Critical Reviews in Biochemistry and Molecular Biology, 42:221–242, 2007

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230701380452

informa
healthcare

Protein Degradation within Mitochondria: Versatile Activities of AAA Proteases and Other Peptidases

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ABSTRACT Cell survival depends on essential processes in mitochondria. Various proteases within these organelles regulate mitochondrial biogenesis and ensure the complete degradation of excess or damaged proteins. Many of these proteases are highly conserved and ubiquitous in eukaryotic cells. They can be assigned to three functional classes: processing peptidases, which cleave off mitochondrial targeting sequences of nuclearly encoded proteins and process mitochondrial proteins with regulatory functions; ATP-dependent proteases, which either act as processing peptidases with regulatory functions or as quality-control enzymes degrading non-native polypeptides to peptides; and oligopeptidases, which degrade these peptides and mitochondrial targeting sequences to amino acids. Disturbances of protein degradation within mitochondria cause severe phenotypes in various organisms and can lead to the induction of apoptotic programmes and cell-specific neurodegeneration in mammals. After an overview of the proteolytic system of mitochondria, we will focus on versatile functions of ATP-dependent AAA proteases in the inner membrane. These conserved proteolytic machines conduct protein quality surveillance of mitochondrial inner membrane proteins, mediate vectorial protein dislocation from membranes, and, acting as processing enzymes, control ribosome assembly, mitochondrial protein synthesis, and mitochondrial fusion. Implications of these functions for cell-specific axonal degeneration in hereditary spastic paraplegia will be discussed.

KEYWORDS Mitochondria, protease, peptidase, AAA protease, paraplegin, rhomboid, ribosome assembly, translation, neurodegeneration, mitochondrial fusion

INTRODUCTION

Cell survival critically depends on the integrity and functionality of mitochondria. These organelles evolved from an endosymbiontic relationship of aerobic bacteria and primordial eukaryotic cells and are the major energy production site in "modern" eukaryotic cells (Wallace, 2005). Besides oxidative phosphorylation, crucial activities in intermediary metabolism, calcium signalling, and cell death pathways highlight the central role of mitochondria for cellular physiology (Chan, 2006a; McBride *et al.*, 2006). The mitochondrion

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generates the majority of cellular reactive oxygen species (ROS), but also provides specialized scavenging systems to protect itself and the cell from oxidative damage (Andreyev *et al.*, 2005). In agreement with the multitude of cellular functions, mitochondrial dysfunction is thought to contribute to cellular ageing and plays an important role in human diseases, such as diabetes, cancer, and prevalent neurodegenerative disorders (Chan, 2006a; Kwong *et al.*, 2006; Lin and Beal, 2006). Moreover, a number of rare genetic diseases are caused by mutations in mitochondrial proteins (DiMauro, 2004; Taylor and Turnbull, 2005; Schapira, 2006).

It is therefore an important cellular task to ensure correct mitochondrial biogenesis and to maintain mitochondrial activities under varying physiological conditions. Mitochondria are composed of ~1000 proteins (Mootha *et al.*, 2003; Sickmann *et al.*, 2003; Taylor *et al.*, 2003). The vast majority of them are nuclearly encoded, synthesized on cytosolic ribosomes, and imported into mitochondria. However, a small number of mitochondrial proteins, almost exclusively subunits of respiratory chain complexes in the inner membrane, are encoded by the mitochondrial genome (Anderson *et al.*, 1981; Foury *et al.*, 1998). They must assemble with nuclearly encoded subunits to constitute a functionally active respiratory chain and to allow oxidative phosphorylation. Maintenance of mitochondrial activities therefore depends on the coordinated expression of two cellular genomes. A number of regulatory circuits have been identified that allow the adjustment of nuclear gene expression to specific demands within mitochondria and ensure a balanced accumulation of nuclearly and mitochondrially encoded proteins (Zhao *et al.*, 2002; Yoneda *et al.*, 2004; Liu and Butow, 2006).

This task is even more demanding when the dynamic nature of mitochondria is taken into account. Mitochondria undergo constant fusion and fission and frequently change their shape in response to altered physiological conditions (Okamoto and Shaw, 2005). This dynamic behavior is crucial for a number of cellular processes, such as apoptosis, the inheritance of mitochondrial DNA, defence against oxidative stress, and development through spermatogenesis (Hales and Fuller, 1997; Chen and Chan, 2005; Chen and Butow, 2005; Cereghetti and Scorrano, 2006), but poses additional challenges for a coordinated nuclear and mitochondrial gene expression.

Mitochondria therefore must be able to eliminate excess, non-assembled polypeptides to avoid potential deleterious effects on organellar functions. Elaborate proteolytic systems which conduct the quality surveillance of mitochondrial proteins are present within different subcompartments of the organelle. A number of these proteases also exert essential housekeeping functions and control crucial steps during mitochondrial biogenesis. The maintenance of the mitochondrial genome, mitochondrial gene expression, and mitochondrial fusion and fission are examples for processes which are under proteolytic control (Van Dyck and Langer, 1999; Escobar-Henriques and Langer, 2006; Rugarli and Langer, 2006). On the other hand, mitochondrial proteases serve as gatekeepers for programmed cell death upon release from the organelle (Garrido and Kroemer, 2004; Saelens *et al.*, 2004).

Here, we will briefly summarize the current understanding of the proteolytic system of mitochondria and then focus on energy-dependent AAA proteases, which represent key players of this system in the inner membrane.

THE PROTEOLYTIC SYSTEM OF MITOCHONDRIA: AN OVERVIEW

Most mitochondrial proteins are stable and exhibit half-times of several days in mammalian cells (Druyan *et al.*, 1969; Ip *et al.*, 1974). In yeast, only 5% to 10% of the mitochondrial proteome is subject to degradation within a generation time (Augustin *et al.*, 2005). However, if mitochondria are damaged or become superfluous due to altered physiological demands, they can be removed by autophagy (Yorimitsu and Klionsky, 2005; Mijaljica *et al.*, 2007). This non-selective process allows the general adjustment of mitochondrial activities in a cell, but cannot explain different turnover rates of mitochondrial proteins (Russell *et al.*, 1980). This observation has therefore suggested from early on the existence of an independent proteolytic system within mitochondria. This conclusion was substantiated when it was realized that many mitochondrial proteins are synthesized in a precursor form on cytosolic ribosomes and undergo proteolytic maturation upon import into mitochondria. Since then, central components of the mitochondrial proteolytic system have been identified. Many of them are highly conserved and ubiquitously distributed in eukaryotic cells. They can be categorized

TABLE 1 **Mitochondrial peptidases**

Name	Location	Yeast	Function in yeast	Mammals	Function in mammals
Processing peptidases					
MPP	M	Mas1, Mas2	Presequence cleavage	α-MPP, β-MPP	See yeast
MIP	M	Oct1	Removal of octapeptides	MIPEP (HMIP)	See yeast
IMP	IM	Imp1, Imp2	Presequence cleavage	IMMP1L, IMMP2L	Unknown
	IMS	Atp23	Atp6 processing and assembly	KUB3	Unknown
Rhomboid	IM	Pcp1	Ccp1 and Mgm1 processing	PARL	Protection against apoptosis
ATP-dependent proteases					
i-AAA	IM	Yme1	Quality control	YME1L	Unknown
m-AAA	IM	Yta10, Yta12	Quality control, protein processing, membrane dislocation	paraplegin, AFG3L1*, AFG3L2	Quality control, cleavage of MrpL32 and OPA1
Lon	M	Pim1	Quality control, mtDNA maintenance and gene expression	LON	Quality control, mtDNA binding
ClpXP	M	—		ClpP, ClpX	Quality control
Oligopeptidases					
	IMS	Mop112	Degradation of peptides and presequences	PreP**	Aβ degradation[1]
	IMS	Prd1	Degradation of peptides and presequences	Neurolysin	Peptide degradation
	M	Lap3	Aminopeptidase, protection against homocysteine[2]	Bleomycin hydrolase	See yeast
Other proteases					
	IM	Oma1	Quality control	OMA1	Unknown
	IMS	—		HtrA2 (Omi)	Pro-apoptotic, cleavage of β-APP

*AFG3L1 is only expressed in mice but encoded by a pseudogene in humans.
**Human PreP has been localized to the matrix.
[1]Falkevall *et al.*, 2006.
[2]Zimny *et al.*, 2006.

into several classes according to their main activities (Figure 1, Table 1):

I. Processing Peptidases

Sorting signals of nuclearly encoded proteins are removed by specific processing peptidases present within different subcompartments of mitochondria (Gakh *et al.*, 2002). These enzymes generally exhibit rather degenerate sequence specificity and, in case of composite targeting sequences, can act sequentially. The mitochondrial processing peptidase (MPP), a conserved hetero-dimeric metallopeptidase, cleaves off sorting sequences in the matrix space (Hawlitschek *et al.*, 1988; Yang *et al.*, 1988; Ou *et al.*, 1989). Subunits of this conserved, hetero-dimeric metallopeptidase are highly homologous to the non-catalytic subunits core 1 and core 2 of cytochrome *c* reductase (complex III) (Schulte *et al.*, 1989; Gencic *et al.*, 1991). In some organisms, MPP subunits are bi-functional proteins or even integrated into complex III in case of plant mitochondria (Glaser and Dessi, 1999). Some mitochondrial precursor proteins are cleaved by a second processing peptidase in the matrix space (Kalousek *et al.*, 1988). The mitochondrial intermediate peptidase (MIP), a monomeric metallopeptidase, removes an octapeptide from preproteins after their processing by MPP (Isaya *et al.*, 1991; Isaya *et al.*, 1992; Kalousek *et al.*, 1992). The physiological role of MIP cleavage is currently poorly understood; however, severe phenotypes upon gene inactivation in yeast and mice suggest important regulatory functions (Isaya *et al.*, 1994; Gakh *et al.*, 2002). The so-called inner membrane peptidase IMP is located within the inner membrane but exposes its active sites to the intermembrane space of mitochondria

