Regulating Mitochondrial Outer Membrane Proteins by Ubiquitination and Proteasomal Degradation

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Abstract

Mitochondrial outer membrane proteins have been found to be ubiquitinated and degraded by the proteasome. This process shares at least one component of the ERAD pathway of ER membrane protein degradation, the AAA ATPase cdc48/p97/VCP, thought to extract integral membrane proteins from the lipid bilayer and chaperone them to the proteasome. Proteasomal degradation of the outer mitochondrial membrane protein Mcl-1 regulates apoptosis whereas Parkin-mediated ubiquitination and degradation of Mitofusins can inhibit mitochondrial fusion and promote mitophagy. The breadth of outer mitochondrial membrane ubiquitin/proteasome substrates and the physiological relevance of their turnover is only beginning to be understood.

1. Introduction: mitochondrial protein quality systems

Mitochondria are the primary cellular sites of energy production. In addition, various vital cellular events including apoptosis, Ca^{2+} buffering, and macromolecule synthesis are also regulated by mitochondria. To eliminate surplus or dysfunctional mitochondrial proteins, or whole damaged organelles that can negatively influence cellular homeostasis, regulated mitochondrial biogenesis and clearance is required. Thus, to counteract continuously occurring accumulation of defective components of mitochondria and functional deterioration of these organelles a number of mitochondrial protein quality control mechanisms operate in the cell.

It has been known for several decades that within the mitochondrial matrix, descendents of bacterial ATP-stimulated mitochondrial proteases, including PIM1/Lon [1,2] i-AAA and the m-AAA proteases (e.g. YME1L1 [3] and paraplegin [4]), mediate the turnover of inner mitochondrial membrane (IMM) proteins. These proteases are essential for various aspects of mitochondrial function, including mtDNA maintenance, mitochondrial fusion and formation of mitochondrial respiratory complexes [1–4]. Since, aging- or disease-linked impairments of these proteases have been suggested to contribute to mitochondrial failure and subsequent cell deterioration (for reviews see [5,6]), they are thought to serve as important mitochondrial protein quality control systems.

Studies published more recently indicate that dynamic remodeling of mitochondrial membranes, mainly through fusion and fission of these organelles, serves as another essential mitochondrial quality system (for reviews see [7–9]). It has been proposed that mixing of mitochondrial contents possibly through cycles of fusion and fission can serve as a mechanism diluting local mitochondrial defects (through fusion) [10,11], as well as eliminating damaged organelles from the mitochondrial network (through fission and inhibition of fusion), and thus priming them for autophagosomal degradation [12, **13,14]. Supporting a critical role of mitochondrial membrane dynamics, impairments of mitochondrial fusion and/or fission lead to mitochondrial and cellular dysfunction (for reviews see [7–9]).

In addition to the above described quality control systems, recent evidence also indicates that the ubiquitin (Ub)/proteasome system controls mitochondrial proteostasis, either by regulating mitochondrial protein turnover, or controlling mitochondrial protein activities, and can therefore be considered as a mitochondrial quality control mechanism. Although, as we will discuss later, some studies support a role for the Ub/proteasome system in regulation of intra-mitochondrial proteins (e.g. those localized in the IMM), the majority of evidence points to the importance of ubiquitination and proteasomal degradation in the control of the outer mitochondrial membrane (OMM) proteostasis.

2. Outer mitochondrial membrane associated degradation (OMMAD)

Given that the OMM serves as a barrier separating mitochondria from the cytosol and plays vital roles for mitochondrial function, including regulation of metabolism, apoptosis and mitochondrial membrane dynamics, the quality control of OMM-associated proteins is likely to be of great importance for cell function. Notably, it has been shown that the majority of known OMM-associated substrates of the Ub/proteasome system are proteins central for the regulation of either apoptosis or mitochondrial membrane dynamics.

