Cell-Autonomous (Cell-Intrinsic) Stress Responses

18

18.1 Introduction

As comprehensively described in Part II, PRMs are specifically involved in the recognition of MAMPs and DAMPs. As will be discussed in Part VI, each of these recognition receptors can trigger distinct signalling cascades in innate immune cells that modify their gene expression to create and execute efferent innate immune responses that involve (1) production of inflammatory mediator substances such as cytokines and chemokines, (2) phagocytosis, and (3) cytotoxicity, as well as, as described in Part VIII, may elicit and shape antigen-specific adaptive immune responses.

Beyond this well-characterized MAMP/DAMP engagement of PRMs leading to a variety of downstream efferent cellular and humoral responses, the innate immune defense program also depends on cell-autonomous, that is, cell-intrinsic, responses which counteract any stressful insult [1]. Constitutive cell-autonomous immunity mobilizes pre-existing molecules and processes in order to primarily and quickly defend the cell and the host against infectious and sterile injury. Hence it can be considered as the very first line of innate immune defense.

Here, the role of constitutive cell-autonomous responses will be examined, whose involvement in the innate immune defense to stress and injury has only been appreciated within the last few years. The focus of this brief overview will be mainly directed toward cellular stress responses.

18.2 Autophagy

18.2.1 Introductory Remarks

The term *autophagy* comes from the Greek words "phagy" meaning eat and "auto" meaning self. Autophagy is an evolutionarily highly conserved self-digestive process in response to environmental stress to eukaryotic cells, by which cytoplasmic

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components such as defective/damaged or redundant organelles or protein aggregates are delivered to the lysosome for recycling and degradation. There is convincing evidence indicating that activation of the autophagic process is promoted by MAMPs and/or DAMPs [2, 3]. In more simple words, autophagy is a classical cell-protective and cell-autonomous process of the innate immune system aimed at maintaining and restoring homeostasis at both the cellular (cell-intrinsic) and organismal (cell-extrinsic) level [4]. Although autophagy was initially identified in mammals, a significant breakthrough in our understanding of how autophagy is controlled came from the analysis in the genetically tractable yeast system. Pioneering work from Ohsumi's group showed that the morphology of autophagy in yeast was similar to that documented in mammals [5]. (As known, Ohsumi received the Nobel Prize in Physiology or Medicine 2016.) In fact, the discovery of the autophagy-related genes in yeast has significantly advanced the understanding of the molecular mechanisms participating in autophagy and the genes involved in regulating the autophagic pathway. Many yeast genes have mammalian homologues, confirming that the basic machinery for autophagy has been evolutionarily conserved along the eukaryotic phylum [6–9].

Notably, a panel of leading experts in the field of autophagy has recently published a new definition of several autophagy-related terms based on specific biochemical features [10]. Accordingly, in the following, three types of autophagy are briefly sketched including macroautophagy, microautophagy, and, in mammals, chaperone-mediated autophagy. Each of them fulfils very specific tasks in intracellular degradation.

18.2.2 Autophagy in General

There is general agreement on two main features that characterize *bona fide*, functional autophagic responses, irrespective of type: (1) they involve cytoplasmic material; and (2) they culminate with (and strictly depend on) lysosomal degradation [10]. Thus, although autophagy substrates can be endogenous such as damaged cellular organelles or exogenous such as viruses or bacteria escaping phagosomes, autophagy acts on entities that are freely accessible to cytosolic proteins. This property is essential in order to distinguish between autophagic responses and branches of vesicular trafficking that originate at the plasma membrane, which also culminates in lysosomal degradation. Such endocytic processes include phagocytosis, receptor-mediated endocytosis, and macropinocytosis, that is, processes which will be dealt with in Part VI, Sect. 22.6. Of note, however, some forms of autophagy and the endocytic pathway interact at multiple levels, and the molecular machinery responsible for the fusion of late endosomes (also known as MVBs) or autophagosomes with lysosomes is essentially the same [11].

As stressed [10], the strict dependency of autophagic responses on lysosomal activity is necessary to discriminate them from other catabolic pathways that also involve cytoplasmic material, such as proteasomal degradation [12]. Thus, the 26S proteasome (Box 18.1) degrades a large number of misfolded cytoplasmic proteins

that have been ubiquitinated (for (poly)ubiquitination, see Box 18.2) as well as properly folded proteins that expose specific degradation signals, such as the so-called N-degrons [13]. On the other hand, the proteasome system shares some substrates with different forms of autophagy whereby these two catabolic pathways differ drastically in their final products. Thus, proteasomal degradation results in short peptides that are not necessarily degraded further but may flow into additional processes including but not limited to antigen presentation/cross-presentation at the plasma membrane, thereby generating MHC-II and MHC-I epitopes (compare Part VIII, Chap. 31). By contrast, lysosomal proteases fully catabolize polypeptides to their constituting amino acids which eventually become available for metabolic reactions or repair processes. Together, as summarized [10], *bona fide* functional autophagic responses navigate cytoplasmic material of endogenous or exogenous origin to degradation within lysosomes (or late endosomes, in specific cases).

Box 18.1 The Proteasome

The proteasome is a common complex for all living cells, needed to recycle and eliminate unwanted proteins. In analogy, it resembles a chaff-cutter. This molecular machine provides a pathway that is involved in many cellular levels such as protein degradation, antigen processing, cell cycle, apoptosis, and DNA repair. The 26S proteasome that is present in the cytoplasm and nucleus is usually formed by one 20S proteasome complex and two 19S proteasome complexes, which are composed of proteases and structural units. The 26S proteasome is a giant protease responsible for the regulated degradation of polyubiquitylated proteins (see Box 18.2). It consists of at least 33 distinct subunits and is arranged into two modules: core particle containing catalytic sites and regulatory particles. The cylinder-shaped proteolytic core is the 20S core particle, which is capped at one or both ends by 19S regulatory particles.

Further reading: Wehmer M, Sakata E. Recent advances in the structural biology of the 26S proteasome. Int J Biochem Cell Biol 2016;79:437–442.

Box 18.2 Ubiquitination–Polyubiquitination

The binding of many ubiquitin molecules to the same target protein. In its simplest form, ubiquitin can be attached to the target protein as a single moiety resulting in monoubiquitination. Ubiquitin itself can be ubiquitinated, resulting in the formation of ubiquitin chains attached to the target protein: polyubiquitination. Polyubiquitination of proteins is the triggering signal that leads to subsequent degradation of the protein in the proteasome. Ligases play a central role in polyubiquitination. Ligases are enzymes that catalyze the synthesis of polyubiquitin chains. Ubiquitin conjugation requires typically

three classes of enzymes. E1 (ubiquitin-activating enzyme) hydrolyzes ATP and forms a thioester-linked complex between itself and ubiquitin. E2 (ubiquitin-conjugating enzyme) receives ubiquitin from E1 and forms a similar thioester-linked intermediate with ubiquitin. E3 (ubiquitin ligase) finally binds both the E2 and a substrate and catalyzes the transfer of ubiquitin to the substrate. Ubiquitin itself is often a substrate for further ubiquitylation, which results in the formation of so-called polyubiquitin chains. Ubiquitin has seven lysine residues, and depending on the lysine residue used for ubiquitin–ubiquitin chain formation, the polyubiquitin chain can signal different functions. Proteins modified by lysine-48 (K48)- or lysine-29 (K29)-linked chains are usually degraded by the proteasome. In contrast, modification by K63-linked chains or by a single ubiquitin moiety (monoubiquitylation) seems to trigger other functions, e.g., protein sorting, gene expression, and DNA repair.

Further reading: Callis J. The ubiquitination machinery of the ubiquitin system. Arabidopsis Book 2014;12:e0174.

18.2.3 Macroautophagy (= Autophagy)

18.2.3.1 General Remarks

Basically, the term macroautophagy is often used when describing autophagy in general. The phenomenon is characterized by its typical morphological features which involve dedicated vesicles that can occupy a considerable part of the cytoplasm. Typically, macroautophagy is one type of autophagic processes in which the substrates are sequestered within cytosolic double-membrane vesicles termed autophagosomes. The substrates of macroautophagy include superfluous and damaged organelles, cytosolic proteins, and invasive microbes. Mechanism of formation and regulation of macroautophagy are very complex and complicated processes that are outlined here in a considerably simplified way.

18.2.3.2 Mechanism and Regulation of Macroautophagy

Macroautophagy involves the sequestration of cytoplasm via a double-membrane intermediate structure termed the phagophore which matures into an autophagosome; the latter compartment fuses with a lysosome allowing degradation and recycling of the cargo [14]. In more detail, the process begins with the formation of a membrane of unknown origin, the initial phagophore or isolation membrane. The phagophore then expands, surrounds proteins or organelles, sequesters cytoplasm, and, on completion, develops into a large double-membrane transport vesicle, the autophagosome. Subsequently, the autophagosome fuses with a lysosome containing acid hydrolases and releases its contents into the lytic acid hydrolases-containing compartment as part of single-membrane vesicles, termed autophagic bodies. The fused compartment where the autophagic body and its contents are degraded is called an autophagolysosome or autolysosome (Fig. 18.1). Notably, the process of phagophore expansion

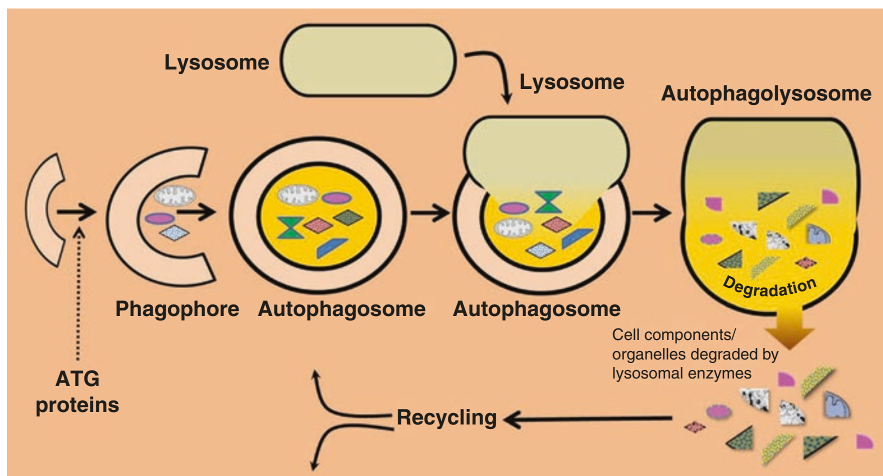

Fig. 18.1 Schematic illustration of the autophagic process. Autophagy is a lysosome-mediated degradation and recycling pathway that involves the formation of multiple membrane structures ranging from phagophores to autophagosomes and autophagolysosomes. The autophagosomes that need intervention of ATG proteins develop to autolysophagosomes through docking and fusion with lysosomes. Internalized material includes all kinds of cell components and organelles which are degraded by lysosomal enzymes for recycling. *ATG* autophagy-regulated. Sources: Refs. [6–9, 14–16]

provides tremendous flexibility and capacity with regard to cargo, allowing entire organelles to be deleted via autophagy; however, this flexibility also means that autophagy must be tightly controlled in order to prevent inappropriate degradation, which could lead to cell death (for relevant papers, see [6–9, 14–16].

Intensive studies have been carried out in the past two decades to understand the mechanism and regulation of autophagy. The biogenesis of autophagosomes needs the ordered intervention of autophagy-regulated (ATG) proteins that act on different modules. Thus, more than 30 ATG genes have been identified in human that orchestrate the complex membrane dynamics involved in autophagic sequestration. These ATG proteins act sequentially in three macromolecular complexes involved in the three successive stages of autophagy. Initiation of autophagy requires the *UNC-51-like kinase 1* (ULK1)-ATG13-FIP200 (also known as *RB1-inducible coiled-coil 1*) complex, whereby the kinase activity of ULK1 is controlled by the kinase *mammalian target of rapamycin* (mTOR) in *mTOR complex 1* (mTORC1), which is sensitive to rapamycin [9]. The next process, membrane nucleation, requires the Beclin1/class III PI3K complex, which also plays a major role in membrane trafficking and restructuring involved in autophagy [15, 17]; the final process refers to the elongation, expansion, and closure of the phagophore membrane/autophagosome which mainly relies on ATG8/microtubule-associated protein *1 light chain 3* (LC3) lipidation. In fact, ATG8/LC3 lipidation is regarded as a hallmark of autophagy and is established by a covalent linkage of cytosolic LC3 to the lipid phosphatidylethanolamine on the surface of the autophagosome [7, 9].

