Role of prohibitins for proteolysis in yeast and murine mitochondria

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Zusammenfassung

Die Prohibitine Phb1 und Phb 2 sind evolutionär konservierte Proteine mit unterschiedlicher zellulärer Lokalisation und Funktion. In Hefe formen Prohibitine einen hochmolekularen Komplex in der inneren Mitochondrienmembran, der physikalisch mit der *m*AAA Protease interagiert und die Stabilität von nicht-assemblierten proteolytischen Substraten beeinflußt. In der vorliegenden Arbeit wurden zwei mögliche Funktionen von Prohibitinen bei der Proteolyse untersucht: 1) Prohibitine als negativer Regulator in der *m*AAA Protease (Steglich *et al.*, 1999) und/oder 2) Prohibitine in der Funktion als molekulare Chaperone für die Assemblierung neu synthetisierter mitochondrialer Proteine (Nijtmans *et al.*, 2000). Um diese beiden Funktionen unterscheiden zu können, wurde die Stabilität der proteolytischen Substrate der *m*AAA Protease in Zellen untersucht, die unterschiedliche Mengen von Prohibitinen enthielten. Während die Überexpression von Prohibitinen die Stabilität von nicht-nativen Proteinen der inneren Membran nicht erhöhte, bewirkte die Abwesenheit von Prohibitinen ihre schnelle Proteolyse. Die Ergebnisse dieser Arbeit lassen also vermuten, daß Prohibitine bei der Stabilisierung von mitochondrialen Proteinen nicht als Chaperone fungieren. Es scheint eher, daß Prohibitine durch die Bindung an *m*AAA Protease in einer Nukleotid-unabhängigen Weise funktionieren und eventuell die Zugänglichkeit von Substraten für die Protease oder die Aktivität der Protease beeinflussen.

*m*AAA Protease ist ein entscheidender Faktor im mitochondrialen Qualitätskontrollsystem in Hefe und menschlichen Zellen (Arlt *et al.*, 1996; Arlt *et al.*, 1998; Nolden *et al.*, 2005). In menschlichen Zellen besteht Protease aus Paraplegin und Afg3l2, die homolog zu den Hefeproteinen Yta10 und Yta12 sind (Atorino *et al.*, 2003). In Mäusen wird eine dritte AAA Untereinheit, genannt Afg3l1 exprimiert (Kremmidiotis *et al.*, 2001) und somit bleibt die Zusammensetzung der murinen *m*AAA Proteaseuntereinheit weiter unbekannt. In dieser Arbeit wurden die Komplexe aus Paraplegin, Afg3l1 und Afg3l2 in den Mitochondrien von Wildtyp- und SPG7$^{-/-}$ Mausstämmen untersucht. Immunpräzipitierungsexperimente zeigten, daß Paraplegin, Afg3l1 und Afg3l2 physikalisch interagieren und in einem hochmolekularen Komplex mit Prohibitinen enthalten sind. Dies deutet auf eine konservierte Rolle von Phb1 und Phb2 für die Proteolyse in murinen Mitochondrien

hin. Co-Immunpräzipitierung und Immunabbauexperimente mit Lebermitochondrien aus SPG7$^{-/-}$ Mausstämmen zeigten, daß Afg3l1 und Afg3l2 ebenfalls hochmolekulare Komplexe in Abwesenheit von Paraplegin bilden können. Darüberhinaus waren diese Komplexe in der Lage, mit Prohibitinen zu interagieren, was darauf hindeutet, daß Paraplegin für diese Interaktion nicht essentiell ist.

Offenbar gibt es in der inneren Membran muriner Mitochondrien *m*AAA Proteasen mit unterschiedlicher Zusammensetzung der Untereinheiten. Die physiologische Relevanz dieser *m*AAA Proteasen mit unterschiedlicher Zusammensetzung der Untereinheiten soll hinsichtlich ihrer Substratspezifität und/oder unterschiedlichen Gewebeverteilung diskutiert werden.

Abstract

Prohibitins, Phb1 and Phb2, are evolutionary conserved proteins with diverse cellular localization and different functions. In yeast, prohibitins form a high molecular weight complex in the inner mitochondrial membrane which physically interacts with the *m*AAA protease and affects the stability of non assembled proteolytic substrates. In the present work, I examined two possible roles of prohibitins in proteolysis: i) role as negative regulators of the *m*AAA protease (Steglich *et al.*, 1999) and/or ii) function as molecular chaperones for the assembly of newly synthesized mitochondrial proteins (Nijtmans *et al.*, 2000). To discriminate between both possibilities, the stability of proteolytic substrates of the *m*AAA protease was examined in cells harboring different levels of prohibitins. While overexpression of prohibitins did not stabilize non-native inner membrane proteins absence of prohibitins resulted in their rapid proteolysis. The results presented in this work suggest that prohibitins do not play a role as chaperones for the stabilization of mitochondrial proteins. Rather, prohibitins function by binding to the *m*AAA protease in a nucleotide independent manner and presumably modulating the accessibility of substrate to the protease or the activity of the protease.

The *m*AAA protease is recognized as a crucial component of the mitochondrial quality control system in yeast and in human (Arlt *et al.*, 1996; Arlt *et al.*, 1998; Nolden *et al.*, 2005). In human, the *m*AAA protease is built up of paraplegin and Afg3l2 which are homologous to the yeast Yta10 and Yta12 proteins (Atorino *et al.*, 2003). In mice, a third AAA subunit called Afg3l1 is expressed (Kremmidiotis *et al.*, 2001) and, therefore, the subunit composition of the murine *m*AAA protease is unknown. Here, the complexes between paraplegin, Afg3l1 and Afg3l2 were examined in mitochondria from wild type and in SPG7$^{-/-}$ mouse strains. Immunoprecipitation experiments showed that paraplegin, Afg3l1 and Afg3l2 physically interact and are contained in a high molecular weight complex with prohibitins suggesting a conserved role of Phb1 and Phb2 for proteolysis in murine mitochondria. Co-immunoprecipitation and immunodepletion experiments using liver mitochondria from a SPG7$^{-/-}$ mouse strain showed that Afg3l1 and Afg3l2 can also form high molecular weight complexes in the absence of paraplegin. These complexes were additionally able to interact with prohibitins suggesting that

paraplegin is not essential for this interaction. Since paraplegin-specific antibodies are able to precipitate Phb1 and Phb2, it is conceivable that also paraplegin-Afg3l1 and paraplegin-Afg3l2 complexes will be able to bind prohibitins.

Thus, *m*AAA proteases with different subunit composition appear to exist in the inner membrane of murine mitochondria. The physiological relevance of *m*AAA proteases with different subunit composition is discussed in view of differences in their substrate specificities and/or different tissue distribution.

I. INTRODUCTION

Prohibitins occur in a wide range of species and exhibit high similarity on both nucleotide and amino acid levels. Their strong conservation along with their abundance and ubiquitous expression point to an important role in the cell. To this moment, however, the molecular function of prohibitins has not been defined. In yeast mitochondria, prohibitins have been linked to the mitochondrial protein quality control system and demonstrated to directly interact with the mitochondrial ATP dependent *m*AAA protease (Steglich *et al.*, 1999). Additional functions of prohibitins in mitochondria and in other cellular compartments have also been described and are discussed below. Since this work focuses on the function of prohibitins during proteolysis, a brief introduction to the mitochondrial proteolytic system is provided.

1. The proteolytic system of mitochondria

Many protein complexes in mitochondria are composed of subunits encoded by both the nuclear and the mitochondrial genome. Since imbalance of nuclearly or mitochondrially encoded subunits can lead to an accumulation of potentially harmful unassembled polypeptides, mitochondria like any other cellular compartment possess a protein degradation system (Kalnov *et al.*, 1979; Goldberg *et al.*, 1985). Major components of this system are proteases which ensure removal of unassembled or damaged proteins and/or function during import of the nuclearly encoded proteins (Figure 1). During protein import, the matrix processing peptidase (MPP) (Brunner *et al.*, 1994), the intermediate peptidase (MIP) (Kalousek *et al.*, 1992; Isaya *et al.*, 1994) and the innermembrane peptidase (IMP) (Behrens *et al.*, 1991; Schneider *et al.*, 1991; Nunnari *et al.*, 1993; Esser *et al.*, 1996) are responsible for the cleavage of targeting presequences of nuclearly encoded proteins.

1.1. ATP-dependent proteases in mitochondria

Improperly folded or unassembled proteins are removed by the action of ATP-dependent proteases within mitochondria (Figure 1) (Kalnov *et al.*, 1979; Desautels and Goldberg, 1982). These proteases are ubiquitously present in prokaryotes and eukaryotes and belong to the AAA+ superfamily of P-loop ATPases (*A*TPase *a*ssociated with a variety of cellular *a*ctivities") (Beyer, 1997; Neuwald *et al.*, 1999;

Vale, 2000; Ogura and Wilkinson, 2001; Frickey and Lupas, 2004; Iyer *et al.*, 2004). ATP-dependent proteases are active in all subcompartments of mitochondria and fall into three subfamilies: Lon-, Clp- and AAA-proteases (Ogura and Wilkinson, 2001, Frickey, 2004 #1361; Nolden *et al.*, 2005).

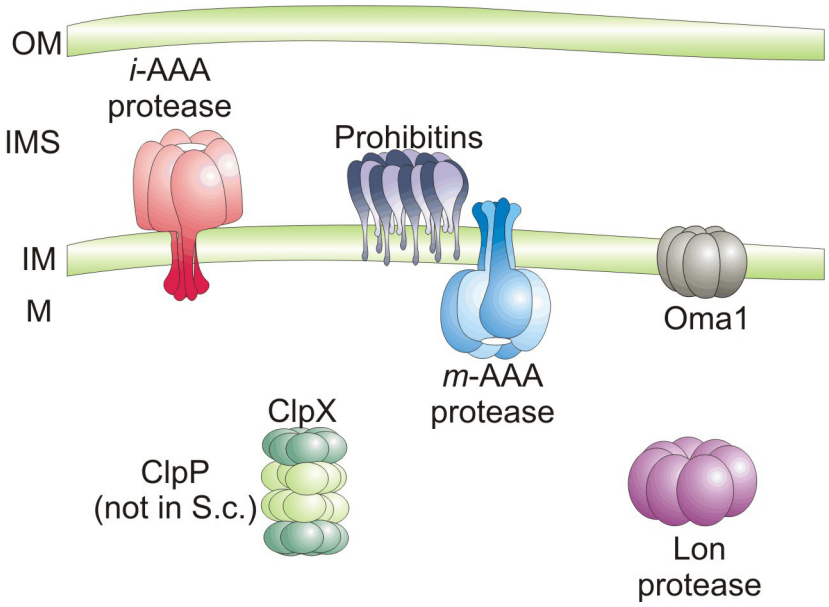

Figure 1. ATP-dependent proteases in the mitochondrial matrix and in the inner mitochondrial membrane. The Lon-protease and ClpXP (ClpP not present in yeast) are responsible for proteolytic breakdown of misfolded polypeptides in the mitochondrial matrix. Integral membrane and peripherally associated proteins are degraded by proteases of the inner mitochondrial membrane, the *i*AAA protease, active on the intermembrane side of the inner membrane; the *m*AAA protease, active in the mitochondrial matrix and the ATP-independent metallo-peptidase Oma1, which is thought to possess catalytic domains at the side of the inner membrane. Prohibitins built up a supercomplex with the *m*AAA protease. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; M, mitochondrial matrix . Reprinted with modifications from (Nolden *et al.*, 2005)

1.1.1. Lon- and Clp-proteases in the mitochondrial matrix

Lon proteases have been identified in the mitochondrial matrix of yeast and mammals (Figure 1) (Watabe and Kimura, 1985; Wang *et al.*, 1993; Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Wang *et al.*, 1994). Lon-proteases are classified as serine proteases as they harbor a catalytic serine-lysine dyad and are presumably homooligomeric, ring-shaped complexes with hexameric or heptameric structure. (Stahlberg *et al.*, 1999; Botos *et al.*, 2004; Rotanova *et al.*, 2004). The Lon-protease in yeast, which is termed PIM1 (Van Dyck *et al.*, 1994), mediates the proteolytic removal of aggregated proteins in collaboration with the chaperone *mt*Hsp70, and its co-factors Mdj1 and Mge1 (Wang *et al.*, 1993; Suzuki *et al.*, 1994; Van Dyck *et al.*,

1994; Wagner *et al.*, 1994). Deletion of PIM1 is associated with inhibited growth on glycerol (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). Mitochondrial matrix proteins are not degraded and tend to accumulate as electron dense inclusions in Δ*pim1* cells (Suzuki *et al.*, 1994). Similarly, downregulation of the human Lon protease leads to accumulation of protein inclusions, impaired mitochondrial function and apoptotic cell death (Bota *et al.*, 2005). Pim1 is additionally required for maintenance of *mt*DNA in yeast by an unknown mechanism probably involving its ability to bind GT-rich DNA sequences (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Fu *et al.*, 1997; Liu *et al.*, 2004).

Clp (*c*aseino-*l*ytic *p*rotease) proteases are also found in the mitochondrial matrix space (Leonhardt *et al.*, 1993; Van Dyck *et al.*, 1998). In contrast to Lon- and AAA-proteases, their ATPase domain and the proteolytic domain are expressed as separate gene products. The ATPase domains (ClpX, ClpA) assemble into hexameric, ring complexes with ATPase and chaperone activity, while the proteolytic domains (ClpP) form heptameric, double ring complexes (Bochtler *et al.*, 1997; Wang *et al.*, 1997; Weber-Ban *et al.*, 1999; Bochtler *et al.*, 2000; Kang *et al.*, 2002; Ortega *et al.*, 2004). The ATPase domains determine the substrate specificity of the Clp-protease and also exert regulatory functions during proteolysis (Schmidt *et al.*, 1999; Weber-Ban *et al.*, 1999; Flynn *et al.*, 2001; Kang *et al.*, 2002; Ortega *et al.*, 2004). While ClpX-like ATPase subunits are present in all organisms, ClpP-like proteins are present in mammals and plants, but have not been detected in yeast (Van Dyck *et al.*, 1998; De Sagarra *et al.*, 1999; Kang *et al.*, 2002).

1.1.2. AAA proteases in the inner mitochondrial membrane

Two AAA proteases, active on both sides of the inner mitochondrial membrane have been identified in yeast and are presumably present in all organisms, the *i*AAA protease and the *mAAA* protease (Figure 1). The *i*AAA protease is presumably a homooligomeric complex of the Yme1 protein in yeast (Leonhard *et al.*, 1996; Weber *et al.*, 1996) and Yme1l/YME1L protein in mouse and human (Shah *et al.*, 2000). In contrast, the *m*AAA protease is a heterooligomeric complex of Yta10 (Afg3) and Yta12 (Rca1) in yeast, and paraplegin and Afg3l2 in human (Arlt *et al.*, 1996; Atorino *et al.*, 2003). Three putative *m*AAA protease subunits have been identified in mice: paraplegin, Afg3l1 and Afg3l2 (Kremmidiotis *et al.*, 2001). Studies in yeast show that

both proteases have different topology into the inner mitochondrial membrane. While the *i*AAA protease exposes catalytic domains to the *i*ntermembrane space, catalytic domains of the *m*AAA protease are exposed to the *m*atrix side of the inner mitochondrial membrane (Arlt *et al.*, 1996; Leonhard *et al.*, 1996).

1.1.2.1. Domain structure of the AAA protease subunits

The subunits of the *i*AAA and the *m*AAA protease share a common domain structure (Figure 2A). Each subunit is anchored to the inner mitochondrial membrane by one (Yme1) or two (Yta10 and Yta12) transmembrane (TM) domains at their N-terminal ends (Pajic *et al.*, 1994; Arlt *et al.*, 1996; Weber *et al.*, 1996). The membrane spanning segments in the FtsH protease, a bacterial homologue of mitochondrial AAA-proteases, have been shown to play a crucial role for the oligomerization of the protease (Akiyama and Ito, 2000). Similarly, subunits of the *m*AAA protease lacking both TM-domains fail to assemble (Korbel *et al.*, 2004). However, deletion of the TM-domain of one of the subunits does not inactivate the *m*AAA protease as cells expressing Yta10ΔTM or Yta12ΔTM can grow on glycerol (Korbel *et al.*, 2004). The TM-domains are also dispensable for the degradation of peripheral membrane proteins (Atp7) and for the presequence removal of Ccp1 (Korbel *et al.*, 2004). Nevertheless, proteolysis of integral membrane proteins is impaired suggesting a specific role of the TM-domains of Yta10 and Yta12 during degradation of integral membrane proteins (Korbel *et al.*, 2004).

