles of DPP9. The antigenic RU131–42 peptide is the first identified natural substrate of DPP9, and it is possible that many more proline-containing antigens present in the cytoplasm are substrates for DPP9 (Geiss-Friedlander et al. 2009). DPP9 influences interferon gamma (IFNγ) secretion and antigen presentation on MHC class

I molecules, suggesting a novel role for DPP9, but not DPP8, in antigen maturation and presentation (Geiss-Friedlander et al. 2009). Two important immune regulators, CXCL10 and IL1RA, have been shown to be DPP9 substrates (Zhang et al. 2015b). Cells and molecules of the immune system are a fundamental component of the tumor microenvironment. The chemokine CXCL10 is converted to its antagonist form by DPP4 in certain ovarian cancer tumors in which the active form of CXCL10 attracts lymphocytes that can attack tumor cells. The putative DPP9 substrates S100-A10, SET, and NUCB1 are mediators of immunity and/or inflammatory response (Zhang et al. 2015b), but a functional or physiological outcome of DPP9 cleavage has not been investigated. In the gki neonatal mouse, which lacks DPP9 enzyme activity, there is less TNF-α (tumor necrosis factor, alpha) and IL-1β (interleukin-1β) and more IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) in liver and gut (Chen et al. 2016), further pointing to a regulatory role for DPP9 in immune responses in vivo.

Summary

The understanding of DPP9 biology has made recent advances. The discovery of the DPP9 long form opens a new page of the DPP9 story revealing the potential regulation by and of DPP9 in nuclear events. Neonatal death of the DPP9 gene knockin mouse indicates a crucial role of DPP9 enzyme activity in survival. Recent examination of the underlying reasons for that neonatal death shows that DPP9 enzyme activity is involved in gluconeogenesis and lipid metabolism. DPP9 may also have important roles in cancers and immunology, and further investigations are needed on these topics.

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DPPIV

▶ Dipeptidyl Peptidase 4

DPRP1

▶ DPP8

DR3L

▶ Tumor Necrosis Factor-Like Weak Inducer of Apoptosis (TNFSFS12)

DR3LG

▶ Tumor Necrosis Factor-Like Weak Inducer of Apoptosis (TNFSFS12)

Drag1

▶ ARAP3

DRAK2

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Synonyms

DAP kinase-related apoptotic kinase 2; STK17B

Historical Background

▶ DRAK2 is a serine/threonine kinase of the death associate protein kinase (DAPK) family. Of this family, DRAK2 is most similar to DRAK1, and these two kinases may represent a unique family. DRAK1 and DRAK2 were originally identified using a polymerase chain reaction (PCR) screen to identify additional DAPK members, and was first thought to be primarily involved in promoting apoptosis (Sanjo et al. 1998). While humans have genes for both DRAK1 and DRAK2, mice lack a DRAK1

gene. Although ectopic expression of DRAK2 in cell lines does induce apoptosis (Sanjo et al. 1998; Matsumoto et al. 2001), it is unlikely that apoptotic induction is its key physiologic function since DRAK2-deficient mice demonstrate no obvious defects in apoptotic signaling (McGargill et al. 2004; Friedrich et al. 2005). Instead, DRAK2 has been shown to negatively regulate calcium signaling in primary T cells. Since its catalytic activity is itself induced by calcium influx, DRAK2 may serve to maintain calcium homeostasis (Friedrich et al. 2007; Newton et al. 2011). DRAK2 (and likely its ortholog DRAK1) has been found to be an important immunomodulatory serine/threonine kinase, serving to a) set the initial threshold for thymic and peripheral T cell activation and later, to maintain the survival of effector T cells. In this capacity, mice lacking DRAK2 are resistant to organ-specific autoimmunity (see below). Thus, the development of small molecule antagonists will be of significant value to efforts aimed at combating such immune system diseases.

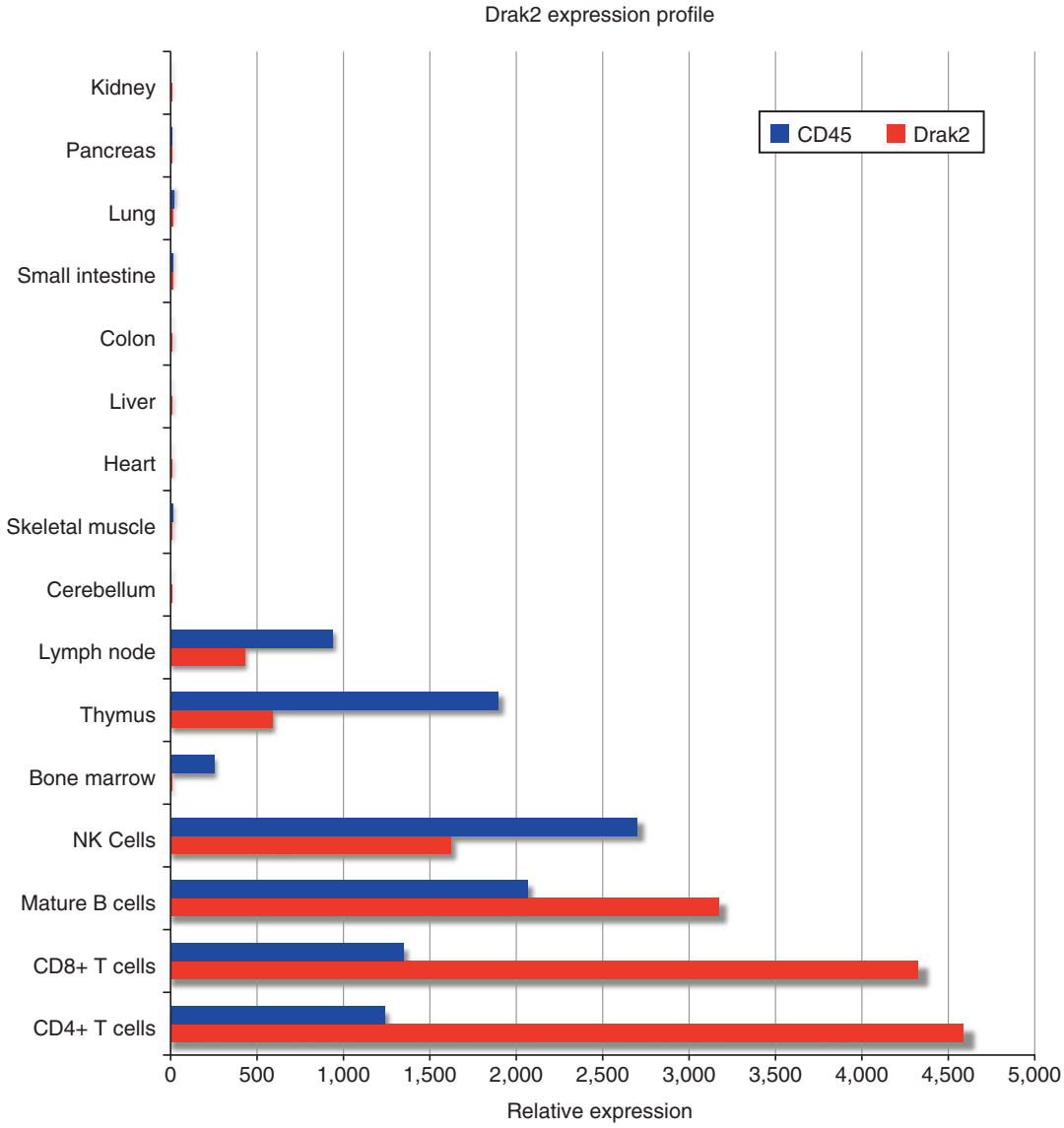
DRAK2 Expression

DRAK2 mRNA expression in humans and mice is fairly limited in adults. DRAK2 is highly enriched in lymphoid tissues including bone marrow, thymus, lymph nodes, and spleen. In particular, T cells and B cells express very high levels of DRAK2 mRNA and protein, and it has been demonstrated to be a critical regulator of T and B cell biology (Gatzka and Walsh 2008). During T cell development in the thymus, DRAK2 expression initiates at the single positive stage and is maintained in the periphery. Stimulation of the T cell receptor (TCR) and co-receptor CD4 leads to acute expression of DRAK2 mRNA and protein in double positive thymocytes (Friedrich et al. 2005). Similar to T cells, B cells also begin to express DRAK2 at the mature B cell stage of development, where it regulates cellular functions in these immune cells also (McGargill et al. 2004; Al-Qahtani et al. 2008).

The original discovery of DRAK2 resulted from screens of human placenta and liver cDNA libraries (Sanjo et al. 1998), although the role of DRAK2 in tissues outside of the lymphoid compartment is not well understood. DRAK2 expression has been found to be very high within parts of the brain, including the Purkinje cells of the cerebellum cortex as well as the olfactory lobe, ventricular zone, pituitary, and superchiasmatic nuclei (Mao et al. 2006). During mouse development, DRAK2 mRNA expression is ubiquitous until mid-gestation stage E14, but wanes by E18 in all tissues except those of the immune system. In addition to healthy tissue expression, colorectal cancer cells have been shown to downregulate DRAK2 expression as a means to enhance transformed cell survival. Treatment with Cyclooxygenase-2 (COX-2) inhibitors resulted in enhanced expression of DRAK2, and induction of apoptosis in the HCA7 colorectal cancer cell line (Doherty et al. 2009). Evaluation of DRAK2 expression using an online expression atlas demonstrates a similar pattern of expression between DRAK2 and DRAK1, with greatest expression found in lymphoid tissues and in B cells and T cells (Wu et al. 2009). DRAK2 also has a similar expression pattern as the hemopoietically restricted phosphatase CD45 (see Fig. 1).

DRAK2's Role in Apoptosis

As a member of the ▶ DAP kinase family, DRAK2 was thought to also be important for initiation of apoptosis. Following isolation from human placental tissue, studies demonstrated that DRAK2 could, in some instances, induce apoptosis. Initial reports suggested that overexpression of DRAK2 in cells lines such as 3T3 fibroblasts, Cos7 cells, rat NRK cells, and human Caco-2 cells all led to enhanced apoptosis (Sanjo et al. 1998; Kuwahara et al. 2006). The requirements for induction of apoptosis by DRAK2 in these various cell lines is not well understood, although in some instances DRAK2 kinase activity and localization to the nucleus seems to be required (Kuwahara et al. 2006). Besides overexpression studies, DRAK2 siRNA treatment in ACL-15 and NRK cells led to diminished apoptosis following exposure to UV-irradiation, further suggesting a



DRAK2, Fig. 1 DRAK2 mRNA expression profile obtained from GeneAtlas (Wu et al. 2009) (http://biogps.gnf.org/#goto=genereport&id=9262) is shown compared to CD45 expression in various mouse tissues. DRAK2 is highly enriched in tissues and cells of the immune system, similar to CD45

role for DRAK2 in apoptosis in cell lines (Kuwahara et al. 2006).

Although apoptosis can be enhanced and prevented in cell lines overexpressing or lacking DRAK2 respectively, several DRAK2 mouse models have been constructed to further elucidate the role of DRAK2 in cellular processes. First, a DRAK2 knockout mouse was created in which DRAK2 expression was abolished from all tissues. Surprisingly, DRAK2 knockout mice did not have any defects in apoptosis induction or embryonic development (McGargill et al. 2004). In particular, mice did not develop any signs of autoimmunity, cancer, or lymphadenopathy, which would be expected upon deletion of proteins involved in apoptosis. On the contrary, deletion of DRAK2 resulted in survival defects particularly in T cells and B cells. In T cells,

DRAK2 has important non-apoptotic functions regulating signals transduced through the T cell receptor, and more detail as to how these processes are regulated will be covered in depth later in this entry.

Besides a DRAK2 knockout mouse, two DRAK2 transgenic mice have been studied. First, DRAK2 expression linked to a human beta actin promoter was constructed, which enhanced expression of DRAK2 roughly fivefold in all adult mouse tissues. As noted before, DRAK2 is not normally expressed in all tissues, and is generally enriched in the immune system. Nonetheless, DRAK2 transgenic mice were shown to have enhanced T cell apoptosis following stimulation, which was dependent on high levels of cytokine exposure. Spleen size was enhanced in these mice, but no other defects were noted (Mao et al. 2006).

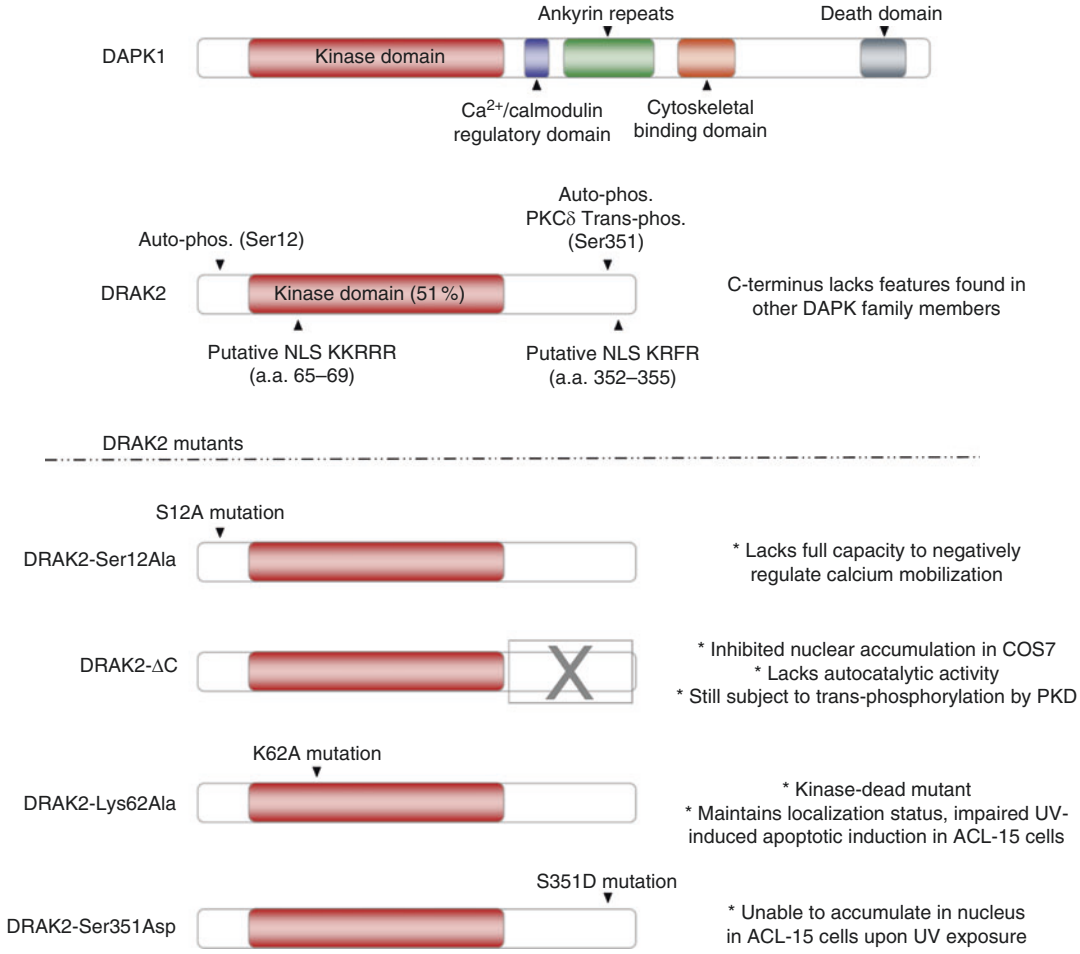
A second DRAK2 transgenic mouse was generated in which overexpression occurred only in the T cell compartment. Specifically, DRAK2 was linked to the lck promoter, which begins to be expressed at the double negative stage of thymocyte development. DRAK2 expression was very high in the thymus during development, and subsequently returned to wild-type levels in the periphery. In this mouse, thymocytes did not exhibit enhanced spontaneous or stimuli-induced apoptosis, and negative selection was diminished. Additionally, apoptosis of peripheral T cells was not enhanced following stimulation, although these cells were hypersensitive to stimuli (Gatzka et al. 2009).

Overall, although DRAK2 is a member of the DAP kinase family, it has controversial roles in apoptosis. Cell lines become sensitized to apoptosis when DRAK2 is overexpressed, yet DRAK2 deletion in mice does not confer resistance to apoptosis or manifest as disease pathology. Additionally, overexpression of DRAK2 in the thymus has no effect on apoptosis, while overexpression in the periphery enhanced apoptosis of stimulated T cells. It is important to note that DRAK2 seems to only enhance apoptosis in cells when overexpressed to very high levels, thus we do not expect DRAK2 to have a specific role in apoptosis under normal physiological conditions.

Structure/Function of DRAK2

Within the DAPK family of Ca2+/calmodulin-regulated serine/threonine kinases, DAPK, DRP-1, and Zip Kinase (ZIPK) comprise a highly homologous subfamily, whereas DRAK1 and DRAK2 represent a more distantly related group (Bialik and Kimchi 2006). DRAK2 has been the focus of intense study not only because orthologs for DRAK1 are present only in higher order primates, but because of its unique role in T cell activation. While DRP-1 and ZIPK share 80% and 83% homology, respectively, to the founding member of this family, ▶ DAPK, DRAK1 and DRAK2 share only 48% and 51%, respectively. Another important attribute that distinguishes the DRAKs from other members of this family is the complete lack of C-terminal features that command the regulation and apoptosis-promoting function of these kinases. Outside of its kinase domain, DRAK2 lacks homology with all other proteins, containing a short N-terminal region subject to autophosphorylation and a C-terminus important for its subcellular localization and its ability to induce apoptosis upon ectopic expression in various carcinoma cell lines.

In stably transfected Jurkat T cells, DRAK2 was localized primarily within the nucleus (Friedrich et al. 2005). This has also been shown to be the case in NIH3T3, NRK, and Caco-2 cell lines, whereas ACL-15, HeLa, and WI-38 cells have exhibited DRAK2 localization within the cytoplasm (Kuwahara et al. 2006). DRAK2 has been shown to contain putative nuclear-localization signals (NLS) in both its kinase domain (Friedrich et al. 2005) and C-terminal region (Kuwahara et al. 2006), the differential regulation of which potentially explaining this cell type-dependent translocation (Fig. 2). Upon stimulation of Jurkat cells with PMA plus PHA to mimic antigen receptor stimulation, DRAK2 translocated to the cytoplasm, whereas in ACL-15 cells, DRAK2 nuclear accumulation could be induced with UV-irradiation. In the latter cell type, this nuclear accumulation was not only dependent on an intact NLS within the C-terminus of DRAK2, but on phosphorylation of Ser350 mediated by protein kinase C (PKC)



DRAK2, Fig. 2 Comparison of DRAK2 and DAPK, and various DRAK2 mutants generated that have provided functional insight. The percentage within the kinase domain indicates the degree of amino acid identity to the kinase domain of DAPK. *NLS* nuclear localization signal

delta, the blockade of which prevented nuclear accumulation of DRAK2 and UV-induced apoptosis (Kuwahara et al. 2008). Interestingly, Ser350 was identified as a prominent site of autophosphorylation in studies (designating this residue as Ser351) aimed at understanding how this kinase is regulated in lymphocytes (Friedrich et al. 2007). These studies also revealed an important role for autophosphorylation of Ser12 in the ability of DRAK2 to affect T cell activation, indicating that, like other members of the DAPK family, DRAK2 autophosphorylation modulates DRAK2 activity and function.