Of note, in addition to the cytoplasmic PTM of various ATG proteins, recent studies have explored the transcriptional and epigenetic control of autophagy [18]. Notably, in human cells, TFEB (for *transcription factor EB*) and ZKSCAN3 (for *zinc finger with KRAB and SCAN domains 3*) were shown to be implicated in playing a crucial role in autophagy regulation [19, 20]. Also, there is growing evidence in support of the notion that histone modification/DNA methylation acts as an alternative approach for long-term autophagy control [21] (for histone modification, see Part VI, Sect. 24.2.3). Also recently, a new AMPK→SKP2→CARM1 (for: *AMP-activated protein kinase; S-phase kinase-associated protein 2 (p45); coactivator-associated arginine methyltransferase 1*) regulatory axis was reported that incorporated cellular nutrient sensing with transcriptional as well as epigenetic control of autophagy [22].

As concluded by Xu and Klionsky [14], "*…this AMPK-SKP2-CARM1 signalling axis integrates the various levels of autophagy regulation including cell signalling, and transcriptional regulation as well as epigenetic modification. Epigenetic and transcriptional regulation provides an energy-saving approach for control and also create an enduring memory in preparation for future adverse events. Thus, this study has deepened our understanding of how autophagy can be controlled in a holistic manner by pathways linking a multitude of regulation mechanisms. Given the extensive involvement of autophagy in human diseases, this work also presents potential directions for novel therapeutic intervention*."

Indeed, besides its beneficial function in controlling cellular homeostasis, macroautophagic pathways when disrupted can have severe consequences leading to major diseases such as cancer, metabolic and neurodegenerative disorders, and cardiovascular and pulmonary diseases [23].

Of note, macroautophagy can be divided into two subtypes depending on the organelle that is targeted for autophagic degradation; thus, the process of mitophagy corresponds to autophagy of mitochondria, whereas the term ER-phagy refers to autophagy of the endoplasmic reticulum (ER). Both processes deserve a few more words in the following subsection.

18.2.3.3 Mitophagy and ER-Phagy

The term mitophagy corresponds to cargo-specific autophagy of mitochondria, a process which mediates the selective removal of mitochondria [24, 25]. The aim of mitophagy is to eliminate mitochondria, either to regulate their number to adjust to metabolic demand or to explicitly remove those that are damaged in terms of a quality control. Mechanistically, mitochondria are selectively recruited into isolation membranes, which seal and then fuse with lysosomes to eliminate the trapped mitochondria. As discussed [24], mitophagy is preceded by so-called mitochondrial fission that divides elongated mitochondria into pieces of manageable size for encapsulation and also controls segregation of damaged mitochondrial material for selective removal by mitophagy.

The term ER-phagy (also called micro-ER-phagy) refers to a process of distinct selective degradation of ER membranes and proteins in the lysosome under stress, and this is independent of the core autophagy machinery [26–28] (for ER stress, see

Sect. 18.5). Studies on yeast showed that ER-phagy is characterized by the fact that stress-induced ER whorls are selectively taken up into the vacuole, the yeast lysosome. Import into the vacuole was found not to involve autophagosomes but occurs through invagination of the vacuolar membrane, indicating that ER-phagy is topologically equivalent to microautophagy [27]. Recent studies on yeast provide evidence suggesting that the ATG proteins Atg39 and Atg40 are specific receptors for this pathway of ER-phagy [28].

At this point, it also appears worthwhile to mention that a more recent study on yeast revealed a novel ER quality-control pathway, namely, the so-called macro-ER-phagy. First results from this study suggest that this pathway delivers an excess of integral-membrane proteins from the ER to the lysosome for degradation and, typically, requires the core autophagy machinery [29].

18.2.3.4 Concluding Remarks

The brief overview about macroautophagy provides another typical example of innate immune responses which, when controlled, operate in a beneficial homeostatic way but, when uncontrolled, may lead to severe pathologies. For other forms of phagocytic responses, this phenomenon has not been investigated sufficiently.

Clearly, mitophagy also plays a key homeostatic role in mitochondrial quality control. Upregulation of mitophagy has been shown to mitigate excessive mitochondrial accumulation and toxicity to safeguard mitochondrial fitness. Hence, mitophagy is a viable target to promote longevity and prevent age-related pathologies [25]. Concerning the two types of ER-phagy (micro- and macro-ER-phagy), one has to state that research in this exciting field has just begun. Several questions remain to be addressed, for example, what is the purpose of ER-phagy and what are the underlying mechanisms. Future studies will probably provide a clue to elucidating the molecular mechanisms and physiologic roles of ER-phagy in other organisms.

Of note, besides mitophagy and ER-phagy, other specific forms of phagocytic pathways have been described. They include

- Pexophagy as a macroautophagic response preferentially targeting peroxisomes
- Nucleophagy as an autophagic response selectively targeting portions of the nucleus
- Ribophagy as a specific autophagic response targeting ribosomes
- Aggrephagy as an autophagic response specific for protein aggregates
- Lipophagy in terms of selective autophagic degradation of neutral lipid droplets
- Bacterial xenophagy as a macroautophagic removal of cytoplasmic bacteria which have escaped the phagosomal compartment upon phagocytosis
- Viral xenophagy as a macroautophagic response targeting fully formed cytoplasmic virions or components thereof
- Proteaphagy in terms of macroautophagic responses specific for inactive proteasomes
- Lysophagy as a specific macroautophagic disposal of damaged lysosomes in mammalian cells

For details of these specific forms of phagocytic responses, the reader is referred to the excellent comprehensive review article of Galluzzi et al. [10].

18.2.4 Microautophagy and Chaperone-Mediated Autophagy

18.2.4.1 General Remarks

In addition to macroautophagy, two other types of autophagy have been described called microautophagy and chaperone-mediated autophagy (CMA). Microautophagy together with macroautophagy plays, for example, a role in nutrient recycling under starvation. On the other hand, CMA is known to contribute to the maintenance of cellular homeostasis by facilitating recycling of amino acids of the degraded proteins and by eliminating abnormal or damaged proteins, thereby exerting major regulatory functions in different pathophysiological scenarios such as metabolic regulation. Here, a few aspects of these two types of autophagic responses are skimpily touched.

18.2.4.2 Microautophagy

By contrast to macroautophagy, the process of microautophagy is much less defined in mammals since most studies have been performed in yeast and plants. According to current models, the term refers to a collection of diverse processes. Unlike autophagy, microautophagy does not involve the autophagosome-dependent degradation of cytoplasmic components but rather and characteristically relies on the direct engulfment of small portions of cytoplasm into lysosomes or late endosomes by invagination and inward budding of the lysosomal/endosomal membrane, a process that leads to their degradation [30–32]. Though microautophagy is the least studied form of autophagy, a molecular signature of the process has begun to emerge and has led to the definition of microautophagy as a type of autophagy in which the cargo is directly internalized in small vesicles that form at the surface of the lysosome/vacuole or late endosomes (multivesicular bodies), respectively [10].

18.2.4.3 Chaperone-Mediated Autophagy

In addition to macroautophagy and microautophagy, there is another type of autophagy experiencing increased attention, the CMA. Characteristically, in CMA, cargo delivery also occurs directly at lysosomes, but it does not require formation of vesicles nor membrane invagination. Instead, the substrate proteins for this autophagic pathway cross the lysosomal membrane through a protein-translocation complex, that is, a process that requires protein interaction with the chaperone HSPA8 (also known as HSC70) and association of HSPA8 with a specific splicing isoform of LAMP-2, that is, the lysosomal protein LAMP-2A. Thus, chaperone-bound autophagy substrates bind LAMP-2A monomers on the cytosolic side of the lysosome, which stimulate the formation of an oligomeric LAMP-2A translocation complex [10, 33–35].

Essential functions that CMA fulfils in cells include a contribution to amino acid recycling during prolonged starvation as well as quality control, directly linked to the ability of this pathway to selectively remove single proteins from the cytosol. For example, CMA is up-regulated during oxidative stress where it contributes to the degradation of oxidized proteins (reviewed in [36]) (see next Sect. 18.3).

Of note, growing evidence demonstrates that malfunction of CMA plays a vital role in the pathogenesis of severe human disorders. Often, the mechanisms underlying the alterations of CMA in these pathologies involve perturbations in the functioning of the CMA translocation complex. Both diminished and enhanced CMA activities have been shown to associate with diseases, an observation that emphasizes the importance of a tight regulation of CMA activity (highlighted in [36]).

18.2.4.4 Concluding Remarks

As argued above, current knowledge about mechanism and physiological relevance of microautophagy in mammalian cells is hard to judge since most findings derive from studies in yeast. However, future studies aimed at identifying proteins controlling microautophagy-related vacuolar membrane changes in yeast will probably allow to search for homologues in mammals and then investigate their contribution to mammalian microautophagy. By contrast, research on CMA has already made substantial progress. For example, the recent identification of a plethora of new CMA substrates and deficiencies in CMA associated with diverse human pathologies has expanded our understanding of the importance of CMA in multiple cellular functions. In fact, the growing number of connections between CMA and human diseases has already generated interest in modulating CMA activity for therapeutic purposes.

18.2.5 Crosstalk Between Autophagic Responses and DAMPs

18.2.5.1 General Remarks

There is a close relationship between autophagy and MAMPs and/or DAMPs in the cellular response to injury. In fact, autophagy cannot be restricted to an innate immune mechanism that controls intracellular homeostasis alone but has to be extended in terms of "immunological autophagy" to a process that is committed to control and regulate efferent innate and potential adaptive immune responses. The "medium" for achieving this goal is the MAMPs and/or DAMPs which operate as a link between intracellular and extracellular events. This scenario is briefly touched in the following.

18.2.5.2 Regulation of DAMP Emission by Autophagy

Growing evidence indicates that autophagy regulates release and degradation of DAMPs—here in terms of inducible DAMPs—including HMGB1, ATP, and DNA in several cell types [37]. For example, autophagic mechanisms reportedly promote and regulate the release and secretion of HMGB1 in a ROS-dependent manner in

fibroblasts, macrophages, and cancer as well as NET-mediated release of HMGB1 in neutrophils [37, 38]. Moreover, autophagy has been shown to be required for the liberation/active secretion of ATP by dying cancer cells [39, 40]. In addition, autophagy was found to contribute to the regulation of the DDR at multiple levels, that is, a process associated with the emission of DAMPs [37, 41] (for DDR, see Sect. 18.6). Via emission of DAMPs, eventually, together with MAMPs, autophagy can amplify or even instigate MAMP/DAMP–PRM signalling leading to efferent innate immune responses.

On the other hand, autophagy can inhibit pro-inflammatory signalling cascades. For example, the Atg5–Atg12 complex, a key regulator of the autophagic process, was shown to negatively regulate RLR signalling by direct binding to CARD domains of RIG-I and *interferon promoter-stimulating factor-1* (IPS-1) [42] (compare Part VI, Sect. 22.3.6). Moreover, as reviewed elsewhere [1], autophagy has been found to inhibit both NLRP3 and AIM2 inflammasome activation and subsequent production of pro-inflammatory cytokines IL-1β and IL-18 (for inflammasomes, see Part VI, Sects. 22.4.2 and 22.4.4). As a possible mechanism, the authors propose that inflammasome components and pro-IL-1β are subjected to ubiquitination and subsequent degradation by autophagy, thereby leading to functional inactivation of inflammasomes.

18.2.5.3 Regulation of Autophagy by Emission of DAMPs

Conversely, an increasing number of studies suggest that DAMPs, including HMGB1, ATP, and DNA, are powerful stimuli and regulators to elicit autophagic responses [3, 43–50]. For example, HMGB1 was demonstrated to be an important regulator of autophagy in various types of cancer cells and keratinocytes. Mechanistically, the reduced form of the HMGB1 protein was proposed to be responsible for the promotion of autophagy in an AGER/RAGE-dependent fashion [47, 48]. Also, and of high interest, in a clinical study on patients with chronic hepatitis B, HMGB1-induced autophagy was found to maintain Treg function during chronic viral infection [49]. Moreover, in studies on a mini pig lung IRI model, evidence was provided indicating that autophagy, when triggered by DAMPs such as HMGB1 and HSP60 during IRI, amplifies the inflammatory response through enhancing K63-linked ubiquitination of TRAF6 and activation of the downstream MAPK and NF-κB signalling (for TRAF6, MAPK, and NF-κB signalling, see Part VI, Sect. 22.3.3).

