Reconstitution of mammalian *m*-AAA protease complexes with variable subunit composition in yeast mitochondria

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Zusammenfassung

Die *m*-AAA-Protease ist ein ATP-abhängiger proteolytischer Komplex in der inneren Mitochondrienmembran, der eine wichtige Rolle bei der Qualitätskontrolle mitochondrialer Proteine und bei der Regulation der Ribosomen-Assemblierung spielt. Mutationen in Paraplegin, einer Untereinheit der humanen *m*-AAA-Protease, führen zu hereditärer spastischer Paraplegie (HSP). Diese Krankheit ist durch eine zellspezifische Degeneration von Axonen gekennzeichnet, die auch in Paraplegin-defizienten Mäusen auftritt. Es wird vermutet, dass Paraplegin die proteolytische Prozessierung von OPA1 vermittelt. OPA1 ist für die Aufrechterhaltung der mitochondrialen Morphologie essentiell und wird mit dominanter optischer Atrophie, einer weiteren neurodegenerativen Erkrankung, in Verbindung gebracht. Paraplegin bildet zusammen mit homologen Afg3l2-Untereinheiten einen heterooligomeren Proteinkomplex. Die Auswirkungen eines Verlustes von Paraplegin auf die Assemblierung und Funktion der *m*-AAA-Protease sind jedoch unklar. Des Weiteren wird eine dritte mutmaßliche Untereinheit der *m*-AAA-Protease, Afg3l1, in Mäusen exprimiert, aber ihre Rolle für die Aktivität der *m*-AAA-Protease ist noch nicht bestimmt worden.

Der Assemblierungszustand von humanem AFG3L2 wurde in Paraplegin-defizienten Mitochondrien aus humanen HSP-Zellen untersucht. Dadurch konnte die Existenz eines homooligomeren AFG3L2-Komplexes nachgewiesen werden, der auch bei heterologer Expression in Hefe gebildet wurde. Dieser AFG3L2-Komplex ist proteolytisch aktiv, da er die *m*-AAA-Protease der Hefe ersetzen konnte. Weitere Komplementationsstudien in der Hefe ergaben, dass die *m*-AAA-Protease-Untereinheiten der Maus in der inneren Mitochondrienmembran verschiedene proteolytische Komplexe mit unterschiedlicher Zusammensetzung bilden. Homooligomere Afg3l1- und Afg3l2-Komplexe sowie heterooligomere Komplexe beider Proteine zusammen mit Paraplegin konnten identifiziert werden. Afg3l1 stellt somit eine echte Untereinheit von *m*-AAA-Proteasen mit proteolytischer Aktivität dar. Alle Komplexe weisen konservierte und überlappende Substratspezifitäten auf, da sie grundlegende mitochondriale Funktionen aufrechterhalten konnten. Diese Ergebnisse lassen vermuten, dass die Abwesenheit von Paraplegin nicht zu einem vollständigen Verlust der *m*-AAA-Protease-Aktivität in betroffenen Mitochondrien führt. Stattdessen könnten *m*-AAA-Proteasen mit unterschiedlicher Untereinheiten-Zusammensetzung gebildet werden und *m*-AAA-Proteasen mit Paraplegin-Beteiligung ersetzen. Durch die Rekonstitution der OPA1-Prozessierung in Hefezellen, die verschiedene *m*-AAA-Protease-Komplexe aus Säugetieren enthielten, konnten erste Hinweise für unterschiedliche Substratspezifitäten von *m*-AAA-

Proteasen mit unterschiedlicher Zusammensetzung erhalten werden. Die Effizienz der OPA1-Prozessierung war abhängig von der Zusammensetzung der Untereinheiten der Säugetier-*m*-AAA-Proteasen. Homooligomere Komplexe bestehend aus murinem Afg3l1, Afg3l2 oder humanem AFG3L2 prozessierten OPA1 mit höherer Effizienz als heterooligomere Komplexe, die Paraplegin enthielten. Diese Ergebnisse bestätigen, dass OPA1 ein weiteres potentielles Substrat der *m*-AAA-Protease darstellt. Die Befunde dieser Arbeit offenbaren eine unerwartete Vielfalt an *m*-AAA-Proteasen in der inneren Mitochondrienmembran von Säugetieren und könnten dazu beitragen, die Pathogenese und Gewebespezifität von HSP zu verstehen.

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1 Introduction

1.1 The proteolytic system of mitochondria

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). 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 maintain mitochondrial activities under varying physiological conditions. Mitochondria are built up of ~1000 proteins (Mootha *et al.*, 2003; Sickmann *et al.*, 2003; Taylor *et al.*, 2003). The vast majority of them are nuclearly encoded, synthesised 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. Moreover, this task is made even more difficult by the fact that mitochondria are highly dynamic organelles. They undergo constant fusion and fission and frequently change their shape in response to altered physiological conditions (Okamoto and Shaw, 2005).

Mitochondria 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. Many of

them are highly conserved and ubiquitously distributed in eukaryotic cells. They can be categorised into several classes according to their main activities (Fig. 1; Tab. 1):

Table 1. Mitochondrial peptidases.

Name	Loc.	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	YME1L1	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 localised to the matrix. [1]Falkevall *et al.*, 2006. [2]Zimny *et al.*, 2006.

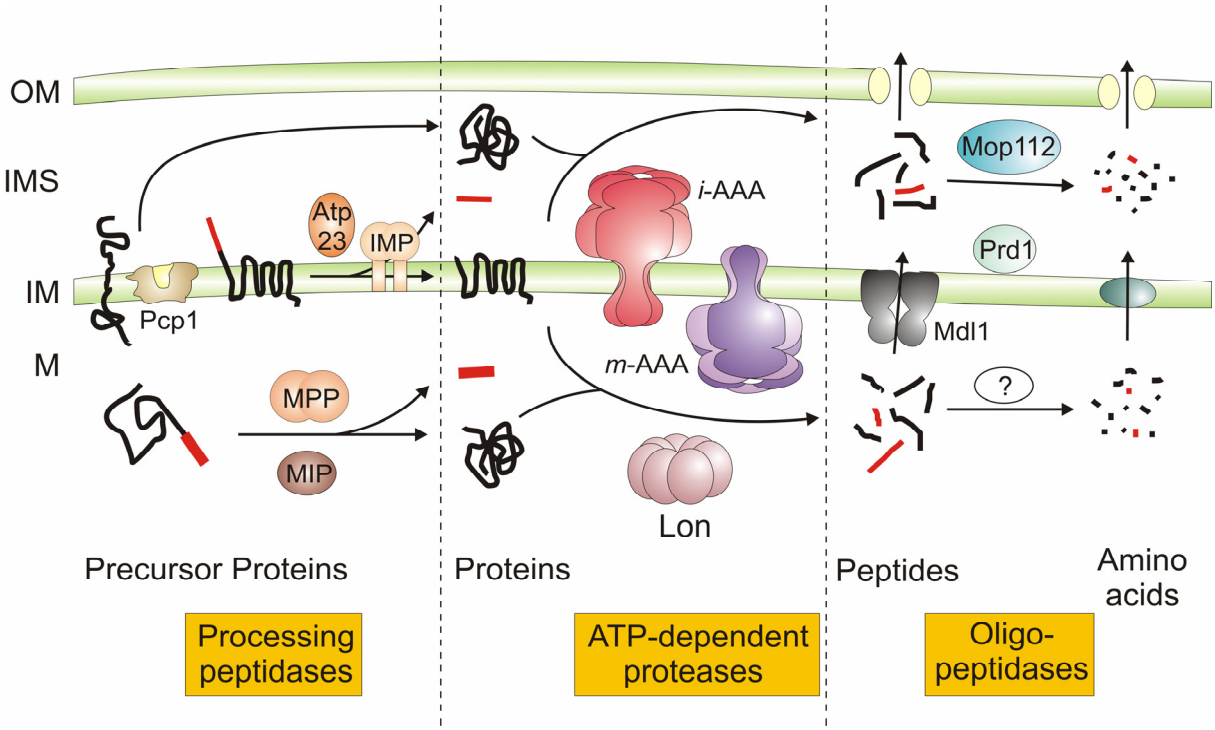

Figure 1. Proteolytic pathways within mitochondria of *Saccharomyces. cerevisiae*.

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.

1.1.1 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). 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 (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). 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 specialised 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). A processing peptidase with a regulatory role for mitochondrial biogenesis is represented by the **rhomboid protease** in the inner membrane. 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 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003a) and cytochrome *c* peroxidase (Ccp1), a mitochondrial scavenger of reactive oxygen species (ROS) (Esser *et al.*, 2002). Mgm1 is a central component of the mitochondrial fusion machinery and accordingly Pcp1-mediated processing affects mitochondrial dynamics (Herlan *et al.*, 2003).

1.1.2 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 utilised 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 understood on the molecular level only in a few cases, 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 (Fig. 1; Tab. 1).

The **Lon protease** (or PIM1 protease in yeast) is localised 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 synthesised 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 recognises misfolded or damaged proteins, mediates their complete proteolysis, and thereby prevents their accumulation and deleterious effects on mitochondrial activities. 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 destabilisation 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). Mitochondrial Lon proteases exhibit a peculiar binding affinity for nucleic acids pointing to a 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 (mtDNA) 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** (AAA: ATPases associated with a variety of cellular activities), are present within the inner membrane of mitochondria and expose their catalytic sites either to the matrix or the intermembrane space (Fig. 1) (Langer, 2000). AAA proteases conduct protein quality surveillance in the inner membrane and exert crucial functions during mitochondrial biogenesis. They are discussed in detail below.

1.1.3 Oligopeptidases

Oligopeptidases are, with few exceptions, the most poorly characterised 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 (Fig. 1). Several proteins with oligopetidase activity *in vitro* have been identified, mostly localised in the intermembrane space. 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 which 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 (Fig. 1) (Young *et al.*, 2001). Nevertheless, a quantitative assessment of mitochondrial peptide export and the mass-spectrometric identification of peptides released from yeast mitochondria provided first evidence for the function of oligopeptidases *in vivo* (Kambacheld *et al.*, 2005). Two oligopeptidases were found to cleave

peptides generated by the *i*-AAA protease in the intermembrane space (Fig. 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). 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 localised in another mitochondrial subcompartment. Several enzymes with proposed oligopeptidase activity have also been identified in the matrix space including the bleomycin hydrolase **Lap3**. It is a cysteine peptidase which is localised 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 characterisation of the *in vivo* function of matrix-localised 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 (Fig. 1).

1.1.4 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 localised 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.

HtrA2 (Omi, Prss25) is localised 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). 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.*, 2006).

1.2 AAA proteases

1.2.1 Domain structure and assembly of AAA proteases

AAA proteases are integral parts of the mitochondrial inner membrane and their catalytic domains are facing either the matrix or the intermembrane space (Langer, 2000). Accordingly, they were termed *m*-AAA protease, active on the matrix side, and *i*-AAA protease, active on the intermembrane side (Fig. 2). AAA proteases form large complexes with a native molecular mass of ~1 MDa, which are composed of closely related or identical subunits of 70-80 kDa (Arlt *et al.*, 1996; Leonhard *et al.*, 1996; Klanner *et al.*, 2001; Atorino *et al.*, 2003; Urantowka *et al.*, 2005). 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 (Fig. 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 characterised 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). Notably, proteolytic activity is only completely abolished if all subunits harbour mutations in their proteolytic centres. 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).

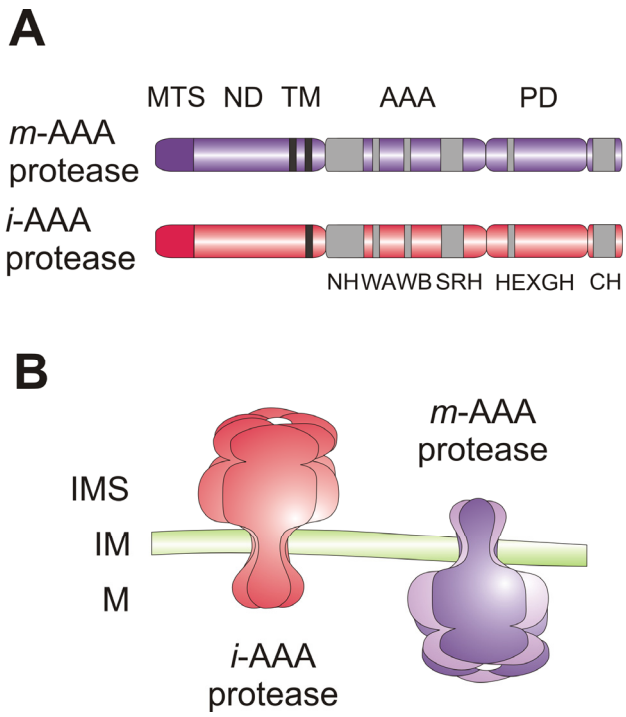

Figure 2. Mitochondrial AAA proteases.

(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. IMS, intermembrane space; IM, inner membrane; M, matrix space.

For two homologous eubacterial FtsH proteases, the crystal structure of the soluble cytosolic region containing the catalytic domains has recently been solved (Bieniossek *et al.*, 2006; Suno *et al.*, 2006). They form a hexameric structure which adopts a flat-cylinder-like shape and consists of two structurally separate rings composed of the proteolytic domains and the AAA domains, respectively (Fig. 3). The proteolytic centres are located within an inner cavity of the hexameric molecule and can be accessed via a narrow central pore formed by the AAA domains facing the membrane. A similar hexameric and ring-like assembly can also be suggested for mitochondrial AAA proteases.

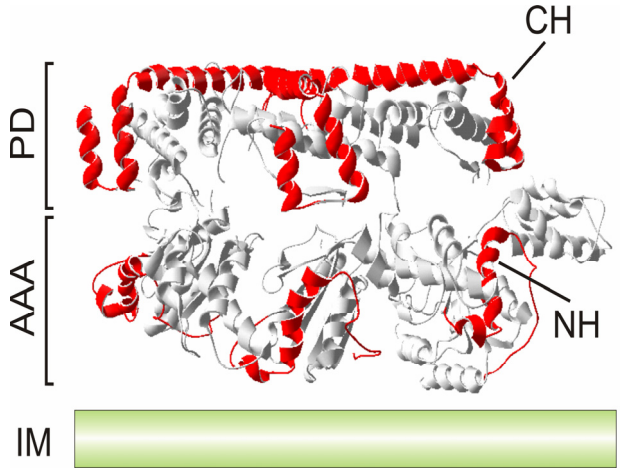

Figure 3. Substrate engagement by the yeast *i*-AAA protease.

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 yeast Yme1 are marked in red in the crystal structure of *Thermotoga maritima* FtsH (Bieniossek *et al.*, 2006). Only three subunits are shown. AAA, AAA domain; PD, proteolytic domain; IM, inner membrane.

Both the *m*- and the *i*-AAA protease recognise and degrade non-native and non-assembled polypeptides to peptides (Fig. 4A) (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). These proteases exhibit degenerate substrate specificity and, similar to molecular chaperone proteins, recognise the folding state of solvent-exposed domains of membrane proteins (Leonhard *et al.*, 1999). Studies on the *i*-AAA protease subunit Yme1 of yeast 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 (Fig. 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). 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), which has been localised to the central pore of other hexameric AAA^+ ring complexes (Wang *et al.*, 2001). Furthermore, AAA protease-mediated degradation of inner membrane proteins involves the extraction of the substrate from the membrane bilayer (Leonhard *et al.*, 2000). Recently, the ability of the *m*-AAA protease to mediate vectorial membrane dislocation of proteins in an ATP-dependent reaction has been directly demonstrated (see below) (Tatsuta *et al.*, 2007). This membrane extraction of substrate proteins is likely to be facilitated by the membrane-embedded parts of AAA protease subunits which might form a pore-like structure or provide at least a more hydrophilic environment (Korbel *et al.*, 2004).

The yeast *m*-AAA protease assembles with the prohibin complex into a large supercomplex (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). Prohibitins appear to modulate proteolysis by the *m*-AAA protease, since deletion of prohibitins in yeast results in accelerated protein degradation by the *m*-AAA protease (Steglich *et al.*, 1999). However, the molecular mechanism of how prohibitins exert this function is not understood.

1.2.2 Functions of AAA proteases within mitochondria

AAA proteases in the inner mitochondrial membrane exert essential housekeeping functions like protein quality control (Fig. 4A) and have crucial roles in mitochondrial biogenesis. Therefore, their deletion or inactivation causes severe pleiotropic phenotypes in various organisms, which are best characterised in the yeast *Saccharomyces cerevisiae*. Here, all observed defects can be attributed to a loss of proteolytic activity as identical phenotypes were observed after deletion of an AAA protease subunit or after inactivation of the proteolytic sites of all subunits of AAA protease complexes (Leonhard *et al.*, 1996; Weber *et al.*, 1996; Arlt *et al.*, 1998). Notably, yeast cells deficient in both the *m*- and the *i*-AAA protease are not viable (Lemaire *et al.*, 2000; Leonhard *et al.*, 2000).

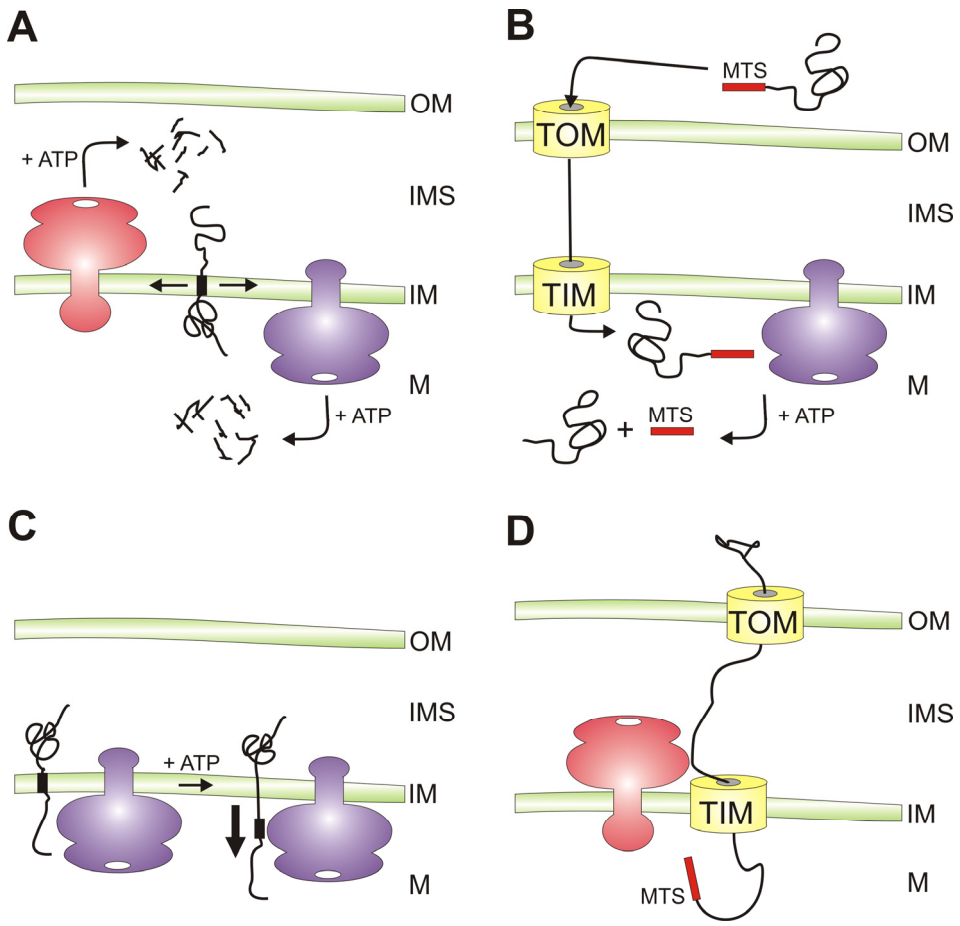

Figure 4. Versatile functions of AAA proteases within mitochondria.

A) Protein turnover. AAA proteases conduct protein quality surveillance and degrade excess and damaged proteins to peptides after their dislocation from the membrane.
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.

The yeast *i*-AAA protease is a homo-oligomeric complex of Yme1 subunits (Leonhard *et al.*, 1996). In the absence of a functional *i*-AAA protease, yeast cells are characterised by a respiratory deficiency at high temperature, an inability to grow on rich glucose medium at low temperature, an increased rate of mtDNA escape to the nucleus (Thorsness and Fox, 1993; Thorsness *et al.*, 1993), and a petite-negative phenotype, i.e. strongly impaired growth in the

absence of mtDNA (Weber *et al.*, 1995). Moreover, Δ*yme1* cells exhibit an accumulation of punctate mitochondria with grossly swollen compartments pointing to a potential role of Yme1 in maintaining normal mitochondrial morphology (Campbell *et al.*, 1994). However, it remains an open question if these phenotypes are caused by the accumulation of misfolded polypeptides or the impaired proteolysis of specific substrate proteins. Only a limited number of protein quality control substrates of the yeast *i*-AAA protease has been identified including non-assembled Cox2 (Nakai *et al.*, 1995; Pearce and Sherman, 1995; Weber *et al.*, 1996), unassembled prohibitin subunits Phb1 and Phb2 (Kambacheld *et al.*, 2005), as well as external NADH dehydrogenase (Nde1) (Augustin *et al.*, 2005). In addition to protein quality surveillance (Fig. 4A), a novel function has recently been associated with the *i*-AAA protease. Polynucleotide phosphorylase (PNPase) has been localised to the mitochondrial intermembrane space in mammals and was shown to be required for the maintenance of mitochondrial homeostasis (Chen *et al.*, 2006). Import of mammalian 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 suggesting a non-proteolytic function (Fig. 4D).

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) and lack assembled respiratory chain and ATP-synthase complexes in the inner membrane (Paul and Tzagoloff, 1995; Arlt *et al.*, 1998; Galluhn and Langer, 2004). As substrates, non-assembled, mitochondrially encoded respiratory chain subunits of complexes III, IV and V (Cob, Cox1, Cox3, Atp6, Atp8, Atp9) were found to be degraded by the yeast *m*-AAA protease (Arlt *et al.*, 1996; Guélin *et al.*, 1996). In addition to these integral membrane proteins, the *m*-AAA protease has also been demonstrated to degrade peripheral membrane proteins such as Atp7 (Korbel *et al.*, 2004). Recently, the processing of specific substrate proteins with regulatory functions has been recognised as a second housekeeping function of the *m*-AAA protease (Fig. 4B). Specific substrates for proteolytic cleavage include the ribosomal subunit MrpL32 (Nolden *et al.*, 2005) and cytochrome *c* peroxidase (Ccp1), a heme-binding ROS scavenger in the intermembrane space (Esser *et al.*, 2002; Tatsuta *et al.*, 2007). These findings have provided a rationale for cellular defects associated with an *m*-AAA protease-deficiency in

yeast. Moreover, they allowed the identification of ATP-dependent membrane dislocation as a novel non-proteolytic function of AAA proteases (Fig. 4C).

1.2.3 Proteolytic processing by AAA proteases

In order to purify specific substrate proteins biochemically, an *m*-AAA protease variant harbouring point mutations in the proteolytic centres 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 (Fig. 5A) (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 rationalises the loss of the respiratory competence of *m*-AAA protease-deficient yeast cells, as essential subunits of the respiratory chain are encoded by the 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 harbours 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 (Fig. 5B). 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 the rhomboid protease Pcp1 was observed in yeast cells harbouring 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 (Fig. 5B) (Tatsuta *et al.*, 2007). This activity most likely ensures the correct positioning of Ccp1 relative to the membrane to allow intramembrane cleavage by Pcp1. It appears that the general

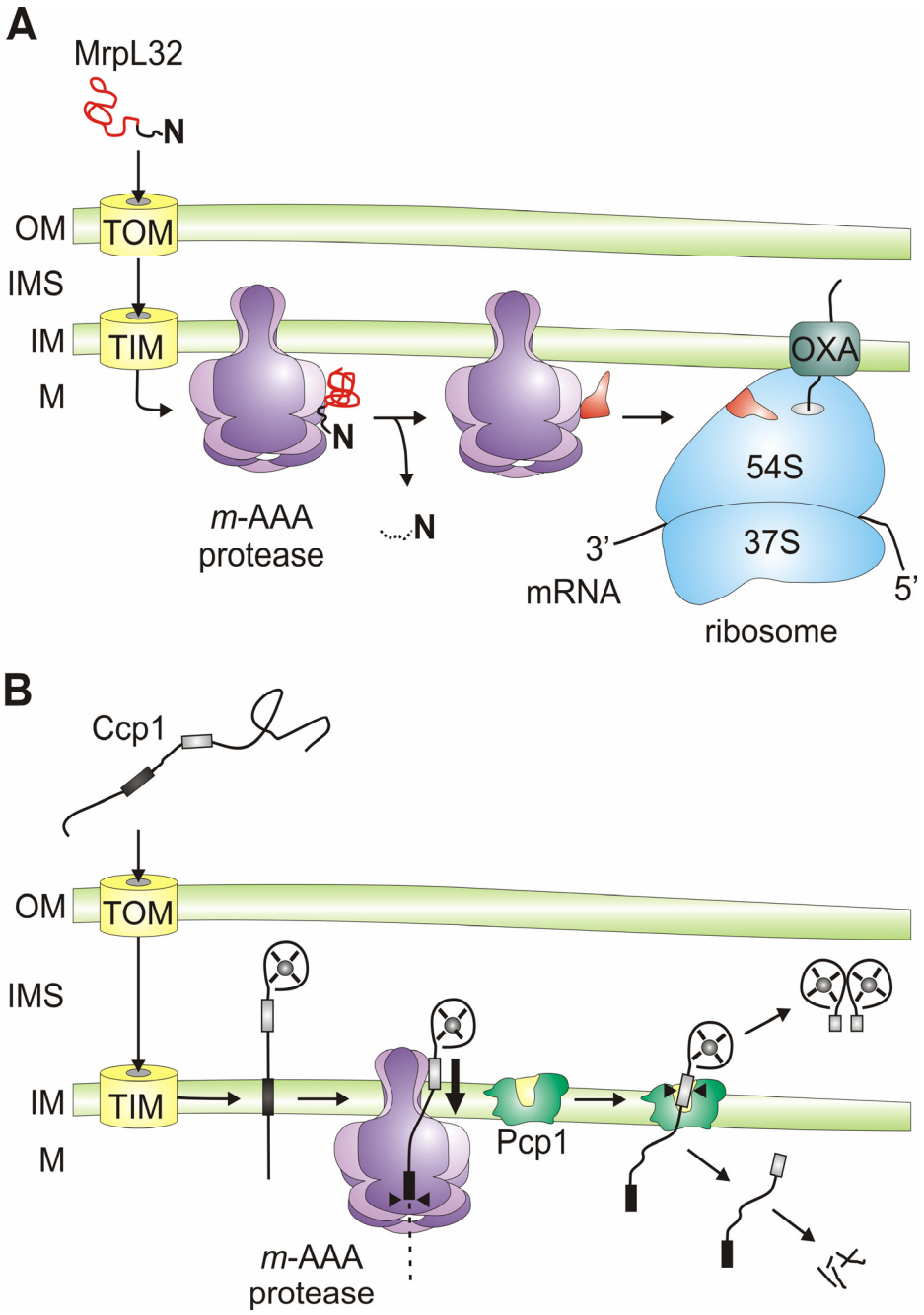

Figure 5. Protein processing by the yeast *m*-AAA protease.
A) Processing of MrpL32, a subunit of mitochondrial ribosomes. Newly imported MrpL32 is matured by the *m*-AAA protease, most likely accompanied by a conformational change. This allows its assembly with pre-assembled ribosomal particles at the 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.

activity of the *m*-AAA protease, namely 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.

1.2.4 Mammalian AAA proteases

The presence of AAA proteases with their catalytic domains exposed to opposite membrane surfaces is conserved in mammals. AAA protease subunits with a high degree of homology to Yta10, Yta12, and Yme1 of yeast have been identified in humans and mice (Fig. 6) and several complementation studies in yeast have demonstrated a functional conservation of *m*- and *i*-AAA proteases (Shah *et al.*, 2000; Atorino *et al.*, 2003; Nolden *et al.*, 2005).

A mammalian homologue of yeast Yme1 has been found in humans and mice, named YME1L1 (Coppola *et al.*, 2000; Shah *et al.*, 2000). Expression of human YME1L1 restores respiratory growth of Δ*yme1* yeast cells at high temperature identifying it as the human *i*-AAA protease (Shah *et al.*, 2000). Thus, the *i*-AAA protease in mammalian mitochondria apparently represents a homo-oligomeric assembly like in yeast. In contrast, next to nothing is known about its substrates and exact function in mammals and the generation of a Yme1l1-deficient mouse line is awaited.

In case of the *m*-AAA protease, three potential subunits have been identified in humans and mice, termed Afg3l1, Afg3l2, and paraplegin (Casari *et al.*, 1998; Shah *et al.*, 1998; Banfi *et al.*, 1999). They are all mitochondrially localised and ubiquitously present in mammalian tissues. However, Afg3l1 which is highly homologous to Afg3l2 is only expressed in mice, whereas it is 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 (Atorino *et al.*, 2003; Nolden *et al.*, 2005). The assembly and function of Afg3l1 has not been analysed. Remarkably, paraplegin was initially identified by its association with a neurodegenerative disease which also provided first insights into the role of *m*-AAA proteases in mammalian mitochondria. Mutations in the gene encoding paraplegin (spastic-paraplegia-gene 7/*SPG7*) are causative for an autosomal recessive form of hereditary

spastic paraplegia (HSP) (Casari *et al.*, 1998). It is noteworthy that up to date disease-causing mutations have not been reported for human AFG3L2 and YME1L1.