As immature thymocytes transit through developmental stages in the thymus to become mature T cells, DRAK2 upregulation occurs and directly affects the degree of calcium mobilization elicited through antigen receptor stimulation. In the mature T cell compartment, where DRAK2 protein levels are highest, loss of DRAK2 leads to substantially enhanced calcium responses (McGargill et al. 2004). Reconstitution of DRAK2-deficient T cells with wild-type DRAK2 restored negative regulation of calcium mobilization, whereas expression of a Ser12Ala DRAK2 mutant was not sufficient to reestablish this negative regulation, indicating the

importance of autophosphorylation on Ser12 for DRAK2 biological function (Friedrich et al. 2007). The generation of DRAK2-transgenic mice in which DRAK2 transgene levels are driven specifically within the immature T cell population also lends support to the role of DRAK2 in directly modulating calcium responses. Whereas immature thymocytes ectopically expressing DRAK2 exhibited dampened calcium responses to antigen receptor stimulation, restoration of normal DRAK2 levels within the peripheral T cell compartment in these mice resulted in normal calcium responses (Gatzka et al. 2009). Finally, knockdown of DRAK2 within the clonal T cell line D10 recapitulated the exacerbated calcium response phenotype seen in DRAK2-deficient T cells, arguing against a developmental defect upon loss of DRAK2, and for a role in DRAK2 signaling to regulate this aspect of T cell activation (Newton et al. 2011).

Autophosphorylation on Ser12 is itself elicited by calcium mobilization, implicating DRAK2 in a negative feedback loop whereby calcium influx is necessary for autocatalytic activity on Ser12 and is required for DRAK2 to limit calcium influx, and not ER calcium store release. This process has been shown to be dependent on protein kinase D (PKD), potentially through direct transphosphorylation of DRAK2 by ▶ PKD, suggested by in vitro kinase assays. Association of DRAK2 with PKD has been demonstrated in T cells in response to stimuli that activate PKD, and is enriched within mitochondria. Immunofluorescence images of endogenous DRAK2 in primary T cells have revealed DRAK2 punctae formation localized to staining of mitochondria in response to thapsigargin to provoke calcium mobilization, and autophosphorylation on Ser12 was induced directly through generation of mitochondrial reactive oxygen species. Together with data showing that association of PKD and DRAK2 is disrupted by molecules that scavenge reactive oxygen species, activation of DRAK2 by PKD is thought to be dependent on calcium-induced mitochondrial reactive oxygen generation in response to antigen receptor stimulation (Newton et al. 2011).

How DRAK2 down-modulates calcium influx is currently unknown, and few substrates have been identified. In vitro, DRAK2 targets myosin light chain (MLC), a result that was anticipated based on its high level of homology in its kinase domain with ▶ MLCK, and based on DAPK's ability to target MLC in vivo. DRAK2 has been shown to interact with calcineurin homologous protein (CHP) in a manner that negatively regulates its autocatalytic activity and its activity toward MLC (Matsumoto et al. 2001). Although this interaction of CHP was not shown to be dependent on calcium, the suppression of DRAK2 catalytic activity by CHP was. S6K1 has been shown to be targeted by DRAK2 on the same residue (Thr389) as targeted by ▶ mTOR, leaving a role for DRAK2 in S6K1 signaling in T cells to be determined (Mao et al. 2009). An incredibly important tool for understanding not only what lies downstream of DRAK2, but for the selective targeting of T cell responses given the unique function of DRAK2 and its role in immune system, will be the development of specific inhibitors to target this kinase. The solved crystal structure with 2.8 Å resolution will undoubtedly aid in the discovery of novel inhibitors that offer the possibility to disrupt a pathway central to the exquisite control of T cell activation and tolerance yet dispensable for key immunological events that maintain resistance toward a multitude of pathological threats.

Role in Immune System

Since DRAK2 is highly expressed in lymphoid tissues, its role in the immune system has been extensively studied. DRAK2 expression is developmentally regulated during thymocyte maturation and its expression is increased following activation of the T cell receptor (TCR) of double positive thymocytes (Friedrich et al. 2005). DRAK2 is involved in setting the threshold for TCR signaling during thymocyte selection as evidenced by the increased calcium flux of DRAK2−/− thymocytes following the double positive stage (Friedrich et al. 2005). To study if DRAK2 plays a role in positive or negative selection, DRAK2-deficient mice have been bred with various TCR transgenic mice (McGargill et al. 2004). DRAK2-deficient mice crossed to OT-II

and AND mice have slight increases in CD4 single positive T cells and slight decreases in double positive T cells. Interestingly, there was no effect on CD8 single positive distribution when DRAK2-deficient mice were crossed to OT-I or P14 mice. Although DRAK2-deficiency led to enhanced positive selection, DRAK2 does not seem to play a role in negative selection as DRAK2 deficiency did not affect the loss of self-reactive T cells under the AND or H-Y TCR transgenic backgrounds. In addition, double positive DRAK2−/− T cells from OT-I and OT-II backgrounds had slight increases in activation markers. Therefore, DRAK2 is necessary for proper TCR activation during thymocyte selection.

In peripheral tissues, CD4+ and CD8+ T cells express similar levels of DRAK2 protein. Not surprisingly, DRAK2−/− peripheral T cells also have an increased calcium flux following TCR stimulation (McGargill et al. 2004; Friedrich et al. 2005). In addition, DRAK2−/− T cells have been observed to hyperproliferate to suboptimal stimulation and to a greater rate with weak agonist. The increased calcium flux and hypersensitivity of DRAK2−/− T cells is characteristic of T cells deficient in a negative regulator. In accord with the increase in proliferation, DRAK2−/− T cells produce higher amounts of IL-2. In addition, activated DRAK2−/− T cells produce higher amounts of IFN-γ and IL-4. Suboptimal stimulation results in higher surface expression levels of the costimulatory markers CD25, IL-7R, ICOS, CD27, OX40, and 41BB in DRAK2−/− T cells compared to wild-type T cells. Interestingly, the addition of exogenous anti-CD28 restores the levels of costimulatory markers and the observed hyper-proliferation of activated DRAK2−/− T cells back to wild-type levels (McGargill et al. 2004; Ramos et al. 2008). These studies support the hypothesis that DRAK2 is a negative regulator of T cell activation.

B cells express the highest level of DRAK2 protein and similar to T cells, DRAK2 expression increases following B cell maturation (McGargill et al. 2004; Friedrich et al. 2005). Following immunization of mice with a T-dependent antigen, the loss of DRAK2 in B cells results in up to a fivefold decrease in germinal centers and, consequently, a decrease in high affinity antibodies (Al-Qahtani et al. 2008). DRAK2−/− B cells proliferate similar to wild-type B cells and have no defects in somatic hypermutation and class switch DNA recombination. Further analysis indicates that the defects in DRAK2−/− B cells is a direct consequence of a loss of DRAK2 in T cells since a T-dependent antigen was used to cause the germinal center reaction. To study any B cell intrinsic defects due to the loss of DRAK2, T-independent antigen immunizations should be conducted.

Deletion of negative regulators of T cell activation often leads to increased sensitivity to autoimmune diseases (Pentcheva-Hoang et al. 2009). Based on studies on T cell negative regulators, it was predicted that DRAK2-deficient mice would also be more vulnerable to autoimmune disease. To the contrary, DRAK2-deficient mice were less susceptible to autoimmunity than wild-type mice. Aged DRAK2-deficient mice were not predisposed to spontaneous autoimmunity since there were no differences in the levels of cellular infiltrates in major organs and autoantibodies compared to wild-type (McGargill et al. 2004). DRAK2-deficient mice were also resistant to MOG-induced experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (McGargill et al. 2004, 2008; Ramos et al. 2007, 2008). Overexpression of DRAK2 in mice via the LCK promoter results in spontaneous autoimmunity and increased susceptibility to EAE (Gatzka et al. 2009). However, this is most likely due to ectopically expressing DRAK2 in double positive thymocytes, which may result in altered thymic selection. In addition, DRAK2−/− mice were resistant to type 1-diabetes when bred to the NOD strain of mice that spontaneously develop autoimmune diabetes (McGargill et al. 2008). The resistance is not due to developmental defects in Th17 or antigen-specific effector T cells (Ramos et al. 2008; McGargill et al. 2008). DRAK2−/− mice were susceptible to collagen-induced arthritis and systemic lupus erythematosus, both of which are mediated by autoantibodies (McGargill et al. 2008). DRAK2−/− mice were also susceptible to

autoimmune diseases that depend on mast cells and neutrophils (McGargill et al. 2008). Hence, DRAK2−/− mice remain susceptible to autoimmune diseases caused by autoantibodies or cells of the innate immune system but are resistant to autoimmune diseases where pathogenesis is primarily mediated by T cells.

The response to virus has also been studied in DRAK2-deficient mice. DRAK2-deficient mice had antiviral responses to Lymphocytic Choriomeningitis Virus (LCMV) (McGargill et al. 2004) and Murine Hepatitis Virus (MHV) (Ramos et al. 2007) that were indistinguishable from wild-type mice. Interestingly, DRAK2−/− mice were also capable of efficiently eliminating West Nile Virus, but did not succumb to the lethal encephalomyelitis, suggesting that while DRAK2 is not required for antiviral responses, it does promote entry of antiviral T cells into the brain. As stated above, activated DRAK2−/− T cells respond similar to activated wild-type T cells following addition of exogenous costimulation. During a viral infection the amount of costimulation is probably maximal and this may explain why DRAK2-deficient mice mount a normal immune response to viral infection. In addition, studies with MHV showed that DRAK2-deficient mice have enhanced memory T cell function on a per cell basis (Schaumburg et al. 2007). Hence, the absence of DRAK2 does not result in generalized suppression of the immune system and blockade of DRAK2 may be useful in treating T cell–dependent autoimmune diseases and enhance antiviral responses. The role DRAK2 plays in T cell survival likely explains the mechanism behind the restoration of DRAK2−/− T cells to a wild-type phenotype with the addition of costimulation (Ramos et al. 2008).

Summary

While the catalytic targets of DRAK2 (and DRAK1) remain to be fully clarified, this serine/threonine kinase offers a unique opportunity to control autoimmunity and potentially cancer. The elucidation of the targets of the kinase, as well as the structural features of the kinase, will be of great value for understanding how DRAK2 controls cellular physiology. While DRAK2 and DRAK1 clearly share significant homology with other members of the DAPK family, ongoing studies should help to determine the functional roles these serine/threonine kinases serve in distinct organ systems. Given the significant autoimmune resistance, but overtly normal antiviral immunity that DRAK2-deficient mice possess, it is likely that small molecule antagonists of the DRAK kinases will be valuable weapons in the arsenal to control autoimmune and other autoinflammatory diseases.

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DREAM

▶ DREAM (Downstream Regulatory Element Antagonist Modulator)

DREAM (Downstream Regulatory Element Antagonist Modulator)

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Synonyms

Calsenilin; Downstream regulatory element antagonist modulator; DREAM; KChIP3; Potassium channel-interacting protein 3

Historical Background

In 1998, Carrión et al. discovered a 110 kDa protein complex present in nuclear extracts of human neuroblastoma cells that could bind to a small region of DNA (+36 to +44 relative to the transcription start site) within the 5′ untranslated region (5′-UTR) of the human *prodynorphin* gene. Occupancy of this DNA element, termed the downstream regulatory element (DRE), by the protein complex caused a significant attenuation in *prodynorphin* gene transcription (Carrión et al. 1998). Utilizing a double-stranded DRE oligonucleotide probe to screen a human caudate expression library, Carrión et al. (1999) subsequently cloned the gene encoding for the DRE-binding protein, which they named downstream regulatory element antagonist modulator (DREAM). Electrophoretic mobility shift assays (EMSAs) confirmed that DREAM can bind to DRE and that the DREAM-DRE interaction is abrogated by increased Ca^{2+} concentration (Carrión et al. 1999). Numerous studies have since been devoted to identifying additional genes that possess

DRE sequences within their 5′-UTRs, as well as the phenotypic abnormalities that are associated with either genetic ablation of *dream* or expression of Ca^{2+}-insensitive mutants of DREAM. It was recognized early on that DREAM has pleiotropic functions beyond Ca^{2+}-dependent transcriptional repression: these functions are reflected in its alternative names of potassium channel-interacting protein 3 (KChIP3) (An et al. 2000) and calsenilin (Buxbaum et al. 1998), the latter referring to its ability to bind to both Ca^{2+} and the Alzheimer's disease (AD) proteins, presenilin 1 (PS1) and presenilin 2 (PS2). DREAM/KChIP3/calsenilin belongs to the neuronal calcium sensor (NCS) superfamily of proteins, which are characterized by the presence of four EF-hand motifs, two or three of which are capable of binding Ca^{2+}, as well as an N-myristoyl group (Burgoyne 2007).

Structure and Expression

In humans, *dream/kchip3/calsenilin* is located on chromosome 2q11.1. The murine *dream/kchip3/calsenilin* gene, also located on chromosome 2, consists of nine exons and can potentially encode up to four protein isoforms (Spreafico et al. 2001). Alternative splicing between exons 2 and 3 can result in the inclusion or exclusion of an ACAG nucleotide tetramer (Spreafico et al. 2001). Additionally, translation can be initiated at one of two alternative ATG start codons that are spaced 85 base pairs apart and are out-of-frame relative to each other (Spreafico et al. 2001). Exons 4, 5, 6, and 8 encode the four Ca^{2+}-binding EF-hand domains that are critical to the functionality of DREAM/KChIP3/calsenilin (Spreafico et al. 2001). Two of the variants, which contain the four EF-hand motifs, are identical in protein sequence except for the presence of 29 additional amino acids at the N-terminus of the variant dubbed DREAM by Spreafico et al. (2001). In that study, the variant with the shorter N-terminus was called KChIP3/calsenilin. In reality, it is unclear if the presence of the extended N-terminus distinguishes DREAM from KChIP3 and calsenilin or if one is dealing with the same protein albeit with different names. The other predicted protein isoforms include an N-terminal truncation variant of DREAM that lacks the EF-hand domains and a variant that contains a unique C-terminus lacking EF-hand domains (Spreafico et al. 2001).

In terms of its expression, *dream/kchip3/calsenilin* mRNA exhibits strong basal expression in the thymus, testis, and thyroid gland (Carrión et al. 1999). DREAM/KChIP3/calsenilin protein is constitutively expressed in the spinal cord, hippocampus, retina, pineal gland, and thyroid gland (Link et al. 2004; Rivas et al. 2004; Cheng et al. 2002). Immunohistochemical analysis revealed that DREAM/KChIP3/calsenilin is very abundant in the cerebellar and retrosplenial granular cortices, as well as moderately present in the optic tract, superior colliculus, olfactory bulb, and various thalamic relay centers, such as the anterior dorsal, medial geniculate, dorsolateral geniculate, ventral posteromedial, and ventral posterolateral nuclei (Hammond et al. 2003). The promoter region of the *dream/kchip3/calsenilin* gene possesses DRE sequences, suggesting that DREAM can regulate its own expression through an autoinhibitory feedback loop that is Ca^{2+} dependent (Mellström et al. 2014).

Molecular and Cellular Functions

When intracellular Ca^{2+} levels are low, Ca^{2+}-free (apo-)DREAM exists as a homotetramer that binds to DRE sequences within target DNA (Osawa et al. 2001). Mg^{2+} ions bind to the second EF-hand domain, and this interaction is critical in allowing DREAM to bind to DNA (Osawa et al. 2005). High levels of intracellular Ca^{2+} result in Ca^{2+} binding to the third and fourth EF-hand domains (Lusin et al. 2008). Ca^{2+} binding promotes the formation of DREAM homodimers, which are unable to interact with DRE sequences, resulting in transcriptional derepression (Lusin et al. 2008). DREAM recognizes and binds to the consensus DRE sequence 3′-PuNGTCAPuPuG-5′ (Carrión et al. 1998). DRE-containing genes that have been shown to be repressed by DREAM include the opioid precursor *prodynorphin*; the proto-oncogene *c-fos* (Carrión et al. 1998); brain-derived neurotrophic

factor (*bdnf*) (Rivera-Arconada et al. 2010); sodium ion (Na^+)-Ca^{2+} exchanger isoform 3 (*ncx3*) (Gomez-Villafuertes et al. 2005); voltage-dependent L-type calcium channel, alpha 1C subunit (*cacna1c*) (Ronkainen et al. 2011); the transcription factors neuronal PAS domain 4 (*npas4*) (Mellström et al. 2014), paired box gene 8 (*pax8*) (D'Andrea et al. 2005), and forkhead box protein E1 (*foxe1*) (D'Andrea et al. 2005); the thyroid protein precursor thyroglobulin (*tg*) (Rivas et al. 2004); calcitonin (Matsuda et al. 2006); the cytokines interleukin-2 (*il-2*), interleukin-4 (*il-4*), and interferon-γ (*ifn-γ)* (Savignac et al. 2005); the deubiquitinase tumor necrosis factor alpha-induced protein 3 (*tnfaip3*) (Tiruppathi et al. 2014); and the pro-apoptotic protein harakiri (*hrk*) (Sanz et al. 2001).

In addition to DRE-mediated transcriptional repression, it has been reported that DREAM is capable of activating gene transcription by directly binding to vitamin D and retinoic acid response elements that are located upstream of the transcription start site (Scsucova et al. 2005). Lastly, as discussed in detail in the next section, DREAM can also influence cAMP-dependent gene transcription (Ledo et al. 2002).

Beyond transcriptional regulation, DREAM is involved in other cellular processes through direct protein-protein interaction. As KChIP3, it binds to the cytoplasmic N-terminal domains of Kv4 potassium channels and promotes channel trafficking to the plasma membrane (An et al. 2000). KChIP3 also modulates the biophysical properties of Kv4 channels, reconstituting several features of native A-type currents (An et al. 2000). Ca^{2+} binding is essential for the ability of KChIP3 to modulate Kv4 channel activity but is not required for the physical association between the two proteins (An et al. 2000). As calsenilin, it interacts with the C-termini of PS1 and PS2 (Buxbaum et al. 1998). Presenilins are components of the γ-secretase protein complex, and the calsenilin-PS interaction has been shown to modulate γ-secretase activity (Jang et al. 2011; Jo et al. 2005).