The transmembrane domains are followed by the AAA domains with ATPase activity (Figure 2A). These domains contain conserved motifs which ensure binding (Walker A) and hydrolysis (Walker B and second region of homology, SRH) of ATP during proteolysis (Figure 2B) (Walker *et al.*, 1982; Whiteheart *et al.*, 1994; Hanson and Whiteheart, 2005). Homology modeling, site directed mutagenesis and crystallographic analysis have led to partial understanding of the mechanism of ATP hydrolysis in AAA+ proteins, including FtsH (Babst *et al.*, 1998; Lenzen *et al.*, 1998; Yu *et al.*, 1998; Bochtler *et al.*, 2000; Sousa *et al.*, 2000; Zhang *et al.*, 2000; Wang *et al.*, 2001; Krzywda *et al.*, 2002). The Walker A motif contains the so called P-loop whose conserved lysine residue is crucial for binding of ATP. The lysine residue (K201) in the Walker A motif of FtsH (corresponding to K334 and K394 in Yta10

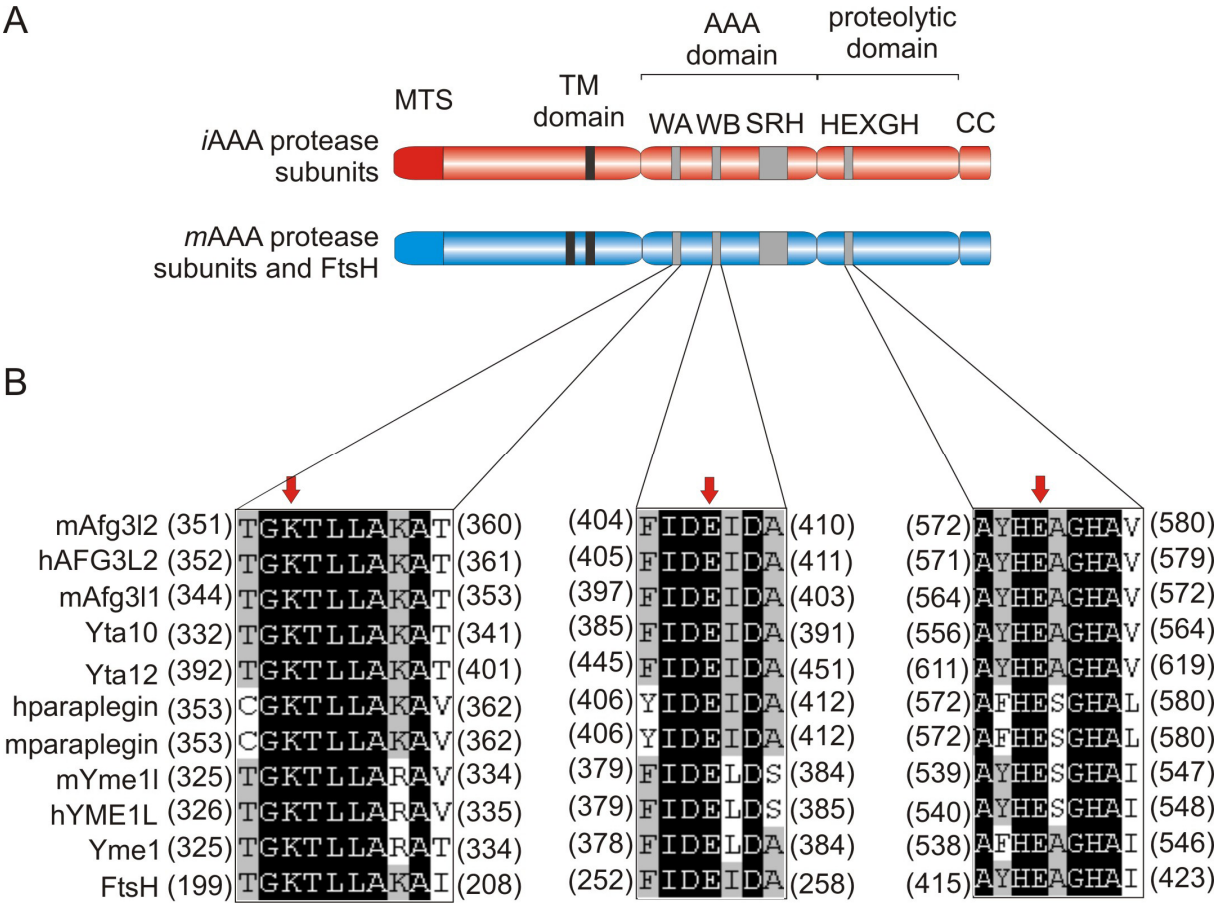

Figure 2. Domain structure of the subunits of the *i*AAA and the *m*AAA protease. A. The nuclear-encoded subunits are imported into mitochondria via an N-terminal mitochondrial targeting sequence (MTS) which is removed after import. One (Yme1) or two (Yta10 Yta12, FtsH) transmembrane (TM) domains are present in each subunit. Towards the C-terminus are AAA-domains containing conserved motifs, characteristic for the AAA protein family; Walker A (WA) and Walker B (WB) motifs as well as the *s*econd *r*egion of *h*omology (SRH). The AAA-domains are followed by proteolytic domains harboring the HE*X*GH metal binding motif. At the most C-terminal end is the coiled-coil region (CC). Reprinted with modifications from (Nolden *et al.*, 2005). **B.** A multisequence alignment of the amino acid regions containing the conserved Walker A, Walker B and the HE*X*GH motif from yeast, mammalian and bacterial AAA-protease subunits. Sequence coordinates of the amino acids are indicated. Alignment of the complete protein sequences is provided in *Appendix I*. m, mouse; h, human; ↑, amino acids crucial for the ATPase and the proteolytic activity of the subunits.

and Yta12, respectively) forms hydrogen bonds with oxygen atoms of the β– and γ–phosphates of ATP (Karata *et al.*, 2001; Krzywda *et al.*, 2002). Mutations of the corresponding lysine residue of other AAA+ proteins, including FtsH, are proposed to inhibit the protein via preventing binding of ATP (Karata *et al.*, 1999; Hanson and Whiteheart, 2005). Together with the Walker A motif, aspartate (D254) and glutamate (E255) amino acids from the Walker B motif are crucial for the ATPase activity (Figure 2B) (Babst *et al.*, 1998; Lenzen *et al.*, 1998; Krzywda *et al.*, 2002; Hanson and Whiteheart, 2005). The aspartate amino acid (D254) forms hydrogen bonds with

water molecules liganded to Mg^{2+} ions while the glutamate amino acid (E255 corresponding to E388 of Yta10 and E448 of Yta12) activates a water molecule for nucleophilic attack on ATP (Krzywda *et al.*, 2002). Mutational exchange of the conserved glutamate renders the AAA+ proteins inactive probably due to defects in ATP hydrolysis but not ATP binding (Whiteheart *et al.*, 1994; Babst *et al.*, 1998; Hanson and Whiteheart, 2005). Similarly, FtsH is inactivated by the mutational exchange of E255 in the Walker B motif (Karata *et al.*, 2001). In addition to the Walker motifs, residues from the SRH function during ATP-hydrolysis as γ–phosphate group sensors and arginine-fingers (Karata *et al.*, 1999; Song *et al.*, 2000; Karata *et al.*, 2001; Krzywda *et al.*, 2002; Ogura *et al.*, 2004). Commonly, the AAA+ proteins assemble into oligomeric complexes with hexameric and sometimes heptameric structure (Lenzen *et al.*, 1998; Yu *et al.*, 1998; Vale, 2000; Hanson and Whiteheart, 2005). In these complexes ATP-binding sites are positioned in the interface between the subunits (Lenzen *et al.*, 1998; Yu *et al.*, 1998; Krzywda *et al.*, 2002). Arginine side groups protrude into the ATP binding pocket of a neighboring subunit and contact the γ–group of an ATP bound there, thereby ensuring intersubunit cooperativity during ATP hydrolysis (Karata *et al.*, 1999; Song *et al.*, 2000; Karata *et al.*, 2001; Krzywda *et al.*, 2002; Ogura *et al.*, 2004). Additional residues within the motifs interact to form a suitable environment for ATP hydrolysis (Krzywda *et al.*, 2002). In addition to its ATPase function the AAA domain also binds unfolded membrane proteins (Leonhard *et al.*, 2000).

The proteolytic domain and a conserved coiled-coil region are present C-terminally from the AAA-domain (Figure 2A) (Nolden *et al.*, 2005). Central feature of the proteolytic domain is the metal binding HE*X*GH motif which is typical for the Zn^{2+}-dependent metalloproteases (Figure 2B) (Vallee and Auld, 1990; Nolden *et al.*, 2005; Nolden *et al.*, 2005a). In this motif a histidine amino acid coordinated the Zn^{2+} ion while the glutamate activates a water molecule for nucleophilic attack on the peptide bond of the substrate (Vallee and Auld, 1990; Hooper, 1994). Mutational exchange of the conserved glutamate abolishes the proteolytic activity of the protease but does not prevent substrate binding and therefore this mutation is often used for the creation of “substrate-trap” mutants (Arlt *et al.*, 1996; Arlt *et al.*, 1998; Nolden *et al.*, 2005a).

Little is known about the function of the coiled-coil region (Figure 2A). In FtsH, the integrity of the coiled-coil region is essential for the activity of the protease (Shotland

et al., 2000). Site directed mutagenesis shows that the coiled-coil region is an important structural element affecting the oligomerization and the ATP-dependant conformational changes in FtsH and other AAA proteins (Babst *et al.*, 1998; Shotland *et al.*, 2000). Additionally, recent studies in our and other laboratories have shown that the coiled-coil region of Yme1 is responsible for binding of substrates during proteolysis (Martin Graef, personal communication)(Shotland *et al.*, 2000).

1.1.2.2. Function of the *m*AAA protease in yeast

Deletion of either Yta10 or Yta12 leads inhibited growth on glycerol (Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994). Similarly, inactivation of Yme1 is associated with respiratory growth phenotypes and tendency to lose *mt*DNA at elevated temperatures (Thorsness and Fox, 1993; Thorsness *et al.*, 1993). Combination of Yme1 deletion with Yta10 or Yta12 deletion is lethal, suggesting overlapping functions of both proteases. Indeed, both proteases have overlapping substrate specificities and integral membrane proteins are degraded by either protease depending on their accessibility from each side of the inner membrane (Leonhard *et al.*, 2000). Mutations in the HE*X*GH motif of the proteolytic domains impair proteolysis without affecting the substrate binding activity of the protease (Arlt *et al.*, 1996; Arlt *et al.*, 1998). While proteolytic inactivation of a single subunit (Yta10 or Yta12) is not sufficient to completely inactivate the *m*AAA protease and cells can grow on glycerol, inactivation of both subunits is associated with an inhibited growth on glycerol (Guelin *et al.*, 1994; Arlt *et al.*, 1996). This suggests an essential proteolytic function of the *m*AAA protease for maintenance of respiratory growth (Arlt *et al.*, 1996; Arlt *et al.*, 1998). Additionally, the assembly of the respiratory chain complexes and of the intermediate form of the F_0 ATPase subunit 9 is impaired in cells expressing proteolytically inactive Yta10 and Yta12 (Tzagoloff *et al.*, 1994; Arlt *et al.*, 1998).

In addition to its role in protein quality control, the *m*AAA protease plays an essential role for the expression of the mitochondrial genome and the processing of intron containing *mt*DNA transcripts in yeast (Arlt *et al.*, 1998; Nolden *et al.*, 2005). Proteolytic inactivation of both Yta10 and Yta12 leads to deficiencies in the processing or stability of the mitochondrially encoded cytochrome oxydase I (*COX I)* and cytochrome *b (COB)* transcripts, which can be suppressed by introduction of intronless *mt*DNA (Arlt *et al.*, 1998). Recent findings revealed that mitochondrial

translation is drastically decreased in Δ*yta10* or Δ*yta12* yeast cells due to defective maturation and assembly of Mrpl32, a subunit of the large ribosomal particle (Nolden *et al.*, 2005a). This function of the *m*AAA protease is sufficient to explain growth defects associated with inactivation of the *m*AAA protease (Nolden *et al.*, 2005a).

1.1.2.3. Mammalian *m*AAA proteases

As previously mentioned the human *m*AAA protease is built up of paraplegin and Afg3l2 which are homologous to Yta10 and Yta12 from yeast (Casari *et al.*, 1998; Banfi *et al.*, 1999; Atorino *et al.*, 2003). Loss of paraplegin causes a recessive form of neuronal degeneration known as hereditary spastic paraplegia (HSP) (Casari *et al.*, 1998; Casari and Rugarli, 2001). The disease is genetically heterogenous and is associated with progressive weakness and spasticity of the lower limbs which sometimes is complicated by the appearance of additional symptoms (pure and complicated HSP) (Harding, 1983; Harding, 1984; Harding, 1993; Fink *et al.*, 1995; Reid, 1997; Bross *et al.*, 2004). Histologically, HSP is associated with axonal degeneration of the sensory neurons and neurons of the corticospinal tract (Schwarz and Liu, 1956). Notably, degeneration is restricted to neurons with the longest axons in the nervous system (Harding, 1984; Deluca *et al.*, 2004; Ferreirinha *et al.*, 2004).
Mouse models for HSP, caused by paraplegin deficiency, exhibit mitochondrial abnormalities in the longest axons suggesting a functional link of HSP to mitochondrial morphology (Ferreirinha *et al.*, 2004). With age the number of abnormal mitochondria increases, but up to 8 months there is no or very small number of degenerating axons (Ferreirinha *et al.*, 2004). Nevertheless, changed mitochondrial morphology does not correlate with changes in the activity of respiratory chain complexes as only senescent mice show decreased activity of complex I and moderately (~15%) reduced ATP synthesis (Ferreirinha *et al.*, 2004). Reduced activity of complex I, due to defective assembly, has also been reported for human HSP-patient fibroblasts (Atorino *et al.*, 2003). These cells also exhibit increased sensitivity to reactive oxygen species (ROS) (Atorino *et al.*, 2003), consistent with the previously observed role of ROS in the development of neurodegenerative diseases (Schapira *et al.*, 1989; Taylor *et al.*, 2003; Manton *et al.*, 2004; Calabrese *et al.*, 2005). Expression of paraplegin in HSP-fibroblasts is able to rescue both complex I assembly and resistance to ROS thereby confirming that

absence of paraplegin is the cause for the disease (Atorino *et al.*, 2003). Interestingly, the HSP-phenotype is also reverted by a proteolytic mutant of paraplegin (*SPG7*^{Q575}) (Atorino *et al.*, 2003). By analogy to yeast, however, the presence of *SPG7*^{Q575} is probably not sufficient to inactivate the protease. Similarly to yeast, mitochondrial translation in hepatocytes from SPG7$^{-/-}$ mice is decreased substantiating the functional conservation between the yeast and the human *m*AAA protease (Nolden *et al.*, 2005). This poses the interesting possibility that the mammalian *m*AAA protease also plays a role in the expression of the mitochondrial genome.

These observations doubtlessly shed light on HSP caused by the absence of paraplegin, but they can not explain why only certain tissues are affected. The molecular explanation of HSP in mouse is additionally complicated by the fact that mice harbor a third putative *m*AAA protease subunit, termed Afg3l1 (Shah *et al.*, 1998; Kremmidiotis *et al.*, 2001). Although the *AFG3L1* gene is expressed in humans it is not translated into a protein product (Kremmidiotis *et al.*, 2001). Therefore, the subunit composition of the murine *m*AAA protease is neither clarified nor is the effect of the absence of either subunit on the assembly and function of the other two subunits. It is also conceivable that the putative AAA-protease subunits differ in their substrate specificity resulting in functional differences of the murine *m*AAA protease(s).

2. Prohibitins

Prohibitins comprise a family of highly conserved and ubiquitous proteins with two family members, Prohibitin 1 (Phb1) and Prohibitin 2 (Phb2) present in all eukaryotic cells. The proteins are highly conserved and have sequence identities between 40% and 100% (Figure 4). Lower sequence similarity can be observed at the N-termini and C-termini of the proteins the most prominent being Prohibitin 1 in *S. cerevisiae*, which has an additional C-terminal amino acid stretch not present in other species. It is still obscure, however, if this structural divergence implicates a functional difference. The present knowledge on prohibitins is summarized below.

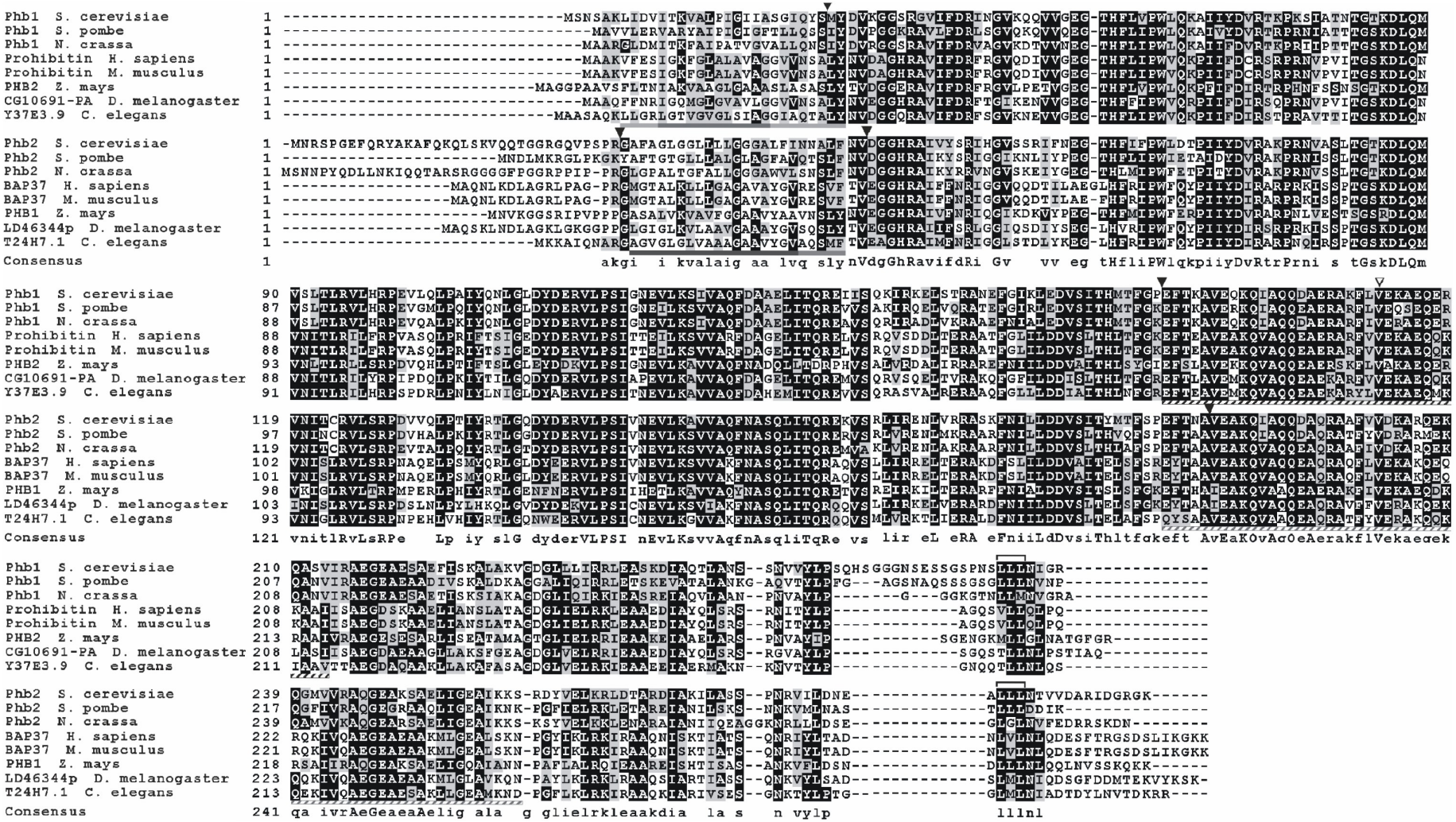

Figure 3. Multisequence alignment of Prohibitin 1 and Prohibitin 2 from different organisms. Alignment was performed with clustalX software using default settings. The accession numers are given in a table format in *Materials and Methods.* Filled boxes show the span of the predictable transmembrane regions. Shadowed boxes show the position of the predictable coiled-coil region. **V** shows the position of a conserved Val residue which is predicted to be essential for the folding of the coiled-coil region. Short highly homologous stretches at the C-terminal end of both, Prohibitin 1 and Prohibitin 2, are also marked.