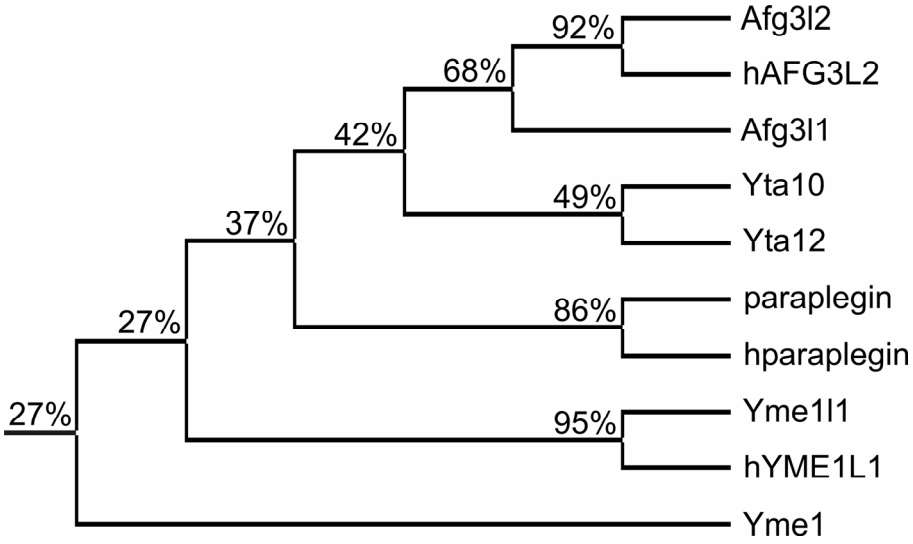

Figure 6. AAA protease subunits of yeast and mammals are evolutionary conserved.

Phylogenetic relationship between mitochondrial AAA protease subunits of yeast (Yta10, Yta12, Yme1), mice (paraplegin, Afg3l1, Afg3l2, Yme1l1), and humans (hparaplegin, hAFG3L2, hYME1L1), including their sequence identity.

1.3 Hereditary spastic paraplegia (HSP)

1.3.1 HSP is a clinically and genetically heterogenous disease

Hereditary spastic paraplegia comprises a diverse group of neurodegenerative disorders whose main clinical features include progressive weakness and spasticity of the lower limbs, loss of the vibratory sense, and urinary urgency (Rugarli and Langer, 2006; Soderblom and Blackstone, 2006). The age of onset is variable, but usually lies between 10 and 40 years of age. A further characteristic of the "pure" forms of HSP, where these symptoms are isolated, is a remarkable tissue-specificity: only motor neurons of the cortico-spinal tracts and sensory neurons of the fasiculus gracilis are selectively affected by axonal degeneration (Rugarli and Langer, 2006; Soderblom and Blackstone, 2006). Interestingly, the axons of these neurons belong to the longest in the central nervous system reaching lengths of ~1 m. The axonal degeneration in HSP starts distally at the synaptic terminals and slowly proceeds towards the neuronal cell bodies which are preserved. Thus, HSP is a member of a new category of neurological disorders and can be classified as a "dying back" axonopathy

(Züchner and Vance, 2005). In the "complicated" forms of HSP, patients have additional neurological abnormalities like cortical and cerebellar atrophy, amyotrophy, peripheral neuropathy, optic atrophy, deafness, short stature, and mental retardation (Harding, 1983).

The clinical heterogeneity of spastic paraplegias is paralleled by a genetic heterogeneity. The inheritance pattern can be X chromosome-linked, autosomal dominant, or autosomal recessive. In total, 33 genetic loci have been associated with different forms of HSP (*SPG1* to *SPG33*). However, causative mutations have been identified only in 15 genes (Tab. 2), which can be divided into three functional categories: Firstly, genes whose products play a role in axonal targeting and pathfinding or myelination. These include the two X chromosome-linked genes *SPG1* and *SPG2* encoding the glycoprotein L1CAM and the proteolipid protein PLP1, respectively (Jouet *et al.*, 1994; Saugier-Veber *et al.*, 1994). Secondly, genes whose products are involved in axonal transport and trafficking (Crosby and Proukakis, 2002; Soderblom and Blackstone, 2006). The kinesin heavy chain gene *KIF5A* (*SPG10*) is the paradigm of this group (Reid *et al.*, 2002). KIF5A is part of a hetero-tetrameric protein complex, the neuronal kinesin-I, that acts as a plus-end-directed microtubule motor involved in anterograde transport of membranous organelles in nerve axons (Goldstein and Yang, 2000). The AAA protein spastin which is encoded by *SPG4* interacts with microtubules and regulates microtubule dynamics (Errico *et al.*, 2002; Errico *et al.*, 2004; Evans *et al.*, 2005). In addition, spastin interacts with one component of the endosomal sorting complex required for transport (ESCRT)-III and with the large GTPase atlastin (*SPG3A*), which also causative for HSP (Reid *et al.*, 2005; Evans *et al.*, 2006; Sanderson *et al.*, 2006). Spartin (*SPG20*), maspardin (*SPG21*), and the recently identified protein ZFYVE27 (*SPG33*) have also been suggested to have a function in intracellular trafficking, but clear evidence is missing (Patel *et al.*, 2002; Simpson *et al.*, 2003; Mannan *et al.*, 2006). The third functional group encompasses genes that have mitochondrial functions (Bross *et al.*, 2004; Rugarli and Langer, 2006). Besides the *m*-AAA protease subunit paraplegin, mitochondrial Hsp60 (heat-shock protein 60; also known as chaperonin) which is encoded by *SPG13* has been linked to HSP (Hansen *et al.*, 2002). Hsp60 mediates the folding of newly imported proteins and protects mitochondrial proteins from heat denaturation (Voos and Röttgers, 2002). Recently, the protein product of the *SPG31* gene, termed REEP1 (receptor expression-enhancing protein 1) has been localised to mitochondria but its function is unknown (Züchner *et al.*, 2006). Conflicting results concerning the subcellular localisation of spartin have been reported (Lu *et al.*, 2006; Robay *et al.*, 2006). In one study, transfected and fluorescent-tagged

spartin was recently found to be mitochondrial and to co-localise with microtubules suggesting a possible role of spartin in transport of mitochondria (Lu *et al.*, 2006).

Table 2. Cloned genes which are linked to HSP.

Locus	Inheritance	Protein	Function	Reference
SPG1	X-linked	L1CAM	axonal targeting and pathfinding, development of corticospinal tract	Jouet *et al.*, 1994
SPG2	X-linked	PLP1	myelination, axonal-glial interactions	Saugier-Veber *et al.*, 1994
SPG3A	AD	atlastin	GTPase in the cis-Golgi, interaction with spastin, involved in axonal develop.	Zhao *et al.*, 2001
SPG4	AD	spastin	microtubule dynamics, vesicular trafficking	Hazan *et al.*, 1999
SPG6	AD	NIPA1	Mg^{2+} transporter	Rainier *et al.*, 2003
SPG7	AR	paraplegin	mitochondrial AAA protease	Casari *et al.*, 1998
SPG8	AD	strumpellin	unknown	Valdmanis *et al.*, 2007
SPG10	AD	KIF5A	kinesin motor protein (axonal transport)	Reid *et al.*, 2002
SPG11	AR	spatacsin	unknown	Stevanin *et al.*, 2007
SPG13	AD	Hsp60	mitochondrial chaperonin	Hansen *et al.*, 2002
SPG17	AD	seipin	ER membrane protein	Windpassinger *et al.*, 2004
SPG20	AR	spartin	microtubule-mediated trafficking of mitochondria?	Patel *et al.*, 2002
SPG21	AR	maspardin	vesicular trafficking?	Simpson *et al.*, 2003
SPG31	AD	REEP1	mitochondrial membrane protein	Züchner *et al.*, 2006
SPG33	AD	ZFYVE27	spastin-binding protein	Mannan *et al.*, 2006

AD, autosomal dominant; AR, autosomal recessive; X-linked, X chromosome-linked

1.3.2 HSP due to lack of paraplegin

Mutations in the *SPG7* gene encoding paraplegin account for ~10% of recessive and sporadic cases of HSP (Casari and Rugarli, 2001). Analysis of the paraplegin mutations that were identified in human HSP patients suggests a complete loss of paraplegin function (McDermott *et al.*, 2001; Wilkinson *et al.*, 2004). What are the consequences of a paraplegin deficiency on mitochondrial activities in HSP patients? Primary fibroblasts from HSP patients showed defects in the assembly and activity of complex I of the respiratory chain, accompanied by decreased ATP levels (Atorino *et al.*, 2003). An impaired activity of

complex I can lead to an elevated production of reactive oxygen species (Barrientos and Moraes, 1999; Li *et al.*, 2003). Consistently, an increased sensitivity of HSP fibroblasts towards oxidative stress was detected (Atorino *et al.*, 2003).

Recently, a paraplegin-deficient mouse line has been generated which recapitulates central features of the human disease and thus can serve as a valuable model to further understand the molecular basis of mitochondrial dysfunction in HSP (Ferreirinha *et al.*, 2004). Similar to HSP patients, the long axons of paraplegin-deficient mice were specifically affected and found to contain enlarged and structurally abnormal mitochondria in the distal regions. These changes in mitochondrial morphology represented the first detectable defect whose appearance was followed by axonal swelling and finally progressive degeneration (Ferreirinha *et al.*, 2004). Thus, the mammalian *m*-AAA protease might also play a role in the regulation of mitochondrial dynamics. Further studies revealed that, similar to *m*-AAA protease-deficient yeast cells, processing of murine MrpL32 and concomitantly mitochondrial translation were impaired in the absence of paraplegin (Nolden *et al.*, 2005). When co-expressed with murine paraplegin and Afg3l2 in yeast, murine MrpL32 was matured identifying it as the first *in vivo* substrate of the mammalian *m*-AAA protease (Nolden *et al.*, 2005). Since essential respiratory chain subunits are mitochondrially encoded (Anderson *et al.*, 1981), mitochondrial-protein-synthesis defects should result in oxidative-phosphorylation deficiencies. However, the activities of respiratory chain complexes were not significantly affected and only moderately defective ATP synthesis became apparent in spinal cord mitochondria of old paraplegin-deficient mice (Ferreirinha *et al.*, 2004).

1.3.3 A possible mechanism for axonal degeneration in HSP

Taken together, significant progress has been made in the recent years to unravel the pathogenetic mechanism of HSP due to a loss of paraplegin. However, 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. One can envision the following general model, which can also be reconciled with the genetic heterogeneity of this neurological disorder. Axons depend on active anterograde transport for the supply of protein and lipid constituents, which are synthesised in the neuronal cell body and on retrograde transport for the degradation and replacement of old and damaged material (Hirokawa and Takemura, 2005). Mitochondria are also transported in an anterograde manner mediated by kinesin motor proteins to the distal regions of axons (Hollenbeck and Saxton, 2005). Neurons with long

axons as those of the corticospinal tracts can be considered to be particularly vulnerable to defects in axonal trafficking as indicated by the axonal length-dependance in HSP. Thus, deficiencies in proteins directly involved in axonal transport such as KIF5A or in mitochondrial proteins like paraplegin and Hsp60 could both result in the absence of functional mitochondria from the synapses triggering degeneration. Consistently, it has been demonstrated that reduced mitochondria content leads to the loss of dendritic spines and synapses (Li *et al.*, 2004). In the absence of paraplegin, synaptic mitochondria undergo morphological changes and accumulate which, in combination with a limited energy supply, might further affect axonal transport and eventually clog the axon. In line with this scenario, a delayed retrograde transport was indeed observed in motor neurons of paraplegin-deficient mice (Ferreirinha *et al.*, 2004).

1.4 Mitochondrial dynamics

1.4.1 Mitochondrial fusion in yeast and mammals

Mitochondria are highly dynamic organelles whose morphology depends on the tissue, on the physiological condition of the cell, and in particular on the functional status of mitochondria (Okamoto and Shaw, 2005). This dynamic behaviour is crucial for a number of cellular processes, such as apoptosis, the inheritance of mtDNA, defence against oxidative stress, and development through spermatogenesis (Hales and Fuller, 1997; Chen and Chan, 2005; Chen and Butow, 2005; Cereghetti and Scorrano, 2006). Mitochondrial morphology is regulated by opposing but balanced fusion and fission events which maintain the normal mitochondrial network (Okamoto and Shaw, 2005). Loss of fusion results in mitochondrial fragmentation due to ongoing fission events. Conversely, loss of fission leads to the formation of elongated and highly interconnected mitochondria. The central components of the mitochondrial fusion and fission machineries have been identified, but the molecular mechanisms of both processes are not completely understood. Most proteins involved are conserved in yeast, flies, mice, and humans indicating that the fundamental mechanisms controlling mitochondrial dynamics have been maintained during evolution.

Mitochondrial fusion includes the fusion of the outer and the inner membrane of two organelles in a coordinated manner. Three proteins are essential for fusion in yeast and interact with each other to form a fusion complex: the two dynamin-related GTPases Fzo1 and Mgm1 as well as Ugo1 (Sesaki *et al.*, 2003b; Wong *et al.*, 2003; Sesaki and Jensen, 2004). Fzo1 is integrated into the outer membrane exposing its functional domains to the

cytoplasm (Hermann *et al.*, 1998; Rapaport *et al.*, 1998; Fritz *et al.*, 2001). Mgm1, on the other hand, resides in the intermembrane space but is present in two forms both of which are required for fusion (Wong *et al.*, 2000; Herlan *et al.*, 2003; Sesaki *et al.*, 2003b; Wong *et al.*, 2003): the large (l-) isoform, which is integrated into the inner membrane, and the short (s-) isoform, which is peripherally associated with inner and/or outer membrane and generated by proteolytic processing of the l-form, as discussed below. The third player, Ugo1, is also embedded into the outer membrane and exposes its N-terminal domain to the cytoplasm and the C-terminal domain to the intermembrane space (Sesaki and Jensen, 2001). Ugo1 has been shown to bind to both Fzo1 and Mgm1 via its N- and C-terminal domains thus linking the fusion components of the outer and inner membrane and possibly the fusion events of both membranes (Sesaki and Jensen, 2004). Outer- and inner-membrane fusion events are tightly coupled *in vivo*, but could be separated by the reconstitution of fusion *in vitro* (Meeusen *et al.*, 2004). Furthermore, these *in vitro* studies revealed that Fzo1 is required for outer membrane fusion, whereas inner membrane fusion depends on Mgm1 (Meeusen *et al.*, 2004; Meeusen *et al.*, 2006). For both mitochondrial membranes, *trans* interactions of either Fzo1 or Mgm1 mediate tethering of opposing membranes and subsequent fusion.

Two homologues of Fzo1 have been identified in humans, called mitofusin (Mfn) 1 and 2, whereas OPA1 is the homologue of Mgm1 (Alexander *et al.*, 2000; Delettre *et al.*, 2000; Santel and Fuller, 2001; Rojo *et al.*, 2002). Interestingly, Ugo1 does not have an obvious counterpart in mammals. The localisation of mitofusins and OPA1 in the outer membrane and intermembrane space, respectively, and their topology has been conserved in mammals (Rojo *et al.*, 2002; Griparic *et al.*, 2004). In addition, different isoforms of OPA1 are also present in mammalian mitochondria. The essential role of mitofusins and OPA1 in mitochondrial fusion has been established through the generation of knockout mice and by knockdown experiments using RNA intereference (RNAi). The absence of either Mfn1 or Mfn2 causes embryonic lethality in mice and isolated mouse embryonic fibroblasts only exhibit greatly reduced levels of mitochondrial fusion leading to a highly fragmented mitochondrial population (Chen *et al.*, 2003; Chen *et al.*, 2005). This indicates that Mfn1 and Mfn2 apparently play similar roles in mitochondrial fusion, which was supported by the finding that they can form both homo- and hetero-oligomeric complexes all competent for fusion (Chen *et al.*, 2003; Eura *et al.*, 2003; Chen *et al.*, 2005). Similarly, depletion of OPA1 from mammalian cells also results in mitochondrial fragmentation due to loss of fusion (Olichon *et al.*, 2003; Cipolat *et al.*, 2004; Griparic *et al.*, 2004; Chen *et al.*, 2005).

1.4.2 Regulation of mitochondrial fusion by proteolytic processes

In yeast, two components of the mitochondrial fusion machinery, Fzo1 and Mgm1, are under proteolytic control. In the case of Fzo1, the maintenance of mitochondrial morphology depends on the tight control of its steady state concentration which is regulated by degradation of Fzo1 via two independent proteolytic pathways. On one hand, Fzo1 turnover can be induced by cell cycle arrest with the mating factor alpha which leads to mitochondrial fragmentation (Neutzner and Youle, 2005). This induced degradation of Fzo1 is mediated by the ubiquitin-proteasome system (UPS) (Escobar-Henriques *et al.*, 2006), the central proteolytic system in the cytosol of eukaryotic cells (Ciechanover, 2005). On the other hand, Fzo1 is degraded in vegetatively growing yeast cells in a constitutive manner which depends on the F-box protein Mdm30 (Escobar-Henriques *et al.*, 2006). In the absence of Mdm30, the steady state concentration of Fzo1 is increased and yeast cells accumulate aggregated and fragmented mitochondria (Fritz *et al.*, 2003). F-box proteins often assemble into Skp1-Cdc53-F-box (SCF) E3 ubiquitin ligase complexes which ensure ubiquitin-dependent degradation by the 26S proteasome (Willems *et al.*, 2004; Petroski and Deshaies, 2005). However, Mdm30-dependent turnover of Fzo1 does not involve SCF complexes or the UPS, but rather proceeds along an alternative proteolytic pathway which remains to be identified (Escobar-Henriques *et al.*, 2006).

In contrast to control of Fzo1, Mgm1 is not subject to complete degradation but is proteolytically processed at its N-terminus yielding two isoforms within the mitochondrial intermembrane space: the large (l-) and the short (s-) isoform. Both Mgm1 isoforms are required for mitochondrial fusion and their balanced formation appears to be crucial for mitochondrial morphology (Herlan *et al.*, 2003). According to the alternative topogenesis model, the ratio of both isoforms is dictated by the level of matrix ATP (Herlan *et al.*, 2004). The Mgm1 protein contains two N-terminal transmembrane segments of which the first one serves as a stop-transfer signal during mitochondrial import of the pre-protein. At low ATP levels, the subsequent removal of the MTS by MPP and lateral membrane insertion result in l-Mgm1, which is anchored to the inner membrane. At high ATP levels, however, Mgm1 can be pulled further into the matrix by the ATP-dependent mitochondrial import motor until the second hydrophobic segment reaches the inner membrane. Thereby, a second cleavage site within this segment gets accessible for the rhomboid protease Pcp1 in the inner membrane. Pcp1-mediated cleavage generates s-Mgm1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003a), which is peripherally associated with the inner and/or outer membrane. This mechanism may link the bioenergetic state of mitochondria and their morphology.

Furthermore, it would allow separating damaged, non-functional mitochondria from the intact mitochondrial network by preventing fusion due to impaired formation of s-Mgm1.

1.4.3 OPA1 and optic atrophy

Mammalian OPA1 has been originally identified by mutations causative for autosomal dominant optic atrophy (ADOA) type I, the most common form of inherited optic neuropathy (Alexander *et al.*, 2000; Delettre *et al.*, 2000). A degeneration of retinal ganglion cells leading to atrophy of the optic nerve is the underlying defect in this disease (Delettre *et al.*, 2002). Thus, optic atrophy is also characterised by an intriguing tissue-specificity, although the OPA1 mRNA and protein are widely distributed in mammalian tissues including various brain areas (Alexander *et al.*, 2000; Delettre *et al.*, 2000; Misaka *et al.*, 2002; Olichon *et al.*, 2002; Aijaz *et al.*, 2004; Bette *et al.*, 2005). Interestingly, mutations in Mfn2 cause a different neuropathy, called Charcot-Marie-Tooth type 2A (CMT2A), whose hallmarks are muscle weakness and sensory loss in the distal limbs (Züchner *et al.*, 2004).

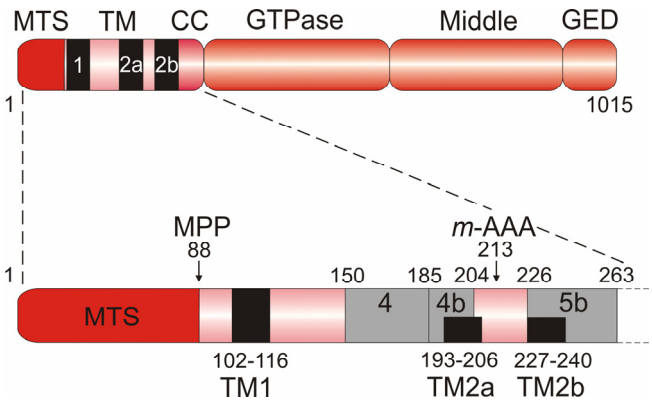

Figure 7. Domain structure of OPA1.

Schematic representation of the domain structure of splice variant 8 of human OPA1, which contains the complete sequence (1015 amino acids) and all three hydrophobic stretches called TM1, TM2a, and TM2b, indicated with black boxes (upper panel). OPA1 splice variants differ from each other by the presence or absence of protein segments encoded by alternatively spliced exons 4, 4b, and 5b, which are indicated by grey boxes (lower panel). Amino acid positions of the hydrophobic stretches and the alternative protein segments are given. The cleavage site of the mitochondrial processing peptidase (MPP) and the putative cleavage site of the *m*-AAA protease (*m*-AAA), as determined by Ishihara *et al.*, 2006, are marked by arrows. In this study, the L1 and S3 isoforms were purified from HeLa cells expressing FLAG-tagged rat OPA1 splice variant 7, which lacks TM2a, and used for protein sequencing. MTS, mitochondrial targeting sequence; TM, transmembrane domain; CC, coiled-coil domain; GTPase, GTPase domain; Middle, middle domain; GED, GTPase effector domain.

OPA1 is a key player in regulating dynamic changes of mitochondrial morphology by promoting mitochondrial fusion (Olichon *et al.*, 2003; Cipolat *et al.*, 2004). Accordingly, downregulation of OPA1 results in fragmentation of the mitochondrial network owing to ongoing fission (Olichon *et al.*, 2003; Cipolat *et al.*, 2004; Griparic *et al.*, 2004; Chen *et al.*, 2005). In addition, cells depleted of OPA1 show an aberrant cristae structure (Olichon *et al.*, 2003; Griparic *et al.*, 2004), which is reminiscent of the role of yeast Mgm1 in maintenance of cristae morphology (Sesaki *et al.*, 2003b; Amutha *et al.*, 2004; Meeusen *et al.*, 2006). Further phenotypes that have been linked to loss of OPA1 are reduction of mitochondrial membrane potential, defects in mitochondrial respiration, accelerated release of cytochrome *c* and concomitantly increased propensity for apoptosis (Olichon *et al.*, 2003; Lee *et al.*, 2004; Arnoult *et al.*, 2005; Chen *et al.*, 2005). Thus, OPA1 seems to have additional functions in mitochondria which are independent from mitochondrial fusion. Indeed, evidence has been obtained for a direct role of OPA1 in the control of cristae remodelling during apoptosis and release of cytochrome *c* which is sequestered in the intra-cristae regions (Cipolat *et al.*, 2006; Frezza *et al.*, 2006).

OPA1 is a dynamin-like GTPase which resides in the mitochondrial intermembrane space (Olichon *et al.*, 2002; Satoh *et al.*, 2003; Griparic *et al.*, 2004) and features the following domain structure (Fig. 7): an N-terminal mitochondrial targeting sequence is followed by one transmembrane segment (TM1) and two further hydrophobic stretches (TM2a and TM2b). A coiled-coil region (or heptad repeat) is located immediately in front of the GTPase domain containing the consensus GTP binding sites. The GTPase domain is followed by a middle domain and a C-terminal coiled-coil domain, known as the GTPase effector domain, which both are generally involved in oligomerisation and regulation of GTPase activity (Praefcke and McMahon, 2004). The mRNAs transcribed from the *OPA1* gene are subject to extensive alternative splicing resulting in eight different splice variants (Delettre *et al.*, 2001; Satoh *et al.*, 2003). The alternative splicing involves the exons 4, 4b, and 5b which encode protein segments of the N-terminal region between the first transmembrane domain and the GTPase domain. Therefore, OPA1 splice variants differ in the presence or absence of the two additional hydrophobic stretches TM2a and TM2b. In addition, the different splice variants are not uniformly expressed in human tissues and appear to have distinct roles in mitochondria such as mitochondrial fusion and apoptosis (Delettre *et al.*, 2001; Olichon *et al.*, 2006). Several protein isoforms of OPA1 can be detected in human cells, e.g. five different isoforms are apparently present in HeLa cells (Olichon *et al.*, 2003; Ishihara *et al.*, 2006; Olichon *et al.*, 2006), which have been designated L1 and L2 for the two

larger isoforms and S3, S4, and S5 for the three smaller isoforms (Fig. 21B) (Duvezin-Caubet *et al.*, 2006). The large and small OPA1 isoforms differ in their membrane association. Whereas the L-isoforms are anchored to the inner membrane, S-isoforms are only peripherally attached to the membrane (Duvezin-Caubet *et al.*, 2006).

It has, however, remained unclear to which extent the different OPA1 isoforms are generated by alternative splicing and/or proteolytic processing similar to yeast Mgm1. Evidence for the involvement of proteolysis has recently been obtained by the observation that upon dissipation of the mitochondrial membrane potential the L-isoforms were converted into S-isoforms (Duvezin-Caubet *et al.*, 2006; Ishihara *et al.*, 2006). This induced conversion was abolished in the presence of protease inhibitors indicating proteolytic cleavage. Moreover, this apparent OPA1 processing was accompanied by mitochondrial fragmentation thus linking mitochondrial dysfunction to changes in mitochondrial morphology (Duvezin-Caubet *et al.*, 2006; Ishihara *et al.*, 2006). Large OPA1 isoforms are generated upon mitochondrial import by MPP (Ishihara *et al.*, 2006). However, the protease mediating further OPA1 cleavage has not been unambiguously identified, yet. Due to the homology of Mgm1 and OPA1, the rhomboid protease PARL (presenilin-associated rhomboid-like) (Pellegrini *et al.*, 2001), the mammalian homologue of yeast Pcp1, has been implicated in OPA1 processing. PARL can functionally replace yeast Pcp1 and mediate processing of Ccp1 and Mgm1 (McQuibban *et al.*, 2003). Furthermore, 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). In support of proteolysis by PARL, it was found to interact with OPA1 and to be involved in the generation of small amounts of a soluble intermembrane space form of OPA1. Deletion of *PARL*, however, did not significantly impair the pattern of OPA1 isoforms (Cipolat *et al.*, 2006). The mammalian *m*-AAA protease has also been linked to OPA1 processing, since over-expression of its subunit paraplegin in HeLa cells led to an increased accumulation of S-isoforms (Ishihara et al., 2006). Vice versa, OPA1 processing was slightly inhibited upon downregulation of paraplegin (Ishihara *et al.*, 2006).

1.5 Aims of the thesis

AAA proteases in the mitochondrial inner membrane are evolutionary conserved components of the protein quality control system and play crucial regulatory roles during organellar biogenesis. Mutations in the mammalian *m*-AAA protease subunit paraplegin are causative for hereditary spastic paraplegia (HSP) in humans and in the mouse model of this disorder. However, the molecular basis of cell-specific axonal degeneration in HSP patients lacking paraplegin is poorly understood. Mammalian paraplegin has been shown to assemble with its homologue Afg3l2 into a hetero-oligomeric *m*-AAA protease complex, but the assembly status of Afg3l2 in the absence of paraplegin has not been assessed. Moreover, murine mitochondria contain a third putative *m*-AAA protease subunit, termed Afg3l1, whose function remains elusive.

Due to these considerations, initial experiments were aiming at analysing the complex formation of human AFG3L2 using mitochondria isolated from paraplegin-deficient HSP fibroblasts. Previous studies have shown that the yeast *S. cerevisiae* can serve as a model system for the biochemical and functional characterisation of mammalian proteins. Therefore, human AFG3L2 and murine *m*-AAA protease subunits were heterologously expressed in yeast cells lacking the endogenous *m*-AAA protease. This should provide further insights into the assembly of human AFG3L2 and allow the identification of the number and subunit composition of murine *m*-AAA protease complexes which potentially can be formed. In addition, these experiments should clarify the role of murine Afg3l1.

Mutations in OPA1, a component of the mitochondrial fusion machinery, lead to another neurodegenerative disorder called autosomal dominant optic atrophy. Mammalian mitochondria contain several protein isoforms of OPA1, but it is unclear to which extent they are derived from alternative splicing and/or proteolytic processing. Paraplegin has recently been linked to OPA1 cleavage, but an involvement of the *m*-AAA protease in this process is still a matter of debate. Therefore, another aim of this thesis was the reconstitution of OPA1 processing in yeast upon co-expression of individual OPA1 splice variants and mammalian *m*-AAA protease subunits. This should help to determine the role of proteolytic processing by the mammalian *m*-AAA protease in the generation of OPA1 isoforms.

2 Material and Methods

2.1 Expression plasmids and cloning procedures

For expression of human *m*-AAA protease subunits in yeast, constructs consisting of the mitochondrial targeting sequence (MTS) of Yta10 (amino acids 1-63 or 1-61) and the assumed mature forms of hparaplegin (amino acids 59-795) or hAFG3L2 (amino acids 36-798), respectively, were excised from the CEN plasmids YCplac111 or pRS316 and cloned into the multicopy plasmids YEplac181 or YEplac112 (Gietz and Sugino, 1988) under the control of the *ADH1* promoter (720 bp) as previously described (Koppen, 2002). In order to facilitate immunodetection, a hemagglutinin (HA) epitope was added to the C-terminus of hparaplegin, whereas a c-Myc epitope was used for hAFG3L2. In case of the murine *m*-AAA protease subunits, the putative mature forms of paraplegin (amino acids 44-781), Afg3l1 (amino acids 25-789), and Afg3l2 (amino acids 36-802) were also fused to the MTS of Yta10 (amino acids 1-61). The corresponding DNA sequences were amplified from either genomic DNA of wild-type yeast cells or murine cDNA and restriction sites were introduced by polymerase chain reaction (PCR) using the oligonucleotides listed in Tab. 4. Subsequently, a *Sbf*I/*Bam*HI DNA fragment containing the *YTA10* promoter (480 bp) and bp 1-183 of *YTA10* and a *Bam*HI/*Eco*RI DNA fragment containing bp 130-2343 of paraplegin were cloned into the corresponding restriction sites of the multicopy plasmid YEplac181. Similarly, a *Hind*III/*Bam*HI DNA fragment of the *YTA10* promoter and MTS and a *Bam*HI/*Kpn*I DNA fragment containing bp 73-2367 of Afg3l1 were cloned into the multicopy vector YEplac195 (Gietz and Sugino, 1988). For cloning of the Afg3l2 expression construct into YEplac112, a *Xma*I/*Hind*III DNA fragment consisting of the *YTA10* promoter and MTS and a *Hind*III/*Kpn*I DNA fragment containing bp 106-2406 of Afg3l2 were used. A c-Myc or an HA epitope were attached to the C-terminus of either Afg3l1 or Afg3l2 to allow immunodetection of the respective hybrid proteins Yta10 (1-61)-Afg3l1 (25-789)-Myc or Yta10 (1-61)-Afg3l2 (36-802)-HA.

m-AAA protease subunits were inactivated by substitution of catalytically active glutamate residues within the proteolytic centres by glutamine residues using the Quick Change site-directed mutagenesis kit (Stratagene). Degenerated oligonucleotides used are listed in Tab. 4. The respective codons of the human and murine expression constructs were mutated as follows: GAA to CAG for hparaplegin (amino acid 575), GAA to CAA for

hAFG3L2 (amino acid 575) and murine Afg3l2 (amino acid 574), and GAG to CAG for murine paraplegin (amino acid 575) and Afg3l1 (amino acid 567). Mutagenesis was always verified by DNA sequencing.