The molecular functions of DREAM/KChIP3/calsenilin are dependent on specific structural properties of the protein as well as posttranslational modifications. For example, there are numerous positively charged amino acid side chains (K87, K90, K91, R98, K101, R60, and K166) that are clustered on one aspect of the protein surface that may mediate electrostatic interactions with target DNAs (Lusin et al. 2008). Nuclear localization of DREAM appears to depend on its sumoylation by the SUMO-conjugating enzyme UBC9 at two specific residues, lys-26 and lys-90 (Palczewska et al. 2011). G protein-coupled receptor kinase 2 (GRK2)-mediated phosphorylation of DREAM/KChIP3 at ser-95 facilitates KChIP3-dependent trafficking of Kv4.2 channels to the plasma membrane but has no effect on DREAM's repressor activity (Ruiz-Gomez et al. 2007). Additionally, DREAM/KChIP3 must be palmitoylated at two adjacent residues, ser-45 and ser-46, in order to effectively traffic Kv4 channels to the plasma membrane (Takimoto et al. 2002).

Involvement in Intracellular Signaling

DREAM is an important downstream effector of Ca^{2+} signaling, as it transduces alterations in intracellular Ca^{2+} levels into changes in gene expression, which have the potential to dramatically alter the functional capability of the cell. In addition to direct transcriptional regulation through its binding to *cis*-acting elements, DREAM can also interact with the transcription factor cAMP response element-binding protein (CREB) and prevent its association with CREB-binding protein (CBP) (Ledo et al. 2002). Furthermore, when the α-isoform of cAMP-responsive element modulator (αCREM) becomes phosphorylated by cAMP-dependent protein kinase (PKA), it interacts directly with DREAM via leucine-rich domains, resulting in transcriptional derepression at DRE sites (Ledo et al. 2000). Thus, it appears that DREAM can coordinate the crosstalk between Ca^{2+} and cAMP signaling to influence gene expression. An example is DREAM-dependent transcriptional activation of the glial fibrillary acidic protein (*gfap*) gene, which requires both cAMP and Ca^{2+} signals (Cebolla et al. 2008).

Transcriptional regulation by DREAM can occur by mechanisms other than those described above. DREAM/calsenilin can bind directly to the

transcriptional corepressor C-terminal binding protein 2 (CtBP2) in a Ca^{2+}-independent manner (Zaidi et al. 2006). The DREAM/calsenilin-CtBP2 complex is suggested to recruit histone deacetylase (HDAC) enzymes to repress the transcription of numerous genes (Zaidi et al. 2006). DREAM can also directly interact with activating transcription factor-6 (ATF6), preventing its release from the endoplasmic reticulum (ER) membrane and nuclear translocation; the DREAM-ATF6 interaction ultimately blocks the expression of genes involved in the unfolded protein response (UPR) (Naranjo et al. 2016).

Several studies have identified a role of DREAM/calsenilin in programmed cell death (apoptosis). Abrogation of the DREAM-DRE interaction in the 3′-UTR of the *hrk* gene has been correlated with increased levels of apoptosis of hematopoietic progenitor cells (Sanz et al. 2001). In neuroblastoma cells, Ca^{2+}-bound DREAM interacts with the anti-apoptotic protein hexokinase 1 (HK1), preventing its localization to the surface of mitochondria and ultimately promoting apoptosis (Craig et al. 2013). Another study showed that DREAM/calsenilin enhances apoptosis by promoting release of intracellular Ca^{2+} stores (Lilliehook et al. 2002). When Ca^{2+} levels are low, battenin/CLN3 protein suppresses apoptosis by binding to and sequestering DREAM/calsenilin (Chang et al. 2007). DREAM/calsenilin is itself a substrate of caspase-3 and is cleaved at the asp-61 and asp-64 residues (DXXD cleavage motif) to produce two small fragments ofundetermined functions (Choi et al. 2001). Phosphorylation of DREAM/calsenilin at ser-63 inhibits caspase-3-mediated cleavage (Choi et al. 2003).

DREAM can also regulate cellular excitability by various means. For instance, DREAM can bind directly to the NR1 subunit of N-methyl-D-aspartate (NMDA) receptors and attenuate cell surface expression of these receptors (Zhang et al. 2010). Through its Ca^{2+}-dependent modulation of Kv4 channel activity, DREAM/KChIP3 can influence the repolarization properties of excitable cells following an action potential (An et al. 2000).

DREAM has been shown to interact with other proteins, including the antioxidant protein peroxiredoxin 3 (Prdx3) (Rivas et al. 2011) and calmodulin (Ramachandran et al. 2012), although the functional significance of these interactions is less clear. The DREAM-calmodulin interaction is mediated by the N-terminus of DREAM, between residues 29 and 44, and enhances calmodulin-dependent activation of the serine/threonine phosphatase calcineurin (Gonzalez et al. 2015; Ramachandran et al. 2012).

D

Physiological Functions

Much of what we know about DREAM's physiological roles have come from studies using three independently generated knockout ($^{-/-}$) mouse strains and transgenic mice expressing Ca^{2+}-insensitive DREAM (TgDREAM) protein. *Dream*$^{-/-}$ mice exhibit a global reduction in pain responses that were attributed to increased expression of *prodynorphin* in the spinal cord (Cheng et al. 2002). The aversive effects of the cannabinoid tetrahydrocannabinol (THC) are also potentiated in these animals, whereas the analgesic effects of THC are reduced (Cheng et al. 2004). *Calsenilin*$^{-/-}$ mice have reduced levels of Aβ peptide, consistent with an alteration in γ-secretase activity (Lilliehook et al. 2003). An observed enhancement of hippocampal long-term potentiation (LTP) in *calsenilin*$^{-/-}$ mice corresponded with reduced Kv4 channel current (Lilliehook et al. 2003). Kv4 channel currents in cortical pyramidal neurons are modestly decreased in *kchip3*$^{-/-}$ mice (Norris et al. 2010). *Dream*$^{-/-}$ and *kchip3*$^{-/-}$ mice exhibit enhanced hippocampal-dependent learning (Alexander et al. 2009; Fontán-Lozano et al. 2009), potentially through facilitation of CREB-dependent transcription (Fontán-Lozano et al. 2009). Estradiol-enhanced memory formation also appears to be modulated by DREAM (Tunur et al. 2013). Ablation of *dream* slows age-dependent cognitive decline and hippocampal gliosis (Fontán-Lozano et al. 2009). In neonatal *dream*$^{-/-}$ mice, GFAP expression and the number of astrocytes are reduced in the cerebral cortex, suggesting a role in astrogliogenesis (Cebolla et a. 2008). TgDREAM mice exhibit impairments in hippocampal-

dependent learning, along with a coincident reduction in *bdnf* expression and NMDA receptor-mediated current in the hippocampus (Mellström et al. 2014; Wu et al. 2010). The hippocampi of TgDREAM mice have reduced dendritic arborisation and spine density of CA1 pyramidal neurons and increased spine density of dentate gyrus granule neurons (Mellström et al. 2016). TgDREAM mice also exhibit reduced viability of cerebellar granule neurons under mild membrane-depolarizing conditions as a result of lower *Ncx3* expression (Gomez-Villafuertes et al. 2005).

The physiological functions of DREAM are not restricted to the brain and spinal cord. TgDREAM mice have markedly enlarged thyroid glands, potentially through alteration of thyroid-stimulating hormone receptor (TSHR) activity (Rivas et al. 2009). *Dream*$^{-/-}$ mice exhibit reduced endotoxin-induced inflammatory injury of the lung and sepsis-induced death as a result of attenuated NF-κB signaling (Tiruppathi et al. 2014). The B cells of TgDREAM transgenic mice are hyperproliferative in vitro but show reduced immunoglobulin (Ig) synthesis (Savignac et al. 2010). siRNA-mediated knockdown of *dream* expression in human embryonic stem cells (hESCs) reduces hESC pluripotency and promotes cellular differentiation (Fontán-Lozano et al. 2016).

Summary

From 1998 to 2000, three independent studies emerged that identified DREAM/KChIP3/calsenilin as a Ca^{2+}-dependent transcriptional repressor, a regulator of Kv4 potassium channels, and a presenilin-interacting protein. The functional pleiotropy of this structurally simple protein, characterized by the presence of four EF-hand motifs, was affirmed in later studies that implicated DREAM in many cellular and physiological processes, including transcriptional regulation by DRE-dependent and DRE-independent mechanisms, protein trafficking, apoptosis, learning and memory, pain modulation, and regulation of thyroid development and immune function. Given the importance of Ca^{2+} signaling in many aspects of cellular function, we may only be witnessing the tip of the proverbial iceberg in terms of the role of DREAM in mammalian physiology.

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Drf1/ASKL1 (A Second Activator of Cdc7 in Human and *Xenopus*)

► Dbf4

Drosophila Su(var)3–9 and "Enhancer of Zeste"

► N-Lysine Methyltransferase SMYD

DRP2

► DPP9

Dscr1

► RCAN

DSCR1, Down Syndrome Critical Region Gene 1

► RCAN

Dscr1l1, Dscr1-Like 1

► RCAN

Dscr1l2

► RCAN

DSCR1L2, DSCR1-Like 2

► RCAN

DSG3

► Desmoglein-3

DSP

► Dual-Specificity Protein Phosphatases

DSPase

► Dual-Specificity Protein Phosphatases

dsRNA-Activated Inhibitor (DAI)

► PKR

DT1P1B11

► PHLDA1 (Pleckstrin Homology-Like Domain, Family A, Member 1)

DTEF-1

► Tead

DTR

► HB-EGF (Heparin-Binding EGF-Like Growth Factor)

DTS

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

DTSF

▶ HB-EGF (Heparin-Binding EGF-Like Growth Factor)

Dual Leucine Zipper Bearing Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Dual Leucine Zipper Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Dual Leucine Zipper-Bearing Kinase

▶ DLK (Dual Leucine Zipper-Bearing Kinase)

Dual Serine/Threonine and Tyrosine Protein Kinase (DSTY Kinase): Dusty Protein Kinase

▶ Receptor-Interacting Protein Kinase

Dual-specificity mitogen-activated protein kinase 5

▶ MEK5/ERK5

Dual Specificity Protein Phosphatase

▶ Dual-Specificity Protein Phosphatases

Dual Specificity Tyrosine-Phosphorylation Regulated Kinase 1A

▶ Dyrk1a

Dual Specificity Yak1-Related Kinase 1A

▶ Dyrk1a

Dual-Specificity Protein Phosphatases

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Synonyms

DSP; DSPase; Dual specificity protein phosphatase; Dual-specificity Protein Tyrosine Phosphatase (PTP); Mammalian class I dual-specificity cysteine-based phosphatase (DSP); MAPK phosphatase; MKP

Historical Background

In the 1980s, the regulatory role of protein phosphorylation in influencing cell growth,

differentiation, and division was found to be mediated by the coordinated action of protein kinases and phosphatases. One particular family of protein ser/thr kinases, known as MAPKs, play key mitogen-activated protein kinases (MAPKs) play key regulatory roles by responding to various extracellular and intracellular stimuli and changes. By phosphorylating downstream targets including protein kinases and transcription factors, activated MAPKs regulate the transcription of MAPK-regulated genes, translation of proteins, and protein activity. The basic physiological processes of the cell growth and survival including cell division, differentiation, metabolism, motility, immunological processes and responses rely on MAPK pathways and cascades. Disruptions of MAPK signaling have been implicated in many diseases especially cancer. Protein kinases and their role in signal transduction and cell cycle regulation were discovered almost 10 years prior to phosphatases and began to be targeted as drug targets. So, what are dual specificity what are dual specificity protein phosphatases (DUSPs) and what is their association with kinases? While kinases attach a phosphate group to a protein, phosphatases remove the phosphate group. In other words, DUSPs appear to serve as negative regulators of MAPKs. DUSPs comprise a family of phosphatases with the unique ability to dephosphorylate both the threonine/serine and tyrosine residues of their substrate. The first phosphatase VH-1 was identified in the *Vaccinia* virus (Guan et al. 1991). Although the function of the protein was unknown, the researchers suggested that the open reading frame of the purified protein expressed in bacteria hydrolyzed substrates containing both phosphotyrosine and phosphoserine. The mechanism of hydrolysis by the vaccinia phosphatase suggested the role of an essential cysteine in the catalytic activity of both substrates. In 1993, a group investigating dual specificity phosphatases analogous to VH-I found highly conserved sequence homology in smallpox variola virus, orthopox virus, and baculovirus *Autographa californica* (Hakes et al. 1993). The role of DUSPs as a key cell cycle regulator was discovered by a study exploring the regulatory mechanism of human cyclin-dependent kinases (CDKs) in CDK/cyclin complexes. The S-phase progression of the cell cycle was regulated by CDK2 via phosphorylation on Thr-14 and Tyr-15, while CDC2/cyclin B1 was activated through treatment with phosphatases. They confirmed that Cdc25 family members comprise a class of dual-specificity phosphatases and that the phosphorylation-dephosphorylation play roles in the cell cycle by serving as check points (Sebastian et al. 1993). In the same year, MKP-1 (3CH134) was identified as a dual specificity phosphatase that dephosphorylates and inactivates p42 MAP kinase both *in vitro* and *in vivo* (Sun et al. 1993). Kinase-associated phosphatase (KAP), representing an additional class of DUSPs, which was able to bind multiple CDKs, was studied in yeast and in mammalian cells (Hannon et al. 1993). The role of DUSP in transcriptional regulation was established when the first human PTPase gene belonging to VH-1 like PTPase subfamily, CL100, was isolated and its promoter characterized. CL 100 gene expression was found to be inducible by mitogen stimulation and oxidative stress (Kwak et al. 1994). The PTPase motif in the catalytic region aligned with the catalytic domain of the aforementioned Cdc25. However, this study showed a second region of sequence homology to cdc25, the CH2 domain of the CL100 gene. The feature of dual-specificity phosphatases, a subfamily of PTPases, was identified as the ability to dephosphorylate phosphotyrosine and phosphoserine/threonine residues. The role of dual-specificity phosphatases in the downregulation of MAP kinase pathways was elucidated (Kortenjann and Shaw 1995) in a study investigating the molecular role of the family of MAP kinases. The enzymes encoded by the DUSPs were related to the PTPs by their possession of the conserved PTP motif, similarities of catalytic mechanism, and similarities in tertiary structure (Barford et al. 1998). In yeast and mammalian cells, KAP interacted with cdc2 and CDK2 however showed a preference for cdc2 in mammalian cells. A proposed model for MAP kinase inactivation by DUSPs showed that rapid transcription of one or more DUSPs were triggered by various factors including growth factors, cytokines and other cellular stresses

(Camps et al. 2000). By 2000, nine mammalian DSP gene family members were identified all of which were implicated in inactivating MAP kinases: CL100/MKP-1 (adult rat brain), PAC1 (hematopoietic cells), hVH-2/MKP-2, hVH3/B23(adult rat brain), hVH-5 (brain, heart and skeletal muscle), MKP-3/PYST1 (adult rat brain) (same as rVH-6), B-59, MKP-4 (placenta, kidney, and embryonic liver), and MKP-5 (liver and skeletal muscle). Further, some of these were localized in the nucleus, while others were exclusively cytosolic. In the 1990s protein kinases were the focus of research, and by 2002, they were seen as major drug targets of the twenty-first century (Cohen 2002). In the first decade of the millennium, the role of protein phosphatases as key regulators in biological processes and therefore as drug targets was increasingly recognized.

Structure, Role, and Classification of DUSPs

Classification of DUSPs

The HUGO Gene Nomenclature Committee (HGNC) currently reports the DUSP gene family comprises 44 genes grouped into any one of the following six classes. There are approximately 18 atypical DUSPs, 4 CDC14 phosphatases, 11 MKPs, 3 PTPs, 5 PTENs, and 3 slingshot protein phosphatases within the DUSP family.

The six classes that DUSPs have been divided into include slingshots, PRLs (phosphatases of regenerating liver), Cdc14 phosphatases, PTENs, myotubularins, MKPs, and atypical DUSPs (Patterson et al. 2009).

Slingshot phosphatases are encoded by three genes and appear to play a role in actin polymerization. They have been found to contain the conserved PTP catalytic domain as well as the 14–3-3 binding motifs, a C-terminal F-actin (filamentous actin)-binding site, and a SH3 (Src homology 3) binding motif.

Phosphatase of regenerating liver (PRL): There are three PRLs shown to be overexpressed in cancer cells especially in metastasis. They are being explored as targets for cancer therapy.

Cdc14 phosphatases: There are four Cdc14 phosphatases which play major roles in the phases of cell cycle including regulation of initiation of mitosis, DNA damage checkpoint control, centrosome maturation, spindle stability, cytokineses with a role in inhibition of cell cycle progression.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylate D3-phosphorylated inositol phospholipids. There are 16 myotubularin phosphatases whose role is not yet fully known and five PTEN-like phosphatases implicated in cancers with effects in proliferative and survival signaling.

Mitogen-activated protein kinase phosphatases (MKPs) are named after the MAPKs. They dephosphorylate MKPs and dephosphorylate MAPKs at both phosphothreonine and phosphotyrosine residues simultaneously. Ten proteins have been identified in this best studied group of DUSPs. Even in resting or unstressed cells, MKPs are known to be expressed at low levels and upon stimulation can increase expression very rapidly. The same MKP can have different effects and levels of effects depending on the type of cell and context. They play key roles as regulators in signaling pathways. MKPs are subject to posttranslational modification, sensitive to reversible oxidation and inactivation, can be phosphorylated directly by their substrates and implicated in many diseases. MKPs have been found to play double roles based on the cancer type. They work as tumor promoters in some and as tumor suppressors in others and are used as biomarkers in specific cancers.

Atypical DUSPs: There are 16 atypical DUSPs characterized by being smaller and lacking the CH2 domain in the N-terminus that is common with typical DUSPs. Atypical DUSPs targeting protein substrates play roles in the MAPK pathway and other pathways. There are others that target nonprotein substrates and some act as scaffold proteins. Atypical DUSPs have been implicated in normal cell functions including cellular proliferation, metabolism, and differentiation and play roles in inflammation, diabetes, and cancer.

A former method of classification used to classify 25 human DUSP genes was based on amino acid alignment. DUSPs have been divided into two groups based on the presence or absence of the MKB/KIM domain into typical DUSPs (or MKPs) and atypical DUSPs, respectively (Huang and Tan 2012). Based on subcellular localization, typical DUSPs have been further grouped into nuclear, cytoplasmic, and dually located DUSPs (Huang and Tan 2012).

Structure

A recent study explored the structure and function of several DUSP family proteins using homology modeling and experimental structure determination. The specificity of the substrate with various DUSPs was found to be a function of the active site loop to strand switch. Further, it was shown that cysteine residues help protect enzyme activity (Jeong et al. 2014).