Moreover, there is already first evidence suggesting a role of ATP in the regulation of autophagy [50]. In addition, there are accumulating data indicating that cytosolic DNA, dislocated as a result of DNA damage, may contribute to the regulation of autophagy whereby the *DNA damage-regulated autophagy modulator 1* (DRAM1) appears to play a mechanistically crucial role [51–53].

The mechanisms involved in MAMP/DAMP-activated autophagic responses have only partially been elucidated. In fact, there is convincing evidence suggesting that many MAMP/DAMP-recognizing PRMs including TLRs (in particular endosomal TLRs), NLRs, and anti-DNA receptors can activate autophagic responses by triggering specific signalling pathways (reviewed or discussed in [1, 2, 54, 55]).

18.2.5.4 Concluding Remarks

The crosstalk between autophagic responses and DAMPs represents a powerful instrument of the innate immune system to integrate and unify various tools for the promotion and regulation of injury-induced inflammation and, in the presence of nonself- or altered self-antigens, injury-induced adaptive immunity. Thus, on the one hand, autophagy is known to promote and regulate the release of DAMPs (though the exact mechanisms are still elusive); subsequently, DAMPs via PRM-triggered pathways participate in the regulation of inflammation. On the other hand, activation of PRMs by MAMPs and/or DAMPs promotes autophagy activation through a mechanism that has been partially elucidated. In fact, an increasing number of findings suggest that this activation process is triggered by PRMs following recognition of MAMPs and/or DAMPs. Nevertheless, the precise molecular mechanisms by which PRMs modulate autophagy remain largely unknown.

18.2.6 Résumé

There is increasing evidence in support of the notion that MAMP/DAMP-activated autophagic responses promote emission of DAMPs which in turn support cellular homeostasis in the course of adaptive stress responses in healthy cells. Notably, this cellular homeostatic effect may spread out and affect the whole organism via emission of autophagy-dependent DAMPs. In other words, via DAMPs, autophagy as a cell-intrinsic stress response can fortify its defending capability by providing a link to promotion and regulation of cell-extrinsic efferent innate immune and eventually subsequent adaptive immune responses.

However, despite the fact that autophagy is one of the best-known cell-autonomous responses in innate immunity and has clearly been shown to counteract dangerous infectious and sterile cell stress, much is left unclear. One such issue concerns the definition of autophagy-dependent cell death. As discussed and summarized [10], autophagy-dependent cell death can be defined as a form of RCD (see next chapter) that can be retarded by pharmacological or genetic inhibition of macroautophagy. In this context, as stressed by Galluzzi et al. [10], it is important to note that (1) specificity issues affect most, if not all, pharmacological agents employed so far for suppressing macroautophagic responses and (2) multiple components of the macroautophagy machinery have autophagy-independent functions. In view of these facts and findings, these authors recommend to favor genetic approaches and to test the involvement of at least two different proteins of the macroautophagy apparatus in a specific instance of RCD before etiologically attributing it to macroautophagy.

Other unclear issues refer to the specific modulation of autophagy by MAMPs and/or DAMPs, the precise interaction of autophagy with innate immune signalling cascades, and the cooperation between autophagy and other physiologic cell-intrinsic and cell-extrinsic processes during scenarios of cell stress and tissue injury. Efforts to solve these problems are of utmost importance in view of the fact that autophagy—when induced by excessive, chronic, or acute-repetitive

emission of DAMPs—can contribute to the pathogenesis of many human diseases, that is, acute and chronic, infectious, or sterile inflammatory disorders. At the respective places, they will often be mentioned in the following chapters as well as in Volume 2.

18.3 The Oxidative Stress Response

18.3.1 Introductory Remarks

Oxidative cell stress and tissue injury reflect most potent and omnipresent threats an organism is exposed to. Though there is a robust defense response continuously operating, this kind of injury is known to contribute to the pathogenesis of many human diseases. How can this be?

Oxidative stress is caused by an imbalance between the production of oxidants such as ROS on one side and the biological antioxidative defense system's ability on the other side to counter the oxidant levels with antioxidants, that is, to readily detoxify the toxic reactive species or easily repair the resulting damage. Thus, it is the excessive production of ROS—overriding the antioxidative capacities—that is pathophysiological and contributes to dysfunction, damage, and even death of cells. By contrast, generation of ROS in physiological low/moderate concentrations—operating as second messenger molecules and causing so-called oxidative "eustress" [56]—assists in intracellular signalling pathways and, thus, is essential for optimal cell functions and homeostasis of an organism. In other words, the biological effects of ROS—beneficial or deleterious—considerably depend on the amounts of ROS present and, in action, a phenomenon that is in agreement with the idea that cellular ROS generation has characteristics of hormesis implying a dose-response phenomenon that is characterized by beneficial effects at low doses and deleterious effectivity at high toxic doses [57].

To guarantee this homeostatic function of ROS, to keep these molecules within physiological limits, and to prevent their deleterious effects, that is, to maintain hormesis, a smooth running of the oxidative stress response is of utmost importance. Accordingly, a few aspects of this critical stress response are addressed in the following.

18.3.2 Sources and Production of Reactive Oxygen Species

18.3.2.1 General Remarks

Reactive oxygen species are produced from molecular oxygen as a result of normal cellular metabolism. To understand any discussion on a role of ROS in host defense or human diseases, one should define free radicals. According to Halliwell and Gutteridge [58], "a free radical is any species capable of independent existence that contains 1 or

more unpaired electrons." An unpaired electron is one that occupies an atomic or molecular orbital by itself. Radicals can be formed by the loss of a single electron from a non-radical, or by the gain of a single electron by a non-radical. In this sense, superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), peroxyl radicals ($RO_2^{\cdot}$), and alkoxyl radicals ($RO^{\cdot}$) are oxygen radicals. Of note, ROS is a collective term often used by scientists to include not only the oxygen radicals but also some non-radical derivatives of oxygen such as H_2O_2, hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$). Nitrogen-containing oxidants, such as $NO^{\cdot}$ are called RNS. Generation of ROS is generally a cascade of reactions that starts with the production of superoxide anions. Superoxide rapidly dismutases to H_2O_2 either spontaneously (especially at the low pH) or catalyzed by SOD. Other elements in the cascade of ROS generation include the reaction of superoxide with NO to form the very toxic peroxynitrite, the peroxidase-catalyzed formation of HOCl from H_2O_2, and the iron-catalyzed Fenton reaction, leading to the generation of hydroxyl radical.

18.3.2.2 Reactive Oxygen Species-Producing Enzyme Systems

The oxidants are produced endogenously as by-products or metabolites of various metabolic processes. Multiple enzyme systems produce superoxide radicals and their derivatives including xanthine oxidoreductase (XOR), the reduced form of NADPH oxidases (NOXes), and mitochondrial electron transport chain (ETC)-associated molecular complexes (Fig. 18.2). Indeed, leakage of electrons from the

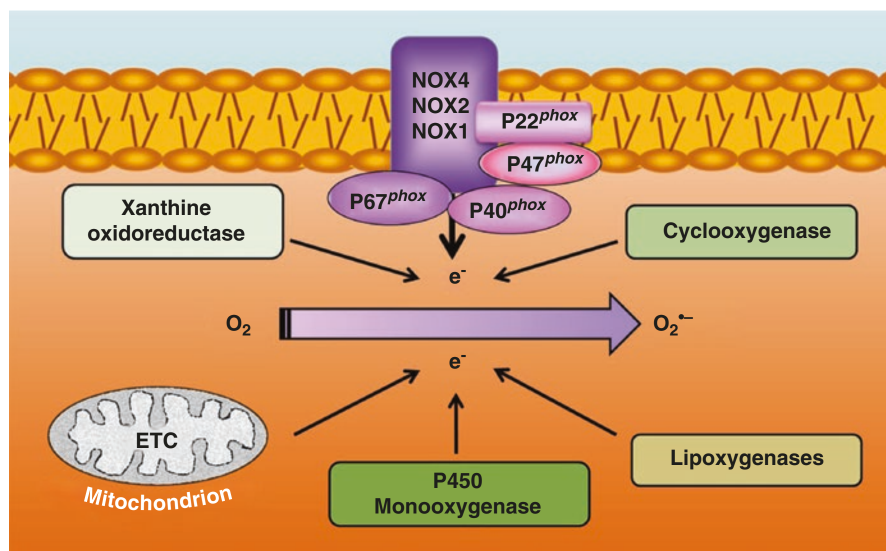

Fig. 18.2 Schematic depiction of enzymatic sources of superoxide radical formation. The major sources include the mitochondrial ETC, members of the NOX enzyme family, lipoxygenases, cyclooxygenase, and P450 monooxygenase. *ETC* electron transport chain, *NOX* nicotinamide adenine dinucleotide phosphate-dependent oxidase. Sources: Refs. [61, 62, 64–70]

mitochondrial ETC is believed to be the main source of ROS. In the following, some aspects of these three systems (other systems not mentioned here) are briefly touched, exemplified by and focused on their role as vascular sources of ROS production as investigated on models of IRI.

Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR), as a housekeeping enzyme, is probably expressed in all cells but primarily in surface epithelia such as capillary endothelial tissue of various organs. Xanthine oxidoreductase, a complex molybdoflavoprotein, is the rate-limiting step in the catabolism of purines, where it catalyzes the last steps of purine metabolism: the conversion of hypoxanthine to xanthine and of xanthine to uric acid, with superoxide/H_2O_2 generated as by-products. There is a definite role of XOR in reperfusion of tissue and organs [59, 60]. For example, experiments performed in isolated rat hearts have demonstrated that radical generation and functional injury are decreased by inhibition of XOR with oxypurinol. Similarly, in human aortic or venous ECs, XOR-mediated ROS generation has been shown to be a central mechanism of oxygen radical generation upon postischemic reoxygenation [61, 62].

The NADPH Oxidases

The NADPH oxidases were initially considered as enzymes expressed only in phagocytic cells involved in host defense and innate immunity; however, recent evidence indicates that there is an entire family of NOXes based on the discovery of gp91phox homologues. The family comprises seven members, including NOX1, NOX2 (formerly termed gp91phox), NOX3, NOX4, NOX5, DUOX1, and DUOX2 [63]. Three members out of the enzyme family are important sources of ROS in the vasculature, namely, NOX1, NOX2, and NOX4 [64–66]. Today, NOXes are perhaps the best-studied enzymes involved in ROS production in the blood vessels. Remarkably, different members of the NOX/DUOX family engaged in IRI are localized in various cells, that is, in vascular cells and phagocytes. This may lead to the notion that NOXes in vascular cells are responsible for the first wave of ROS production because vascular cells are first confronted with reintroduced molecular oxygen. As generally believed, there is, in fact, no vascular specific NOX isoform but rather a complex expression of various NOX isoforms in different cells and regions of the vascular system. Nevertheless, in arteries from humans and animals, NOX2, NOX4, and a shallow level of NOX1 have been consistently found to be present both as messenger RNA and as protein [63]. Altogether, the findings and data briefly described here make clear that vascular cells are equipped with efficient machinery able to efficiently produce ROS. Regarding the different enzymatic sources, superoxide radicals appear to be predominantly generated compared, for example, to hydroxyl radicals.

Mitochondrial Electron Transport Chain and Its Associated Enzyme Systems

Mitochondria have been implicated as potential oxygen sensors by increasing the generation of ROS which regulate a variety of hypoxic responses [67–70]. In fact,

mitochondria are increasingly recognized as lynchpins in the evolution of tissue injury during postischemic reperfusion. It is generally acknowledged that the majority of intracellular ROS production is generated in the mitochondrial ETC and its associated metabolic enzymes. However, very little is known about which mitochondrial sites are involved in physiological or pathological ROS production under native conditions. Of note, using inhibitors to manipulate the redox states of particular sites and prevent superoxide generation from others, at least ten different locations of superoxide/H_2O_2 production in the ETC and associated enzymes (Krebs cycle, β-oxidation, etc.) have been identified in mammalian mitochondria. In fact, the relative and absolute contributions of specific sites to the production of ROS in isolated mitochondria depend very strongly on the substrates being oxidized, and the same is likely valid in cells and in vivo [71]. For example, superoxide formation occurs on the outer mitochondrial membrane, in the matrix, and on both sides of the inner mitochondrial membrane (Fig. 18.3). Complex I (NADH-ubiquinone oxidoreductase) accepts electrons from NADH; these electrons are carried to complex II (the succinate dehydrogenase-CoQ oxidoreductase), where they are used to oxidize succinate to fumarate. Afterward, electrons continue to travel down their electrochemical gradient to complex III (the cytochrome bc1 complex (ubiquinol-cytochrome c oxidoreductase)), and subsequently to complex IV (cytochrome c oxidase); finally, the electrons are used to reduce molecular oxygen to water. Thus, complex I and complex II oxidize the energy-rich molecules NADH and flavin adenine dinucleotide H_2, respectively, and then transfer the resulting electrons to ubiquinol that carries it up to complex III (for competent articles, see [72, 73]).