2.1. Prohibitins are conserved SPFH-domain proteins

Based on their limited homology with stomatin and stomatin-like proteins, prohibitins are proposed to fall within the so called stomatin protein superfamily (Tavernarakis *et al.*, 1999; Nadimpalli *et al.*, 2000). This family is also designated PID after **p**roliferation, **i**on, and **d**eath. In addition, proteins like the bacterial plasma membrane HflK and HflC, close bacterial homologues of prohibitins, as well as flotillins and plant disease response proteins (HIR) fall within this superfamily. A common feature of the aforementioned proteins is the so called SPFH domain (after **S**tomatin, **P**rohibitin, **F**lotillin and **H**flK/C) (*Appendix II*). The role of the SPFH-domain and the functions of the proteins carrying SPFH-domain are discussed bellow.

2.1.1. Functions of the SPFH domain

Most of the characterized SPFH family members are membrane proteins with their SPFH-domain exposed to a hydrophilic environment (Tavernarakis *et al.*, 1999). In addition, stomatin and its homologues are associated with lipid microdomains called lipid rafts. These are tight associations between sphingolipids and cholesterol with distinct biophysical properties within the surrounding lipid bilayer (Brown and Rose, 1992; Simons and Ikonen, 1997; Brown and London, 2000; London and Brown, 2000). Association within membrane subcompartments makes sense in the light of protein sorting or concentrating molecules involved in processes like control of signal transduction, antigen representation and etc. (Anderson *et al.*, 2000; Anderson and Jacobson, 2002; Bickel, 2002). Constant flotation, separation and fusion of such domains and their docked proteins allows for more flexible spatial and temporal regulation of signaling cascades according to the requirements of the cell (Simons and Toomre, 2000; Golub *et al.*, 2004; Razzaq *et al.*, 2004).

2.1.1.1. Role of the SPFH-domain for localization to the plasma membrane and lipid rafts

Proteins are targeted to lipid rafts via different mechanisms of which lipid modification (like palmitoylation) is the most common (Melkonian *et al.*, 1999). In some stomatin homologues the SPFH domain contains sequences responsible for proper targeting to the plasma membrane and to lipid rafts (Salzer and Prohaska, 2001). Flotillin-1/reggie-2, enriched in lipid rafts from erythrocytes (Salzer and Prohaska, 2001), is

targeted to the plasma membrane via a combination of two hydrophobic regions embedded within the SPFH domain (Liu *et al.*, 2005). A cysteine residue (cys34) from the SPFH-domain of flotillin-1/reggie-2 is palmitoylated and functions together with the hydrophobic sequences in targeting to the plasma membrane (Morrow *et al.*, 2002). Mutational exchange of cysteine at position 34 to alanine prevents the association of flotillin-1/reggie-2 with the plasma membrane suggesting a crucial role of palmitoylation for the proper localization (Morrow *et al.*, 2002). Similarly to flotillin-1/reggie-2, palmitoylation of cys29 targets stomatin to lipid rafts (Wang *et al.*, 1991; Snyers *et al.*, 1999). Mutations, which prevent the proper localization to the plasma membrane have been detected in the SPFH-domain of podocin. In cells, podocin is normally localized to the plasma membrane and mutations in the SPFH domain cause retaining in the endoplasmic reticulum (Roselli *et al.*, 2004).

2.1.1.2. Role of the SPFH-domain for protein-protein interactions and as a structural scaffold

The SPFH-domain functions as a mediator of protein-protein interactions in some stomatin homologues. The SPFH-domain of MEC-2, the *C. elegans* homologue of human stomatin, is involved in the interaction with MEC-4 and MEC-10 subunits of the degenerin channel in the plasma membrane of the touch-sensory neurons (Huang *et al.*, 1995; Zhang *et al.*, 2004). Mutations in the SPFH-domain of MEC-2 lead to loss of touch sensitivity due to an impaired interaction of MEC-2 with the degenerin channel (Zhang *et al.*, 2004). In this case, the SPFH domain ensures proximity between the regulatory N- and C-terminal ends of MEC-2 and the channel subunits, but does not play a role in the proper distribution of MEC-2 along the sensory neurons (Zhang *et al.*, 2004). Rather, this function is attributed to the MEC-2-specific N-terminal sequence (Huang *et al.*, 1995).

In stomatin and podocin, the SPFH domain can form a hairpin conformation thereby properly localizing the N- and C- terminal ends to the cytoplasm where interaction with other molecules takes place (Snyers *et al.*, 1999; Roselli *et al.*, 2002). Thus, the SPFH domain not only provides proper targeting to the plasma membrane and lipid rafts, but also serves as a structural scaffold (Goodman *et al.*, 2002; Zhang *et al.*, 2004).

Prohibitins and bacterial HflK and HflC proteins were also proposed to possess SPFH-domains (Tavernarakis *et al.*, 1999). HflK and HflC proteins form a high molecular weight complex which is anchored to the plasma membrane with the N-termini of the molecules and exposes C-terminal domains to the periplasmic side of the membrane (Kihara *et al.*, 1997). However, no data exists whether HflK/C complexes are associated with lipid rafts or related subdomains in the bacterial plasma membrane. In contrast, mammalian prohibitins have been shown to localize to the plasma membrane in B-lymphocytes and several cancer cell lines (Terashima *et al.*, 1994; Mengwasser *et al.*, 2004). Prohibitins associate with the IgM antigen receptor in a TritonX-100 resistant manner (Terashima *et al.*, 1994) in murine B-cell lines and purified lipid rafts are positive for Prohibitin 1 and Prohibitin 2 (Saeki *et al.*, 2003; Mielenz *et al.*, 2005) suggesting that prohibitins are associated with lipid rafts. However, due to their different cellular localization it is difficult to assess whether association of prohibitins with lipid rafts is a rule or exception. In yeast, prohibitins are localized exclusively to mitochondria and no association with lipid rafts has been reported.

2.1.2. Functions of the proteins harboring SPFH-domains

2.1.2.1. Regulation of channels across the plasma membrane

SPFH-domain proteins of clinical importance are the close homologues stomatin and podocin. Podocin (*NPHS2*) is exclusively expressed in kidney podocytes and regulates the function of the glomerular filtration barrier (Boute *et al.*, 2000; Roselli *et al.*, 2002). Mutations in the *NPHS2* gene cause the familial steroid-resistant nephric syndrome in which the filtration function of podocytes is disturbed and a condition known as protein-urea occurs.

The human stomatin protein (erythrocyte band 7 protein) has long been shown to be affected in overhydrated stomatocytosis (OHSt, spherocytosis of the erythrocytes and leakage of Na and K ions) (Lande *et al.*, 1982; Stewart *et al.*, 1992; Stewart *et al.*, 1993). The protein is absent in the most severe cases of OHSt, but seems not to be the cause for the disease (Stewart *et al.*, 1992; Fricke *et al.*, 2003). Although the exact cause for OHSt is unknown, stomatin has been shown to interact with the actin cytoskeleton in erythrocytes (Stewart *et al.*, 1992), with the glucose transporter GLUT-1 regulating the uptake of glucose (Zhang *et al.*, 2001) and with ASIC-related

DEG/ENaC subunits in rat mechanosensory neurons (Price *et al.*, 2004). Therefore, the interaction and regulation of channels across the plasma membrane is emerging as a common function of most of the so far characterized stomatin-like proteins.

2.1.2.2. Regulation of proteases

In contrast to stomatin-like proteins, HflK/C interacts with the membrane protease FtsH thereby modulating its proteolytic activity (Kihara *et al.*, 1996; Saikawa *et al.*, 2004). In the absence of HflK/C, the nonassembled subunit SecY of the membrane protein translocase (SecY-SecE) is rapidly degraded (Kihara *et al.*, 1996). In contrast, the λ phage CII protein is stabilized under identical conditions (Kihara *et al.*, 1997).
Similarly to HflK/C, prohibitins associate with the *m*AAA protease and were proposed to negatively regulate its activity in yeast [see chapter 2.6.3. and (Steglich *et al.*, 1999)] .
Based on the involvement of HflK/C and prohibitins in proteolysis by direct interaction with proteases it was proposed that the region including the SPFH domain functions as an association factor for the formation of complexes with membrane associated proteases (Tavernarakis *et al.*, 1999).

2.3. Expression of the prohibitin gene in mammals

Prohibitin genes are constitutively expressed in all mammalian cells and tissues with expression levels varying as a function of the cell cycle (Roskams *et al.*, 1993). How the expression of prohibitins is regulated is presently unknown. Analysis of the promoter regions of rat and human *PHB1* gene shows that the promoter has features similar to those of house keeping genes, e.g. lack of a clearly identifiable TATA box (McClung, 1995). Endogenous prohibitin mRNA and protein levels decrease in S-phase and rise again in the G_2-phase. Interestingly, two forms of *PHB1* mRNA, with molecular size of 1.2 kb and 1.9 kb, have been detected in human normal and immortalized cells and in rats (see below) (Nuell *et al.*, 1991; Jupe *et al.*, 1996).

2.4. Cellular localization of prohibitins

Prohibitins have been localized to various cellular compartments in different organisms. While in yeast Phb1 and Phb2 are localized exclusively to mitochondria, in mammalian cells they are also detectable in the nucleus (Wang *et al.*, 2002;

Fusaro *et al.*, 2003) and at the plasma membrane (Mengwasser *et al.*, 2004). In mitochondria from yeast, *C. elegans* and mammals Phb1 and Phb2 form a high molecular weight complex (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000; Coates *et al.*, 2001; Artal-Sanz *et al.*, 2003). Moreover, Phb1 and Phb2 are interdependent in yeast i.e. in the absence of Phb1 Phb2 is rapidly degraded and vice versa (Berger and Yaffe, 1998). Therefore, it is of interest to note that extramitochondrial localization is shown separately for Phb1 and Phb2 in mammalian cells. Since this thesis focuses on the mitochondrial localization and function of prohibitins, their localization and function in other cellular compartments will be discussed in less detail.

2.5. Prohibitins function as regulators of cell proliferation and are important for normal development

Both Phb1 and Phb2 are necessary for embryonic development in flies (Eveleth and Marsh, 1986), *C. elegans* (Artal-Sanz *et al.*, 2003) and mice (Park *et al.*, 2005). Mutations in the Prohibitin 1 gene are recessive lethals in late larvae development or during the progression to pupae in *Drosophila* (Eveleth and Marsh, 1986). Similarly, down regulation of prohibitins by short interfering RNAs in *C. elegans* leads to embryonic arrest (Artal-Sanz *et al.*, 2003). Furthermore, homozygous Phb2 knock-out mice die early in development (before E9.0) (Beal, 2003). Interestingly, Prohibitin 1 has also been recently shown to play a role during plant development (Chen *et al.*, 2005). Although the exact role of prohibitins for development is not understood, it is conceivable that it is linked to their function as transcriptional regulators in the nucleus. Therefore, the role of both proteins in transcriptional regulation, cell proliferation and apoptosis is discussed below.

2.5.1 Function of Prohibitin 1 as a regulator of cell proliferation and apoptosis

PHB1 mRNA is capable to block proliferation in normal and HeLa human fibroblasts (McClung *et al.*, 1989; Nuell *et al.*, 1991; Liu *et al.*, 1994). Transfected *PHB1* mRNA has maximum antiproliferative activity in G_0 to G_1 transition phase by maintaining high prohibitin levels prior to entering in S-phase and blocking DNA synthesis (Roskams *et al.*, 1993). The *PHB1* gene gives rise to major (1.9 kb) and minor (1.2 kb) transcripts in human cells and in rats (McClung *et al.*, 1989; Jupe *et al.*, 1995;

Thompson *et al.*, 2001). The level of the major transcript in immortalized cell lines is increased while the level of the 1.2 kb transcript remains constant (Jupe *et al.*, 1995) suggesting functional role for the major transcript. Indeed, the antiproliferative effect of *PHB1* mRNA is contained within the 3`-untranslated region (UTR) of the 1.9 kb transcript in a region absent in the minor transcript as indicated by two lines of evidence: i) the 3`-UTR alone is able to suppress cellular proliferation and ii) single nucleotide exchange (C/T) in this region or the removal of the 3`-UTR abolishes the antiproliferative effect of *PHB1* mRNA. Moreover, the UTR/T allele is associated with increased risk of breast cancer (Jupe *et al.*, 1996; Manjeshwar *et al.*, 2003).

The Phb1 protein can also affect proliferation (Wang *et al.*, 1999; Wang *et al.*, 1999a; Wang *et al.*, 2002; Wang *et al.*, 2002a; Gamble *et al.*, 2004; Wang *et al.*, 2004). Phb1 binds to retinoblastoma-family members (Rb, p107 and p130) thereby repressing the activity of E2F-transcription factors (E2F1-5), which results in inhibition of cell proliferation (Wang *et al.*, 1999; Wang *et al.*, 1999a) (Figure 4A). To repress transcription, Phb1 also binds the co-repressor NcoR and recruits chromatin remodeling complexes, such as HDAC1 and Brg-1/Brm, to the promoter element (Wang *et al.*, 2002; Wang *et al.*, 2002a; Wang *et al.*, 2004). Recruitment of HDAC1 and Brg-1/Brm is presumably independent of binding to Rb as Phb1/Brg-1/Brm complexes exist in the absence of Rb (Wang *et al.*, 2002a). Binding of Phb1 to Rb is, however, compulsory for the activity of these complexes since Phb1 exerts no effect on E2F-mediated transcription in the absence of Rb (Wang *et al.*, 1999; Wang *et al.*, 2002a). Therefore, it is imaginable that binding of Phb1 to Rb is the rate limiting step which ensures recruitment to the promoter and specificity of the Phb1-mediated transcriptional repression.

Notably, stimulation of the IgM signaling network can reverse the Phb1-mediated suppression of cell proliferation (Figure 4B) (Wang *et al.*, 1999a; Wang *et al.*, 2002). This effect is attributed to an activated Raf-1 kynase which translocates to the nucleus and binds to Phb1 thereby interfering with the formation of the Phb1/Rb or Phb1/E2F complexes (Wang *et al.*, 1999a).

What is the role of Phb1 for development? E2F activity is essential for early development in *Drosophila* (Duronio *et al.*, 1995; Myster *et al.*, 2000) and *Xenopus* (Suzuki and Hemmati-Brivanlou, 2000). In mice, simultaneous deletions of E2F family members are lethal and deletion of a single E2F gene results in a specific developmental defect (Humbert *et al.*, 2000; Rempel *et al.*, 2000). In mammalian

cells, loss of E2Fs repression or deregulation of E2Fs protein levels have been shown to alter the expression of various genes involved in cell proliferation, apoptosis (APAF1, caspase3, caspase 7, Bcl-3), development (homeobox genes and genes from the TGFβ and Wnt pathways) and etc. (Hunt *et al.*, 1997; Gaubatz *et al.*, 2000; Humbert *et al.*, 2000a; Muller *et al.*, 2001). It is conceivable that similarly to the inactivation of Rb (Knudsen and Wang, 1996) absence of Phb1 would result in higher transcription levels from E2F promoters thereby affecting the expression of E2F-responsive genes. Therefore, it is plausible that Phb1 balances the timely expression of E2F responsive genes which are necessary for normal development.