Catalytic Mechanism

All DUSPs contain a common phosphatase domain with conserved aspartic acid, cysteine, and arginine residues in the catalytic site. The catalytic mechanism of DUSP was first investigated in a study that used VHR as a model enzyme for the DUSP family (Zhou et al. 1994). Experimental evidence suggested that the putative active site cysteine, Cys 124, is required for forming a thiol-phosphate-enzyme intermediate during catalysis. A recent review of DUSP structure presents a clear mechanism of catalytic activity (Rios et al. 2014). In the first step of dephosphorylation, a thiol-phosphate covalent transient intermediate is formed using the catalytic cysteine. In the second step, the conserved Asp acts a general base activating a water molecule which hydrolyzes the intermediate complex, releasing the phosphate. Amino acid sequence alignment of 10 catalytically active MKPs, 13 atypical DUSPs, and 3 PRLs reveal three structural loops: the P-loop (which includes the conserved catalytic Cys), the WPD-loop which includes the catalytic Asp, and the TI/Q-loop which includes the residues that interact with the structural water molecule (Rios et al. 2014). However, there is variability in the length and amino acid composition of the WPD and the TI/Q-loop. For example, in PTENs and PRL-3, the WPD-loop Asp residue does not interact/participate with nonproteinaceous substrates. As noted above, understanding the structural similarities and differences in DUSP structure is critical in designing DUSP-catalytic inhibition strategies.

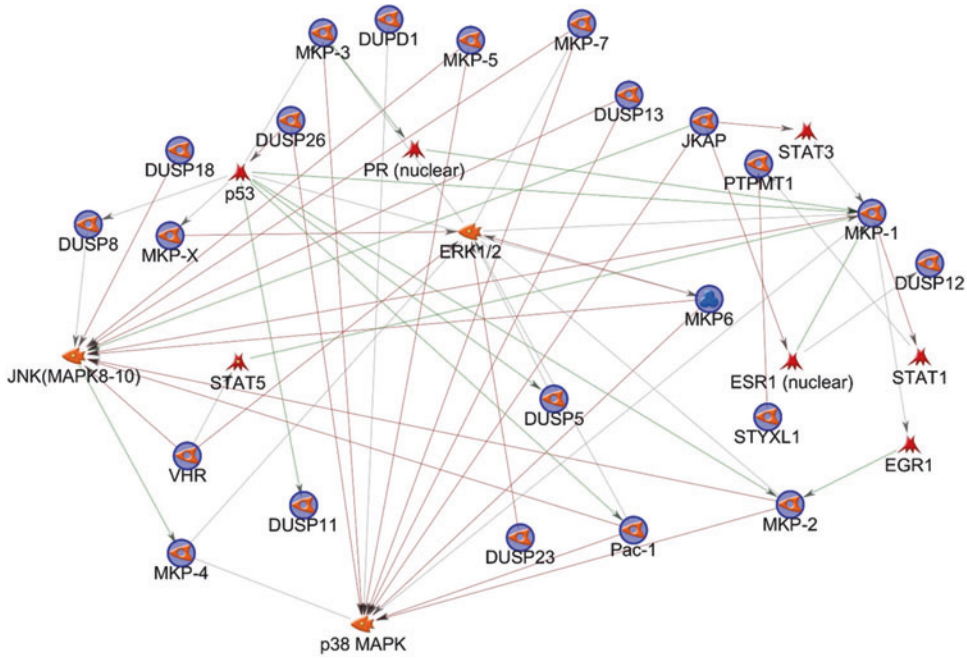
Regulation of DUSPs

The DUSPs that play a role in the three major MAPK pathways are depicted below (Fig. 1). As observed from the diagram, MKPs play pivotal roles as coordinators of MAPK signaling and crosstalk. They are not simple feedback regulators, as previously thought. Some of the areas still unclear include how MKPs maintain spatial control of MAPK signaling, the interplay between delayed transcriptional feedback by MKPs in the nucleus and posttranslational feedback loops like ERK phosphorylation of RAF in the cytoplasm (Caunt and Keyse 2013). DUSPs have also been implicated as regulators of non-MAPK protein targets including STAT, histone, and FAK. Atypical DUSPs are known to have the most varied substrate specificity and as such have other protein and nonprotein (RNA) substrates. Some atypical DUSPs such as DUSP 19, 22, and 23 do not directly effect their targets through catalytic activity but are scaffold proteins affecting interaction of signaling proteins (Patterson et al. 2009) (Fig. 1).

The three main mechanisms of DUSP regulation include (Patterson et al. 2009; Huang and Tan 2012)

Regulation at the transcriptional level – DUSPs are often regulated through a negative feedback mechanism, which is directly dependent on MAPK activation. One of the most important regulators of nuclear DUSPs is p53, which regulates the transcription of all four nuclear DUSPs. Epigenetic modifications and microRNA-medicated gene silencing also serve to regulate DUSPs.

Regulation at the post-translational level via chemical modification – Myristoylation and/or phosphorylation are examples of the post-translational modifications that serve to



Dual-Specificity Protein Phosphatases, Fig. 1 Various DUSPs and their roles in the major MAPK pathways and non-MAP kinase targets in mammalian cells. The direction of the arrows indicate the pathway the DUSP is implicated in and the color of the arrows suggest the role as a transcriptional activator/upregulation (*green*) or down-regulation (*red*)

stabilize DUSP expression levels. When MAP kinases phosphorylate DUSPs, they are protected from degradation by the proteasomes and half-life can be prolonged.

Regulation of protein activity via catalytic activation/inhibition – The catalytic activity of DUSPs can be enhanced or inhibited as an additional form of regulation. Activity of some DUSPs is enhanced upon binding to the MAPK substrate. DUSPs can compete with MAP kinase substrates for binding with MAP kinases. The cysteine residue in the catalytic site, which is subject/sensitive to reversible oxidation of the DUSP, often renders it inactive. During this process, there is the production of reactive oxygen species (ROS).

One of the interesting features of DUSP regulation is a dramatic transcriptional induction by growth factors or factors that induce cellular stress, seen especially in leucocytes. DUSPs respond to various stimuli differently depending upon the type of cell and context. Another level of regulation occurs through the control of protein expression. Activation of some DUSPs occurs following binding to their substrates. Reversible oxidation provides a mode for activation or deactivation of some DUSPs. Some DUSPs can control the subcellular localization of MAPKs. DUSPs appear to show substrate specificity although this may depend upon the cell type and type of stimulus. The efficacies of any two DUSPs in dephosphorylating a given MAPK may differ.

Role of DUSPs in Human Health and Disease

As observed earlier, MAPKs are implicated in physiological processes of cell growth and function including embryogenesis, immunity, cardiac function, neuronal plasticity, and metabolism. By dephosphorylating and therefore inactivating MAPKs, DUSPs (MKPs) play a critical role in the MAPK pathways, which in turn affects the cell's response to external and internal stimuli. When the expression and/or function of DUSPs

is pathologically altered, or when DUSPs are mutated, it leads to disease. Although it may seem simple to target a specific DUSP implicated in a specific disease, the growing body of knowledge on DUSPs suggests a complex web of stringent regulation, which affects multiple signal transduction partners and feedback control mechanisms, making their validation as drug targets difficult. Understanding the full extent of DUSPs implicated in human health and disease has led to the proposal/development of drugs that target DUSPs. The disorders that DUSPs have been implicated in include tumor-related, neurological, and muscle disorders, and cardiovascular and inflammatory diseases (Table 1).

Inhibitors of DUSPs – Targets for Therapy

As the knowledge of DUSPs grew, the potential use of DUSP inhibitors became a viable option for treating diseases caused by overexpression. Therefore, MAPK-dephosphorylating DUSPs are emerging as targets in treating diseases implicated by DUSP regulation. Sodium orthovanadate was the first DUSP inhibitor used in the 1990s. By 2002, several natural product inhibitors, synthetic derivatives of natural products, and synthetic inhibitors were identified as potent inhibitors of DUSPs (Lyon et al. 2002). The initial challenge of the geometrically shallow and hydrophilic nature of the catalytic domain of DUSPs was overcome by targeting the KIM-MAPK interactions using small-molecule inhibitors. Other approaches proposed for DUSP inhibition have included RNA interference and antisense approaches (Jeffrey et al. 2007). The first study to assess individual DUSP-knockout to test the effects on ERK2 regulation revealed a surprisingly large number of DUSPs responsible for the independent regulation of the ERK2 signaling pathway. Because many MKP-DUSPs are bivalent (i.e., having oncogenic or tumor-suppressing functions depending on cell type and signaling context), direct inhibition of DUSPs in cancer seems challenging. A list of MKP-DUSP expression by cancer type, specific inhibitors used in therapy, and their sensitivity to therapy was compiled by Nunes-Xavier et al. (2011). In an extensive review on DUSPs published in 2012, the authors highlighted reasons why DUSPs make novel targets in medical research and treatment of conditions implicated by DUSPs: their small size and simple domain structure makes pharmacological inhibition of DUSPs successful and effects are likely to be safer, milder, and less dramatic, due to compensatory effects from other DUSPs (Huang and Tan 2012).

Kinases from three major MAPK pathways, including MAPK ERK1/2, p38s, and JNKs, are rendered inactive through the dephosphorylation by DUSPs. Therefore, DUSP enzyme inhibitors seem to be promising targets for use in diseases such as cancer, which involves the overexpression or hyperactivity of a DUSP. Rios et al. (2014) listed the active MKPs and small-sized atypical DUSPs, their substrates, and their representative inhibitors projected to have therapeutic benefits in human disease. This review also discusses the emerging alternative strategies used for DUSP inhibition. Oxidation, differential redox regulation, reversible oxidation, antibody and gene therapies, farnesylation inhibition, and PRL-inhibitory strategies all exist to inhibit DUSP. These inhibition strategies can be used singly or as combination therapies. A recent review provides an updated list of inhibitors of DUSPs that belong to MAPK phosphatases, which can be used to prevent or mitigate human disease. However, several challenges are yet to be overcome before DUSP-inhibitor-based treatments can be clinically validated. Knockout mice, structure-affinity studies, and chemical library screening were used to identify small-molecule inhibitors of Vaccinia H1-related (VHR) phosphatase, also known as dual-specificity phosphatase (DUSP) 3. Examples of small-molecule inhibitors include SA3, RK-682, GATPT, and MLS 0437605 (Pavic et al. 2015). Korotchenko et al. (2014) explored the inhibition of DUSP by a specific inhibitor via structure-activity relationship. They were able to synthesize a series of 26 analogs with modifications in four functional groups of the pharmacophore. *In vivo* studies using transgenic zebrafish were confirmed by in vitro studies.

Dual-Specificity Protein Phosphatases, Table 1 Role of DUSPs in Human Health and Disease

DUSP/s	General category of disease	Details of DUSP implicated in specific diseases	Reference
DUSP1	Allergies	Allergies to house dust mite	Golebski et al. 2015
DUSP1, DUSP 5	Bone-related disorders	DUSP1 (osteolytic lesions in arthritis), DUSP 5 (rheumatoid arthritis)	Vattakuzhi et al. 2012; Moon et al. 2014
DUSP1, DUSP4, DUSP6, DUSP26	Cancers	Brain cancers	Prabhakar et al. 2014
DUSP1, DUSP2, DUSP6, DUSP7, DUSP26		DUSP1 (breast, prostate, ovarian) DUSP2 (ovarian cancer and leukemia), DUSP6 (pancreatic cancer, DUSP7 (AML), DUSP26 (certain cancers)	Jeffrey et al. 2007
DUSP28		human hepatocellular carcinoma	Wang et al. 2014
DUSP 6		Stomach cancer	Liu et al. 2013
DUSP4 and DUSP10		Colorectal cancer	DeVriendt et al. 2013; Zhang et al. 2014
DUSP2		Various cancers	Wei et al. 2013
DUSP5 and 6	Endocrine/ hormonal function	severe hyperparathyroidism	Roman-Garcia et al. 2012
DUSP1 (MKP1), DUSP6 (MKP2), PTEN, PTP4A1 (PRL-1), MTM1, MTMR2, MTMR3, MTMR7, MTMR9, MTMR13, MTMR14	Hereditary disorders	DUSP1 (MKP1), DUSP6 (MKP2), PTEN, PTP4A1 (PRL-1), MTM1, MTMR2 (Charcot-Marie-Tooth Disease), MTMR3 (lung and gastric cancer and early onset inflammatory bowel disease), MTMR7 (risk to variant Creutzfeudlt-Jakob disease), MTMR9 (susceptibility to metabolic syndrome and obesity), SBF2 or MTMR 13 (Charcot-Marie Tooth Disease 4B2), MTMR14 (Autosomal centronuclear myopathy), EPM2A (laforin) – myoclonic epilepsy of Lafora	Hendriks and Pulido 2013
Cdc25A and B MKP-1	Neurodegenerative	Alzheimer's Disease and cancers Huntington's Disease	Ducruet et el. 2005; Taylor et al. 2013
DUSP 5	Vascular development and disease	infantile hemangioma	Pramanik et al. 2009

Salubrinal acts as a DUSP2 inhibitor and suppresses inhibition in anticollagen, antibody-induced arthritis (Hamamura et al. 2015). The crystal structure of DUSP7, a potential drug target for certain types of cancers, has been been determined (Lountos et al. 2015). A further understanding of the challenges arising from specific structural features associated with the active site is needed to develop DUSP7 inhibitors (Lountos et al. 2015). This class of enzymes was considered "undruggable" due to the highly conserved structure and charged and reactive catalytic site but has now become a focus of drug targets (Hoekstra et al. 2016).

Summary

Protein kinases, including the MAPKs have long been implicated in cellular growth, differentiation, division, and metabolism. It was only in the early 1990s that the critical role of new group of enzymes, protein phosphatases, and more specifically, DUSPs, was discovered as playing

regulatory roles by dephosphorylating kinases part of MAPK pathways. The human genome encodes about 44 DUSPs named for their ability to dephosphorylate both tyrosine and serine/threonine residues within one substrate. DUSPs are subdivided into six subgroups based on sequence similarity, substrate specificity, and physiological role. The six groups are as follows: Slingshots, PRLs, CDC14s, PTENs and Myotubularins, MKPs, and atypical DUSPs. Some DUSPs are known to occur in the nucleus, others in the cytoplasm, while some are dually located. In cells that are at rest, they are known to be expressed at low levels, but upon stimulation, can be very rapidly transcribed. The mechanisms of DUSP regulation suggest that DUSPs are regulated at the transcriptional level, post-translational level, and protein activity level. The phosphatase domain of a DUSP contains a conserved aspartic acid, cysteine, and arginine residues in the catalytic site. Dephosphorylation occurs when the catalytic cysteine participates in the formation a thiol-phosphate covalent transient intermediate. Ever since the role of DUSPs as key regulators of MAPKs began to emerge, researchers explored the role of DUSPs in diseases. Research of DUSPs implicated in diseases till date has revealed that specific DUSPs serve as tumor promoters or tumor suppressors in various cancers depending on the type of cell or signaling context. This feature makes it difficult to target DUSPs in cancer treatment by direct inhibition. DUSPs have also been implicated in neurodegenerative diseases including Alzheimer's disease, Huntington's disease, various brain cancers, hereditary disorders, and endocrine disorders and even in allergic responses. Although DUSPs began to be targeted in treating diseases associated with MAPK-phosphatase regulation a decade later than when kinases were prominent, they seem to be more desirable for targeting because of features including their diversity in structure, specificity, and function and small-size and safe application with fewer side effects. It must be noted that DUSPs not only target MAPKs but other regulators including STATs, histones, etc., and their complexity in signal regulation is likened to that of a neuron network. Many strides have been made in the field of development of DUSP-inhibitors so far, and current work continues to focus on *in vivo* efforts to validate the *in vitro* results. Knock-out studies, structure-affinity, and chemical screening methods are used in identifying inhibitors. Understanding the catalytic activity of DUSPs helps exploit them as drug targets through oxidation, differential redox regulation, reversible oxidation, antibody and gene therapies, farnesylation inhibition, and PRL-inhibitory strategies. Some of these inhibition strategies can be used singly or as combination therapies. In conclusion, this review provides a general overview of the classification, structure, function, and the regulatory role of DUSPs in human health and disease and presents a short update on how DUSPs are being explored as targets in cancer treatment and therapy.

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Dual-Specificity Protein Tyrosine Phosphatase (PTP)

▶ Dual-Specificity Protein Phosphatases

DUET

▶ Kalirin

Duo (Human Kalirin-7)

▶ Kalirin

DUSP24

▶ MK-STYX

DXS6984E

▶ BEX3

DYNLT1/Tctex-1 (AGS2)

▶ Activators of G-Protein Signaling (AGS)

Dyrk1a

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Synonyms

Dual specificity tyrosine-phosphorylation regulated kinase 1A; Dual specificity Yak1-related kinase 1A; hMNB; Minibrain; mnb; Mnb; MNB; MNBH; Protein kinase minibrain homolog

Historical Background

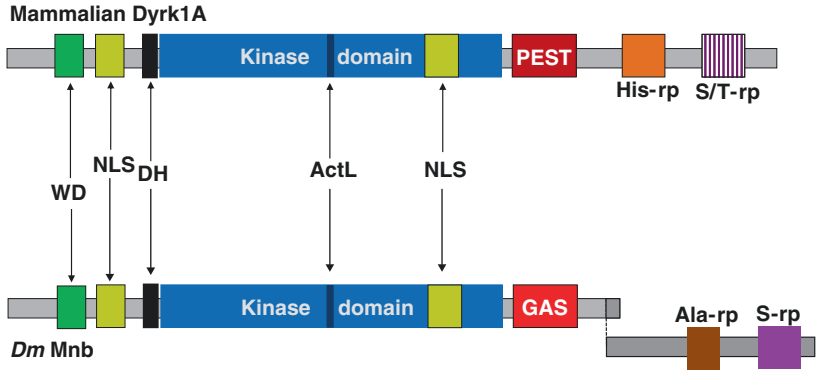
DYRK1A is a member of the Mnb/DYRK subfamily of protein kinases, which falls within the CMGC Ser/Thr family to which cyclin-dependent kinases (CDKs), CDC-like kinases (CLKs), glycogen synthase kinase (GSK3), and mitogen-activated protein kinase (MAPK) also belong. The founding member of the Mnb/DYRK subfamily in multicellular organisms was identified in *Drosophila melanogaster* (Dm) and due to the reduced brain size associated with its loss of function it was named *minibrain (mnb*). This phenotype is caused by altered proliferation during brain development, suggesting a key function for this kinase in the regulation of neural proliferation and neurogenesis (Tejedor et al. 1995). In addition, *mnb* mutant flies have behavioral defects, indicative of alterations to neuronal activity. The rat Mnb ortholog was independently cloned, identifying unusual structural features and biochemical properties of this novel protein kinase (Kentrup et al. 1996). This protein was called Dyrk1 (Dual specificity tyrosine (Y)-phosphorylation Regulated Kinase) due to its ability to perform Tyr autophosphorylation and Ser/Thr phosphorylation in exogenous substrates. Interestingly, the human *MNB/DYRK1A* ortholog maps to the Down syndrome (DS) critical region (Guimera et al. 1996), and it is overexpressed in the fetal and adult DS brain. These seminal publications have greatly influenced research into this protein kinase and thus, most initial efforts were oriented toward understanding its role in neurodevelopment, its implications in DS neurobiology and its unique biochemical properties (reviewed by Becker and Sippl 2011; Tejedor and Hammerle 2011). The highly conserved protein structure of the Mnb/Dyrk1A kinase orthologs (reviewed in Aranda et al. 2011) prompted extensive studies in mouse mutants and DS mouse models. Remarkably, transgenic mice overexpressing *Dyrk1A* experience neurodevelopmental delay and cognitive deficits (Altafaj et al. 2001), and

haploinsufficient *Dyrk1A*$^{+/-}$ mice have smaller brains (Fotaki et al. 2002), strongly suggesting evolutionary conserved functions in brain development.