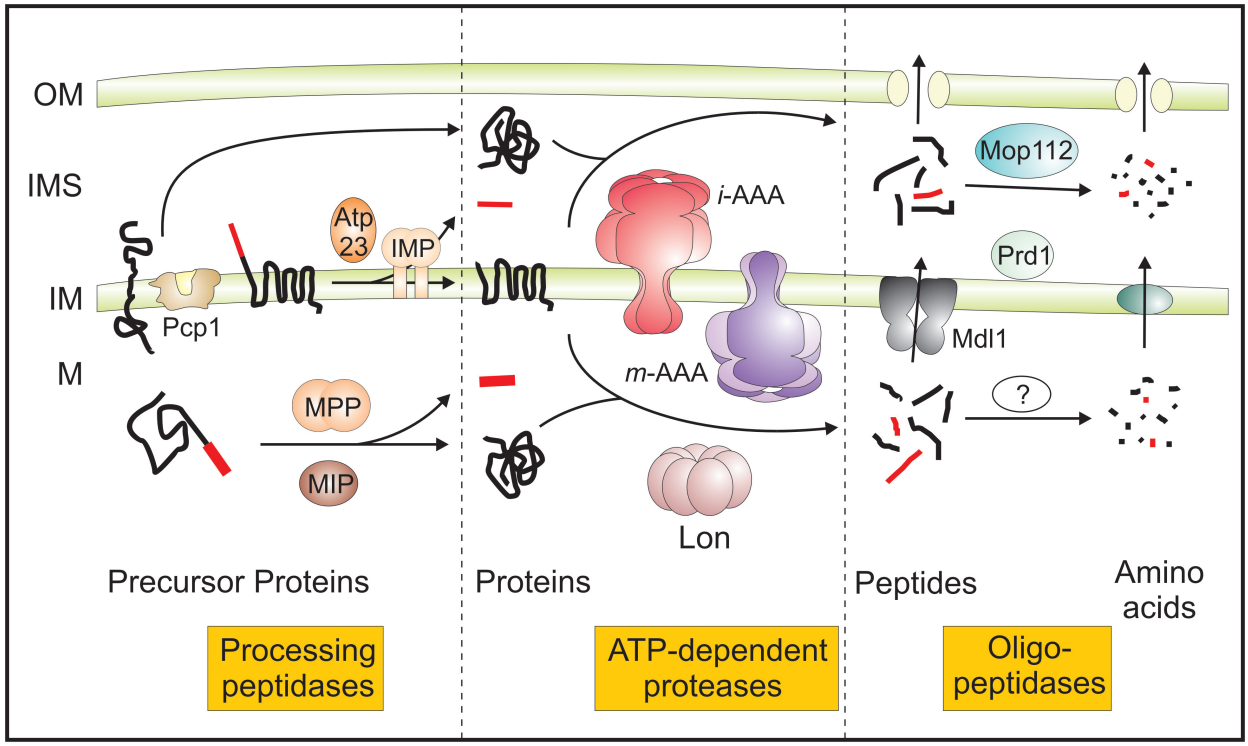

Figure 1 **Proteolytic pathways within mitochondria of *S. cerevisiae*. Mitochondrial proteases can be functionally classified into processing peptidases, ATP-dependent proteases, and oligopeptidases. Proteins residing in the intermembrane space (IMS), the inner membrane (IM), or the matrix (M) can be degraded by the consecutive action of ATP-dependent proteases and oligopeptidases to amino acids. The oligopeptidases Prd1 and Mop112 have been demonstrated to degrade peptides generated by the *i*-AAA protease. Although oligopeptidases are present in the matrix, it remains to be determined which of them act in concert with ATP-dependent proteases during protein turnover. See text for details. (OM, outer membrane).**

(Gakh *et al.*, 2002). This hetero-oligomeric complex is composed of a non-catalytic subunit, Som1, and two catalytic subunits containing serine/lysine dyads (Nunnari *et al.*, 1993; Schneider *et al.*, 1994; Jan *et al.*, 2000). Both catalytic subunits, Imp1 and Imp2, exert non-overlapping substrate specificity and are homologous to bacterial leader peptidase and thylakoidal processing peptidase (Halpin *et al.*, 1989; Behrens *et al.*, 1991). IMP mediates the maturation of intermediate forms of several nuclearly encoded proteins generated by MPP in the matrix space (Daum *et al.*, 1982; Nunnari *et al.*, 1993; Burri *et al.*, 2005), as well as mitochondrially encoded subunit 2 of cytochrome *c* oxidase (Cox2) (Pratje *et al.*, 1983).

In addition to these generic enzymes, several other mitochondrial peptidases can act as processing peptidases and regulate the biogenesis of mitochondria. These include energy-dependent AAA proteases, which are discussed in detail below, but also enzymes with apparently more specialized functions. The conserved metallopeptidase Atp23 cleaves off the presequence of the mitochondrially encoded subunit Atp6 of the F_1F_O-ATP synthase complex in the intermembrane space after its insertion into the inner membrane (Osman *et al.*, 2007; Zeng *et al.*, 2007). Independent of its proteolytic activity, Atp23 acts as a chaperone protein and promotes the assembly of Atp6 into the membrane-embedded F_O-moiety of the ATP synthase complex (Osman *et al.*, 2007; Zeng *et al.*, 2007). Atp6 is the only known substrate of Atp23. However, the presence of an Atp23 homologue in mammalian cells, which synthesize mitochondrially encoded Atp6 without a cleavable presequence, suggests the existence of additional substrate proteins. A processing peptidase with a regulatory role for mitochondrial biogenesis is represented by the rhomboid protease in the inner membrane (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003). Rhomboid proteases comprise a conserved family of membrane-embedded serine peptidases, which possess catalytic residues within their hydrophobic, membrane-spanning domains and which generate soluble, biologically active protein fragments by cleaving membrane-anchored proteins within transmembrane segments (Urban, 2006). The yeast rhomboid Pcp1 cleaves the dynamin-like GTPase Mgm1, a central component of the mitochondrial fusion machinery, and generates a

short isoform of Mgm1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003). Such cleavage occurs after removal of the mitochondrial targeting sequence of newly imported Mgm1 by MPP (Herlan *et al.*, 2003). As mitochondrial fusion depends on both the long and the short isoform of Mgm1, Pcp1-mediated processing affects mitochondrial dynamics (Herlan *et al.*, 2003). Interestingly, Mgm1 cleavage by Pcp1 was found to depend on mitochondrial Hsp70 and the cellular ATP levels (Herlan *et al.*, 2004). It may therefore function as an energy sensor regulating mitochondrial fusion and allowing the segregation of damaged mitochondria from the network of intact mitochondria (Herlan *et al.*, 2004; Duvezin-Caubet *et al.*, 2006). The second known substrate of Pcp1 is the mitochondrial ROS-scavenger cytochrome *c* peroxidase (Ccp1) (Esser *et al.*, 2002). A bipartite presequence targets Ccp1 to the mitochondrial intermembrane space and is removed by rhomboid after its vectorial membrane dislocation by the ATP-dependent *m*-AAA protease (Tatsuta *et al.*, 2007) (see below). The function of mammalian rhomboid PARL (presenilin-associated rhomboid-like) (Pellegrini *et al.*, 2001) in mitochondria is less clear. PARL can functionally replace yeast Pcp1 and was found to directly interact with the mammalian Mgm1-homologue OPA1 (McQuibban *et al.*, 2003; Cipolat *et al.*, 2006). The analysis of PARL-deficient mice revealed a protective role of PARL against mitochondria-dependent apoptosis in a pathway which also depends on OPA1 (Cipolat *et al.*, 2006). However, deletion of PARL did not significantly impair OPA1 processing suggesting that at most a small fraction of OPA1 is processed by rhomboid (Cipolat *et al.*, 2006). Notably, RNAi-mediated downregulation of the murine *m*-AAA protease subunit paraplegin (see below) has also linked this protease to OPA1 processing (Ishihara *et al.*, 2006). It remains therefore a hotly debated question which mitochondrial protease(s) are involved in the cleavage of OPA1. The answer to this question will be also of significant medical relevance as mutations in OPA1 are causative for neurodegeneration in dominant optic atrophy type 1 (Alexander *et al.*, 2000; Delettre *et al.*, 2000).

II. ATP-Dependent proteases

Proteases powered by ATP have been identified in various subcompartments of mitochondria. They generally form multimeric protein complexes, which constitute sequestered, proteolytic microcompartments, and contain P-loop ATPase domains characteristic of the AAA^+-family of ATPases (Sauer *et al.*, 2004; Hanson and Whiteheart, 2005). The energy derived from ATP-hydrolysis is utilized to unfold specific substrate proteins and to transport them into this internal proteolytic cavity (Baker and Sauer, 2006). The importance of ATP-dependent proteases for mitochondrial functions is illustrated by severe phenotypes associated with the loss of these proteases in yeast (Van Dyck and Langer, 1999). Although these phenotypes are only in a few cases understood on the molecular level, they appear to reflect two activities of the proteases: protein quality surveillance and proteolytic control of regulatory steps during mitochondrial biogenesis.

Mitochondrial ATP-dependent proteases are closely related to bacterial enzymes and can be assigned to highly conserved protease families, the Lon proteases, the Clp proteases, and FtsH-like AAA proteases (Figure 1; Table 1).