Table 3. List of plasmids used in this study.

Plasmid	Reference
YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin (59-795)	Koppen, 2002
YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin^{E575Q} (59-795)	this study
YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc	Koppen, 2002
YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2^{E575Q} (36-798)-Myc	this study
YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781)	this study
YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781)	this study
YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc	this study
YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc	this study
YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA	this study
YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA	this study
pYES2-OPA1-Sp.1, -Sp.4, -Sp.7, and -Sp.8 (*URA3*)	Duvezin-Caubet *et al.*, 2007
pYES2-OPA1-Sp.1, -Sp.4, -Sp.7, and -Sp.8 (*ura3::TRP1*)	this study
pUT11	Cross, 1997
pGEM4-Yme2^{F114S}ΔC15+1 (-Yme2ΔC)	Korbel *et al.*, 2004
pGEM4-Atp7	Korbel *et al.*, 2004
pFA6a-kanMX6	Longtine *et al.*, 1998

Plasmids for expression of human OPA1 splice variants (Sp.) 1, 4, 7, and 8 were described previously (Duvezin-Caubet *et al.*, 2007). The OPA1 splice variant 8 encodes the polymorphism A210V (Yao *et al.*, 2006). In order to change the *URA3* selection marker of the pYES2-OPA1-Sp.X (X: 1, 4, 7, or 8) plasmid series to *TRP1*, the 'marker swap' plasmid pUT11 was employed. By using the restriction endonuclease *Sma*I, a disrupting DNA fragment of ~4 kb was generated which contains the *TRP1* gene and flanking regions homologous to the *URA3* gene (Cross, 1997). Wild-type yeast cells harbouring pYES2-OPA1-Sp.X were transformed with this *Sma*I DNA fragment to allow homologous recombination resulting in pYES2-OPA1-Sp.X (*ura3::TRP1*). After selection of Trp1^{+} but

Ura3$^-$ yeast cells, the pYES2-OPA1-Sp.X (*ura3::TRP1*) plasmids were re-isolated and used for further transformations.

Table 4. List of oligonucleotides used in this study.

Oligonu-cleotide	Description	Sequence
TL209	Deletion of *YTA12*	5'-ACTATACCGGCTAACTGGAAAGAACAGAAAC GCAAAATTGATGAACGTACGCTGCAGGTCGAC-3'
TL210	Deletion of *YTA12*	5'-GTCACCTGTCTCGTCCGAAAATGGTTTTGTCA AATCGCTATCATCATCGATGAATTCGAGCTCG-3'
TL1048	3'-hparaplegin^{E575Q}	5'-GGCGTGGCCCGACTGATGAAACGCAACCAC-3'
TL1049	5'-hparaplegin^{E575Q}	5'-GTGGTTGCGTTTCATCAGTCGGGCCACGCC-3'
TL1050	3'-hAFG3L2^{E575Q}	5'-CCGCATGGCCTGCTTGGTGGTATGCCACAG-3'
TL1051	5'-hAFG3L2^{E575Q}	5'-CTGTGGCATACCACCAAGCAGGCCATGCGG-3'
TL1616	5'-*Sbf*I-*YTA10* promoter and MTS	5'-TTTTTCCTGCAGGGTGTGTCCAATTACGCGCAC GT-3'
TL1617	3'-*Bam*HI-*YTA10* promoter and MTS	5'-AAAAGGATCCTATTTGGGTAGAACGGTGTATT GTG-3'
TL1618	5'-*Hind*III-*YTA10* promoter and MTS	5'-TTTTTAAGCTTGTGTGTCCAATTACGCGCACGT TGGG-3'
TL1619	5'-*Xma*I-*YTA10* promoter and MTS	5'-TCCCCCCGGGGTGTGTCCAATTACGCGCACGT TGG-3'
TL1620	3'-*Hind*III-*YTA10* promoter and MTS	5'-AAAAAAGCTTTATTTGGGTAGAACGGTGTATT GTGTT-3'
TL1621	5'-*Hind*III-Afg3l2	5'-TTTTAAGCTTCTCCGTACGCTCTATCAATATGC TACTG-3'
TL1622	3'-*Kpn*I-Afg3l2	5'-AAAAGGTACCTCAAGCGTAATCTGGAACATCG TATGG-3'
TL1642	5'-*Bam*HI-paraplegin	5'-TTTTGGATCCGCGGCCCGCGGGACTCCGGTTG-3'
TL1643	3'-*Eco*RI-paraplegin	5'-AAAAGAATTCCTAGGGAGCCGGAGCCTCCTCC TCCCCTG-3'
TL1644	5'-*Bam*HI-Afg3l1	5'-TTTTGGATCCGGCGGATGCGCAGGGAGCGGCG GGAC-3'
TL1645	3'-*Kpn*I-Afg3l1	5'-AAAAGGTACCCTACAGATCCTCCTCAGAAATC AGCTTTTG-3'
TL2061	5'-Afg3l2^{E574Q}	5'-CGGTGGCTTACCACCAAGCAGGCCATGCGG-3'
TL2062	3'-Afg3l2^{E574Q}	5'-CCGCATGGCCTGCTTGGTGGTAAGCCACCG-3'
TL2501	5'-paraplegin^{E575Q}	5'-GGTCGCCTTCCATCAGTCTGGCCATGCC-3'
TL2502	3'-paraplegin^{E575Q}	5'-GGCATGGCCAGACTGATGGAAGGCGACC-3'
TL2503	5'-Afg3l1^{E567Q}	5'-CTGTAGCCTACCACCAGGCTGGGCATGCAG-3'
TL2504	3'-Afg3l1^{E567Q}	5'-CTGCATGCCCAGCCTGGTGGTAGGCTACAG-3'

The plasmids pGEM4-Atp7 and pGEM4-Yme2^{F114S}ΔC15+1 (-Yme2ΔC) for SP6-polymerase-driven expression *in vitro* were described previously (Korbel *et al.*, 2004).

2.2 Yeast strains and growth conditions

All *S. cerevisiae* strains used in this study are derivatives of W303 (Rothstein and Sherman, 1980; genotype: *MATa/α ade2-1 his3-11,15 leu2,112 trp1-1 ura3-52 can1-100*) and listed in Tab. 5. The strain Δ*yta10*Δ*yta12* (YKO200) was obtained by deletion of *YTA12* in the strain Δ*yta10* (YGS101) (Arlt *et al.*, 1998) by PCR-targeted homologous recombination using the *kanMX6* deletion cassette and the oligonucleotides TL209 and TL210 (Longtine *et al.*, 1998). Δ*yta10*Δ*yta12* strains expressing human or murine *m*-AAA proteases (see Tab. 3) were generated by transformation of YKO200 cells with the plasmids YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin (59-795), YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc, YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781), YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc, and YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA or plasmids encoding the respective proteolytic site variants. To obtain yeast strains harbouring human OPA1 splice variants 1, 4, 7, or 8, W303 *MATa* cells, YKO200 cells, or YKO200 cells expressing mammalian *m*-AAA protease subunits were transformed with the pYES2-OPA1-Sp.X (*URA3*) plasmid series. For co-expression of OPA1 splice variants in Δ*yta10*Δ*yta12* cells containing YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc, the pYES2-OPA1-Sp.X (*ura3::TRP1*) plasmids were employed. Δ*pcp1* and Δ*phb1* strains expressing the OPA1 splice variants were generated by transformation of either YTT407 cells (T. Tatsuta, unpublished) or YGS410 cells (Steglich *et al.*, 1999) with pYES2-OPA1-Sp.X (*URA3*). All transformations were carried out using the lithium acetate method described by (Gietz *et al.*, 1995).

Yeast cells were grown according to standard procedures at 30°C either in YP medium or synthetic minimal medium supplemented with the required auxotrophs (Sherman, 2002; Tatsuta and Langer, 2007). 2% (w/v) glucose or, for isolation of mitochondria, 2% (w/v) galactose and 0.5% (w/v) lactate or, for examining respiratory growth, 3% (w/v) glycerol were added as carbon sources. In the latter case, 5 x 10^4 yeast cells were spotted onto glycerol-containing YP plates. To induce *GAL1* promoter-driven OPA1 expression, yeast cells harbouring pYES2-OPA1-Sp.X plasmids were grown in minimal medium containing 1.5% (w/v) galactose and 0.5% (w/v) glucose.

Table 5. List of yeast strains used*. All YKO strains were generated in this study.

Name	Description	Genotype	Plasmids
WT	Wild-type	W303 *MATa*	
YKO200	Δ*yta10*Δ*yta12*	W303 *MATα yta10::HIS3MX6 yta12::kanMX6*	
YKO201	Δ*yta10*Δ*yta12* + hparaplegin	YKO200	YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin (59-795)
YKO203	Δ*yta10*Δ*yta12* + hAFG3L2	YKO200	YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc
YKO204	Δ*yta10*Δ*yta12* + hAFG3L2^{EQ}	YKO200	YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2^{E575Q} (36-798)-Myc
YKO205	Δ*yta10*Δ*yta12* + hparaplegin + hAFG3L2	YKO200	YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin (59-795); YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc
YKO206	Δ*yta10*Δ*yta12* + hparaplegin^{EQ} + hAFG3L2	YKO200	YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin^{E575Q} (59-795); YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc
YKO207	Δ*yta10*Δ*yta12* + hparaplegin + hAFG3L2^{EQ}	YKO200	YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin (59-795); YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2^{E575Q} (36-798)-Myc
YKO208	Δ*yta10*Δ*yta12* + hparaplegin^{EQ} + hAFG3L2^{EQ}	YKO200	YEplac181^{ADH1}-Yta10 (1-63)-hparaplegin^{E575Q} (59-795); YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2^{E575Q} (36-798)-Myc
YKO209	Δ*yta10*Δ*yta12* + paraplegin	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781)
YKO211	Δ*yta10*Δ*yta12* + Afg3l1	YKO200	YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc
YKO212	Δ*yta10*Δ*yta12* + Afg3l1^{EQ}	YKO200	YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc
YKO213	Δ*yta10*Δ*yta12* + Afg3l2	YKO200	YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA
YKO214	Δ*yta10*Δ*yta12* + Afg3l2^{EQ}	YKO200	YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA
YKO215	Δ*yta10*Δ*yta12* + paraplegin + Afg3l1	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc
YKO216	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l1	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc
YKO217	Δ*yta10*Δ*yta12* + paraplegin + Afg3l1^{EQ}	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc
YKO218	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l1^{EQ}	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc
YKO219	Δ*yta10*Δ*yta12* + paraplegin + Afg3l2	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA

Name	Description	Genotype	Plasmids
YKO220	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l2	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA
YKO221	Δ*yta10*Δ*yta12* + paraplegin + Afg3l2^{EQ}	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA
YKO222	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l2^{EQ}	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA
YKO400	WT + OPA1-Sp4	W303 *MATa*	pYES2-OPA1-Sp4 (*URA3*)
YKO401	Δ*yta10*Δ*yta12* + OPA1-Sp4	YKO200	pYES2-OPA1-Sp4 (*URA3*)
YKO402	Δ*pcp1* + OPA1-Sp4	W303 *MATα* *pcp1::kanMX6*	pYES2-OPA1-Sp4 (*URA3*)
YKO404	Δ*yta10*Δ*yta12* + paraplegin + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781) pYES2-OPA1-Sp4 (*URA3*)
YKO405	Δ*yta10*Δ*yta12* + Afg3l1 + OPA1-Sp4	YKO200	YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc pYES2-OPA1-Sp4 (*ura3*::*TRP1*)
YKO406	Δ*yta10*Δ*yta12* + Afg3l1^{EQ} + OPA1-Sp4	YKO200	YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc pYES2-OPA1-Sp4 (*ura3*::*TRP1*)
YKO407	Δ*yta10*Δ*yta12* + Afg3l2 + OPA1-Sp4	YKO200	YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA pYES2-OPA1-Sp4 (*URA3*)
YKO408	Δ*yta10*Δ*yta12* + Afg3l2^{EQ} + OPA1-Sp4	YKO200	YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA pYES2-OPA1-Sp4 (*URA3*)
YKO409	Δ*yta10*Δ*yta12* + paraplegin + Afg3l1 + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc; pYES2-OPA1-Sp4 (*ura3*::*TRP1*)
YKO410	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l1 + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1 (25-789)-Myc; pYES2-OPA1-Sp4 (*ura3*::*TRP1*)
YKO411	Δ*yta10*Δ*yta12* + paraplegin + Afg3l1^{EQ} + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc; pYES2-OPA1-Sp4 (*ura3*::*TRP1*)
YKO412	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l1^{EQ} + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac195^{YTA10}-Yta10 (1-61)-Afg3l1^{E567Q} (25-789)-Myc; pYES2-OPA1-Sp4 (*ura3*::*TRP1*)

Name	Description	Genotype	Plasmids
YKO413	Δ*yta10*Δ*yta12* + paraplegin + Afg3l2 + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA; pYES2-OPA1-Sp4 (*URA3*)
YKO414	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l2 + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2 (36-802)-HA; pYES2-OPA1-Sp4 (*URA3*)
YKO415	Δ*yta10*Δ*yta12* + paraplegin + Afg3l2^{EQ} + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA; pYES2-OPA1-Sp4 (*URA3*)
YKO416	Δ*yta10*Δ*yta12* + paraplegin^{EQ} + Afg3l2^{EQ} + OPA1-Sp4	YKO200	YEplac181^{YTA10}-Yta10 (1-61)-paraplegin^{E575Q} (44-781); YEplac112^{YTA10}-Yta10 (1-61)-Afg3l2^{E574Q} (36-802)-HA; pYES2-OPA1-Sp4 (*URA3*)
YKO470	Δ*yta10*Δ*yta12* + hAFG3L2 + OPA1-Sp4	YKO200	YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2 (36-798)-Myc; pYES2-OPA1-Sp4 (*URA3*)
YKO471	Δ*yta10*Δ*yta12* + hAFG3L2^{EQ} + OPA1-Sp4	YKO200	YEplac112^{ADH1}-Yta10 (1-61)-hAFG3L2^{E575Q} (36-798)-Myc; pYES2-OPA1-Sp4 (*URA3*)
YKO477	Δ*phb1* + OPA1-Sp4	W303 *MATα phb1::HIS3MX6*	pYES2-OPA1-Sp4 (*URA3*)

*This list only contains the strains expressing OPA1 splice variant 4 (OPA1-Sp.4). The corresponding strain names for OPA1 splice variants 1, 7, and 8 are as follows: YKO417-YKO433, YKO468, YKO469, and YKO476 for OPA1-Sp.1; YKO434-450, YKO472, YKO473, and YKO478 for OPA1-Sp.7; YKO451-YKO469, YKO474, YKO475, and YKO479 for OPA1-Sp.8.

2.3 Cell culture

Primary human fibroblasts are derived from a control individual and an HSP patient who is homozygous for a 9.5 kb deletion in the *SPG7* gene encoding hparaplegin (De Michele *et al.*, 1998; Atorino *et al.*, 2003). Fibroblasts were cultured in minimal essential medium supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, and 20% fetal bovine serum at 37°C in 95% humidified air and 5% CO_2. For HeLa cells, minimal essential medium with Earle's salts supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, 1x non-essential amino acids, and 10% fetal bovine serum was used.

2.4 Preparation of total extracts from S. cerevisiae and HeLa cells

In order to prepare total cell extracts from yeast cells expressing human OPA1 splice variants, alkaline lysis was carried out according to (Yaffe and Schatz, 1984). 1 OD_{600} unit yeast cells was sedimented by centrifugation for 2 min at 16,000 *g* and washed once with H_2O. Subsequently, the cell pellet was resuspended in 500 µl H_2O and 75 µl alkaline lysis buffer [1.85 M NaOH, 7.4% (v/v) β-mercaptoethanol, 10 mM PMSF, 5% (v/v) ethanol] were added. After incubation for 10 min at 4°C, the sample was subjected to TCA precipitation (Tatsuta and Langer, 2007) and analysed by SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). For SDS-PAGE, precipitated cellular proteins corresponding to 0.25 OD_{600} units yeast cells were loaded for cells expressing OPA1 splice variants 4, 7, and 8, whereas proteins of 1 OD_{600} unit yeast cells were used in case of OPA1 splice variant 1.

HeLa cells were resuspended in SDS-PAGE sample buffer [1.6% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 50 mM Tris-HCl pH 6.8, 8% (w/v) glycerol, 0,005% (w/v) bromphenol blue]. After addition of 1 µl of the nuclease benzonase (25 U/µl, Novagen), the sample was mixed for 30 min at 1,400 rpm and RT and analysed as described above.

2.5 Preparation of cellular membranes from S. cerevisiae

For the fast preparation of cellular membrane fractions including mitochondrial fractions, yeast cells were lysed using glass beads. 10 OD_{600} units of a yeast culture were centrifuged for 5 min at 3,000 *g* and RT. The yeast cells were then resuspended in 300 µl ice-cold SHKCl buffer (0,6 M sorbitol, 50 mM HEPES/KOH pH 7.4, 80 mM KCl, 2 mM PMSF) and glass beads (∅ 0,5 mm) equivalent to a volume of 200 µl were added. The sample was mixed four times for 30 s with a Vortex mixer and each time cooled on ice for another 30 s. Upon the addition of 400 µl SHKCl buffer, glass beads and non-lysed cells were removed by centrifugation for 3 min at 500 *g* and 4°C. The cellular membrane fraction was separated from the soluble supernatant by centrifugation for 10 min at 16,000 *g* and 4°C. In addition, 50 µl of the supernatant containing the cytosolic fraction were subjected to TCA precipitation (Tatsuta and Langer, 2007) and together with the total membrane fraction immunochemically analysed after SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). The presence of mitochondrial membranes in the cellular membrane fraction was verified by immunodecoration with an antibody recognising the mitochondrial ADP/ATP carrier Aac2 (Tab. 6).

2.6 Alkaline extraction of mitochondrial membranes

The membrane insertion of mitochondrial proteins can be examined using alkaline extraction of membranes in a sodium carbonate buffer (Fujiki *et al.*, 1982). This extraction allows the separation of integral membrane proteins from soluble proteins and proteins peripherally associated with mitochondrial membranes by centrifugation. These latter proteins can be recovered from the supernatant fraction, whereas integral membrane proteins remain in the insoluble pellet fraction. Isolated mitochondria (100 µg mitochondrial protein) were resuspended in 1 ml 100 mM sodium carbonate (pH 11.5) and incubated for 30 min at 4°C. A sample of 500 µl (total) was withdrawn and TCA-precipitated (Tatsuta and Langer, 2007). The remaining 500 µl were subjected to centrifugation for 30 min at 125,000 g and 4°C. The resulting insoluble pellet fraction was resuspended in 500 µl 100 mM sodium carbonate (pH 11.5) and like the supernatant fraction supplemented with TCA. Precipitated proteins of the total, the supernatant, and the pellet were analysed by SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). The separation of integral membrane proteins from soluble proteins was surveyed by immunodetection using antibodies directed against the multi-spanning inner membrane protein Aac2 and the soluble matrix protein Mge1 (Tab. 6).

2.7 Isolation of mitochondria from human fibroblasts

Mitochondria of human primary fibroblasts from a control individual or a paraplegin-deficient HSP patient were isolated by differential centrifugation of fibroblast homogenates. Human fibroblasts were washed in PBS and resuspended in 5 ml isotonic homogenisation buffer (250 mM sucrose, 5 mM Tris-HCl pH 7.5, 0.1 mM PMSF). The cell suspension was homogenised by 35 strokes in a Dounce glass-homogeniser. Nuclei and unbroken cells were removed by centrifugation for 10 min at 4,500 *g* and 4°C. The supernatant was centrifuged for 20 min at 16,000 *g* and 4°C to collect the mitochondria. Subsequently, the mitochondrial pellet was washed and resuspended with homogenisation buffer supplemented with 1 mM EDTA (pH 8.0). Finally, the protein concentration of the suspension was determined using the Bradford assay according to manufacturer's instruction (Bio-Rad). Mitochondria were diluted, frozen as 100 µg aliquots in liquid nitrogen, and stored at -80°C.

2.8 Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE is a method to analyse native protein complexes of mitochondrial membranes (Schägger, 2001; Reisinger and Eichacker, 2006). It is based on the usage of an anionic dye (Coomassie blue G-250), which is added after the solubilisation of mitochondria and binds to the surface of proteins. Thereby, a negative net charge is introduced into the protein complexes allowing an electrophoretic separation. A high resolution according to the mass of the protein complexes is achieved by the decreasing pore size of an acrylamide-gradient gel. The gradient gels used in this study were composed of a separation gel with a linear gradient of 3-13% polyacrylamide [3-13% (w/v) acrylamide, 0.09-0.4% (w/v) bisacrylamide, 0-20% (w/v) glycerol, 0.5 M ε-amino-*n*-caproic acid, 25 mM imidazole-HCl pH 7.0, 0.1% (w/v) APS, 0.1% (v/v) TEMED] and a stacking gel [3% (w/v) acrylamide, 0.09% (w/v) bisacrylamide, 0.5 M ε-amino-*n*-caproic acid, 25 mM imidazole-HCl pH 7.0, 0.1% (w/v) APS, 0.1% (v/v) TEMED]. Mitochondrial proteins (150 µg) isolated from yeast or human primary fibroblasts were solubilised by shaking (15 min; 1,400 rpm; 4°C) at a concentration of 5 mg/ml in 1% (w/v) digitonin, 30 mM Tris-HCl pH 7.4, 4 mM Mg-acetate, 5 mM ε-amino-*n*-caproic acid, 50 mM NaCl, 1 mM ATP. After a clarifying spin for 30 min at 125,000 *g* and 4°C, mitochondrial extracts were supplemented with 2 µl 50% (w/v) glycerol and 1 µl sample buffer [5% (w/v) Coomassie blue G-250 in 0.5 M ε-amino-*n*-caproic acid] and loaded onto the polyacrylamide-gradient gel. The electrophoretic separation was carried out in Mini-Protean-3-gel chambers (Bio-Rad) at 4°C [deep blue cathode buffer B: 50 mM Tricine, 7.5 mM imidazole, 0.02% (w/v) Coomassie blue G-250; anode buffer: 25 mM imidazole-HCl pH 7.0] using a constant voltage of 50 V and a current of 15 mA for ~30 min, followed by 300 V and 15 mA for ~30 min. Subsequently, the deep blue cathode buffer B was exchanged for a cathode buffer of identical composition but lacking Coomassie blue G-250 and the separation was continued at 300 V and 15 mA. Finally, the proteins were transferred from the gradient gel onto a PVDF membrane for 2 h at 200 mA. Thyroglobulin (669 kDa) and apoferritin (443 kDa) were used for calibration. Protein complexes containing hAFG3L2 were detected by immunodecoration with a specific antiserum (Tab. 6).

2.9 Gel filtration analysis of mitochondrial extracts from yeast

Gel filtration analysis (size exclusion chromatography) using a Superose 6 column and the Äkta Purifier-LC system (GE Healthcare) was employed to analyse the complex formation of mammalian *m*-AAA protease subunits and to determine their native molecular

mass. Isolated yeast mitochondria (900 µg mitochondrial protein) were solubilised by shaking (15 min; 1,400 rpm; 4°C) at a concentration of 5 mg/ml in 1% (w/v) digitonin, 30 mM Tris-HCl pH 7.4, 4 mM Mg-acetate, 150 mM K-acetate pH 7.4, 1 mM PMSF, 1 mM ATP. Non-solubilised or aggregated material was removed by centrifugation for 30 min at 125,000 *g* and 4°C and the supernatant was loaded onto a Superose 6 column, which was equilibrated with digitonin-containing buffer lacking PMSF. The flow rate was set to 0.3 ml/min. Eluted proteins were collected in 500 µl fractions, TCA-precipitated (Tatsuta and Langer, 2007), and analysed by SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). Proteins present in eluate fractions were quantified by laser densitometry (Kodak Image Station 440) and normalised to the amount of the respective protein in the total eluate. The calibration of the Superose 6 column was carried out with the following marker proteins: Hsp60 (840 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumine (66 kDa).

2.10 Degradation of newly imported, radiolabelled polypeptides in isolated yeast mitochondria

The SP6-polymerase-driven *in vitro* transcription of pGEM4-Atp7 and pGEM4-Yme2ΔC, the cell free synthesis of radiolabelled precursor proteins, and the subsequent analysis of their degradation upon import into isolated yeast mitochondria were performed as previously described except for the following changes (Tatsuta and Langer, 2007). For mitochondrial import, the import buffer was supplemented with 5 mM NADH, 2.5 mM ATP, 10 mM creatine phosphate, 100 µg/ml creatine kinase, and 0.5 mg/ml isolated yeast mitochondria. After incubation for 3 min at 25°C, the reticulocyte lysate (Promega) containing radiolabelled precursor proteins was added to the import reaction at a final concentration of 2% (v/v), followed by incubation for 10 min at 25°C. Mitochondrial import was stopped by the addition of 0.5 µM valinomycin. After removal of non-imported precursor proteins by trypsin treatment (Tatsuta and Langer, 2007), the import reaction was divided into 5 aliquots of 60 µl (corresponding to 30 µg mitochondrial protein), which were incubated for 0, 5, 10, 20, and 30 min at 37°C to allow proteolysis. Mitochondria were re-isolated by centrifugation for 5 min at 16,000 *g* and 4°C and washed once with SHKCl. The samples were analysed by autoradiography after SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). For the quantification of radiolabelled proteins, a phosphorimaging plate (BAS-SR 127, Fuji) was used and the phosphorescence signals were detected with a

reader (Molecular Imager, Bio-Rad) and quantified (Quantity One, Bio-Rad). The integrity of the mitochondria was monitored by immunodetection with antisera specific for the intermembrane space protein Ccp1 and the matrix protein Mge1 (Tab. 6).

2.11 Co-immunoprecipitation

Co-immunoprecipitation experiments were performed to investigate a direct interaction between murine *m*-AAA protease subunits following expression in yeast. In order to couple antibodies to Protein A Sepharose (PAS) beads (GE Healthcare), 5 mg PAS beads were equilibrated with washing buffer [0.5% (w/v) digitonin, 30 mM Tris-HCl pH 7.4, 4 mM Mg-acetate, 150 mM K-acetate pH 7.4, 1 mM PMSF, 1 mM ATP] and then incubated with 15 µl of c-Myc-specific or 10 µl of HA-specific antisera (Tab. 6) for 1.5 h at 4°C under gentle shaking in 400 µl washing buffer. Subsequently, the PAS beads with coupled antibodies were washed once with washing buffer and used for co-immunoprecipitation.

Isolated yeast mitochondria (400 µg mitochondrial protein) harbouring murine *m*-AAA protease subunits were solubilised by shaking (15 min; 1,400 rpm; 4°C) at a concentration of 1 mg/ml in lysis buffer [2% (w/v) digitonin, 30 mM Tris-HCl pH 7.4, 4 mM Mg-acetate, 150 mM K-acetate pH 7.4, 1 mM PMSF, 1 mM ATP supplemented with EDTA-free protease inhibitor cocktail (Roche)]. After centrifugation for 15 min at 125,000 *g* and 4°C, 300 µl of the supernatant were loaded onto the prepared PAS beads (5 mg) with coupled c-Myc- or HA-specific antibodies and incubated under gentle shaking for 2.5 h at 4°C. The PAS beads were then washed twice with washing buffer and once with 10 mM Tris-HCl pH 7.4. Antibody-antigen complexes were eluted from the PAS beads with SDS-PAGE sample buffer and analysed by SDS-PAGE (Laemmli, 1970) and Western blot (Towbin *et al.*, 1979). 10% of the mitochondrial extract obtained by solubilisation and centrifugation were used as input control and analysed in parallel.

2.12 Immunological detection of proteins

Nitrocellulose or PVDF membranes were incubated after protein transfer for at least 30 min at RT in blocking solution [5% (w/v) milk powder in TBS buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl)]. This was followed by immunodecoration for 60-120 min with a specific antiserum (Tab. 6) diluted in 5% (w/v) milk powder in TBS buffer. Upon removal of the antiserum, the membranes were washed for 10 min with TBS, TBS supplemented with 0.05% (v/v) Triton X-100, and again with TBS, respectively. To recognise bound antibodies,

horseradish peroxidase-conjugated antibodies specific for immunoglobulins G of rabbit, mouse, or rat were employed in a dilution of 1:10,000 in 5% (w/v) milk powder in TBS. Membranes were incubated for 60 min with these solutions and subsequently washed twice for 10 min with TBS. The bound peroxidase was detected after incubating the membranes with 2 ml of a 1:1 mixture of chemoluminiscence reagents [solution 1: 10 ml 1 M Tris-HCl pH 8.5, 1 ml luminol (44 µg/ml in DMSO), 440 µl p-coumaric acid (15 mg/ml in DMSO), 88.56 ml H_2O; solution 2: 10 ml Tris-HCl pH 8.5, 60 µl 30% hydrogen peroxide, 89.94 ml H_20]. Subsequently, the membranes were exposed to light-sensitive X-ray films (Super RX, Fuji).