Proteins regulating mitochondrial steps in apoptosis

The most extensively studied OMM associated substrate of the Ub/proteasome system is an anti-apoptotic protein in the Bcl-2 family, Mcl-1. Under normal growth conditions the half-life of Mcl1 has been estimated to be in the range of ~40–60 min [15,16]. Upon induction of apoptosis Mcl1 is rapidly degraded in a Ub and proteasome-dependent manner [15,17]. The apoptotic degradation of Mcl1, as well as its turnover in non-apoptotic cells, is regulated by the counteracting activities of the HECT-domain-containing Ub ligase ARF-BP1/Mule (Mcl-1 Ub ligase E3) [18], and the deubiquitinase Usp9x [19]. Expression levels of ARF-BP1/Mule and Usp9x appears to be critical for the maintenance of proper cellular balance of anti- and pro-apoptotic proteins, and contributes to cell sensitivity to apoptosis and is linked to tumor formation [18, *19].

In addition to Mcl1, turnover of other mitochondria-associated Bcl-2 family proteins, including Bax and Bcl-2 [20, *21,22,23], is also under Ub/proteasome control. Bax, a pro-apoptotic Bcl-2 family protein, is mainly localized in the cytosol in an apoptotically inactive form (6A7-epitope negative) and it moves to mitochondria upon pro-apoptotic trigger-induced change in its conformation (6A7-positive) [24–26]. Proteasome-dependent degradation of Bax occurs specifically on the mitochondria [23], suggesting that the apoptotic conformation of Bax might be recognized by the Ub conjugation machinery, and serve as a degradation signal preventing the accumulation of potentially dangerous apoptotically-active Bax in healthy cell mitochondria. Baxβ, a 24-kD splice variant of Bax that has shorter half-life, and is a more efficient pro-apoptotic protein than the more abundant 21-kD Baxα, is continuously degraded in a proteasome-dependent manner in non-apoptotic cells [22]. Furthermore, as shown by Benard et al., degradation of 6A7-positive

Baxα is also proteasome-dependent and is regulated by IBRDC2, an IBR-type RING domain E3 Ub ligase [*21]. Based on these data, it has been proposed that a Ub-dependent apoptosis checkpoint safeguards mitochondria from Bax-dependent damage, and cells from unprompted apoptosis [*21,22]. Aside from Ub/proteasome-dependent regulation of Mcl-1 and Bax, several examples of other mitochondria-localized and mitochondria-interacting E3 Ub ligases, or Ub/proteasome-dependent regulatory events that influence mitochondrial steps in apoptosis have been described. BRCA1-associated RING domain 1 (BARD1) partially localizes to mitochondria, and it has been proposed that the apoptotic function of BARD1 is associated with stimulation of Bax oligomerization at mitochondria [27]. Furthermore, ARTS a pro-apoptotic mitochondrial protein is regulated through Ub/proteasome degradation [28]. Like Bax, high cellular levels of ARTS protein sensitize cells toward apoptosis, and in healthy cells ARTS levels are kept low through constant Ub-mediated degradation [28]. Moreover, in addition to Mcl-1 and Bax, ubiquitination of other Bcl-2 family proteins, including Bcl-2 [29], and a truncated form of Bid [30], regulates their expression and activity. Altogether, these data indicate a direct role for the Ub/proteasome system in the regulation of mitochondrial steps in apoptosis, and therefore place the Ub/proteasome system as a critical mitochondrial quality control system.

Mitochondrial membrane dynamics and mitochondrial autophagy

In Eukaryotic cells, turnover of Mitofusins (Mfn), integral GTPases of the OMM required for mitochondrial fusion (reviewed in [8,9]), is also mediated by the Ub/proteasome system [31–35]. Fzo1p, a yeast homologue of Mfn [36] is modified with proteasome-targeting Lys-48-linked Ub chains [31,32,34], and the proteasome inhibitor, MG132 [33] as well as aberrations in proteolytic activity (pre1 and pre2) of the 20S proteasomal core particle or the ATPase subunit (cim3 and cim5) of the 19S regulatory complex of the proteasome [34,35] suppressed the degradation of Fzo1p. In mammalian cells Mfn1 is relatively unstable, with a half-life estimated in a range of ~4–6hr [**37]. Mammalian cell culture studies reveal that, like Fzo1p in yeast, Mfn1 and Mfn2 are stabilized by proteasome inhibition [**13,**37]. In addition, consistent with Ub-dependence, accumulation of ubiquitinated forms of these proteins is detected in proteasome inhibitor-treated cells [**13,**37].