The Lon protease (or PIM1 protease in yeast) is localized in the mitochondrial matrix space (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Wang *et al.*, 1994). This serine protease forms homo-oligomeric complexes, which, based on cryoelectron microscopy studies on purified PIM1 protease, represent flexible, ring-shaped heptamers (Stahlberg *et al.*, 1999). The subunits are nuclearly encoded and synthesized in a prepro-form. After import into mitochondria, maturation and thereby activation of the subunits occurs in a two-step process: the mitochondrial targeting sequence is removed by MPP followed by protease assembly and autocatalytic cleavage of the pro-region (Wagner *et al.*, 1997). The PIM1/Lon protease conducts protein quality control in the matrix space, a function which is of particular importance under stress conditions, when thermally denatured or oxidatively damaged proteins accumulate (Bota and Davies, 2002). The protease recognizes misfolded or damaged proteins, mediates their complete proteolysis, and thereby prevents their accumulation and deleterious effects on mitochondrial activities. PIM1 requires a large and exposed non-native protein segment for selective recognition and degradation, but can also degrade folded substrates *in vitro* (Ondrovicova *et al.*, 2005; von Janowsky *et al.*, 2005). Mapping of peptides generated by Lon-mediated proteolysis revealed that initial cleavages occur between hydrophobic amino acids, which are exposed at the surface of folded proteins and surrounded by highly

charged environments (Ondrovicova *et al.*, 2005). A prerequisite for proteolysis appears to be that substrates are kept in a soluble conformation and do not aggregate. This is ensured by molecular chaperones of the Hsp70 family which were shown to cooperate with PIM1/Lon protease in the proteolytic degradation of non-native polypeptides (Wagner *et al.*, 1994; Sav'lev *et al.*, 1998). However, additional functions of molecular chaperone proteins, perhaps for substrate recognition, are possible. The ClpB-homologue Hsp78 is required for the protection of mitochondrial activities against thermal stress (Schmitt *et al.*, 1996; Germaniuk *et al.*, 2002; Lewandowska *et al.*, 2006). Hsp78 has been linked to the Lon-mediated degradation of model substrates not prone to aggregation (Bateman *et al.*, 2002; Röttgers *et al.*, 2002). In addition to its function in protein quality control, regulatory functions of the Lon protease during mitochondrial biogenesis are suggested by phenotypes associated with the loss of PIM1 protease in yeast. Deletion of the *PIM1* gene causes the destabilization of the mitochondrial genome, impairs mitochondrial gene expression, and results in respiratory deficiency (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Van Dyck *et al.*, 1998a). Although in the absence of Pim1 deleterious effects of accumulating, non-native polypeptides on the inheritance of mitochondrial DNA cannot be excluded, the peculiar binding affinity of mitochondrial Lon proteases for nucleic acids points to a more specific role of these proteases for DNA maintenance (Fu and Markovitz, 1998; Lu *et al.*, 2003; Liu *et al.*, 2004). It is conceivable that Lon proteases affect mitochondrial DNA stability by complete proteolysis or by processing of a regulatory protein. The autocatalytic maturation of newly imported Pim1 subunits in yeast illustrates that Lon proteases, like the *m*-AAA protease (see below), can indeed act as a processing peptidase.

A second ATP-dependent protease, the ClpXP protease, is present in the matrix of mammalian mitochondria (De Sagarra *et al.*, 1999; Santagata *et al.*, 1999). This hetero-oligomeric protease is built up of proteolytic ClpP subunits with serine peptidase activity and of ClpX subunits, which exert ATPase and, most likely, chaperone activity and confer substrate specificity to the complex (Kang *et al.*, 2002; Kang *et al.*, 2005). Notably, yeast mitochondria harbour only ATPase but no proteolytic ClpP subunits suggesting non-proteolytic functions (Van Dyck *et al.*, 1998b). The role of Clp proteases within mitochondria is currently not understood. The ClpP-mediated turnover of a misfolded model substrate and the increased expression of ClpP under cellular stress in mammalian cells suggest a similar role of mitochondrial Clp and Lon proteases for protein quality control (Zhao *et al.*, 2002).

Members of a third class of ATP-dependent proteases, the AAA proteases, are present within the inner membrane of mitochondria and expose their catalytic sites either to the matrix or the intermembrane space (Figure 2) (Langer, 2000). Accordingly, they were termed *m*-AAA protease, active on the matrix side, and *i*-AAA protease, active on the intermembrane side (Figure 2). AAA proteases form large complexes, which are composed of closely related or identical subunits of 70 to 80 kDa (Arlt *et al.*, 1996; Leonhard *et al.*, 1996;

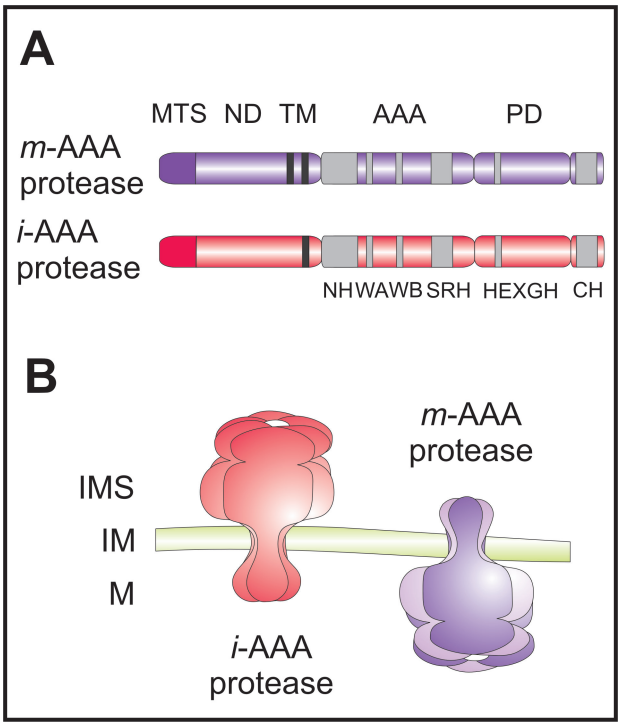

Figure 2 **Mitochondrial AAA proteases. (*A*.) Domain structure of AAA protease subunits. Subunits of *i*- and *m*-AAA protease complexes harbour one or two transmembrane domains (TM), respectively. Conserved sequence motifs within domains are indicated with grey boxes. MTS, mitochondrial targeting sequence; ND, N-terminal domain; AAA, AAA domain; PD, proteolytic domain; NH, N-terminal helices; WA, Walker A-motif; WB, Walker B-motif; SRH, second region of homology; HEXGH, proteolytic centre; CH, C-terminal helices. (*B*.) Membrane topology of AAA proteases. The stoichiometry and arrangement of mitochondrial AAA protease subunits within a complex is still speculative. *m*-AAA proteases with variable subunit composition are present in the inner membrane of mammalian mitochondria (see Figure 5). (IMS, intermembrane space; IM, inner membrane; M, matrix space).**

Klanner *et al.*, 2001; Atorino *et al.*, 2003; Urantowka *et al.*, 2005) and which, based on the crystal structure of the catalytic domains of homologous eubacterial FtsH proteases, represent hexameric, cylinder-like structures (Bieniossek *et al.*, 2006; Suno *et al.*, 2006). An N-terminal mitochondrial sorting signal targets the nuclearly encoded subunits to mitochondria. While *m*-AAA protease subunits are anchored to the inner membrane by two transmembrane segments present in the N-terminal part, *i*-AAA protease subunits span the inner membrane only once (Figure 2). Accordingly, catalytic domains following the membrane-embedded domains are exposed to the intermembrane or matrix space. The AAA domain contains Walker A and Walker B motifs involved in ATP binding and hydrolysis, respectively, and the so-called second region of homology (SRH) (Hanson and Whiteheart, 2005). Two conserved arginine residues in this region protrude into the catalytic site of adjacent subunits and thereby stimulate ATP hydrolysis (Hanson and Whiteheart, 2005). Mutational alterations of these arginine fingers in bacterial and mitochondrial *m*-AAA protease subunits impaired ATPase activity (Karata *et al.*, 1999; Karata *et al.*, 2001; Korbel *et al.*, 2004). Therefore, assembly of AAA protease subunits is a prerequisite for ATP-dependent proteolysis. AAA proteases belong to the M41 family of metallopeptidases characterized by the consensus metal-binding motif HExxH (x represents a variable amino acid residue) (Rawlings and Barrett, 1995). Mutation of the glutamate residue within the proteolytic centre inhibits protein degradation by AAA proteases (Arlt *et al.*, 1996; Guélin *et al.*, 1996; Leonhard *et al.*, 1996; Weber *et al.*, 1996; Atorino *et al.*, 2003; Koppen *et al.*, 2007). Notably, proteolytic activity is only completely abolished if all subunits harbor mutations in their proteolytic centres (Arlt *et al.*, 1998). Hetero-oligomeric mutant *m*-AAA proteases still containing wild type subunits exhibit residual activity and are capable of processing protein substrates (Arlt *et al.*, 1998; Koppen *et al.*, 2007).

Taken together, it can be concluded that energy-dependent proteases homologous to Lon, Clp, and AAA proteases have been identified in all mitochondrial subcompartments except the outer membrane. Proteins in this compartment appear to be degraded by the cytosolic ubiquitin-proteasome system as suggested by recent studies in yeast and mammalian cells (Neutzner and Youle, 2005; Escobar-Henriques *et al.*, 2006; Nakamura *et al.*, 2006; Yonashiro *et al.*, 2006).

