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Review

Proteolytic control of mitochondrial function and morphogenesis ☆

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ABSTRACT

Mitochondrial proteostasis depends on a hierarchical system of tightly controlled quality surveillance mechanisms. Proteases within mitochondria take center stage in this network. They eliminate misfolded and damaged proteins and ensure the biogenesis and morphogenesis of mitochondria by processing or degrading short-lived regulatory proteins. Mitochondrial gene expression, the mitochondrial phospholipid metabolism and the fusion of mitochondrial membranes are under proteolytic control. Furthermore, in response to stress and mitochondrial dysfunction, proteolysis inhibits fusion and facilitates mitophagy and apoptosis. Defining these versatile activities of mitochondrial proteases will be pivotal for understanding the pathogenesis of various neurodegenerative disorders associated with defective mitochondria-associated proteolysis. This article is part of a Special Issue entitled: Mitochondrial dynamics and physiology.

1. Introduction

Mitochondria are essential organelles housing the respiratory chain complexes, which generate most of the cellular energy. They are the sites of many biosynthetic events, buffer cellular calcium and play an integral role in numerous cellular signaling pathways including programmed cell death [1–4]. Dysfunction of mitochondria is detrimental to cellular viability, has been linked to many diseases, and is associated with aging [5,6].

Recent proteomics and bioinformatics approaches defined a comprehensive inventory of mitochondrial proteins [7,8]. Numerous cellular pathways are emerging that maintain the mitochondrial proteome, ensuring a healthy and functional mitochondrial network. These pathways counteract the many challenges that mitochondria face and that may influence their well-being. The first challenge arises from the unique property of the organelle in that their proteome is encoded by two distinct genomes. While the vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized in the cytosol, mitochondria also harbor their own genome, encoding for 13 polypeptides in mammalian cells (8 in yeast). All mitochondrial-encoded proteins constitute core components of the respiratory chain complexes. In order to achieve efficient respiratory chain complex assembly, it is imperative that there is coordinated expression and import of nuclear-encoded proteins to assemble with the mitochondrial-encoded proteins. Failure to coordinate this process may result in unpartnered proteins that are prone to misfolding or aggregation. The second challenge imposed upon mitochondria arises due to production of reactive oxygen species (ROS), which is an inevitable byproduct of the generation of ATP through oxidative phosphorylation. ROS can react with proteins, DNA and lipids resulting in accumulation of oxidatively damaged products. Hence, mitochondrial proteins, lipids and DNA are more prone to oxidative damage, which can lead to depolarization of the mitochondrial inner membrane. This may ultimately trigger the permeabilization of the outer membrane, the release of pro-apoptotic proteins from the intermembrane space into the cytosol and the initiation of the apoptotic cascade [9,10].

2. Mitochondrial quality control at a glance

In order to maintain a functional mitochondrial network, elaborate quality control mechanisms have evolved that remove damaged proteins or sequester and remove damaged organelles (Fig. 1) [11–15]. Impaired quality control of mitochondria is detrimental to cell health and has been linked to aging and various diseases, including prevalent neurological disorders like Parkinson's disease and spinocerebellar ataxia, highlighting the relevance of these defense pathways for cellular homeostasis and survival [16].

Mitochondrial proteases and the cytosolic ubiquitin–proteasome system (UPS) comprise the first line of cellular defense by facilitating the removal of damaged, oxidized or misfolded mitochondrial proteins (Figs. 1A and 2). Two membrane-bound AAA protease complexes conduct quality control surveillance across the mitochondrial

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inner membrane [17]. The *m*-AAA protease exposes its catalytic domain towards the matrix and the *i*-AAA protease towards the intermembrane space. Additional peptidases, such as the metallopeptidase OMA1, extend the capacity of this proteolytic system and contribute to the quality control of inner membrane proteins [18,19]. The Lon protease (Pim1 in yeast) eliminates denatured or oxidatively damaged proteins from the mitochondrial matrix [20–22]. Substrates of another matrix-localized peptidase, the ClpXP protease, remain to be identified. ClpXP from *Caenorhabditis elegans* is involved in the mitochondrial unfolded protein response (mtUPR), a compartment-specific stress response, regulating the levels of mitochondrial proteases and molecular chaperones to accommodate the unfolded protein load [23–25]. In addition to proteases localized within mitochondria, it is becoming clear that the cytosolic UPS also contributes to mitochondrial quality control (Fig. 2). Mitochondrial proteins that become mistargeted or misfolded *en route* to mitochondria are recognized and removed by the UPS [26,27]. Moreover, the UPS degrades proteins residing in the outer membrane of mitochondria in a process resembling ER-associated protein degradation (ERAD) and is therefore referred to as OMMAD (Fig. 2) (for outer mitochondrial membrane-associated degradation) [26,28].

Mitochondria are highly dynamic organelles, constantly undergoing fission and fusion events. This property of mitochondria offers a second line of defense against mitochondrial dysfunction (Fig. 1B) [29,30]. Mitochondrial fusion allows content mixing helping organelles deficient in certain components to replenish their stores (Fig. 1Bii) [31–33]. Consistently, mtDNA mutations accumulate upon inhibition of fusion, finally triggering the loss of the mitochondrial genome [32]. Moreover, the mitochondrial network becomes hyper-fused under certain stress conditions, for instance upon starvation, following inhibition of autophagy or protein synthesis and under oxidative stress conditions (Fig. 1Bii) [34–36]. This protects mitochondria against autophagy and increases cellular ATP production [35,36]. However, severe mitochondrial damage can trigger loss of the membrane potential across the mitochondrial inner membrane, which impairs mitochondrial fusion. Ongoing fission events then result in fragmentation of the mitochondrial network, a prerequisite for the mitochondria-specific form of autophagy [37], termed mitophagy [38], which represents another important form of mitochondrial quality control (Fig. 1Bi). Therefore, the fragmentation of mitochondria facilitates the segregation of damaged organelles from the healthy network and their removal by mitophagy. Moreover, fragmentation of the mitochondrial network precedes programmed cell death [28,39,40]. Although the precise reason that mitochondria fragment during apoptosis remains undefined, perhaps this facilitates the release of pro-apoptotic components ensuring turnover of cells unable to cope with high levels of mitochondrial dysfunction.