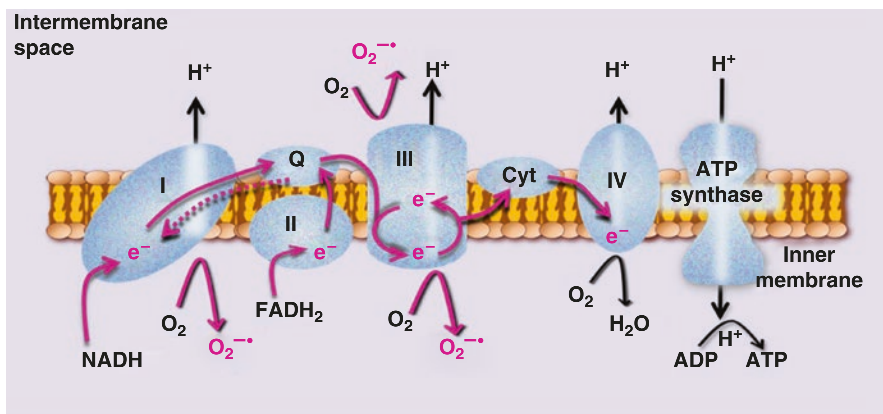

Fig. 18.3 Schematically depicted simplified model of the electron transport chain (ETC) inside mitochondria involved in generation of reactive oxygen species. In the course of this scenario, the hydrogen protons (or a pair of electrons) are transported from one carrier to another, and they are finally used to reduce oxygen to water. During this transfer of electrons, a lot of energy in the form of ATP is released. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, *Cyt* cytochrome c, e^-, electrons, *FADH2* flavin adenine dinucleotide(reduced form), H^+ hydrogen proton, *NADH* nicotinamide adenine dinucleotide (reduced form), $O_2^{-\cdot}$ superoxide anions, *Q* coenzyme Q. Sources: Refs. [67–69, 71–73, 247]

Notably, complexes I and II generate superoxide within the mitochondrial matrix, whereas complex III produces superoxide at the Qo site, resulting in the release of superoxide into either the intermembrane space or the matrix. Regarding complex I, it was recently demonstrated that inhibition of ND5, a subunit of complex I, suppresses the activity of this complex and thus ROS production [74]. Furthermore, data from another set of studies on complex I showed that stable down-modulation of its subunits GRIM-19 and NDUFS3 decreased complex I activity that was associated with a significant reduction in the overall NADH oxidation rate but with an increased production of ROS by the target cells [75]. Similar results have been found in studies on complex II: there is evidence suggesting that inhibition of complex II on the level of subunits even leads to an increase in ROS production. The phenomena can be explained by assuming that, if electrons provided in the course of the ETC cannot efficiently be transferred to the next complex, they would leak out from the inhibited complex and generate ROS [76].

The complex III subunits *Rieske iron–sulfur protein* (RISP) encoded by *ubiquinol-cytochrome C reductase, Rieske iron–sulfur polypeptide 1* (UQCRFS1), and *ubiquinol-cytochrome c reductase binding* (UQCRB) protein appear to play a crucial role in hypoxia-triggered mitochondrial ROS generation (for Rieske, see Box 18.3). Thus, it was shown that RISP promotes the hypoxic stabilization of the transcription factor HIF-1α protein [77] and UQCRB was found to mediate hypoxia-induced tumor angiogenesis via mitochondrial ROS-mediated signalling [78, 79]. Also, and of note, a mouse model to permit conditional deletion of the nuclear-encoded RISP gene was recently developed to assess its role in hypoxia-induced ROS signalling in the pulmonary circulation [80]. It was found that depletion of RISP abolishes the ROS response to hypoxia in isolated pulmonary

Box 18.3 What Is the Rieske Fe-S Protein?

The Rieske protein is an iron–sulfur protein (ISP) component of the cytochrome bc1 complex that was first discovered and isolated by John S. Rieske and coworkers in 1964. The Rieske iron–sulfur protein is an essential subunit of mitochondrial cytochrome *bc1* complexes and, like the majority of mitochondrial proteins, is encoded by a nuclear gene and synthesized on cytoplasmic ribosomes as a precursor with a 32-residue amino-terminal extension. The iron–sulfur protein is then post-translationally imported into the mitochondria where it is inserted into the bc1 complex in the inner mitochondrial membrane. At first, the precursor is translocated via translocation contact sites into the matrix. There, cleavage to an intermediate containing an 8-residue extension occurs. The intermediate is then redirected across the inner membrane, processed to the mature subunit, and assembled into complex III.

Further reading: Conte L, Zara V. The Rieske Iron-Sulfur Protein: Import and Assembly into the Cytochrome bc(1) Complex of Yeast Mitochondria. Bioinorg Chem Appl 2011;2011:363941.

arterial SMCs and isolated pulmonary artery segments. Further, in this article, it was discussed that mitochondria are not the only source of ROS during hypoxia. Thus, studies using a genetic knockout of p47phox suggested that cytosolic NADPH oxidase systems may also contribute to a hypoxic pulmonary vasoconstriction response during acute hypoxia [81, 82]. According to the authors' conclusion, the blockade of hypoxia-induced ROS responses (in these studies observed with depletion of RISP) suggests that the mitochondria may act as the initiators of ROS production, which could be amplified by engagement of NADPH oxidase systems elsewhere in the cell. Such "ROS-induced ROS release" might permit small ROS signals generated by mitochondria to activate ROS signalling throughout the cell, thereby avoiding mitochondrial damage that might arise if the entire cellular oxidant signal originated from that organelle [83] (or even leading to excessive ROS production?).

18.3.2.3 Concluding Remarks

Indeed, the substantial advances in oxidative stress research of recent times, in particular, the specification of hypoxia-sensing ROS-producing enzyme systems, will contribute to new therapeutic strategies to be applied in acute and chronic human diseases known to be influenced by oxidative stress. For example, discrimination of oxidative eustress, a fundamental process in maintaining health, from oxidative damage will improve clarity in developing "redox medicine" [56].

18.3.3 Antioxidative Defense Systems

18.3.3.1 General Remarks

When the redox equilibrium of a cell is upset by pro-oxidant environmental stimuli, that is, when oxidative stress exists, an adaptive stress response takes place which can result in upregulation of antioxidant proteins and detoxification enzymes. These antioxidative defense molecules comprise the following [58]: (1) agents that catalytically remove free oxygen radicals and other reactive species, for example, SOD, catalase, peroxidase, and thiol-specific antioxidants; (2) proteins that minimize the availability of pro-oxidants such as iron ions, copper ions, and heme, for example, transferrins, haptoglobins, hemopexin, and metallothionein; (3) proteins that protect biomolecules against damage (including oxidative damage) by other mechanisms, for example, HSPs; and (4) low-molecular-mass agents that scavenge ROS and RNS, for example, glutathione, α-tocopherol, and (possibly) bilirubin and uric acid.

18.3.3.2 The Three Tiers of Antioxidative Defense Response

In the past, it was fashionable to divide the oxidative stress response into three main tiers: (1) antioxidant enzymes including SOD, catalase, glutathione peroxidase, and glutathione; (2) detoxifying enzymes such as glutathione peroxidase, glutathione S-transferase, aldo-keto reductase, and aldehyde dehydrogenase; and (3) energy-dependent efflux pumps. As a fourth defense system, the antioxidant

nutrients such as vitamins E and C as well as carotenoids were appreciated [84]. It was generally accepted that the first line of enzymes is of enormous importance in limiting ROS-mediated damage to intracellular macromolecules. For example, among the most important regulators of ROS levels were the SOD enzymes: Cu/ZnSOD in the cytoplasm and outer mitochondrial space and MnSOD exclusively in the inner mitochondrial space. Mechanistically, superoxide is converted to H_2O_2 and oxygen ($O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) by SOD. Peroxiredoxins and abundant catalase enzyme then scavenge H_2O_2, converting it to molecular oxygen and water. Another example of a first-line defense molecule is TRX. Thioredoxin contains two adjacent –SH groups in its reduced form which are converted to a disulfide in oxidized TRX. Notably, it can undergo redox reactions with multiple proteins using the reaction TRX (SH)2 + protein − S2 ↔ TRX − S2 + protein − (SH)2.

However, it then turned out that these antioxidative principles were clearly not 100% effective at performing this task, as under normal physiological conditions, lipid and DNA oxidation products can be detected in blood and urine. Because certain compounds of the chemicals generated after an interaction of ROS with macromolecules are highly reactive, there must be an equal necessity to detoxify these secondary oxidation products to prevent them from also damaging DNA, proteins, and lipids. Without the adequate detoxification of such products, an extended chain reaction will occur resulting in the degradation of cellular components and the ultimate death of the cell. This second line of defense against ROS is provided by those detoxifying enzymes. Finally, detoxified metabolites produced by these enzymes are eliminated from the cell by energy-dependent efflux pumps such as the glutathione S-conjugate transporter, also called the multidrug resistance-associated protein (MRP) [58].

Today, one must state that these previous notions are incomplete. At first, it became apparent that members of the so-called CNC (for *cap 'n' collar*)–*basic region-leucine zipper* (bZIP) family of transcription factors are principal mediators of defense responses to redox stress. In mammals, the CNC family members Nrf1 and Nrf2 were shown to be involved in the transcriptional upregulation of cytoprotective genes encoding a large number of diverse detoxification, antioxidant, and anti-inflammatory proteins (e.g., glutamate cysteine ligase, NADPH–quinone oxidoreductase, glutathione S-transferases, and aldo-keto reductases) as well as enzymes with essential roles in cell metabolism [85].

More recent studies then revealed that these transcription factors, notably Nrf2, are activated by Keap1 as the primary negative regulator of Nrf2, that is, a molecule that simultaneously operates as a sensor protein able to perceive dyshomeostatic Subclass IIC-4 DAMPs, for example, in terms of redox changes reflecting electrophilic stress. It is worth to add here that six critical domains have been defined in Nfr2 (Neh1-Neh6), and it is Neh2, located at the N terminus of Nrf2 that acts as the regulatory domain for the cellular stress response. Actually, Neh2 interacts with the cytoplasmic protein Keap1 [86].

In the following, this very important DAMP-induced and gene-based antioxidative and cytoprotective system is addressed a bit more in detail.

18.3.3.3 Oxidant-Promoted, Keap1 ↔ Nrf2-Triggered Antioxidant Response Element Pathway

Increasing evidence indicates that several redox-regulated gene products serve to protect cells from ROS damage. The *antioxidant response element* (ARE), a cis-acting DNA regulatory element or enhancer sequence is known to be activated by oxidative stress and to be responsible for the transcriptional regulation of several redox-regulated gene products. Both Nrf 1 and 2 bind to ARE and regulate ARE-mediated gene expression and induction. The molecule Nrf2 is more potent than Nrf1 in activation of ARE-regulated gene expression and is regarded as the principal transcription factor that binds to the ARE. This transcription factor is ubiquitously expressed and present in various organs and tissues including the kidney, muscle, lung, heart, liver, and brain (for reviews, see [87–91]). As touched in Part II, Sect. 5.3.2 and Part IV, Sect. 13.4.5, the Nrf2-triggered antioxidant response is initiated by activation of Keap1 that functions as a substrate adaptor protein for the degradation of Nrf2 and serves as an intracellular sensor for redox changes reflecting the presence of Subclass IIC-4 DAMPs (for reviews see [92–94]).

Earlier Studies

Earlier studies had already shown that Nrf2 is a bZIP transcription factor that translocates to the nucleus after liberation under oxidative stress conditions from its cytosolic inhibitor Keap1 [86]. In the nucleus, Nrf2 was found to form dimers with the proteins Maf, Jun, Fos, *activating transcription factors 4* (ATF4), and/or *CREB binding protein* (CBP) and, in addition, regulates transcription by binding to the ARE upstream of a variety of cytoprotective and detoxification target genes to combat the oxidative stress [95]. Thus, established Nrf2-regulated genes reportedly included Cu/Zn SOD, catalase, TRX, TRX reductase, glutathione reductase (GR), glutathione peroxidase (GPX), and ferritin L (FTL) [96] (FTL and ferritin H (FTH) subunits are responsible for intracellular iron storage). All of these genes are involved in the response to oxidative stress. There are several other genes also known to be engaged in the response to oxidative stress that are not described here.