Table 6: Antibodies used in this study are listed in the order of their appearance.

Antibody	Antigen	Dilution	Reference
α-hAFG3L2	amino acids 413-828 of human AFG3L2	1:2000	Atorino *et al.*, 2003
α-pMrpL32	peptide corresponding to amino acids 45-59 of MrpL32 from *S. cerevisiae*	1:1000	Nolden *et al.*, 2005
α-mMrpL32	peptide corresponding to amino acids 131-145 of MrpL32 from *S. cerevisiae*	1:500	Nolden *et al.*, 2005
α-Mge1	C-terminal peptide of Mge1 from *S. cerevisiae*	1:10000	Schneider *et al.*, 1994
α-Ccp1	purified protein, cytochrome *c* peroxidase from *S. cerevisiae*	1:1000	
α-Tom40	C-terminal peptide of Tom40 from *S. cerevisiae*	1:20000	
α-paraplegin*	peptide corresponding to amino acids 121-139 of murine paraplegin	1:100	Koppen *et al.*, 2007
α-Myc (9B11)	peptide corresponding to amino acids 410-419 of human c-Myc (EQKLISEEDL)	1:1000	Cell Signalling Technology
α-HA (3F10)	synthetic peptide corresponding to amino acids 76-111 of X47 hemagglutinin 1 (recognises the sequence YPYDVPDYA)	1:1000	Roche
α-Aac2	C-terminal peptide of the ADP/ATP carrier from *S. cerevisiae*	1:10000	
α-OPA1	amino acids 708-830 of human OPA1	1:500	BD biosciences

p, precursor; m, mature form; *, this antiserum raised against an epitope of murine paraplegin also recognises human paraplegin.

2.13 Miscellaneous

The following procedures used in this study have been described previously: isolation of mitochondria from yeast (Tatsuta and Langer, 2007); molecular biology and genetics methods (Sambrook and Russell, 2001).

3 Results

3.1 Identification of the human AFG3L2 complex, a novel homo-oligomeric m-AAA protease

Human paraplegin (hparaplegin) and AFG3L2 (hAFG3L2) assemble into a hetero-oligomeric *m*-AAA protease, which was identified in mitochondria of human primary fibroblasts by co-immunoprecipitation experiments and gel filtration analysis (Atorino *et al.*, 2003). Complementation studies in the yeast *S. cerevisiae* assigned functional activity to this complex, as it could substitute for the endogenous *m*-AAA protease (Atorino *et al.*, 2003). The formation of this hetero-oligomeric assembly is impaired in HSP patients lacking hparaplegin. Therefore, initial experiments were aiming at the analysis of the assembly status of the remaining *m*-AAA protease subunit hAFG3L2 in mitochondria of hparaplegin-deficient HSP fibroblasts. In parallel, hAFG3L2 was heterologously expressed in yeast in order to investigate its activity and function in the absence of hparaplegin.

3.1.1 A homo-oligomeric hAFG3L2 complex in HSP fibroblasts and upon expression in yeast

To assess the assembly status of hAFG3L2, mitochondria were isolated from control and HSP fibroblasts, solubilised in digitonin and subjected to blue-native gel electrophoresis (BN-PAGE) (Schägger, 2001; Reisinger and Eichacker, 2006). The complete absence of hparaplegin in HSP fibroblasts was verified by SDS-PAGE and immunoblotting of isolated mitochondria using a hparaplegin-specific antiserum (Fig. 8A). In mitochondria of control fibroblasts, hAFG3L2 was detected with specific antibodies in a high molecular mass complex of ~900 kDa (Fig. 8A). Surprisingly, the loss of hparaplegin in mitochondria of HSP patients did not impair the formation of this complex, which showed a similar size as the putative hetero-oligomeric *m*-AAA protease observed in control mitochondria (Fig. 8A). This finding is in contrast to previous results showing that hAFG3L2 is only present in a complex with a native molecular mass of ~250 kDa in hparaplegin-deficient HSP fibroblasts (Atorino *et al.*, 2003). This apparent discrepancy, however, can be explained by the usage of the milder detergent digitonin in this work instead of the previously used NP-40.

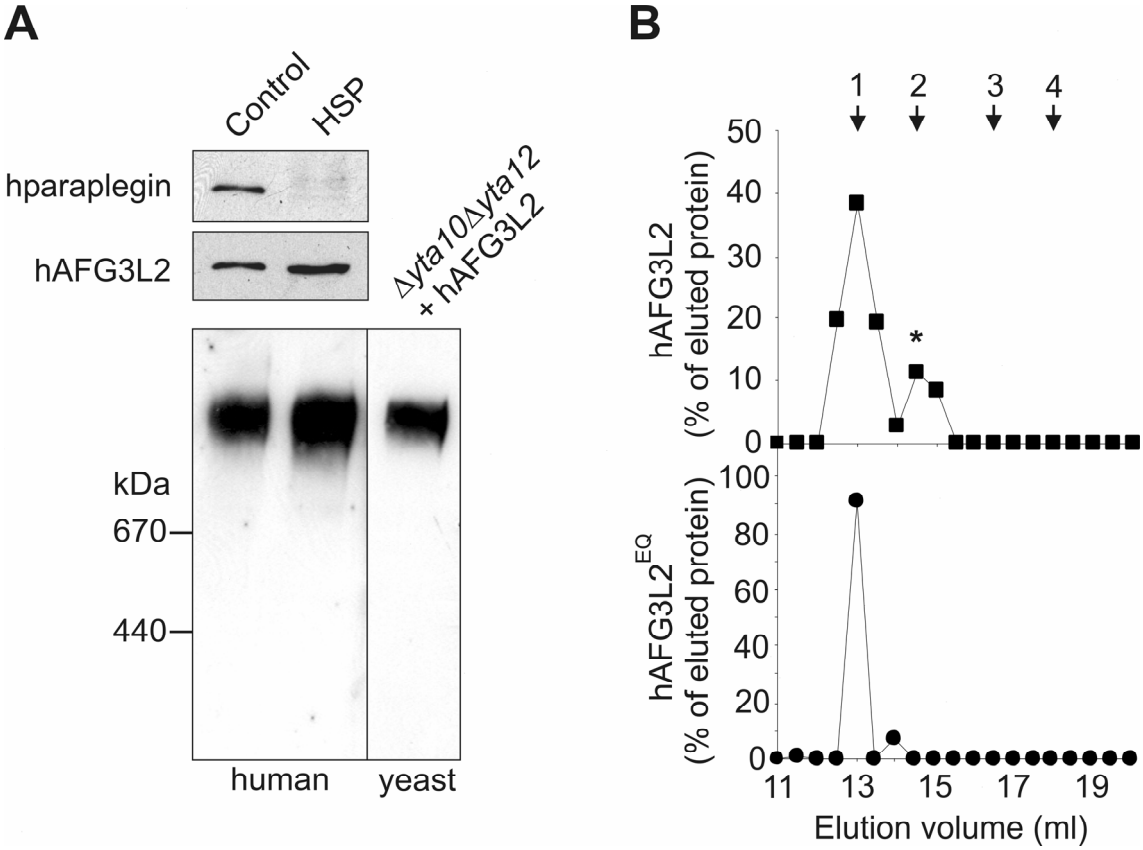

Figure 8. hAFG3L2 forms a homo-oligomeric *m*-AAA protease complex in HSP fibroblasts and upon expression in the yeast *S. cerevisiae*.

(A) A homo-oligomeric hAFG3L2 complex in mitochondria isolated from HSP fibroblasts. Mitochondria from control fibroblasts and hparaplegin-deficient HSP fibroblasts were either subjected to SDS-PAGE (50 µg mitochondrial protein; upper panel) or solubilised in digitonin and analysed by BN-PAGE (150 µg mitochondrial protein; lower panel), followed by immunoblotting using hparaplegin- (upper panel) and hAFG3L2-specific (upper and lower panel) antisera. For control, yeast Δ*yta10*Δ*yta12* mitochondria containing hAFG3L2 were fractionated by BN-PAGE in parallel. Thyroglobulin (669 kDa) and apoferritin (443 kDa) were used for calibration.
(B) Homo-oligomeric hAFG3L2 complexes in Δ*yta10*Δ*yta12* mitochondria. hAFG3L2 or hAFG3L2^{E575Q} (hAFG3L2^{EQ}) were expressed in Δ*yta10*Δ*yta12* cells and mitochondria were isolated. After solubilisation in digitonin-containing buffer, mitochondrial extracts (900 µg mitochondrial protein) were fractionated by Superose 6 sizing chromatography. Eluate fractions were TCA-precipitated and analysed by SDS-PAGE and immunoblotting using antibodies specific for the C-terminal c-Myc-epitope of hAFG3L2. hAFG3L2 (filled squares) or hAFG3L2^{E575Q} (hAFG3L2^{EQ}; filled circles) present in eluate fractions were quantified by laser densitometry and are given as percent of the respective protein in the total eluate. A smaller hAFG3L2-containing complex, marked with an asterisk, most likely results from partial dissociation of the large complex. The following marker proteins were used for calibration: 1, Hsp60 (840 kDa); 2, apoferritin (443 kDa); 3, alcohol dehydrogenase (150 kDa); 4, bovine serum albumine (66 kDa).

The complex formation of hAFG3L2 in the absence of hparaplegin could be due to a homo-oligomerisation or to its assembly with another AAA protease subunit. To distinguish between these possibilities, plasmid-borne hAFG3L2 was expressed under the control of the yeast *ADH1* promoter in Δ*yta10*Δ*yta12* yeast cells lacking any *m*-AAA protease subunit.

Mitochondrial targeting of hAFG3L2 was ensured by replacing its putative mitochondrial targeting sequence (amino acids 1-35) with the sorting signal of Yta10 (amino acids 1-61). Expression and mitochondrial localisation of the resulting hybrid protein were confirmed previously (Koppen, 2002). Mitochondria were isolated from these cells and digitonin extracts were again analysed by BN-PAGE. When expressed in *S. cerevisiae*, hAFG3L2 assembled into a complex of ~900 kDa corresponding in size to the hAFG3L2 containing complex observed in mitochondria of HSP fibroblasts (Fig. 8A).

To substantiate these findings and to determine the native molecular mass of the hAFG3L2 complex by an additional method, digitonin-solubilised mitochondria of Δ*yta10*Δ*yta12* yeast cells expressing C-terminally c-Myc-tagged hAFG3L2 were loaded onto a Superose 6 gel filtration column and eluate fractions were analysed after TCA precipitation by Western blot analysis using an antibody directed against the c-Myc epitope. hAFG3L2 was detected in eluate fractions corresponding to a native molecular mass of ~850 kDa confirming the results obtained by BN-PAGE (Fig. 8B). In addition, hAFG3L2 was found to be part of an additional smaller complex of ~370 kDa which could represent an assembly intermediate of the hAFG3L2 complex or may be caused by a partial disassembly of the high molecular mass complex upon solubilisation of mitochondrial membranes (Fig. 8B).

Taken together, hAFG3L2 assembles with hparaplegin and, in addition, can form a homo-oligomeric complex. Moreover, this hAFG3L2 complex shows a similar size as hetero-oligomeric *m*-AAA proteases in humans and yeast, consisting of either hparaplegin and hAFG3L2 or Yta10 and Yta12, respectively (Arlt *et al.*, 1996; Atorino *et al.*, 2003).

3.1.2 The hAFG3L2 complex can substitute for the yeast *m*-AAA protease

To determine if the homo-oligomeric hAFG3L2 complex is functionally active, it was tested if hAFG3L2 expression can complement the growth phenotype of Δ*yta10*Δ*yta12* cells. Yeast cells lacking the *m*-AAA protease are respiratory deficient and cannot grow on non-fermentable carbon sources like glycerol (Fig. 9) (Guélin *et al.*, 1994; Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994; Arlt *et al.*, 1998). Upon expression of hAFG3L2, growth on glycerol-containing medium (YPG) was comparable to wild-type cells, thus the respiratory competence of Δ*yta10*Δ*yta12* cells was restored (Fig 9).

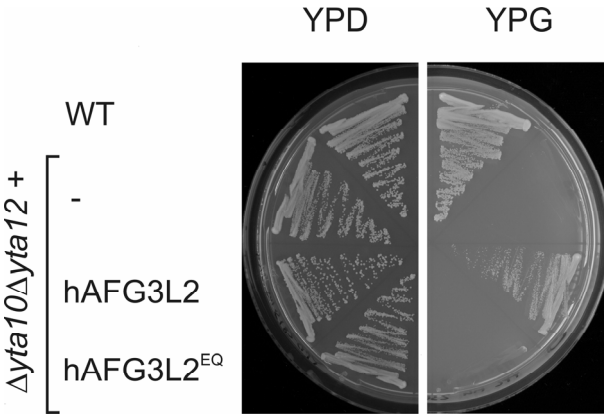

Figure 9. Maintenance of respiratory growth of Δ*yta10*Δ*yta12* yeast cells by hAFG3L2.

Wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, and Δ*yta10*Δ*yta12* cells expressing hAFG3L2 or the proteolytically inactive variant hAFG3L2^{E575Q} (hAFG3L2^{EQ}), were grown on fermentable (YPD) and non-fermentable (YPG) carbon sources at 30°C.

In order to assess if the proteolytic activity of hAFG3L2 is responsible for the observed respiratory growth, a point mutation which was shown to inactivate AAA protease subunits of yeast was introduced into its proteolytic centre (Arlt *et al.*, 1996; Guélin *et al.*, 1996; Leonhard *et al.*, 1996). Glutamate residue 575 of the consensus metal-binding motif was replaced by glutamine and the mutant variant of hAFG3L2 (hAFG3L2^{E575Q}) was expressed in Δ*yta10*Δ*yta12* cells. To rule out the possibility that this mutation impairs the assembly of hAFG3L2 into a high molecular mass complex, mitochondria were isolated from the respective strain and analysed as above by Superose 6 gel filtration after solubilisation in digitonin. As observed for hAFG3L2, its variant harbouring the proteolytic site mutation was detected as part of a complex of ~850 kDa (Fig. 8B). Therefore, this mutation does not interfere with the formation of the hAFG3L2 complex. However, growth of Δ*yta10*Δ*yta12* cells expressing hAFG3L2^{E575Q} on glycerol-containing medium was completely abolished (Fig. 9).

In summary, these experiments reveal that human AFG3L2 confers respiratory competence to Δ*yta10*Δ*yta12* yeast cells, which apparently is dependent on proteolysis by hAFG3L2. Thus, hAFG3L2 alone can substitute for the yeast *m*-AAA protease showing that the homo-oligomeric assembly constitutes a functionally active complex. This contradicts previous findings that only co-expression of hparaplegin together with hAFG3L2 can complement the respiratory growth phenotype of Δ*yta10*Δ*yta12* cells (Atorino *et al.*, 2003). As another Δ*yta10*Δ*yta12* yeast strain was used in that study, it is likely that strain-specific differences account for the observed discrepancy.

3.1.3 Proteolysis by homo-oligomeric hAFG3L2 complexes

a) hAFG3L2 can mediate processing of MrpL32 and Ccp1

The experiments described suggest proteolytic activity of the homo-oligomeric hAFG3L2 complex, since proteolysis by the *m*-AAA protease is required for maintenance of the respiratory competence of yeast cells (Arlt *et al.*, 1998). This notion is further supported by the effect of the mutation of the glutamate residue in the proteolytic centre of hAFG3L2 preventing suppression of the growth phenotype of Δ*yta10*Δ*yta12* cells (Fig. 9). To obtain direct evidence for the proteolytic activity of hAFG3L2, the processing of two mitochondrial preproteins, the ribosomal subunit MrpL32 and cytochrome *c* peroxidase (Ccp1), which are both cleaved by the yeast *m*-AAA protease upon import into mitochondria, was examined (Esser *et al.*, 2002; Nolden *et al.*, 2005).

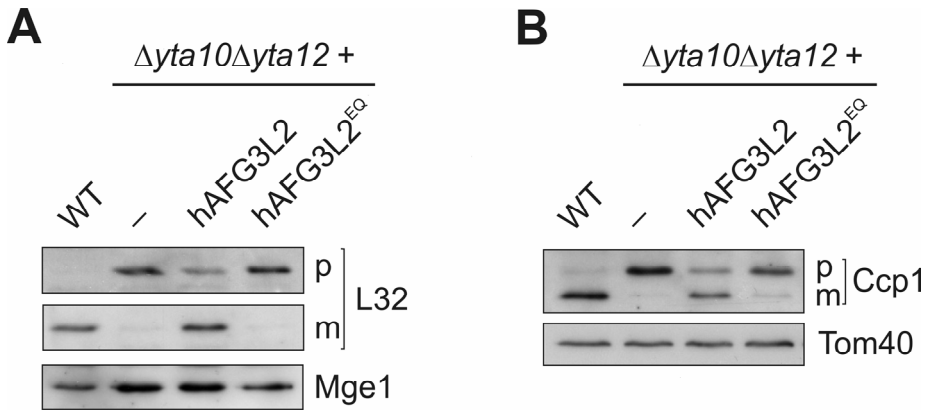

Figure 10. Processing of yeast MrpL32 and Ccp1 by hAFG3L2.

Protein processing was analysed in wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, and Δ*yta10*Δ*yta12* cells expressing either hAFG3L2 or hAFG3L2^{E575Q} (hAFG3L2^{EQ}) by SDS-PAGE. **(A)** Maturation of MrpL32 (L32) was monitored in isolated mitochondria (30 µg mitochondrial protein) by immunoblotting using polyclonal antisera directed against the mature and the precursor form of MrpL32 and, as a loading control, against matrix-localised Mge1. **(B)** Ccp1 processing was examined in cell extracts using antisera directed against Ccp1 and, for control, the outer membrane protein Tom40. p, precursor; m, mature form.

The maturation of MrpL32 was monitored in isolated yeast mitochondria by Western blot analysis using antibodies directed against either the precursor or the mature form of MrpL32. Whereas wild-type mitochondria only contained mature MrpL32, its precursor accumulated in the absence of the *m*-AAA protease and processed MrpL32 could not be detected as previously observed (Fig. 10A) (Nolden *et al.*, 2005). However, upon expression of hAFG3L2 in Δ*yta10*Δ*yta12* cells, the processing of MrpL32 was largely restored with only

little precursor protein remaining in mitochondria isolated from these cells (Fig. 10A). In contrast, expression of the mutant variant hAFG3L2^{E575Q} resulted in the complete absence of mature MrpL32 from Δ*yta10*Δ*yta12* mitochondria (Fig. 10A). These findings correlate with the observed respiratory growth phenotypes of Δ*yta10*Δ*yta12* cells expressing either hAFG3L2 or proteolytically inactive hAFG3L2^{E575Q} (Fig. 9), as the maturation of MrpL32 is required to allow completion of ribosomal assembly and thereby initiation of mitochondrial translation (Nolden *et al.*, 2005). Mitochondrial protein synthesis in turn is a prerequisite for the formation of the respiratory chain complexes in the inner membrane, since in *S. cerevisiae* seven subunits are encoded by the mitochondrial genome (Foury *et al.*, 1998).

To assess processing of Ccp1, a ROS scavenger in the mitochondrial intermembrane space, cell extracts were prepared from yeast cells and subjected to Western blot analysis with an antibody recognising both the precursor and the mature form of Ccp1. In contrast to wild-type cells which accumulated the mature form, processing of Ccp1 was completely blocked in yeast cells lacking the *m*-AAA protease (Fig. 10B) (Esser *et al.*, 2002). The expression of hAFG3L2 in these cells led to a partial restoration of Ccp1 maturation (Fig. 10B). In the presence of hAFG3L2^{E575Q}, Ccp1 processing in Δ*yta10*Δ*yta12* cells was still impaired and only residual amounts of mature Ccp1 accumulated (Fig. 10B). The latter observation is in agreement with a recent report showing that the maturation of Ccp1 in yeast is not strictly dependent on the proteolytic activity of the *m*-AAA protease (Tatsuta *et al.*, 2007).

b) hAFG3L2 can degrade integral and peripheral non-assembled membrane proteins

The processing of MrpL32 and Ccp1 by hAFG3L2 provides further evidence for its proteolytic activity. Subsequently, it was investigated whether hAFG3L2 can also completely degrade misfolded and unassembled model substrates of the yeast *m*-AAA protease and thereby functions in protein quality control (Arlt *et al.*, 1996; Leonhard *et al.*, 2000). To this end, the following approach was exploited: two substrate proteins, a Yme2 derivative and Atp7, were synthesised in a cell-free system in the presence of ^{35}S-methionine and imported into isolated Δ*yta10*Δ*yta12* mitochondria harbouring hAFG3L2 or proteolytically inactive hAFG3L2^{E575Q}. Mitochondria were then incubated at 37°C and the stability of the imported radiolabelled proteins was followed at different time points by SDS-PAGE and autoradiography.

The first substrate, Yme2^{F114S}ΔC15+1 (in the following referred to as Yme2ΔC), is localised in the inner membrane of mitochondria (Korbel *et al.*, 2004). This Yme2 variant exposes its N-terminal domain to the matrix, but lacks its C-terminal intermembrane space

domain leading to the destabilisation of Yme2ΔC and subsequent degradation by the *m*-AAA protease (Fig. 11A) (Korbel *et al.*, 2004). Degradation of newly imported Yme2ΔC is largely inhibited in Δ*yta10*Δ*yta12* mitochondria, but was observed in the presence of hAFG3L2, although with slightly slower kinetics compared to wild-type mitochondria (Fig. 11A). In Δ*yta10*Δ*yta12* mitochondria harbouring hAFG3L2, ~40% of imported Yme2ΔC could be detected after 30 min at 37°C, whereas only ~20% were still present in wild-type mitochondria at the same time point. On the other hand, the expression of hAFG3L2^{E575Q} in Δ*yta10*Δ*yta12* mitochondria had no effect on the degradation of Yme2ΔC confirming the dependence of the observed degradation on the integrity of the proteolytic centre of hAFG3L2 (Fig. 11A).

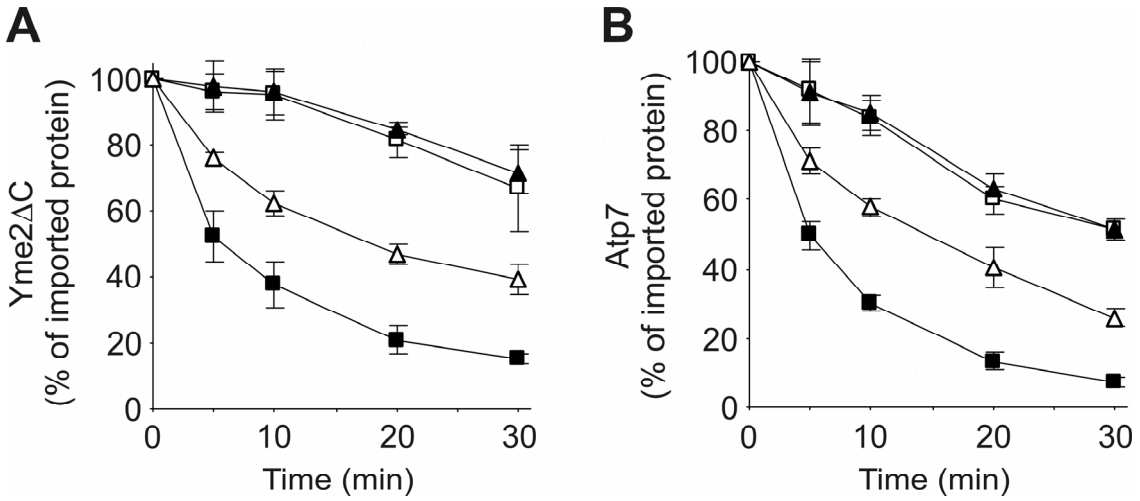

Figure 11. Degradation of non-assembled membrane proteins by hAFG3L2 expressed in yeast.

Radiolabelled Yme2ΔC **(A)** or Atp7 **(B)** were imported for 10 min at 25°C into mitochondria isolated from yeast strains used in Fig. 10. The stability of newly imported proteins at 37°C was determined by SDS-PAGE and autoradiography. The average of three independent experiments (± SEM) is shown. Filled squares, wild-type; filled triangles, Δ*yta10*Δ*yta12*; open triangles, Δ*yta10*Δ*yta12* + hAFG3L2; open squares, Δ*yta10*Δ*yta12* + hAFG3L2^{E575Q}.

Atp7 was chosen as an example for a peripheral membrane protein, whose degradation is mediated by the *m*-AAA protease (Korbel *et al.*, 2004). Atp7 is a stalk subunit of the F_1F_O-ATP synthase and peripherally associated with the inner surface of the mitochondrial inner membrane (Ackerman and Tzagoloff, 2005). Radiolabelled Atp7 was rapidly degraded after import into wild-type mitochondria, but was partially stabilised in Δ*yta10*Δ*yta12* mitochondria. Apparently, another, yet unidentified mitochondrial protease can mediate the degradation of Atp7 in the absence of the *m*-AAA protease (Fig. 11B) (Korbel *et al.*, 2004).

hAFG3L2 was found to significantly accelerate the degradation of Atp7 in Δ*yta10*Δ*yta12* mitochondria with ~30% of newly imported protein present after 30 min, while ~60% of Atp7 were still detected without hAFG3L2 (Fig. 11B). However, similar to the results for Yme2ΔC, degradation by hAFG3L2 was not as efficient as observed for the yeast *m*-AAA protease resulting in less than 10% of imported Atp7 detectable after 30 min (Fig. 11B). In contrast, Atp7 remained rather stable in Δ*yta10*Δ*yta12* mitochondria harbouring proteolytically inactive hAFG3L2^{E575Q} and its degradation kinetics was indistinguishable from Δ*yta10*Δ*yta12* mitochondria (Fig. 11B).

Thus, hAFG3L2 also possesses the ability to mediate the complete turnover of both integral and peripheral membrane proteins. Taken together, it can be concluded, that the homo-oligomeric hAFG3L2 complex is proteolytically active and can substitute for two activities of the yeast *m*-AAA protease, protein quality surveillance in the inner membrane and protein processing, respectively. In each case, mutating the proteolytic centre of hAFG3L2 rendered it inactive towards its proteolytic substrates.

3.1.4 hAFG3L2 is active upon homo-oligomerisation and assembly with hparaplegin

The growth phenotype of the Δ*yta10*Δ*yta12* yeast strain YKO200 used in this work could be suppressed by expression of human AFG3L2 alone (Fig. 9), whereas previous experiments with another *m*-AAA protease-deficient yeast strain indicated that the respiratory competence is only restored upon co-expression of human paraplegin (Atorino *et al.*, 2003). Therefore, the effect of hparaplegin expression and co-expression with hAFG3L2 on respiratory growth of Δ*yta10*Δ*yta12* cells was re-examined in the YKO200 strain. For expression of plasmid-borne hparaplegin in yeast, the mitochondrial targeting sequence of Yta10 (amino acids 1-63) was fused to the putative mature form of hparaplegin (amino acids 59-795). *ADH1* promoter-driven expression and correct targeting of the corresponding hybrid protein has already been confirmed (Koppen, 2002).

When hparaplegin was expressed in *m*-AAA protease-deficient YKO200 cells, growth on glycerol-containing medium was still prevented (Fig. 12), which is in line with the previous observation that hparaplegin does not build up homo-oligomeric high molecular mass complexes (Koppen, 2002). Co-expression of hparaplegin together with hAFG3L2 resulted in respiratory growth of Δ*yta10*Δ*yta12* cells, which was indistinguishable from cells expressing only hAFG3L2 (Fig. 12). However, it remained open if the observed respiratory

activity is conferred only by homo-oligomeric hAFG3L2 complexes or by hetero-oligomeric complexes containing hparaplegin. To discriminate between these two possibilities, proteolytically inactive variants of hparaplegin (hparaplegin^{E575Q}) and hAFG3L2 (hAFG3L2^{E575Q}) were co-expressed. Studies on the yeast *m*-AAA protease have demonstrated that the hetero-oligomeric protease complex is still active and maintains respiratory growth if it is composed of proteolytically active and inactive subunits, whereas it is completely inactivated when all subunits harbour mutations in their proteolytic centre (Arlt *et al.*, 1998). Consistently, co-expression of hparaplegin^{E575Q} and hAFG3L2 in Δ*yta10*Δ*yta12* cells did not impair respiratory growth (Fig. 12). As it cannot be excluded that active homo-oligomeric hAFG3L2 complexes are still formed under these conditions, hAFG3L2^{E575Q} was also expressed in combination with hparaplegin. In contrast to Δ*yta10*Δ*yta12* cells expressing only hAFG3L2^{E575Q}, cells co-expressing hparaplegin showed a partial restoration of growth on glycerol-containing medium, which was abolished if both human *m*-AAA protease subunits were inactivated (Fig. 12).

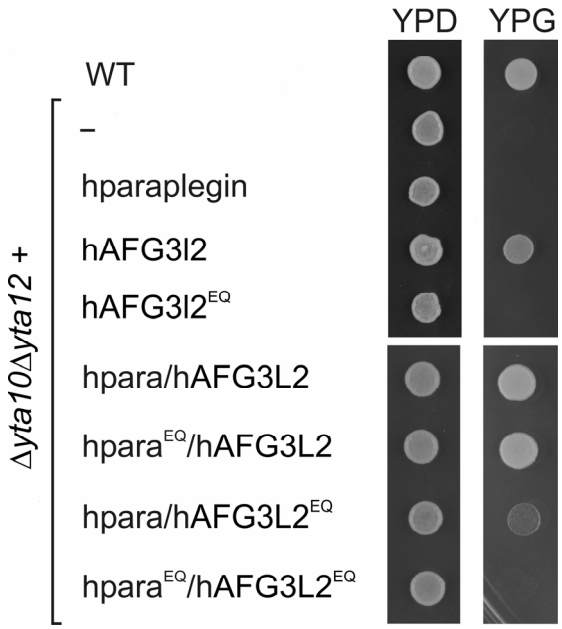

Figure 12. Respiratory growth of Δ*yta10*Δ*yta12* cells co-expressing hparaplegin and hAFG3L2.

Wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, and Δ*yta10*Δ*yta12* cells expressing either hparaplegin, hAFG3L2, or the proteolytically inactive variant hAFG3L2^{E575Q} (hAFG3L2^{EQ}) were spotted on glucose- (YPD) and glycerol-containing media (YPG) and grown at 30°C. To examine respiratory activity of Δ*yta10*Δ*yta12* cells harbouring hetero-oligomeric complexes, hparaplegin, hAFG3L2, or their mutant variants hparaplegin^{E575Q} (hpara^{EQ}) and hAFG3L2^{EQ} were co-expressed in all four possible combinations (hpara$^{+/-EQ}$/hAFG3L2$^{+/-EQ}$) and cells were grown as described above.

Thus, human paraplegin and AFG3L2 can assemble into a hetero-oligomeric *m*-AAA protease complex with proteolytic activity also in the YKO200 strain, thereby maintaining respiratory competence. These results, therefore, confirm the previously observed interaction of hparaplegin and hAFG3L2 upon expression in yeast (Atorino *et al.*, 2003). Similar to the yeast *m*-AAA protease, the hetero-oligomeric complex still exerts proteolytic activity when it consist of catalytically active and inactive subunits, which is contrary to the former observation that the catalytic activity of both subunits is essential (Atorino *et al.*, 2003). Furthermore, the limited respiratory growth of Δ*yta10*Δ*yta12* cells containing hparaplegin and hAFG3L2^{E575Q}, compared to expression of hAFG3L2 with proteolytically active or inactive hparaplegin, suggests that upon co-expression in yeast both subunits are not quantitatively assembled with each other. Rather, the homo-oligomeric hAFG3L2 complex and the hetero-oligomeric assembly might co-exist. Alternatively, the proteolytic activity of the hetero-oligomeric complex could be different depending on if either the hparaplegin or the hAFG3L2 subunits harbour a mutation in the proteolytic site. Last but not least, these observations could also reflect a general limited activity of hparaplegin subunits upon assembly into hetero-oligomeric complexes in yeast.

Notably, human AFG3L2 has been identified to be part of two potential proteolytic complexes in human mitochondria and when expressed in yeast, either by its assembly with hparaplegin into a hetero-oligomeric *m*-AAA protease or by its homo-oligomerisation resulting in a hAFG3L2 complex.

3.2 *Murine* m-*AAA protease subunits assemble into protease complexes with variable subunit composition*

The proteolytic system of the inner mitochondrial membrane is conserved in mammals as demonstrated by complementation studies in yeast (Shah *et al.*, 2000; Atorino *et al.*, 2003; Nolden *et al.*, 2005). Putative *m*-AAA subunits sharing a high degree of sequence homology with their human counterparts have also been identified in mice. Interestingly, a mouse line with a paraplegin deficiency reproducing central features of HSP has been generated (Ferreirinha *et al.*, 2004). Thus, mice can serve as a valid model to study the molecular basis of the human disease necessitating a detailed understanding of the number and subunit composition of murine *m*-AAA protease complexes. This is underlined by the results for the human *m*-AAA protease subunit hAFG3L2 which displays variability in its assembly. Furthermore, in addition to paraplegin and Afg3l2, a third putative *m*-AAA protease subunit, termed Afg3l1, is expressed in mice, whereas it is encoded by a pseuodgene in humans (Kremmidiotis *et al.*, 2001). A sequence comparison of Afg3l1 and Afg3l2 shows 68% amino acid identity between both murine proteins suggesting similar activities. However, the assembly of murine *m*-AAA protease subunits into proteolytic complexes and their functions have not been analysed, yet. Especially, the role of the additional putative subunit Afg3l1 remains to be established. Heterologous expression in *S. cerevisiae* has been proven to be a powerful tool for studying assembly and proteolytic activity of *m*-AAA protease subunits. Hence, murine paraplegin, Afg3l1, and Afg3l2 were introduced into Δ*yta10*Δ*yta12* yeast cells in order to perform complementation analysis as well as biochemical and functional characterisation.

3.2.1 Murine *m*-AAA protease subunits show mitochondrial localisation and correct membrane insertion when expressed in yeast

For expression in yeast, the sorting signal of Yta10 (amino acids 1-61) was fused to the assumed mature forms of murine paraplegin (amino acids 44-781), Afg3l1 (amino acids 25-789), and Afg3l2 (amino acids 36-802) to ensure mitochondrial targeting. The DNA sequences coding for these hybrid proteins were cloned into multicopy vectors under the control of the *YTA10* promoter. After transformation of Δ*yta10*Δ*yta12* cells with the obtained plasmids, expression and mitochondrial localisation of the hybrid proteins were examined by Western blot analysis of cellular membrane fractions using antisera specific for paraplegin or

the C-terminal c-Myc and hemagglutinin (HA) epitopes of Afg3l1 and Afg3l2, respectively. All three murine *m*-AAA protease subunits were exclusively detected in the membrane fraction containing mitochondrial membranes as shown by the presence of the mitochondrial ADP/ATP carrier Aac2 (Fig 13).

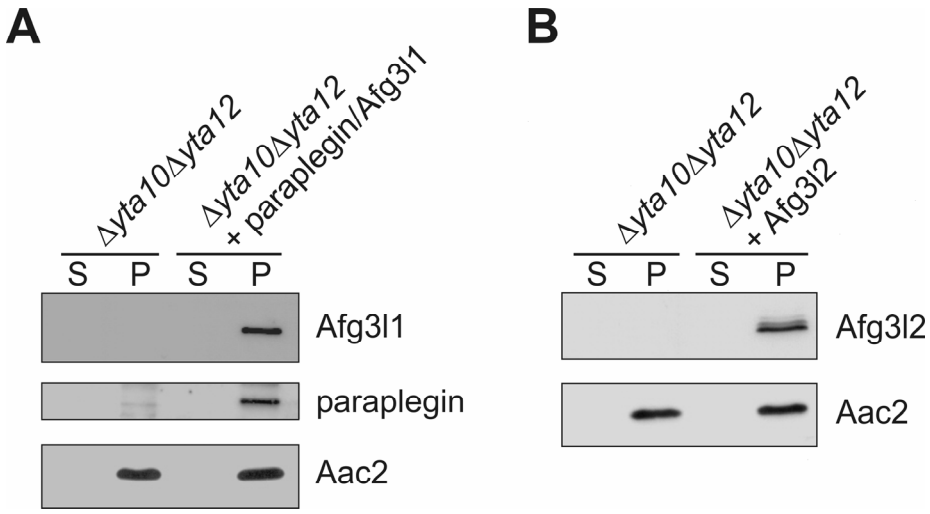

Figure 13. Murine *m*-AAA protease subunits co-localise with mitochondrial membranes in yeast.

Δ*yta10*Δ*yta12* yeast cells expressing either paraplegin and Afg3l1 **(A)** or Afg3l2 **(B)** were lysed and separated by centrifugation into a supernatant fraction (S) and a pellet fraction (P) containing cellular membranes. Fractions were analysed by SDS-PAGE and immunoblotting using specific antisera for paraplegin, the c-Myc epitope of Afg3l1, the HA epitope of Afg3l2, or, as a marker for mitochondrial membranes, an antibody directed against the integral inner membrane protein Aac2. To confirm the specificity of the antibodies, Δ*yta10*Δ*yta12* cells were subjected to Western blot analysis in parallel.

To confirm membrane insertion of the hybrid proteins, alkaline extraction at pH 11.5 was performed with mitochondria isolated from Δ*yta10*Δ*yta12* cells containing paraplegin, Afg3l1, and Afg3l2. All three murine *m*-AAA protease subunits remained in the insoluble pellet fraction and thus behaved like the integral inner membrane protein Aac2 containing three transmembrane domains (Fig. 14). In contrast, the matrix protein Mge1 lacking any transmembrane domain was exclusively recovered from the soluble fraction (Fig. 14). From these experiments, one can conclude that the paraplegin-, Afg3l1-, and Afg3l2-hybrid proteins expressed in yeast localise to mitochondrial membranes.

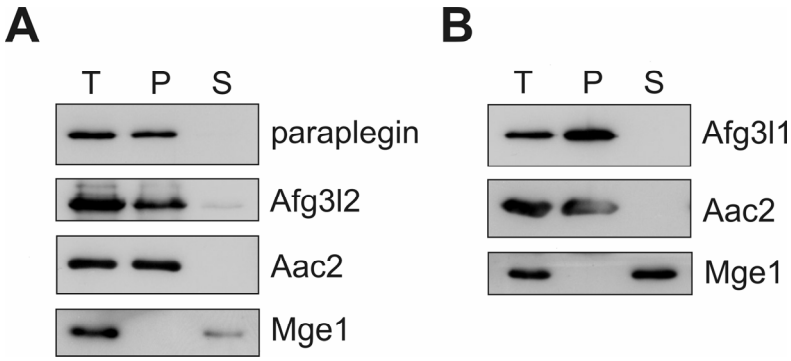

Figure 14. Murine *m*-AAA protease subunits expressed in yeast are integral membrane proteins.

Mitochondria (100 µg mitochondrial protein) isolated from Δ*yta10*Δ*yta12* cells co-expressing paraplegin and Afg3l2 **(A)** or expressing Afg3l1 **(B)** were treated with 100 mM sodium carbonate (pH 11.5) (T) and subjected to ultracentrifugation. The obtained soluble (S) and insoluble (P) fractions were analysed by SDS-PAGE and immunoblotting using specific antibodies described in Fig. 13. Antibodies directed against the integral inner membrane protein Aac2 and the soluble matrix protein Mge1 were used as controls.

3.2.2 Murine Afg3l1 and Afg3l2 can assemble into homo-oligomeric complexes able to substitute for the yeast *m*-AAA protease

By expression in yeast cells lacking any *m*-AAA protease subunit, hAFG3L2 was found to assemble into a homo-oligomeric and proteolytically active complex (see chapter 3.1.2). To investigate if murine *m*-AAA protease subunits also possess the ability to homo-oligomerise and substitute for the yeast *m*-AAA protease, paraplegin, Afg3l1, and Afg3l2 were individually expressed in Δ*yta10*Δ*yta12* cells and respiratory growth was examined (Fig. 15). Whereas expression of paraplegin did not suppress the respiratory deficiency, expression of either Afg3l1 or Afg3l2 completely restored the growth of Δ*yta10*Δ*yta12* cells on glycerol-containing medium, compared to wild-type cells (Fig. 15). This indicates that Afg3l1 and Afg3l2 indeed can build up homo-oligomeric complexes which are functionally active and confer respiratory competence. To further support the notion of proteolytic activity of both proteins upon assembly, the catalytic glutamate residues in the metal binding sites of Afg3l1 and Afg3l2 were changed to glutamine using site directed mutagenesis. Consistently, expression of the corresponding proteolytically inactive variants Afg3l1^{E567Q} or Afg3l2^{E574Q} in Δ*yta10*Δ*yta12* cells did not allow respiratory growth (Fig. 15).

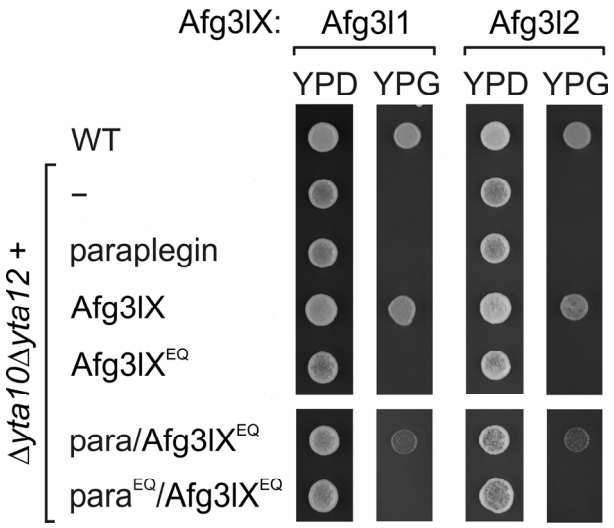

Figure 15. Respiratory growth of Δ*yta10*Δ*yta12* cells expressing murine *m*-AAA protease subunits.

Wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, and Δ*yta10*Δ*yta12* cells expressing either paraplegin, Afg3l1 (Afg3lX), Afg3l2 (Afg3lX), or their mutant variants Afg3l1^{E567Q} or Afg3l2^{E574Q} (Afg3lX^{EQ}) were grown at 30°C on glucose- (YPD) or glycerol-containing media (YPG) to examine the respiratory competence of the cells. To assess the activity of hetero-oligomeric complexes, the mutant variants Afg3l1^{E567Q} or Afg3l2^{E574Q} were co-expressed with paraplegin (para/Afg3lX^{EQ}) or with paraplegin^{E575Q} (para^{EQ}/Afg3lX^{EQ}) in Δ*yta10*Δ*yta12* cells and cell growth was analysed as above.

In order to reveal this proposed complex formation biochemically and to determine the native molecular mass, mitochondria were isolated from Δ*yta10*Δ*yta12* cells expressing individual murine *m*-AAA protease subunits, solubilised in digitonin, and fractionated by sizing chromatography using a Superose 6 column. Eluate fractions were analysed via SDS-PAGE and immunoblotting using specific antisera. In agreement with the results of the complementation analysis, murine paraplegin was only detected as part of a low molecular mass complex of ~300 kDa (Fig. 16). Furthermore, the gel filtration analysis confirmed that Afg3l2 homo-oligomerises into a high molecular mass complex. Similar to hAFG3L2, its native molecular mass was estimated to be ~850 kDa (Fig. 16). Murine Afg3l1, however, exhibited a different elution pattern and was already detected in the first eluate fractions corresponding to a molecular mass of >2 MDa pointing to its homo-oligomerisation (Fig. 16). It remains unclear whether this large Afg3l1 complex represents the correct assembly or rather an aggregated species. Alternatively, the interaction with other protein complexes in the mitochondrial inner membrane of yeast, e.g. the prohibitin complex, could account for the observed elution profile of Afg3l1.

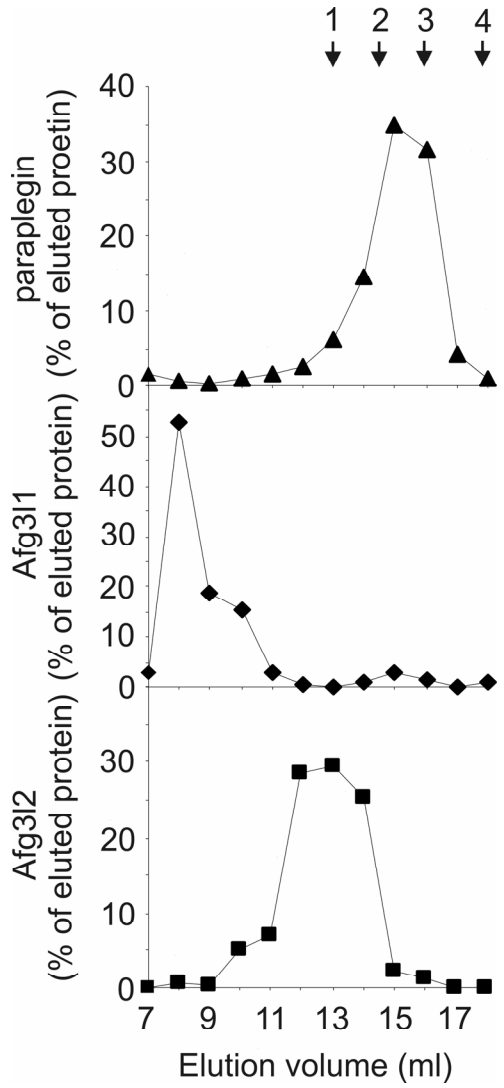

Figure 16. Homo-oligomerisation of murine Afg3l1 and Afg3l2 into high molecular mass complexes in Δ*yta10*Δ*yta12* mitochondria.

Murine paraplegin, Afg3l1, or Afg3l2 were expressed in Δ*yta10*Δ*yta12* cells and mitochondria were isolated. After solubilisation in digitonin-containing buffer, mitochondrial extracts (900 µg mitochondrial protein) were subjected to Superose 6 size exclusion chromatography. Eluate fractions were TCA-precipitated and examined by Western blot analysis using antibodies specific for paraplegin, the c-Myc epitope of Afg3l1, or the HA epitope of Afg3l2. Paraplegin (filled triangles), Afg3l1 (filled rectangles), and Afg3l2 (filled squares) present in eluate fractions were quantified by laser densitometry and are given as percent of the respective protein in the total eluate. The following marker proteins were used for calibration: 1, Hsp60 (840 kDa); 2, apoferritin (443 kDa); 3, alcohol dehydrogenase (150 kDa); 4, bovine serum albumine (66 kDa).

Taken together, these findings show that assembly into a high molecular mass complex is restricted to Afg3l1 and Afg3l2. These homo-oligomeric Afg3l1 and Afg3l2 complexes can apparently both conduct proteolytic functions of the yeast *m*-AAA protease resulting in respiratory competence. Furthermore, this suggests that Afg3l1 is a functional *m*-AAA protease subunit in yeast and presumably as well in mice.

3.2.3 Murine paraplegin can assemble with either Afg3l1 or Afg3l2 into hetero-oligomeric protease complexes

Mammalian paraplegin lacks the ability to form homo-oligomeric complexes, but human paraplegin has been already shown to assemble into hetero-oligomeric complexes with human AFG3L2, both in human fibroblasts and upon co-expression in yeast (Atorino *et al.*, 2003). This suggests a similar complex formation of murine paraplegin with its potential assembly partners Afg3l1 and Afg3l2. Therefore, paraplegin was co-expressed with Afg3l1 or Afg3l2 in *m*-AAA protease-deficient yeast cells and co-immunoprecipitation experiments were performed to demonstrate a direct interaction of paraplegin with either protein. After solubilisation of mitochondria in digitonin, extracts were incubated with antibodies recognising the C-terminal epitope tags of Afg3l1 and Afg3l2. The two precipitates were subsequently analysed by SDS-PAGE and immunoblotting using an antiserum specific for murine paraplegin. Paraplegin could be detected in both immunoprecipitates (Fig. 17). In

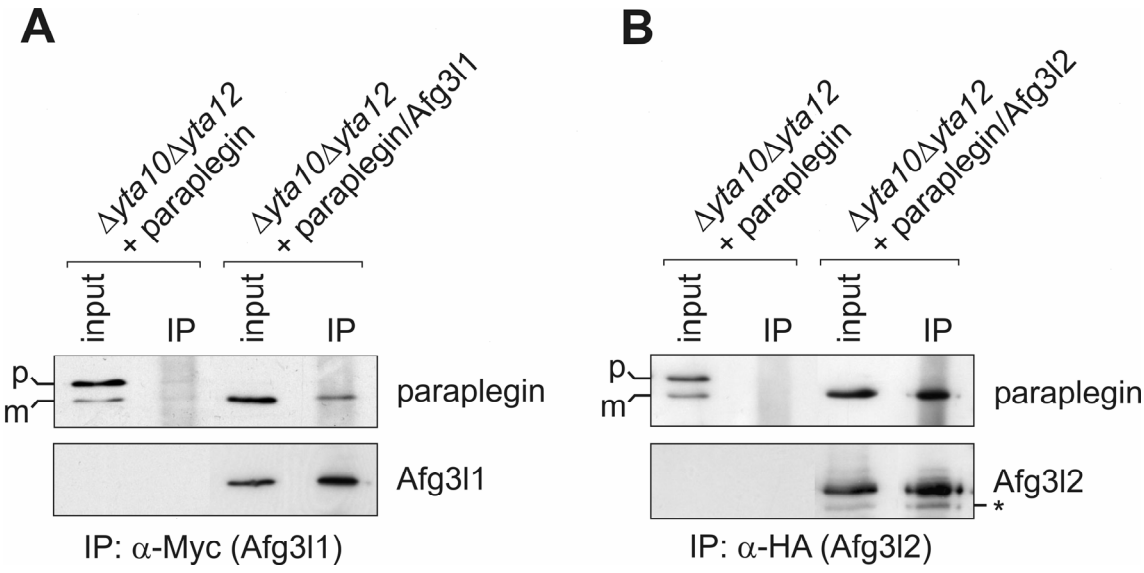

Figure 17. Direct interaction of paraplegin with Afg3l1 or Afg3l2 resulting in hetero-oligomeric *m*-AAA protease complexes.

Mitochondria (400 µg mitochondrial protein) isolated from Δ*yta10*Δ*yta12* cells co-expressing murine paraplegin and either Afg3l1 **(A)** or Afg3l2 **(B)** were solubilised with digitonin. 10% of the samples were removed for control (input). Co-immunoprecipitations were carried out using antibodies directed against the c-Myc epitope **(A)** and the HA epitope **(B)** fused to the C-terminus of Afg3l1 and Afg3l2, respectively. Immunoprecipitates (IP) were analysed by SDS-PAGE and immunoblotting with a paraplegin-specific antiserum and the antibodies used for precipitation. For negative control, mitochondria isolated from Δ*yta10*Δ*yta12* cells harbouring only paraplegin were employed. The two paraplegin-specific bands observed in these mitochondria presumably correspond to the precursor (p) and the mature (m) form of paraplegin. A putative degradation product of Afg3l2 is marked with an asterisk.

contrast, paraplegin was not precipitated with the same antibodies when Afg3l1 and Afg3l2 were not co-expressed verifying the specificity of the observed interaction (Fig. 17). Hence, one can conclude that paraplegin directly interacts with Afg3l1 and Afg3l2 resulting in the formation of hetero-oligomeric, high molecular mass complexes. These findings were confirmed by gel filtration analysis of mitochondrial extracts (data not shown).

The proteolytic activity of the paraplegin containing complexes was again assessed by complementation analysis in yeast. Similar to cells containing only Afg3l1 or Afg3l2, the respiratory growth of Δ*yta10*Δ*yta12* cells was completely restored if paraplegin was co-expressed with either protein (data not shown). To distinguish whether this phenotype stems from hetero-oligomerisation with paraplegin rather than only from homo-oligomerisation of Afg3l1 or Afg3l2, respectively, proteolytically inactive variants of the murine subunits were employed as described for human paraplegin and AFG3L2 in chapter 3.1.4. Introduction of the proteolytic site mutation into paraplegin (paraplegin^{E575Q}) and co-expression with Afg3l1 or Afg3l2 in Δ*yta10*Δ*yta12* cells did not affect respiratory growth, which was comparable to wild-type cells (data not shown). However, co-expression of paraplegin and proteolytically inactive Afg3l1^{E567Q} or Afg3l2^{E574Q} led to a partial restoration of respiratory growth, which was not observed when paraplegin, Afg3l1^{E567Q}, or Afg3l2^{E574Q} were expressed individually (Fig 15). This residual growth on glycerol-containing medium was lost when two proteolytically inactive subunits were co-expressed, either paraplegin^{E575Q}/Afg3l1^{E567Q} or paraplegin^{E575Q}/Afg3l2^{E574Q} (Fig. 15).

These observations indicate that hetero-oligomeric complexes composed of murine paraplegin and either Afg3l1 or Afg3l2 exhibit proteolytic activity, which is sufficient to promote respiratory competence of yeast cells lacking the *m*-AAA protease. In addition, they confirm the functional conservation of the murine proteins and support the notion that Afg3l1 is a *bona fide* subunit of *m*-AAA proteases. The formation of a possible hetero-oligomeric Afg3l1/Afg3l2 complex was not investigated in Δ*yta10*Δ*yta12* cells, as Afg3l1 and Afg3l2 both homo-oligomerise after expression in yeast, and due to the lack of a functional assay to distinguish between both complexes.

3.2.4 Proteolysis by homo- and hetero-oligomeric murine *m*-AAA protease complexes

a) Processing of MrpL32 and Ccp1 by homo- and hetero-oligomeric complexes

As proteolysis by the *m*-AAA protease is indispensable for respiratory competence of yeast cells, proteolytic activity can be assigned to the murine homo- and hetero-oligomeric *m*-AAA proteases (Arlt *et al.*, 1998). The human AFG3L2 complex has been shown in this work to substitute for the yeast *m*-AAA protease in one of its housekeeping functions, namely the processing of certain precursor proteins after import into mitochondria (Fig. 10). In order to establish if the murine protease complexes show a similar activity, the processing of the

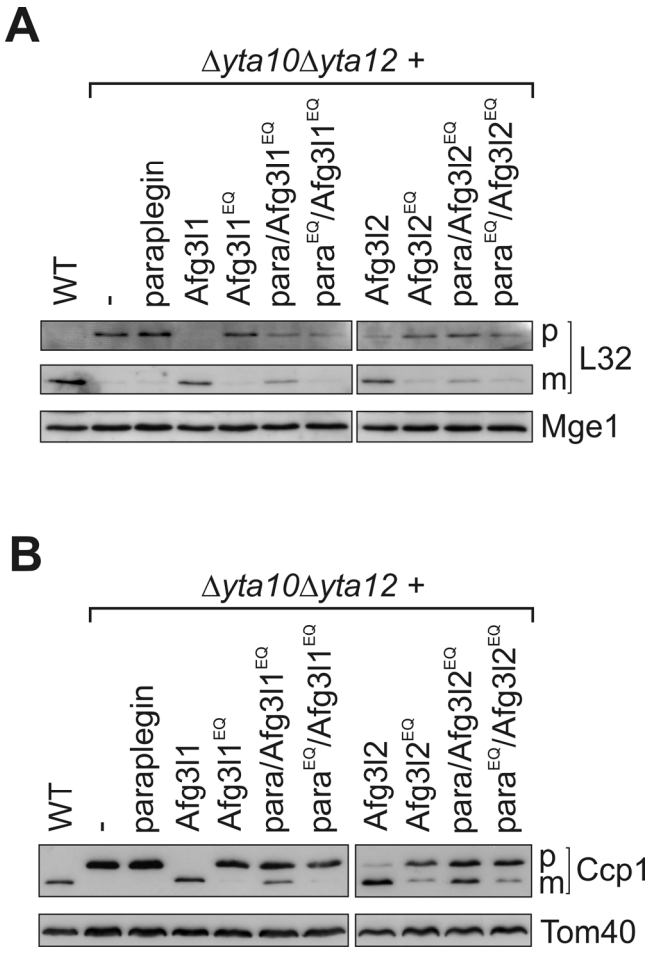

Figure 18. Processing of yeast MrpL32 and Ccp1 by murine *m*-AAA proteases.

Protein processing was analysed in Δ*yta10*Δ*yta12* cells harbouring murine *m*-AAA protease subunits as described in Fig. 15 by SDS-PAGE and immunoblotting of **(A)** isolated mitochondria (30 µg mitochondrial protein) or **(B)** cell extracts using **(A)** MrpL32 (L32)-specific antisera or **(B)** Ccp1-specific antisera. p, precursor forms; m, mature forms. Mge1 and Tom40 were used to control for equal gel loading.

ribosomal subunit MrpL32 and the ROS scavenger Ccp1 was analysed in isolated mitochondria or cell extracts, respectively.

When murine paraplegin was individually expressed in Δ*yta10*Δ*yta12* cells, mature forms of neither MrpL32 nor Ccp1 could be detected (Fig. 18). In line with their observed homo-oligomerisation, both Afg3l1 and Afg3l2 could mediate processing of either substrate (Fig. 18). Notably, processing by Afg3l1 appeared to be slightly more efficient compared to Afg3l2, as no precursor forms were detected. In contrast, residual amounts of MrpL32 and Ccp1 preproteins were present in Afg3l2-containing Δ*yta10*Δ*yta12* cells (Fig. 18). Processing was largely inhibited upon expression of the mutant variants Afg3l1^{E567Q} or Afg3l2^{E574Q} (Fig. 18). However, co-expression of paraplegin with either proteolytically inactive protein partially restored the maturation of MrpL32 and Ccp1, which was not observed when paraplegin also contained the point mutation in its proteolytic centre (paraplegin^{E575Q}) (Fig. 18). Since the generation of mature MrpL32 is a prerequisite for mitochondrial translation and assembly of respiratory chain complexes (Nolden *et al.*, 2005), this reduced processing of MrpL32 in the presence of hetero-oligomeric complexes is consistent with the observed limited respiratory growth of the respective yeast strains (Fig. 15).

In summary, both the homo-oligomeric Afg3l1 and Afg3l2 complexes as well as the hetero-oligomeric *m*-AAA proteases containing paraplegin can mediate the proteolytic removal of MrpL32 and Ccp1 presequences.

b) Degradation of integral and peripheral non-assembled membrane proteins by homo-oligomeric complexes

Degradation of misfolded or non-assembled membrane proteins represents another housekeeping function of the *m*-AAA protease in yeast (Arlt *et al.*, 1996; Leonhard *et al.*, 2000). To investigate if the homo-oligomeric protease complex formed by murine *m*-AAA protease subunits can also mediate the complete turnover of proteins, the stability of the two model substrates Yme2ΔC and Atp7 was monitored using the assay described in chapter 3.1.3.

Degradation of newly imported Yme2ΔC, an integral protein of the inner membrane, was observed in Δ*yta10*Δ*yta12* mitochondria containing Afg3l1 and Afg3l2, whereas it remained stable in the presence of paraplegin (Fig. 19A). Interestingly, Afg3l1-mediated degradation occurred with faster kinetics compared to Afg3l2 and was similar to turnover by the yeast *m*-AAA protease. Similar results were obtained for newly imported Atp7, a matrix-localised and peripheral inner membrane protein (Fig. 19B). Whereas the presence of

paraplegin in Δ*yta10*Δ*yta12* mitochondria had no effect on degradation of Atp7, Afg3l1 and Afg3l2 accelerated its degradation in a similar manner, resembling the degradation kinetics observed in wild-type mitochondria (Fig. 19B).