Recently, mitochondrial-derived vesicles (MDVs) were reported to facilitate mitochondrial quality control by delivery of selective mitochondrial cargo to lysosomes (Fig. 1Biii) [41–43]. These MDVs are generated as an early response to oxidative stress [41]. Their formation and delivery to lysosomes does not require mitochondrial depolarization or Drp1-mediated mitochondrial fission and is independent of the general autophagy machinery [41]. These findings suggest a quality control route parallel to mitophagy for the selective degradation of mitochondrial proteins in lysosomes.

While the complexity of mitochondrial quality control mechanisms is unfolding, it is becoming increasingly clear that mitochondrial proteases take center stage in these pathways. In addition to their conventional role in degrading damaged or misfolded mitochondrial proteins, many proteases have additional functions as processing peptidases and control the stability of proteins that regulate crucial steps during mitochondrial biogenesis, morphogenesis and turnover (Fig. 3). In this review, we describe how proteolysis can influence various mitochondrial functions and can impinge on mitochondrial quality control.

3. Proteolytic control of mitochondrial biogenesis

3.1. Lon protease regulates mitochondrial transcription

Lon is an ATP-dependent serine protease found in the mitochondrial matrix that mediates the degradation of misfolded and oxidatively damaged proteins [20–22]. It has also been recently illustrated that Lon plays a pivotal role in the regulation of mitochondrial gene expression [44,45]. In yeast, Lon/Pim1 is required for the expression of intron-containing genes encoded by mitochondrial DNA (mtDNA), including cytochrome *c* oxidase subunit 1 and cytochrome *b* [46]. In *Drosophila* cells, depletion of Lon increases the protein levels of mitochondrial transcription factor A (TFAM) and mtDNA copy number, resulting in enhanced transcription of mtDNA-encoded genes [44,47]. When the

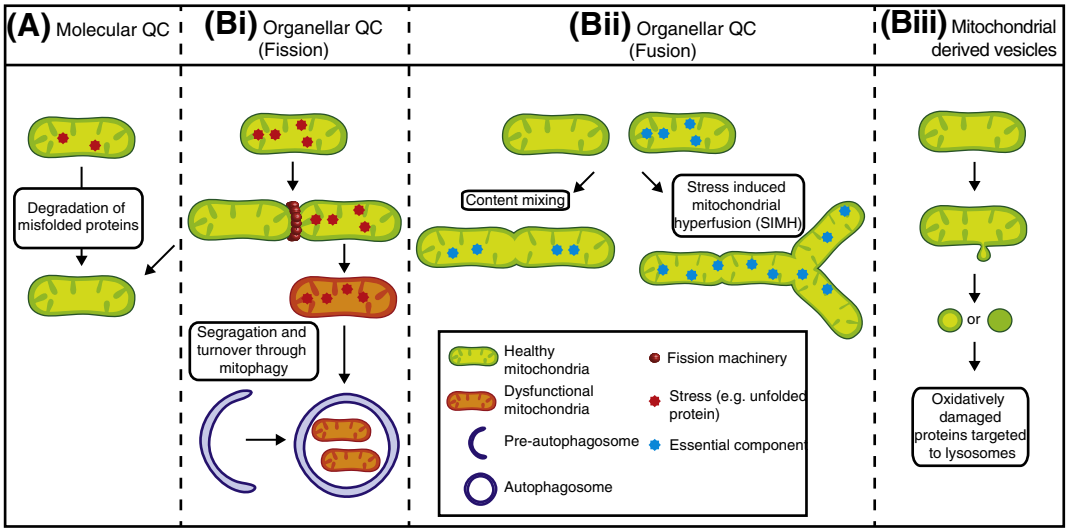

Fig. 1. Mechanisms of mitochondrial quality control. (A) Mitochondrial proteases and chaperones localized in different subcompartments of mitochondria degrade misfolded and damaged proteins. (B) Fusion and fission of mitochondria contribute to mitochondrial quality surveillance. (Bi) Mitochondrial dysfunction inhibits fusion and ongoing fission events segregate non-functional mitochondria from the healthy mitochondrial network. During mitophagy, autophagosomal membranes encapsulate dysfunctional mitochondria and fuse subsequently with lysosomes. (Bii) Mitochondrial fusion serves as a pro-survival mechanism. Content mixing allows complementing functional deficiencies within the mitochondria network. Stress conditions induce fusion (stress-induced mitochondrial hyperfusion; SIMH), resulting in elongation of mitochondrial tubules and protection against mitophagy. (Biii) Various populations of mitochondrial-derived vesicles (MDVs) containing selective cargo are budded from mitochondria during oxidative stress and targeted to lysosomes for degradation. QC, quality control.