Under mitochondrial stress, Ub/proteasome-dependent degradation of mammalian Mfns [**13,*38] and the *D. melanogaster* homologue dMfn [39,40] is mediated by Parkin, an IBR-type RING domain E3 Ub ligase. Parkin translocates to functionally impaired mitochondria [41], and prior to initiating their removal by mitochondria-specific autophagy, Mfns are ubiquitinated and degraded in a proteasome-dependent manner [**13,**37,42]. Yet, since in cells deficient in Parkin expression the turnover of Mfns is also regulated by Ub/proteasome system [**13,*38], it is likely that, in addition to Parkin, another E3 Ub ligase mediates ubiquitination of these proteins. Interestingly, since inhibition of the proteasome suppresses Parkin-dependent autophagy of dysfunctional mitochondria [**13], it is likely that degradation of certain OMM-associated protein(s) initiates mitochondrial assembly of autophagy components (Fig. 1). Since Parkin can initiate mitochondria-specific autophagy in Mfn1/Mfn2$^{-/-}$ DKO cells [**13], OMM-associated substrates of the Ub/proteasome system other than Mfns likely serve this purpose. Consistent with this, it has been proposed that mitochondrial autophagy requires proteasomal degradation of the OMM associated voltage-dependent anion-selective channel 1 (VDAC1) [43]. Yet, since this process occurs in VDAC deficient cells [44], it is likely that degradation of other OMM-associated substrate(s) of the Ub/proteasome system is critical for initiation of mitochondria-specific autophagy.

In addition to Mfns, Drp1 a large GTPase essential for mitochondrial fission (for reviews see [9,21]) is also targeted by Ub-conjugation. Based on co-immunoprecipitation experiments, it has been proposed that MARCH5 (also known as Mitol or MARCH-V) [45–47], a

mitochondria-associated RING-finger E3 Ub ligase promotes ubiquitination of Drp1. Yet, in contrast to Mfns, MARCH5-mediated ubiquitination of Drp1 is not required for Drp1 degradation [45,46], but rather regulates Drp1 activity. Although RNAi downregulation, as well as overexpression of wild type or RING-inactive mutants of MARCH5, did not induce any detectable changes in the levels of Drp1 [45,46], expression of RING-inactive mutants of MARCH5 inhibited subcellular trafficking of Drp1 associated with abnormal elongation and interconnection of mitochondria [45]. It is important to examine the possibility that degradation of other mitochondrial protein(s), is regulated by MARCH5-dependent ubiquitination, and that this in turn affects cellular trafficking of Drp1. Notably, a large-scale proteomic study of Ub-modified proteins in yeast revealed that Dnm1p, a yeast homologue of Drp1 is also ubiquitinated [48], suggesting that Ub-dependent regulation of Drp1 might be evolutionarily conserved. Clearly, further mechanistic studies are required to reveal the significance of the Ub/proteasome system in Drp1/Dnm1p and mitochondrial fission regulation.

Non-OMM Ub/proteasomal substrates

Mammalian sperm mitochondria that are preordained for degradation during normal development are tagged with Ub inside the oocyte cytoplasm and later subjected to proteolysis [49]. Notably, prohibitin, an IMM-associated protein, is ubiquitinated in sperm mitochondria [50]. Prohibitins regulate the stability of IMM proteins by protecting them from degradation by the IMM associated, Ub-independent protein degradation systems. Therefore, the Ub-dependent prohibitin turnover regulation might coordinate Ub-dependent and Ub-independent proteolytic quality control mechanisms in the mitochondria. Margineantu et al. [51] shows that a number of non-OMM mitochondrial proteins, including the OSCP subunit of mitochondrial F_1F_0-ATPase can be detected as Ub conjugates. Furthermore, since proteasome inhibition also induced an accumulation of certain IMM-localized proteins (e.g. vital for the respiratory function of mitochondria: COXI, III, IV, OSCP) [51] one might suggest that in addition to the OMM-associated proteins, the Ub/proteasome system also frequently regulates the turnover of IMM-associated proteins.