Although Mnb/Dyrk1A homologs have been identified from yeast to humans (reviewed in Aranda et al. 2011), the deletion of *mbk-1* (the closest homologue in *Caenorhabditis elegans*) does not cause morphological alterations in any tissue, and if we also consider that there are also some differences in protein structure (see below), it would appear that most relevant cellular functions of Mnb/Dyrk1A emerged during evolution from insects on. Accordingly, only data from Dm Mnb to human DYRK1A will be discussed here and the structurally distinguishable (see below) vertebrate paralog involved in muscle development and tumorigenesis, Dyrk1B/Mirk, will not be considered either.

Protein Structure and Biochemical Properties of DYRK1A

The Mnb/DYRK kinase subfamily, which includes mammalian DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4 (see Aranda et al. 2011 for a detailed phylogenetic analysis), is characterized by specific sequence motifs in the catalytic domain. Nevertheless, Mnb/DYRK1A orthologs display several unique structural features outside the kinase domain (Fig. 1, see also http://www.uniprot.org/uniprot/P49657 and http://www.uniprot.org/uniprot/Q13627 for details of the *Dm* Mnb and Human DYRK1A proteins, respectively). Thus, in addition to the DYRK homology (DH) box, specific to all DYRK kinases, the N-terminal of Mnb/DRK1A contains a bipartite nuclear localization signal (NLS) that targets DYRK1A to the nucleus. There is another NLS harbored within the kinase domain of DYRK1A (and possibly in *Dm* Mnb too) but not in that of the DYRK1B paralogs. The amino-terminal also contains a motif for binding to adaptor WD40 repeat domain proteins like DCAF7/HAN11, WDR68. It has also been proposed recently that a basic patch comprising part of the N-term NLS domain may bind directly to tubulin. A PEST motif (rich in proline, glutamic acid, serine, and threonine), putatively involved in rapid protein degradation, is located at the carboxy-terminal of the kinase domain. A histidine repeat (His-rp), possibly involved in localization to nuclear splicing speckles, and a serine/threonine repeat (S/T-rp) of unknown function are also located in the C-terminal of vertebrate Mnb/DYRK1A orthologs but not in DYRK1Bs. The *Dm mnb* gene encodes several alternative C-terminal splice isoforms (Tejedor et al. 1995: see updated information at http://flybase.org/cgi-bin/gbrowse2/dmel/?Search=1;name=FBgn0259168), all of which show significant sequence homology in the C-terminus to mammalian DYRK1As



Dyrk1a, Fig. 1 Schematic representation of the protein domain structure of mammalian DYRK1A and Dm Mnb. *ActL* activation loop, *Ala-rp* Alanine repeat, *DH* DYRK homology box, *His-rp* Histidine repeat, *NLS* nuclear localization signal, *PEST* proline-, glutamic acid-, serine-, threonine-rich region, *S-rp* serine repeat, *S/T-rp* serine/threonine repeat, *WD* binding motif to adaptor WD40 repeat domain proteins

although lack the His and S/T repeat domains. They also contain a GAS domain (rich in Glycine, Alanine, and Serine) which fulfills a similar function to the vertebrate PEST domain. In addition, the Dm Mnb long isoforms contain an Alanine repeat (Ala-Rp) and a Serine rich (Ser-Rp) motif of unknown function. By contrast, although the *Caenorhabditis elegans* homolog, Mbk-1, is highly conserved in the catalytic domain and DH-box, it differs in its nuclear localization and C-terminal structure.

A very unusual property of Mnb/DYRK1A kinases is that they are switched on by autophosphorylation of a Tyr-X-Tyr motif in the activation loop located within the catalytic domain (Kentrup et al. 1996). Remarkably, this Tyr-phosphorylation is irreversible and independent of regulatory signals. Moreover, it takes place only on a transitory intermediate form of the kinase during protein translation, leading to the constitutive activation of the kinase (Lochhead et al. 2005). Subsequently, the mature active kinase only phosphorylates substrates at Ser/Thr residues (reviewed by Becker and Sippl 2011).

Pioneering work with a peptide library designed according to putative substrate sequences defined DYRK1A as a proline-directed kinase with a RPX (S/T)P phosphorylation consensus sequence and accordingly, a peptide (DYRKtide) with the optimal substrate sequence was designed (Himpel et al. 2000). Nevertheless, the identification of non-consensus phosphorylation sites in several putative substrates suggests that DYRK1A is not strictly a proline-directed kinase. Thus, DYRK1A appears to be a pleiotropic protein kinase capable of phosphorylating a plethora of substrates *in vitro* or when overexpressed in diverse cell systems. Nevertheless, only a fraction of these substrates have been verified *in vivo*, and therefore, the majority should only be considered as putative substrates (Table 1). As described below, these substrates are involved in quite diverse cell processes.

Cellular Functions of DYRK1A

Based on the molecular nature of its putative substrates and interactors, as well as the cellular phenotypes caused by its mutations, Mnb/DYRK1A would appear to participate in the regulation of a wide repertoire of cell functions (Table 1), some of which deserve particular mention.

Given the phenotype of *Dm mnb* mutants, the regulation of the cell cycle of neural progenitors by Mnb/DYRK1A has been studied extensively, identifying several targets and underlying mechanisms (Table 1, Fig. 2). Thus, Dyrk1A appears to control the duration of G1 in neural progenitors by promoting Cyclin D1 turnover through its direct phosphorylation (Soppa et al. 2014). A similar action on CycD3 has been implicated in lymphoid development. On the other hand, the phosphorylation of p27Kip1, the main cyclin-dependent kinase inhibitor (CKI) in the mammalian CNS, results in its stabilization, possibly facilitating the differentiation of neuronal precursors (Soppa et al. 2014). Remarkably, Mnb/DYRK1A also promotes the expression of p27Kip1 through the regulation of a transcriptional network to trigger the cell cycle exit of neuronal precursors (Hammerle et al. 2011; Shaikh et al. 2016). Antiproliferative effects of DYRK1A in neural progenitors have been also proposed to be mediated through p53 phosphorylation and the subsequent expression of p21cip1, another CKI. Similarly, Dyrk1A appears to control the G1 entry/exit decision in fibroblasts by regulating the relative levels of CycD1 and p21cip1. Interestingly, DYRK1A was seen to facilitate cell cycle exit of tumor cells by phosphorylating the DREAM complex subunit LIN52, thereby promoting the assembly of this repressor complex, leading to the silencing of E2F cell cycle target genes and driving their entry into quiescence/senescence (Tschöp et al. 2011).

Although Mnb/Dyrk1A has mainly been attributed an antiproliferative role (reviewed by Tejedor and Hammerle, 2011), there are some cases in which it seems to promote growth. This is the case in *Drosophila* larval imaginal discs (the eye, wing, and leg primordia), where Mnb promotes Salvador-Warts-Hippo (SWH) signaling (Degoutin et al. 2013). These apparently conflicting data might be due to distinct functions of Mnb/DYRK1A in neural and non-neural progenitors. There have also been a few, yet

Dyrk1a, Table 1 Substrates and interactors of Dyrk1A arranged into functional subgroups

Cell function subgroup	Protein	Molecular nature	Molecular Relation with Dyrk1A	Biological function
Cell cycle regulation	Cyclin D1	Regulatory subunit of cyclin-dependent kinases (CDKs)	S (**iv**)	Proliferation of neural progenitors
	Cyclin D3	Regulatory subunit of cyclin-dependent kinases (CDKs)	S	Proliferation of lymphocyte precursors
	LIN52	Subunit of the DREAM complex	S	Quiescence/ senescence of tumor cells
	p27Kip1	Cyclin-dependent kinase inhibitor (CKI)	S (**iv**) ExpR (**iv**)	Neurogenesis and neuronal differentiation
Cell death regulation	ASK1	Apoptosis signal regulation kinase	S	UF
	Caspase 9	Cysteine aspartyl protease	S (**iv**)	Programed cell death of neurons
	SIRT1/Sir2	NAD-dependent protein deacetylase	S	Food intake
Cytoskeleton Regulation	ABLIM1	Actin regulatory protein	S	UF
	MAP1B	Microtubule associated protein	S (**iv**)	Neuronal differentiation
	N-WASP	Regulator of actin cytoskeleton	S	Neuronal differentiation
	SEPT4	GTPase and cytoskeleton scaffolding protein	S (**iv**)	Neuronal differentiation
	TAU	Microtubule-associated protein	S, $ (**iv**)	Neuronal differentiation, neurodegeneration
	β-tubulin	Microtubules component	S (**iv**)	Dendritogenesis
Gene Expression Regulation	Arip4	Steroid hormone receptor cofactor, chromatin remodeling	I, S	UF
	Capicua (Cic)	Transcriptional repressor of receptor tyrosine kinase/ERK pathway	I, S	Tissue growth
	CREB	cAMP responsive transcription factor	S	Neuronal differentiation
	CRY2	Cryptochrome TF	S (**iv**), $	Circadian regulation
	FKHR / FOXO1	Transcription factor	S, I	UF
	GLI1	Transcription factor, oncogene	S	Proliferation
	Histone H3	Chromatin remodeling	S	UF
	INI1/SNF5, SNR1	Component of the SWI/SNF chromatin remodeling complex	S, I	UF
	NFAT	Transcription factor	S (**iv**), $	Tissue growth, cell proliferation
	NRSF/REST	Neuron-restrictive transcriptional regulator	S, ExpR (**iv**)	Cell proliferation, differentiation
	p53	Transcription factor	S (**iv**)	Neural proliferation
	RNAPII	RNA polymerase	S	UF
	STAT3	Transcription factor	S (**iv**)	Astrogliogenesis

(*continued*)

Dyrk1a, Table 1 (continued)

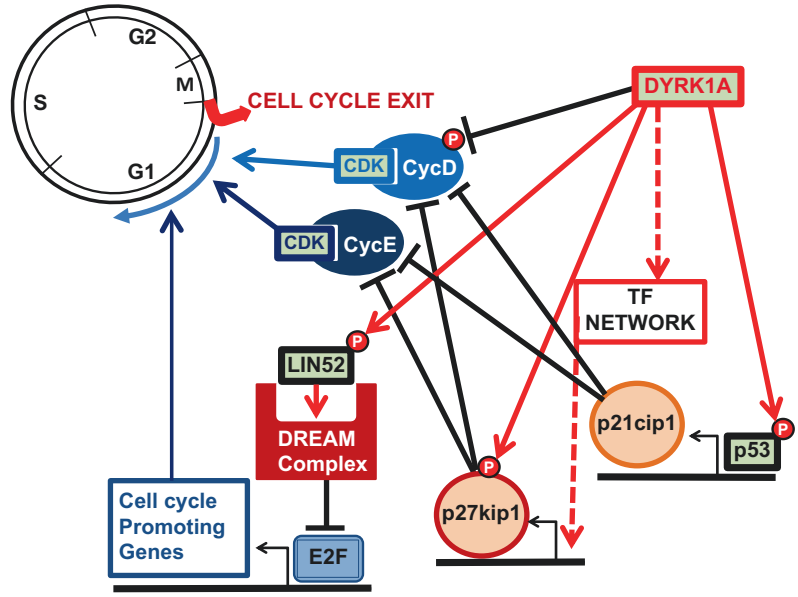
Cell function subgroup	Protein	Molecular nature	Molecular Relation with Dyrk1A	Biological function
Membrane trafficking	Amphiphysin	Protein of synaptic vesicles	S	Synaptic function
	DNM1	GTPase	S (**iv**)	Dendritogenesis, synaptic function
	Endophilin 1	Endocytosis regulatory protein	I (**iv**)	Synaptic function
	Munc18–1	Regulator of exocytosis	S (**iv**)	Synaptic function
	Synaptojanin 1 (Synj)	Endocytosis regulatory protein	S (**iv**)	Synaptic function
	α-synuclein (SNCA)	Possible regulator of synaptic vesicle release	S	UF
mRNA processing	ASF	Splicing factor	S, I (**iv**)	UF
	Cyclin L2	Splicing factor	S	UF
	SF3b1/ SAP155	Splicing factor	S	UF
	SRp55	Splicing factor	S	UF
	9G8	Splicing factor	S	UF
Transmembrane receptor signaling	APP	Amyloid precursor protein	S	UF
	GluN2A	Subunit of NMDA receptors	S	Synaptic function
	Notch	Cell-cell signaling transmembrane receptor protein	S, (**iv**)	Neuronal differentiation
	P120-catenin	Component of non-canonical WNT signaling	S	UF
	Presenilin1 (PS1)	Component of the γ-secretase complex	S	UF
	Sprouty 2 (SPRY2)	Modulator of growth factor receptor signaling	S (**iv**)	Adult neurogenesis
	Warts (Wts)	Protein kinase of the Hippo pathway	S	Tissue growth
Miscellaneous	eIF2B	protein-synthesis initiation factor	S,$	Protein translation
	Glycogen synthase	Glycogen synthase	S	Metabolism regulation
	Parkin	E3 ubiquitin ligase	S	UF
	RCAN1/ DSCR1	Regulator of calcineurin	S, $ (**iv**)	UF
	SIRT1/Sir2	NAD-dependent protein deacetylase	S, (**iv**)	Food intake

Since Mnb/Dyrk1A is a very pleiotropic protein kinase, only substrates/interactors for which at least cellular or functional evidence were reported are included in this table. Among them, those confirmed in proper *in vivo* studies are highlighted (**iv**). Abbreviations: *ExpR* expression regulation, *I* interactor, *S* substrate, *$* priming of GSK3 phosphorylation, *UF* unknown function

consistent, reports on how DYRK1A could regulate cell death/survival by phosphorylating well-known effectors like ASK1, Caspase 9, and SIRT1/Sir2 (Table 1).

Another prominent function of DYRK1A is in the regulation of the cytoskeleton. A number of regulators of both actin (ABLIM1, N-WASP) and microtubules (MAP1B, TAU, and β-tubulin) are phosphorylated by Mnb/DYRK1A, and there are compelling data showing that these actions may influence neuritogenesis, dendritogenesis, synaptogenesis, and synaptic plasticity (reviewed by Tejedor and Hammerle 2011).

There is also compelling evidence for the involvement of DYRK1A in the regulation of gene expression. Thus, DYRK1A can phosphorylate



Dyrk1a, Fig. 2 Schematic summary of main molecular actions of Mnb/DYRK1A on cell cycle regulation as described in the text. DYRK1A exerts positive effects (*red arrows*) on negative cell cycle regulators and negative effects (*black* T shaped *arrows*) on positive regulators

several transcription factors (CREB, FKHR/FOXO1, GLI1, NFAT, NRSF/REST, p53, STAT3), regulating their activity, stability/degradation, or nuclear-cytoplasmic trafficking. These actions can affect specific functions such as growth, proliferation, differentiation, or precursor cell specification, although detailed functional studies are scarce (Table 1; reviewed by Tejedor and Hammerle 2011). In addition, it has been reported that Mnb/DYRK1A may have a broad effect on gene transcription by acting on chromatin remodeling regulators like the SWI/SNF complex and Histone 3, or by regulating RNA polymerase II. However, the functional consequences of these actions remain to be elucidated. Furthermore, there is increasing evidence for the involvement of DYRK1A in the regulation of mRNA splicing through the phosphorylation of several splicing factors (see Table 1). Nevertheless, with the exception of the effect on the splicing of Tau mediated by ASF, again very little is known about their functional consequences.

Mnb/DYRK1A has also emerged as a key regulator of membrane trafficking (Table 1), particularly at the synapse. Remarkably, key components of the endocytic machinery like amphiphysin, dynamin 1, endophilin 1, and synaptojanin (Synj) have been identified as substrates/interactors of Mnb/DYRK1A, possibly allowing these interactions to be modulated in an activity dependent manner (Xie et al. 2012 and refs. therein). Remarkably, Dm Mnb is required for synaptic growth and synaptic vesicle endocytosis in vivo through the regulation of Synj activity (Chen et al. 2014).