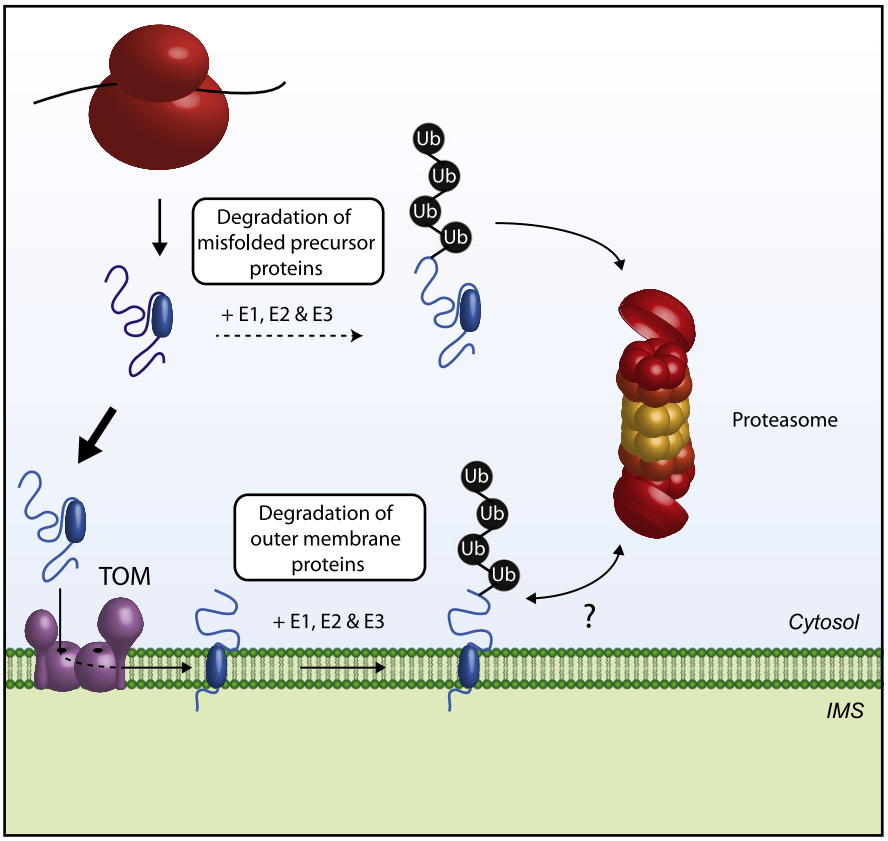

Fig. 2. The cytosolic UPS contributes to mitochondrial quality control. The vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes. Newly synthesized proteins are either efficiently imported into mitochondria (bold line) or degraded by the UPS if they are mistargeted or misfolded prior to import (dashed line). Proteins present in the outer mitochondrial (OM) membrane can be ubiquitylated (Ub) and degraded by a cascade of E1, E2 and E3 enzymes. The proteolysis of OM proteins shares similarities with the turnover of ER membrane proteins but the detailed mechanism remains to be elucidated (indicated by ?). TOM, protein translocase of the outer membrane; IMS, intermembrane space.

mtDNA copy number is low, Lon degrades TFAM to maintain the TFAM:mtDNA ratio and to control the rate of mitochondrial transcription. As a corollary, TFAM is not degraded upon depletion of mtDNA in cells lacking Lon, resulting in increased TFAM:mtDNA ratio and inhibition of mtDNA transcription [47]. The cellular levels of TFAM vary in concert with Lon levels, suggesting that Lon-mediated degradation of TFAM levels may adjust mtDNA levels and concomitantly mtDNA transcription under physiological conditions [44,45,47]. It remains to be determined whether this regulatory circuit explains early observations that Lon can bind both DNA and RNA *in vitro* [48,49].

3.2. The m-AAA protease controls mitochondrial translation

Another protease regulating mitochondrial gene expression is the *m*-AAA protease, a central component of the protein quality control system in the mitochondrial inner membrane. The *m*-AAA protease consists of six subunits, each harboring an ATPase domain and a proteolytic domain. Mutations in subunits of the *m*-AAA protease, AFG3L2 and paraplegin, are associated with various neurological disorders, including spinocerebellar ataxia, hereditary spastic paraplegia and a spastic ataxia neuropathy syndrome [50–52]. The *m*-AAA protease degrades misfolded and non-assembled proteins that are integrated or peripherally associated with the inner membrane. Moreover, the yeast *m*-AAA protease serves as a processing peptidase mediating the maturation of newly imported MrpL32, a nuclear-encoded subunit of the large mitochondrial ribosome [53,54]. Yeast cells lacking the *m*-AAA protease accumulate the precursor form of MrpL32, which does not assemble into mitochondrial ribosomes, inhibiting mitochondrial translation. The dual activity of the *m*-AAA protease as a processing peptidase and quality control enzyme degrading misfolded polypeptides to peptides raises the intriguing question why the *m*-AAA protease does not completely degrade particular substrates. In the case of MrpL32, the *m*-AAA protease initiates proteolysis from the N-terminus, however stable folding of a cysteine-rich domain prevents further degradation after the maturation site [53]. Mutations in MrpL32 that destabilize the tightly folded domain result in the complete degradation of MrpL32 by the *m*-AAA protease.

MrpL32 processing by the *m*-AAA protease and its requirement for the synthesis of mitochondrially encoded respiratory chain subunits explains the respiratory deficiency of yeast cells lacking the *m*-AAA protease. Indeed, targeting MrpL32 to mitochondria by a heterologous presequence that is cleaved off by the general mitochondrial processing peptidase restores respiratory growth of *m*-AAA protease deficient yeast cells, demonstrating that MrpL32 processing represents the central function of the *m*-AAA protease in yeast mitochondria [54]. Notably, MrpL32 maturation and mitochondrial translation was impaired in liver mitochondria of a mouse model for hereditary spastic paraplegia caused by the loss of the *m*-AAA protease subunit paraplegin, suggesting that impaired mitochondrial translation may contribute to the pathogenesis of this neurodegenerative disorder [54,55].