The Keap1 ↔ Nrf2 Pathway Today

Recently, the molecular signalling mechanism involved in the Keap1 ↔ Nrf2 pathway has been further elucidated and specified. The core can be seen in an axis consisting of redox change (Subclass IIC-4 DAMPs)-initiated → Keap1-induced → Nrf2-triggered → ARE-driven expression of antioxidant and detoxifying genes (Fig. 18.4) (discussed in [92–94, 97, 98]). The complex and complicated sequelae of the pathway are simplified in the following text.

Under homeostatic and stress-free conditions, binding of Keap1 to the Nrf2 molecule leads to its polyubiquitination and subsequent degradation by the proteasomal pathway, thereby maintaining a consistent generation of Nrf2 and retaining its very low levels in the cytoplasm. In this scenario, Keap1 homodimer binds to a single Nrf2 protein via a high-affinity so-called “ETGE” motif and low-affinity so-called “DLG” motif. The two-site recognition of Nrf2 by the Keap1 dimer is essential for polyubiquitination of Nrf2 (also see Part II, Sect. 5.3.2) (for polyubiquitination, see Box 18.2). In this sense, the Keap1 ↔ Nrf2 system can be regarded as a vital part of regulating cells under a

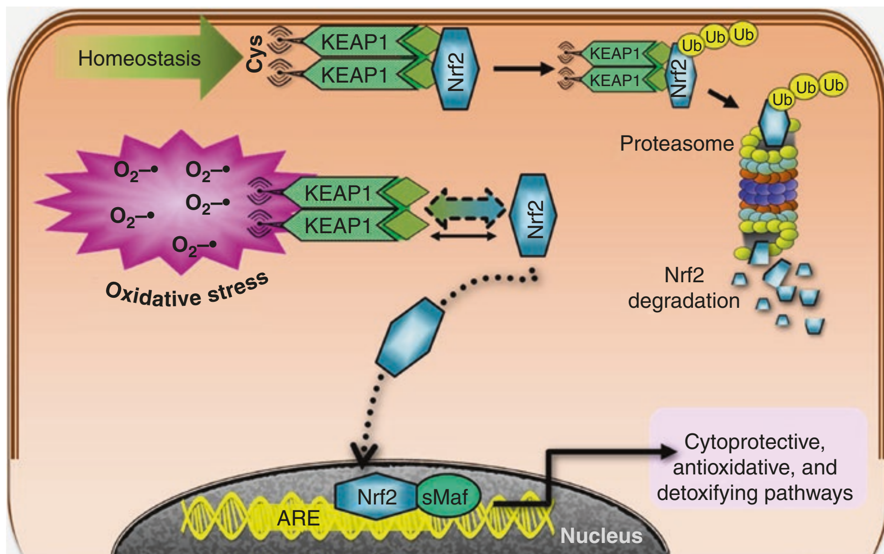

Fig. 18.4 The oxidative stress-induced Keap1 ↔ Nrf2 pathway. Under non-oxidative homeostatic conditions, the sensor Keap1is bound to the Nrf2 molecule resulting in its polyubiquitination and subsequent degradation via the proteasomal pathway. Oxidative stress modifies the ROS-sensing cysteine residues of Keap1 leading to loss of its polyubiquitination and degradation activity, dissociation of Nrf2 that becomes stabilized and accumulates. Then, Nrf2 translocates to the nucleus and forms a heterodimer with the sMAF transcription factor. The Nrf2/sMAF heterodimer binds to ARE and induces transcription of numerous cytoprotective antioxidant and detoxification genes. *ARE* antioxidant response element, *Keap1* Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1, *Nrf2* nuclear factor-erythroid 2 p45- related factors 2, *sMAF* small musculoaponeurotic fibrosarcoma, *Ub-Ub-Ub* poly-ubiquitin chain. Sources: Refs. [92–94, 97, 98]

homeostatic environment. However, in case of dangerous and threatening oxidative (and xenobiotic) stress, the system instigates a stress response that is characterized by a rapid and dramatic cessation of the Keap1-dependent polyubiquitination process resulting in a rapid increase of Nrf2 abundance. In fact, exposure to ROS-mediated stress (or electrophilic stress) is thought to modify the reactive "IIC-4 DAMPs-sensing" cysteine residues in Keap1, which is associated with a conformational change of the protein resulting in a loss of Keap1 ubiquitination activity (Fig. 18.4). Notably, as a cysteine-rich protein, the human Keap1 possesses 27 cysteine residues, and they are all reactive to stress to varying degrees. Among these sensor cysteines of Keap1, C151 is best characterized. Evidence from a number of studies has suggested that C151 is the most reactive and critical to the Keap1 ↔ Nrf2 stress-sensing response. Even, there is already evidence from a first atomic-level view suggesting that the unique environment of Cys 151 (besides Cys171, Cys273, and Cys288) appears to be the critical residue of Keap1 responsible for detecting increased levels of oxidative stress [91, 92, 97].

Oxidative modification of cysteine sensors of Keap1 leads to a loss of its polyubiquitination and degradation activity thereby stabilizing Nrf2. Consequently,

stabilized Nrf2 accumulates in the cytoplasm, translocates into the nucleus, and forms a heterodimer with a sMAF transcription factor (sMAF, *small musculoaponeurotic fibrosarcoma*).

Thus, this accumulation of Nrf2 in response to ROS (and electrophiles) cannot be regarded as an induction in a strict sense but instead is a mechanism referred to as derepression, that is, from the rapid degradation-based repression.

As highlighted and discussed [93], there are two models of how to explain the cytoplasmic accumulation process of Nrf2. The "hinge-and-latch" model holds that the modification of the sensing cysteine residues of Keap1 reduces its affinity for Nrf2 but does not result in release. Instead, newly synthesized Nrf2 is translocated to the nucleus to trigger the transcription of Nrf2-dependent genes. The other model denoted as the "conformation cycling" model claims that Keap1 uses a cyclic mechanism to target Nrf2 for polyubiquitination and proteasomal degradation. An important feature of this cyclic mechanism is that it ensures regeneration of Keap1 which allows the cycle to proceed. Modification of specific reactive cysteine residues of Keap1 may block the cycle of Keap1-dependent Nrf2 degradation allowing de novo synthesized Nrf2 to accumulate.

The subsequent transcriptional process in the nucleus has been specified as well. As partially mentioned above, the Nrf2-sMAF heterodimer binds to ARE or electrophile-responsive element (EpRE) and induces transcription of numerous cytoprotective genes. Of note, recently, an extensive genome-wide analysis of the Nrf2-sMAF-binding sequence, that is, the ARE/EpRE, and the MAF homodimer-binding sequence (the so-called MAF responsive element or "MARE") was conducted, and the differences between these elements were clarified. As a result, it was proposed that ARE, EpRE, and the NF-E2 binding sequence be collectively named CNC-sMAF-binding elements (CsMBE) [99].

Interestingly, PRMs appear to be co-players in this scenario. Thus, TLRs have been observed to induce Nrf2 activation. Remarkably, in a recent study, TLR agonists were shown to activate Nrf2 signalling via reduction of Keap1 [100]. The authors could demonstrate that TLR signalling-induced Keap1 reduction promotes Nrf2 translocation from the cytoplasm to the nucleus, where it activated transcription of its target genes. Further, TLR agonists were found to modulate Keap1 at the protein post-translation level through autophagy. In fact, TLR signalling increased the expression of autophagy protein p62 and LC3-II and induced their association with Keap1 in the autophagosome-like structures.

18.3.3.4 Concluding Remarks

The Keap1 ↔ Nrf2 system as briefly described here is a robust oxidative stress response. Its regulatory mechanisms, for example, stress-sensing mechanism, proteasome-based regulation of Nrf2 activity, and selection of target genes have been elucidated mainly in mammals (for proteasome, see Box 18.1). Nevertheless, the pathway is now regarded as an evolutionarily conserved defense mechanism against oxidative and xenobiotic stress across the tree of life. Thus, the Keap1 ↔ Nrf2 system has been found to be also present in zebrafish, fruit fly, and *Caenorhabditis elegans* indicating that its roles in cellular defense are conserved throughout

evolution among vertebrates and suggesting that analogous defense systems are widely conserved throughout the animal kingdom [101, 102].

18.3.4 Résumé

As briefly demonstrated in this subchapter, aerobic organisms have integrated antioxidant systems, which include DAMP-promoted generation of enzymatic and nonenzymatic antioxidants that are usually effective in blocking harmful effects of ROS. However, when ROS is produced in excess causing pathological conditions, the stress response against oxidative damage can be overridden. Thus, oxidative stress is known to contribute to many pathological conditions, including cancer, neurological disorders, atherosclerosis, hypertension, ARDS, and chronic obstructive pulmonary disease, just to mention a few of them. Plausibly, these disorders are motivation enough to search for new effective therapeutic options by harnessing the new insights into mechanisms of the Keap1 ↔ Nrf2 system. Certainly, intense research is essential for a detailed understanding of the precise consequences of targeting Keap1 for disease prevention and treatment. All the more so as the oxidative stress response is integrated into other forms of innate stress responses that will be further outlined in the following subchapters.

18.4 The Heat Shock Response

18.4.1 Introductory Remarks

The heat shock response—one of the most ancient and evolutionarily conserved cytoprotective mechanisms found in nature—is induced upon exposure of living cells to acute, subacute, or chronic stress conditions. This defense response is characterized by the expression of a group of phylogenetically conserved intracellular HSPs, which possess the capacity to recognize structures commonly found in the interior of proteins and to bind such structures. Thanks to this property, they form a chaperone network involved in correct protein folding, trafficking, and complex assembly (for reviews, see [103–109]). In order to be released from engagement with proteins after folding and take part in further rounds of activity, HSP70 and other chaperones utilize an intrinsic ATPase domain to hydrolyze ATP and assume a free conformation [110]. Once released, HSPs mediate protective cellular defense mechanisms including regulation of apoptosis, the aim being to maintain and restore cellular protein homeostasis (proteostasis).

18.4.2 Heat Shock Proteins in Their Function as DAMPs

Exposure of almost any cell to heat shock most often leads to the rapid transcription, translation, and accumulation of a variety of HSPs that increase to quite considerable levels when the stress is pronounced. In fact, these molecules are induced by various environmental insults that can cause protein denaturation and unfolding

within the cells, leading to the formation of nonnative proteins and protein aggregates, thereby emitting dyshomeostatic DAMPs.

Of note, besides their protective role in different intracellular compartments, HSPs in terms of inducible DAMPs can translocate to the cell surface to get exposed or are actively secreted via non-canonical pathways. In case of necrotic cell death, they are passively released in large amounts into the extracellular space to act as constitutively expressed DAMPs (compare Part IV, Sect. 12.2.3 and Sects 12.3.2.3 and 14.2.2.2). Once emitted, HSPs are sensed by classical recognition receptors and/or non-classical receptors (e.g., CD 91) [111–117]. Interestingly, recent knowledge about similarities between allograft and tumor rejection has visualized that the processes of both IRI to allografts [108, 118, 119] and therapy-mediated injury to tumors [120–123] are characteristically associated with emission of HSPs.

In all eukaryotes, the HSR is primarily regulated and controlled by the HSFs, in particular, HSF1, a sequence-specific factor that binds upstream to heat shock elements in the promoters of target genes [124] (Fig.18.5). For example, HSF1 is activated by environmental stress including oxidative and tumor-associated stress [125, 126].