III. Oligopeptidases

Oligopeptidases are, with few exceptions, the most poorly characterized mitochondrial proteases. Their existence was suggested by the observation that polypeptides can be completely degraded to amino acids within mitochondria (Desautels and Goldberg, 1982; Young *et al.*, 2001). As ATP-dependent proteases are known to degrade proteins processively to peptides, proteolysis was expected to be completed by other proteins with oligopeptidase activity (Figure 1). Thimet oligopeptidases were the first to be identified in the mitochondrial intermembrane space in various organisms, some of them representing isoforms of cytosolic enzymes (Garcia-Alvarez *et al.*, 1987; Büchler *et al.*, 1994; Serizawa *et al.*, 1995; Krause *et al.*, 1997; Serizawa *et al.*, 1997). *In vitro* experiments documented oligopeptidase activity and revealed broad substrate specificity (Büchler *et al.*, 1994; Serizawa *et al.*, 1995; Krause *et al.*, 1997). However, evidence for their *in vivo* function is scarce. Yeast mutant cells lacking mitochondrial oligopeptidases show usually no or only mild phenotypes, most likely due to the redundant activity of various enzymes (Garcia-Alvarez *et al.*, 1987; Büchler *et al.*, 1994; Kambacheld *et al.*, 2005). Moreover, mitochondria contain a peptide export system that allows the efficient extrusion of peptides and prevents their accumulation in case of an impaired proteolysis. The ABC-transporter protein Mdl1 has been linked to this transport process (Figure 1) (Young *et al.*, 2001). It has been speculated that peptides generated by the proteolytic breakdown of mitochondrial proteins may serve a signalling function to coordinate mitochondrial and nuclear gene expression (Young *et al.*, 2001; Arnold *et al.*, 2006), but direct evidence is still lacking. Nevertheless, a quantitative assessment of mitochondrial peptide export and the mass-spectrometric identification of peptides released from yeast mitochondria allowed an estimation of the stability of the mitochondrial proteome and provided first evidence for the function of oligopeptidases *in vivo* (Augustin *et al.*, 2005; Kambacheld *et al.*, 2005). Two oligopeptidases were found to cleave peptides generated by the *i*-AAA protease in the intermembrane space (Figure 1) (Kambacheld *et al.*, 2005): the thimet oligopeptidase Prd1 (or saccharolysin) and a second metallopeptidase Mop112/Cym1 (or PreP1 and PreP2 in *Arabidopsis thaliana* and PreP in human) (Jonson *et al.*, 2004; Kambacheld *et al.*, 2005; Falkevall *et al.*,

2006; Glaser *et al.*, 2006). A large cavity occurred in the crystal structure of PreP1 of *A. thaliana*, which allows peptide cleavage with a preference for basic and small uncharged amino acids (Stahl *et al.*, 2005; Johnson *et al.*, 2006). Peptides degraded by this group of peptidases also include various mitochondrial targeting signals cleaved off by specific processing peptidases (Stahl *et al.*, 2002; Moberg *et al.*, 2003; Kambacheld *et al.*, 2005). Interestingly, the yeast oligopeptidases Prd1 and Mop112 also mediate the proteolytic breakdown of peptides, which were initially generated in the matrix and then transported into the intermembrane space (Kambacheld *et al.*, 2005). They thus can contribute to the complete degradation of proteins localized in another mitochondrial subcompartment. Although accordingly oligopeptidases in the matrix space appear to be dispensable, several enzymes with proposed oligopeptidase activity have been identified in this compartment. This also includes the bleomycin hydrolase Lap3, a cysteine peptidase which is localized both in the cytosol and the mitochondrial matrix space in yeast (Kambouris *et al.*, 1992; Enenkel and Wolf, 1993; Magdolen *et al.*, 1993; Huh *et al.*, 2003; Sickmann *et al.*, 2003). A functional characterization of the *in vivo* function of matrix-localized oligopeptidases, however, is still awaited. It is likely that they act in concert with ATP-dependent proteases in the proteolysis of matrix proteins and are thus part of a similar pathway as present in the intermembrane space (Figure 1).

IV. Other Mitochondrial Proteases

A number of additional proteases are present within mitochondria but their functional classification into the above mentioned categories remains unclear. The metallopeptidase Oma1 has been identified in a genome-wide screen for mitochondrial peptidases in yeast and was localized to the inner membrane of mitochondria with its catalytic site facing the matrix space (Käser *et al.*, 2003). Oma1 is a member of a conserved and widespread family of membrane-integrated metallopeptidases and was shown to cleave a misfolded polytopic membrane protein at multiple sites (Käser *et al.*, 2003). It therefore exerts a function similar to the *m*-AAA protease for quality control of inner membrane proteins and has been proposed to be part of a salvage system under conditions of limited AAA protease activity. This is reminiscent of the homologous protease HtpX, a stress-controlled protease in the plasmamembrane of *Escherichia coli*, whose activity overlaps with the AAA protease FtsH (Shimohata *et al.*, 2002).

HtrA2 (Omi, Prss25) is localized in the intermembrane space of mammalian mitochondria (Suzuki *et al.*, 2001; Hegde *et al.*, 2002). It is a member of a conserved family of oligomeric serine peptidases, which were functionally linked to protein quality control in various organisms (Clausen *et al.*, 2002; Kim and Kim, 2005). Mitochondrial HtrA2 has been identified as a pro-apoptotic XIAP-binding protein in the cytosol and was demonstrated to be released from mitochondria in apoptotic cells (Suzuki *et al.*, 2001; Hegde *et al.*, 2002; Martins *et al.*, 2002; van Loo *et al.*, 2002; Verhagen *et al.*, 2002). A pro-apoptotic function of HtrA2, however, was recently challenged as targeted deletion of HtrA2 in mice did not impair the rate of apoptosis, but led to a selective loss of neurons in the striatum (Martins *et al.*, 2004). Consistently, a missense mutation inactivating HtrA2 in mice causes a neuromuscular disorder (Jones *et al.*, 2003). The molecular basis of these phenotypes, as the function of HtrA2 within mitochondria, is currently not understood. Murine embryonic fibroblasts lacking HtrA2 exhibit an increased sensitivity to stress-induced cell death (Martins *et al.*, 2004). It is therefore conceivable that, in analogy to homologous bacterial proteases, HtrA2 conducts protein quality control and protects mitochondrial activities under stress conditions (Clausen *et al.*, 2002; Kim and Kim, 2005). HtrA2 may directly degrade misfolded polypeptides in the intermembrane space or, acting as stress-sensor like bacterial DegS (Walsh *et al.*, 2003), may be part of a cellular transcriptional response toward protein misfolding. Evidence for a processing activity of HtrA2 was recently provided analysing cleavage of the β-amyloid precursor protein *in vivo* and *in vitro* (Park *et al.*, 2006a).

QUALITY CONTROL OF MEMBRANE PROTEINS BY AAA PROTEASES

The mitochondrial inner membrane represents the protein-richest cellular membrane and harbors a large fraction of the mitochondrial proteome, including the respiratory chain. The surveillance of protein quality is therefore of major importance for organellar functions. Non-native and non-assembled polypeptides are recognized and degraded by two membrane-embedded

AAA proteases, the *m*- and the *i*-AAA protease (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). These proteases exhibit degenerate substrate specificity and, similar to molecular chaperone proteins, recognize the folding state of solvent-exposed domains of membrane proteins (Leonhard *et al.*, 1999). The involvement of one or the other protease therefore appears to be mainly driven by the membrane topology of substrate proteins. Surprisingly, biochemical and genetic evidence in yeast demonstrates overlapping specificity of both AAA proteases, although they expose their catalytic sites to opposite membrane surfaces (Lemaire *et al.*, 2000; Leonhard *et al.*, 2000). Proteins exposing unfolded domains at both membrane surfaces were found being degraded by either protease. As no proteolytic fragments accumulated after inactivation of one protease, it has been concluded that complete degradation of these proteins to peptides can be mediated by only one protease, irrespective on which side of the membrane its catalytic sites are located. N- or C-tails of ~20 amino acids protruding from the membrane bilayer are sufficient to allow the proteolytic attack by an AAA protease and the complete degradation of domains located at the opposite side of the membrane (Leonhard *et al.*, 2000). These experiments therefore provided first evidence for a dislocation of substrate proteins from the membrane during proteolysis. A recent study on the processing of cytochrome *c* peroxidase (Ccp1) directly demonstrated the ability of the *m*-AAA protease to mediate vectorial membrane dislocation of proteins in an ATP-dependent reaction (see below) (Tatsuta *et al.*, 2007). While this can be considered as the reversal of protein insertion during mitochondrial biogenesis, protein translocases residing in the inner membrane are apparently not involved in proteolysis by AAA proteases (Korbel *et al.*, 2004). It is conceivable that the membrane-embedded parts of AAA protease subunits facilitate the membrane extraction of substrate proteins by forming a pore-like structure or by providing at least a more hydrophilic environment. Consistently, mutational analysis of the yeast *m*-AAA protease indicated that the membrane-spanning segments of all subunits are essential for the degradation of integral membrane proteins, but not for proteolysis of a peripheral model substrate or for protein processing (Korbel *et al.*, 2004).