3.3. Mitochondrial proteases regulate the phospholipid metabolism

The functional integrity of mitochondrial membranes depends on the coordinated supply of proteins and membrane lipids. These lipids often exert specific functions in mitochondrial biogenesis [3]. For instance, the dimeric glycerophospholipid cardiolipin (CL), the signature

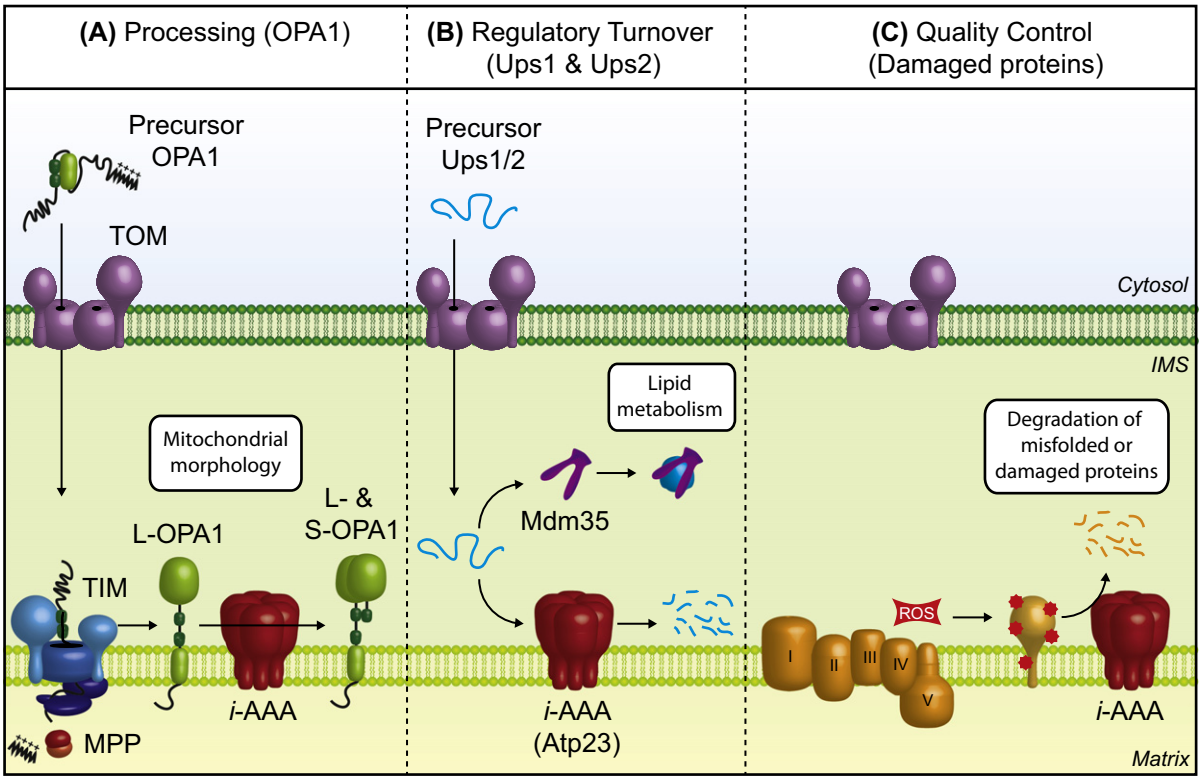

Fig. 3. Versatile activities of *i*-AAA proteases. ATP-dependent proteases within mitochondria have versatile activities as exemplified by the *i*-AAA protease. (A) *i*-AAA proteases regulate the mitochondrial morphology by proteolytic processing of the dynamin-like GTPase OPA1 in the mitochondrial inner membrane. After import of newly synthesized OPA1 precursor proteins via the TOM and TIM translocases, the mitochondrial targeting sequence of OPA1 is cleaved off by the mitochondrial processing peptidase (MPP) generating long OPA1 forms (L-OPA1). Approximately half of the L-OPA1 molecules are converted into short OPA1 forms (S-OPA1) by proteolytic processing at site S1 by OMA1 and at site S2 by the *i*-AAA protease. Both L- and S-OPA1 are required for mitochondrial fusion. (B) *i*-AAA proteases determine the stability and completely degrade Ups1 and Ups2, which regulate the accumulation of cardiolipin and phosphatidylethanolamine, respectively. Ups1/2 are unstable proteins that are degraded by the *i*-AAA protease if they do not assemble with Mdm35 in the intermembrane space. (C) *i*-AAA proteases completely degrade misfolded or damaged proteins to peptides. TOM, protein translocase of the outer membrane; TIM, protein translocase of the inner membrane; IMS, intermembrane space; MPP, mitochondrial processing peptidase.

lipid of mitochondrial membranes, is required for the stability of the mitochondrial genome, the respiratory chain complexes and the protein translocases. Furthermore, CL also affects mitochondrial fusion and the activation of apoptosis [3,56–58]. Mutations in tafazzin, a mitochondrial transacylase for CL synthesis, are associated with Barth syndrome highlighting the importance of CL in maintaining mitochondrial functions [59–61].

Increasing evidence points to a central role of proteases for the maintenance of the lipid composition of mitochondrial membranes. A conserved regulatory circuit in the intermembrane space determines the levels of CL and of phosphatidylethanolamine (PE) in the mitochondrial membranes [62]. Central components of this regulon are Ups1 and Ups2, members of the conserved family of Ups1/PRELI-like proteins [63,64], which ensure the accumulation of CL and PE, respectively (Fig. 3B) [65,66]. Both proteins are intrinsically unstable and subjected to turnover if they do not assemble with Mdm35 in the intermembrane space [66]. The *i*-AAA protease, a homo-oligomeric proteolytic complex composed of Yme1 subunits [67], degrades both Ups1 and Ups2, while the stability of Ups1 is additionally affected by the inner membrane peptidase Atp23 [62]. Moreover, the *i*-AAA protease was found to determine the stability of phosphatidylserine decarboxylase 1 (Psd1) [68], which mediates the conversion of phosphatidylserine into PE in the inner membrane. The *i*-AAA protease thus appears to affect the composition of mitochondrial membranes in multiple ways.