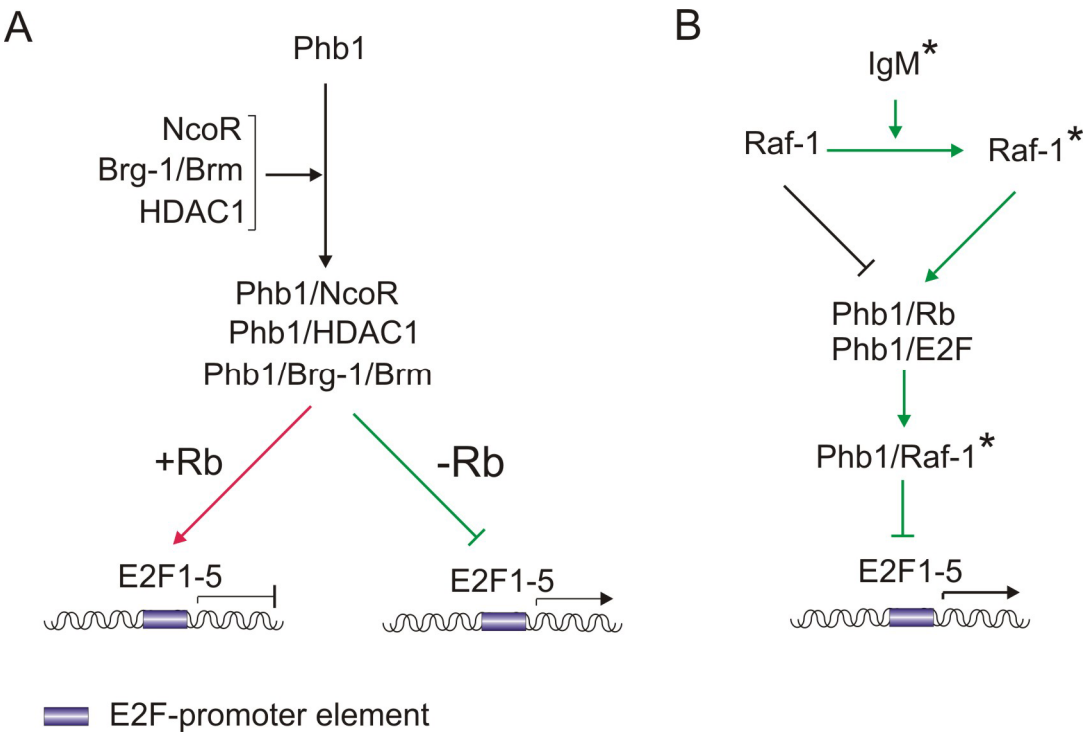

Figure 4. Phb1 functions as a negative regulator of cell proliferation by repressing the activity of E2F transcription factors. A. In the presence of Rb, Phb1 binds to E2F1-5 transcription factors and represses the expression from E2F responsive promoters. This is mediated by binding of Phb1 to the transcriptional co-repressor NcoR (Phb1/NcoR) and concomitant recruitment of histone deacetylase HDAC1 (Phb1/HDAC1). Alternatively, recruitment of the chromatin remodeling complexes Brg-1/Brm by Phb1 (Phb1/Brg-1/Brm) can repress E2F-mediated transcription. Although Phb1/Brg-1/Brm and presumably Phb1/HDAC1 complexes are formed upstream of Rb binding (not shown), they can repress transcription only in the presence of Rb. Requirement of Rb is shown as +Rb. **B.** The antiproliferative effect of prohibitins is reverted in the presence of activated Raf-1 kinase. Stimulation of the IgM-receptor (IgM*) activates the Raf-1 kinase (Raf-1*), which can bind Phb1 (Phb1/Raf-1*) and thereby interferes with the formation of the Phb1/Rb of Phb1/E2F complexes. Phb1/Raf-1* complexes can not repress E2F-mediated transcription. green arrow or crossed line, pathway that allows E2F-mediated transcription, red arrow lines, pathway that represses E2F activity.

These studies, however, focus only on Phb1 and the role of Phb2 for the regulation of E2F promoters remains unclear.

2.5.2. Role of Phb2 as a regulator of the estrogen signaling pathway

Independent studies have revealed that Phb2 can also function as a transcriptional regulator in the nucleus (Montano *et al.*, 1999; Sun *et al.*, 2004). Phb2 was identified in yeast two hybrid studies as a novel co-regulator of the nuclear estrogen receptor (ER) and was accordingly designated *R*epressor of *E*strogen receptor *A*ctivity (REA) (Montano *et al.*, 1999). When expressed in cell culture, Phb2 selectively represses the transcription from consensus and non-consensus ER responsive promoters (Delage-Mourroux *et al.*, 2000). This is achieved by a direct binding of Phb2 to the liganded ER and recruitment of HDAC1 to the promoter element (Delage-Mourroux *et al.*, 2000; Kurtev *et al.*, 2004). A model was proposed according to which Phb2 represses transcription by mutually competing with the transcriptional co-activator SRC-1 for binding to the liganded ER (Figure 5) (Delage-Mourroux *et al.*, 2000; Kurtev *et al.*, 2004).

The function of Phb2 in the estrogen signaling cascade is further substantiated in mice (Park *et al.*, 2005). Although knock-out of Phb2 is embryonic lethal early in development, heterozygous mice lacking one PHB2 allele are viable. Notably, genes normally stimulated by estrogen (complement C3, PTα and etc.) are upregulated in the ovary from such animals (Park *et al.*, 2005).

The similarities between the emerging functions of Prohibitin 1 and Prohibitin 2 as transcriptional regulators are interesting. Both proteins serve as transcriptional repressors and both can utilize HDAC1. It seems plausible that binding to transcription factors (E2F and ER) or repressors (Rb) ensures specificity of Phb1 or Phb2 mediated repression of transcription. Recent findings suggest a functional interaction between the ER pathway and the Phb1-mediated regulation of E2F (Wang *et al.*, 2004). In particular, binding of anti-estrogen to the ER-receptor can target Phb1 and thereby induce repression the activity of E2F transcription factors (Wang *et al.*, 2004). It remains unclear though, if both proteins act as a complex in this process.

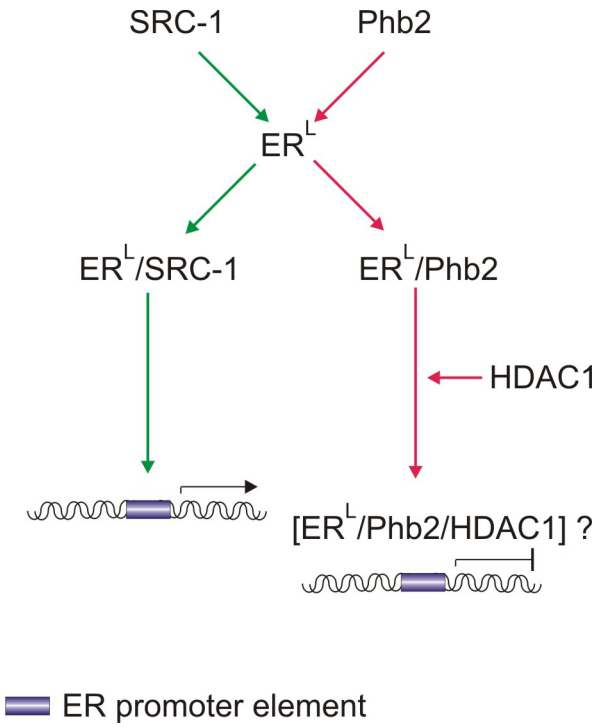

Figure 5. ER-mediated transcription is repressed by Phb2. A. Phb2 competes with the transcriptional activator SRC-1 for the liganded ER (ER^L). ER^L bound to SRC-1 (ER^L/SRC-1) promotes transcription from ER-responsive elements. In contrast, binding of Phb2 to ER^L (ER^L/Phb2) inhibits the expression from ER-responsive promoters via recruitment of HDAC1. green green arrow lines, pathway that allows E2F-mediated transcription, red arrow lines, pathway that represses E2F activity, ?, unknown complex.

2.6. Mitochondrial localization and function of prohibitins

Phb1 and Phb2 were localized to mitochondria in plants (Snedden and Fromm, 1997; Takahashi *et al.*, 2003), yeast (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000), mammals (Nijtmans *et al.*, 2000) and *C. elegans* (Artal-Sanz *et al.*, 2003). Their biogenesis, possible function and molecular interactions in yeast mitochondria are discussed below.

2.6.1. Assembly and structure of the yeast prohibitin complex in the inner mitochondrial membrane

Phb1 and Phb2 are nuclear-encoded and posttranslationally imported in mitochondria where they assemble into a high molecular weight complex in the inner mitochondria membrane (Steglich *et al.*, 1999; Tatsuta *et al.*, 2005). Targeting of both Phb1 and Phb2 to mitochondria in yeast occurs via non-cleavable N-terminal presequences. In Phb2, this presequence contains positively charged residues

capable of forming amphipathic α-helix, followed by a hydrophobic amino acid stretch which may serve as a membrane anchor (Ikonen *et al.*, 1995; Tatsuta *et al.*, 2005). Deletion of each of both stretches drastically reduces the import of Phb2 (Tatsuta *et al.*, 2005). Unlike Phb2, the N-terminal region of Phb1 lacks characteristics of mitochondrial presequences. Nevertheless, the first 28 amino acids have been shown to be essential and sufficient for mitochondrial import in yeast (Tatsuta *et al.*, 2005). The stable insertion of Phb1 and Phb2 in the inner membrane depends on the Tim23-translocase, which is also responsible for the insertion of other presequence carrying proteins (Neupert, 1997; Pfanner and Geissler, 2001). The import of Phb1 and Phb2 is drastically reduced in mitochondria from cells harboring decreased levels of Tim23 (*tim23^{ts})* and depletion of Tim23 results in no detectable levels of Phb1 and Phb2 *in vivo* (Tatsuta *et al.*, 2005).

The newly imported Phb1 and Phb2 subunits form assembly intermediates of ~120 kDa (Tatsuta *et al.*, 2005). These intermediates are detectable on BN-SDS PAGE of imported radio labeled Phb1 or Phb2 in wild type cells but their exact composition is unclear. Along with Phb1 and Phb2, Tim13 can also be detected within such an intermediate although it is not essential for import of Phb1 and Phb2 in mitochondria (Tatsuta *et al.*, 2005). The 120 kDa intermediates oligomerize into the mature Phb1-Phb2 complex. Within this complex, Phb1 and Phb2 are anchored to the inner mitochondrial membrane by their N-terminal ends and expose C-terminal ends to the intermembrane space (Figure 6C) (Ikonen *et al.*, 1995; Berger and Yaffe, 1998; Coates *et al.*, 2001). As judged by gel filtration analysis and BN-SDS PAGE the complex has a molecular weigh between 1.000 kDa and 1.200 kDa (Steglich *et al.*, 1999; Tatsuta *et al.*, 2005). Therefore, it was proposed that the Phb1-Phb2 complex is built up of 12 to 18 subunits (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000; Back *et al.*, 2002). Additionally, crosslinking experiments show that Phb1 and Phb2 molecules are alternatingly assembled (Figure 6A and 6B) as no crosslinks can be observed between two Phb1 or two Phb2 molecules (Back *et al.*, 2002). Most of the crosslinks are concentrated in the C-terminal region of Phb1 and Phb2 delineating it as the interaction site. Indeed, the C-terminal regions of Phb1 (amino acids 180–224) and Phb2 (amino acids 212–253) form computationally predictable coiled-coil regions whose disruption prevents formation of the mature Phb1-Phb2 complex, but do not affect the formation of the 120 KDa intermediates (Tatsuta *et al.*, 2005). Thus, interactions between the coiled-coil regions of Phb1 and Phb2 are necessary for the

complex formation. Single particle electron microscopy revealed that the Phb1-Phb2 complex has a roughly a circular equatorial projection with a central cavity and dimensions ~260-170 Å (Tatsuta *et al.*, 2005) (Figure 6D). The crystal structure of the Phb1-Phb2 complex as well as that of the subunits are currently unknown.

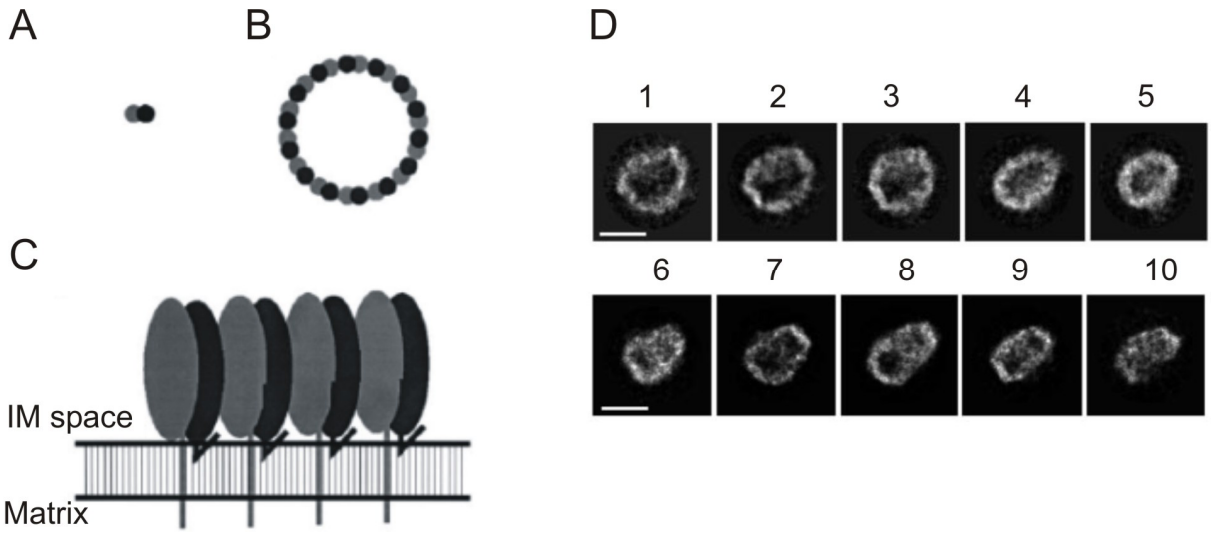

Figure 6. A representation of the superstructure of the prohibitin complex. A. Dimeric building block of Phb1 and Phb2 (top view). **B**. Proposed circular arrangement of the building blocks (top view). **C**. A section of four building blocks and the mitochondrial inner membrane (side view). IM space, intermembrane space. reprinted from (Back *et al.*, 2002). **D** Single particle EM-analysis of purified Phb1-Phb2 complex. Dataset is split into elliptical ring-like (top, 1-5) and rectangular-like (bottom, 6-10) subsets. Bar, 200 Å**.** reprinted from (Tatsuta *et al.*, 2005).

2.6.2. Role of prohibitins for mitochondrial morphology and inheritance

The function of the Phb1-Phb2 complex in the inner mitochondrial membrane is only partially understood. In contrast to lethal phenotypes in *C. elegans* and mammals (Artal-Sanz *et al.*, 2003; Park *et al.*, 2005), yeast cells lacking prohibitins grow as wild type cells on different carbon sources (Berger and Yaffe, 1998; Steglich *et al.*, 1999). Mitochondrial morphology and inheritance are not affected in prohibitin null mutants (Berger and Yaffe, 1998). Only in aged cells, the absence of prohibitins leads to shortening of the replicative life span and an overall decrease in the mitochondrial membrane potential (Coates *et al.*, 1997). These effects are attributed to a delayed, but not absent, segregation of mitochondria from old mother to daughter cells (Piper *et al.*, 2002). However, deletion of prohibitins is lethal in combination with mutations

in components of the mitochondrial maintenance system, including *MMM1*, *MDM10* and *MDM12* (Berger and Yaffe, 1998).

In *S. cerevisiae*, Mmm1, Mdm10 and Mdm12 function for the regulation of mitochondrial morphology and inheritance (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Boldogh *et al.*, 1998). The three proteins seem to form a complex in the outer mitochondrial membrane responsible for linking mitochondria to the actin cables during cell division (Boldogh *et al.*, 1998; Boldogh *et al.*, 2003). Additionally, this complex is functionally linked to the stability and/or assembly of *mt*DNA nucleoids as the respective deletion mutants are characterized with high levels of petite formation due to loss of *mt*DNA (Hobbs *et al.*, 2001; Hanekamp *et al.*, 2002; Boldogh *et al.*, 2003). Therefore, the Mmm1-Mdm10-Mdm12 complex (MMM) has been proposed to function as membrane integrated machinery for simultaneous inheritance of mitochondria and *mt*DNA (Hobbs *et al.*, 2001; Boldogh *et al.*, 2003).

Deletion of either *PHB1* or *PHB2* in Δ*mmm1*, Δ*mdm10* and Δ*mdm12* cells is lethal (Berger and Yaffe, 1998). As single deletions are viable it is possible that prohibitins function in a pathway redundant to the function of the Mmm1-Mdm10-Mdm12 complex. Phb1-Phb2 may serve as a structural scaffold which stabilizes, but is not essential for the contact between components of the MMM-machinery from the inner and the outer mitochondrial membrane. However, the molecular mechanism by which Phb1 and Phb2 affect mitochondrial morphology is not known since stable interaction between prohibitins and any of the MMM-components have not been reported.

A role of prohibitins in mitochondrial morphology has also been shown in *C.elegans*. siRNA knock-down of Phb1 results in altered mitochondrial morphology and reduced oxygen consumption in the body wall muscle (Artal-Sanz *et al.*, 2003).

2.6.3 Role of prohibitins during proteolysis in mitochondria

The first direct indication for the molecular function of prohibitins came with the observation that Phb1 and Phb2 co-elute with Yta10 and Yta12 at apparent molecular weight of ~2.000 kDa in wild type cells (Steglich *et al.*, 1999). In line with this, both Phb1 and Phb2 can be precipitated with Yta10- or Yta12-specific antibodies indicating the formation of a supercomplex between the Phb1-Phb2 complex and the *m*AAA protease (Steglich *et al.*, 1999). Moreover, deletion of

prohibitins affects the stability of unassembled mitochondrial proteins further accentuating a role of prohibitins during proteolysis.