While the folding state has been recognized as being important for recognition by AAA proteases, further determinants ensuring the specificity of proteolysis are likely to exist. Striking differences in substrate specificity have been observed between orthologous *i*-AAA proteases from yeast and the filamentous fungus *Neurospora crassa* which are built up of Yme1 and IAP-1 subunits, respectively (Leonhard *et al.*, 1996; Weber *et al.*, 1996; Klanner *et al.*, 2001). Non-assembled subunits of mitochondrially encoded Cox2 in yeast were degraded by the yeast (Nakai *et al.*, 1995; Pearce and Sherman, 1995; Weber *et al.*, 1996) but not the *N. crassa* enzyme (Graef *et al.*, 2007). Mutagenesis studies on Yme1 and domain-swap experiments between the homologous *i*-AAA protease subunits allowed the identification of two helical binding regions, which form a lattice-like structure at the surface of the proteolytic cylinder and mediate the initial encounter of substrate proteins with the protease (Figure 3) (Leonhard *et al.*, 1999; Graef *et al.*, 2007): helices C-terminal of the proteolytic domain (CH) and N-terminal helices of the AAA domain (NH), which are located in close proximity to the membrane surfaces and highly negatively charged. Thus, substrate proteins initially interact with the *i*-AAA protease at the outer surface of the proteolytic cylinder, before they enter the proteolytic chamber (Graef *et al.*, 2007). Interestingly, the binding properties of the surface-exposed interaction sites vary suggesting that alternative pathways for substrate entry into the proteolytic chamber of *i*-AAA proteases exist. The CH-region is only required for the binding and degradation of a subset of proteins. Its involvement

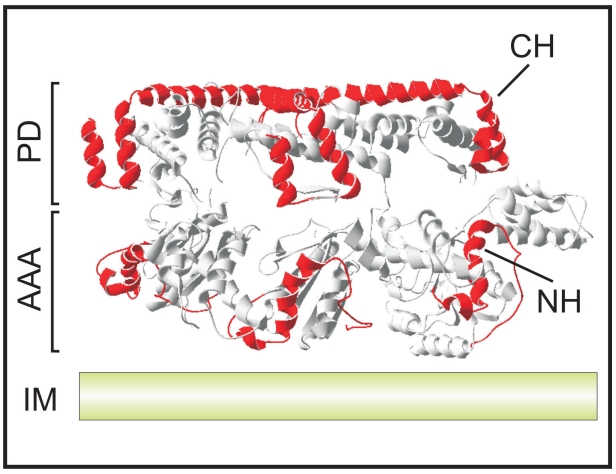

Figure 3 **Substrate engagement by the *i*-AAA protease. A lattice-like arrangement of the substrate binding regions CH (C-terminal helices) and NH (N-terminal helices) at the surface of the proteolytic cylinder of an AAA protease. Helices homologous to CH and NH of Yme1 are marked in red in the crystal structure of *T. maritima* FtsH (Bieniossek *et al.*, 2006). Only three subunits are shown. AAA, AAA domain; PD, proteolytic domain; IM, inner membrane.**

depends on the membrane insertion of substrates, their folding state as well as substrate-specific co-factors. For instance, degradation of newly imported, non-assembled subunits of the prohibitin complex requires the CH-region of Yme1, but this dependence is alleviated if these subunits are integrated into the inner membrane (Graef *et al.*, 2007). The distance of an unfolded domain from the membrane surface, therefore, is most likely one parameter determining the involvement of the CH-region for substrate binding. In case of non-assembled Cox2, destabilization of the solvent-exposed Cox2 domain at high temperatures by incorporation of amino acid analogues renders proteolysis by the *i*-AAA protease CH-dependent (Graef *et al.*, 2007). In contrast to the CH-region, the NH-region appears to be generally involved in proteolysis. It is therefore likely that CH-dependent substrates bind in a sequential manner at CH- and NH-regions before they enter the proteolytic chamber through the central pore formed by the AAA domains.

First evidence for substrate translocation into a proteolytic chamber through the central pore of mitochondrial AAA proteases has been obtained by mutational analysis of a conserved loop motif YVG (aromatic-hydrophobic-glycine) in Yme1 (Graef and Langer, 2006). This loop motif has been localized to the central pore of other hexameric AAA^+ ring complexes (Wang *et al.*, 2001). Proteolytic activity of Yme1 depends on the presence of hydrophobic amino acids at position 354 (Graef and Langer, 2006). Mutations of Y354 impaired binding of membrane proteins to the *i*-AAA protease in a substrate-specific manner, but did not prevent binding of unfolded substrates to the CH-region (Graef and Langer, 2006; Graef *et al.*, 2007). The effect of central pore mutations at a post-binding step suggests an essential function for the ATP-dependent substrate translocation into the proteolytic chamber of the *i*-AAA protease.

Increasing evidence suggests that substrate binding and proteolysis by AAA proteases is modulated by additional factors within mitochondria. Some of them appear to act in a substrate-specific manner as exemplified by Cox20, which affects proteolysis of Cox2 by the *i*-AAA protease (Graef *et al.*, 2007). Cox20 has been originally identified as a chaperone in the inner membrane, which is required for IMP-mediated processing and assembly of newly synthesized Cox2 (Hell *et al.*, 2000). While dispensable for proteolysis, Cox20 modulates recognition of non-assembled Cox2 subunits by the *i*-AAA protease and appears to exert a function overlapping with the CH-region of Yme1. Proteolysis (and binding) of non-assembled Cox2 was found to be strictly dependent on the CH-region in Cox20-deficient mitochondria, but not in the presence of Cox20 (Graef *et al.*, 2007). How Cox20 acts on a molecular level remains to be elucidated; however, it is conceivable that Cox20 is a holdase for Cox2 facilitating its assembly or, if Cox2 folding is impaired, its proteolysis by the *i*-AAA protease.

Another co-factor of the *i*-AAA protease was identified in a genome-wide screen for yeast mutants whose cell growth depends on the presence of mitochondrial DNA (Dunn *et al.*, 2006). Similar to Δ*yme1* cells, cells lacking the mitochondrial intermembrane space protein Mgr1 were not viable if mitochondrial DNA is lost. Mgr1 is part of the Yme1 complex but is not essential for proteolysis (Dunn *et al.*, 2006) suggesting that it does not represent a *bona fide* subunit of the *i*-AAA protease. Rather, available data indicate that Mgr1 may act as an adaptor-like protein which targets specific substrates in the intermembrane space for degradation by the *i*-AAA protease.

Substrate-specific co-factors of the *m*-AAA protease are currently not known. However, the yeast *m*-AAA protease is part of a large supercomplex with prohibitins which modulate proteolysis (Steglich *et al.*, 1999). Two ubiquitous and highly conserved subunits, Phb1 and Phb2, assemble into a multimeric complex which is exposed to the intermembrane space and anchored N-terminally to the inner membrane (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000; Artal-Sanz *et al.*, 2003). Similarly, *E. coli* FtsH assembles quantitatively with a complex built up of HflK and HflC, which both are distantly related to mitochondrial prohibitins (Kihara *et al.*, 1996). Single particle analysis of purified prohibitin complexes revealed ring-like assemblies with a diameter of ~20 to 25 nm suggesting that they may exert a scaffolding function in the mitochondrial inner membrane (Tatsuta *et al.*, 2005). How they affect proteolysis by the *m*-AAA protease is currently not understood. Deletion of prohibitins results in accelerated protein degradation by the *m*-AAA protease (Steglich *et al.*, 1999). It is conceivable that prohibitins modulate proteolysis by affecting substrate access or the lipid environment of the *m*-AAA protease. Although yeast cells lacking prohibitins do not exert strong growth defects (Coates *et al.*, 1997; Berger and Yaffe, 1998), severe synthetic growth defects with several

mutations have been reported (Berger and Yaffe, 1998; Birner *et al.*, 2003). These include mutations in the subunits of the yeast *m*-AAA protease, Yta10 and Yta12, as well as mutations in Atp23, a chaperone and processing peptidase for Atp6 in the intermembrane space (Steglich *et al.*, 1999; Osman *et al.*, 2007). The molecular characterization of these genetic interactions in yeast may also shed new light on the function of prohibitins in mammalian cells, which have been linked to a large variety of mitochondrial and non-mitochondrial processes (Mishra *et al.*, 2006).

AAA PROTEASES AND THE REGULATION OF MITOCHONDRIAL BIOGENESIS

The central role of AAA proteases for protein quality control within mitochondria raises the intriguing question whether phenotypes associated with mutations in AAA proteases are caused by misfolded polypeptides accumulating in the absence of the proteases. Inactivation of individual AAA proteases in yeast has severe impact on mitochondrial functions, while yeast cells lacking both proteases are not viable (Lemaire *et al.*, 2000; Leonhard *et al.*, 2000). In general, identical phenotypes have been observed after deletion of AAA proteases or mutating the proteolytic sites of all subunits of AAA protease complexes, suggesting that the observed defects result from impaired proteolytic functions of AAA proteases within mitochondria (Leonhard *et al.*, 1996; Weber *et al.*, 1996; Arlt *et al.*, 1998).