The Lon protease affects the biosynthesis of steroid hormones in cells of the adrenal cortex, placenta and gonads, as it degrades the steroidogenic acute regulatory protein (StAR) [69,70]. StAR facilitates the transfer of cholesterol from the outer to the inner mitochondrial membrane, making it available for steroid hormone synthesis [71]. StAR is thought to release cholesterol to the outer membrane and is subsequently degraded rapidly upon import into the mitochondrial matrix. Lon has been identified as the primary peptidase degrading StAR [69,70,72]. It is conceivable that the release of its substrate impairs refolding of StAR in the matrix, which is degraded as a misfolded polypeptide by Lon. Alternatively, Lon might specifically recognize StAR and initiates proteolysis at hydrophobic amino acids that are surface exposed and situated within a highly charged environment [73].

3.4. Presequence processing regulates mitochondrial protein stability

The majority of matrix-localized proteins contain an N-terminal, amphipathic presequence that is enriched in positively charged amino acids and ensures correct targeting [74–76]. Most of these presequences are cleaved off by mitochondrial processing peptidases, MPP, upon import into mitochondria and degraded [77]. Some mitochondrial preproteins are further processed by additional peptidases. Icp55 (Intermediate cleaving peptidase 55) cleaves a single amino acid from a precursor protein, whereas Oct1 (Octapeptidyl aminopeptidase 1) has been reported to remove an octapeptide [78–80]. Recent findings showed an unexpected link of these processing events to a protein's half-life [78–80]. Icp55 and Oct1 act specifically on those intermediates, which expose N-terminal amino acids upon processing by MPP that are destabilizing according to the N-end rule [78–80]. The N-end rule has been originally defined for bacterial proteins and links the half-life of a protein to the type of the N-terminal amino acid [81,82]. Accordingly, processing by Icp55 and Oct1 was found to increase the half-life of