3. Molecular steps of OMMAD

CDC48/p97/VCP-mediated retrotranslocation of the OMM proteins

Since most known OMM-associated substrates of the Ub/proteasome system are integral membrane proteins, with one (e.g. Mcl1) or more (e.g. Mfns have two) transmembrane domains inserted in the OMM, these proteins likely need to be extracted from the OMM prior to proteasomal degradation. Consistently, in addition to ubiquitination machinery and the proteasome, participation of other factors is required for OMMAD.

Recent studies have revealed that the cytosolic AAA ATPase CDC48/p97/VCP, that is required for extracting ubiquitinated proteins from the ER and other cellular membranes [52,53], regulates OMMAD both in mammalian and yeast cells [**13,**37, **54]. In mammals p97 is required for turnover of Mcl-1 and Mfn1, two OMM-associated proteins with relatively short half-lives [**13,**37]. Both of these proteins are stabilized on mitochondria in cells depleted of p97 activity [**37], suggesting that p97 acts directly on the OMM. A number of proteomic studies revealed that p97 associates with mitochondria in unstressed mammalian cells [55–57], further suggesting a widespread role for p97 in mitochondrial proteostasis. p97 is also required for Parkin-dependent stress-induced degradation of Mfns and subsequent autophagy of dysfunctional mitochondria [**13]. Notably, in Parkin expressing cells p97 accumulates on dysfunctional mitochondria [**13], confirming a direct mitochondrial role for this protein. Consistent with this, in response to mitochondrial stress, Cdc48 a yeast homologue of p97 is also recruited to the OMM [54].

Mitochondrial translocation of Cdc48 depends on, and occurs subsequently to mitochondrial translocation of Vms1 (VCP/Cdc48-associated mitochondrial stress-responsive 1) [**54]. Yeast cells depleted of Vms1, and therefore deficient in mitochondrial translocation of Cdc48, show progressive mitochondrial failure that is associated with increased sensitivity to mitochondrial stress, as well as a significant delay in the degradation rate of Fzo1p [**54]. Thus, the role for Cdc48/p97 in the regulation of OMM protein turnover appears to be conserved. Interestingly, the Cdc48/Vms1 protein complex also contained Npl4 protein. Since Npl4 is required for Cdc48/p97 mediated retrotranslocation of ubiquitinated proteins from membranes, including those following the endoplasmic reticulum associated degradation (ERAD) pathway [58], it appears that OMMAD shares a number of critical components with other Ub/proteasome dependent protein degradation pathways.

Mitochondria-associated deubiquitinases

Proteasomal degradation of polyubiquitinated proteins, as well as activities of mono- or Lys-63-chain poly- ubiquitinated proteins can be affected by activities of deubiquitinating proteases (DUBs) (for review see [59]). Until now, two mitochondria-associated DUBs have been identified [*19,60]. As discussed above Usp9x mediates deubiquitination, and regulates stability of Mcl1 [*19]. Usp9x partially localizes to the mitochondria where it binds Mcl1 and removes the proteasome targeting Lys 48-linked polyubiquitin chains [*19]. Notably, increased Usp9x expression correlates with increased Mcl1 protein in human follicular lymphomas and diffuse large B-cell lymphomas [*19]. Unlike Usp9x, Usp30, another mitochondria associated DUB [60] is specifically associated with the OMM. The mitochondrial substrates of Usp30 are currently unknown. Yet, since RNAi downregulation of Usp30 induces abnormal elongation and interconnection of mitochondria [60], it is likely that Usp30 mediates deubiquitination of certain proteins implicated in the regulation of mitochondrial membrane dynamics.

4. Future perspectives

As in the case of any developing research field, the studies summarized here are raising a number of questions regarding the scope and mechanism of Ub/proteasome system in mitochondrial homeostasis. For example, some published reports indicate a role for the Ub/proteasome in regulation of mitochondrial proteins localized in the inner mitochondrial compartments (e.g. IMM). However, the OMM is the barrier separating mitochondria from the cytosol, and the cytosol localized components of Ub/proteasome system. Thus, the mechanism of ubiquitination and subsequent movement of Ub-conjugated proteins from the IMM to the cytosol are currently unknown. Undoubtedly, further studies addressing this exciting research topic are needed. Furthermore, it is currently unknown whether misfolded proteins of the OMM, are processed by OMMAD in the same manner as misfolded ER proteins are targeted for proteasomal degradation by ERAD pathway. For example, the mechanisms by which mitochondrial proteins are recognized by Ub/proteasome system, as well as factors mediating this recognition and other steps of OMMAD need to be identified. Revealing additional molecular components as well as more detailed understanding of protein targets, mechanisms and roles of known mitochondrial components of the OMMAD pathway will likely help to address these issues.