The *i*-AAA protease subunit Yme1 was first identified in a genetic screen for yeast mutants resulting in an increased rate of DNA escape from mitochondria to the nucleus (Thorsness and Fox, 1993). Yme1 is required for respiratory growth of yeast cells at high temperatures and for fermenting growth at low temperature (Thorsness and Fox, 1993). Moreover, *yme1* mutant cells are petite negative, *i.e.*, exhibit a strong growth phenotype when the mitochondrial DNA is deleted (Thorsness *et al.*, 1993). The identification of specific suppressors for individual phenotypes of Δ*yme1* cells may indicate that the impaired proteolysis of different substrate proteins, rather than deleterious effects of misfolded polypeptides, are causative for these deficiencies (Weber *et al.*, 1995; Hanekamp and Thorsness, 1996; Park *et al.*, 2006b). Similar to the situation in yeast, inactivation of the *i*-AAA protease subunit IAP-1 of *N. crassa* causes impaired respiratory growth at high temperature, pointing to conserved functions of *i*-AAA proteases within mitochondria (Klanner *et al.*, 2001). This conclusion is further substantiated by a series of complementation studies: expression of IAP-1 in yeast restored fermentative growth at low temperature and converted Δ*yme1* into petite positive cells (Klanner *et al.*, 2001). Similarly, the petite negative yeast *Schizosaccharomyces pombe* can be converted to petite positive by the expression of yeast Yme1 (Kominsky and Thorsness, 2000). Moreover, the expression of the human homologue YME1L allowed growth on non-fermentable carbon sources at high temperature (Shah *et al.*, 2000; Klanner *et al.*, 2001). While these studies demonstrate functional conservation, differences in both the roles of *i*-AAA proteases within mitochondria and their substrate specificity appear to exist. Mitochondria do not form a reticulated network in Yme1-deficient yeast cells and accumulate in a punctuate form with grossly swollen compartments suggesting a role of Yme1 in the maintenance of mitochondrial morphology in yeast (Campbell *et al.*, 1994). This phenotype can be suppressed by IAP-1 although the morphology of mitochondria is not affected in *N. crassa* cells lacking IAP-1. On the other hand, IAP-1 does not restore respiratory growth at elevated temperature in yeast Δ*yme1* cells nor does it allow the proteolysis of non-assembled Cox2 (Klanner *et al.*, 2001; Graef *et al.*, 2007). Although these observations suggest the existence of specific substrates of the *i*-AAA protease within mitochondria, whose impaired proteolysis explains phenotypes of protease-deficient cells, their identification is still awaited. The impaired proteolysis of specific substrates may also underlie the synthetic lethality of double mutant yeast cells lacking both AAA proteases. A first hint to the identity of such substrates came from the observation that the lethality can be suppressed by mutations in Atp3, the γ-subunit of the ATP synthase, (Dunn *et al.*, 2006). These mutations increase the ATPase activity of the F_1 portion and therefore may ensure the maintenance of the mitochondrial membrane potential in cells lacking both AAA proteases.

Significant progress has been obtained in the understanding of growth defects associated with mutations in the yeast *m*-AAA protease. In yeast, the *m*-AAA protease is hetero-oligomeric and composed of two closely related subunits, Yta10 (Afg3) and Yta12 (Rca1), which assemble in an ATP-dependent manner into *m*-AAA

protease complexes (Arlt *et al.*, 1996). Yeast cells lacking either subunit or expressing proteolytically inactive variants of both subunits are respiratory deficient (Guélin *et al.*, 1994; Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994; Arlt *et al.*, 1998), lack assembled respiratory chain and ATP-synthase complexes in the inner membrane (Paul and Tzagoloff, 1995; Arlt *et al.*, 1998; Galluhn and Langer, 2004), and show an increased tendency to lose mitochondrial DNA. In order to purify specific substrate proteins biochemically, an *m*-AAA protease variant harboring point mutations in the proteolytic centers was employed as substrate-trap (Nolden *et al.*, 2005). This approach has led to the identification of MrpL32, a conserved nuclearly encoded subunit of mitochondrial ribosomes (Grohmann *et al.*, 1991). Surprisingly, the *m*-AAA protease does not affect the stability of this protein but cleaves off the N-terminal targeting sequence upon import into mitochondria (Figure 4A) (Nolden *et al.*, 2005). MrpL32 processing is a prerequisite for its assembly into ribosomes, which is thus completed in close proximity to the inner membrane. An impaired translation within mitochondria rationalizes the loss of the respiratory competence of *m*-AAA protease-deficient yeast cells, as essential subunits of the respiratory chain are encoded by mitochondrial DNA (Foury *et al.*, 1998). That the processing of MrpL32 indeed represents a key function of the *m*-AAA protease in yeast is demonstrated by a complementation experiment. Expression of an MrpL32 variant, which harbors an unrelated presequence and is matured by MPP, maintains respiratory growth of *m*-AAA protease-deficient cells (Nolden *et al.*, 2005). Therefore, it can be concluded that cellular defects associated with an inactivation of the yeast *m*-AAA protease are largely explained by an impaired processing of a matrix protein rather than the deleterious effects of accumulating non-native membrane proteins in mitochondria.

Another substrate whose processing depends on the yeast *m*-AAA protease is the ROS-scavenger protein Ccp1 (Figure 4B). A bipartite presequence targets this nuclearly encoded protein into the intermembrane space and is cleaved off by the consecutive action of *m*-AAA and rhomboid proteases in the inner membrane (Esser *et al.*, 2002). Strikingly, although strictly dependent on the presence of the *m*-AAA protease, maturation of Ccp1 by rhomboid was observed in yeast cells harboring a proteolytically inactive variant of the *m*-AAA protease (Tatsuta *et al.*, 2007). In contrast, Ccp1 cleavage by rhomboid was abolished if mutations were introduced into the AAA domains of *m*-AAA protease subunits. Subsequent experiments revealed that the *m*-AAA protease is required to mediate the ATP-dependent vectorial dislocation of Ccp1 from the membrane bilayer (Figure 4B) (Tatsuta *et al.*, 2007). This activity most likely ensures the correct positioning of Ccp1 relative to the membrane to allow intramembrane cleavage by rhomboid. It appears that the general activity of the *m*-AAA protease to extract non-native substrate proteins from the membrane bilayer for proteolysis is recruited to ensure the biogenesis of Ccp1. Maturation of Ccp1 within mitochondria thus represents the first non-proteolytic function of the *m*-AAA protease within mitochondrial biogenesis. It is tempting to speculate that additional substrates of the *m*-AAA protease exist whose biogenesis depend on the ATP-dependent dislocase rather than the proteolytic activity of the *m*-AAA protease.

Mammalian polynucleotide phosphorylase (PNPase) may represent such a substrate for the *i*-AAA protease. PNPase has recently been localized to the mitochondrial intermembrane space and was shown to be required for the maintenance of mitochondrial homeostasis (Chen *et al.*, 2006). Import of PNPase into yeast mitochondria critically depends on the presence of the *i*-AAA protease Yme1 in mitochondria (Rainey *et al.*, 2006). Yme1 binds to the precursor form of PNPase and mediates its translocation across the outer membrane, but does not degrade it (Figure 6D). This suggests a non-proteolytic function, although it remains to be clarified why mutations in the proteolytic site of Yme1 abolish PNPase import. Regardless, together with the role of the *m*-AAA protease for Ccp1 biogenesis, a more general role of AAA proteases for the vectorial membrane dislocation is emerging. Notably, AAA proteases appear to share this ability with a number of other AAA proteins mediating protein dislocation from different cellular membranes (Platta *et al.*, 2005; Carlson *et al.*, 2006).

MAMMALIAN AAA PROTEASES AND NEURODEGENERATION

Subunits of mitochondrial AAA proteases are highly conserved and ubiquitously distributed in eukaryotic cells. First insights into their role in mammalian mitochondria came from the identification of disease-causing mutations in the gene paraplegin, coding for an *m*-AAA protease subunit in humans (Rugarli and