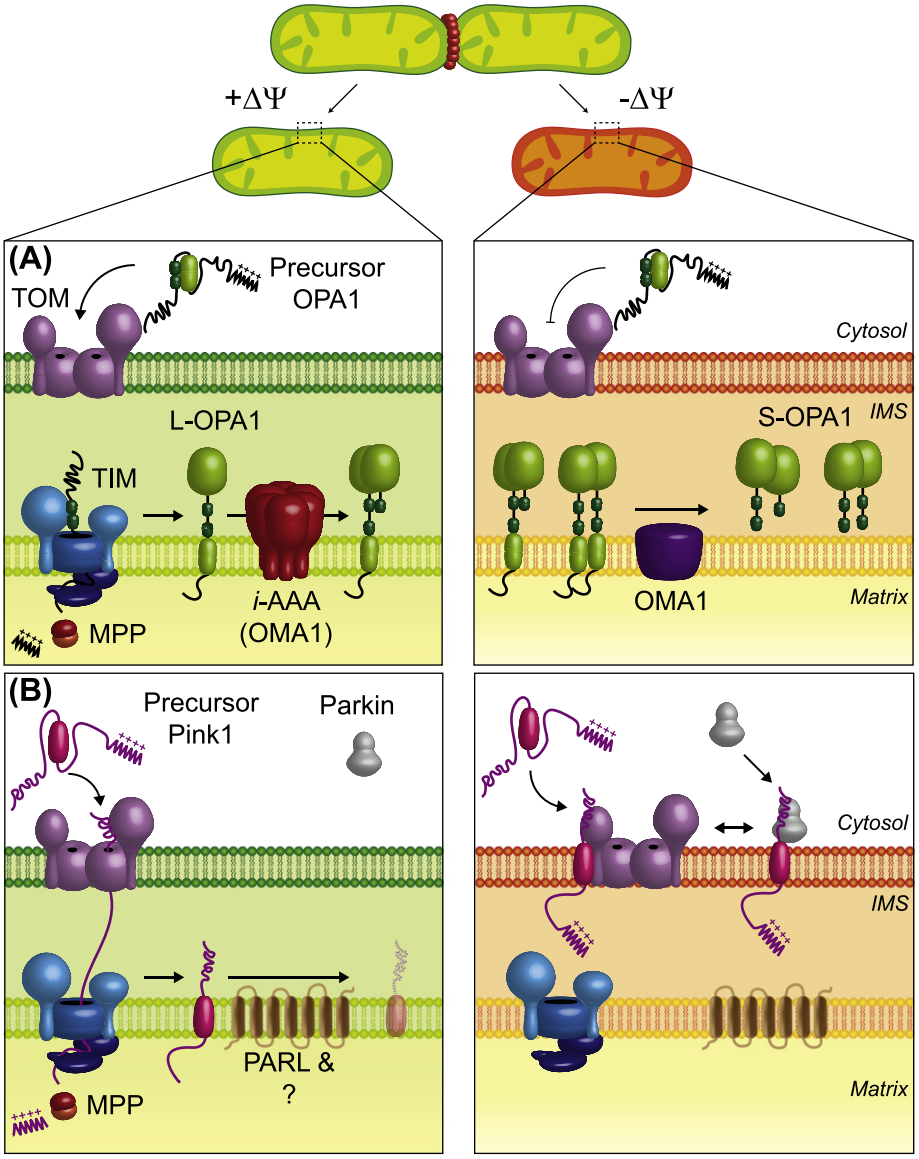

Fig. 4. Proteolytic control of mitochondrial morphogenesis and turnover. (A) Mitochondrial fusion depends on the balanced formation of L- and S-OPA1. In healthy mitochondria, approximately half of L-OPA1 molecules are converted to S-OPA1 by proteolytic processing. A fraction of L-OPA1 is processed at S2 site by the *i*-AAA protease and at the S1 site by OMA1 (constitutive cleavage). Stress conditions or mitochondrial dysfunction (e.g. dissipation of $\Delta\psi$) activate OMA1 which mediates the complete conversion of L-OPA1 to S-OPA1 and inhibits fusion. (B) Regulation of mitophagy by PINK1. Newly synthesized PINK1 is transported across the outer membrane via the TOM complex and inserted in the inner membrane in a $\Delta\psi$-dependent manner. Upon import, the mitochondrial processing peptidase cleaves off the targeting sequence. Subsequent processing of PINK1 by the rhomboid protease PARL generates a 52 kDa fragment that is further degraded by a MG132-sensitive, yet to be identified protease. Mitochondrial depolarization inhibits PINK1 import and degradation but does not interfere with the targeting of newly synthesized PINK1 molecules to mitochondria. PINK1 accumulates on the OM membrane and associates with the TOM complex. PINK1 present at the mitochondrial surface can recruit Parkin and initiate mitophagy. TOM, protein translocase of the outer membrane; TIM, protein translocase of the inner membrane; IMS, intermembrane space; $\Delta\psi$, membrane potential; MPP, mitochondrial processing peptidase.

newly imported proteins [78–80], thus representing a novel quality control mechanism for newly imported proteins.

3.5. Regulation of mitochondrial dynamics by proteolysis

Mitochondria form reticular networks, which are maintained by fusion and fission events mediated by conserved dynamin-like GTPases in both the mitochondrial inner and outer membrane [29,30]. Outer membrane fusion depends on mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), while OPA1 (Mgm1 in yeast) is required for the fusion of the inner membrane. The dynamin related protein 1 (Drp1) (Dnm1 in yeast) mediates mitochondrial fission. It has been well documented that the stability and activity of these dynamin-like GTPases is tightly controlled by proteolysis.

3.6. Ubiquitylation determines the accumulation of mitofusins

Studies in yeast and mammalian cells revealed that proteolysis regulates the steady state protein level of mitofusins, allowing fusion to proceed. In yeast, the mitofusin Fzo1 interacts with Mdm30, an F-box protein associated with the Skp1-Cdc53-Fbox (SCF) ubiquitin ligase complex [83–87]. Mdm30 regulates mitochondrial fusion by mediating Fzo1 ubiquitylation and degradation in an F-box-dependent manner. Fzo1 ubiquitylation and degradation occurs only after trans tethering and GTP hydrolysis and ongoing Mdm30-dependent degradation of Fzo1 is important for outer mitochondrial membrane fusion [83,84]. Ubiquitylation and clearance of Fzo1 might overcome the steric hindrance imposed by a tethering structure to facilitate membrane closeness for efficient fusion of the two membranes.

Mammalian mitofusins are ubiquitylated by the E3 ubiquitin ligases Parkin (see below) and March5/MITOL. Depletion of March5/MITOL that ubiquitylates Mfn1 induces highly interconnected and elongated mitochondria and increased senescence [88–91]. The significantly increased level of Mfn1 (but not of Mfn2) in cells depleted of March5/MITOL suggests Mfn1 is a target of March5/MITOL-dependent proteasomal degradation [90]. Consistently, expression of a GTPase deficient mutant of Mfn1 in March5/MITOL-depleted cells can restore a reticular mitochondrial morphology and reverts cellular senescence phenotypes [90]. However, the physiological role of March5/MITOL-mediated degradation of Mfn1 remains elusive. As March5/MITOL also affects trafficking of Drp1, it is possible that it serves as an upstream regulator of mitochondrial dynamics [88,90].

3.7. Regulation of Drp1 and mitochondrial fission by proteolysis

The cytosolic dynamin-like GTPase Drp1 mediates mitochondrial fission. Drp1 translocates to the sites of mitochondrial fission [92,93], where it self-assembles into ring-shaped oligomers around the mitochondrial constriction site and uses energy from GTP hydrolysis to divide mitochondria [94]. The steady state levels of Drp1 and its activity is under the control of various posttranslational modifications, including ubiquitylation, sumoylation, nitrosylation and phosphorylation [95].

The E3 ubiquitin ligase March5/MITOL ubiquitylates Drp1 [89,91]. However, unlike Mfn1, ubiquitylation of Drp1 does not trigger its degradation but rather regulates its activity [90]. Although, the levels of Drp1 remain unchanged upon downregulation or overexpression of March5/MITOL, the trafficking of Drp1 to mitochondria is affected upon overexpression of mutant variant of March5/MITOL harboring a mutation in the E3-RING domain [88]. The mechanism how March5/MITOL affects Drp1 remains to be elucidated and it is possible that March5/MITOL might regulate degradation of another mitochondrial protein that in turn affects Drp1 trafficking.

Drp1 is also a substrate of the mitochondrial E3 SUMO ligase MAPL [96]. Fluorescently tagged SUMO1 (YFP:SUMO) co-localizes with endogenous Drp1, and is found at sites of mitochondrial fission and at the tip of fragmented mitochondria [97]. Overexpression of MAPL or downregulation of the SUMO protease SENP5 results in hypersumoylation of Drp1 and mitochondrial fragmentation [96,98]. Depletion of MAPL, on the other hand, leads to reduced levels of Drp1 suggesting that sumoylation protects Drp1 against degradation [96,97]. However, mitochondrial morphology is not grossly affected under these conditions, suggesting that MAPL is not essential for mitochondrial fission. It is conceivable that MAPL equally affects both fission and fusion, as its downregulation also results in reduced rates of fusion [96].

3.8. Processing of OPA1 by mitochondrial proteases

The fusion of the mitochondrial inner membrane and cristae morphogenesis depends on the dynamin-like GTPase OPA1 (Mgm1 in yeast), which is anchored to the inner membrane by a single transmembrane domain with the bulk of the protein facing the mitochondrial intermembrane space [99–102]. Approximately half of the long (L-) OPA1 molecules are cleaved to generate short (S-) OPA1 forms that lack the transmembrane anchor (Fig. 4A) [101]. L- and S-OPA1 forms assemble together in a CL-dependent manner, which stimulates OPA1 GTPase activity [103]. The balanced accumulation of L- and S-OPA1 forms is a prerequisite for the function of OPA1 in fusion and cristae morphogenesis [104–106], which are thus both under proteolytic control.