Regulation of DYRK1A and its Involvement in Cell Signaling

In contrast to the information available about its downstream targets, very little is known about the upstream pathways that regulate the gene expression, activity, and subcellular localization of DYRK1A (reviewed by Becker and Sippl 2011, Table 2). As the Mnb/DKRY1A kinase is constitutively activated during translation, it is currently assumed that changes in its expression are translated into changes in its activity. Thus, Mnb/DKRY1A expression appears to be tightly regulated, although very few underlying mechanisms have been proposed to date, and even fewer have been assessed *in vivo* (Table 2). Thus, two homologous neuropeptides, NPY/sNPF, modulate Mnb/DYRK1A expression through the PKA-CREB axis, playing a key role in the

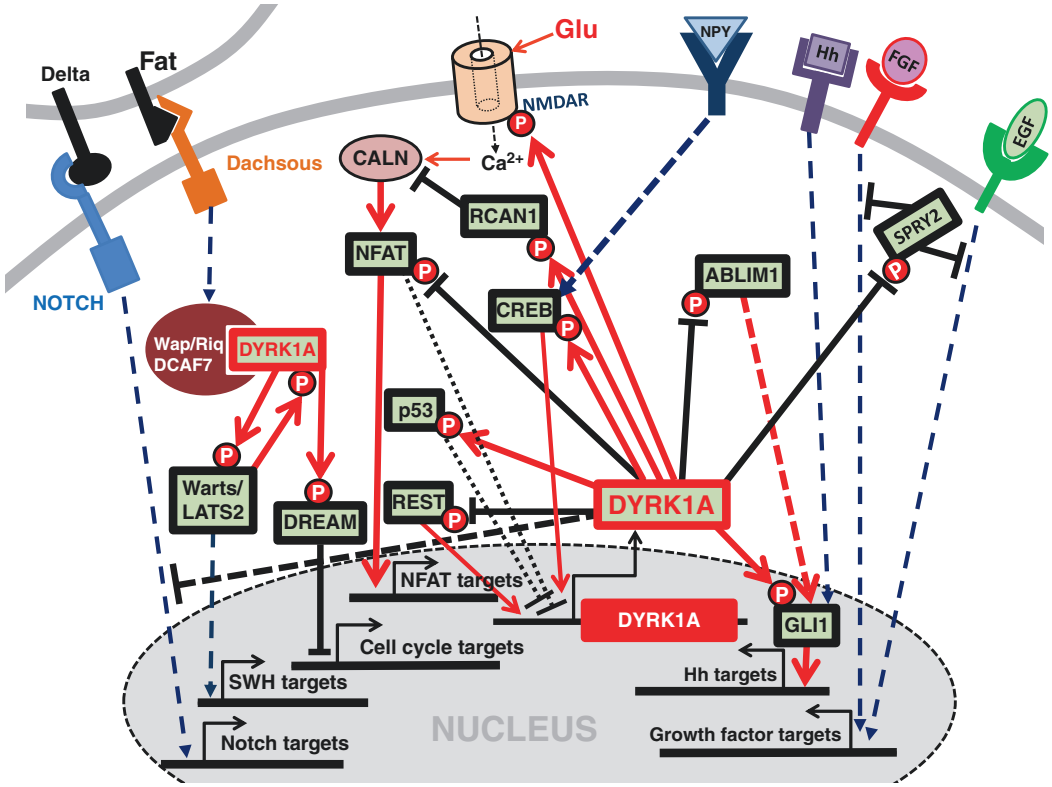
Dyrk1a, Table 2 Regulators of activity and expression of DYRK1A

Regulator group	Protein	Molecular nature or function	Molecular action	Possible cell function
Regulators of DYRK1A kinase activity	DCAF7/ HAN1/ WDR68 Wap/Riq	WD40 repeat domain proteins	Interactor (**iv**)	Cell proliferation,
	E1A	Viral oncogene	Interactor	Oncogenic transformation
	LATS2,	Protein kinase of the Hippo pathway	Phosphorylation	Cell proliferation
	14–3-3	14–3-3 family of regulating proteins	Interactor	UF
Regulators of DYRK1A expression	β-amyloid (Aβ)	Peptide derived from APP. Main component of amyloid plaques in Alzheimer disease	ND	Neurodegeneration
	CREB	Transcription factor	Transcription downstream of NPY/sNPF signaling (**iv**)	Food intake
	E2F1	Transcription factor	Transcription	Cell cycle
	miR-199b	MicroRNA	mRNA regulation downstream of NFAT (**iv**)	Cell proliferation
	miR-1246	MicroRNA	mRNA regulation downstream of p53	UF
	NRSF/ REST	Neuronal transcriptional repressor	Transcription (**iv**)	Neuronal differentiation

Actions assessed in proper *in vivo* studies are highlighted (**iv**). Abbreviations: *ND* not determined, *UF* unknown function

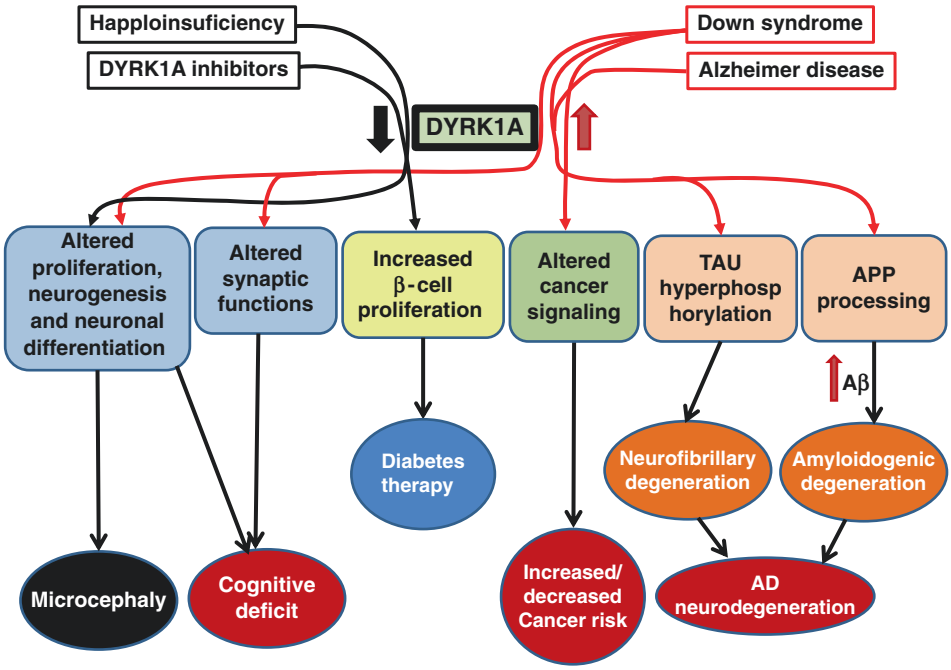
regulation of food intake (Hong et al. 2012). Mnb/Dyrk1A expression is also regulated by miR-199b, a direct calcineurin/NFAT target involved in cardiomyocyte growth (Da Costa Martins et al. 2010). Interestingly, some regulators of its expression (CREB, NFAT, p53, and REST) are direct targets of Mnb/DYRK1A (Table 1), which points to possible feedback and feedforward mechanisms (Fig. 3).

There is also little information on the regulation of Mnb/DYRK1A activity. There are indications that the intracellular distribution and compartmentalization of DYRK1A may depend on its differential phosphorylation, although the kinases that putatively regulate these events are unknown. Possible regulators of Mnb/DYRK1A could be found among the interactors identified in various high-throughput analyses. Thus, a summary of the genetically generated interactome of Dm Mnb can be found in Flybase (http://flybase.org/cgi-bin/get_interactions.html?items=FBgn0259168&mode=ppi) and putative DYRK1A interacting proteins can be found in the MINT database (http://mint.bio.uniroma2.it/index.py). Unfortunately, there are currently no functional assessments available, except for that of DCAF7 that has repeatedly been shown to be a substrate recruiting subunit of DYRK1A. Interestingly, its *Drosophila* ortholog Wap/Riq associates with Mnb in response to signaling from the atypical cadherins Fat and Dachsous, thereby inducing phosphorylation-dependent inhibition of the Hippo pathway kinase Warts and promoting tissue growth (Fig. 3, Degoutin et al. 2013). Strikingly, LATS2, the mammalian Warts ortholog, appears to phosphorylate DYRK1A and enhances its antiproliferative action through the DREAM complex (Fig. 3, Tschöp et al. 2011). These apparently conflicting results might be due to differences in cell context, evolutionary functional divergence or to a possible regulatory feedback loop.



Dyrk1a, Fig. 3 Involvement of Mnb/DYRK1A in cell signaling. DYRK1A exerts positive (*red arrows*) and negative (*black* T *shaped arrows*) effects on several factors as described in the main text. Signaling cascades are indicated by *blue dotted arrows*

Although Mnb/DYRK1A has not been clearly associated with a particular signaling pathway, there are abundant data showing that it can interact with and modulate diverse cell signaling mechanisms (summarized in Fig. 3). Unfortunately, only some of these have been assessed in clear functional contexts. Thus, the inhibition of NFAT signaling by its direct phosphorylation by Mnb/DYRK1A or through phosphorylation of RCAN1/DSCR1, an inhibitor of the calcium/calmodulin-dependent phosphatase calcineurin (Caln) that regulates NFAT nuclear translocation, alters a range of developmental processes (Arron et al. 2006; Wang et al. 2015). Interestingly, DYRK1A promotes EGFR stability by preventing its endocytosis-mediated degradation through the phosphorylation of SPRY2, participating in the regulation of self-renewal and cell-fate of adult neural stem cells (Ferron et al. 2010). A similar action on SPRY2 has been described for FGF–MAPK signaling although its function is unknown. DYRK1A represses Delta/Notch signaling to facilitate neuronal differentiation through a yet unknown mechanism (Hammerle et al. 2011). Two DYRK1A-modulated arms within the mammalian Hh signaling cascade have also been described. On one hand, DYRK1A can stimulate Hh signaling by phosphorylating and retaining GLI1 in the nucleus; on the other, DYRK1A can inhibit Hh signaling by destabilizing GLI1 through a complex cascade that begins with the actin regulator ABLIM (Schneider et al. 2015 and references therein). These opposing effects of DYRK1A could be cell-type specific or depend on its level of expression. Additionally, DYRK1 can modulate Ca^{2+} signaling in neurons by phosphorylating NMDA receptors. Finally, the capacity of DYRK1A to act



Dyrk1a, Fig. 4 Increased (*red arrow*) or decreased (*black arrow*) activity/expression of DYRK1A can alter several pathogenic pathways. See description in the main text

as a priming kinase for several GSK3 substrates (e.g., CRY2, eIF2B, MAP1B, NFAT, RCAN1, and TAU) strongly suggests that DYRK1A might also modulate GSK3 mediated pathways.

Pathological Implications of DYRK1A Dysfunction

The evidence of several roles for DYRK1A in neurodevelopment (neural proliferation, neurogenesis, and neuronal differentiation) and neuronal functions (particularly, synaptic functions) have sustained the hypothesis that DYRK1A overexpression has a major contribution to DS mental retardation (Tejedor and Hammerle 2011, Fig. 4). This idea has been reinforced by evidence from DS mouse models and transgenic mice overexpressing Dyrk1A, which display several neurobiological alterations reminiscent of DS. Thus, DYRK1A is presently considered a suitable drug target for DS therapy (reviewed by Becker et al. 2014). Remarkably, happloinsuficiency of *DYRK1A* causes an intellectual disability syndrome characterized by microcephaly (see an overview in GeneReviews® http://www.ncbi.nlm.nih.gov/books/NBK333438/). Indeed, reduced expression or activity of Mnb/DYRK1A provokes neuronal deficits in the developing *Drosophila* and vertebrate brain (Tejedor et al. 1995; Fotaki et al. 2002), inducing cell death after the failure of neuronal precursor to exit the cell cycle and differentiate (Hammerle et al. 2011, Shaikh et al. 2016). Together these data highlight how sensitive neurodevelopmental processes are to *DYRK1A* dosage imbalance (Fig. 4).

The fact that DS individuals develop Alzheimer's disease (AD) precociously and that increased DYRK1A expression has been found in AD brains prompted intense research into the implications of DYRK1A in neurodegeneration. Significantly, several neurodegeneration related proteins (APP, Parkin, Presenilin 1, SEPT4, α-Synuclein, and TAU) have been identified as DYRK1A substrates. Furthermore, Tau is hyperphosphorylated in transgenic mice that overexpress Dyrk1A, and Aβ levels are elevated. As mentioned before, DYRK1A could also contribute to

neurofibrillary degeneration by dysregulating TAU splicing. Thus, there is compelling evidence that DYRK1A overexpression can contribute to neurofibrillary and amyloidogenic degeneration by deregulating multiple pathways (Fig. 4, reviewed by Wegiel et al. 2011).

Again, the observation that DS individuals have an increased risk of leukemia and a decreased risk of solid tumors, in conjunction with the functions of DYRK1A in cell cycle regulation and its interaction with tumor related signaling pathways (e.g., EGFR, Hh, Hippo, and NOTCH) make DYRK1A a focal point in cancer research. Thus, it has been proposed that DYRK1A may contribute to leukemogenesis by dysregulating NFAT and to glioblastoma growth by enhancing EGFR signaling. There is also evidence that DYRK1A may modulate the activity of viral oncoproteins.

Finally, it should be noted that DYRK1A has also been implicated in the deregulation of pathogenic pathways involved in heart failure (i.e., NFAT: Da Costa Martins et al. 2010), and that DYRK1A inhibitors promote NFAT mediated β-cell proliferation (Wang et al. 2015). Accordingly, DYRK1A is now being considered a possible target for certain therapeutic approaches aimed at managing diabetes.

Summary

DYRK1A is a pleiotropic protein kinase capable of phosphorylating a large and molecularly diverse repertoire of substrates, although the true cell context and biological function of these events often remain to be elucidated or confirmed *in vivo*. Nevertheless, compelling data implicate DYRK1A in the regulation of quite diverse cellular functions like cell cycle, cell death, cytoskeleton dynamics, gene expression, membrane trafficking, mRNA processing, and transmembrane receptor signaling. The functional diversification reflecting its molecular pleiotropicity is further enhanced by the capacity of DYRK1A to modulate quite diverse cell signaling pathways (EGFR, Hh, Hippo, Notch, etc).

The highly conserved structure and the picture emerging from the still limited comparative studies make it likely that most biological functions of DYRK1A are to a large extent evolutionarily conserved. By contrast, it is possible that some cellular functions might work in divergent (even opposing) directions in different tissues, as is the case for the antiproliferative effects in the larval brain relative to the promotion of growth in larval imaginal discs of *Drosophila*. It remains to be determined whether these divergent effects could be due to different levels of expression, or to the presence/absence of certain targets or regulators of DYRK1A activity.

The functional diversity of DYRK1A is producing an increasing number of reports on its pathological implications (cognitive deficit, neurodegeneration, cancer, etc.), which is driving efforts to identify and develop new inhibitors for therapeutic strategies. Nevertheless, as several cell functions have been found to be particularly sensitive to DYRK1A dosage and some dysfunctions have been described for both excessive and defective activity/expression of DYRK1A, progress in this area will be particularly challenging. An additional difficulty in this regard will be to design inhibitors sufficiently specific for DYRK1A and that do not interfere with structurally related kinases like DYRK1B and CLKs. Finally, further efforts to increase the scarce information on the upstream mechanisms that regulate DYRK1A expression and activity will greatly help to understand better the functional framework of this increasingly important signaling molecule.

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Dystroglycan

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Synonyms

156DAG; A3a; AGRNR; DAG; DAG1; Dystroglycan 1; Dystrophin-associated glycoprotein 1; MDDGA9; MDDGC7; MDDGC9

Historical Background

Thirty years ago, the biochemical studies of plasma membranes of rodent NG108–15 neural hybrid cells, 14-day embryonic chicken brain, and mouse 3 T3 fibroblasts had led to the identification of cranin (LBP120), a glycosylated laminin-binding protein. Later, by sucrose-gradient centrifugation following purification of proteins from heavy microsomes of rabbit skeletal muscles using wheat germ agglutinin and DEAE-sepharose, four novel glycoproteins associated with dystrophin were purified and labelled as the "dystrophin-glycoprotein complex (DGC)" (Ervasti et al. 1990). At the center of this complex, the dystroglycan (DG) has been identified as a glycan component whose amino acid sequence is identical to cranin. Since then, other members of the DGC were identified. Thus, the DGC provides a link between proteins of the extracellular matrix (ECM) and the internal actin-cytoskeletal machinery (Fig. 1; Table 1). Also the cytoplasmic domain of DG interacts with several mediators of signaling pathways (Table 1; Fig. 4).

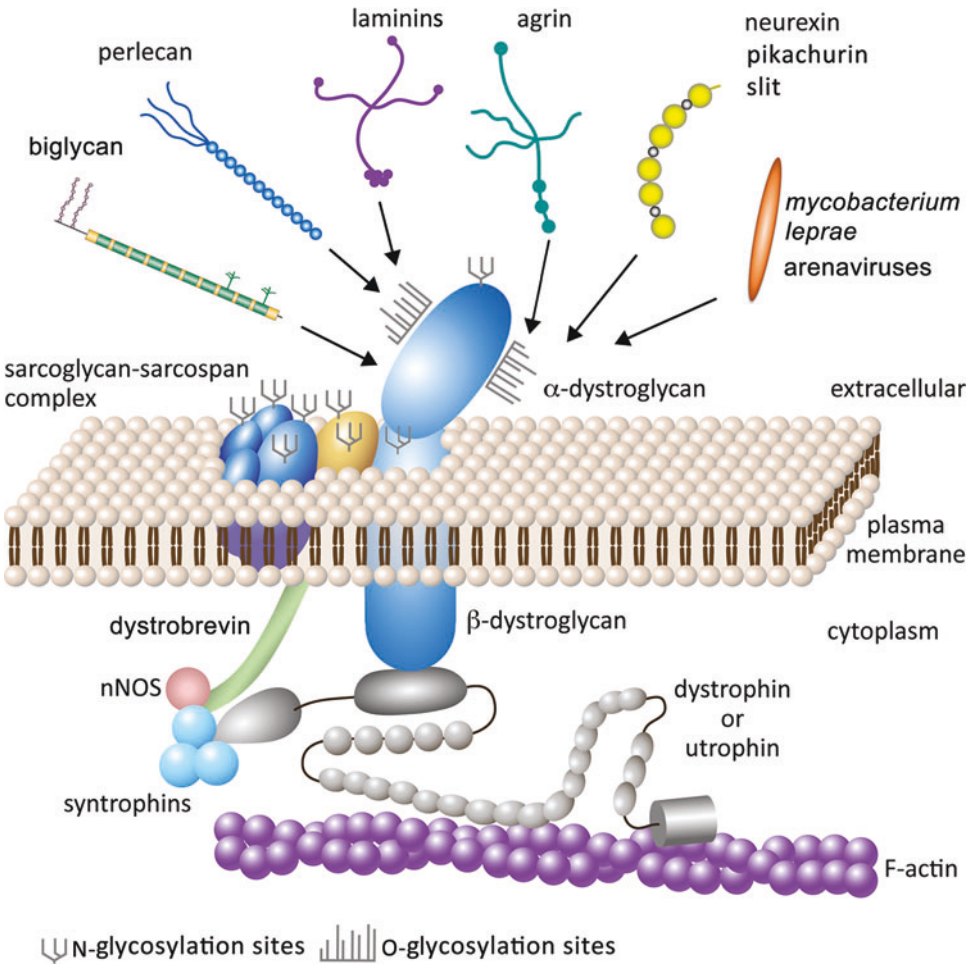
Gene, Protein Structure, Expression and Distribution

DG genes have been cloned and identified in a broad diversity of phyla. Analyses of the phylogenetic distribution of *DG* and its partners have identified that *DG* originated after the divergence of ctenophores from porifera and eumetazoa (see Table 2 in Adams and Brancaccio 2015).

Gene and Protein Structure

Using somatic cell hybrids and fluorescence in situ hybridization, the human gene (*dystroglycan 1, DAG1*) has been mapped to chromosome 3p21.1–21.31, the mouse gene to chromosome 9 in a region of conserved synteny with human 3p. The coding sequence is organized over a small and a large exon (2.4 kb) separated by a broad intron. The mature 5.8 kb transcript that contains an 895-residue open reading frame is translated in a protein with a molecular mass of 96 kD. Posttranslational modifications, including proteolysis, phosphorylation, and glycosylations, result in the mature form of DG (Fig. 2). The mature form is a type I transmembrane protein composed of two noncovalently interacting subunits: α-DG, an extracellular protein of 653 amino acids; and β-DG, a protein of 242 amino acids which contains extracellular, transmembrane, and cytoplasmic domains.