The stability of Cox3, but not Cox2, is decreased after deletion of *PHB1* and *PHB2* in *Δcox4* cells (Steglich *et al.*, 1999). Since the assembly of the cytochrome *c* oxydase (COX complex) is impaired in Δ*cox4* cells, the newly synthesized Cox2 and Cox3 subunits are subjected to proteolytic removal by the mitochondrial proteases (Nakai *et al.*, 1994). While Cox2 is a substrate for the *i*AAA protease (Nakai *et al.*, 1995), Cox3 is degraded by the *m*AAA protease (Arlt *et al.*, 1996; Guzelin *et al.*, 1996), indicating a specific function of prohibitins for the stability of substrates for the *m*AAA protease (Steglich *et al.*, 1999). Similarly, unassembled Atp6, Atp8 and Atp9, which are also substrates for the *m*AAA protease (Guzelin *et al.*, 1996), are rapidly degraded in the absence of prohibitins (Steglich *et al.*, 1999). Therefore, together with structural and functional similarities to the bacterial HflK/C proteins it was proposed that prohibitins function as modulators of the activity of the *m*AAA protease (Steglich *et al.*, 1999).

This hypothesis, however, can not explain why prohibitins are needed for growth in the absence of either the *i*AAA protease or the *m*AAA protease. Δ*yta10*Δ*phb1* and Δ*yta10*Δ*phb2* cells are unviable (Steglich *et al.*, 1999) whereas Δ*yta10* and Δ*yta12* cells have only moderately affected growth on glucose (Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994). Synthetic lethal are also Δ*yme1*Δ*phb1* and Δ*yme1*Δ*phb2* cells while *Δyme1* grows normally under identical conditions (Steglich *et al.*, 1999). Therefore prohibitins must have an additional function in mitochondria.

Interestingly, the prohibitin complex can bind substrates of both the *i*AAA protease and the *m*AAA protease. Co-immunoprecipitation and BN-SDS page analysis of mitochondrial lysates identified Cox2 and Cox3 as binding partners for prohibitins (Nijtmans *et al.*, 2000). Moreover, both substrates are stabilized in cells overexpressing prohibitins when compared to Δ*phb1*Δ*phb2* cells. The ability to bind and stabilize substrates and the very low similarity to members of the Hsp60 family has led to the proposal that prohibitins function as novel membrane bound chaperones (Nijtmans *et al.*, 2000). According to this scenario, prohibitins may function as a holdase which assists protein folding. Such a chaperone may provide a crossroad between the assembly of properly folded substrates into the COX complex and proteolytic breakdown by passing the missfolded substrate directly to the

protease (Nijtmans *et al.*, 2000). However, prohibitins are not essential for the assembly of the respiratory chain complexes in yeast as no growth phenotype or assembly defects can be observed in Δ*phb1*Δ*phb2* cells.

II. AIMS OF THESIS

1. Role of prohibitins during proteolysis in yeast

Two main possibilities emerge to explain the functions of prohibitins in proteolysis.

i) Prohibitins act as negative regulators of the activity of the *m*AAA protease through direct interaction with it (Steglich *et al.*, 1999).

ii). Prohibitins are general chaperones for the assembly of the respiratory chain complexes or chaperone system acting during proteolysis (Nijtmans *et al.*, 2000).

To examine these possibilities further, model substrates of the *m*AAA protease will be overexpressed and their stability will be assessed in cells harboring different levels of prohibitins.

Additionally, the possibility that prohibitins regulate the proteolytic activity of the *m*AAA protease by binding in a nucleotide dependant manner will be examined. Therefore, mutant subunits which stabilize the *m*AAA protease in nucleotide-free or ATP-bound conformation will be generated. The ability of the *m*AAA protease, harboring such subunits, to bind prohibitins will be examined by glycerol gradient centrifugation and BN-SDS PAGE.

2. Assembly of prohibitins in mammalian mitochondria

The conservation of prohibitins between yeast and mammals suggests that the mammalian Phb1-Phb2 complex may also play a role during proteolysis. Therefore, the interaction of prohibitins with the murine *m*AAA protease(s) will be examined by sizing chromatography and co-immunoprecipitation experiments using mitochondria isolated from murine liver.

These experiments should also provide an insight into the subunit composition of murine *m*AAA protease(s).

III Materials and methods

1. Nomenclature of prohibitins

Prohibitins are termed differently in various organisms. The table below summarizes the names commonly used for both proteins. For clarity, in this work the two homologues will be designated as Prohibitin 1 (Phb1) and Prohibitin 2 (Phb2), respectively.

HOMOLOG	NAME	ORGANISM / KINGDOM	REFERENCE
Prohibitin 1	Phb1	Yeast	(Coates *et al.*, 1997; Berger and Yaffe, 1998)
	Prohibitin, BAP32	Mammalia	(Terashima *et al.*, 1994)
	I(2)37Cc (*Cc*)	*Drosophila*	(Eveleth and Marsh, 1986)
Prohibitin 2	Phb2	Yeast	(Coates *et al.*, 1997; Berger and Yaffe, 1998)
	Prohibiton BAP37, REA	Mammalia	(Terashima *et al.*, 1994; Montano *et al.*, 1999)

2. Materials

2.1. Yeast strains

STRAIN	GENOTYPE	PLASMID	SOURCE / COMMENT
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100*		
YGS402	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 phb1::HIS3*		(Steglich, 2000)
YHA101	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 YTA10::URA3*		(Arlt *et al.*, 1996)
YHA 201	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta12::HIS3*		(Arlt *et al.*, 1996)

YMM1	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 PHB1::HIS3*	pESC-URA3-P_{GAL1}*oxa1^{ts}* pYX142-*TPI* pYX143-*TPI*	This study *oxa1^{ts}* overexpression
YMM2	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100*	pESC-URA3 P_{GAL1}*oxa1^{ts}* pYX142-*TPI-PHB1* pYX143-*TPI-PHB2*	This study *oxa1^{ts}* overexpression Phb1 and Phb2 overexpression
YMM3	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 PHB1::HIS3*	pESC-URA3-P_{GAL1}*oxa1^{ts}* pYX142-*TPI* pYX143-*TPI*	This study *oxa1^{ts}* overexpression
YMM4	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta10::URA3*	Ycplac22 – *EP* YTA10*	This study *YTA10* endogenous promoter
YMM5	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta10::URA3*	Ycplac22 – *EP* yta10^{E388Q}*	This study *yta10^{E388Q}* endogenous promoter
YMM6	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta10::URA3*	Ycplac22 – *EP* yta10^{K334A}*	This study *yta10^{K334A}* endogenous promoter
YMM7	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100*	pESC-URA3 P_{GAL1}*yme2ΔC* pYX142-*TPI* pYX143-*TPI*	This study *yme2ΔC* overexpression
YMM8	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100*	pESC-URA3 P_{GAL1}-*yme2ΔC* pYX142-*TPI-PHB1* pYX143-*TPI-PHB2*	This study *yme2ΔC* overexpression Phb1 and Phb2 overexpression
YMM9	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 PHB1::HIS3*	pESC-URA3-P_{GAL1}-*yme2ΔC* pYX142-*TPI* pYX143-*TPI*	This study *yme2ΔC* overexpression
YMM10**	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta12::HIS3*	YCplac111:*ADH1 -YTA12*	This study
YMM11**	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta12::HIS3*	YCplac111:*ADH1 - yta12^{K394A}*	This study
YMM12**	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 yta12::HIS3*	YCplac111:*ADH1 -12^{E448Q}*	This study

YTT69	*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 phb1::HIS3, phb2:: KAN*	pESC-URA3 P_{GAL1}*PHB1* P_{GAL10} *PHB2*	*PHB1 PHB2* overexpression
oxa1^{ts} (pet ts1402)	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 oxa1^{ts} L240S*		(Hell *et al.*, 1997)
YGS418	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 oxa1^{ts} L240S*		G. Steglich, unpublished
YGS507	*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100 phb1::HIS3, phb2:: KAN*	pESC-URA3	(Steglich *et al.*, 1999)

*, endogenous promoter (*EP*)

**, YHA201 background

ADH1-yeast alcohol dehydrogenase promoter

TPI - yeast triosephosphate isomerase promoter

2.2. Mouse strains

Mouse strains from 129Sv, C57BL/6 or mixed C57BL/6-129Sv genetic background were used as wild type or paraplegin-deficient (SPG7$^{-/-}$) (Ferreirinha *et al.*, 2004). The SPG7$^{-/-}$ mouse strain was generated by targeting of exon 1 and exon 2 of the SPG7 locus with a cassette containing a neomycin resistance gene flanked by two lox sites and homologous recombination (Ferreirinha *et al.*, 2004).

2.3. *E. coli* strains

The strains DH5α [*supE*44 Δ*lac*U169(ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] and MC1061 [hsdR *mcr*B *araD*139Δ(*araABC-leu*)7697 Δ*lacX*74 *gal*U *gal*K *rps*L *thi*] were used during the cloning procedures transformation competent cells.

The previously described *E. coli* B strain BL21(DE3)/pLysS [F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal*λ(DE3) (pLysS Camr)] (Stratagene, La Jolla, CA) (Studier *et al.*, 1990) was used for expression of Yta12MD, Phb1 and Phb2 and isolation of inclusion bodies. The advantage of this strain is that pLysS weakly expresses T7 lysozyme to facilitate lysis and to reduce activity of T7 RNA polymerase produced before induction (Studier *et al.*, 1990). The cells are grown in media containing chloramphenicol.

2.4. Plasmids

PLASMID NAME	ORF	SOURCE / COMMENT
pKM263-HIS$_6$(*TEV*)GST		pKM263 vector allowing IPTG inducible expression of 6xHIS and GST fusion proteins in *E. coli* (Melcher, 2000) . 6HIS and GST are separated by a *TEV* cleavage site
pKM263-HIS$_6$(*TEV*)GST	*yta12MD*	This work
	PHB1	Dr. T.Tatsuta (unpublished)
	PHB2	
pGEM4	*yme2ΔC*	(Leonhard *et al.*, 2000)
pGEM-T		Promega
pGEM-T	*oxa1^{ts}*	This work
pESC-URA3		Multi copy yeast expression plasmid for galactose inducible overexpression, P_{GAL1} (*GAL1* promoter) and P_{GAL10} (*GAL10* promoter)
pESC-URA3	*oxa1^{ts}*	This study *GAL1* promoter
	yme2ΔC	
	PHB1, PHB2	inducible expression under the *GAL1* and *GAL10* promoters (Tatsuta *et al.*, 2005)
YCplac22; YCplac22:*ADH1* YCplac111:*ADH1*		Centromeric yeast expression plasmid without or with yeast alcohol dehydrogenase (*ADH1*) promoter
pYX142:*TPI* pYX132:*TPI*		Centromeric yeast expression plasmid with triosephosphate isomerase (*TPI*) promoter
pYX142:*TPI*	*PHB1*	(Tatsuta *et al.*, 2005)
pYX132:*TPI*	*PHB2*	(Tatsuta *et al.*, 2005)
YCplac22	*EP- YTA10*	This study EP, endogenous promoter of *YTA10*
	EP- yta10^{K334A}	
	EP- yta10^{E388Q}	
YCplac22:*ADH1*	*YTA10*	Wild type (Steglich, 2000)
	yta10^{K334A}	ATP binding mutant in the Walker A motif (Steglich, 2000)
	yta10^{E388Q}	ATP hydrolysis mutant in the Walker B motif (Steglich, 2000)
	yta10^{E559Q}	Proteolytic mutant of Yta10 (Arlt *et al.*, 1998)
YCplac111:*ADH1*	*YTA12*	Wild type (Steglich, 2000)
	yta12^{K394A}	ATP binding mutant in the Walker A motif (Steglich, 2000)
YCplac111:*ADH1*	*yta12^{E448Q}*	ATP hydrolysis mutant in the Walker B motif (Steglich, 2000)
	yta12^{E614Q}	Proteolytic mutant of Yta12 (Arlt *et al.*, 1998)

2.5. Oligonucleotides

ORF	PRIMER NAME	SEQUENCE (5' - 3')
yta12MD	TL1280	CGG GAT CCC GTC AGT TTG TAG ATG GCT TAG G
	TL1281	CTA GAC CAT GGG AGG CAT ATT TGG TCT GAG
oxa1^{ts}	TL1386	CGC GGA TCC CGA TGT TCA AAC TCA CCT CTC
	TL1388	CCG CTC GAG TCA TTT TTT GTT ATT AAT GAA G
yme2ΔC	TL713	GGG GGA TCC ATG TTG CTA GTA CGA ACG
	TL714	GGG AAG CTT TCA GCA TTT GTG AGT GAT CTT CTG
EP-YTA10	TL680	GGG AAG CTT GTG TGT CCA ATT ACG CGC AC
	TL1561	CCC CTC TAG AGT GTG TCC AAT TAC GCG CAC
	TL601	GGG GAA TTC TTA ATT TGT TGC TGC AGG TGC

2.6. Enzymes and chemicals

Enzymes and chemicals were commercially obtained and used according to the suppliers' recommendation.

2.7. Buffers

BUFFER	COMPOSITION
10 x PBS	80 g/l NaCl 2 g/l KCl 26,8 g/l Na_2HPO_4-$7H_2O$ 2,4 g/l KH_2PO_4 pH 7,4 adjusted with HCl
10 x TBS	12 g/l Tris 90 g/l NaCl pH 7,4 adjusted with HCl
1M potassium phosphate buffer (KPi)	(Sambrook *et al.*, 1989)
1M sodium phosphate buffer (Na-phosphate)	(Sambrook *et al.*, 1989)
SDS-PAGE sample buffer	50 mM Tris/HCl, pH 6,8 1% (v/v) β-mercaptoethanol 2% (w/v) SDS 0,01% (w/v) bromphenol blue 10% (w/v)glycerin
10 x SDS-PAGE buffer	10 g/l SDS 30,3 g/l Tris 144,1 g/l glycine
Blotting buffer	0,2 l/l methanol 0,2 g/l SDS 2,42 g/l Tris 11,3 g/l glycine
10 x ponceauS	0,2 g/l ponceauS

	0,3 g/l TCA 0,3 g/l sulfosalicylic acid
SHKCl	0,6 M Sorbitol 50 mM HEPES 80 mM KCl

The composition of assay specific buffers is indicated elsewhere.

2.8. Yeast and *E.coli* growth media

MEDIA	COMPOSITION
LB medium	1% (w/v) bacto-trypton 0,5% (w/v) bacto-yeast extract 1% (w/v) NaCl for LB-agar plates 1,5% (w/v) bactoagar** for LB-ampicillin-medium 100 µg/ml ampicillin added to sterile LB-medium
Synthetic complete (SC) medium	6,7 g/l yeast nitrogen base (YNB) with ammonium sulfate 1,5 g/l drop-out mix Carbon source: 2% (w/v) glucose* 2% (w/v) raffinose* 2% (w/v) galactose* Selection markers were added to sterilized media: adenine (40 µg/ml) histidine (20µg/ml) leucine (60 µg/ml) lysine (30 µg/ml) tryptophan (40µg/ml) uracil (20 µg/ml) 2% (w/v) glucose or 2% (w/v) galactose for SC-agar plates 2% (w/v) bactoagar
Drop-out mix	asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, methionine, isoleucine, phenylalanine, proline, serine, threonine, tyrosine, valine, myo-inositol [all 6,12% (w/w)], alanine [1,53% (w/w)], and p-aminobenzoic acid [0,61% (w/w)]
YP medium	1% (w/v) bactoyeast extract 2% (w/v) bactopepton pH adjusted to 5,5 with HCl carbon source: 2% (w/v) glucose, galactose or glycerol up to 0,05% lactate* pH adjusted to 5,5 with NaOH for YP-agar plates 2% (w/v) bactoagar**
* **Carbon source**	40% (w/v) glucose 20% (w/v) raffinose 30% (w/v) galactose 40% (w/v) lactate pH adjusted to 5,5 with NaOH prepared separately and added to sterilized media

** **Bactoagar**	Bactoagar was added to media and pH was adjusted before sterilization

3. Methods

3.1. Molecular biology and genetics methods

3.1.1. DNA cloning, manipulation and purification

Cloning of DNA fragments, PCR, gel electrophoresis and isolation of DNA fragments were done according to (Sambrook and Russell, 2001). When commercially available materials were used, the manufacturers' recommendations were followed. chemically competent *E. coli* cells (3.2.2.) were used.

Cloning of the matrix-exposed domain of Yta12 (Yta12MD)

For expression in *E.coli*, DNA fragment, corresponding to amino acid region 324-825 of the Yta12 protein, was amplified from genomic DNA by primers TL1280 and TL1281 and cloned into the *NcoI-BamHI* sites of pKM263-HIS_6(*TEV*)GST resulting in pKM263-HIS_6(*TEV*)GST-*yta12MD*

Cloning of *oxa1^{ts}* in pESC-URA3

For galactose inducible overexpression of *oxa1^{ts}*, the corresponding gene was amplified from genomic DNA with primer pair TL1386/TL1388. The purified DNA fragments were cloned into the *BamHI-XhoI* sites of pESC-URA (Stratagene, La Jolla,CA) generating pESC-URA-*oxa1^{ts}*

Cloning of *yme2ΔC* in pESC-URA3

yme2ΔC was amplified from pGEM4-*yme2ΔC* (Leonhard *et al.*, 2000) by primer pair TL713/TL714. The purified DNA fragments were cloned into the *BamHI-HindIII* sites of pESC-URA generating pESC-URA-*yme2ΔC*.