The yeast homologue of OPA1, Mgm1, is processed by the rhomboid type protease Pcp1 in the inner membrane to yield one long and one short form [104,107]. Interestingly, Mgm1 cleavage was found to depend on cellular ATP levels [108]. It was therefore proposed that nucleotide dependent Mgm1 processing acted as an energetic sensor to regulate mitochondrial fusion through the balanced formation of both Mgm1 isoforms. In mammalian cells, the situation is more complex due to the presence of eight OPA1 splice variants, which are expressed in a tissue-specific manner, and two processing sites [105]. Alternate splicing can occur at exons 4, 4b and 5b and proteolytic cleavage at sites S1 and S2 encoded by exons 5 and 5b, respectively [105]. Newly synthesized OPA1 precursor molecules are targeted to mitochondria by an N-terminal mitochondrial targeting sequence that is cleaved off upon import into mitochondria generating L-OPA1. Subsequent processing results in the accumulation of three different short forms, which assemble with two long forms (constitutive cleavage) (Fig. 4A). Different cellular insults that result in mitochondrial dysfunction induce the complete cleavage of L-OPA1 to S-OPA1 (stress-induced cleavage) (Fig. 4A). Such stresses include decreased ATP levels, loss of mtDNA, dissipation of the mitochondrial membrane potential and induction of apoptosis. Stress-induced OPA1 processing occurs exclusively at the site S1. Mitochondrial fusion is inhibited under these conditions due to the lack of L-OPA1 and ongoing fission events cause fragmentation of the mitochondrial network [109–111]. Stress-induced OPA1 processing is emerging as a central mechanism to monitor mitochondrial integrity and conduct quality surveillance, as mitochondrial fragmentation is associated with mitophagy and apoptosis.

Various mitochondrial proteases were screened for their role in OPA1 processing. The rhomboid protease PARL is orthologous to yeast Pcp1 and therefore was considered to be the prime candidate that mediates OPA1 processing. However, mouse embryonic fibroblasts (MEFs) lacking PARL fail to show any difference in mitochondrial morphology or in OPA1 processing, suggesting that PARL does not directly process OPA1 [112,113]. In contrast, depletion of the *i*-AAA protease, YME1L, decreases processing at the S2 site, indicating constitutive cleavage of OPA1 at the S2 site by YME1L [105,113]. In accordance with this, cells that have depleted levels of YME1L display a highly fragmented mitochondrial phenotype. However, loss of additional YME1L functions may contribute to the altered mitochondrial morphology. The mammalian *m*-AAA protease was able to cleave OPA1 at the S1 site when both were expressed in yeast [114]. However, depletion of the *m*-AAA protease in mammalian cells did not stabilize long forms of OPA1. Instead, it led to enhanced OPA1 processing accompanied by increased mitochondrial fragmentation [115]. In the absence of the *m*-AAA protease, OMA1 was found to be the protease responsible for induced OPA1 processing [115,116]. OMA1 is a metallopeptidase found in the mitochondrial inner membrane, originally identified in yeast due to its overlapping activity with the *m*-AAA protease [18]. Experiments in OMA1-deficient mouse embryonic fibroblasts revealed that OMA1 mediates both constitutive and stress-induced processing of OPA1 at S1 [117]. The mitochondrial network appears hyperfused in OMA1-deficient MEFs [117]. Furthermore, depletion of OMA1 also prevents stress-induced OPA1 processing and mitochondrial fragmentation [115,116]. OMA1 therefore takes center stage in monitoring mitochondrial integrity under stress conditions, however how dysfunction of mitochondria is sensed and how OMA1 is activated remains elusive. Stress-sensing by OMA1 appears to be of fundamental importance under metabolic stress conditions. Loss of OMA1 increases the susceptibility to obesity and causes defective thermogenesis in mice accompanied by marked metabolic alterations, suggesting that OMA1 mediates adaptive responses to different metabolic stressors [117].

3.9. Proteolytic control of mitophagy

Severe mitochondrial damage can induce the selective autophagic removal of mitochondria by mitophagy [37,118]. Specific receptor proteins have been identified at the mitochondrial surface, which target the general autophagic machinery to mitochondria and ensure

specificity [119–121]. A central role for the autophagic removal of depolarized mitochondria has been assigned to the E3 ubiquitin ligase Parkin and the PTEN-induced kinase 1 (PINK1) [122–127]. Mutations in both genes have been associated with autosomal recessive forms of Parkinson's disease, suggesting that accumulating dysfunctional mitochondria may contribute to neurodegeneration. Strikingly, the stability of PINK1 was found to be important for the recruitment of Parkin to depolarized mitochondria, where Parkin mediates the ubiquitylation of mitochondrial outer membrane proteins and initiates mitophagy. Thus, mitochondrial proteolysis precedes and regulates the removal of severely damaged mitochondria by autophagy.