The α-DG subunit possesses two globular domains flanking a central mucin domain that is characterized by a high concentration of proline residues and more than 40 serine/threonine residues. In the reticulum, in addition to proteolysis and N-glycosylation, the *O*-glycosylation on serine/threonine residues begins in the N-terminal half of the mucin domain leading to three types of *O*-mannosyl glycan structures (Endo 2015 for a review). In the endoplasmic reticulum, the glycosylation, that is shared among the three structures, starts by the addition of mannoses to hydroxyl groups on Ser/Thr residues by protein *O*-mannosyltransferase 1 and 2 (POMT-1, POMT-2). Then, the protein β-1,2-N--acetylglucosaminyltransferase (POMGnT1) transfers an N-acetylglucosamine residues to *O*-linked mannoses leading to the M1 glycan structure. A subset of M1 structures are modified by the *N*-acetylglucosaminyltransferase Vb (GnT-Vb, also referred to GnT-IX) that branches the mannose with a β1,6-linked N-acetyl glucosamine generating the M2 glycan structure. Subsequently, enzymes add galactose, fucose, glucuronic acid, neuraminic acid, and sulfate groups to M1 and M2 glycan structures in the Golgi apparatus. On a subset of mannoses, the glycosylation is extended to form an O-mannosyl trisaccharide leading to the M3 glycan structure (Fig. 3). Then possibly, a phosphorylation of mannoses allows the addition of xylosyl-glucuronyl polymers, named matriglycan, required to bind extracellular ligands (Fig. 3; Yoshida-Moriguchi and Campbell 2015). The glycosylation of α-DG is species specific, developmentally regulated, and tissue specific. It dictates the specificity of ligand binding. The extracellular ligands interact with α-DG in a Ca^{2+}-dependent manner through laminin globular



Dystroglycan, Fig. 1 Schematic representation of the dystroglycan and its main partners. The scheme brings together all the known partners of the DG in different types of tissues. O-mannosylated α-DG serves as binding partner for ECM proteins, such as laminins, perlecan, agrin, neurexin, pikachurin, slit, biglycan, and the causative organism of leprosy and arenaviruses. Laminins are major components of the basal lamina. Perlecan is a proteoglycan that binds and crosslinks ECM components. Agrin is a large proteoglycan of the neuromuscular junction. Neurexin is a presynaptic protein connecting neurons at the synapse. Pikachurin is localized to the synaptic cleft in the photoreceptor ribbon synapse. Slit is a chemorepellent protein, preventing the axon crossing of commissural neurons through the midline of the central nervous system. Biglycan is a leucine-rich proteoglycan found in a variety of ECM. *Mycobacterium leprae* and arenavirus are pathogens which cause leprosy and hemorrhagic fever respectively. The transmembrane and extracellular domains of β-DG interact with the sarcoglycan-sarcospan subcomplex. The cytoplasmic tail of β-DG binds the actin cytoskeleton via direct interaction with dystrophin/utrophin. Other intracellular molecules being a part of, or associated with, DGC are dystrobrevin, the syntrophin adapter proteins, and neural nitric oxide synthase (nNOS) (not drawn to scale)

domains (LG modules), motifs of 177 amino acids in length presents in many ECM proteins (Fig. 1; Table 1).

The β-DG has a single domain spanning the plasma membrane and an amino-terminal extracellular domain that binds to the carboxy-terminal globular domain of α-DG. The transmembrane domain of the β-DG interacts with sarcoglycans (α, β, γ, and δ), asparagine-linked glycosylated proteins containing a single transmembrane domain and with sarcospan that contains four transmembrane spanning domains with both

Dystroglycan, Table 1 The dystroglycan and its associated partners

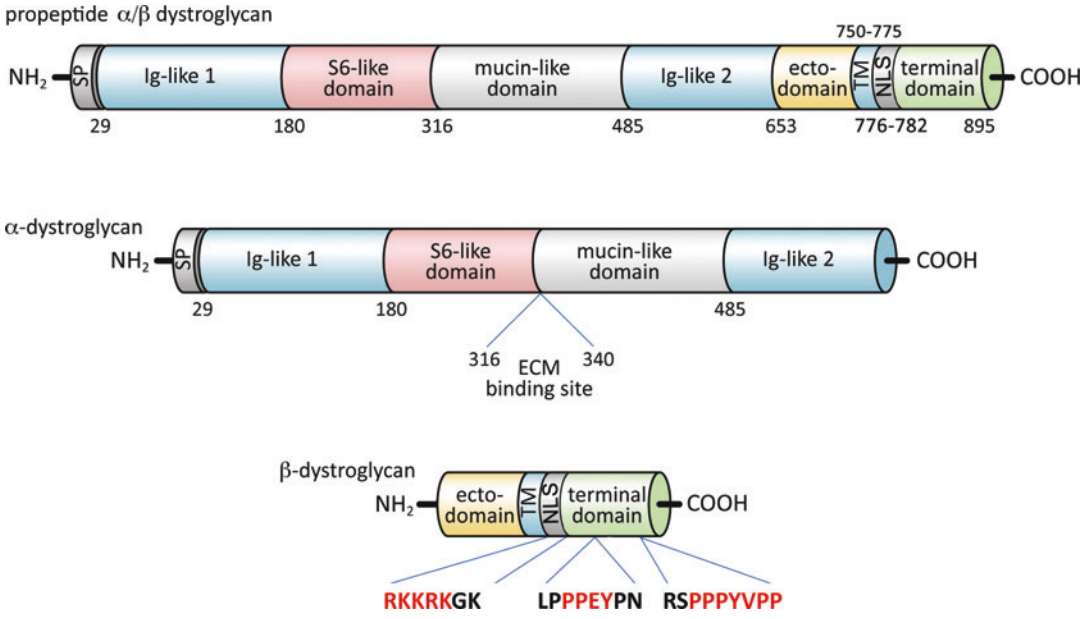
Subunits	Ligands	Binding sites in ligands	Binding sites in DG
α-Dystroglycan	Agrin	Laminin G-like modules	O-mannosylglycan protruding from the mucin-like region
	Biglycan	Protein core of the COOH-terminal third of α-DG	The protein core of the COOH-terminal third of α-dystroglycan
	Laminins	Laminin G modules	O-mannosylglycan protruding from the mucin-like region
	Neurexins	Laminin G-like modules	O-mannosylglycan protruding from the mucin-like region
	Perlecan	Laminin G-like modules	Mucin-like region
	Pikachurin	Laminin G-like modules	O-mannosylglycan protruding from the mucin-like region
	Slit proteins	C-terminal domain	Mucin-like region
	Mycobacterium leprae	Phenolic glycolipid-1 (PGL-1) and 21 kDa laminin-2-binding protein (ML-LBP2)	O-mannosylglycan protruding from the mucin-like region
	Arenavirus	Unknown	O-mannosylglycan protruding from the mucin-like region
	Caveolin-3	WW domain	Phosphorylated Y in the C-terminal motif: $PPxY^{892}$
	Dystrophin/ utrophin	WW domain	Nonphosphorylated Y in the C-terminal motif: $PPxY^{892}$
	ERK-MAP	Unknown	The juxtamembrane portion of the cytoplasmic domain
	Ezrin	Unknown	The juxtamembrane portion of the cytoplasmic domain RKKRK
β-Dystroglycan	F-actin	Unknown	Cytoplasmic tail
	Grb2	SH3 domain	The C-terminal motif: PxxP
	Myosin IIA	unknown	Cytoplasmic tail
	Plectin	N-term: Plakin domain. C-term: IF-binding domain	The C-terminal binding domain
	Importins	IBB domain	The juxtamembrane portion of the cytoplasmic domain RKKRK
	Rapsyn	Ring-H2-domain	The juxtamembrane portion of the cytoplasmic domain: RKKRK
	Src family kinases	WW domain	Phosphorylated Y in the C-terminal motif: $PPxY^{892}$
	Vinexin	SH3 domain	The C-terminal motif: PxxP

ERK extracellular signal-regulated kinase, *Grb2* growth factor receptor-bound protein 2, *IF* intermediate filament

N- and C-terminal regions located intracellularly. They form a subcomplex that stabilizes the α-DG association with β-DG at the cell surface. The cytoplasmic tail of β-DG interacts with signaling partners and with the proteins dystrophin, utrophin, syntrophins, and α-dystrobrevin, thereby to F-actin (Figs. 1 and 4).

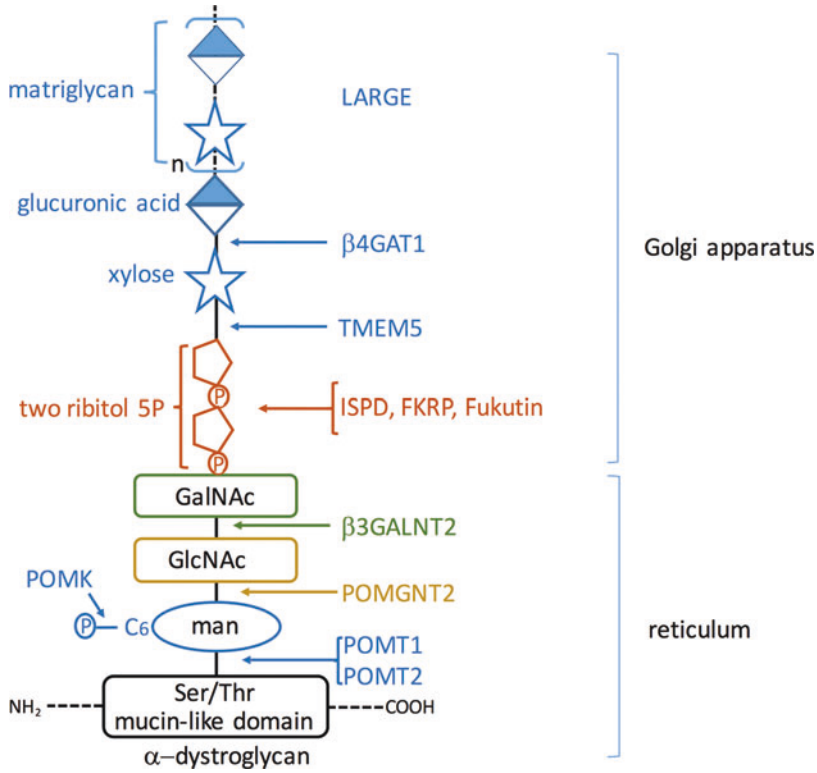
Dystroglycan Expression and Distribution

In humans, DG transcripts are detected in cells of the brain, kidney, liver, lung, diaphragm, placenta, pancreas, and stomach and with the most important level in cardiac and skeletal-muscle cells. In adult mice, Dg exhibits the same expression profile. In early mouse development, transcripts are



Dystroglycan, Fig. 2 Organization of the dystroglycan domains. Dystroglycan consists of two subunits, which are translated from a single mRNA as a propeptide. The first 29 amino acids represent a signal peptide (SP). Posttranslational processing through cleavage at Ser 654 yields the two noncovalently associated proteins α-DG and β-DG. The α-DG subunit contains two Ig-like domains, a domain similar to the S6 protein present in the small ribosomal subunit (S6-like domain) and a mucin-like domain that has a complex and still not fully characterized pattern of glycosylation. The β-DG exhibits an ectodomain that extends into the extracellular space with two cysteines (669 and 713) participating in disulfide bonds. It also exhibits a transmembrane domain (TM), a putative nuclear localization signal (NLS), and a cytoplasmic domain that contains three binding motifs for cytoplasmic proteins (see also Fig. 4)

first detected in cells around the Reichert's membrane. Later during development, the protein is present within the plasma membranes of cells of the notochord, neural tube, myotomes, myocardium, spinal cord, lung buds, sex cords, mesonephric duct and tubules, and otic and optic vesicles. In *Danio rerio*, DG-maternal mRNAs are detected at the 128-cell stage and ubiquitously expressed throughout gastrulation. By the tailbud stage, transcripts are present in cells of the developing neural tube, throughout the paraxial mesoderm, in the notochord and hypochord. In *Xenopus laevis*, the DG-maternal mRNAs are detected from the four-cell stage. The protein is present on notochord cells and remains expressed throughout its differentiation process. It is also present on cells of the hypochord, brain, otic vesicles, eyes, visceral arches, somites, pronephros, skin, and heart. Several *Drosophila melanogaster* DG isoforms are generated via alternative splicing. Only one of these contains the full mucin-like domain. During oogenesis, DG is expressed in nurse, follicle, and female germline cells. During development, DG is detected in salivary gland, foregut, hindgut, brain primordium, peripheral nervous system, ventral nerve cord, longitudinal visceral muscle, tracheal system, heart, and aorta primordia. A high level of DG is detected on axons of photoreceptor cells in the optic stalk, lamina plexus, and medulla neuropil, and on glial cells of brain and optic lobes. In *Caenorhabditis elegans*, three genes encode three different isoforms of DG (*dgn-1*, *dgn-2*, and *dgn-3*). The *dgn-1* gene is the most similar in sequence and structural organization to vertebrate. It encodes a protein that contains the N-terminal immunoglobulin-like domain and a reduced mucin-like domain. The protein lacks the proteolytic cleavage region and the residues involved in binding WW and SH3



Dystroglycan, Fig. 3 The steps of glycosylation pathways of the M3 glycan structure. The M3 glycan structure is synthesized in the endoplasmic reticulum. Protein O-mannosylation is initiated by POMT1/2 and extended by the protein O-linked mannose N-acetylglucosaminyltransferase 2 (POMGNT2) that adds an N-acetyl glucosamine (GlcNAc) to the mannose. The resulting disaccharide is modified by the β-1,3-N-acetylgalactosaminyltransferase 2 (β3GALNT2) that adds an N-acetyl galactosamine (GalNAc) to form the M3 structure, an O-mannosyl trisaccharide. If the 6-position of mannose is phosphorylated by protein-O-mannose kinase (SGK196, POMK), imperfectly known modifications of M3 glycan structures occur in the Golgi apparatus involving sequential enzymatic activities. The isoprenoid synthase domain-containing protein (ISPD), fukutin and fukutin-related protein (FKRP), sequentially act to transfer a tandem repeat of ribitol 5-phosphate (a phosphate ester of pentose alcohol) to the terminal GalNAc of M3 glycan structures (Gerin et al. 2016). Then, the transmembrane protein 5 (TMEM5), a xylose transferase, creates a linkage between a xylose and ribitol 5 phosphate. And then, the β1,4 glucuronyltransferase (β4GAT1) transfers a glucuronic acid residue onto the xylose. It thereby forms a glucuronyl-β1,4-xylosyl disaccharide. This disaccharide is required by the glycosyltransferase LARGE to catalyze the extension of a xylosyl–glucuronyl polysaccharide chains called matriglycan. These chains function as receptors for ECM ligands and their length correlates with the affinity of α-DG for its ligands (modified from Taniguchi-Ikeda et al. 2016; Praissman et al. 2016)

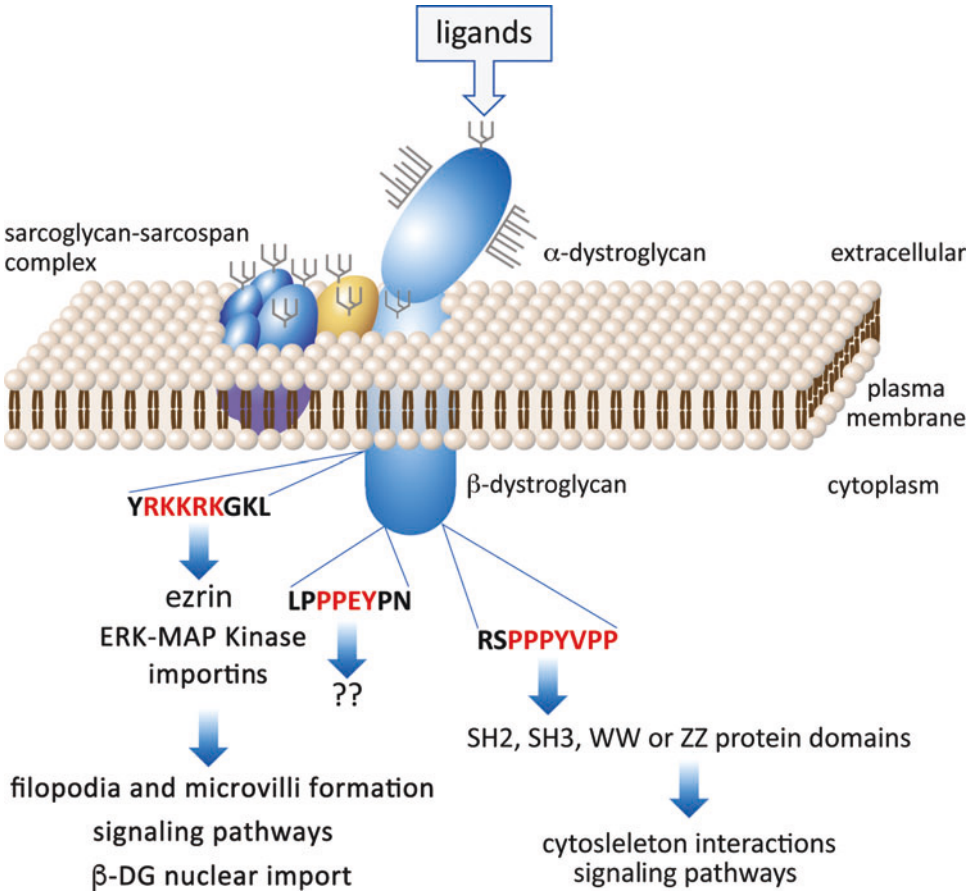
domain-containing proteins. The *dgn-2* and *dgn-3* genes share only the α-DG C-terminus and the β-DG N-terminus domains with vertebrate. The *dgn-1* is the unique nematode ortholog of vertebrate *DG* that is not expressed in muscle but expressed in gonads, epithelia, and neurons.

Intriguingly, the presence of β-DG in the nucleus of human carcinoma cells as well as in normal cell lines like mouse C2C12 myoblasts has been described.

Dystroglycan Functions

The analyses of the DG functions were mainly performed on various organisms, tissues, and cell cultures and based on knockout and specific knockout of DG, on inhibition of translation via morpholino antisense, on RNAi knockouts, and overexpression of DG or truncated forms of DG.