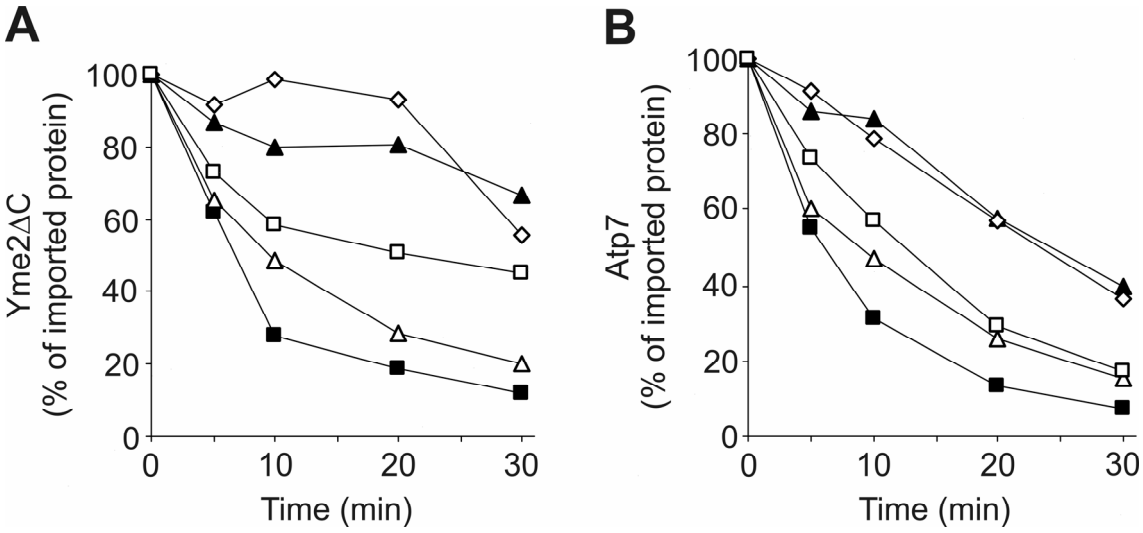

Figure 19. Degradation of non-assembled membrane proteins by homo-oligomeric Afg3l1 and Afg3l2 complexes.

Radiolabelled Yme2ΔC **(A)** or Atp7 **(B)** were imported for 10 min at 25°C into mitochondria isolated from wild-type (WT, filled squares) cells, Δ*yta10*Δ*yta12* cells (filled triangles), and Δ*yta10*Δ*yta12* cells expressing either paraplegin (open rectangles), Afg3l1 (open triangles), or Afg3l2 (open squares). The stability of newly imported proteins at 37°C was determined by SDS-PAGE and autoradiography.

Hence, the homo-oligomeric Afg3l1 and Afg3l2 complexes mediate the complete turnover of non-assembled or misfolded substrate proteins, which are integral parts or peripherally associated with the matrix-exposed surface of the inner mitochondrial membrane. Like the human AFG3L2 complex, they can execute both housekeeping functions of the yeast *m*-AAA protease. Notably, murine paraplegin does not exhibit any activity, unless it assembles with Afg3l1 or Afg3l2 into a hetero-oligomeric *m*-AAA protease. However, degradation of Yme2ΔC and Atp7 by paraplegin-containing complexes, though likely to occur, was not analysed. Δ*yta10*Δ*yta12* cells harbouring paraplegin and either Afg3l1^{E567Q} or Afg3l2^{E574Q} only showed partial restoration of respiratory growth and protein processing, questioning if degradation by these complexes can be visualised.

3.2.5 Evidence for autocatalytic processing of murine *m*-AAA protease subunits during mitochondrial import

For the present analysis in yeast, murine *m*-AAA protease subunits were expressed as hybrid proteins. They consist of the first 61 amino acids of Yta10 serving as a mitochondrial sorting signal and the putative mature forms of paraplegin, Afg3l1, or Afg3l2, whose exact N-termini have not been determined. The presequence of Yta10 should also allow processing of the hybrid proteins by MPP. Interestingly, in the course of the co-immunoprecipitation experiments, two bands which correspond to murine paraplegin were detected in Δ*yta10*Δ*yta12* mitochondria containing only this subunit (Fig. 17). On the other hand, only one form of paraplegin was observed when Afg3l1 or Afg3l2 were co-expressed (Fig. 13, 14, and 17). To further characterise these different paraplegin forms and to investigate if similar observations can be obtained for Afg3l1 and Afg3l2, mitochondria isolated from Δ*yta10*Δ*yta12* cells expressing only one of the murine hybrid proteins were again fractionated by SDS-PAGE followed by immunoblotting.

In contrast to two protein forms of paraplegin, only one major protein form of Afg3l1 or Afg3l2 was present in Δ*yta10*Δ*yta12* mitochondria (Fig. 20A). This observation correlates with the assembly of the latter two murine proteins into homo-oligomeric complexes with proteolytic activity. To discern a role of proteolysis, Δ*yta10*Δ*yta12* mitochondria harbouring the proteolytically inactive variants of paraplegin (paraplegin^{E575Q}), Afg3l1 (Afg3l1^{E567Q}), or Afg3l2 (Afg3l2^{E574Q}) were analysed in the same way. Again two bands were observed for paraplegin, irrespective of the integrity of the proteolytic centre (Fig. 20A). Introduction of the proteolytic site mutation in Afg3l1 or Afg3l2 resulted in the accumulation of an additional band with an apparently higher molecular weight, which was already present in low amounts in mitochondria containing active Afg3l2 (Fig. 20A). This suggests that for all three murine *m*-AAA protease subunits the bigger form corresponds to the precursor, whereas the smaller form presumably represents the mature protein lacking the presequence. Moreover, the proteolytic activity of Afg3l1 and Afg3l2 upon assembly coincides with the predominant presence of processed forms. Thus, murine *m*-AAA protease subunits could have a role in their own maturation.

Subsequently, this proposed processing was examined in Δ*yta10*Δ*yta12* cells co-expressing paraplegin and either Afg3l1 or Afg3l2. In mitochondria isolated from these two strains, paraplegin was found to be almost completely converted to the putative mature form (Fig. 20B). In order to determine if this can be attributed to the proteolytic activity of the

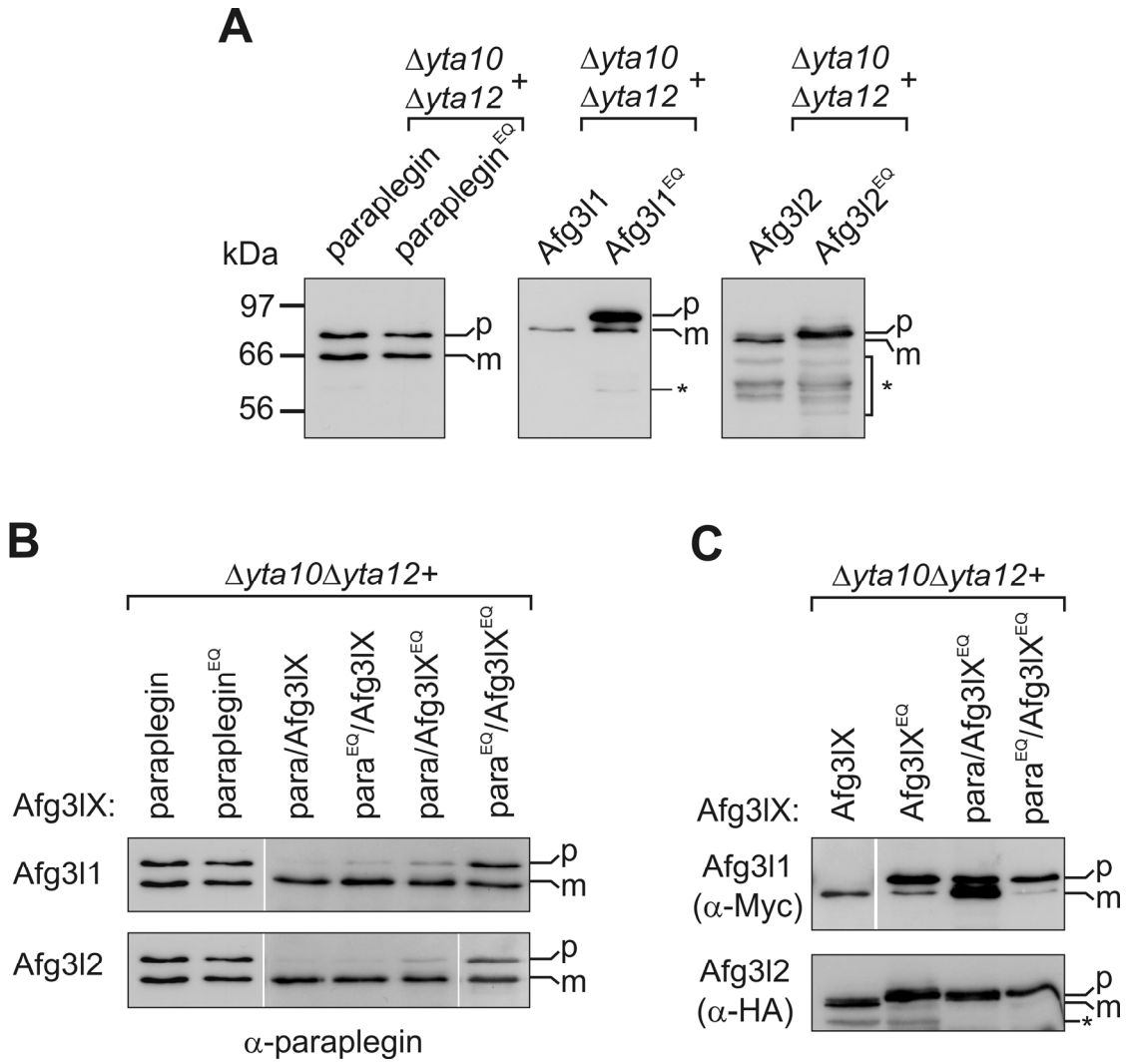

Figure 20. Murine homo- and hetero-oligomeric *m*-AAA protease complexes are involved in the maturation of their subunits in yeast.

(A) Processing of individual murine subunits in yeast. Murine paraplegin, Afg3l1, Afg3l2, or their proteolytically inactive variants paraplegin^{E575Q} (paraplegin^{EQ}), Afg3l1^{E567Q} (Afg3l1^{EQ}), and Afg3l2^{E574Q} (Afg3l2^{EQ}) were individually expressed in Δ*yta10*Δ*yta12* cells. Mitochondria isolated from these strains were used for SDS-PAGE and immunodetection using antibodies recognising paraplegin, the c-Myc epitope of Afg3l1, or the HA epitope of Afg3l2. Bands marked with an asterisk presumably represent degradation products of Afg3l1 and Afg3l2, respectively. p, putative precursor of murine *m*-AAA protease subunits; m, putative mature form of murine *m*-AAA protease subunits.
(B) Processing of paraplegin following co-expression with Afg3l1 or Afg3l2. Mitochondria isolated from Δ*yta10*Δ*yta12* cells harbouring paraplegin (para), paraplegin^{E575Q} (para^{EQ}), or all possible combinations of both proteins with Afg3l1 (upper panel) or Afg3l2 (lower panel, para$^{-/+EQ}$/Afg3lX), or their mutant variants Afg3l1^{E567Q} (upper panel) and Afg3l2^{E574Q} (lower panel, para$^{-/+EQ}$/Afg3lX$^{-/+EQ}$) were examined as in (A) with paraplegin-specific antibodies (α-paraplegin).
(C) Processing of Afg3l1 and Afg3l2 in the presence of paraplegin. Mitochondria were isolated from Δ*yta10*Δ*yta12* cells containing Afg3l1, Afg3l2 (Afg3lX), or the inactive proteins Afg3l1^{E567Q} or Afg3l2^{E574Q} (Afg3lX^{EQ}), as well as from cells co-expressing the mutant forms of both proteins with either paraplegin (para/Afg3lX^{EQ}) or paraplegin^{E575Q} (para^{EQ}/Afg3lX^{EQ}) and analysed as in (A) using antibodies directed against the C-terminal epitopes of Afg3l1 (α-Myc, upper panel) and Afg3l2 (α-HA, lower panel).

respective homo-oligomeric *m*-AAA proteases or rather to the hetero-oligomeric assemblies with paraplegin, proteolytically inactive subunits were employed as described previously (see chapter 3.2.4). Only one predominant band for paraplegin corresponding to the putative mature form was detected after co-expression of paraplegin^{E575Q} and Afg3l1 or paraplegin and Afg3l1^{E567Q} (Fig. 20B). However, a faint band corresponding to the putative precursor form of paraplegin could additionally be observed in these mitochondria and a slightly higher amount of precursor accumulated in mitochondria containing paraplegin and Afg3l1^{E567Q} (Fig. 20B). Upon co-expression of paraplegin^{E575Q} and Afg3l1^{E567Q}, both forms of paraplegin were present in a ratio indistinguishable from mitochondria containing only paraplegin or its mutant variant (Fig. 20B). Analysis of Afg3l1 maturation showed that co-expression of paraplegin concomitantly increased the amount of the putative mature form compared to mitochondria harbouring only Afg3l1^{E567Q} (Fig 20C). This was not observed when paraplegin^{E575Q} was co-expressed with Afg3l1^{E567Q} (Fig 20C). Notably, very similar results concerning the processing of paraplegin and Afg3l2 were observed when proteolytically active and inactive variants of Afg3l2 were co-expressed with paraplegin variants (Fig. 20B and C). In contrast to individual expression of paraplegin or Afg3l2^{E574Q}, co-expression of both murine proteins led to an increase of their mature forms, which was dependent on the proteolytic activity of paraplegin (Fig. 20B and C).

Taken together, these findings indicate that not only homo-oligomeric Afg3l1 and Afg3l2 protease complexes but also hetero-oligomeric complexes with paraplegin are involved in the efficient processing of their subunits. This maturation correlates with the proteolytic activity of the different *m*-AAA proteases. However, significant amounts of mature paraplegin and Afg3l1 are present in the absence of a functional murine *m*-AAA protease suggesting either that it is not the proteolytic activity itself which is essential or that in yeast another protease can also mediate processing though with reduced efficiency. Finally, it has to be considered that this analysis was carried out with hybrid proteins of the murine *m*-AAA protease subunits containing the mitochondrial targeting sequence of Yta10. It is, therefore, possible that these hybrid proteins are processed in a different manner than the murine full length proteins.

3.3 *Mammalian homo-oligomeric* m-*AAA proteases mediate OPA1 processing in yeast*

OPA1 is a dynamin-like GTPase and the mammalian homologue of yeast Mgm1 which are both required for mitochondrial fusion (Okamoto and Shaw, 2005; Chan, 2006b). Notably, mutations in the *OPA1* gene cause hereditary autosomal dominant optic atrophy (ADOA) type I (Alexander *et al.*, 2000; Delettre *et al.*, 2000). Up to five different isoforms of the OPA1 protein have been found in mammalian mitochondria indicating proteolytic processing of OPA1 (Olichon *et al.*, 2003; Duvezin-Caubet *et al.*, 2006; Ishihara *et al.*, 2006; Olichon *et al.*, 2006). However, the fact that eight alternatively spliced mRNAs transcribed from the *OPA1* gene have been reported complicates the analysis of OPA1 processing in human cells and it remains unclear to which extent the different OPA1 isoforms are generated by alternative splicing and/or limited proteolysis (Delettre *et al.*, 2001; Satoh *et al.*, 2003). For Mgm1, it has been shown that the conversion of the large (L-) isoform to the short (S-) isoform is mediated by the rhomboid protease Pcp1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003a). In contrast, the protease(s) responsible for processing of OPA1 have not been unambiguously identified. Recently, cleavage of OPA1 has been linked to the *m*-AAA protease subunit paraplegin whose downregulation in human cells resulted in impaired OPA1 processing (Ishihara *et al.*, 2006). Therefore, the aim of the following experiments was to investigate if homo- and hetero-oligomeric mammalian *m*-AAA proteases are indeed capable of cleaving OPA1 using yeast as a model. This allows the expression of a single human OPA1 splice variant in cells harbouring human and murine *m*-AAA protease subunits. The reconstitution of OPA1 processing in yeast could also provide insights which splice variants give rise to the observed five OPA1 isoforms.

3.3.1 OPA1 processing can be reconstituted in yeast and is mediated by the endogenous *m*-AAA protease

To initially investigate if OPA1 processing can be reconstituted in yeast, human OPA1 splice variants 1, 4, 7, and 8 were separately expressed in wild-type cells under the control of the galactose-inducible *GAL1* promoter using the multicopy plasmid pYES2. These OPA1 variants differ in the N-terminal region between the first transmembrane domain and the GTPase domain and are characterised by the presence or absence of protein segments

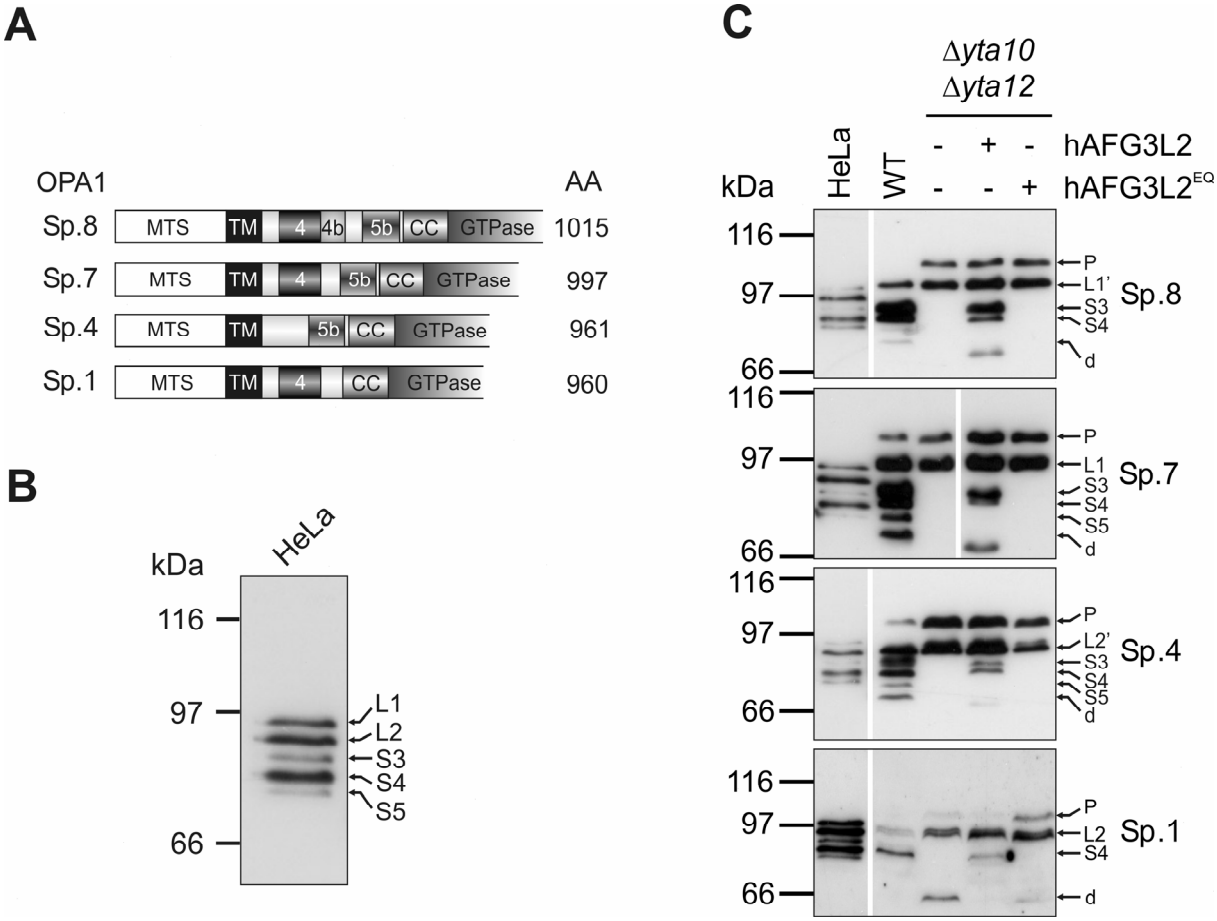

Figure 21. Reconstitution of human OPA1 processing in yeast: cleavage can be mediated by the yeast *m*-AAA protease or the homo-oligomeric hAFG3L2 complex.

(A) Schematic representation of the primary structures of the human OPA1 splice variants (Sp.) 1, 4, 7, and 8. The N-terminal regions upstream of the GTPase domain are depicted including the mitochondrial targeting sequence (MTS), the first transmembrane domain (TM), protein segments encoded by alternatively spliced exons 4, 4b, and 5b, and a coiled-coil domain (CC). The number of amino acids (AA) is indicated for each splice variant on the right side.
(B) OPA1 isoforms present in HeLa cells. A HeLa cell lysate (50 µg protein) was separated by SDS-PAGE followed by immunodetection with an antiserum recognising human OPA1. The resulting different OPA1 isoforms are indicated by arrows and named L1, L2, S3, S4, and S5 according to (Duvezin-Caubet *et al.*, 2006).
(C) Processing of human OPA1 in yeast. OPA1 splice variants (Sp.) 1, 4, 7, or 8 were expressed in wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, and Δ*yta10*Δ*yta12* cells harbouring either hAFG3L2 or its proteolytically inactive variant hAFG3L2^{E575Q} (hAFG3L2^{EQ}). Total cell extracts from these strains were analysed by Western blotting with anti-OPA1 antibodies. HeLa total extracts (50 µg protein) were used as reference and bands are labelled by arrows according to the apparent corresponding size of OPA1 isoforms in the HeLa cell lysate (L1, L2, S3, S4, and S5). L1' and L2' refer to L1 and L2, respectively, but show a slight mobility shift. Putative precursor proteins (P) and yeast-specific degradation products (d) are also indicated.

encoded by exons 4, 4b, and 5b of the *OPA1* gene (Fig. 21A) (Delettre *et al.*, 2001). The mitochondrial localisation of OPA1 variants expressed in yeast was verified by SDS-PAGE and immunoblotting of cellular membrane preparations containing mitochondrial membranes

(data not shown). In these experiments, a specific antiserum was used which has been raised against a C-terminal region of human OPA1 present in all splice variants. To study OPA1 processing, total cell extracts were prepared from wild-type cells harbouring a single OPA1 splice variant and subjected to Western blot analysis. For comparison, HeLa cell lysates were analysed in parallel, which contain five different OPA1 isoforms, named L1 and L2 for the two larger isoforms and S3, S4, and S5 for the three smaller isoforms (Fig. 21B) (Duvezin-Caubet *et al.*, 2006). Moreover, mRNAs of OPA1 splice variants 1, 7, and 8 belong to the most abundant OPA1 mRNAs in HeLa cells (Satoh *et al.*, 2003; Olichon *et al.*, 2006).

Upon expression of OPA1 in wild-type yeast cells, several OPA1 isoforms could be detected for each splice variant (Fig. 21C). These forms corresponded in size to OPA1 isoforms present in HeLa cell lysates (Fig. 21C). For splice variant 8, a band similar but not identical in size to isoform L1 of HeLa cells, thus named L1', as well as bands identical in size to S3 and S4 of HeLa cells were observed (Fig. 21C). Expression of splice variant 7 gave rise to bands with a molecular weight corresponding to the isoforms L1, S3, S4, and S5 (Fig. 21C). Expression of splice variant 4 led to the accumulation of a large isoform slightly bigger in size than L2 of HeLa cells, thus named L2', and short isoforms S3, S4, and to a lesser extent S5 (Fig. 21C). Splice variant 1 showed a reduced expression level in yeast cells compared to the other three variants suggesting that it might be not as stable and subject to complete degradation. Nevertheless, its expression resulted in the distinct presence of L2 and S4 isoforms (Fig. 21C). In addition, a band larger than the OPA1 isoforms in HeLa cells was detected in wild-type yeast cells containing splice variants 4 and 7 (Fig. 21C). It presumably represents the precursor of human OPA1 accumulating due to overexpression in yeast. Smaller OPA1 bands present at least in case of splice variants 4, 7, and 8 are likely to be yeast-specific degradation products (Fig. 21C).

These findings show that OPA1 splice variants expressed in wild-type yeast cells receive proteolytic processing leading to short OPA1 isoforms, which are apparently indistinguishable in size from short OPA1 isoforms present in HeLa cells. Thus, OPA1 processing was reconstituted in yeast, which can be used as a valid model for further investigations. The validity of this approach was confirmed by mass spectrometric analysis of OPA1 isoforms purified from HeLa and yeast cells (Duvezin-Caubet *et al.*, 2007). Furthermore, both alternative splicing and limited proteolysis seem to contribute to the pattern of OPA1 isoforms, as S3, S4, and to a limited extent S5 could be generated by expression of splice variants 4, 7, and 8, though in different ratios.

Apparently, yeast cells possess a proteolytic system in mitochondria capable of cleaving heterologously expressed human OPA1. In a recent study, the human *m*-AAA protease subunit paraplegin has been implicated in OPA1 cleavage in HeLa cells (Ishihara *et al.*, 2006). In addition, processing of rat OPA1 splice variants1 and 7 in yeast was found to be dependent on the *m*-AAA protease composed of Yta10 and Yta12 subunits (Ishihara *et al.*, 2006). To confirm these observations, human OPA1 splice variants 1, 4, 7, and 8 were expressed in Δ*yta10*Δ*yta12* cells and processing was examined in total cell extracts. For each OPA1 splice variant, only two bands corresponding to the putative precursor and the large isoform were detected, whereas short isoforms were completely absent from Δ*yta10*Δ*yta12* cells (Fig. 21C). This clearly indicates that the *m*-AAA protease is responsible for OPA1 processing in yeast. In another study, a role of PARL, the human homologue of the yeast rhomboid protease Pcp1, in the generation of a soluble OPA1 isoform of lower molecular weight has been reported (Cipolat *et al.*, 2006). However, OPA1 processing was not affected in PARL-deficient mouse cells (Cipolat *et al.*, 2006; Duvezin-Caubet *et al.*, 2007). Upon expression of OPA1 splice variants in Δ*pcp1*cells, a band pattern identical to that observed in wild-type cells was obtained for each OPA1 splice variant excluding processing by Pcp1 (data not shown).

3.3.2 Deletion of yeast *PHB1* can enhance *m*-AAA protease mediated OPA1 processing

In yeast, the *m*-AAA protease assembles with the prohibitin complex, which consists of Phb1 and Phb2, into a supercomplex in the inner mitochondrial membrane (Steglich *et al.*, 1999). A negative regulatory effect of prohibitins on *m*-AAA protease activity has been suggested, as the degradation of non-assembled membrane proteins was accelerated in the absence of prohibitins (Steglich *et al.*, 1999). Therefore, experiments were carried out in order to investigate if the prohibitin complex has an effect on OPA1 processing by the yeast *m*-AAA protease. Total cell extracts were prepared from Δ*phb1* cells expressing OPA1 splice variants 1, 4, 7, or 8, followed by Western blot analysis with OPA1-specific antibodies. Due to the interdependence of Phb1 and Phb2, both proteins are absent in Δ*phb1* cells (Berger and Yaffe, 1998). Essentially, no difference in the band pattern could be observed for OPA1 splice variants 1 and 7 compared to wild-type cells (Fig. 22). However, in contrast to wild-type cells, the L1' isoform was not detected in Δ*phb1* cells containing splice variant 8, suggesting a complete conversion of large OPA1 isoforms to small isoforms (Fig. 22). Similarly, the

amount of the L2' isoform was strongly reduced for splice variant 4 when prohibitins were lacking (Fig. 22). In parallel, an increase in the S3 isoform was observed for this splice variant (Fig. 22). Apparently, OPA1 processing is enhanced in the absence of prohibitins, although their role in OPA1 cleavage is restricted only to certain OPA1 splice variants. These findings suggest a possible negative regulatory function of the prohibitin complex also for processing by the *m*-AAA protease. Alternatively, this increased generation of short isoforms of OPA1 may also reflect a presumed chaperone-like function of the prohibitin complex protecting OPA1 from *m*-AAA protease mediated cleavage (Nijtmans *et al.*, 2000).

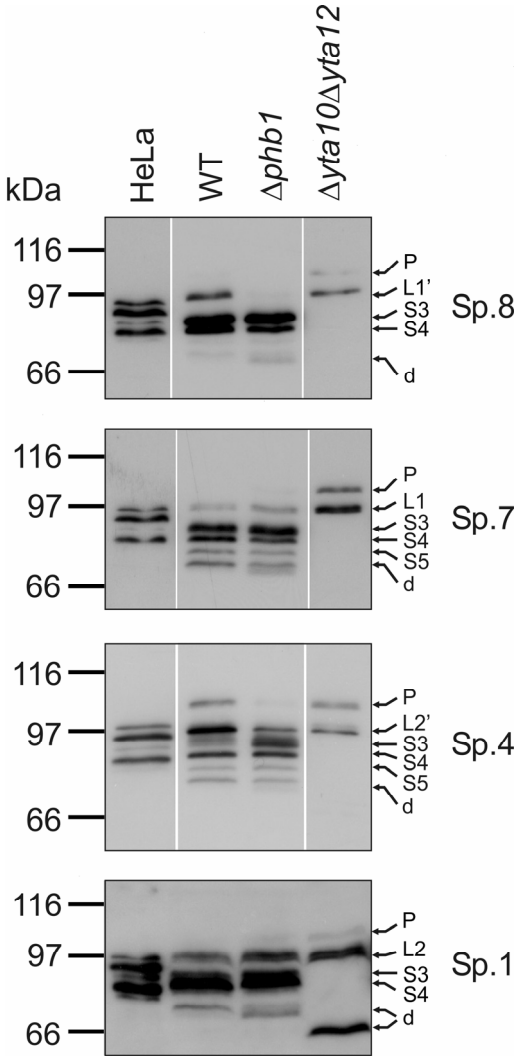

Figure 22. Processing of human OPA1 in Δ*phb1* yeast cells lacking the prohibitin complex.

The human OPA1 splice variants (Sp.) 1, 4, 7, and 8 were expressed in wild-type (WT), Δ*phb1*, or Δ*yta10*Δ*yta12* cells. Total cell extracts were analysed as described in Fig. 15C using an antiserum recognising human OPA1. HeLa cell extracts (50 µg protein) were used for comparison and bands are labelled by arrows as described for Fig. 15C.

