JB Minireview—Quality Control of the Cellular Protein Systems

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Protein Quality Control in Mitochondria

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Mitochondria are crucial for both life and death of eukaryotic cells. Compromised mitochondrial integrity has severe cellular consequences and is linked to senescence and neurodegenerative disorders in humans. To maintain the functionality of proteins in mitochondria, quality-control mechanisms including signal transduction pathways counteracting mitochondrial stress have evolved. A network of molecular chaperones and proteases monitors protein integrity and prevents accumulation of damaged proteins. In this review, the current knowledge of elaborate defence strategies within mitochondria is summarized.

Key words: mitochondria, ATP-dependent proteases, chaperones, mitochondrial unfolded protein response, protein aggregation.

Abbreviations: QC, quality control; ROS, reactive oxygen species; IM, inner membrane; OM, outer membrane; IMS, intermembrane space; TOM, translocator of outer membrane; TIM, translocator of inner membrane; NH, n-terminal helices; CH, c-terminal helices; ERAD, endoplasmic reticulum associated degradation; SCF complex, Skp1-Cullin-F-box complex; UPR, unfolded protein response; OTC, ornitine transcarbamylase; MURE, mitochondrial unfolded protein response element.

Mitochondrial functions depend on the integrity of protein machineries making the quality control (QC) of mitochondrial proteins an essential task. Mainly, two troublesome issues challenge protein homeostasis in mitochondria. First, mitochondria are a major source of reactive oxygen species (ROS), an inevitable by-product of oxidative phosphorylation (*1*). Therefore, mitochondrial proteins are continuously in danger of oxidative modification. Second, problems arise from the complex biogenesis of mitochondrial proteins and protein complexes. Mitochondria are surrounded by two membranes—the inner and outer membrane (IM and OM)—and are separated into the intermembrane (IMS) and the matrix space. Most of mitochondrial proteins are synthesized in the cytosol and delivered to their final destination within mitochondria through various protein translocation pathways. Moreover, mitochondria contain their own genome and synthesize a set of proteins including subunits of respiratory chain complexes (RCs), which need to assemble with nuclear encoded subunits. Due to such a complex biogenesis, mitochondrial proteins are at risk of falling into non-functional state especially under stress conditions. Mitochondrial QC systems composed of chaperones and proteolytic machineries deal with these organelle-specific problems and maintain mitochondrial integrity.

QC OF PROTEINS IN THE MATRIX

Almost all matrix proteins are synthesised in the cytosol and imported into mitochondria through protein translocases in the OM and IM (TOM and TIM23, respectively) in an unfolded conformation (*2*). Thus, post-import folding and maturation must be ensured by the help of molecular chaperones. ROS or other external stresses such as heat can cause irreversible damage of proteins and result in the formation of insoluble protein aggregates in the matrix. ATP-dependent proteases degrade such aggregation-prone, damaged proteins. In line with the endosymbiotic origin of mitochondria, components of QC systems in the matrix resemble those in the cytosol of α-proteobacteria (Fig. 1).

Chaperones Assist Protein Translocation and Post-Import Folding—The matrix contains chaperone machineries including members of Hsp70, Hsp60 and Hsp100 family (*3*). mtHsp70 (Ssc1 in yeast) exerts versatile functions in the matrix. mtHsp70 is recruited to TIM23 translocase and forms a motor complex which promotes protein translocation into the matrix. The transient binding of mtHsp70 to incoming polypeptides as well as to fully translocated polypeptides keeps the polypeptide in a partially folded state thereby assisting its proper folding (the so-called holdase function). J-type co-chaperones (in the motor complex associated with the TIM23 translocase or Mdj1 in the matrix) and the nucleotide exchange factor Mge1 coordinate mtHsp70 activities. Recently, specialized Hsp70 chaperone and co-chaperone (Ssq1 and Jac1 in yeast) have been implicated in the biogenesis of Fe-S clusters (*4*, *5*). Additional Hsp70 family proteins and J-type co-chaperones have been identified in mitochondria, but their physiological roles are still largely obscure.