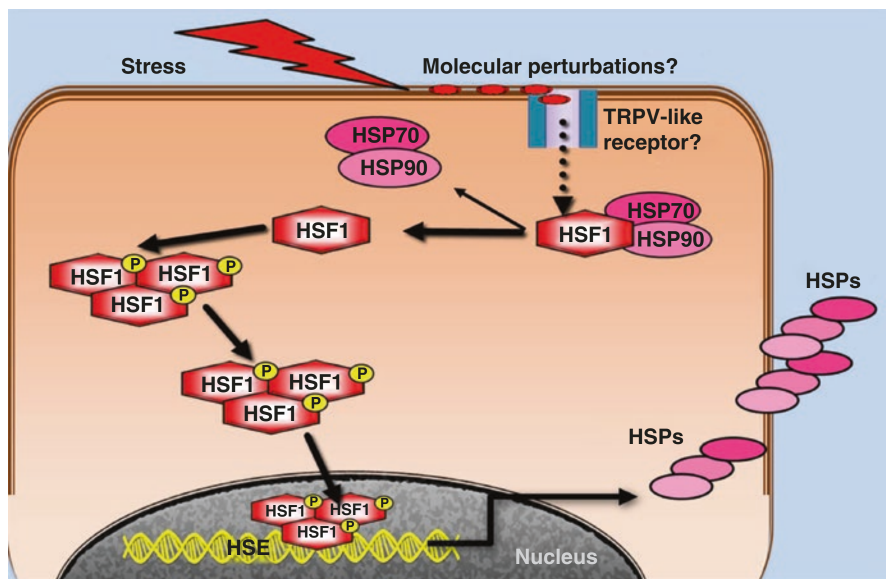

Fig. 18.5 Simplified scenario model illustrating the heat shock response. Under homeostatic conditions, the transcription factor HSF1 exists as an inert monomer in a complex with Hsp90 and Hsp70 that block its transcriptional activity. In response to stress, HSPs dissociate from the complex activating HSF1 that then trimerizes; undergoes a series of posttranslational modifications, including phosphorylation; and translocates to the nucleus. There is preliminary evidence suggesting that stress-induced perturbations reflecting dyshomeostatic DAMPs may be sensed by TRPV calcium channel-like receptors to activate HSF1. Following nuclear translocation, activated HSF1 trimer binds to specific heat shock elements to activate transcription of *hsp* genes resulting in the translation of HSPs, including Hsp90 and Hsp70. The HSPs are subsequently secreted. *HSE* heat shock elements, *HSF1* heat shock transcription factor 1, *HSPs* heat shock proteins, *P* phosphorylation, *TRPV* a family of transient receptor potential cation channels. Sources: Refs. [125–131]

Notably, in all eukaryotes, HSF1 responds to such stress conditions by undergoing a monomer to trimer transition and becomes massively phosphorylated, leading to its acquiring ability to bind to DNA rapidly and activate transcription [127].

There is at least preliminary evidence suggesting that intracellular perturbations reflecting dyshomeostatic DAMPs may activate HSFs [128–131]. Such molecular alterations reportedly include changes in cytosolic Ca^{2+} concentration, for example, caused by increase of fluidity in specific membrane domains [128, 129]. Interestingly, a recent study in support of these earlier findings provided evidence of the existence of a plasma membrane-dependent mechanism of HSF1 activation in animal cells, which is initiated by specific membrane-dependent TRPV calcium channel-like receptors [130]. These findings lend support to the notion that heat sensing and signalling in mammalian cells are dependent on TRPV channels, suggesting that these receptors may act as a major HSR sensor in different epithelial non-cancerous and cancerous cells, capable of triggering the cellular HSR [130]. In another line of experiments, TRPV2 was demonstrated to mediate the effects of transient heat shock on endocytosis of human monocyte-derived DCs, suggesting a central role of TRPV2 in mediating the cellular action of heat shock on these important cells of the innate immune system [131] (for TRPV channel receptors, also compare Part II, Sect. 5.3.6).

18.4.3 Infection-Induced Heat Shock Response

Induction of an HSR is not only mediated by sterile stress conditions but is also believed to be promoted by cell-invading viruses or bacteria. Consequent emission of HSPs in their role as DAMPs may reflect a mechanism by which pathogens may contribute to sterile inflammation. For example, exacerbation of hepatitis B virus (HBV)-associated liver injury is reportedly characterized by an abnormal immune response that not only mobilizes specific antiviral effects but also poses a potentially lethal non-specific sterile inflammation to the host [132].

Heat shock proteins may be the most extensively studied DAMPs in the context of HBV infection. A number of HSPs such as HSP70 and HSP90 have been reported to be supportive factors in the process of HBV replication, and selective inhibition of these HSPs was proposed to be host-based anti-HBV strategies [133–135]. Thus, as argued [132], both infective and sterile inflammation may synergistically contribute to the exaggeration of chronic hepatitis, if the HBV cannot be cleared entirely. Likewise, bacterial infections were also reported to promote induction of the HSR [136, 137]. For example, in studies on *H. pylori* and *E. coli* infection models, the initiation of an HSP70 stress response could be demonstrated [138, 139].

18.4.4 Résumé

The HSR is recognized and accepted as a classical stress response in nearly all species across the tree of life. It reflects the desperate efforts of a cell to restore

homeostasis and survive upon both infectious and sterile insults. Its products, the HSPs, operate as DAMPs in commission of the innate immune system to reach this goal. Notably, via this mechanism, an HSR, induced by infectious damage to a cell, can promote sterile inflammation.

Whereas a successful HSR upon stress leads to restoration of cellular homeostasis and cell survival, an unsuccessful HSR may result in RCD such as apoptosis [109]. This phenomenon will be resumed in the following subchapters.

18.5 Endoplasmic Reticulum Stress and the Unfolded Protein Response

18.5.1 Introductory Remarks

The ER is a continuous membrane system that forms a series of flattened sacs within the cytoplasm of eukaryotic cells. As a subcellular organelle in the control of proteostasis, it is responsible for calcium storage and lipid biosynthesis as well as the synthesis, correct folding, processing, and maturation of proteins as well as for the orchestration of their transport along the classical/conventional secretory pathway. The ER delivers these components to their destination compartments which include the ER itself, the Golgi apparatus, the plasma membrane, and the extracellular milieu or the endocytic and autophagic pathways. Plausibly, the multifunctional nature of this organelle requires a myriad of proteins, unique physical structures, and coordination with and response to perturbations in the intracellular environment. A series of chaperones, folding enzymes, glucosidases, and carbohydrate transferases support and execute these processes.

Perturbation of ER-associated functions such as accumulation of unfolded/misfolded proteins, excessive ROS production, hypoxia, calcium and glucose depletion, or viral and bacterial infections reflect stress of the organelle and result in activation of an ER stress-coping response, the evolutionary conserved UPR [140–143]. For example, the processes of both IRI-mediated cell damage/cell death [144–146] and induction of the ICD of cancer cells [147–149] are characterized by demonstration of ER stress that is almost always associated with oxidative stress and vice versa [150].

18.5.2 The Successful and Unsuccessful Outcome of the Unfolded Protein Response

18.5.2.1 General Remarks

To cope with ER stress, cells have the unique possibility to activate the UPR, a dynamic signalling network that orchestrates the recovery of homeostasis or triggers RCD modalities, depending on the level of damage. Perception of any perturbation of the ER is provided by three sensor molecules of the UPR embedded in the ER membrane: the PERK, IRE-1, and ATF6. The UPR-mediated recovery of ER

homeostasis mainly occurs through the PERK-eIF2α-mediated temporary shutdown of protein translation and the activation of a complex genetic program that aims to improve ER quality control and adaptive responses [140–143] (Fig. 18.6). Accordingly, PERK, devoted to perceiving dyshomeostatic DAMP- emitting ER perturbations (so far, no clear evidence for IRE-1 and ATF6 in this respect), may be regarded as a new family of non-classical PRMs, at least in a broader sense. In non-stressed conditions, the three sensors of the ER homeostasis are kept in an inactive state by the ER-luminal *binding immunoglobulin protein* (BiP). The protein BiP, also known as *glucose regulated protein 78* (GRP78), is a member of the HSP70 family of proteins (specifically HSPA5). It functions as a chaperone to selectively bind unfolded proteins in the ER lumen by interacting with exposed hydrophobic

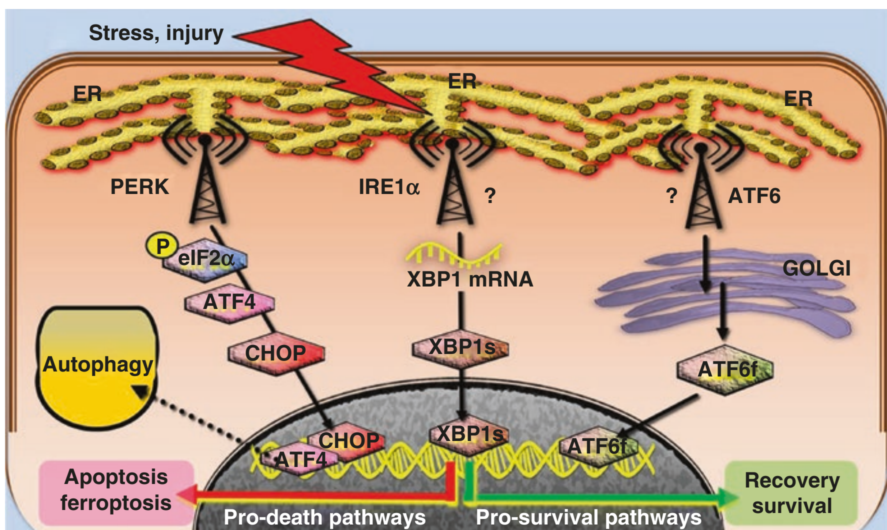

Fig. 18.6 Simplified scenario model illustrating the ER stress-induced three arms of the unfolded protein response. Perception of any perturbation of the ER is provided by three sensor molecules of the unfolded protein response embedded in the ER membrane: the PERK, IRE-1, and ATF6. PERK perceives dyshomeostatic DAMP (Subclass IIC-4 DAMP)—emitting ER perturbations (IRE1 and ATF6 still questionable). PERK phosphorylates eIF2a to up-regulate transcription factor ATF4 that induces the expression of transcription factor CHOP. IRE1a signals through its RNase via the splicing of XBP1 mRNA. The active transcription factor XBP1s translocates to the nucleus. ATF6 is exported from the ER to the Golgi complex to enter the nucleus as a potent transcription factor. Together, these transcription factors of the unfolded protein response determine the cell fate by the regulation of distinct subsets of target genes toward recovery of ER homeostasis and cell survival or the induction of regulated cell death in form of apoptosis and ferroptosis. In addition, the transcription factor ATF4 is differentially translated, up-regulating genes participating in autophagy and other homeostatic pathways. *ATF* activating transcription factor, *CHOP* cytidine-cytidine-adenosine-adenosine thymidine-enhancer-binding homologous protein, *eIF2α* eukaryotic translational initiation factor 2α, *ER* endoplasmic reticulum, *IRE1α* inositol-requiring transmembrane kinase/endoribonuclease 1α, *PERK* protein kinase-like eukaryotic initiation factor 2α kinase, *UPR* unfolded protein response, *XBP1* X-box binding protein 1. XBP1s, X-box binding protein 1 whereby the “s” stands for the spliced form of XBP1. Sources: Refs. [142, 153–161]

residues on nascent peptides [151]. However, in conditions of ER stress, BiP is detached from these sensors allowing their activation to trigger pathways, collectively included in the term of UPR. This stress response acts as a corrective path, capable of both increasing the ER folding capacity and decreasing the incoming polypeptide load.

Of note, this downstream pathway of each of the three UPR sensors appears to have an innate preference for a particular type of ER stress. Moreover, as reviewed [152], upon dissociating from BiP, each of the three sensors modifies the ER to mitigate stress in its own unique way. For example, ATF6 is often the first sensor to respond to ER stress. Once ATF6 dissociates from BiP, it is translocated to the Golgi apparatus for cleavage. The cytosolic domain of ATF6 is then free to move to the nucleus, where it moderates increased expression of several proteins involved in lipid biosynthesis and chaperones. This allows an increase in the volume of the ER and provides more chaperone proteins to aid in folding, thus relieving some of the ER stress. The other two sensors, IRE-1 and PERK, remain as integral ER proteins but oligomerize and autophosphorylate following BiP disassociation (autophosphorylation, a type of post-translational modification of proteins (see Part VI, Sect. 24.3); typical for this biochemical process, a phosphate is added to a protein kinase by itself).

18.5.2.2 Remediable Endoplasmic Reticulum Stress

Under remediable ER stress conditions, the three sensors trigger signalling pathways to resolve ER stress aiming at maintaining cellular integrity (Fig. 18.6). They are briefly touched in the following (for reviews and original articles, see Refs [142, 153–162].).