Cloning of *YTA10, yta10^{K334A} and yta10^{E388Q}* in YCplac22

For expression of *YTA10* under the control of *YTA10* endogenous promoter, *YTA10* DNA fragment was amplified from genomic DNA by primer pairs: TL680/TL601 or TL1561/TL601 and cloned into pGEM-T vector (Promega, USA) resulting in pGEM-T-*EP-YTA10*. *EP-YTA10* was isolated by endonuclease treatment with *HindIII or XbaI-*

HindIII and cloned into the *HindIII* or *XbaI-HindIII* cloning sites of YCplac22, resulting in YCplac22-*EP-YTA10*(*HindIII*) and YCplac22-*EP-YTA10*(*XbaI-HindIII*). Endonuclease treatment of YCplac22:*ADH1-yta10*^{K334A} and YCplac22:*ADH1-yta10*^{E388Q} (Steglich, 2000) generated DNA fragments containing K334A and E388Q mutations in the Walker motifs of *YTA10*. The fragments were cloned into the internal *NcoI* and *StuI* sites of YCplac22-*EP-YTA10*(*XbaI-HindIII*) resulting in YCplac22-*EP-yta10*^{K334A} and YCplac22-*EP-yta10*^{E388Q}.

3.1.2. Preparation and transformation of chemically competent *E. coli* cells

Single *E. coli* (DH5α, MC1061 or BL21(DE3)/pLysS) colony, grown on LB-agar plate, was inoculated in 5 ml liquid LB medium and incubated for 12 hrs, 37°C. The culture was next diluted in 100 ml LB medium and further incubated to an OD_{600} ~0,8. After cooling down of the culture on ice the cells were collected by centrifugation (5.000 rpm, 10 min, 4°C) and resuspended in 40 ml RF1 buffer. The cells were next isolated by centrifugation (5.000 rpm, 10 min, 4°C) and resuspended in 4 ml RF2 buffer. 100 µl aliquots were distributed in 1,5 ml eppendorf tubes and frozen in liquid nitrogen. The competent cells were stored at -70°C.

BUFFER	COMPOSITION
RF1-Buffer	100 mM RbCl 50 mM $MnCl_2$ 30 mM Potassium acetate 10 mM $CaCl_2$ 15% (w/v) glycerin pH adjusted to 5,8 with HCl filter-sterilized
RF2-Buffer	10 mM MOPS 10 mM RbCl 75 mM $CaCl_2$ 15% (w/v) glycerin pH adjusted to 6,8 with NaOH. filter-sterilized.

Transformation of competent cells

Competent cells were incubated with 0,1 µg plasmid DNA for 20 min on ice. After incubation for 90 sec at 42°C, 1ml LB medium without antibiotic was added and the

cells were further incubated for 60 min at 37°C with gentle mixing. Finally, the cells were plated on LB-agar plates with antibiotic.

3.1.3. High efficiency yeast transformation

Plasmid DNA or DNA for gene disruption were transformed in yeast cells by the LiAc procedure according to (Gietz *et al.*, 1995; Gietz and Woods, 2005).

3.1.4. Isolation of genomic DNA from *S. cerevisiae*

A single yeast colony, grown on YPD plate, was inoculated in 5 ml YPD liquid medium and incubated to an OD_{600} ~10. The cells were isolated by centrifugation (4.000 rpm, 5 min), washed with 10 ml 1,2 M sorbitol and resuspended in 10 ml buffer A supplied with 0,5 ml β-mercaptoethanol. After incubation for 15 min at room temperature the cells were reisolated (4.000 rpm, 5 min) and washed with buffer A without β-mercaptoethanol. The cells were next resuspended in 10 ml buffer A containing 2% (w/v) zymolyase and incubation for 30 min at 30°C. The cells were isolated by centrifugation (4.000 rpm, 5 min), resuspended in 0,5 ml buffer B and incubated for 15 min at 70°C. 5M K-acetate was added to final concentration 0,5 M followed by incubation on ice for 30 min. Precipitated SDS and proteins were removed by centrifugation (13.000 rpm, 15 min, 4°C). The supernatant, containing DNA, was transferred to new tube and the precipitation with K-acetate was repeated. Finally, DNA was precipitated from the supernatant with 1ml 95% (v/v) ethanol and isolated by centrifugation (13.000 rpm, 5 min). The DNA was dried for ~10 min at room temperature and dissolved in 250 µl of TE buffer supplemented with RNAse A (10 mg/ml) to final concentration 0,04 mg/ml. After incubation for 30 min at 37°C, 20 µl 3M Na-acetate and 220 µl isopropanol (kept at -20°C) were added to the suspension, mixed by inversion and centrifuged (13.000 rpm, 5 min). The pellet, containing genomic DNA, was dried for 30 min at 37°C and resuspended in 50 µl TE buffer. The quality of the purification was estimated by agarose gel electrophoresis.

BUFFER	COMPOSITION
Buffer A	1,2 M sorbitol 10 mM EDTA 100 mM Na-citrate pH 8,0

Buffer B	50 mM EDTA 0,2% (w/v) SDS pH 8,0
TE buffer	10 mM Tris/HCl 1 mM EDTA pH 8,0

3.2. Biochemical methods

3.2.1. Estimation of protein concentration

Protein concentration was estimated according to (Bradford, 1976) and with Protein Assay System (BioRad Laboratories). Alternatively, concentration of proteins in inclusion bodies (3.2.4) was determined by SDS-PAGE and densitometry. Serial dilutions of the preparations together with BSA samples with known concentrations were subjected to SDS-PAGE. Proteins were visualized by staining with PageBlue™ protein staining solution (Fermentas) and quantified by densitometry (LI-COR® Biosciences).

3.2.2. TCA precipitation

Proteins were precipitated with 3-chloracetic acid [TCA, final concentration ~12 % (w/v)] for at least 60 min on ice. The samples were centrifuged at 4°C, 18,000 rpm for 20 min and washed two times with ice cold Acetone. Pellets were dried at 56°C for 5 min and resuspended in SDS-PAGE sample buffer (Laemmli *et al.*, 1970).

3.2.3. SDS-PAGE and western blotting

Preparative or analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and semi-dry western blotting were carried out as described previously (Laemmli *et al.*, 1970; Sambrook and Russell, 2001). The BioRad mini-gel system was used.

3.2.4. Expression of proteins in *E. coli* and isolation of inclusion bodies

HIS_6(*TEV*)GST-*yta12MD*, HIS_6(*TEV*)GST-*PHB1* and HIS_6(*TEV*)GST-*PHB2* fusion proteins were expressed in *E. coli* BL21(DE3)/pLysS cells and isolated as inclusion bodies.

Single colony of BL21(DE3)/pLysS *E.coli* strain, transformed with the corresponding expression plasmids, was inoculated into 20 ml LB medium supplemented with 0.1 mg/ml chloramphenicol and incubated for 24 hrs at 37°C. 5 ml of the culture were diluted in 40 ml LB medium containing chloramphenicol (0.1 mg/ml) and further incubated at 37°C to an OD_{600} ~0,8. IPTG (0.1 M) was added to final concentration 1 mM and the cells were incubated at 24°C for 3 hrs.

Cells were collected by centrifugation and the cell pellet was frozen in liquid nitrogen. The cells were then thawed at room temperature and resuspended in 1 ml buffer A supplied with lysozyme (10 mg/ml) to a final concentration of 0,2 mg/ml. After incubation for 30 min on ice, Triton X-100 was added to a final concentration of 0,5 % (w/v). The samples were next supplied with 5 μl DNAse I and incubated on ice for 30 min followed by sonication (5 min, level 6, 50% duty cycle, microtip).

Inclusion bodies were isolated by centrifugation (22,000 xg, 60 min, 4°C). Three consecutive washings were performed by resuspending of the inclusion bodies in 1 ml buffer B and centrifugation (22.000 xg, 30 min, 4°C). The inclusion bodies were next washed with buffer C and resuspended in 2 ml buffer D. After clarifying ultracentrifugation (100.000 xg, 60 min, 4°C) the supernatant was transferred to new tubes and stored at -80°C.

Determination of protein concentration

To estimate the protein concentration serial dilutions of the preparations were subjected to SDS-PAGE and densitometrically compared with known amounts of BSA (3.2.1.).

BUFFER	COMPOSITION
Buffer A	50 mM Tris-HCl 100 mM EDTA 0,2 mM PMSF pH 8,0
Buffer B	100 mM NaCl 0,2 mM PMSF 50 mM Na-phosphate buffer 1 mM EDTA 1 mM β-mercaptoethanol 1 M urea 1% Triton X100 pH 7,4

Buffer C	100 mM NaCl 0,2 mM PMSF 50 mM Na-phosphate buffer 1 mM EDTA 1 mM β-mercaptoethanol pH 7,4
Buffer D	20 mM HEPES 8 M urea pH 7,4

3.2.5. BN-PAGE and second dimension SDS-PAGE

Blue native PAGE (BN-PAGE) was performed essentially as described previously (Schagger, 2001). Briefly, isolated mitochondria (100 μg) were lysed in lysis buffer at concentration 5 mg/ml by vigorous mixing for 20 min at 4°C. Samples were pre-cleared by centrifugation (18.000 rpm, 10 min, 4°C) and the supernatant was supplied with coomassie loading buffer to achieve a detergent : coomassie ratio of 1:10. Next, the protein complexes were separated according to their native molecular weight within a 3%–16% native polyacrylamide gel without stacking gel (first dimension). When second dimension was intended (BN-SDS PAGE), lanes from the first dimension were cut out, incubated for 10 min in SDS-PAGE sample buffer without β-mercaptoethanol and subjected to SDS-PAGE. The migration of the ATP synthase dimer (~750 kDa), ATP-synthase monomer (~1.500 kDa), Complex I monomer (~1.000 kDa) and Complex I - Complex III supercomplex (~1.500 kDa) were used for calibration (Schagger and Pfeiffer, 2000).

BUFFER	COMPOSITION
Lysis buffer	2% (w/v) digitonin 50 mM NaCl 10% (w/v) glycerol 5 mM 6-aminohexanoic acid 50 mM imidazole pH 7,0
Coomassie loading buffer	50 mM NaCl 10% (w/v) glycerol 5 mM 6-aminohexanoic acid 50 mM imidazole pH 7,0 5% (w/v) coomassie® brilliant blue G250 (SERVA)

3.2.6. Gel filtration chromatography

Gel filtration chromatography of mitochondrial extracts was performed as described previously (Arlt *et al.*, 1996; Steglich *et al.*, 1999) with modifications. Mouse liver mitochondria (1 mg) were lysed in lysis buffer at concentration 5 mg/ml by vigorous vortexing (20 min, 4^{o}C). After clarifying ultracentrifugation (45.000 rpm, 15 min, 4^{o}C) the supernatant was loaded on Superose-6 gel filtration column equilibrated with buffer A. Fractions (0,5 ml) were collected and subjected to TCA precipitation. The proteins of interest were detected immunologically after SDS-PAGE and western blotting. Laser densitometry (LI-COR Biosciences) was used to quantify the signals in each elution fraction. To avoid the previously reported broad elution profiles of paraplegin and Afg3l2 (Atorino *et al.*, 2003), elution profiles were calculated for 1 ml elution fractions (the sum of the signals for every two 0,5 ml fractions). Protein standards [Thyroglobulin, 669 kDa (13,1 ml); Appoferritin, 443 kDa (14,9 ml); Alcohol dehydrogenase (ADH), 150 kDa (16,9 ml) and BSA, 66 kDa (20,4 ml)] together with the monomeric and dimeric ATP-synthase [monomer ~ 750 kDa (13 ml) and dimer ~1.500 kDa (11,5 ml) (Schagger and Pfeiffer, 2000)] were used for calibration.

BUFFER	COMPOSITION
Lysis buffer	2% (w/v) digitonin or 0,5% (w/v) Triton-X100 50 mM NaCl 50 mM potassium phosphate buffer (KPi) 4 mM Mg-acetate 10% (w/v) glycerol 1 mM EDTA 1 mM PMSF protease inhibitor mix pH 7,0
Buffer A	0,5% (w/v) digitonin or 0,05% (w/v) Triton-X100 50 mM NaCl 50 mM KPi 4 mM Mg-acetate 10% (w/v) glycerol 1 mM EDTA 1 mM PMSF pH 7,0

3.2.7. Glycerol gradient ultracentrifugation

Glycerol gradients were prepared by overlaying 2 ml 10% glycerol solution on top of 2 ml 30% glycerol solution and incubation in horizontal position for 5 hrs at 4°C. Mitochondria were lysed with vigorous shaking in lysis buffer at concentration 2 mg/ml (20 min, 4°C). After clarifying ultracentrifugation (45.000 rpm, 15 min, 4°C), the supernatant was applied on top of the gradient.

Ultracentrifugation of the gradients was performed for 2,5 hrs at 50,000 rpm, 2°C. Ten 400 μl fractions were collected from the top of the gradients and subjected to TCA precipitation and SDS-PAGE. The fractions were analyzed by immunoblotting.

BUFFER	COMPOSITION
10% and 30% glycerol gradient solutions	0,2% (w/v) digitionin PBS 1 mM ATP 4 mM Mg-acetate 1 mM EDTA 10% or 30% (w/v) glycerol 1 mM PMSF complete protease inhibitor
Lysis buffer	1,5% (w/v) digitionin PBS 1 mM ATP 4 mM Mg-acetate 1 mM EDTA 10% (w/v) glycerol 1 mM PMSF complete protease inhibitor

3.2.8. Analysis of protein stability

3.2.8.1. Pulse chase analysis in isolated mitochondria (in organello)

Pulse-chase in isolated mitochondria was used to examine the stability of unassembled mitochondrial translation products.

Pulse: Radioactive labeling of mitochondrially encoded proteins was performed as described previously (McKee *et al.*, 1984; McKee and Poyton, 1984; McKee and Poyton, 1984a; Langer *et al.*, 1995) in the presence of [^{35}S]-labeled Methionine (MP Biomedicals). The reaction was stopped by addition of puromycin to a final concentration of 50 μg/ml and non-radioactive L-methionine.

Chase: Mitochondrial pellets were resuspended in translation buffer and further incubated at 30°C for different time lengths. Aliquots were withdrawn at each time point and put on ice. Mitochondria were reisolated after centrifugation (13.000 rpm, 10 min, 4°C), washed twice with SHKCl and resuspended in 100 µl SHKCl. Incorporated radioactivity was isolated within the TCA-insoluble fraction after TCA-precipitation. [^{35}S]-labeled methionine and peptides generated during proteolysis were in the TCA-soluble fraction (Augustin *et al.*, 2005). The pellets after TCA-precipitation were resuspended in SDS-PAGE sample buffer or Ultima-Gold scintillation cocktail (Perkin Elmer, USA) and analyzed by autoradiography or scintillation counting, respectively.

BUFFER	COMPOSITION
Translation buffer	225 mM potassium phosphate buffer 0,9 M sorbitol 30 mM Tris-HCl 19 mM $MgSO_4$ 6 mM ATP 2 mM NADH 7,5 mM α-ketoglutarate 7,5 mM phosphoenolpyruvate 150 mM of all amino acids except methionine 4,5 mg/ml fatty acid free BSA pH 7,4

3.2.8.2. *in vivo* pulse-chase analysis

in vivo pulse-chase analysis was used to determine stability of overexpressed model substrates.

Pulse: Yeast cells were grown until mid-log phase (OD_{600} ~0,8) in synthetic media and cells were isolated by centrifugation (3.000 rpm, 5 min). Cell pellets were next washed with sterile water and resuspended in synthetic media containing 2% (w/v) galactose. Induction was performed for 12 hours at 24°C (Oxa1^{ts}) or 30°C (Yme2ΔC).

Chase: To block cytoplasmic translation, cycloheximide (final concentration 0,1 mg/ml) was added to the cells. The cells were next incubated at the appropriate temperature for 5 min and culture volume corresponding to 10 OD units was

withdrawn as time point 0. Cells were isolated by centrifugation and immediately frozen in liquid nitrogen. All following time points were processed in identical manner.

Analysis: Proteins in the cell pellets from different time points were isolated by mechanical lysis with glass beads (3.2.9.) or by alkaline cell lysis (3.2.10.). The method of protein isolation depended on the membrane topology of the model substrate. Integral membrane proteins were isolated along with mitochondrial membranes during glass beads procedure while peripheral membrane proteins were isolated by alkaline extraction. Stability of the proteins of interest was estimated after immunological detection with specific antisera and laser densitometry (LI-COR® Biosciences).

3.2.9. Cell lysis by glass beads

Cells (10 OD units) were collected by centrifugation (5.000 rpm, 5 min, 4°C), washed once with ice cold distilled water and resuspended in 300 µl ice cold lysis buffer (SHKCl, 2 mM PMSF). After addition of 200 µg glass beads the cells were mechanically ruptured by repetitive cycles of 30 sec vortexing followed by 30 sec incubation on ice for four times. 400 µl of lysis buffer were added and after brief vortexing unbroken cells were removed by centrifugation (2.400 rpm, 3 min, 4°C). The supernatant was placed in a new tube and mitochondrial membranes were isolated by centrifugation (12.000 rpm, 10 min, 4°C). Cytoplasmic proteins were TCA-precipitated from the supernatant (3.2.2.), subjected to SDS-PAGE and analyzed by immunoblotting. The pellet, containing mitochondrial and other cellular membranes was resuspended in sample buffer and used directly for SDS-PAGE and immunoblotting.

3.2.10. Alkaline lysis of cells

1 OD-unit of cells was collected by centrifugation (5.000 rpm, 5 min, 4°C) and washed with 500 µl TE buffer. Cell pellet was resuspended in 500 µl ice cold distilled water and 75 µl of lysis buffer were added. After brief vortexing, the samples were incubated for 10 min on ice. TCA [50% (w/v)] was next added to a final concentration of ~25% (w/v) and the samples were further incubated on ice for at least 20 min. TCA precipitation was carried out as described in 3.2.2. Samples were resuspended in 50

µl SDS-PAGE sample buffer and 1/5 to 1/10 of the sample was subjected to SDS-PAGE. Proteins of interest were detected immunologically.