3.10. PARL regulates PINK1 stability

Nuclear-encoded PINK1 is synthesized on cytosolic ribosomes and imported into mitochondria where it is subject to proteolytic degradation (Fig. 4B) [125,128–131]. An N-terminal mitochondrial targeting sequence directs PINK1 to mitochondria, where it is transported across the outer membrane via the TOM (translocase of outer membrane) complex and inserted into the inner membrane in a membrane potential dependent manner. The general mitochondrial processing peptidase, MPP, cleaves off the mitochondrial targeting sequence upon import. Depletion of the ß-subunit of MPP impairs mitochondrial import of PINK1 pointing to an unexpected role of MPP-ß for PINK1 biogenesis [129]. Subsequent to its integration into the inner membrane, PINK1 is processed within its transmembrane segment by the rhomboid protease PARL, resulting in the formation of a 52 kDa fragment of PINK1 that is further degraded by an unknown protease (Fig. 4B) [128,130–132]. As degradation of PINK1 can be inhibited by MG132, it is conceivable that degradation is mediated by the 26S proteasome in the cytosol. This suggests that either the entire 52 kDa fragment of PINK1 is exported from the organelle or, more likely, incompletely imported PINK1, which remains associated with the TOM complex in the mitochondrial outer membrane, is released into the cytosol [133]. *In vitro* import assays of PINK1 in human mitochondria indicate that the cleaved PINK1 fragment is protease-accessible and thus might be located on the mitochondrial outer membrane [134]. Depletion of PARL results in stabilization of mature PINK1 with a molecular mass of 60 kDa, which accumulates in the mitochondrial inner membrane [130]. Despite overall compelling support for this model (Fig. 4B), many of these localization studies rely on overexpression of PINK1, as endogenous PINK1 is detected poorly by the available antibodies. Indeed, some puzzling observations have been reported recently upon import of substoichiometric amounts of PINK1 *in vitro* [134]. Both full-length and the 52 kDa fragment of PINK1 were localized to the outer membrane and PINK1 processing, though with strongly reduced efficiency, was found to occur in mitochondria isolated from *Parl*$^{-/-}$ MEFs. Moreover, additional peptidases, including AFG3L2, have been linked to PINK1 processing [129]. It thus appears that the biogenesis of PINK1 is more complex than anticipated and may still bear surprises.

3.11. Parkin mediates ubiquitylation of mitochondrial outer membrane proteins

Loss of the mitochondrial membrane potential inhibits PINK1 import into the mitochondrial inner membrane, thereby blocking its degradation [122,125,126]. Instead, PINK1 assembles into the mitochondrial outer membrane and associates with the TOM complex (Fig. 4B) [133]. Parkin is subsequently recruited to the outer membrane by PINK1, a prerequisite for the initiation of mitophagy.

Upon translocation to depolarized mitochondria, Parkin mediates K-63 or K-48 linked polyubiquitylation of outer mitochondrial membrane proteins [135]. K-63 linked ubiquitylation has been proposed to activate autophagic machinery by recruiting ubiquitin-binding adaptors like HDACs and p62/SQSTM1 [136,137]. However, the importance of this pathway for mitophagy has been challenged, as cells lacking VDAC and p62/SQSTM1 have no defect in mitochondrial turnover [138,139]. On the other hand, K-48 linked ubiquitylation by Parkin is associated with activation of the UPS and degradation of various mitochondrial outer membrane proteins, including mitofusins, Miro, and Tom20 [135,140–142].

UPS-dependent degradation of mitochondrial outer membrane proteins precedes mitophagy but it is currently discussed controversially whether or not it is essential for the process to occur [135]. Parkin-mediated degradation of outer mitochondrial proteins can occur independent of mitophagy in *Atg5*$^{-/-}$ cells [143]. Moreover, Parkin-independent pathways for mitophagy, e.g. the NIX-dependent removal of mitochondria in reticulocytes, have been described [146–148]. On the other hand, the widespread proteolysis of mitochondrial outer membrane proteins might remodel the membrane and make it suitable for engulfment by autophagosomes [135]. Turnover of mitochondrial outer membrane proteins may be required to remove a negative regulator of mitophagy that is present on the mitochondrial surface [135]. Mitochondrial fusion is inhibited upon degradation of mitofusins allowing segregation of the dysfunctional mitochondria for mitophagy [144]. Another substrate ubiquitylated by Parkin is the Ca^{2+}-dependent GTPase Miro, a component of an adaptor complex that anchors kinesins to the mitochondrial surface to facilitate mitochondrial motility. Miro is phosphorylated by PINK1, ubiquitylated by Parkin and degraded by the 26 S proteasomes [145].

Parkin-mediated ubiquitylation triggers recruitment of the proteasome and p97 (UPS components). The presence of p97 (VCP/Cdc48 homolog in yeast), which has a pronounced role in ER-associated degradation (ERAD) for dislocation of ER membrane proteins, suggests that a similar mechanism might exist for recognition and degradation of mitochondrial outer membrane proteins [28,144,149]. The yeast protein, Vms1 (VCP/Cdc48-associated mitochondrial stree-responsive1) is identified as an adaptor for the p97-orthologue Cdc48, which re-localizes to mitochondria upon mitochondrial stress and stably interacts with Cdc48 [150]. Loss of Vms1 in yeast results in impairment of ubiquitin-dependent mitochondrial protein degradation. Similar to Vms1, ERAD uses Npl4 and Ufd1 for recruitment of Cdc48/p97 to the ER membrane [151]. Interestingly, the Cdc48/Vms1 complex also contains Npl4 (not Ufd1) suggesting selective sharing of critical UPS components.

4. Concluding remarks

Many mitochondrial proteases have been originally identified as quality control enzymes that degrade misfolded or damaged proteins and therefore protect against mitochondrial dysfunction. However, it is becoming increasingly clear that the same proteases mediate the processing or determine the stability of mitochondrial proteins with regulatory functions in diverse processes, such as mitochondrial gene expression, phospholipid homeostasis, mitochondrial dynamics or mitophagy. Moreover, central roles of the cytosolic UPS for mitochondrial activities have been recognized. Proteolytic processes thus emerge as central regulators in an interconnected network of mechanisms that maintain mitochondrial activities. Impairment of mitochondrial proteolysis is often associated with diseases. Future studies will be pivotal to clarify whether the impaired clearance of damaged proteins or a general mitochondrial dysfunction underlie such diseases.

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