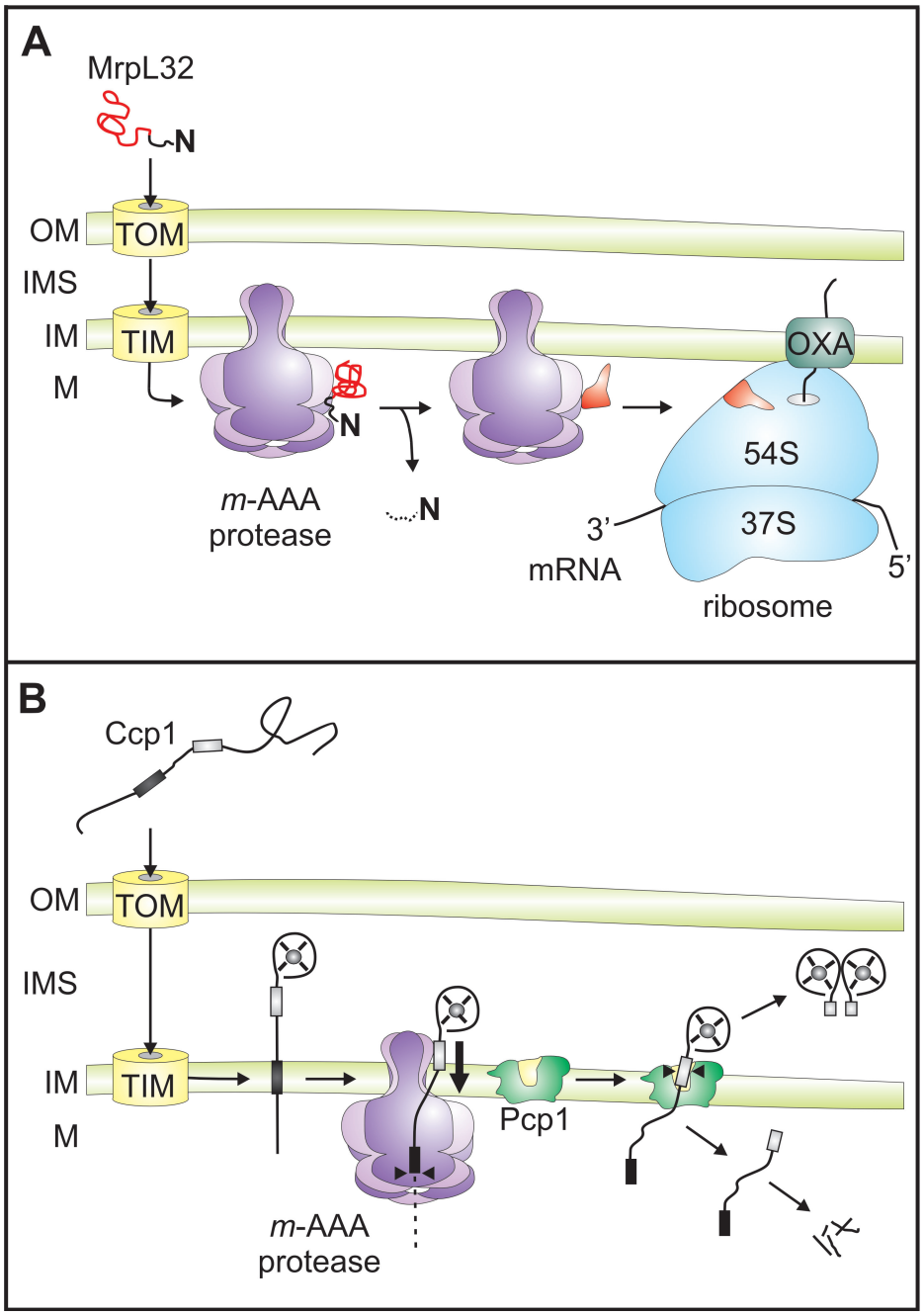

Figure 4 **Protein processing by the yeast *m*-AAA protease. (*A*.) Processing of MrpL32, a subunit of mitochondrial ribosomes. Newly imported MrpL32 is matured by the *m*-AAA protease, most likely accompanied by a conformational change. This allows its assembly with pre-assembled ribosomal particles at the inner surface of the inner membrane. The presequence of MrpL32 is either degraded within mitochondria or released from the organelle (Kambacheld *et al*., 2005). (*B*.) Processing of cytochrome *c* peroxidase (Ccp1), a ROS scavenger in the intermembrane space. Ccp1 is targeted to mitochondria by a bipartite presequence which is cleaved off in a two-step process by the *m*-AAA protease and rhomboid (Pcp1). The first hydrophobic stretch triggers lateral sorting of the precursor protein in the inner membrane. The *m*-AAA protease mediates the ATP-dependent vectorial dislocation and positions the precursor protein within the lipid bilayer for intramembrane cleavage by rhomboid. Mature Ccp1 is released as a soluble protein into the intermembrane space. (OM = outer membrane; IMS = intermembrane space; IM = inner membrane; M = matrix space; TOM = translocase of the outer membrane; TIM = translocase of the inner membrane; OXA = membrane insertion machinery containing Oxa1.)**

Langer, 2006). Nonsense mutations in paraplegin are causative for an autosomal recessive form of hereditary spastic paraplegia (HSP) (Casari *et al*., 1998). HSP is a genetically heterogeneous disease, which has been linked to 33 loci and is characterized by degeneration of motor axons of the cortico-spinal tracts and of sensory axons of the fasciculus gracilis (Fink, 2003; Rugarli and Langer, 2006; Soderblom and Blackstone, 2006).

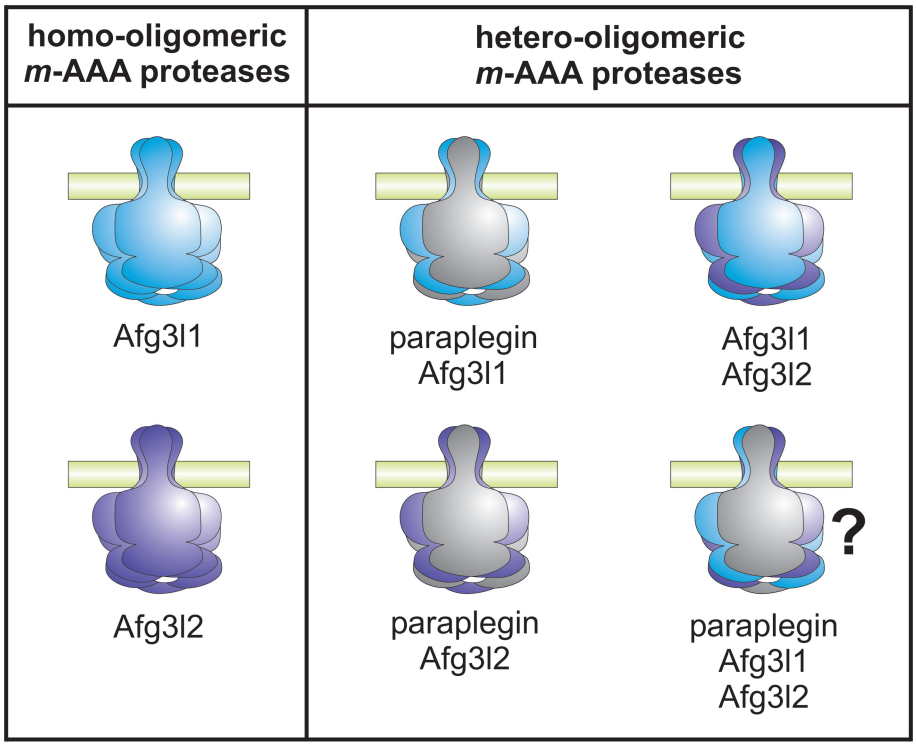

Figure 5 **Isoforms of murine *m*-AAA proteases. Evidence for five isoforms of the *m*-AAA proteases with different subunit composition was obtained by coimmunoprecipitation and immunodepletion experiments using brain and liver mitochondria as well as by yeast complementation studies. The existence of an *m*-AAA protease complex containing paraplegin, Afg3l1, and Afg3l2 is speculative. Only two subunits, AFG3L2 and paraplegin, are expressed in human mitochondria and assemble into homo-oligomeric AFG3L2 or hetero-oligomeric AFG3L2/paraplegin complexes.**

Main clinical features include weakness and spasticity of the lower limbs, loss of vibratory sense, and urinary urgency. The identification of paraplegin as an HSP gene suggests that impaired mitochondrial activities are the basis of some forms of HSP.

How can the clinical phenotype be reconciled with the function of the *m*-AAA protease within mitochondria? Complementation studies in yeast revealed functional conservation of *m*- and *i*-AAA proteases from yeast to mammals (Shah *et al.*, 2000; Atorino *et al.*, 2003; Nolden *et al.*, 2005; Koppen *et al.*, 2007). Human mitochondria contain two *m*-AAA protease subunits, Afg3l2 and paraplegin (Casari *et al.*, 1998; Banfi *et al.*, 1999). A third subunit, Afg3l1, is expressed in mice but encoded by a pseudogene in humans (Kremmidiotis *et al.*, 2001). Both in human and murine mitochondria, paraplegin assembles with Afg3l2 into a hetero-oligomeric complex which can functionally substitute for the yeast *m*-AAA protease when expressed in yeast suggesting that essential housekeeping functions are conserved (Atorino *et al.*, 2003; Nolden *et al.*, 2005). A complex of paraplegin and Afg3l2 can mediate the processing of yeast MrpL32 and therefore maintain ribosome assembly, mitochondrial protein synthesis, and respiratory growth (Nolden *et al.*, 2005). Similarly, mammalian MrpL32 when co-expressed with paraplegin and Afg3l2 in yeast is matured identifying MrpL32 as the first mammalian substrate of the *m*-AAA protease. Thus, the ability of the *m*-AAA protease to act as a processing enzyme is preserved from yeast to mammals. Processing of MrpL32 and mitochondrial protein synthesis were also found to be impaired in liver mitochondria isolated from paraplegin-deficient mice (Nolden *et al.*, 2005). This mouse line represents a valuable animal model to examine consequences of a paraplegin deficiency on mitochondrial activities, as it recapitulates important features of the human disease caused by the loss of paraplegin, like progressive and cell-specific axonal degeneration (Ferreirinha *et al.*, 2004).

Although these studies reveal important functions of the mammalian *m*-AAA protease, several caveats for the pathogenic relevance of these findings remain. Given that essential respiratory subunits are mitochondrially encoded, mitochondrial protein synthesis defects should result in OXPHOS deficiencies. This