For example, misfolded proteins are dislocated in the cytosol where degradation processes such as the *ER-associated degradation* (ERAD) and autophagy will clear them, thereby reducing their potential toxicity. Characteristically, ERAD is a protein quality control mechanism conserved in all eukaryotic cells and represents a critical arm of the UPR, necessary to alleviate ER stress. The ERAD mechanism results in the selective dislocation of unfolded and misfolded proteins from the ER to the cytosol via specific membrane machinery. The ERAD targets are subsequently degraded by the cytosolic *ubiquitin proteasome system* (UPS). The whole process includes transcriptional activation of a variety of ER-associated chaperones and folding enzymes which include but are not limited to BiP and the lectins CALR, calmodulin (CAM), and calnexin (CNX).

A pathway that represents the most conserved branch of the UPR is mediated by IRE-1, a multifunctional protein that possesses kinase and endonuclease activities. Upon activation, IRE-1 aggregates and autophosphorylates, thereby activating its endonuclease activity to catalyze the unconventional splicing of *X-box binding protein 1* (XBP1) via removal of a 26-nucleotide intron. This processing event changes the open reading frame of the mRNA, resulting in the translation of the transcription factor now termed XBP1s ("s" stands for the spliced form of XBP1) (for splicing, see Box 18.4). Production of XBP1s leads to upregulation of several genes involved in the UPR's adaptive phase, for example, expression of ER-resident molecular chaperones and protein folding enzymes.

Box 18.4 The Process of Splicing

In molecular biology, splicing is a modification of an RNA after transcription in which introns are removed and exons are joined. Thus, an intron is any non-coding nucleotide sequence within a gene that is removed by RNA splicing during maturation of the final RNA product; an exon is any part of a gene that encodes a part of the final mature RNA produced by that gene after introns have been removed by RNA splicing. This process is needed for the typical eukaryotic messenger RNA before it can be used to produce a correct protein through translation. For many eukaryotic introns, splicing is

done in a series of reactions catalyzed by the spliceosome, a complex of small nuclear ribonucleoproteins (snRNPs), but there are also self-splicing introns.

Further reading: Sanford JR, Caceres JF. Pre-mRNA splicing: life at the center of the central dogma. J Cell Sci. 2004;117(Pt 26):6261–3.

Activation of PERK leads to the phosphorylation of eIF2α that is required for the initiation of translation. This factor inhibits global protein synthesis by inhibiting the assembly of the 80S ribosome, thereby reducing ER load and promoting cellular survival.

At the same time and under these conditions, the transcription factor ATF4 is differentially translated, up-regulating genes participating in protein folding, amino acid metabolism and transport, autophagy, and oxidative stress resistance/redox homeostasis. Under conditions of prolonged or severe ER stress that the UPR cannot resolve (see below), ATF4 also contributes to apoptosis through the induction of the transcription factor CHOP (for *cytidine-cytidine-adenosine-adenosine-thymidine-enhancer-binding homologous protein*) and by enhancing oxidative stress and protein synthesis.

Finally, the third branch of the UPR is initiated by ATF6. The sensor ATF6 is retained at the ER under homeostatic conditions but translocates to the Golgi apparatus under ER stress where it is cleaved by the Golgi-resident proteases *site-1 protease* (SP1) and SP2. This event leads to the release of ATF6 N-terminal fragment, a potent transcription factor that moves to the nucleus, where it binds the ER stress response element upstream of a subset of UPR genes to activate their transcription. Together with XBP1, this fragment regulates the expression of several genes with functions in protein folding, protein transport, and lipid biosynthesis, that is, genes involved in re-establishing ER homeostasis.

The Unfolded Protein Response, Crosstalks, and Emission of DAMPs

Strikingly, there is a considerable interconnectedness of the ER stress-promoted UPR with other innate immune processes. For example, an increasing number of studies support the view that oxidative stress has a strong connection with ER stress. During the protein folding process, ROS are produced as by-products, leading to impaired redox balance conferring oxidative stress. As the protein

folding process is dependent on redox homeostasis, the oxidative stress can disrupt the protein folding mechanism and facilitate the production of misfolded proteins, causing further ER stress [163]. Moreover, this stress response functions as a productive source of DAMPs. Typically, HSPs and CALR, like other ER chaperones, can translocate to the cytosol and eventually to the surface of cells. Once exposed, these molecules can operate as Subclass IB-1 DAMPs to facilitate engulfment of antigens as mostly described in the context of induction of ICD [148, 164–168]. Via these mechanisms and by crosstalk with other molecular machines of the innate immune system including NLRP3 inflammasome activation via TXNIP (see Part IV, Sect. 13.4.6.3 and Part VI, Sect. 22.4.2.2), the UPR may contribute to sterile inflammation and immunity (for articles, see Refs [153–157, 169–172]). Also, several signalling cascades triggered by the three sensors are apparently potent inducers of autophagy at a cell-wide level that—as mentioned above—normally has an adaptive/protective function and consists of the three major types: chaperone-mediated, macro- and microautophagy. Interestingly, recent evidence has indicated that UPR-induced autophagic processes are capable of alleviating the UPR pointing to a crosstalk between these two innate immune defense mechanisms [173, 174]. Strikingly, a complex relationship reportedly exists between autophagy and DAMPs in cellular adaption to stress and injury and cell death characterized by a crosstalk between autophagy induction and secretion or release of DAMPs. In fact, growing evidence indicates that autophagic mechanisms are involved in regulating release and degradation of DAMPs including CALR, HMGB1, ATP, and DNA in several cell types [37, 148, 175]. This scenario may contribute to the observation that autophagy is also able to shape a supportive cellular immune response [176, 177]. This kind of innate immune interrelationships is, for example, involved in mechanisms of both reperfusion-mediated cell injury and ICD of cancer cells (see Refs [40, 178–190]).

18.5.2.3 Irremediable Endoplasmic Reticulum Stress

Overall, depending on the duration and intensity of the stress, the UPR engages different cellular pathways to restore and maintain cell survival, on the one hand, or trigger apoptosis, on the other hand. In cases of severe irremediable ER stress, however, the balance is tipped in favor of pro-death signalling; that is, the UPR, now mediated by different biochemical pathways, may lead to pro-inflammatory and pro-apoptotic responses resulting in catastrophic RCD (Fig. 18.6). While the precise pathways of apoptosis induced by ER stress are not known, the up-regulated PERK → eIF2α → ATF4 → CHOP pathway plays an essential role by reversing translational arrest, increasing generation of ROS, and promoting calcium efflux from the ER. Together, these signals lead to cytochrome C release from mitochondria and loss of membrane potential, resulting in apoptosis [191].

In this context, it is interesting that one pathway of RN (ferroptosis; see Sect. 19.3.3) apparently shares a partially overlapping machinery with ER stress, suggesting a molecular interconnectivity between these two events [192].

18.5.2.4 Concluding Remarks

The issue of ER-stress-promoted UPR is a parade example of an injury-induced innate immune response that can decide besides life and death of a cell. Placed at the very beginning of defense processes of multicellular organisms upon injury, this stress response nicely reflects a hierarchy of DAMP emission (see Part IV, Chap. 16). Striking is also the existence of an inter-organelle communication, for example, between UPR, inflammasome activation, and autophagic pathways which emerge as a homeostatic network determining the switch from adaptive life-saving programs to cell death under stress conditions, where specialized sentinels are localized at organelle membranes to induce the core apoptosis pathway. As briefly sketched in the next section, this innate immune defense response is not only directed against sterile stress but also against pathogen-mediated stress.

18.5.3 Virus- and Bacteria-Induced Stress of the Endoplasmic Reticulum

18.5.3.1 General Remarks

In the previous section, induction of a UPR was mainly exemplified by referring to reperfusion-mediated cell damage and ICD of cancer cells, that is, instances of sterile stress. However, growing evidence is coming to light clearly indicating that viruses and bacteria also induce ER stress, thereby activating a robust UPR. In fact, the constitution of cellular stress responses is meanwhile regarded as the first line of defense against both viral and bacterial infection. However, the outcome is not always in favor of the host; the pathogen may also profit from this stress response, at least under certain circumstances. An increasing number of reports have recently been published on this emerging topic (such as Refs [192–197]), the quintessence briefly being addressed here.

18.5.3.2 Virus Infections

There are several mechanisms described of how a virus can induce ER stress. The central mechanisms of perturbation of the ER during virus infection can be seen in the production of large amounts of viral proteins by the virus concerned. Such accumulation of viral proteins in the ER implies a challenge to the protein folding machinery which may cause ER stress and, in turn, activate the UPR resulting in restoration of the ER homeostasis or apoptosis. So far, at least 36 viruses have been found to be able to induce ER stress and activate the three UPR stress signalling pathways [198]. Moreover, ER stress can be caused by viruses via other mechanisms, for example, as a result of ER membrane exploitation, imbalance of calcium concentration, or sabotage/depletion of the ER membrane during virion release.

Viral infections may activate these pathways resulting in the inhibition or promotion of viral replication. For example, the PERK-mediated global translation shutdown is a very efficient antiviral mechanism, and a similar shutdown by PKR has been used in the interferon pathway to defend against viral infection [199]. Also, the virus-related UPR was found to trigger host inflammatory signalling cascade through innate immune signalling pathways that activate NF-κB and AP-1

transcription factors as a result of chronic ER stress. In fact, overexpression of viral proteins in the ER has long been known to activate these transcription factors which induce expression of pro-inflammatory cytokines such as IL-6 [200, 201]. Increasing evidence supports the notion that the UPR signalling synergistically interacts with virus-induced signalling to produce inflammatory cytokines and type I IFNs. In addition, other lines of studies have shed light on a role of NOD1 and NOD2 receptors in transducing virus-related ER stress signals to elicit inflammation [197]. So far, however, a possible contribution of DAMPs to the promotion of these signalling pathways has not been investigated.

Importantly, however, the effects of virus-induced UPR have been observed not only to inhibit but also to potentiate viral infection. In fact, manipulation of the UPR response has become an asset for many viruses to promote their translation, thereby leading to chronic ER stress. In other words, during infection, viruses are capable of hijacking the host translational machinery and fill the ER with viral proteins. For example, this is the case for many positive-strand RNA viruses, which house the virus replication machinery in the protective ER-membrane. In fact, viruses need host ER to produce increased quantities of viral proteins to continue replication.

Intriguingly, as discussed [194], many viruses have evolved strategies aimed at continuing the replication cycle. Thus, viruses were shown to manipulate the host UPR in various ways to stimulate protein synthesis capacity and to improve cell survival by inhibiting cellular apoptosis. In particular, the link between the UPR and autophagy are intensely discussed to be involved in this scenario. These two systems may act dependently, or the induction of one system may interfere with the other [202]. Thus, experimental studies could demonstrate that different viruses modulate these mechanisms to allow them to circumvent and bypass the host immune response or, worse, to exploit the host's defense to their advantage. According to current knowledge, RNA viruses including influenza virus, poliovirus, coxsackievirus, enterovirus, Japanese encephalitis virus, HCV, and dengue virus were shown to regulate these processes. For example, recent studies on HCV-infected hepatocytes confirmed the evidence that virus-associated ER stress and UPR are linked to cellular autophagy. Thus, induction of the cellular autophagic response is reportedly required to improve survival of infected cells by inhibition of cellular apoptosis. Moreover, the autophagic response was demonstrated to inhibit the cellular innate antiviral program that usually inhibits virus replication. Nevertheless, as argued by the authors [194], though HCV induces ER stress and autophagy, their cause–effect relationship is not clear.

18.5.3.3 Bacterial Infections

Notably, the UPR is not only modulated by viruses! Recent evidence indicates that this stress response plays multiple roles during bacterial infections as well. Thus, as comprehensively reviewed by Celli and Tsolis [203], the UPR has been shown to be induced in murine lungs by *M. tuberculosis* (associated with apoptotic events) and also be correlated with *Helicobacter*-induced gastric carcinogenesis. Similarly, in vitro infectious models have revealed UPR induction in macrophages and epithelial cells infected with either *Brucella melitensis* and *B. abortus* or *Listeria monocytogenes*.

On the other hand, there is evidence suggesting that bacteria can subvert the UPR for their own advantage. Nevertheless, indications that bacteria can modulate this response are still somewhat sparse. One example refers to *L. pneumophila* that recruits components of the ERAD on its vacuole to mediate turnover of bacterial effectors on the vacuolar surface and uses the proteasome to generate amino acids necessary for its intracellular growth.

18.5.3.4 Concluding Remarks

Interestingly, like with virus-induced UPR, a bacteria-initiated UPR may turn out to be of advantage for the host or in favor of the bacteria, but it is not clear in each case whether this response benefits the host or the pathogen. Thus, as argued by Celli and Tsolis [203], "*modulation of ER function during infection by intracellular bacteria can promote bacterial infection by providing a replicative niche, but at the same time the resulting disruption of the secretory pathway can provide a pattern of pathogenesis that aids the innate immune system in recognizing intracellular infection and in mounting an appropriate defence. However, considering the more rapid evolution of bacterial pathogens compared to their hosts, it is likely that bacteria have evolved to modulate the UPR to their advantage during infection*."