3.3.3 Proteolytic processing of OPA1 splice variants by the human AFG3L2 complex

The yeast *m*-AAA protease mediates proteolytic processing of human OPA1 suggesting that mammalian *m*-AAA proteases have a similar activity. To examine this possibility for the human homo-oligomeric AFG3L2 complex, OPA1 splice variants 1, 4, 7, and 8 were individually expressed in Δ*yta10*Δ*yta12* cells containing either hAFG3L2 or proteolytically inactive hAFG3L2^{E575Q}. The analysis of OPA1 processing by SDS-PAGE and immunoblotting of total cell extracts revealed that short isoforms were generated for each OPA1 splice variant in the presence of hAFG3L2 (Fig. 21C). Co-expression of hAFG3L2^{E575Q} with one of the four OPA1 splice variants did not restore its processing in Δ*yta10*Δ*yta12* cells demonstrating that cleavage depends on the proteolytic activity of hAFG3L2 (Fig. 21C). It is noteworthy, that hAFG3L2 seems to have a different preference or enzymatic activity for the different splice variants, as a larger amount of short isoforms was observed for splice variants 7 and 8 than for variants 1 and 4 (Fig. 21C). However, cleavage by the hAFG3L2 complex was always less efficient than observed for the yeast *m*-AAA protease and only S3 and S4 isoforms were generated, whereas S5 isoforms were lacking (Fig. 21C). Taken together, the homo-oligomeric *m*-AAA protease built up of hAFG3L2 can promote cleavage of human OPA1 upon reconstitution in yeast.

3.3.4 Variable efficiencies of OPA1 processing by homo- and hetero-oligomeric murine *m*-AAA proteases

The analysis of the proteolytic complexes formed by the three murine *m*-AAA protease subunits has demonstrated a high variability in the assembly of *m*-AAA protease complexes. Homo-oligomeric Afg3l1 and Afg3l2 complexes as well as hetero-oligomeric paraplegin/Afg3l1 and paraplegin/Afg3l2 complexes can be formed, which all exert proteolytic activity. To assess if these complexes can promote OPA1 cleavage or display varying activities, human OPA1 splice variants 1, 4, 7, and 8 were expressed in Δ*yta10*Δ*yta12* cells containing single murine subunits or various combinations of them. Human OPA1 splice variants are highly homologous to their murine counterparts and thus should also be recognised by murine *m*-AAA proteases. In the case of splice variant 1, the human and the murine protein share 96% identity over the whole sequence and 86% identity for the N-terminal 208 amino acids comprising the mitochondrial targeting sequence and the variable protein segments caused by differential splicing.

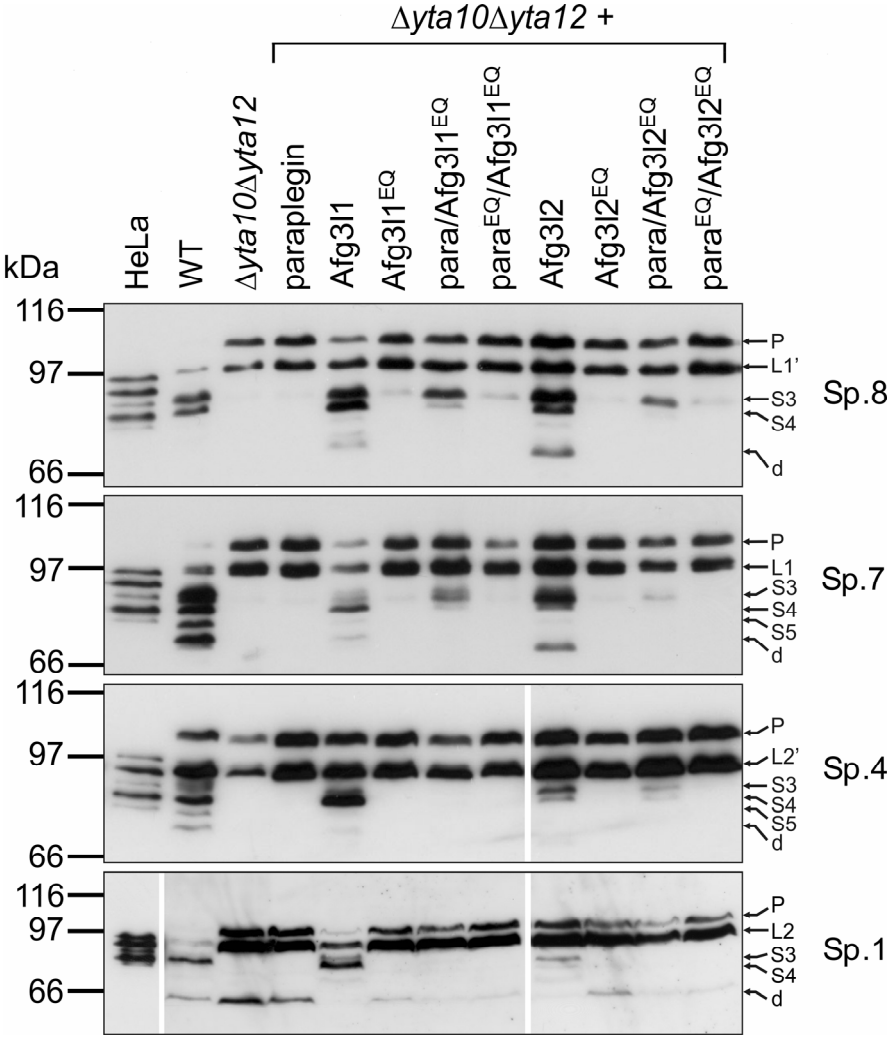

Figure 23. Processing of human OPA1 in yeast by murine homo- and hetero-oligomeric *m*-AAA protease complexes.

The human OPA1 splice variants (Sp.) 1, 4, 7, and 8 were expressed in wild-type (WT), Δ*yta10*Δ*yta12* cells, or Δ*yta10*Δ*yta12* cells containing either murine paraplegin (para), Afg3l1, Afg3l2, or their mutant variants paraplegin^{E575Q} (para^{EQ}), Afg3l1^{E567Q} (Afg3l1^{EQ}), or Afg3l2^{E574Q} (Afg3l2^{EQ}) or combinations of them. Total cell extracts were prepared from these strains and subjected to SDS-PAGE and immunoblotting using human OPA1-specific antibodies. Cell extracts (50 µg protein) from HeLa cells were used as reference and bands are labelled by arrows as described for Fig. 21C.

Expression of paraplegin in these yeast cells did not compensate for the lack of the *m*-AAA protease, as short isoforms of OPA1 were not detected for any splice variant (Fig. 23). This is in line with the observation that paraplegin, in contrast to Afg3l1 and Afg3l2, does not assemble into a homo-oligomeric complex with proteolytic activity. Accordingly, the short isoforms S3 and S4 were present for all four splice variants when either Afg3l1 or Afg3l2 were individually expressed in Δ*yta10*Δ*yta12* cells (Fig. 23). S5 isoforms were hardly detectable under these conditions (Fig. 23). On the other hand, OPA1 cleavage was almost completely abolished when the proteolytic centres of Afg3l1 and Afg3l2 were mutated

(Afg3l1^{E567Q} and Afg3l2^{E574Q}) (Fig. 23). Hence, similar to hAFG3L2, the homo-oligomeric Afg3l1 and Afg3l2 complexes are capable of converting large OPA1 isoforms, derived from splice variants 1, 4, 7, and 8, into small isoforms due to their proteolytic activity.

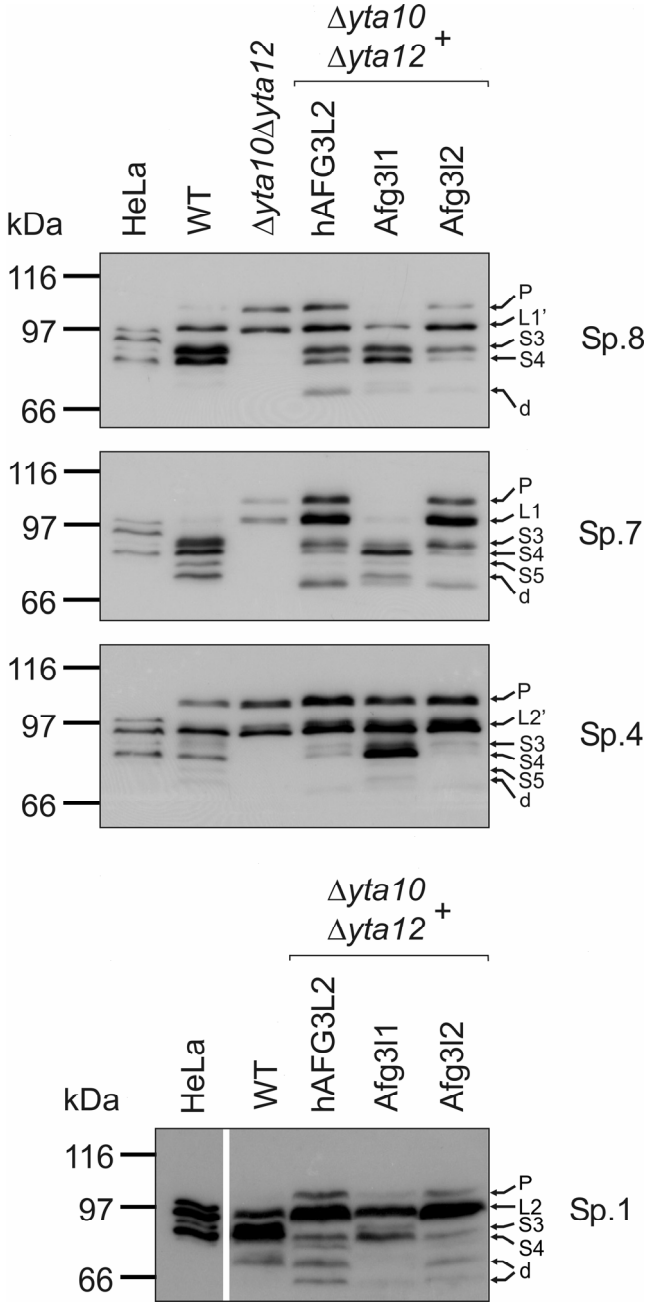

Figure 24. Different efficiencies of OPA1 processing by homo-oligomeric *m*-AAA proteases composed of hAFG3L2, murine Afg3l1, or murine Afg3l2 in yeast.

The human OPA1 splice variants (Sp.) 1, 4, 7, and 8 were expressed in wild-type (WT) cells, Δ*yta10*Δ*yta12* cells, or Δ*yta10*Δ*yta12* cells harbouring hAFG3L2, murine Afg3l1, or murine Afg3l2. Total cell extracts were analysed as described in Fig. 21C using an antiserum recognising human OPA1. HeLa cell lysates (50 µg protein) were used as reference and bands are labelled by arrows as described for Fig. 21C.

This initial analysis of OPA1 processing by murine Afg3l1 and Afg3l2 complexes pointed to slight differences between both homo-oligomeric *m*-AAA proteases with respect to processing efficiency and the ratio of short isoforms generated. Therefore, the investigation of OPA1 processing was repeated with OPA1 splice variants 1, 4, 7, and 8, and total extracts of Δ*yta10*Δ*yta12* cells harbouring hAFG3L2, Afg3l1, or Afg3l2 were loaded in adjacent lanes to facilitate a more careful analysis of the different OPA1 band patterns. For comparison, HeLa cell lysates and total extracts from wild-type and Δ*yta10*Δ*yta12* cells were also included. For each OPA1 splice variant, the generation of short isoforms was apparently more pronounced in the presence of Afg3l1 when compared to hAFG3L2 and Afg3l2, which both showed a similar amount of short isoforms (Fig. 24). Moreover, OPA1 splice variants 1, 7, and 8 were nearly as efficiently cleaved by Afg3l1 complexes as observed for the yeast *m*-AAA protease, while splice variant 4 was cleaved even more efficiently (Fig. 24). In addition, expression of Afg3l1 preferentially led to the formation of S4 isoforms, whereas more of the isoform S3 accumulated in the presence of hAFG3L2 and Afg3l2, at least for splice variants 4, 7, and 8 (Fig. 24). These observations support the notion that the homo-oligomeric murine Afg3l1 and Afg3l2 complexes differ in their substrate specificity towards OPA1 splice variants as exemplified by a different processing efficiency and subtle differences in the pattern of short isoforms.

Co-expression of murine paraplegin with either Afg3l1 or Afg3l2 in Δ*yta10*Δ*yta12* cells harbouring splice variants 1, 4, 7, or 8 did not significantly affect OPA1 processing compared to expression of Afg3l1 or Afg3l2 alone (data not shown). To be able to distinguish between OPA1 cleavage by hetero-oligomeric *m*-AAA protease complexes containing paraplegin and a contribution of the homo-oligomeric complexes, paraplegin was co-expressed with the proteolytic site mutants Afg3l1^{E567Q} and Afg3l2^{E574Q} in Δ*yta10*Δ*yta12* cells. As described in chapter 2.4, homo-oligomeric complexes built up of mutant subunits are inactivate, whereas hetero-oligomeric complexes containing mutant and wild-type subunits are still functional due to the proteolytic activity of their paraplegin subunit. Processing of OPA1 splice variants 4, 7, or 8 was rather inefficient in these cells and in some cases barely detectable (Fig. 23). However, the accumulation of small amounts of shorter isoforms indicates OPA1 cleavage by paraplegin-containing *m*-AAA protease complexes. Furthermore, a preference for the generation of the respective S3 isoform could be observed for both hetero-oligomeric assemblies, whereas S4 isoforms were largely absent (Fig. 23). Notably, small OPA1 isoforms were not detected with splice variant 1 suggesting that it does not receive any processing by hetero-oligomeric complexes (Fig. 23). As short OPA1 isoforms

did not accumulate for any splice variant if both, Afg3l1 or Afg3l2, and paraplegin contained point mutations in their proteolytic centres, the observed processing can indeed be attributed to the proteolytic activity of paraplegin and thus of the hetero-oligomeric complexes (Fig. 23).

In conclusion, OPA1 splice variants 4, 7, and 8 can be processed not only by homo-oligomeric *m*-AAA proteases composed of either Afg3l1 or Afg3l2 subunits, but also by hetero-oligomeric paraplegin/Afg3l1 and paraplegin/Afg3l2 complexes upon reconstitution in yeast. However, the processing efficiency of the latter is limited and lower than in the presence of Afg3l1 and Afg3l2 complexes. Thus, *m*-AAA proteases with a variable subunit composition are apparently able to mediate OPA1 processing with different efficiencies.

4 Discussion

4.1 Mammalian m-*AAA protease subunits assemble into proteolytic complexes with variable subunit composition*

The *m*-AAA protease represents a hetero-oligomeric assembly in the inner mitochondrial membrane of yeast, which is composed of Yta10 and Yta12 subunits (Arlt *et al.*, 1996). Complementation studies revealed a functional conservation of hetero-oligomeric *m*-AAA proteases in yeast and mammals, where it is built up of the subunits paraplegin and hAFG3L2/Afg3l2 (Atorino *et al.*, 2003; Nolden *et al.*, 2005). In the absence of either Yta10 or Yta12, yeast cells lose their respiratory competence and the remaining subunit assembles only into a low molecular weight complex lacking proteolytic activity (Arlt *et al.*, 1996). Similarly, a paraplegin deficiency leads to severe phenotypes in humans and mice including degeneration of long axons (Casari *et al.*, 1998; Ferreirinha *et al.*, 2004). However, the assembly status of the remaining hAFG3L2 and Afg3l2 subunits, respectively, has not been established. In mice, the situation is complicated by the presence of a third putative *m*-AAA protease subunit, termed Afg3l1, which has been localised to mitochondria, but whose assembly and function were still unclear (Kremmidiotis *et al.*, 2001). In order to address these open questions, human and murine *m*-AAA protease subunits were separately expressed and co-expressed in Δ*yta10*Δ*yta12* yeast cells allowing biochemical and functional characterisation.

Upon expression in yeast, human and murine *m*-AAA protease subunits assembled into various proteolytic complexes which differ in their subunit composition. In addition to its assembly with human paraplegin, hAFG3L2 has the ability to homo-oligomerise into a high molecular mass complex when expressed in the absence of paraplegin. This hAFG3L2 complex is functionally active and could substitute for the yeast *m*-AAA protease in maintaining respiratory growth. Homo-oligomerisation appears to be restricted to hAFG3L2, as human paraplegin was previously shown to be only part of low molecular weight complexes, which are proteolytically inactive (Koppen, 2002). Similar to hAFG3L2, murine Afg3l1 and Afg3l2 can homo-oligomerise in yeast and maintain respiratory activity. In line with the results for human paraplegin, no evidence for homo-oligomerisation was obtained for murine paraplegin, which only assembled into hetero-oligomeric *m*-AAA proteases containing either Afg3l1 or Afg3l2. The complementation studies in yeast thus reveal that

murine *m*-AAA protease subunits can build up at least four distinct complexes with proteolytic activity: homo-oligomeric Afg3l1 and Afg3l2 complexes or hetero-oligomeric paraplegin/Afg3l1 and paraplegin/Afg3l2 complexes (Fig. 25).

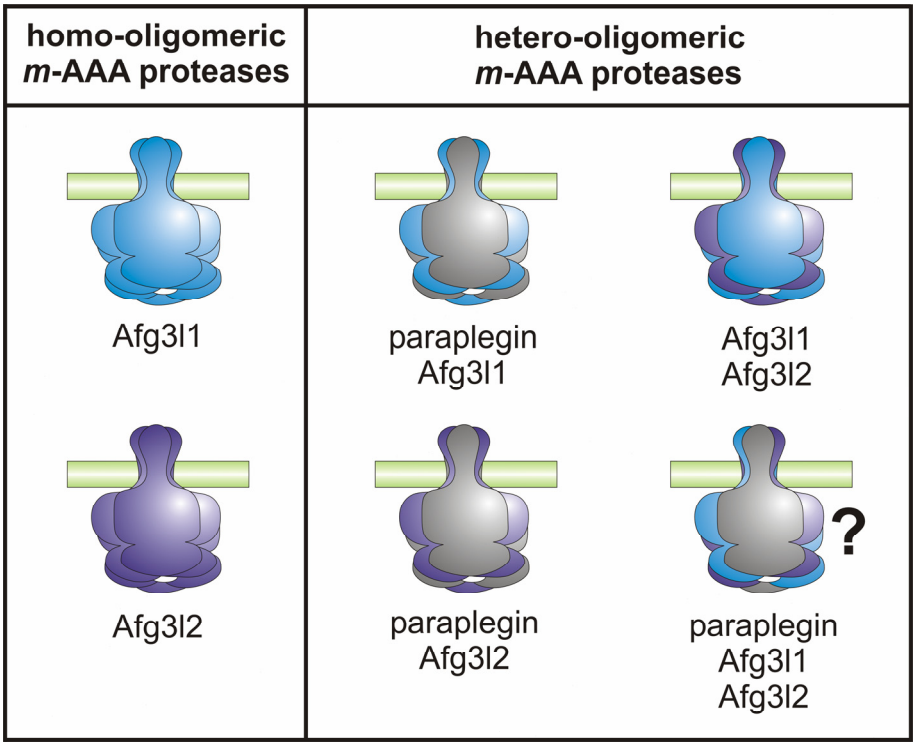

Figure 25. Isoenzymes of the murine *m*-AAA protease.

Evidence for five isoenzymes of the murine *m*-AAA protease 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, hAFG3L2 and hparaplegin, are expressed in human mitochondria and assemble into homo-oligomeric hAFG3L2 or hetero-oligomeric hparaplegin/hAFG3L2 complexes.

The formation of homo-oligomeric proteolytic complexes by hAFG3L2 as well as by murine Afg3l1 and Afg3l2 raises the question why paraplegin subunits do not have the ability to homo-oligomerise. Crystal structures of FtsH, the bacterial AAA protease, have revealed that intersubunit contacts in the hexamer mainly occur between the proteolytic domains of the FtsH monomers (Bieniossek *et al.*, 2006; Suno *et al.*, 2006). Despite the high degree of sequence conservation, significant variations between *m*-AAA protease subunits exist in C-terminal regions. It is therefore conceivable that sequence differences of AAA proteases in this region determine their ability for homo-oligomerisation. In addition, a role in oligomerisation of FtsH from *Escherichia coli* and the yeast *m*-AAA protease has been

attributed to the N-terminal domain of the respective subunits, which contains the two transmembrane segments (Akiyama *et al.*, 1995; Makino *et al.*, 1999; Korbel *et al.*, 2004). This domain is less conserved between the mammalian *m*-AAA protease subunits than the catalytic domains suggesting that sequence differences in paraplegin subunits could contribute or even account for the observed oligomeric status. Besides, it has to be considered that the mammalian *m*-AAA protease subunits were expressed in yeast as hybrid proteins containing the MTS of Yta10 fused to the putative mature forms of the subunits. Thus, a negative effect on the assembly and activity of human and murine paraplegin subunits due to the absence of critical residues at the extreme N-terminus cannot be ruled out.

Do *m*-AAA proteases with variable subunit composition exist in mammalian cells? Evidence for this versatile assembly has been obtained by the analysis of mitochondria isolated from murine liver and brain. Co-immunoprecipitation experiments of murine wild-type mitochondria have revealed that paraplegin, Afg3l1, and Afg3l2 directly interact with each other (Koppen *et al.*, 2007). The formation of three independent complexes containing two types of subunits each, either paraplegin and Afg3l1, paraplegin and Afg3l2, or Afg3l1 and Afg3l2, can rationalise the observed interactions (Fig. 25). However, three different types of subunits may also be part of one *m*-AAA protease complex, although direct evidence for such an isoenzyme is still missing (Fig. 25). The formation of a hetero-oligomeric Afg3l1/Afg3l2 complex has been demonstrated using liver and brain mitochondria from paraplegin-deficient mice (Koppen *et al.*, 2007). Both subunits were found to directly interact with each other in co-immunoprecipitation experiments resulting in the formation of a high molecular weight complex. In line with these observations, the steady state concentrations of Afg3l1 and Afg3l2 were not altered in the absence of paraplegin (Koppen *et al.*, 2007). Taken together, different *m*-AAA protease isoenzymes can be formed in murine mitochondria. Moreover, results from both yeast and mouse establish Afg3l1 as a *bona fide* subunit of *m*-AAA proteases which can assemble into homo- and hetero-oligomeric complexes both exerting proteolytic activity.

The formation of *m*-AAA protease isoenzymes differing in their subunit composition raises the question if they co-exist with each other in the inner membrane of mammalian mitochondria. Proteomic studies have demonstrated a large variability in the protein composition of mitochondria isolated from different murine and rat tissues (Mootha *et al.*, 2003; Forner *et al.*, 2006). Consistently, Afg3l2 was found to be ~10-fold and paraplegin ~4-fold more abundant in murine brain mitochondria than in liver mitochondria compared to the relative abundance of Afg3l1 in these tissues (Koppen *et al.*, 2007). Differences in the relative

abundance of *m*-AAA protease subunits were also reflected in immunodepletion experiments. Paraplegin and Afg3l1 could be almost completely immunodepleted from mitochondrial extracts of wild-type brain with Afg3l2-specific antibodies (Koppen *et al.*, 2007). Thus, the three murine proteins assemble with each other in a virtually quantative manner. In contrast, a large fraction of paraplegin and Afg3l2 was still present in the depleted supernatant when Afg3l1-specific antibodies were employed indicating that Afg3l1 is apparently present at lower molecular concentrations than those of paraplegin and Afg3l2 in murine brain (Koppen *et al.*, 2007). The different results of these immmunodepletion experiments therefore point to the co-existence of at least two different *m*-AAA proteases in brain mitochondria: paraplegin/Afg3l2 complexes and less abundant Afg3l1 complexes containing paraplegin, Afg3l2, or both. Using brain mitochondria isolated from paraplegin-deficient mice, Afg3l1 could be nearly entirely immunodepleted with antibodies directed against Afg3l2, which was not observed vice versa (Koppen *et al.*, 2007). This strongly suggests that, in the absence of paraplegin, a hetero-oligomeric Afg3l1/Afg3l2 complex and a predominant homo-oligomeric Afg3l2 are present in murine brain. The existence of homo-oligomeric *m*-AAA proteases in mammalian mitochondria was further substantiated in this study by the identification of a hAFG3L2 complex in mitochondria of HSP fibroblasts lacking human paraplegin. However, it remains elusive if both the hetero-oligomeric paraplegin/hAFG3L2 and the homo-oligomeric hAFG3L2 complex co-exist in human mitochondria, since immunodepletion experiments could not be performed.

In summary, these data demonstrate the versatile assembly of mammalian *m*-AAA protease subunits into proteolytic complexes with variable composition apparently depending on subunit availability, which is determined by the tissue-specific expression of different subunits. The absence of one *m*-AAA protease subunit does not lead to a complete loss of proteolytic complexes. Instead, different isoenzymes of the *m*-AAA protease can be formed as exemplified by a paraplegin deficiency in HSP patients and the corresponding mouse model.

4.2 Mammalian m-*AAA proteases have conserved and overlapping functions in mitochondria*

Different human and murine *m*-AAA protease isoenzymes are functionally active as they could substitute for the yeast *m*-AAA protease in complementation studies. This holds true for the homo-oligomeric assemblies of hAFG3L2, Afg3l1, and Afg3l2 as well as for the

hetero-oligomeric isoenzymes containing paraplegin and demonstrates functional conservation of *m*-AAA proteases from yeast, mouse, and humans.

The homo-oligomeric hAFG3L2 complex was shown to exert proteolytic activity in yeast. Like the yeast *m*-AAA protease, it could recognise and degrade misfolded polypeptides that are either integrated into the inner membrane or peripherally associated with its matrix-exposed surface. Moreover, the precursor form of the ribosomal subunit MrpL32 was cleaved by hAFG3L2 which is required for its activation and the concomitant initiation of mitochondrial translation (Nolden *et al.*, 2005). Thus, the hAFG3L2 complex can substitute for crucial housekeeping functions of the yeast *m*-AAA protease. An additional non-proteolytic function of the yeast enzyme during mitochondrial biogenesis has recently been identified. Removal of the bipartite presequence of the ROS-scavenger Ccp1 by the consecutive action of the *m*-AAA protease and the rhomboid protease Pcp1 requires its ATP-dependent dislocation from the inner membrane by the *m*-AAA protease (Tatsuta *et al.*, 2007). The proteolytic activity of the *m*-AAA protease is dispensable as maturation of Ccp1 by Pcp1 was observed in yeast cells harbouring a proteolytically inactive *m*-AAA protease (Tatsuta *et al.*, 2007). Ccp1 processing in *m*-AAA protease-deficient yeast cells was also restored in the presence of proteolytically active and inactive hAFG3L2 complexes. This indicates that hAFG3L2 can mediate membrane dislocation and functionally interact with the yeast rhomboid protease. Notably, similar observations regarding protein degradation, processing, and membrane dislocation were also made for murine Afg3l1 or Afg3l2 but not for paraplegin, demonstrating conserved activities of homo-oligomeric complexes. Furthermore, complementation studies in yeast point to overlapping substrate specificities of these homo-oligomeric assemblies with hetero-oligomeric *m*-AAA proteases containing paraplegin. Indeed, hetero-oligomeric complexes of Afg3l1, Afg3l2, and hAFG3L2 with paraplegin mediated processing of MrpL32 and Ccp1 in Δ*yta10*Δ*yta12* yeast cells as shown in this and previous studies (Koppen, 2002; Nolden *et al.*, 2005).

Hence, it can be concluded that heterologously expressed mammalian *m*-AAA protease complexes, either homo- or hetero-oligomeric, maintain mitochondrial housekeeping functions in yeast like protein quality control, protein processing, and membrane dislocation demonstrating conserved and overlapping substrate specificities.

4.3 *Mammalian homo-oligomeric* m*-AAA protease complexes mediate OPA1 processing reconstituted in yeast*

The dynamin-like GTPase OPA1 is a central component of the mitochondrial fusion machinery in mammals (Chan, 2006b). Eight alternatively spliced mRNAs are transcribed from the *OPA1* gene resulting in the accumulation of several large (L-) and short (S-) isoforms of the OPA1 protein in mitochondria. The shorter OPA1 isoforms are generated by proteolytic processing of the larger isoforms (Duvezin-Caubet *et al.*, 2006; Ishihara *et al.*, 2006). To support the notion of OPA1 cleavage and to identify the protease(s) involved, yeast was employed as a model system in this study. This offered the advantage to express OPA1 splice variants individually and to use Δ*yta10*Δ*yta12* yeast cells harbouring mammalian *m*-AAA protease subunits. Hence, homo- and hetero-oligomeric *m*-AAA proteases with different subunit composition could be assessed for their ability to mediate human OPA1 processing in yeast.

Upon expression of single human OPA1 splice variants in yeast, OPA1 was recognised and cleaved by yeast and mammalian *m*-AAA proteases. Several lines of evidence support the validity of the yeast model for studying OPA1 processing. For all four splice variants, the OPA1 isoforms obtained after expression in yeast were similar in size to those detected in HeLa cells demonstrating that OPA1 was specifically processed and not completely degraded by the *m*-AAA protease. In particular, similar OPA1 isoforms could be observed when splice variants 1 and 4 were expressed in yeast cells harbouring a functional *m*-AAA protease or after transfection of HeLa cells (Olichon *et al.*, 2006). The OPA1 isoforms obtained for splice variants 1 and 7 in yeast are also comparable to those reported by Ishihara *et al.*, 2006, upon expression of the corresponding splice variants from rat in HeLa cells. Last but not least, the mass spectrometric analysis of OPA1 isoforms purified from either HeLa cells or wild-type yeast cells containing OPA1 splice variants 7 or 8 showed a consistent peptide pattern for large and small isoforms (Duvezin-Caubet *et al.*, 2007). Therefore, it can be concluded that OPA1 is processed in a similar manner in yeast and human mitochondria.