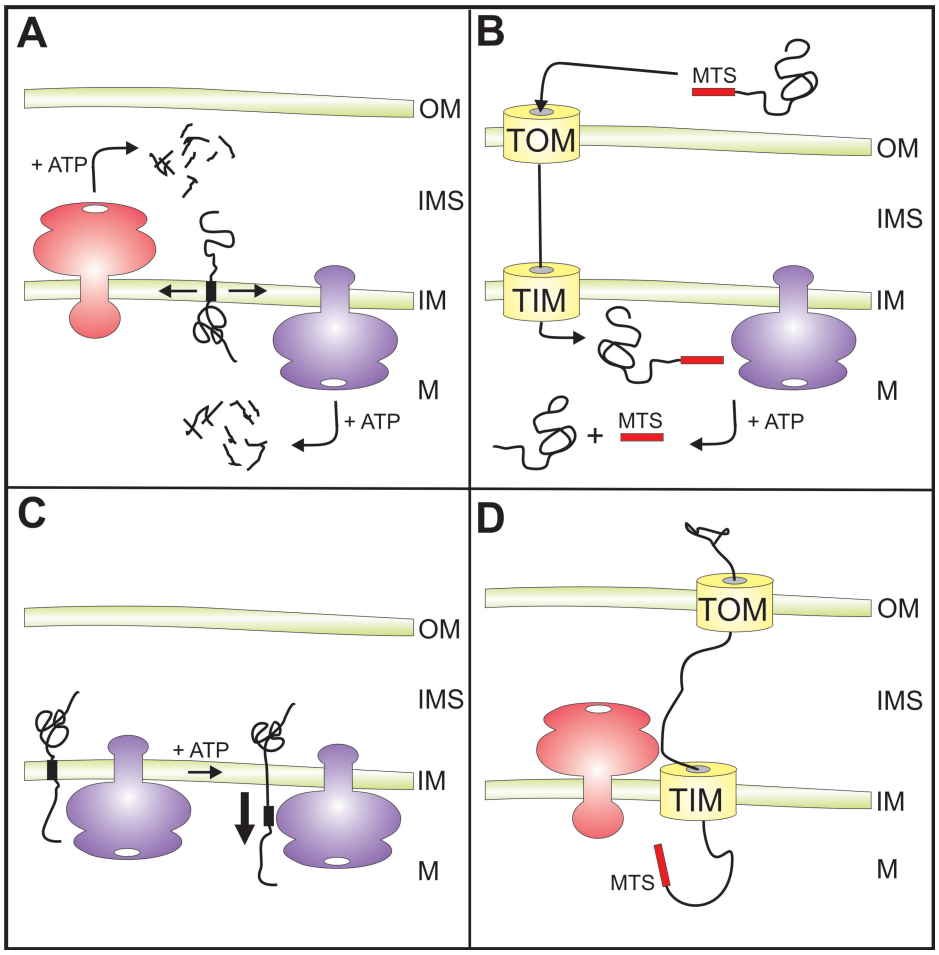

Figure 6 **Versatile functions of AAA proteases within mitochondria. (*A*.) Protein turnover. AAA proteases conduct protein quality surveillance and degrade excess and damaged proteins after their dislocation from the membrane to peptides. (*B*.) Protein processing. AAA proteases can cleave mitochondrial proteins resulting in their activation. This is exemplified by the maturation of newly imported MrpL32 by yeast and mammalian *m*-AAA proteases, but additional substrates are likely to exist. (*C*.) ATP-dependent membrane dislocation. Independent of its proteolytic activity, the *m*-AAA protease mediates the vectorial dislocation of Ccp1 precursor proteins to allow intramembrane cleavage by rhomboid. It is conceivable that this activity is required for the correct sorting of additional mitochondrial proteins residing in the inner membrane or the intermembrane space. (*D*.) Protein import into mitochondria. The yeast *i*-AAA protease is required for the import of heterologously expressed PNPase into the mitochondrial intermembrane space. The role of the proteolytic activity of the *i*-AAA protease in this process is currently not understood. (OM = outer membrane; IMS = intermembrane space; IM = inner membrane; M = matrix space; TOM = translocase of the outer membrane; TIM = translocase of the inner membrane; MTS = mitochondrial targeting sequence.)**

is illustrated by mutations in mitochondrial tRNA or in nuclearly encoded components of the mitochondrial translation machinery causative for a number of inherited human diseases (Jacobs and Turnbull, 2005; Taylor and Turnbull, 2005). However, only moderately defective ATP synthesis became apparent in spinal cord mitochondria of old paraplegin-deficient mice (Ferreirinha *et al.*, 2004). Decreased ATP levels were also detected in fibroblasts from HSP patients with known paraplegin mutations, but they were linked to a complex I deficiency rather than a general mitochondrial protein synthesis defect (Atorino *et al.*, 2003). An impaired complex I activity, due to defective synthesis or assembly, may cause an increased ROS production that contributes to damage mitochondrial proteins that cannot be properly degraded due to lack of paraplegin. This scenario is in line with the observed increased sensitivity of HSP fibroblasts to oxidative damage (Atorino *et al.*, 2003). Therefore, it remains an open question whether accumulating non-native polypeptides, an impaired mitochondrial translation, or the impaired processing of another mitochondrial protein in the absence of paraplegin results in axonal degeneration in HSP.

A defect in a housekeeping function in paraplegin-deficient mitochondria has to be reconciled with the

tissue specificity of the phenotype in HSP. Therefore, it will be crucial to examine respiratory and other mitochondrial activities in organelles isolated from affected axons and synapses, a rather challenging experimental task. In view of large variations in the mitochondrial proteome in different murine tissues (Mootha *et al.*, 2003) and the variability of mitochondria within neurons (Hollenbeck, 2005) it is conceivable that tissue-, cell-, or even subcellular-specific threshold effects exist. Moreover, specific substrates of the paraplegin-containing *m*-AAA protease might be differentially expressed leading to variable sensitivity toward the loss of paraplegin. Finally, additional members of the mitochondrial AAA protease family have to be considered. This includes the *i*-AAA protease, which, at least in yeast, functionally overlaps with the *m*-AAA protease (Leonhard *et al.*, 2000), but also other mammalian *m*-AAA protease subunits. Murine and human Afg3l2 as well as murine Afg3l1 assemble with paraplegin, but also form homo-oligomeric complexes (Figure 5) (Koppen *et al.*, 2007). This contrasts paraplegin, which has only been identified in hetero-oligomeric complexes with other *m*-AAA protease subunits. Thus, a variety of *m*-AAA protease isoforms can be formed in mammalian mitochondria (Figure 5). Yeast complementation studies demonstrated proteolytic activity of homo-oligomeric *m*-AAA proteases (Koppen *et al.*, 2007). They conduct protein quality surveillance and mediate protein processing. Strikingly, the expression of *m*-AAA protease subunits relative to each other varies between murine liver and brain (Koppen *et al.*, 2007). Afg3l1 is less abundant in brain when compared to Afg3l2 and paraplegin explaining why the loss of paraplegin leads to similar neuronal defects in human and mice despite the presence of an additional subunit in the later organism (Koppen *et al.*, 2007). Immunodepletion experiments indicate that homo-oligomeric Afg3l2-complexes are present in brain mitochondria of mice lacking paraplegin (Koppen *et al.*, 2007). Homo-oligomeric, proteolytically active Afg3l2-complexes may partially substitute for the loss of Afg3l2/paraplegin-complexes in HSP mitochondria and lessen the phenotypic consequences of the loss of paraplegin. Indeed, both homo- and hetero-oligomeric *m*-AAA protease complexes can cleave MrpL32 and maintain housekeeping functions in yeast demonstrating overlapping substrate specificity (Koppen *et al.*, 2007). Defects observed upon loss of paraplegin could therefore result from a reduced amount of functionally active *m*-AAA protease or, more likely, from differences in substrate specificities between homo-oligomeric Afg3l2- and hetero-oligomeric Afg3l2/paraplegin complexes.

A prediction of this hypothesis is that the impaired proteolysis of specific substrate proteins of hetero-oligomeric, paraplegin-containing *m*-AAA proteases leads to cell-specific neurodegeneration in the absence of paraplegin. Interestingly, paraplegin has recently been linked to the processing of the dynamin-like GTPase OPA1 (Ishihara *et al.*, 2006), a central component of the mitochondrial fusion machinery (Chan, 2006b). RNAi-mediated downregulation of paraplegin resulted in a modestly impaired processing of over-expressed OPA1, while over-expression of paraplegin but not Afg3l2 led to mitochondrial fragmentation (Ishihara *et al.*, 2006). Moreover, a complex of co-transfected paraplegin and OPA1 has been detected. Impaired OPA1 processing and, as a consequence, an impaired mitochondrial morphology may therefore be of pathogenic relevance in HSP. Notably, one of the most intriguing features of paraplegin-deficient mice is the presence of enlarged and structurally abnormal mitochondria in the synaptic terminal of motor axons (Ferreirinha *et al.*, 2004). An aberrant mitochondrial morphology could also explain axonal transport defects observed in these mice (Ferreirinha *et al.*, 2004). Interestingly, mutations in OPA1 lead to neurodegeneration in dominant optic atrophy type I suggesting that impaired OPA1 processing is linked to two neurodegenerative disorders (Alexander *et al.*, 2000; Delettre *et al.*, 2000). The different tissue specificities of both disorders, however, suggest that tissue-specific consequences of a paraplegin deficiency cannot simply be reconciled with an impaired OPA1 function by itself.

CONCLUDING REMARKS

Recent years have seen significant advances in our understanding of the proteolytic system of mitochondria. Key components, often highly conserved, have been identified and important functions were unravelled. The ATP-dependent AAA proteases are perhaps one of the best understood mitochondrial proteases but central questions concerning their physiological functions are still poorly characterized. Studies in yeast characterized AAA proteases as versatile molecular machines which affect mitochondrial biogenesis and homeostasis in many ways (Figure 6). First, they ensure

the quality control of inner membrane proteins and degrade non-native polypeptides processively to peptides. Second, acting as specific processing peptidases they regulate crucial steps during mitochondrial biogenesis. The assembly of ribosomes, mitochondrial translation, and the regulation of mitochondrial dynamics appear to be important processes controlled by mitochondrial AAA proteases. Third, AAA proteases can mediate the membrane dislocation of proteins independent of their proteolytic activity and thereby control the biogenesis and sorting of newly imported mitochondrial proteins. These findings will pave the way for further studies and many surprises can be awaited. The association of various human diseases with mitochondrial proteases and phenotypes of murine disease models illustrates the importance of the mitochondrial proteolytic system for cellular activities. Strong links appear to exist to apoptotic processes as exemplified by the mitochondrial proteases PARL and HtrA2, but still await further characterization. Inactivation of mitochondrial proteases, like the paraplegin-containing *m*-AAA protease or HtrA2, leads to cell-specific neurodegeneration, suggesting a high vulnerability of neurons for disturbances in mitochondrial proteolysis. The further analysis of the versatile functions of mitochondrial proteases is therefore likely to shed also new light on neuron-specific mitochondrial activities.

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Editor: Elizabeth A. Craig