BUFFER	COMPOSITION
Lysis buffer	1,85 M NaOH 10 mM PMSF 7,4% (v/v) β-mercaptoethanol
TE buffer	10 mM Tris 1 mM EDTA pH 7,4

3.3. Immunological methods

3.3.1. Antibodies

ANTIBODY	ANTIGEN	COMMENTS
α-Yta10-C	peptide N/A	(Pajic *et al.*, 1994), polyclonal
α-Yta12	Matrix-exposed domain of Yta12	(Arlt *et al.*, 1996), polyclonal affinity purified small scale
α-Phb1 yeast	Amino acids 27-287	(Steglich *et al.*, 1999), polyclonal
α-Phb2 yeast	Amino acids 61-315	(Steglich *et al.*, 1999), polyclonal
α-Phb1 (BAP32)	Recombinant rat prohibitin protein, epitope N/A	polyclonal, affinity purified LabVision Corp. (USA)
α-Phb2 (BAP37)	C-terminal domain	Polyclonal, affinity purified BioLegend (SanDiego, CA)
α-Oxa1	C-terminal peptide CDNEKKLQESFKEKR	(Herrmann *et al.*, 1997)
α-Yme2	C-terminal peptide	(Leonhard *et al.*, 2000)
α-paraplegin human	Amino acids 243-844	(Atorino *et al.*, 2003)
α-paraplegin mouse	N-terminal peptide PEDDEEEKRRKEREDQMYR	This study, polyclonal, affinity purified

α-Afg3l1 mouse	N-terminal peptide NAGPGGDGGNRGGKG	This study, polyclonal, affinity purified
α-Afg3l2 human	Amino acids 413-828	(Atorino *et al.*, 2003)
α-Afg3l2 mouse	N-terminal peptide KEAVGEKKEPQPSG	This study, polyclonal, affinity purified
α-F_1α	Epitope N/A, Atp5a1 protein	Monoclonal affinity purified Molecular Probes (Eugene, OR, USA)
HSP60/GroEL	Isolated protein GroEL	
Ccp1	Isolated protein, cytochrome *c* peroxidase	
α-Ndufa9 (39 kDa)	Epitope N/A, Ndufa9 protein	Monoclonal affinity purified Molecular Probes (Eugene, OR, USA)

3.3.2. Affinity purification of antisera

3.3.2.1. Purification of paraplegin-, Afg3l1- and Afg3l2-specific antisera

Paraplegin, Afg3l1 and Afg3l2-specific IgGs were purified from serum by immunoabsorption on a peptide-conjugated Sulfo-Link™ (PIERCE) matrix.

Peptide conjugation to the matrix

2 ml matrix solution were transferred to a 10 ml Poly-Prep® chromatography column (BioRad) and equilibrated to room temperature. 1 ml peptide solution (1 mg/ml) in buffer A was added and incubated with the matrix for 15 min at room temperature while mixing, followed by an incubation in vertical position without mixing for 30 min at room temperature. Next the matrix was washed with 6 ml buffer A. 1 ml buffer A supplemented with 50 mM L-cysteine was added to the matrix and incubated for 15 min (with mixing) and 30 min (without mixing). The matrix was then washed with 32 ml 1M NaCl.

Immunoabsorption of antigen specific IgGs

The matrix, conjugated with peptide, was equilibrated by consecutive washings with 10 ml of 10 mM Tris-HCl, pH 7,5; 100 M glycine-HCl, pH 2,5; 10 mM Tris-HCl, pH 8,8; 100 mM Na-phosphate, pH 11,5; and 10 mM Tris-HCl, pH 7,5. After the last washing step antiserum, diluted in buffer B to a ratio of 1:1 and final volume of 30 ml, was applied on the column at flow rate 0,1 ml/min. This was repeated three times. The column was then washed with 10 ml Tris-HCl, pH 7,5 followed by a washing with 10 ml buffer C.

Peptide specific IgGs were eluted in three steps.

i) Elution with 10 ml 100 mM Na-citrate, pH 4,5. Elution fractions (1 ml) were collected and pH was adjusted to 7,0 with 1M Tris-HCl, pH 8,8.

ii) Elution with 10 ml 100 mM glycine-HCl, pH2,5. 1 ml fractions were collected and pH was adjusted to 7,0 with 1M Tris-HCl, pH 8,8.

The column was washed by 10 ml 10 mM Tris-HCl, pH 8,8.

iii) Elution with 100 mM Na-phosphate buffer, pH 11,5. Fractions (1ml) were collected and pH was adjusted to 7,0 with 1M glycine-HCl, pH 2,5.

The elution fractions were examined for the presence of IgGs by SDS-PAGE and immunoblotting with HRP-conjugated secondary antibodies (3.3.3.). Elution fractions, containing the majority of IgGs, were pooled and concentrated to a volume of ~500 μl with Centricon YM-100 (Millipore) centrifugal filter concentrator according to manufacturer's recommendations.

Test of the specificity of purified antibodies

Immunoprecipitations were carried out to test the specificity of affinity purified paraplegin-, Afg3l1- and Afg3l2- antibodies. Mitochondria from Δ*yta10*Δ*yta12* yeast cells expressing either paraplegin (amino acids 44-781), Afg3l2 (amino acids 44-781) (Nolden *et al.*, 2005) or Afg3l1 (amino acids 25-789) fused to the mitochondrial targeting sequence of Yta10 (amino acids 1-61) were used. Immunoprecipitations were carried out as described in 3.3.5.

BUFFER	COMPOSITION
Buffer A	50 mM Tris-HCl 5 mM EDTA pH 8,5

Buffer B	10 mM Tris-HCl 1 mM PMSF 1 mM EDTA 2 mM EGTA complete protease inhibitor
Buffer C	10 mM Tris-HCl 500 mM NaCl pH 7,5

3.3.2.2. Purification of antisera by an antigen transferred to a nitrocellulose membrane (small scale)

The matrix exposed domain of Yta12 (Yta12MD) N-terminally fused to HIS_6(*TEV*)GST was expressed in *E. coli* BL21(DE3)/pLysS cells and isolated as inclusion bodies (3.2.4). Protein concentration in the preparations was determined and ~0,2 mg protein was subjected to a preparative SDS-PAGE followed by western blotting. The position of Yta12MD was identified after staining of the nitrocellulose membranes with ponceauS and excised. The nitrocellulose band was then incubated for 60 min with blocking solution [5% (w/v) milk powder in TBS buffer]. Polyclonal Yta12-speciffic antiserum, diluted in blocking solution, was applied and the band was incubated for 24 hrs at 4^{o}C. The band was washed three times with PBS buffer (10 min) and incubated with 0,1 M Na-citrate pH, 4,5 for 10 min at room temperature followed by incubation with 0,1 M glycine-HCl, pH 2,5 (glycine elution) for 10 min at room temperature. The glycine elution was collected, neutralized by 1M Tris-HCl, pH 8,8 and kept on ice. The nitrocellulose band was incubated with PBS for neutralization (30 min at room temperature). After incubation with blocking solution (60 min at room temperature), Yta12-speciffic antiserum was applied followed by incubation for 24 hrs at 4^{o}C. The elution procedure was repeated. The fractions from 3 repetitive glycine elutions were pooled and concentrated to a volume of ~500 μl (Centricon YM-100). Immunological detections were carried out after dilution of the affinity purified Yta12 specific antisera with blocking solution.

3.3.3. Immunological detection of proteins

After western blotting the nitrocellulose membranes were incubated in blocking solution [5% (w/v) milk powder in TBS buffer] for 60 min. Primary antibody (diluted in blocking solution) was applied and incubated at room temperature for 60 min while

shaking. Membranes were incubated with PBS buffer for 10 min and the procedure was repeated 3 times (washing). Horse-radish peroxidase (HRP) (BioRad), IRDye™800- or AlexaFluor®680-conjugated affinity purified anti-Rabbit IgG (Biomol, Germany) was applied for 60 min. After washing with PBS (3 times, 10 min), membranes were incubated with Lumi-light Plus reagent (Roche) and exposed to a light sensitive X-ray film. In the case of IRDye™- or AlexaFluor® -conjugated secondary antibodies, detection was performed by scanning of the membranes for emission in the infrared spectra with Odyssey imaging system (LI-COR Biosciences).

3.3.4. Elution of primary and secondary antibodies

Primary and secondary antibodies were eluted from the nitrocellulose membranes by incubation in 0,1 M Na-citrate, pH 4,5 for 10 min followed by an incubation in 0,1M glycine-HCl, pH 2,5 for 10 min. Next, membranes were washed with distilled water and incubated for 60 min with 0,2 M NaOH. New immunological detections were carried out after incubation of the membranes with blocking solution (3.4.3).

3.3.5. Immunoprecipitation

Mitochondria (400 μg) were lysed in lysis buffer at concentration 1 mg/ml and mixed vigorously for 20 min at 4°C. After clarifying ultracentrifugation (45.000 rpm, 15 min, 4°C), 300 μl of the supernatant were applied onto 20 μl protein-A sepharose slurry (100 mg/ml) coupled with affinity purified antibodies or preimmune antisera and further incubated at 4°C with gentle mixing (12 hrs). The beads were washed 2 times with buffer A followed by a washing step with 10mM Tris-HCl, pH 7,4. Antibody-antigen (Ab-Ag) complexes were eluted from the beads according to (Bordier, 1981; Knittler *et al.*, 1998). In detail, the beads were supplied with 1 ml buffer B and incubated with gentle mixing for 10 min at 4°C. The samples were then incubated at 37°C followed by centrifugation at 13.000 rpm (room temperature). The hydrophobic antigens were isolated in the detergent phase while hydrophilic IgGs were recovered from the aqueous phase. To remove remaining IgGs from the detergent phase 1 ml ice cold distilled water was added, samples were mixed and incubated at 37°C followed by centrifugation at 13.000 rpm. Finally, the detergent phase was resuspended in 300 μl SHKCl and subjected to TCA precipitation, SDS-PAGE and immunoblotting.

BUFFER	COMPOSITION
Lysis buffer	2% (w/v) digitonin 50 mM NaCl 50 mM potassium phosphate buffer (KPi) 4 mM Mg-acetate 10% (w/v) glycerol protease inhibitor cocktail 1 mM PMSF pH 7,0
Buffer A	0,5% (w/v) digitonin 50 mM NaCl 50 mM KPi 4 mM Mg-acetate 1 mM PMSF pH 7,0
Buffer B	1% Triton-X114 1% acetic acid pH ~3

3.3.6. Immunodepletion

Mitochondria were lysed in lysis buffer (3.4.5) at concentration 0,5 mg/ml and processed as in 3.4.5. Saturating cencentrations of affinity purified antibodies were coupled to 100 μl protein-A sepharose slurry (10 mg/ml) and used for the precipitation reaction. Precipitations were carried out for 12 hrs at 4°C. Beads were removed by centrifugation (13.000 rpm, 15 sec) and a volume corresponding to 75 μg protein was withdrawn from the supernatant and subjected to TCA precipitation followed by SDS-PAGE. The presence of the protein(s) of interest was examined by immunoblotting with affinity purified antibodies. The beads, containing Ab-Ag complexes, were processed as described in 3.3.5.

3.4. Cell biology methods

3.4.1. Isolation of mitochondria from *S. cerevisiae*

Mitochondria were isolated essentially according to (Herrmann *et al.*, 1994) and (Zinser and Daum, 1995) Yeast culture from a glycerol stock was streaked out on YP or selective glucose media and grown at the appropriate temperature. A single colony was inoculated in 20 ml liquid media and further grown to OD_{600} ~0,8. This was repeated within several days with increasing culture volumes. Cells were next isolated by centrifugation (3.000 rpm, 5 min) and inoculated in YP or selective media

supplemented with galactose [2% (w/v)] and with lactate [0,5% (w/v)]. Upon growing to an OD_{600} of ~0,8, cells were collected and inoculated in fresh medium containing 2% (w/v) galactose and 0.5% (w/v) lactate, and grown for 12 hrs to an OD_{600} between 0,8 and 1,5.

Cells were collected by centrifugation (3.000 rpm, 5 min). After washing with distilled water the cell wet weight was determined. The cells were resuspended in freshly prepared DTT buffer at 2 ml/g cell mass followed by incubation for 10 min at 30°C. Cells were collected and washed with 50 ml 1,2 M sorbitol. Finally, the cell pellet was resuspended in zymolyase buffer at 6,7 ml/g pellet and incubated with 4 mg/g pellet zymolyase or lyticase for 40 min at 30°C. Spheroplasted cells were collected by centrifugation (3.000 rpm, 5 min, 4°C), resuspended in homogenization buffer at 6,7 ml/g pellet and homogenized in Dounce glass homogenizer (13 slow strokes). Unbroken cells were removed by two centrifugation steps at 3.000 rpm, for 5 min, 4°C. Mitochondria were isolated from the supernatant by centrifugation (10 000 rpm, 10 min, 4°C), resuspended in 20 ml SEM buffer and centrifuged at 3.500 rpm for 5 min to completely remove membranes not associated with the mitochondrial fraction. Mitochondria were reisolated by centrifugation of the supernatant (12.000 rpm, 12 min, 4°C) and were resuspended in 500 μl SEM buffer. Protein concentration was measured with Bradford protein assay (3.2.1.) and was adjusted to 10 mg/ml. Aliquots (300 μg) were prepared, frozen in liquid nitrogen and stored at -70°C.

BUFFER	COMPOSITION
DTT buffer	100 mM Tris no adjustment of pH 10 mM DTT
Zymolyase buffer	1,2 M sorbitol 20 mM potassium phosphate buffer pH 7,4 4 g zymolyase (or lyticase) / gram pellet
Homogenization buffer	1,2 M sorbitol 10 mM Tris-HCl, pH 7.4 1 mM EDTA 1mM PMSF 0,3% (w/v) BSA, fatty acid free
SEM buffer	250 mM saccharose 10 mM MOPS-KOH 1 mM EDTA pH 7,4

3.4.2. Isolation of mitochondria from mouse liver (Mattiazzi *et al.*, 2002)

After sacrificing a mouse by cervical dislocation the liver was explanted and washed with ice cold isolation buffer with protease inhibitor mix. Next the liver was cut into small pieces in 10 ml isolation buffer with protease inhibitor mix and was homogenized with Teflon homogenizer (10 strokes, 700 rpm). Tissue debris was removed by centrifugation (1.000 xg, 10 min, 4°C) and the supernatant was filtered through gauze and kept on ice. The pellet was resuspended in 5 ml isolation buffer and recentrifuged (1.000 xg, 10 min, 4°C). After filtering, the supernatants were pooled and mitochondria were isolated by centrifugation (8.000 xg, 10 min, 4°C). The lipids on top of the supernatant were removed and the supernatant discarded. The mitochondrial pellet was resuspended in 300 µl isolation buffer without protease inhibitor mix. Protein concentration was estimated by the Bradford procedure and adjusted to 10 mg/ml with isolation buffer. Mitochondria were stored at -70°C after freezing in liquid nitrogen. For import and other *in organello* experiments mitochondria were used directly after the preparation without freezing.

BUFFER	COMPOSITION
Isolation buffer	220 mM mannitol 20 mM HEPES 70 mM saccharose 2 mM EGTA 0,1% (w/v) BSA pH 7,4 adjusted with KOH with or without complete protease inhibitor cocktail

3.5. Protein and DNA sequence analysis

Primer design and analysis were performed with SE Central Clone Manager Professional Suite (Clone Manager 6, v.6.00, Align Plus 4, v.4.10, Primer Designer 4, v.4.20).

Protein sequence alignments were performed with the multisequence alignment software “alignX”. Alignments were visualized in GeneDoc (Multiple sequence alignment editor and shading utility, Karl Nicholas Copyright©2000 www.pesc.edu/biomed/genedoc) or with BoxShade visualization tool available at http://www.ch.embnet.org/software/BOX_form.html

Accession numbers of the protein sequences used for the multiple sequence alignment in Figure 3. Sequences were extracted from the national center for biotechnology information (NCBI, http://www.ncbi.nlm.nih.gov/).

PROTEIN/ORGANISM	ACCESSION NUMBER
Phb1 *S. cerevisiae*	CAA97145.1
Phb1 *S. pombe*	CAB76268.1
Phb1 *N. crassa*	CAD71006.1
Phb1 Human	AAB21614.1
Phb1 Mouse	CAA55349.1
PHB2 *Z. mays*	AAF68385.1
CG10691-PA *D. melanogaster*	AAN11026.1
Y37E3.9 *C. elegans*	AAK27865.1
Phb2 *S. cerevisiae*[1]	CAA97259.1
Phb2 *S. pombe*	CAA22869.1
Phb2 *N. crassa*	EAA35251.1
Phb2 Human	AAF17231.1
Phb2 Mouse	CAA55350.1
PHB1 *Z. mays*	AAF68384.1
LD46344p *D. melanogaster*	AAL29056.1
T24H7.1 *C. elegans*	AAA68353.1

[1] -Two sequences are annotated for Phb2 *S. cerevisiae*. One has a 5 amino acid residue truncation. The correct, full length sequence was used for the alignment and is given in the table.

IV. RESULTS

1. Function of Phb1 and Phb2 during proteolysis in yeast

Two possible functions of prohibitins, which are not mutually exclusive, have been proposed to explain the decreased stability of substrates in Δ*phb1*Δ*phb2* cells: i) prohibitins function as negative regulators of the activity of the *m*AAA protease (Steglich *et al.*, 1999) and ii) prohibitins function as chaperones for the assembly of newly synthesized mitochondrial translation products (Nijtmans *et al.*, 2000). These possibilities were further examined by assessing the stability of proteolytic substrates in cells harboring different levels of prohibitins. In a first step, the molar ratio was determined between prohibitins and the *m*AAA protease. Additionally, the possibility was examined that prohibitins regulate the activity of the *m*AAA protease by binding in a nucleotide dependent manner.