In addition to Hsp70, a set of proteins requires the Hsp60 chaperone system to reach the functional state. Hsp60 represents a class of chaperone proteins, which assemble with the co-chaperone Hsp10 and form large cage-like structures (*6*). Hsp60 interacts with partially

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folded polypeptides after the release from Hsp70 and completes their folding (*7*). Consistent with its major role in protein folding in mitochondria, yeast cells lacking functional Hsp60 are inviable (*8*).

In fungal mitochondria, a chaperone protein of the Hsp100 family (Hsp78 in yeast) has been identified in the matrix (*3*). Similar to other Hsp100 proteins, Hsp78 can dissolute aggregated proteins upon heat stress (*9*). However, no mitochondrial Hsp100 homologue has been identified in the genome of higher eukaryotes (*10*).

ATP-dependent Proteolysis in the Matrix—Two ATP-dependent proteases have been found in the matrix. Lon (Pim1 in yeast), a serine protease and member of the AAA+ protein family, eliminates denatured or oxidatively damaged proteins in the matrix (*11*). Especially, iron–sulfur-containing proteins are prone to be oxidized and targeted to Lon-dependent turnover (*12*, *13*). Deterioration of Lon may contribute to cellular aging since age-dependent progressive decline of Lon activity is accompanied by the accumulation of electron-dense aggregates in mitochondria (*14*).

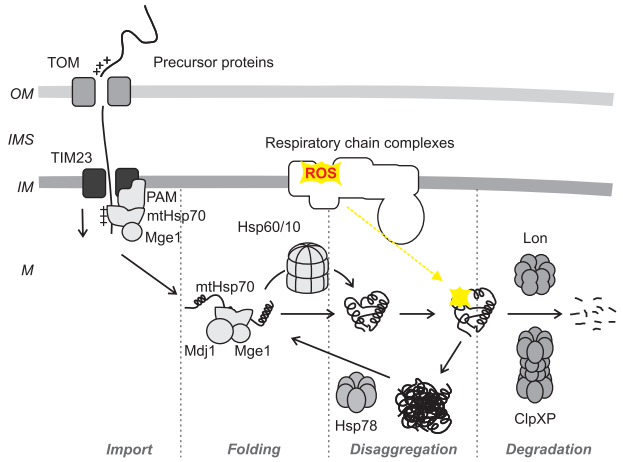

Fig. 1. **QC network in the matrix.** Import of proteins into the matrix is driven by TIM23 translocase and a complex (PAM) containing mtHsp70 and co-factors. Post-import folding of proteins is assisted by mtHsp70 and Hsp60 chaperones. ROS-induced damage leads to protein aggregation. In yeast mitochondria, Hsp78 dissolutes protein aggregates. ATP-dependent proteases degrade aggregation-prone, damaged proteins.

A second ATP-dependent protease, ClpXP, has been identified in the matrix space of mammalian mitochondria (*15*). ClpXP is build up of the subunit ClpX and the proteolytic subunit ClpP, forming double-donut like ring complexes (*16*). Prokaryotic ClpXP has extensively been studied; however, the physiological role of mitochondrial ClpXP remains largely elusive.

Although Lon selectively degrades misfolded and oxidized proteins, the molecular basis of substrate recognition has not been well defined. Analysis of model substrates demonstrated that yeast Lon (Pim1) recognizes an unfolded segment of polypeptides (*17*). On the other hand, human Lon can initiate proteolysis at solvent-exposed sites of a folded protein (*18*). It is conceivable that substrate recognition by Lon is determined by several factors including the stability of folded domains. Oxidative modification may destabilize the folding of proteins, making them accessible for Lon. In this regard, the cooperation of chaperones and proteases in the turnover of aggregation-prone proteins is intriguing (*3*). Chaperones may prevent irreversible aggregation of aggregation-prone proteins and keep them in a soluble state, allowing Lon-mediated proteolysis.

QC OF PROTEINS IN THE INNER MEMBRANE

The mitochondrial IM is considered as the protein richest biological membrane and houses a large part of the mitochondrial proteome including RCs and metabolite carriers. Since a set of RC subunits are encoded by the mitochondrial genome, balanced synthesis of nuclear- and mitochondrial-encoded proteins is demanded and its disturbance may cause accumulation of unassembled subunits and assembly intermediates of RC. Moreover, proteins integral to the IM are the prime target of oxidative modification. Various proteolytic sytems in the IM ensure the selective elimination of non-functional and potentially harmful proteins (Fig. 2).