Finally, the overall physiological significance of the OMMAD system should also be established. How the Ub/proteasome facilitates mitophagy, for example, remains enigmatic and important. Considering that mitochondrial dysfunctions are hallmarks of aging and various aging-linked diseases, one may assume that Up/proteasome system might be also important for elimination of misfolded mitochondrial proteins expected to accumulate in aging cells. The degrees to which aging-associated decline in Ub/proteasome activities contribute to mitochondrial dysfunctions need to be investigated.

Answering these, and many other emerging questions will likely shed more light on the significance of Ub/proteasome system in the regulation of mitochondrial function. Given the important role of mitochondria for cellular homeostasis, these should also improve general knowledge of mitochondrial biology, and reveal how mitochondrial dysfunctions contribute to disease.

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Highlights

1. E3 ligases that can localize to mitochondria such as Parkin ubiquitinate outer mitochondrial membrane proteins.

2. Membrane spanning proteins localized to the outer mitochondrial membrane can be degraded by the ubiquitin proteosomal system.

3. p97, an AAA ATPase involved in membrane protein retrotranslocation mediates outer mitochondrial membrane protein degradation.
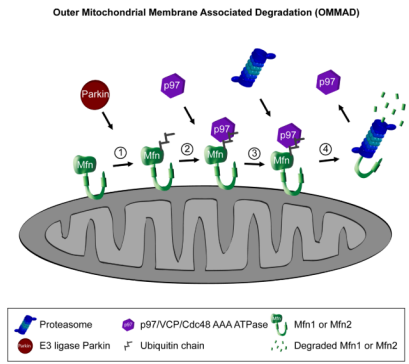
Figure.

Table 1

Proteins implicated in the OMMAD pathway

Protein	Biological function	Role in OMMAD/Targets	References
Parkin	IBR-domain E3 Ub ligase	ubiquitination of Mfn1, Mfn2 and VDAC1; induces mitochondria-specific auophagy	[13,41–43]
PINK1	mitochondrial kinase	recruits Parkin to the mitochondria	[61,62]
MARCH5/MARCH-V/Mitol	mitochondrial RING-domain E3 Ub ligase	Binds and ubiquitinate Drp1 (mitochondrial fission factor) and Mitofusins (mitochondrial fusion factors); mitochondrial dynamics regulation	[45–47]
MULAN/MAPL	mitochondrial RING-domain E3 Ub ligase	mitochondrial dynamics regulation; reported also as E3 SUMO ligase targeting Drp1	[63,64]
IBRDC2	IBR-domain E3 Ub ligase	apoptosis regulation; targets Bax, a proapoptotic protein in Bcl2 family	[21]
Mule/ARF-BP1	HECT domain E3 Ub ligase	ubiquitinates and regulates turnover of Mcl1, an antiapoptotic protein in Bcl2 family	[18]
USP9x	deubiquitinase	deubiquitinates and regulates turnover of Mcl1	[19]
USP30	mitochondrial deubiquitinase	regulates mitochondrial dynamics; substrates unknown	[60]
p97/CDC48	AAA-ATPase; protein dislocase	regulates OMM protein turnover and mitochondria-specific autophagy in yeast and mammals	[13,37,54]
Vms1p	adaptor protein	cofactor of CDC48; regulates OMM protein and mitochondrial degradation in yeast	[54]
Npl4	adaptor protein	cofactor of CDC48; regulates OMM protein and mitochondrial degradation in yeast	[54]
Proteasome	protein degradation complex	translocates to the mitochondria upon activation of Parkin-dependent, mitochondrial stress-induced mitophagy	[38]