18.5.4 Résumé

The function of the ER stress-induced UPR appears to provide another impressive evidence in support of the notion that immunity is induced by both sterile and infectious stressful injury and not primarily by invading nonself. In fact, this stress response is at the forefront of any stressful injury and is dedicated to initiating involvement of further DAMP-promoted defense mechanisms. However, like all the other innate immune processes, the UPR may become uncontrolled. Together, this may be reason enough that this stress response is involved in the pathogenesis of many human systemic and organ-specific disorders. In fact, the list of human diseases that are pathogenetically associated with ER stress and activation of the UPR is steadily growing [204–206]. They include neurodegenerative and metabolic diseases, autoimmune disorders, and atherosclerosis. Given such an ever-increasing list of diseases, it is not a surprise that chemical compounds and inhibitors targeting the UPR signalling pathways with a high degree of specificity have been and will be further developed [207–209].

18.6 The DNA Damage Response

18.6.1 Introductory Remarks

Maintaining genome integrity and transmission of intact genomes is a condition sine qua non for cellular, organismal, and species survival [210]. This homeostatic integrity is threatened by DNA damage that occurs in a variety of conditions

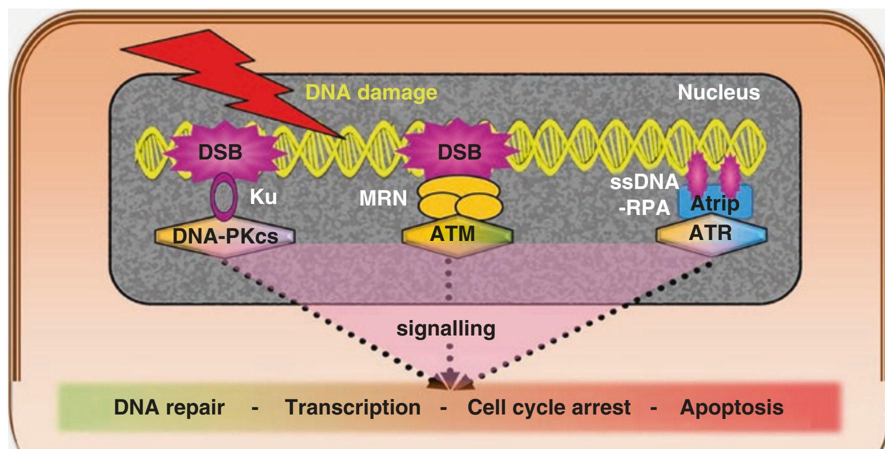

Fig. 18.7 Simplified scenario model illustrating the DNA damage response. In response to DNA damage, the three phosphoinositide 3-kinase-related kinases are recruited and activated. DNA-PKcs is recruited and activated by Ku-bound DSB ends; ATM is recruited and activated by DSBs through the MRN complex; ATR is recruited to RPA-coated ssDNA by its stable binding partner ATRIP. The cellular response to damage may involve activation of a cell cycle checkpoint, commencement of transcriptional programs, execution of DNA repair, or, when the damage is too severe, initiation of apoptosis. Upon sensing DNA damage, cell cycle checkpoints are activated to arrest cell cycle progression to allow time for repair before the damage is passed on to the cell. *ATM* ataxia telangiectasia mutated, *ATR* ataxia telangiectasia, and Rad3-related, *DNA-PKcs* DNA protein kinase catalytic subunit, *DSB* double-strand DNA breaks, *Ku* Ku70–Ku80 sensor, *MRN* Mre11–RAD50–NBS1 complex, *ssDNA-RPA* replication protein A-coated single-strand DNA. Sources: Refs. [220–229]

including but not limited to ionizing radiation, chemical reactions, and viral infections, two of the most dominant conditions being oxidative stress and replication stress. These exogenous and endogenous factors induce diverse lesions in the DNA such as nucleotide alterations (substitution, deletion, and insertion), bulky adducts, collapsed DNA replication forks, SSBs, and DSBs (see Refs [57, 211–219]). In response to DNA damage, cells initiate and activate a complex network of cellular signalling cascades that cooperate to sense and repair lesions in DNA, denoted as the DDR. This stress response plays an important role in fighting against detrimental effects of cell stress and injury. It orchestrates many processes, including not only DNA repair but also regulation of cell-cycle checkpoints, transcription of DDR genes, and autophagy (Fig. 18.7).

18.6.2 Role of Phosphoinositide 3-Kinase-Related Kinases

The DDR is controlled by three PI3K-related kinases (PIKKs): the ATM, the DNA-PK, and the ATR kinases (mostly nuclear proteins). All three PIKKs are enormous polypeptides with similar domain organizations and various common structural features. Equipped with the capability to autophosphorylate (see above

explanation), they govern this complex network of effector pathways promoting DNA repair, checkpoint activation, transient cell cycle arrest, cellular senescence, or apoptosis [220–226].

18.6.3 Potential Sensors of DNA Damage

However, at the beginning of this dance, sensors have recently been identified which, as PRMs in a wider sense or even as a new group of recognition receptors, are capable of detecting DNA damage—as manifested by the toxic DSBs or generation of ssDNA to activate these three PIKKs. As already briefly touched in Part II, Sect. 5.2.6.4, two highly conserved multiprotein complexes, MRN and Ku, are considered the primary sensors of DSBs to subsequently activate ATM and DNA-PK [221, 227, 228]. In addition, ssDNA is sensed by the recognition molecule RPA that then, analogous to Ku, acts as a signalling and repair platform for downstream factors such as the PRP19, an E3 ubiquitin ligase involved in pre-mRNA splicing, and ATR kinase [229]. Other lines of studies have provided evidence suggesting that the protein PRP19 itself may act as the primary sensor of RPA-ssDNA to subsequently activate ATR [224] (Fig. 18.7).

In brief, upon DNA damage, DSBs are recognized by the MRN complex. Following recognition, MRN recruits ATM to this DNA lesion where it binds to the C-terminus of NBS1 as a component of MRN [230]. Following binding, ATM kinase is activated. However, the exact mechanism whereby MRN activates ATM is still not fully understood (discussed in [226]). Recognition of DSBs is also realized by Ku70/80 heterodimer that has been shown to bind broken dsDNA ends preferentially [231]. It is the Ku70/80 then that recruits the catalytic subunit of DNA-PK (DNA-PKcs) at the site of DSBs to form the DNA-PK holoenzyme. The major role of activated DNA-PK is to promote a peculiar DNA repair mechanism called *non-homologous end joining* (NHEJ), a pathway that repairs double-strand breaks in DNA [231]. In fact, NHEJ repairs most DSBs in mammalian cells. As its name implies, NHEJ involves ligation of two broken DNA ends without needing a repair template [232].

In contrast to ATM and DNA-PKcs which respond primarily to DSBs, ATR is activated by a much wider range of genotoxic stresses, for example, reflected by exposure of increasing amounts of ssDNA as a consequence of compromised activity of replication proteins or nucleolytic processing of various forms of damaged DNA. In fact, first evidence suggests that ssDNA is initially sensed by RPA which then recruits and activates PRP19 [229]. In turn, PRP19 facilitates the accumulation of *ATR-interacting protein* (ATRIP, the regulatory partner of the ATR kinase) at DNA damage sites, thereby activating ATR [233, 234].

Plausibly, the recent discovery of DNA damage-sensing molecules such as MRN, Ku70/80, and RPA calls for the definition of those molecules they recognize, that is, broken DNA ends at the site of DSBs and ssDNA. As outlined in Part IV, Sect. 13.4.2, they have been tentatively sorted into a subclass of cell-intrinsically emitted DAMPs (Subclass IIC-1). Future studies will have to assign their exact place in the world of DAMPs.

18.6.4 Generation of DAMPs in the Course of the DNA Damage Response

Apart from those DAMPs emitted in the nucleus, other lines of studies lend support to the suggestion that DNA damage or DNA replication stress, in case of unsuccessful DNA repair, promotes release of aberrant DNA structures into the cytosol in the form of ssDNA and dsDNA breaks/fragments which operate as cell-intrinsically emitted dislocated DAMPs. They are sensed by the DNA receptor cGAS and probably other as-yet-not-identified DNA receptors to activate STING-dependent pathways to promote defense pathways [235, 236] (compare Part II, Sect. 5.2.6; Part IV, Sect. 13.4.3; as well as Part VI, Sect. 22.3.7). As discussed by the authors [236], it is conceivable that cytosolic DNA is released by dysfunctional mitochondria upon DNA damage or generated during repair of damaged genomic DNA.

Moreover, as shown in studies on tumor models, the DDR—through the activation of STING-mediated pathways—induces the expression of another class of constitutive DAMPs which are exposed at the cell surface, namely, the MICs and different ULBPs [236–240] (compare Part IV, Sect. 12.3.3).

Of note, however, the DDR does not always result in a happy end. In fact, when unsuccessful in repairing DNA damage, the DDR can lead to cellular senescence or—like a UPR in case of irremediable ER stress—ultimately induces an RCD, most often in the form of apoptosis, less frequently in the form of necrosis. Such subroutines of RCD are presumably aimed at mitigating the propagation of potentially mutated cells leading to cancer or other age-related pathologies (Fig. 18.7) [222, 223, 241].

18.6.5 Résumé

The DNA damage response must be regarded as an efficient cell-intrinsic defense process, which is sophistically connected with other stress responses such as autophagy that also plays a significant role in maintaining genome stability [242] and the ER stress-induced UPR [243]. Also, of particular interest is the observation that three tiers of DAMPs are involved in this pathway, starting with broken DNA ends at the site of DSBs and ssDNA in the nucleus immediately generated upon DNA damage; followed by DNA fragments dislocated in the cytosol and operating as class IIC-2 DAMPs to promote intracellular innate immune signalling; and finishing with the exposure of Subclass IB-2 DAMPs (e.g., MICs) able to activate NK, NKT, and $\gamma\delta$ T cells (compare Part VII, Sects. 27.2.2, 28.2.2 and 28.4.2). This phenomenon may again reflect a particular hierarchy in the work of DAMPs to maintain homeostasis.

In fact, genomic integrity is of utmost importance and key to human health, and this is retained by the DDR. This stress response, however, may fail in maintaining and restoring homeostasis. Accordingly, DNA damage has been observed to play a causal role in numerous human pathologies associated with genome instability or aberrant PIKK function such as cancer, leukemia, premature ageing, and certain

chronic inflammatory conditions. Furthermore, there is growing evidence suggesting that uncontrolled DDR signalling is associated with various neurodegenerative diseases, as documented by recent work showing that ATM inhibition alleviates pathologies in models of Huntington's disease [244]. This is reason enough that DDR pathways have been and are being explored therapeutically to induce freedom from these diseases. In particular, small molecule inhibitors of ATM, DNA-PK, and ATR are regarded as potential therapeutic agents, for example, as innovative drugs in cancer treatment [245, 246].

18.7 Outlook

An increasing amount of publications in the international literature provides convincing evidence that cell-intrinsic, cell-autonomous stress responses are initiated by any damage to a cell, regardless of being of sterile or infectious in nature. The ultimate goal of those responses, which are all highly conserved among eukaryotic species, is to maintain cellular homeostasis and ensure cell integrity. Again this knowledge is in support of the concept that any form of host defense is primarily directed against injury and not against microbes. Autophagy in terms of "immunological autophagy" appears to act as the center of all stress responses that is committed to control and regulate efferent innate and potential adaptive immune responses. The "medium" for achieving this goal is the MAMPs and/or DAMPs which operate as a link between intracellular and extracellular events.

Another characteristic feature of cell-intrinsic stress responses is their interconnectivity and interdependency. A typical example is represented by oxidative stress and ER stress responses, which appear to act mutually in any form of cellular damage. Strikingly, this kind of interconnectedness is not restricted to a collaboration between the stress responses but expands to the elicitation of innate and adaptive immune processes. Again, the DAMPs—in terms of a particular hierarchy in their emission –appear to take center stage in this scenario. A better understanding of these multiple connections between cell-autonomous processes, on the one hand, and innate/adaptive immune responses, on the other hand, will undoubtedly stimulate new approaches to therapeutic interference with injury-induced pathologies.

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