Proteolytic Systems in the IM—Two membrane-embedded metalloprotease complexes, termed AAA proteases, exert a major role for protein QC in the IM. The *m*-AAA protease exposes its catalytic sites to the *m*atrix space, whereas the *i*-AAA protease is active on the intermembrane space side of the membrane. AAA proteases

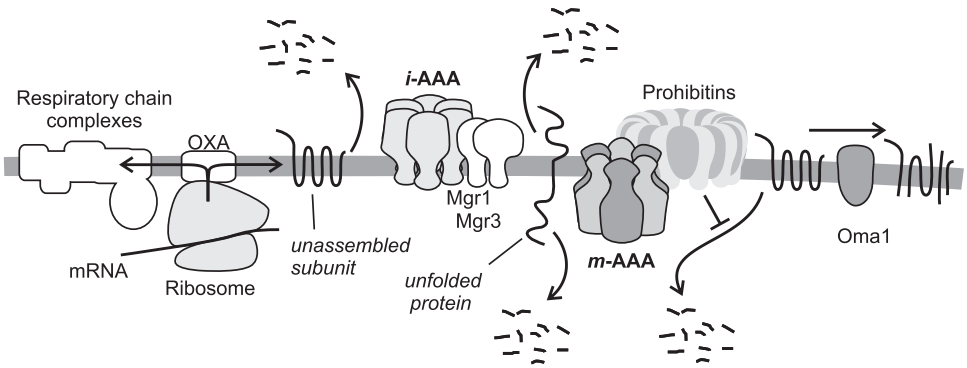

Fig. 2. **Proteolytic systems in the IM.** Newly synthesized subunits of RC are inserted into the IM by an insertion machinery (OXA). Unassembled subunits and unfolded proteins are degraded by AAA proteases. Assembly partners of AAA proteases modulate proteolytic activities. Oma1 mediates processing of membrane proteins.

form oligomeric complexes composed of subunits, which contains one or two transmembrane segments, an ATPase domain conserved throughout the AAA+ protein family of ATPases and a C-terminal Zn^{2+}-dependent metalloprotease domain. The *i*-AAA protease forms homooligomeric complexes of identical subunits (Yme1 in yeast), while homo- and heterotypic assemblies of *m*-AAA proteases do exist. In yeast, the *m*-AAA protease is found exclusively as a heterooligomeric complex of two closely related subunits, Yta10 and Yta12 (*19*). In human mitochondria, in addition to heterooligomeric assemblies of two subunits (AFG3L2 and paraplegin), homooligomeric *m*-AAA complexes composed of AFG3L2 can be formed (*20*). Both homo- and hetero-oligomeric human *m*-AAA protease complexes can substitute for the *m*-AAA protease in yeast, showing functional conservation (*20*).

Substrates of AAA proteases include unassembled subunits of various macromolecular complexes and non-native membrane proteins. Not only integral membrane proteins, but also peripherally associated proteins like subunit 7 of ATP synthase are targeted for proteolysis by the *m*-AAA protease (*21*). On the other hand, the *m*-AAA protease processes and thereby activates specific substrates with regulatory roles in mitochondria. Such a substrate is MrpL32, a subunit of the mitochondrial ribosome whose assembly into ribosomal particles depends on processing by the *m*-AAA protease (*22*).

Another protease has been linked to QC in the IM; Oma1 (*23*). Oma1 is a member of metallopeptidase family M48 and is conserved in eukaryotes. In the absence of the *m*-AAA protease, Oma1 degrades a model substrate of the *m*-AAA protease, suggesting that Oma1 has overlapping function with *m*-AAA protease in QC of IM proteins.