Expression of a single OPA1 splice variant in yeast cells gave rise to several S- and L-isoforms of OPA1 directly demonstrating the proteolytic conversion of OPA1 to short isoforms. In wild-type yeast cells, the respective large isoforms of OPA1 splice variants 4, 7, and 8 were found to be converted mainly to S3 and S4 isoforms. Additionally, the OPA1 splice variants 4 and 7 were processed to the S5 isoform, whereas expression of splice variant 1 almost exclusively resulted in the generation of the S4 isoform. Thus, both

alternative splicing and proteolytic processing apparently contribute to the pattern of OPA1 isoforms as observed in HeLa cells. This was confirmed by mass spectrometric analysis of purified OPA1 isoforms from HeLa cells, which revealed that some isoforms could be derived from several OPA1 splice variants (Duvezin-Caubet *et al.*, 2007). In the absence of Yta10 and Yta12, short isoforms were not generated upon expression of OPA1 splice variants indicating that the yeast *m*-AAA protease cleaves OPA1. This is consistent with a recent report about *m*-AAA protease-mediated processing of rat OPA1 in yeast (Ishihara *et al.*, 2006). The observation that the generation of L-isoforms is not affected is in agreement with the removal of the OPA1 presequence upon import into mitochondria by MPP (Ishihara *et al.*, 2006). In summary, these data suggest that S-isoforms of OPA1 are generated by *m*-AAA protease-mediated processing of L1- and L2-isoforms, which both originate from different splice variants.

Furthermore, OPA1 processing by homo- and hetero-oligomeric mammalian *m*-AAA proteases was reconstituted in yeast. The mammalian *m*-AAA protease has previously been linked to proteolytic processing of OPA1, since RNAi-mediated downregulation of human paraplegin in HeLa cells led to a modestly impaired processing of endogenous and over-expressed rat OPA1 (Ishihara *et al.*, 2006). Upon co-transfection of rat OPA1 and human paraplegin, an increased accumulation of S-isoforms as well as a direct interaction of both over-expressed proteins has been observed in HeLa cells (Ishihara *et al.*, 2006). However, the over-expression of hAFG3L2 had no effect on processing of rat OPA1. In apparent contrast to these results, cleavage of OPA1 was not affected in fibroblasts of paraplegin-deficient mice under steady-state conditions and after dissipation of the membrane potential, respectively (Duvezin-Caubet *et al.*, 2007). Moreover, upon reconstitution in yeast, homo-oligomeric *m*-AAA proteases composed of murine Afg3l1, Afg3l2, or human AFG3L2 mediated processing of all four OPA1 splice variants analysed in this study. Thus, OPA1 processing in yeast can occur independently of murine paraplegin. Expression of paraplegin did not restore OPA1 processing in *m*-AAA protease-deficient yeast cells, which is in line with the observation that this subunit lacks the ability to homo-oligomerise. Paraplegin could cleave OPA1 only when assembled into hetero-oligomeric *m*-AAA protease complexes containing Afg3l1 or Afg3l2. These findings in yeast provide a rationale for the normal pattern of OPA1 isoforms in paraplegin-deficient mice, since Afg3l1 and Afg3l2 can apparently substitute for the loss of paraplegin, which is not essential for OPA1 processing. The complete lack of short OPA1 isoforms in the presence of proteolytically inactive mammalian *m*-AAA proteases, either

homo- or hetero-oligomeric, further supports the notion that OPA1 processing depends on the proteolytic activity of *m*-AAA proteases.

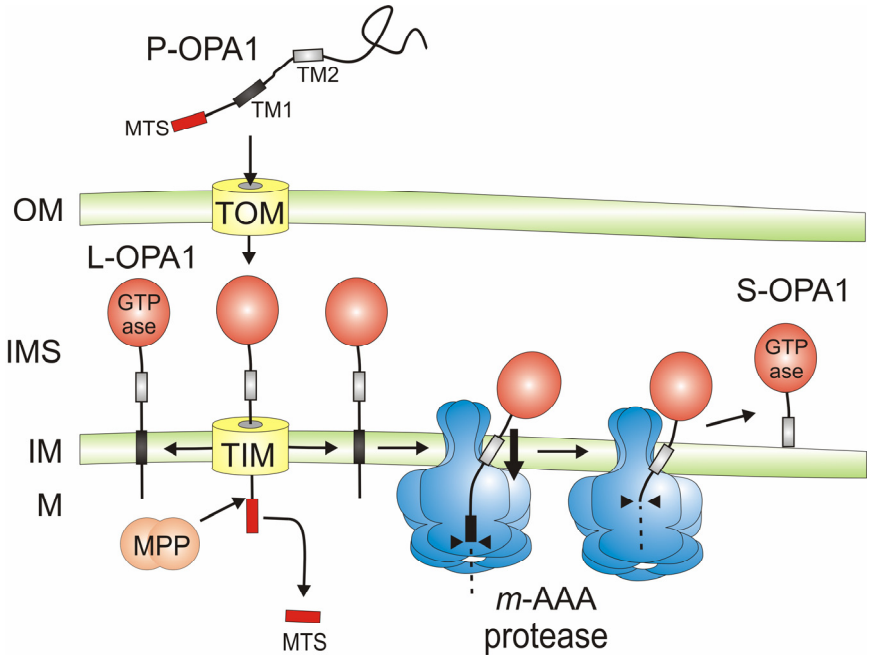

Figure 26. Model of OPA1 processing in mammalian mitochondria.

The newly imported precursor of OPA1 (P-OPA1) is matured by the mitochondrial processing peptidase (MPP) leading to the large isoform of OPA1 (L-OPA1). This isoform is integrated into the inner membrane (IM) and can receive further proteolytic processing mediated by the *m*-AAA protease, which can occur independently of paraplegin. Finally, short OPA1 isoforms (S-OPA1) are released into the intermembrane space (IMS), where they are associated with the inner membrane. The first transmembrane domain (TM1) is present in all OPA1 splice variants, whereas the presence of one or two additional hydrophobic stretches, termed TM2a and TM2b, varies between different splice variants. Only one additional hydrophobic segment is depicted here (TM2). *m*-AAA protease-mediated OPA1 processing occurs in close proximity to TM2a or TM2b (Duvezin-Caubet *et al.*, 2007). OM, outer membrane; M, matrix space; MTS, mitochondrial targeting sequence; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane.

The mammalian rhomboid protease PARL, the homologue of yeast Pcp1, has also been suggested to be involved in OPA1 processing, as both proteins were co-immunoprecipiated in murine mitochondria (Cipolat *et al.*, 2006). PARL regulates OPA1-dependent cristae remodelling and plays a protective role against cytochrome *c* release and apoptosis. In this context, PARL was found to be responsible for the generation of low amounts of a soluble OPA1 isoform (Cipolat *et al.*, 2006). OPA1 processing, however, was not significantly impaired upon RNAi-mediated downregulation in HeLa cells or in PARL-deficient mouse cells (Cipolat *et al.*, 2006; Ishihara *et al.*, 2006; Duvezin-Caubet *et al.*, 2007).

Consistently, neither the absence of yeast Pcp1 nor the presence of human PARL had an effect on processing of several OPA1 splice variants in the reconstituted yeast system (Duvezin-Caubet *et al.*, 2007). Overexpression of PARL in *m*-AAA protease-deficient yeast cells did not lead to the generation of short OPA1 isoforms either. These results indicate that PARL is dispensable for OPA1 processing in mammals and that at most a small fraction of S-isoforms is generated by rhomboid.

In summary, OPA1 biogenesis and proteolytic processing in mammalian mitochondria apparently proceed according to the following model (Fig. 26): Upon import into mitochondria via the TOM and TIM complexes, the matrix targeting signal of the OPA1 precursor is cleaved off by MPP resulting in the L-isoform of OPA1, which is embedded into the inner membrane by its first transmembrane domain (Olichon *et al.*, 2002). Further proteolytic processing is mediated by the *m*-AAA protease, either homo-or hetero-oligomeric, and generates S-isoforms, which are released into the intermembrane space, but stay peripherally associated with the inner membrane (Duvezin-Caubet *et al.*, 2006). This cleavage occurs after the first transmembrane domain and most likely involves the *m*-AAA protease-driven and ATP-dependent dislocation of this OPA1 segment from the membrane bilayer to allow proteolysis on the matrix side. However, a role of the Hsp70 import motor in this dislocation process cannot be excluded at the moment. Moreover, there is so far no evidence for a functional interplay between the *m*-AAA protease and the rhomboid protease as shown for the maturation of Ccp1 in yeast.

4.4 OPA1 processing provides first evidence for different substrate specificities of m*-AAA protease isoenzymes*

The efficiency of OPA1 processing and the resulting pattern of short isoforms in yeast were dependent on the splice variant and the subunit composition of the mammalian *m*-AAA protease. In particular, homo-oligomeric *m*-AAA proteases converted OPA1 more efficiently to short isoforms than observed for hetero-oligomeric *m*-AAA proteases containing paraplegin. Regarding the homo-oligomeric enzymes, Afg3l1 complexes mediated OPA1 processing more efficiently than human AFG3L2 and murine Afg3l2 complexes. In addition, the presence of Afg3l1 complexes preferentially led to S4 isoforms, whereas S3 isoforms were predominantly generated by hAFG3L2 and Afg3l2 complexes and paraplegin-containing, hetero-oligomeric *m*-AAA proteases. These results provide first evidence for a varying substrate specificity of *m*-AAA proteases with different subunit composition and

suggest that certain short isoforms of OPA1 are potentially generated by certain *m*-AAA protease assemblies. However, additional factors might influence OPA1 recognition and processing in mammalian cells. For instance, the yeast prohibitin complex apparently had a weak inhibitory effect on *m*-AAA protease-mediated cleavage of some OPA1 splice variants in the reconstituted system. As a supercomplex consisting of the *m*-AAA protease and the prohibitin complex has also been identified in mammalian mitochondria (M. Metodiev and T. Langer, unpublished observation), an influence of prohibitins on OPA1 processing can be envisioned.

Potential limitations of the reconstituted system have to be considered. It is possible that the hybrid proteins containing mammalian *m*-AAA protease subunits are not fully active in the heterologous environment. Especially, this might be the case for mammalian paraplegin which does not homo-oligomerise and on its own exert proteolytic activity in yeast. Moreover, the observed decreased efficiency of OPA1 processing by paraplegin-containing *m*-AAA proteases is based on the analysis of paraplegin/Afg3l1^{E567Q} and paraplegin/Afg3l2^{E574Q} complexes. Thus, this limited OPA1 cleavage could be attributed to a potential negative effect of proteolytic site mutations in the Afg3l1 and Afg3l2 subunits on the proteolytic activity of paraplegin.

Different efficiencies of OPA1 cleavage by mammalian *m*-AAA proteases in yeast could be on one hand explained by different binding specificities to OPA1. On the other hand, it is conceivable that *m*-AAA protease-mediated OPA1 processing involves the ATP-dependent membrane dislocation of OPA1 segments from the inner membrane to facilitate cleavage on the matrix side. Thus, differences in the ATPase activities of *m*-AAA proteases with variable subunit composition could account for the different efficiencies of OPA1 cleavage or the preferential formation of certain short isoforms. In case of the yeast *m*-AAA protease, a correlation of the efficiency of Ccp1 processing with the ATPase activities of *m*-AAA protease variants has been demonstrated (Tatsuta *et al.*, 2007). Similarly, *m*-AAA protease isoenzymes with a lower ATPase activity could promote dislocation of the first transmembrane and following OPA1 segments only to a lesser extent than proteolytic assemblies with high ATPase activity.

In conclusion, mammalian *m*-AAA protease subunits can assemble into several proteolytic complexes with different subunit composition mediating OPA1 processing with varying efficiencies. It is conceivable that this variable assembly of *m*-AAA proteases in addition to the expression of alternatively spliced OPA1 variants allows a tissue-specific adjustment of OPA1 isoforms needed for mitochondrial dynamics.

4.5 The versatile assembly of the mammalian m-AAA protease and its implications for the pathogenesis of HSP

HSP due to a paraplegin deficiency is a neurodegenerative disease, which is characterised by an intriguing tissue specificity. Only a subset of motor and sensory neurons is affected by axonal degeneration, although paraplegin is ubiquitously expressed. In the current model, HSP is caused ultimately by deficiencies of mitochondrial activities in axonal endings due to impaired axonal transport, which is of special importance for neurons with long axons. The variable assembly of mammalian *m*-AAA proteases and the varying relative abundance of murine *m*-AAA protease subunits in different tissues shed new light on the pathogenesis of HSP.

Remarkably, the lack of paraplegin *per se* does not cause a loss of *m*-AAA protease activity in mammalian cells. Instead, *m*-AAA proteases with a different subunit composition can be formed by the remaining *m*-AAA protease subunits depending on the tissue or cell type. In the mouse model of HSP, a hetero-oligomeric Afg3l1/Afg3l2 complex and due to the decreased relative abundance of Afg3l1 a predominant homo-oligomeric Afg3l2 complex are present in brain mitochondria. Similarly, human mitochondria isolated from HSP fibroblasts contain a homo-oligomeric hAFG3L2 complex in the absence of paraplegin, which is also expected to be present in human brain. The apparent lower molar concentration of Afg3l1 compared to other *m*-AAA protease subunits in murine brain can thus also explain why a paraplegin-deficiency leads to similar phenotypic consequences in humans and mice, despite the presence of a third proteolytic subunit in mice. Taken together, these findings could at least partially explain the tissue specificity of mitochondrial defects causing HSP when paraplegin is absent. Similarly, a varying abundance of several mitochondrial translation factors in different tissues has also been identified to form the basis of tissue-specific oxidative phosphorylation defects due to mutations in EFG1, the mitochondrial translation factor (Antonicka *et al.*, 2006). Another neurodegenerative disease, called Charcot-Marie-Tooth disease type 2A (CMT2A), is caused by autosomal-dominant mutations in the mitofusin Mfn2 (Züchner *et al.*, 2004). Homo- and hetero-oligomeric mitofusin complexes of Mfn1 and Mfn2, both mediating mitochondrial fusion, can be formed (Chen *et al.*, 2003; Eura *et al.*, 2003; Detmer and Chan, 2007). A low Mfn1 expression in specific cells has recently been suggested to underlie cell type-specific mitochondrial fusion defects in CMT2A as the ratio and number of functional mitofusin complexes is perturbed (Detmer and Chan, 2007). Thus, protein complexes with variable composition and tissue-specific differences in the

expression of their subunits may represent a general principle in mammalian cells to regulate mitochondrial processes in a tissue-specific manner.

A different substrate abundance or dependence represents another aspect of tissue specificity. However, the ribosomal subunit MrpL32 is currently the only known substrate of the mammalian *m*-AAA protease (Nolden *et al.*, 2005). Defective MrpL32 processing in paraplegin-deficient mice leads to impaired mitochondrial protein synthesis, but it is unclear if this is the reason for axonal degeneration in HSP. Complementation studies in yeast have demonstrated the proteolytic activity of homo-oligomeric hAFG3L2 and Afg3l2 complexes and overlapping substrate specificity with hetero-oligomeric *m*-AAA proteases containing paraplegin. These homo-oligomeric *m*-AAA proteases can apparently exert important housekeeping functions in mitochondria challenging the view that defects in mitochondrial translation and/or protein quality control can sufficiently explain the development of HSP due to mutations in paraplegin. Consistently, the analysis of HSP fibroblasts lacking paraplegin has only revealed an impaired assembly of complex I but no general mitochondrial protein synthesis defect (Atorino *et al.*, 2003). Mitochondrial translation was found to be reduced by 50% in liver mitochondria of paraplegin-deficient mice (Nolden *et al.*, 2005), but ATP synthesis was only modestly affected in spinal cord mitochondria of old mice (Ferreirinha *et al.*, 2004). These observations could be simply explained by a reduced number of functionally active *m*-AAA proteases in the absence of paraplegin, but additionally suggest the existence of specific substrates of *m*-AAA proteases differing in their subunit composition, e.g. for paraplegin-containing *m*-AAA proteases. These paraplegin-specific substrates could be expressed in a tissue-specific manner providing a further rationale for cell-specific consequences of a loss of paraplegin in HSP. Alternatively, the impaired proteolysis of a specific substrate could only affect the mitochondrial functions of certain cells.

Apart from the identification of MrpL32, specific substrates of mammalian *m*-AAA proteases have remained elusive. However, the dynamin-like GTPase OPA1, which is required for mitochondrial fusion, may represent such a substrate. In mammals, OPA1 is proteolytically processed by *m*-AAA proteases with varying efficiencies depending on their subunit composition and the OPA1 splice variant as suggested by the results of the yeast model. Mutations in OPA1 lead to neurodegeneration in dominant optic atrophy type 1, which selectively affects retinal ganglion cells thus exhibiting a tissue-specificity different from HSP (Alexander *et al.*, 2000; Delettre *et al.*, 2000). Interestingly, optic atrophy has been found as an additional symptom in some paraplegin-deficient patients suffering from a complicated form of HSP (Casari *et al.*, 1998). More significantly, enlarged and structurally abnormal

mitochondria appear at early stages at the synaptic terminals of motor axons in paraplegin-deficient mice (Ferreirinha *et al.*, 2004). Changes in the morphology of mitochondria are known to affect axonal transport of these organelles (Li *et al.*, 2004; Chen and Chan, 2005; Verstreken *et al.*, 2005). Consistently, mitochondria with aberrant morphology accumulate in axons of paraplegin-deficient mice further impairing axonal transport (Ferreirinha *et al.*, 2004). Thus, impaired OPA1 processing and, as a consequence, altered mitochondrial morphology might also play a role in the pathogenesis of HSP. On the contrary, OPA1 processing is not significantly affected in paraplegin-deficient mice (Duvezin-Caubet *et al.*, 2007) and paraplegin-containing, hetero-oligomeric *m*-AAA proteases process OPA1 splice variants only to a minor extent in yeast. As tissue-specific differences in the expression and assembly of their subunits are hallmarks of mammalian *m*-AAA proteases, paraplegin-containing *m*-AAA proteases might be crucial for OPA1 processing in specific tissues or cell types.

In view of the versatile assembly of mammalian *m*-AAA proteases and their potential differences in substrate specificity, the findings of this study further suggest that mutations in Afg3l1 or Afg3l2 may be linked to phenotypes different from HSP and may possibly affect different tissues. In line with this prediction, Afg3l2-deficient mice which recently have been generated exhibit a much more severe phenotype than paraplegin-deficient mice exemplified by a postnatal death (G. Casari, personal communication).

5 Abstract

The *m*-AAA protease, an ATP-dependent proteolytic complex in the mitochondrial inner membrane, controls protein quality and regulates ribosome assembly within mitochondria. Mutations in the human *m*-AAA protease subunit paraplegin cause hereditary spastic paraplegia (HSP) characterised by cell-specific axonal degeneration which is also observed in a paraplegin-deficient mouse model. It has been proposed that paraplegin mediates proteolytic processing of OPA1 which is essential for mitochondrial morphology and linked to dominant optic atrophy, another neurodegenerative disease in humans. Paraplegin assembles with homologous Afg3l2 subunits into hetero-oligomeric complexes, but the consequences of a loss of paraplegin for *m*-AAA protease assembly and function remain unclear. Moreover, a third putative *m*-AAA protease subunit, termed Afg3l1, is expressed in mice but its role for *m*-AAA protease activity has not been determined.

The assembly status of human AFG3L2 was monitored in paraplegin-deficient mitochondria from human HSP cells revealing the existence of a homo-oligomeric AFG3L2 complex which was also formed upon heterologous expression in yeast. This AFG3L2 complex is proteolytically active as it could substitute for the yeast *m*-AAA protease. In related complementation studies in yeast, murine *m*-AAA protease subunits were found to assemble into several proteolytic complexes with variable subunit composition in the mitochondrial inner membrane. Homo-oligomeric Afg3l1 and Afg3l2 complexes and hetero-oligomeric complexes of both proteins with paraplegin could be identified. Afg3l1 was established as a *bona fide* subunit of *m*-AAA proteases with proteolytic activity. All assemblies have conserved and overlapping substrate specificities as they were able to maintain mitochondrial housekeeping functions in yeast. These results suggest that the lack of paraplegin does not lead to a complete loss of *m*-AAA protease activity in affected mitochondria. Instead, *m*-AAA protease complexes with a different subunit composition could be formed which may substitute for paraplegin-containing *m*-AAA proteases. First evidence for different substrate specificities of *m*-AAA proteases differing in their subunit composition was obtained by reconstitution of OPA1 cleavage in yeast cells harbouring different mammalian *m*-AAA protease complexes. The efficiency of OPA1 processing was dependent on the subunit composition of mammalian *m*-AAA proteases. Homo-oligomeric complexes composed of murine Afg3l1, Afg3l2, or human AFG3L2 cleaved OPA1 with higher efficiency than hetero-oligomeric complexes containing paraplegin. This also confirms OPA1 as a novel potential substrate of the *m*-AAA protease. Taken together, these findings reveal an unexpected variety of mammalian *m*-AAA proteases in the inner mitochondrial membrane and may help to understand the pathogenesis and tissue-specificity of HSP due to a loss of paraplegin.

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7 List of Abbreviations

AAA	ATPases associated with a variety of cellular activities
Aβ	amyloid β–peptide
ABC	ATP-binding cassette
ADOA	autosomal dominant optic atrophy
ADP	adenosine diphosphate
APP	amyloid precursor protein
APS	ammoniumperoxo disulfate
ATP	adenosine triphosphate
bp	base pairs
BN	blue native
cDNA	complementary DNA
CEN	centromere
CMT2A	Charcot-Marie-Tooth type 2A
DMSO	dimethyl sulfoxide
C-terminal	carboxyterminal
C-terminus	carboxy terminus
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
Fig.	Figure
g	standard gravity
GTP	guanosine triphosphate
h	hour(s)
HA	hemagglutinin
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic-acid
HSP	hereditary spastic paraplegia
IMP	inner membrane peptidase
K	potassium
kb	kilobase pairs
KCl	potassium chloride
kDa	kilodalton
KOH	potassium hydroxide
m	meter
M	molar (mole per liter)
mA	milliampere
MDa	megadalton
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
Mg	magnesium
min	minute(s)
MIP	mitochondrial intermediate peptidase
mM	millimolar
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
mtDNA	mitochondrial DNA

MTS	mitochondrial targeting sequence
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide (reduced form)
NaOH	sodium hydroxide
NP-40	Nonidet P-40
N-terminal	aminoterminal
N-terminus	amino terminus
OD_{600}	optical density at a wavelength of 600 nanometer
PAGE	polyacrylamide gel electrophoresis
PAS	Protein A Sepharose
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
s	second(s)
SDS	sodium dodecyl sulfate
SPG	spastic paraplegia gene
SRH	second region of homology
Tab.	Table
TBS	Tris buffered saline
TCA	trichloracetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIM	translocase of the inner membrane
TM	transmembrane domain
TOM	translocase of the outer membrane
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiole
U	unit(s)
UPS	ubiquitin-proteasome system
v/v	volume per volume
V	volt
WT	wild-type
w/v	weight per voulme
XIAP	X chromosome-linked inhibitor of apoptosis protein
YP	yeast extract-peptone
YPD	yeast extract-peptone with glucose
YPG	yeast extract-peptone with glycerol

8 Appendix

Teilpublikationen im Rahmen dieser Arbeit:

Atorino, L., Silvestri, L., Koppen, M., Cassina, L., Ballabio, A., Marconi, R., Langer, T., and Casari, G. (2003)

"Loss of *m*-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia."

Journal of Cell Biology 163: 777-787.

Abstract:

Mutations in paraplegin, a putative mitochondrialmetallopeptidase of the AAA family, cause an autosomal recessive form of hereditary spastic paraplegia (HSP). Here, we analyze the function of paraplegin at the cellular level and characterize the phenotypic defects of HSP patients' cells lacking this protein. We demonstrate that paraplegin coassembles with a homologous protein, AFG3L2, in the mitochondrial inner membrane. These two proteins form a high molecular mass complex, which we show to be aberrant in HSP fibroblasts. The loss of this complex causes a reduced complex I activity in mitochondria and an increased sensitivity to oxidant stress, which can both be rescued by exogenous expression of wild-type paraplegin. Furthermore, complementation studies in yeast demonstrate functional conservation of the human paraplegin–AFG3L2 complex with the yeast *m*-AAA protease and assign proteolytic activity to this structure. These results shed new light on the molecular pathogenesis of HSP and functionally link AFG3L2 to this neurodegenerative disease.

Nolden, M., Ehses, S., Koppen, M., Bernacchia, A., Rugarli, E. I., and Langer, T. (2005)

"The *m*-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria."

Cell 123: 277-289.

Abstract:
AAA proteases comprise a conserved family of membrane bound ATP-dependent proteases that ensures the quality control of mitochondrial inner-membrane proteins. Inactivation of AAA proteases causes pleiotropic phenotypes in various organisms, including respiratory deficiencies, mitochondrial morphology defects, and axonal degeneration in hereditary spastic paraplegia (HSP). The molecular basis of these defects, however, remained unclear. Here, we describe a regulatory role of an AAA protease for mitochondrial protein synthesis in yeast. The mitochondrial ribosomal protein MrpL32 is processed by the *m*-AAA protease, allowing its association with preassembled ribosomal particles and completion of ribosome assembly in close proximity to the inner membrane. Maturation of MrpL32 and mitochondrial protein synthesis are also impaired in a HSP mouse model lacking the *m*-AAA protease subunit paraplegin, demonstrating functional conservation. Our findings therefore rationalize mitochondrial defects associated with *m*-AAA protease mutants in yeast and shed new light on the mechanism of axonal degeneration in HSP.

Koppen, M.*, Metodiev, M. D.*, Casari, G., Rugarli, E. I., and Langer, T. (2007)

"Variable and tissue-tpecific subunit composition of mitochondrial *m*-AAA protease complexes linked to hereditary spastic paraplegia."

Molecular and Cellular Biology 27: 758-767.

*gleichberechtigte Erstautoren

Abstract:

The *m*-AAA protease, an ATP-dependent proteolytic complex in the mitochondrial inner membrane, controls protein quality and regulates ribosome assembly, thus exerting essential housekeeping functions within mitochondria. Mutations in the *m*-AAA protease subunit paraplegin cause axonal degeneration in hereditary spastic paraplegia (HSP), but the basis for the unexpected tissue specificity is not understood. Paraplegin assembles with homologous Afg3l2 subunits into hetero-oligomeric complexes which can substitute for yeast *m*-AAA proteases, demonstrating functional conservation. The function of a third paralogue, Afg3l1 expressed in mouse, is unknown. Here, we analyze the assembly of paraplegin into *m*-AAA complexes and monitor consequences of paraplegin deficiency in HSP fibroblasts and in a mouse model for HSP. Our findings reveal variability in the assembly of *m*-AAA proteases in mitochondria in different tissues. Homo-oligomeric Afg3l1 and Afg3l2 complexes and hetero-oligomeric assemblies of both proteins with paraplegin can be formed. Yeast complementation studies demonstrate the proteolytic activity of these assemblies. Paraplegin deficiency in HSP does not result in the loss of *m*-AAA protease activity in brain mitochondria. Rather, homo-oligomeric Afg3l2 complexes accumulate, and these complexes can substitute for housekeeping functions of paraplegin-containing *m*-AAA complexes. We therefore propose that the formation of *m*-AAA proteases with altered substrate specificities leads to axonal degeneration in HSP.

Koppen, M. and Langer, T. (2007)

"Protein degradation within mitochondria: Versatile activities of AAA proteases and other peptidases."

Critical reviews in Biochemistry and Molecular Biology 42: 221-242.

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.

Duvezin-Caubet, S.*, Koppen, M.*, Wagener, J.*, Zick, M., Israel, L., Bernacchia, A., Jagasia, R., **Rugarli, E. I., Imhof, A., Neupert, W., Langer, T., and Reichert A. S.(2007)**
"OPA1 processing reconstituted in yeast depends on the subunit composition of the *m*-AAA protease in mitochondria."
Molecular Biology of the Cell in press

*gleichberechtigte Erstautoren

Abstract:

The morphology of mitochondria in mammalian cells is regulated by proteolytic cleavage of OPA1, a dynamin-like GTPase of the mitochondrial inner membrane. The mitochondrial rhomboid protease, PARL, as well as paraplegin, a subunit of the ATPdependent *m*-AAA protease, were proposed to be involved in this process. Here, we characterized individual OPA1 isoforms by mass spectrometry and reconstituted their processing in yeast to identify proteases involved in OPA1 cleavage. The yeast homolog of OPA1, Mgm1, was processed both by PARL and its yeast homolog Pcp1. Neither of these rhomboid proteases cleaved OPA1. The formation of small OPA1 isoforms was impaired in yeast cells lacking the *m*-AAA protease subunits Yta10 and Yta12 and was restored upon expression of murine or human *m*-AAA proteases. OPA1 processing depended on the subunit composition of mammalian *m*-AAA proteases. Homooligomeric *m*-AAA protease complexes composed of murine Afg3l1, Afg3l2, or human AFG3L2 subunits cleaved OPA1 with higher efficiency than paraplegincontaining *m*-AAA proteases. OPA1 processing proceeded normally in murine cell lines lacking paraplegin or PARL. Our results provide evidence for different substrate specificities of *m*-AAA proteases composed of different subunits and reveal a striking evolutionary switch of proteases involved in the proteolytic processing of dynamin-like GTPases in mitochondria.

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Eidesstattliche Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Thomas Langer betreut worden.

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Nolden, M., Ehses, S., **Koppen, M.**, Bernacchia, A., Rugarli, E. I., and Langer, T. 2005. The *m*-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. *Cell* 123: 277-289.

Koppen, M.*, Metodiev, M. D.*, Casari, G., Rugarli, E. I., and Langer, T. 2007. Variable and tissue-specific subunit composition of mitochondrial *m*-AAA Protease complexes linked to hereditary spastic paraplegia. *Mol Cell Biol* 27: 758-767.

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Duvezin-Caubet, S.*, **Koppen, M.***, Wagener, J.*, Zick, M., Israel, L., Bernacchia, A., Jagasia, R., Rugarli, E. I., Imhof, A., Neupert, W., Langer, T., and Reichert, A. S. 2007. OPA1 processing reconstituted in yeast depends on the subunit composition of the *m*-AAA protease in mitochondria. *Mol Biol Cell* in press

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