In mouse, the DAG1-null allele results in heterozygous animals that appear healthy and bred normally. However, the DG knockout is lethal for



Dystroglycan, Fig. 4 Schematic representation of the β-dystroglycan binding motifs for cytoplasmic proteins. The RKKRKGK site interacts with members of the ERM protein family (ezrin, radixin, and moesin), the Extracellular signal-related kinase-Mitogen-activated protein (ERK-MAP) kinase and importins. The LPPPETPN site is a potential site of interactions with actually unknown ligands. The RSPPPYVPP site interacts with various proteins depending on its phosphorylation. The non-phosphorylated tyrosine (Y892) allows the association of β-DG with the cytoskeletal adaptor proteins dystrophin/utrophin or proteins containing a SRC homology 3 domain (SH3 domain) as Grb2. Tyrosine phosphorylation of the PPxY recruits proteins with an SRC homology 2 domain (SH2 domain) (FYN, C-src tyrosine kinase, Src, NCK1, SHC1, Tks5, or Grb2). It also recruits Caveolin, an integral membrane protein which is the principal components of flask-shaped plasma membrane invaginations in muscle cells (not drawn to scale)

homozygous embryos that died at the embryonic day 6.5 because of the disorganization of Reichert's membrane, the first extraembryonic basement membrane. The absence of DG causes a patchy distribution of laminins that precludes its assembly in the ECM and in addition embryos show gastrulation and mesoderm patterning defects. To overcome the embryonic lethality, chimeric mice were generated with embryonic stem cells targeted for both DAG1 alleles. Skeletal muscles differentiate normally but they develop a progressive muscular dystrophy characterized by centrally nucleated fibers, connective tissue infiltration, and significant differences in fiber size. At the sarcolemma, the entire DGC complex is disassembled, with dystrophin and sarcoglycans absent in fibers. The neuromuscular junctions are grossly disorganized and disrupted. The heart appears dilated because of an extensive connective tissue hyperplasia. In the kidney, different conditional knockout results in no aberrant phenotypes except an increase of the glomerular

basement membrane thickness. In conditional DG knockouts in the cerebral cortex, the basal lamina of the glia limitans is severely disrupted leading to an overabundance of glia and the overextended migration of neurons in the developing brain. Also, the fissure between the hemispheres is lost. In peripheral nerves, selective absence of DG in Schwann cells causes structurally abnormal myelin sheaths around the neurons and throughout the internodal segments associated with a slow nerve conduction. Laminins are not deposited around the cells, and the sarcospan, sarcoglycan, and dystrobrevin are lost. Retinal photoreceptor-specific DG knockout inhibits pikachurin (a retinal ECM protein) accumulation on tips of photoreceptor synapses. In the neonatal brain of mouse, Dg regulates proliferation of neural stem and progenitor cells. It suppresses Notch activation in neural stem cells to promote the maturation of ependymal cells and the formation of stem cell niche. Additionally, it modulates oligodendrogenesis by regulating Notch activities to promote cell differentiation and myelination timely (McClenahan et al. 2016). All these findings point to the crucial role of DG during mouse development in the organization of the basement membranes, in the stability of the DGC, in the structural integrity of the sarcolemma, in the myelin integrity, in the node of Ranvier structure, in the formation of proper photoreceptor ribbon synaptic structures, and in oligodendrogenesis.

In *Danio rerio*, antisense morpholino oligonucleotide approach results in the destabilization of the embryonic muscles, with loss of sarcomere organization and necrosis of the developing muscle. The central nervous system and the neuromuscular junctions develop normally. A zebrafish mutant, designated *patchytail*, contains a point mutation resulting in a missense amino acid change of valine to aspartic acid within the Ig-like domain in the C-terminal region of α-DG. The muscle plasma membrane of *patchytail* fish appears disorganized and detached from the ECM. The mutant shows only subtle defects in cell organization in tectum and cerebellum and no significant neuromuscular junction defects. By contrast abnormal development of ganglion, lens and cornea layers in eyes are observed and the embryos do not survive more than ten day–post fertilization. On the other hand, in a mutation leading to a complete loss of DG, both myogenesis and myofibrillogenesis are unaffected while muscular dystrophy becomes apparent by 36 hours post fertilization, shortly after the elongation and fusion of myofibers. Therefore, in zebrafish, DG is dispensable for basement membrane formation during early development and for muscle formation while it is required for long-term survival of muscle cells.

The DG functions in *Xenopus laevis* development were analyzed using the morpholino knockout approach. It was shown that Dg is required for ECM organization, for somitogenesis, and for myoblast alignment during myogenesis. Also, the loss of DG precludes epithelial differentiation during the retinal, renal, and skin development. Interestingly, the Notch signaling pathway controls the transcription of DG during skin morphogenesis. Furthermore, point mutations in the Dg-cytoplasmic domain lead to the disruption of cell-mediolateral intercalation required for notochord formation and/or cytoskeleton integrity needed for vacuolation of notochord cells. Moreover, an overexpression of DG by microinjection of rabbit DG mRNA into embryos corrupts the aggregation of acetylcholine receptors and the structure of neuromuscular junctions.

In *D. melanogaster*, DG is required cell-autonomously to polarize both follicular epithelial cells and the oocyte. Mutations of protein O-mannosyl transferases, encoded by *rotated abdomen* (rt) or *twisted* (tw) genes, result in developmental failures of larval muscles, leading to defective muscles in adults characterized by a rotated abdomen phenotype. Genetic and RNAi-induced perturbations of *DG* specifically in mesoderm-derived tissues cause decreased cell mobility, age-dependent progressive deficits, and severe muscle degeneration in adult flies. DG depletion also leads to defective photoreceptor axon adhesion and migration during differentiation resulting in stunted photoreceptors in the adult. DG protein is present in ectodermal cells, but is absent in the ones that differentiate into tendon cells. These ones express miR-9a. Upon *miR-9a* deficiency, the DG is detected not only in

muscle cells but also within the membrane of tendon cells leading to the alteration of muscle-tendon matrix assembly and disorganization of musculature assembly. Thus, DG is crucial for proper muscle-tendon attachments and its proper expression is adjusted by miR-9a (Yatsenkoet al. 2014). In the brain, the accuracy of neuronal proliferation, differentiation, and axon pathfinding depend on DG levels. It has been shown that the microRNA complex miR-310 s acts as an executive mechanism to buffer DG (Yatsenko et al. 2014).

In *Caenorhabditis elegans*, a deletion which removes a large coding region and partially the 3′ untranslated region of *dgn-1* gene leads to viable but sterile worms. They show normal muscles and a severe disorganization of the somatic gonad epithelia, defects in vulval and excretory cell epithelialization, and impaired axon guidance of motoneurons.

In C2C12 myoblast cells, electron microscopy observation reveals β-DG localized in the inner nuclear membrane, the nucleoplasm, and nucleoli. Also, β-Dg interacts with nuclear envelope proteins emerin and lamins. This finding suggest that DG serves as a nuclear scaffolding protein involved in nuclear organization and nuclear envelope structure or that it interacts with transcriptionally active regions of the nucleus (Martínez-Vieyra et al. 2013).

All of this evidence, although conflicting in some areas, imply that DG is indispensable for normal development and is a vital contributor in maintaining cell and tissue integrity in adults.

Dystroglycan and Signaling Pathways

The C-terminal domain of β-DG is a short unfolded cytoplasmic tail that contains binding sites for numerous proteins at the juxtamembrane region and at the carboxy terminus (Figs. 2 and 4).

The juxtamembrane region of β-DG interacts with ezrin, and ERK-MAP kinase through a cluster of basic residues and possesses a nuclear localization signal. The interaction with ezrin, a member of the ERM protein family that crosslink actin filaments with plasma membranes, mediates actin cytoskeleton remodeling and induces peripheral filopodia and microvilli formation. These processes are controlled by complexes containing DG, ezrin, and members of the Dbl family of guanine nucleotide exchange factors. They are targeted to the membrane by DG and drive local activation of cell division cycle 42 protein (Cdc42), which in turn influences signaling events that initiate the formation of actin-rich surface protrusions. The juxtamembrane domain also interacts with protein kinases MEK and ERK, downstream components of the Extracellular signal-Regulated Kinase/Mitogen-Activated Protein Kinase (ERK-MAP) cascade. The link between β-DG and MEK is localized to membrane ruffles, while the link with ERK is found in focal adhesion in fibroblasts. Using the protein domain prediction program PSORT II, analysis unveils a putative nuclear localization signal (NLS) located in the cytoplasmic domain of β-DG within residues 776–782 (^{776}RKKRKGK^{782}) (Fig. 4). This putative NLS is totally conserved among the orthologous proteins of different species. It mediates the nuclear import of β-DG through a process dependent on importin α/β and Ran proteins. Interestingly, the NLS overlaps with the domain that interacts with ezrin suggesting a binding competition between importins and ezrin to regulate β-DG nuclear import. Instead it has been shown that cytoskeletal reorganization mediated by ezrin activation enhances the nuclear trafficking of β-DG through the importin nuclear import pathway (Vásquez-Limeta et al. 2014).

The carboxy terminus domain contains the PPxY motif, a motif governing interactions with proteins containing WW, SH2, and SH3 domains. DG-ECM interactions may phosphorylate tyrosine 892 in human and tyrosine 890 in mouse within the cytoplasmic domain of β-DG. The tyrosine kinase involved is the SH2 domain containing protein c-Src. Once tyrosine is phosphorylated, the β-Dg is no longer able to interact with proteins containing SH3 and WW domains leading to a loss of interaction with dystrophin/utrophin and thus with the cytoskeleton. Thus, the phosphorylation of tyrosine regulates β-DG cytoplasmic interactions, functioning as a balance between interaction with proteins containing SH3 or WW domains when it is not phosphorylated, and with proteins containing SH2 domain when it is phosphorylated (Fig. 4).

Other binding partners for β-DG include growth factor receptor-bound protein 2 (Grb2), caveolin, dynamin, and rapsyn. Grb2 is an adaptor protein composed of a single Src homology 2 domain (SH2) surrounded by two Src homology 3 domains (SH3) which permit Grb2 association with proteins containing tyrosine-phosphorylated residues and proline-rich regions, respectively. DG-Grb2 interaction may participate in the ERK-MAP kinase pathway involving MEK and ERK. The carboxy terminus of β-DG is associated with caveolin, a scaffolding protein and the main component of caveolae in muscle cells. The WW-like domain within caveolin directly recognizes the C-terminus PPxY motif. As the WW domain of dystrophin recognizes the same site, caveolin can block the interaction of dystrophin with β-DG suggesting that caveolin may competitively regulate the recruitment of dystrophin to the sarcolemma. Dynamin, a GTPase implicated in endocytosis, interacts directly with β-DG suggesting that this interaction regulates endocytosis in cells. The interaction with rapsyn, a Src-like kinase, leads to signaling events required for the formation of a specific complex essential for the agrin-mediated clustering of acetylcholine receptors at the neuromuscular junction.

Thus, DG and cytoplasmic proteins may be of biological importance in transducing signals arising from the binding of DG to ECM proteins. They contribute to regulate cytoskeletal assembly, cell shape, and intracellular signaling pathways.

Dystroglycanopathies

The dystroglycanopathies forms a heterogeneous group of human rare diseases that have been classified as muscular dystrophy-dystroglycanopathy by Online Mendelian Inheritance in Man (OMIM), an Online Catalog of Human Genes and Genetic Disorders. They concern malformations during central nervous system and ocular development. These diseases are characterized by DG dysfunctions and classified into two groups. The primary dystroglycanopathies result from mutations in the *DAG1* gene, the secondary dystroglycanopathies from the hypoglycosylation of α-DG.

Primary Dystroglycanopathies

The first case of primary dystroglycanopathy has been described in a Turkish woman with limb-girdle muscular dystrophy (LGMD) and severe mental retardation (Hara et al. 2011; OMIM #128239). It is caused by a homozygous missense mutation in *DAG1* that generates a threonine-to-methionine substitution at amino acid residue 192. Using knock-in mice as model systems and in vitro binding assays, it has been shown that the mutation precludes activities of LARGE leading to defects in DG-laminin binding in skeletal muscle and brain. Another report describes a muscle-eye-brain (MEB) disease associated with an extended bilateral multicystic leucodystrophy in two Libyan siblings (Geis et al. 2013). This disease originates from a mutation identified in the extracellular domain of β-DG that substitutes a cysteine-to-phenylalanine at amino acid residue 669. It affects the highly conserved cysteine residue predicted to form a covalent intra-chain disulphide. The substitution changes the conformational structure of β-DG that disrupts its interaction with α-DG. Also, a homozygous loss-of-function mutation has been described in five female infants from a consanguineous Israeli-Arab family with intracranial calcifications associated with a Walker-Warburg syndrome (WWS, OMIM #236670) resulting in death soon after birth (Riemersma et al. 2015). A homozygous deletion, resulting in a frameshift and premature termination, causes this syndrome because a premature stop codon leads to the complete absence of both α- and β-DG.

Secondary Dystroglycanopathies

Secondary dystroglycanopathies are characterized by reduced glycosylation of DG generated by mutations in known or putative genes involved in the O-mannosyl-glycosylation of α-DG (Fig. 3; Yoshida-Moriguchi and Campbell 2015 for a complete list and references). The resulting defective glycosylation of α-DG impairs DG interactions with its extracellular partners, disrupting the link between the ECM and the cytoskeleton and also signaling pathways. These mutations are the underlying causes of a wide clinical spectrum including severe structural brain involvement

resembling Walker–Warburg syndrome (WWS), muscle-eye-brain disease (MEB; OMIM #253280), Fukuyama congenital muscular dystrophy (FCMD; OMIM #253800), congenital muscular dystrophy types 1C and 1D (MDC1C; OMIM #606612; MDC1D; OMIM #608840), and some forms of autosomal recessive adult-onset limb-girdle muscular dystrophy (LGMD2I-2 N; OMIM #607155). Although the function of the isoprenoid synthase domain containing protein (ISPD; OMIM #614631) in mammals is not yet perfectly known, mutations in this protein have been found in nine patients from seven families who have phenotypes ranging from congenital muscular dystrophy to limb-girdle muscular dystrophy (Cirak et al. 2013). Mutations of all these genes coding for proteins involved in the glycosylation pathways of α-DG are responsible for at least 50% of dystroglycanopathies suggesting that many of the secondary dystroglycanopathies remain unsolved and that additional mutations in genes await discovery.

Animal Models of Dystroglycanopathies

As DG is conserved in vertebrates and invertebrates, this offers a wide range of possible animal models to understand its role during embryogenesis, in different adult tissues and in pathogenesis of dystroglycanopathies. Thereby, several animal models including mouse, zebrafish, xenopus, drosophila, and worm have been generated. They are listed in Tables 1 and 2 in the review by Sciandra et al. 2015. Although dystroglycanopathies show various symptoms and anomalies, enzymatic corrections have been proposed based on studies using theses animal models. For example, this concerns antisense therapy for FCMD and gene therapy for FCMD, FKRP, and LARGE (Taniguchi-Ikeda et al. 2016 for a review).

Dystroglycan in Other Pathologies

In addition to the pathologies described above, DG is also involved in bacteria and virus infections and appears to be a suppressor of tumors.

DG is a cellular receptor for the causative organism of leprosy, *Mycobacterium leprae*, and for arenaviruses such as Lassa fever virus (LFV), African arenaviruses Mobala and Mopeia, and lymphocytic choriomeningitis virus (LCMV), which cause hemorrhagic fever. Schwann cells are targets for infection by *Mycobacterium leprae*, leading to the breakdown of the myelin sheath and consequently to neuronal and tissue death. *Mycobacterium leprae* interacts with α-DG on Schwann cells via the G domain of laminins that forms a bridge between bacteria and α-DG. The specificity of infection might be a result of Schwann-cell-specific glycosylation of α-DG. The arenaviruses bind to purified α-DG at a site mapped to the 18-amino acid domain spanning residues 316–334 of the mucin-like domain that overlaps with the ECM ligand-binding domain. Arenaviruses use α-DG for viral entry in a laminin-independent mechanism. The infection is due to competition between laminins and viruses for their shared binding site on α-DG leading to enhanced membrane fragility and instability (Oldstone and Campbell 2011). The attachment of viruses to DG induces tyrosine phosphorylation of β-DG at tyrosine 892 leading to the dissociation of DG from dystrophin/utrophin thereby facilitating the subsequent endocytosis of the virus-DG complex (Oppliger et al. 2016).

The role for DG in tumor metastasis was first demonstrated in prostate and breast cancers. Without describing here all data, it is known that mutations in DG are not yet described to be associated with cancer. While DG transcription appears largely unaltered in the majority of carcinomas, posttranscriptional mechanisms, including hypoglycosylation and proteolysis, are involved in the observed loss of DG function in cancer cell lines and primary tumors. They generate increased tumor aggressiveness, loss of extracellular matrix integrity and/or loss of intracellular signaling. For example, in adenocarcinoma, although α-DG is correctly expressed and trafficked to the cell surface, it is not functionally glycosylated caused by transcriptional silencing of LARGE. This abolishes DG ability to interact with ECM components leading to a failure of ECM-induced cell polarization thereby the invasiveness is promoted. Also, in adenocarcinoma cells, a cell density-dependent γ-secretase and furin, which could be Notch stimulated, lead to the degradation of β-DG

in cytoplasmic fragments that are targeted and accumulated in the nucleus (Leocadio et al. 2016). Knowing that DG mediates transduction of signals, data point toward an effect on signal transduction pathways resulting in alterations of metabolism and growth rate in tumorigenic cells.

Summary

DG is a widely expressed transmembrane glycoprotein that requires complex and actually imperfectly known posttranslational processing to function as an ECM receptor. Synthesized as a single polypeptide, DG is cleaved to yield a cell-surface α-subunit and a transmembrane β-subunit. An extensive glycosylation of α-DG is required to function as a receptor for ECM proteins containing LG-domains. The phosphorylation of β-DG acts as a molecular switch to regulate DG interactions with various binding partners and different cellular adhesion and signaling functions. It also acts to determine its internalization by endocytosis and trafficking to the nucleus. Mutations of α, β-Dg or abnormalities in the posttranslational processing of α-DG disrupt its interactions with components of the ECM that result in human cancers and various congenital muscular dystrophies, referred as dystroglycanopathies. Phenotypic analyses, in both patients with dystroglycanopathies and animal models, show that DG is required in developmental and a variety of physiological processes: basement membrane assembly, muscle maintenance, peripheral-nerve myelination, neuromuscular-junction formation, neuronal migration in the brain, axon guidance, synapse formation and plasticity, and development of eye and brain. Also, DG serves as a cellular receptor for Old World arenaviruses, including the pathogenic Lassa fever virus. Interestingly, the requirements of DG for viral infections look like those for bindings to ECM proteins. In the future, a deeper understanding of both the molecular structure and cellular functions of DG, in particular in the nucleus, promises to realize the coming of new methods for treating viral infections and dystroglycanopathies.

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Dystroglycan 1

► Dystroglycan

Dystrophin-Associated Glycoprotein 1

► Dystroglycan