Mechanism of Substrate Selection for Proteolysis by AAA Proteases—IM proteins are degraded rapidly when either unassembled or malfunctioning (e.g. damaged proteins or mutant variants). An important question is therefore how AAA proteases monitor the status of IM proteins and select non-functional polypeptides for degradation. Analyses using model substrates revealed that the membrane topology of substrates determines involvement of *m*-AAA and/or *i*-AAA proteases for their turnover (*24*). An unfolded domain or a terminal segment with only ~20 amino acids protruding from the membrane is sufficient to allow proteolytic attack by an AAA protease. Amino acid residues involved in substrate recognition have recently been identified in the *i*-AAA protease: C-terminal helices of the proteolytic domain (CH) and N-terminal helices of the AAA domain (NH) (*25*). Whereas the NH is required for degradation of all substrates, the CH is involved in the degradation of a subset of proteins only. The distance of the recognition site from the membrane surface is most likely one parameter determining the involvement of the CH-region for substrate recognition.

In prokaryotic Clp proteases, various adaptor proteins associate with proteases and modulate their substrate specificity. Recently, a similar role for degradation by the yeast *i*-AAA protease was assigned to Mgr1 and Mgr3 (*26*, *27*). Both are integral membrane proteins facing the IMS and associate with the *i*-AAA protease. Deletion of either Mgr1 or Mgr3 impairs the turnover of a set of model substrates by the *i*-AAA protease, pointing an adaptor-like function. Related co-factors of the *m*-AAA protease are currently not known. *m*-AAA proteases associate with prohibitins which modulate proteolysis (*28*). Prohibitins form ring-like assemblies with a diameter of 20–25 nm which are composed of multiple copies of two highly conserved subunits, Phb1 and Phb2 (*29*). The loss of prohibitins results in an accelerated proteolysis of non-assembled IM proteins by the *m*-AAA protease in yeast (*28*). Although the precise function of prohibitins is still unclear, recent genome-wide analysis of the genetic interactome of prohibitins in yeast revealed its close relation to the phospholipid homeostasis in mitochondria (*30*). Considering the large ring-like assembly of prohibitins, they may act as a scaffold to maintain a certain membrane domain with a specific lipid composition and consequently affect proteolysis of membrane-bound proteins. Alternatively, prohibitins may sequester the *m*-AAA protease and control its access to substrates. In this respect, the recent observation that the *m*-AAA protease and one of its substrate, Ccp1, are preferentially found in the inner boundary membrane is intriguing (*31*). If the IM is more mosaic than previously anticipated, compartmentalization of proteases and their substrates could serve as an additional control mechanism for proteolytic events.

QC IN IMS AND OM

Although IMS and OM house many proteins essential for organelle functions, QC mechanisms in these subcompartments have not been well characterized yet. Several proteins have been implicated in QC in the IMS. One is the *i*-AAA protease which is active on the IMS side and degrades proteins residing in the IMS (*25*, *32*). The other one is HtrA2/Omi, a highly conserved serine protease which has been shown to play both pro-apoptotic and cytoprotective roles (*33*). HtrA2 knockout mice show neuronal degeneration with a parkinsonian phenotype accompanied by severe mitochondrial defects, suggesting that HtrA2 plays an important role in maintenance of mitochondrial integrity (*34*). Analogous to its bacterial counterparts (DegP and DegS), a role as a stress sensor or chaperone/protease for unfolded proteins has been suggested. In line with this assumption, the recent finding that the activity of HtrA2 can be modulated by PTEN-induced putative kinase 1 (PINK1)-dependent phosphorylation is intriguing and may point to a signalling pathway through phosphorylation of HtrA2 in the IMS (*35*).

By analogy to ER-associated degradation (ERAD), it has been assumed that the ubiquitin-proteasome system (UPS) might be involved in the protein QC in the OM since it allows direct access from the cytosol. Consistent with this idea, several RING-finger ubiquitin ligases as well as F-box proteins for Skp1-Cullin-F-box (SCF) complexes have recently been implicated in the proteolytic regulation of mitochondrial membrane dynamics by UPS (*36*). Thus, UPS conducts the turnover of at least a subset of mitochondrial proteins, although

its direct role in protein QC is still obscure. Surprisingly, several mitochondrial proteins residing in the IM or the matrix have been shown to be conjugated with ubiquitin and accumulated upon inhibition of the proteasome (*37*). Moreover, many mitochondrial proteins are found to be conjugated to ubiquitin (*38*, *39*). The important question is whether ubiquitin conjugation and proteasomal degradation of mitochondrial proteins can occur upon localization to mitochondria or it only happens at the pre-import stage. Direct evidence for UPS-mediated degradation of mitochondrial proteins in the cytosol has been provided for apo-cytochrome *c* or the E2 component of 2-oxoglutarate dehydrogenase complex (*40*, *41*). Further analysis is required to clarify the role of UPS and its underlying mechanisms in QC of mitochondrial proteins.

MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE

Environmental stresses may perturb the balance between the activity of QC systems and the client protein load in the cell. To cope with such a situation, signalling pathways to enhance the capacity of organellar QC systems have evolved. In mitochondria, signal transduction mechanisms in response to metabolic conditions in the cell have been characterized. For instance, transcriptional regulatory pathways act via PGC-1 in mammalian cells or HAP/RTG transcription factors in yeast (*42*, *43*). Until recently, however, it was not clear whether there is a specific response to accumulation of misfolded proteins within mitochondria (termed mtUPR). First direct evidence for a response to mitochondrial stress was obtained by the analysis of genes response to overexpression of mutant ornitine transcarbamylase (OTC). In COS-7 cells, expression of mutant OTC, but not wild-type OTC induces mitochondrial QC components (*44*). Similar response has been reported in *Caenorhabditis elegans* which is triggered by non-assembled proteins but independent of either membrane potential across the IM or oxidative stress (*45*).

Recent progress in the discovery of elements involved in mtUPR revealed differences in the signalling pathway between mammals and nematodes (Fig. 3). In mammalian cells, the upregulation of the transcriptional activator complex CHOP-C/EBPβ through the MEK/JNK2 pathway appears to be required for mtUPR (*46*). Since CHOP-C/EBPβ are upregulated by ER stress, distinct transcriptional regulatory elements must control activation during mtUPR. A bioinformatic analysis of promoters of mtUPR responsive genes identified two conserved elements, MURE1 and MURE2 (*47*). These elements may be recognized by transcription factors specific for mtUPR.

By an RNAi-based genome-wide screen, Ron and co-workers (*48*, *49*) identified four genes which are responsible for mtUPR in *C. elegans*: *ubl-5*, *dve-1*, *clpp* and *rheb-1*. Ubl-5 is a highly conserved ubiquitin-like protein. Its depletion causes mitochondrial defects

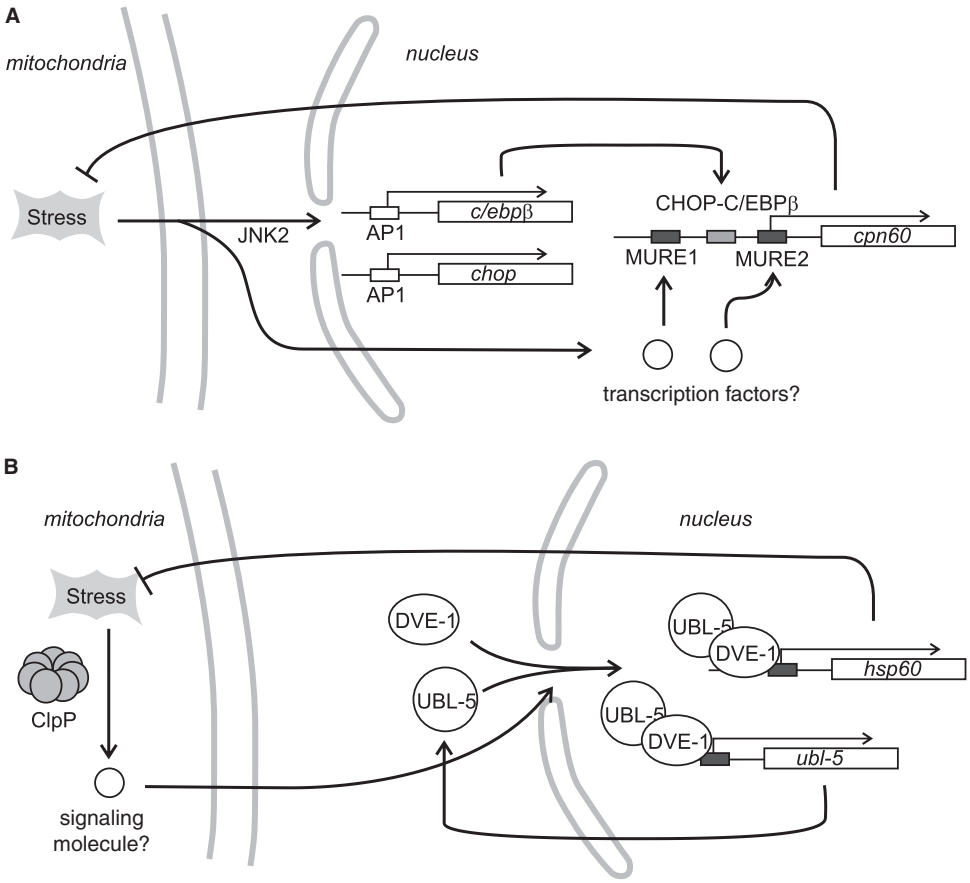

Fig. 3. **Proposed model for mtUPR in mammalian cells (A) and in nematodes (B).** See text for details.

associated with protein quality disturbance in mitochondria. Dve-1 is a homeobox transcription factor, which can bind the promoter region of the *hsp60* gene. Upon mtUPR, Ubl-5 is upregulated and translocates to the nucleus together with Dve-1. Recruitment of the Ubl-5-Dve-1 complex to the promoter of mtUPR responsive genes has been proposed.

However, a critical question remains to be determined: How do mitochondria transmit the stress signal to nucleus? Factors in mitochondria must contribute to mtUPR. In this regard, the finding of ClpP as a component of mtUPR is intriguing (*49*). It can be assumed that depletion of ClpP leads to accumulation of unfolded proteins and induces mtUPR. However, it blocks mtUPR in *C. elegans*. Therefore, ClpP may play a key role in mtUPR, most probably in the signal transmission cascade. Consistently, depletion of ClpP blocks induction of Ubl-5 upon mitochondrial stress, placing ClpP upstream of Ubl-5 and Dve-1. One possibility is that the Clp protease degrades a repressor protein for mtUPR in the matrix, which is reminiscent of the regulatory role of bacterial ClpXP during the extracytoplasmic stress response (*50*).

Another hypothesis is that degradation products (peptides) produced by ClpXP may work as a signal transmitter. If this is the case, only a set of specific peptides produced by ClpP may have signal transmission activity since depletion of LON or the *m*-AAA protease, which are major producers of peptide in the matrix, does not suppress the mtUPR. In *Saccharomyces cerevisiae*, it has been shown that peptides produced by proteases in the matrix are exported to the IMS in a process depending on the ABC-transporter Mdl1 (*32*, *51*). Since the OM is permeable for molecules less than ~1 kDa in size, small peptides in the IMS may reach the cytosol by passive diffusion. Although no evidence exists for an mtUPR-like stress response in *S. cerevisiae* (*52*), an orthologous peptide transport system in mitochondria of mammals and nematodes might play an essential role in mtUPR. Further investigation is necessary to find the missing link between mitochondria and the nucleus in mtUPR.

CONCLUDING REMARKS

Recent progress in the study of mitochondrial QC pathways has unveiled how cells maintain the protein quality in this organelle. It should be noted, however, that intramitochondrial QC is only a part of cellular strategies against degeneration of mitochondria. Dynamic regulation of mitochondrial morphology by fusion and fission plays a pivotal role in the maintenance of mitochondrial activities (*53*). Moreover, damaged mitochondria can be removed selectively by autophagy (termed mitophagy) (*54*). Thus, an intimate network of different cellular mechanisms is emerging, ensuring mitochondrial QC. In this regard, the involvement of AAA proteases in the maintenance of mitochondrial morphology is intriguing since it might link intramitochondrial QC and organelle-level QC events (*53*). Regulatory roles of ATP-dependent proteases by proteolysis of specific substrates may contribute to the maintenance of mitochondrial activities in different levels. The recent identification of many covalently modified proteins (e.g. ubiquitinated or phosphorylated) as well as putative E3 ligases or phosphatases/kinases in mitochondria may open a new area for the study of mitochondrial QC.

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CONFLICT OF INTEREST

None declared.

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