1.1. The *m*AAA protease is quantitatively assembled with prohibitins

To determine the molar ratio between the Phb1-Phb2 complex and the *m*AAA protease in mitochondria, Phb1, Phb2 and the matrix exposed protein domain of Yta12 (Yta12-MD) were expressed in *E. coli* and isolated (*Materials and Methods*). The protein concentration in these preparations was determined and serial dilutions of Yta12MD, Phb1, Phb2 and mitochondrial extracts were subjected to SDS-PAGE and analyzed by immunoblotting. The molar concentrations of Yta12, Phb1 and Phb2 were calculated after quantification of the immunoblots. These experiments revealed that in steady-state Phb1 and Phb2 are present in approximately 2-3 fold molar excess to Yta12 and presumably to Yta10 within mitochondria (data for Yta12 and Phb2 are summarized in Table 1).

The molar ratio between oligomeric *m*AAA protease- and the Phb1-Phb2 complexes was determined based on their apparent molecular weights in BN-SDS PAGE and sizing chromatography. It has to be noted that the stoichiometry of the subunits in the complexes and therefore their exact native molecular weights are not known and can only be estimated in detergent extracts. Phb1 and Phb2 were proposed to form a complex with a molecular weight between 1.000 and 1.200 kDa (Steglich *et al.*, 1999; Back *et al.*, 2002; Tatsuta *et al.*, 2005). Taken together with the molecular weights of the individual subunits it was estimated that the complex is composed of 15 to 18

subunits. A number of AAA proteins have been shown to form oligomers with hexameric and sometimes heptameric structure (Lenzen *et al.*, 1998; Yu *et al.*, 1998; Vale, 2000; Hanson and Whiteheart, 2005). Homooligomers with hexameric structure have indeed been proposed for FtsH, a close bacterial homologue of the *m*AAA protease (Karata *et al.*, 2001; Krzywda *et al.*, 2002). Therefore, it is conceivable that the *m*AAA protease is also a hexameric complex of Yta10 and Yta12 subunits in a presumably equimolar ratio (Arlt *et al.*, 1996). Hence, the Phb1-Phb2 complex contains approximately three times more subunits than the Yta10-Yta12 complex. Together with the molar ratio between the individual subunits, determined above, this suggests that the Phb1-Phb2 complex and the *m*AAA protease are present in approximately equimolar concentrations within mitochondria. This is also in agreement with the observation that Yta10 and Yta12 exclusively co-elute with prohibitins during sizing chromatography (Steglich *et al.*, 1999). It can therefore be concluded that the *m*AAA protease is quantitatively assembled with prohibitins in yeast mitochondria

Protein	Amount in 20µg mitochondria (ng)	Number of molecules in 20µg mitochondria (f mol)
Yta12	6.9	79
Phb2	7.8	224
Ratio (Yta12=1)	1 : 1.1	1 : 2.8

Table 1. Molar ratio between Yta12 and Phb2 proteins in mitochondria. The molar ratio of Yta12 and Phb2 in mitochondria was estimated by densitometric comparison of known amounts of purified Yta12-MD and Phb2 protein. The number of molecules was calculated according to the molecular weights of the proteins [Yta10, apparent molecular weight of ~73 kDa (Tauer *et al.*, 1994); Yta12, ~80 kDa; Phb1, ~31 kDa; Phb2, ~34 kDa (Steglich *et al.*, 1999)]. The number of Yta12 molecules was set to 1. Yta12-MD, matrix domain of Yta12.

1.2. Role of prohibitins for the turnover of mitochondrial proteins

In view of the quantitative assembly between prohibitins and the *m*AAA protease it is conceivable that overexpression of prohibitins will result in the accumulation of Phb1-Phb2 complexes unassembled with the *m*AAA protease. Given a direct interaction with proteolytic substrates, an excess of Phb1-Phb2 complexes should result in a stabilization of these substrates. On the other hand, if prohibitins function only as negative regulators of the *m*AAA protease, their overexpression should not affect

proteolysis. Therefore, the stability of both mitochondrially and nuclear-encoded polypeptides was assessed in yeast cells harboring different levels of prohibitins.

1.2.1. Degradation of non-assembled, mitochondrially encoded polypeptides

Mitochondrially encoded proteins, the majority of which represent subunits of the respiratory chain complexes, can be synthesized in isolated mitochondria *in vitro* (McKee *et al.*, 1984; McKee and Poyton, 1984; McKee and Poyton, 1984a). However, nuclear-encoded assembly partners are not present under these conditions and the unassembled translation products are therefore subject to proteolytic removal (Black-Schaefer *et al.*, 1991). This assay was used to examine the effect of prohibitin overexpression on the stability of non-assembled, mitochondrially encoded proteins in wild type, Δ*phb1*Δ*phb2* cells and cells overexpressing prohibitins. *PHB1* and *PHB2* were expressed from a multi copy plasmid under the control of galactose inducible promoters in Δ*phb1*Δ*phb2* cells. After induction in galactose, Phb1 and Phb2 accumulated more than 10 fold compared to wild type cells (Figure 7A). Mitochondria were next isolated from wild type, Δ*phb1* cells and cells overexpressing prohibitins and used in pulse-chase experiments in the presence of [^{35}S]-methionine. The stability of newly synthesized mitochondrial proteins was estimated after scintillation counting of the TCA-insoluble fraction, containing the incorporated radioactivity.

It was thereby estimated that non-assembled translation products are degraded with similar rates in mitochondria from wild type cells and from cells overexpressing prohibitins (Figure 7B). This result suggests that prohibitins do not function as chaperones for the stabilization of mitochondrial translation products. On the other hand, deletion of *PHB1* and *PHB2* resulted in a modest, but statistically significant destabilization of mitochondrial translation products in agreement with previously reported data (Steglich *et al.*, 1999).

1.2.2. Stability of overexpressed model substrates

The fact that only a modest acceleration in proteolysis was observed in the absence of prohibitins can be explained with the limiting substrate concentration. Therefore,

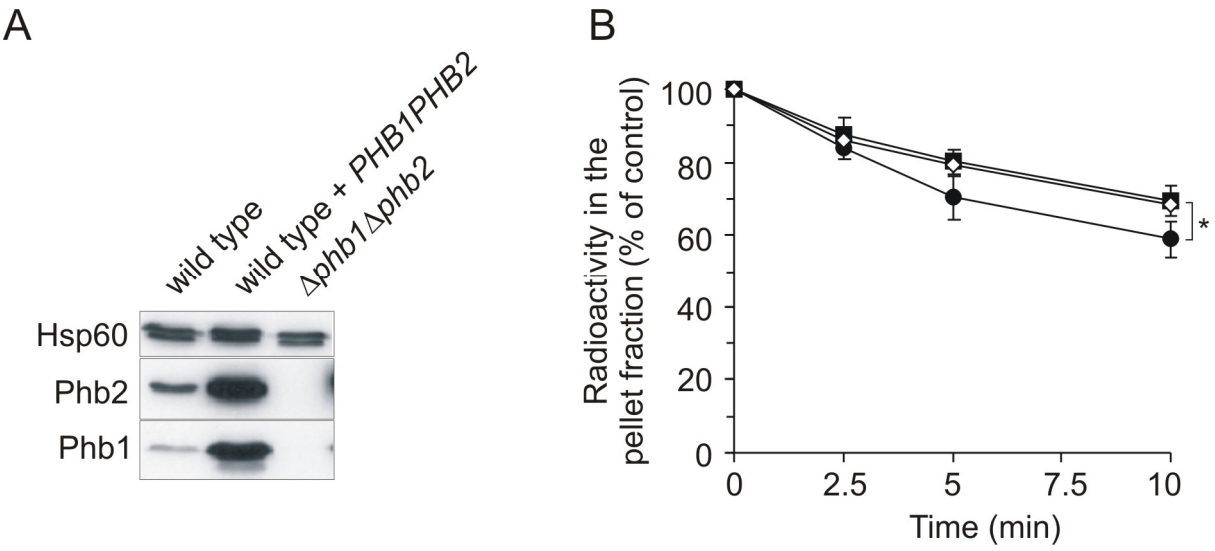

Figure 7. Stability of mitochondrially encoded polypeptides in presence of varying levels of prohibitins. A. Steady-state levels of Phb1 and Phb2 in wild type cells overexpressing prohibitins. Wild type cells and Δ*phb1*Δ*phb2* cells transformed with vector only are shown for comparison. Phb1 and Phb2 were immunologically detected with specific polyclonal antibodies (Steglich *et al.*, 1999). Gel loading was assessed by immunoblotting with Hsp60-specific polyclonal antibodies. *PHB1PHB2*, overexpression of *PHB1* and *PHB2* genes. **B.** Mitochondrial translation products were radioactively labeled for 5 min at 30°C. After stopping of the labeling reaction, the samples were further incubated at 30°C for the indicated time. Radioactivity in the TCA-insoluble fraction at each time point was measured by scintillation counting. The TCA-soluble fraction, containing [^{35}S]-methionine and peptides generated during proteolysis (Augustin *et al.*, 2005), is not shown. The average of four independent experiments is shown together with the deviation from the mean. Time point 0 was set as 100%. *, p≤0.5. ■, wild type; ◊, *PHB1* and *PHB2* overexpression; ●, Δ*phb1*Δ*phb2*.

an inhibition of the proteolytic activity of the *m*AAA protease by prohibitins will be only apparent under conditions of substrate saturation.

To achieve substrate saturation, two known substrates of the *m*AAA protease, Oxa1^{ts} and Yme2ΔC, were overexpressed (Leonhard *et al.*, 2000; Käser *et al.*, 2003). *oxa1^{ts}* and *yme2ΔC* were cloned under the control of a galactose inducible promoter in a multi copy plasmid and expressed in wild type, Δ*phb1* and *PHB1PHB2* overexpressing cells. In these experiments, *PHB1* and *PHB2* genes were expressed under the control of the yeast triosephosphate isomerase (*TPI*) promoter which resulted in ~ 5 fold overexpression in wild type cells (Figure 8C). The stability of Oxa1^{ts} and Yme2ΔC, accumulating in mitochondria, was assessed *in vivo* in cycloheximide-chase experiments (*Materials and Methods*).

1.2.2.1. Stability of Oxa1^{ts}

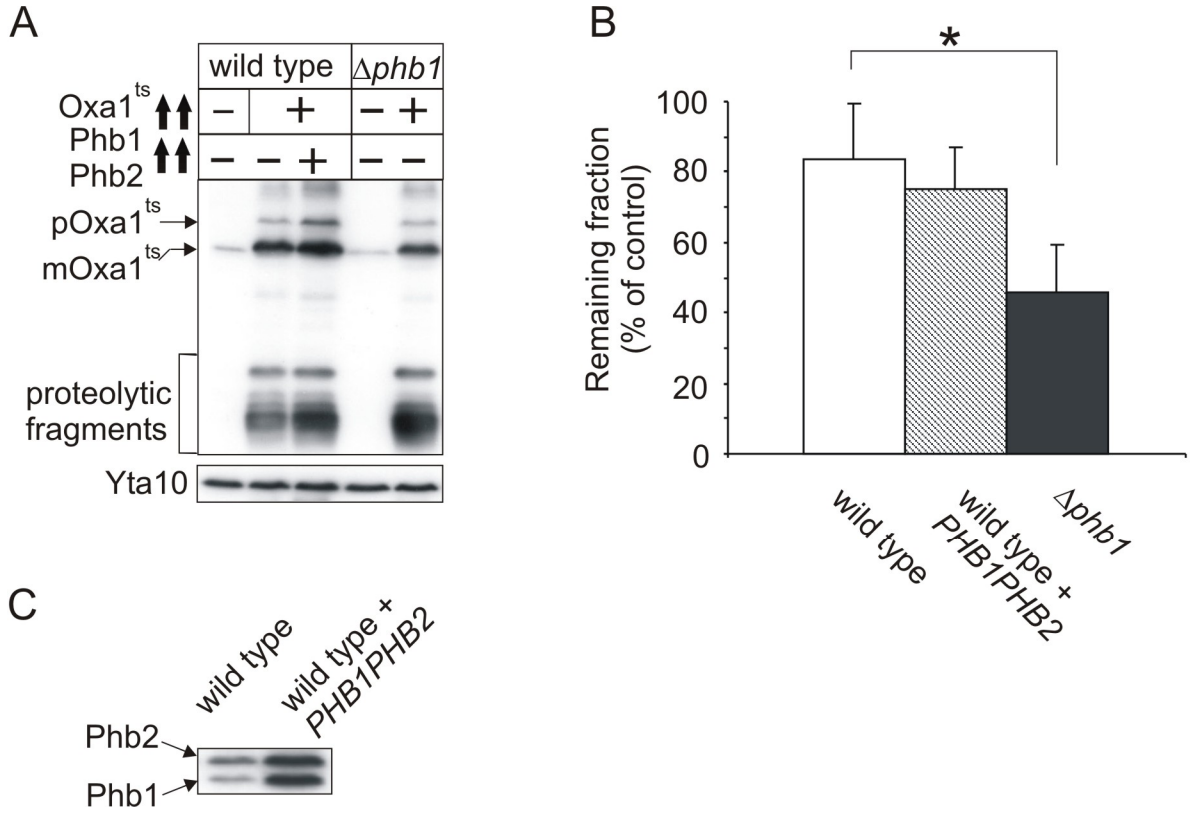

Figure 8. Substrate saturation of the *m*AAA protease and stability of Oxa1^{ts} in the presence of different levels of prohibitins. A. Overexpression of Oxa1^{ts} in galactose containing medium. Oxa1^{ts} and Oxa1^{ts} proteolytic fragments (upper panel) were immunologically detected with polyclonal antibodies raised against the C-terminal domain of Oxa1^{ts} (Herrmann *et al.*, 1997). Gel loading was assessed by immunoblotting with polyclonal Yta10-specific antibodies (Pajic *et al.*, 1994). **B.** *S*tability of overexpressed Oxa1^{ts} at 24°C. Oxa1^{ts} present at time point 0 was set to 100%. Three independent experiments were performed and the standard deviation is shown. *, p≤0.05. **C.** Steady-state levels of Phb1 and Phb2 in wild type cells overexpressing prohibitins. Wild type cells transformed with the vector only are shown for comparison. pOxa1^{ts}, precursor form; mOxa1^{ts}, mature form; ↑↑, overexpression; *PHB1PHB2*, overexpression of prohibitins.

Oxa1 is a polytopic membrane protein that mediates protein insertion into the inner mitochondrial membrane (Bauer *et al.*, 1994; Altamura *et al.*, 1996; Hell *et al.*, 1997; Hell *et al.*, 1998; Hell *et al.*, 2001; Nargang *et al.*, 2002). Replacement of leucine at position 240 to serine, in Oxa1, results in a thermosensitive protein (Oxa1^{ts}) (Bauer *et al.*, 1994). Newly imported Oxa1^{ts} is completely degraded at high temperature by the *m*AAA protease (Käser *et al.*, 2003). The absence of a functional *m*AAA protease (Δ*yta10* or Δ*yta12*) leads to partial stabilization of Oxa1^{ts}, accompanied by accumulation of proteolytic fragments, which are generated by the metallopeptidase Oma1 (Käser *et al.*, 2003). Therefore, accumulation of proteolytic fragments, when

Oxa1^{ts} is overexpressed in wild type, is indicative for substrate saturation of the *m*AAA protease.

Cultivation of Oxa1^{ts} overexpressing cells in the presence of galactose led to the formation of proteolytic fragments of Oxa1^{ts} by Oma1 (Figure 8A). The stability of overexpressed Oxa1^{ts} was next assessed after incubation of the cells in the presence of cycloheximide (Figure 8B). Under these conditions, Oxa1^{ts} was rapidly degraded in Δ*phb1* cells. On the other hand, overexpression of prohibitins did not stabilize Oxa1^{ts} as this substrate was degraded to a similar extent in wild type cells and in cells overexpressing prohibitins. This result suggests that prohibitins do not bind proteolytic substrates, and therefore argues against a chaperone-like function of prohibitins. Additionally, this result shows that the effect of prohibitins is not limited to substrates encoded by the mitochondrial genome and nuclear-encoded substrates of the *m*AAA protease are also destabilized in the absence of prohibitins.

1.2.2.2. Stability of the membrane protein Yme2ΔC

It was found that Oxa1^{ts} is more rapidly degraded in the absence of prohibitins indicating a role of prohibitins for proteolysis of this substrate. Additionally, stable binding of the substrate by excess of prohibitins is unlikely to occur as overexpression of Phb1 and Phb2 did not stabilize Oxa1^{ts}. However, an interaction of prohibitins assembled with the *m*AAA protease and proteolytic substrates can not be excluded. Oxa1^{ts} exposes domains into the intermembrane space (IMS) rising the possibility that prohibitins interact with these domains. It is therefore conceivable that prohibitins exert their effect during proteolysis by interacting with the IMS-domains of the substrates. Substrates lacking IMS-domains will therefore be degraded similarly in wild type and in Δ*phb1* cells. To test this hypothesis, the proteolysis was analyzed of Yme2ΔC, whose C-terminal IMS-domain was deleted (Yme2ΔC) (Figure 9A) (Leonhard *et al.*, 2000). While Yme2 is degraded by the combined action of both the *i*AAA protease and the *m*AAA protease, Yme2ΔC cannot be degraded by the *i*AAA protease (Leonhard *et al.*, 2000). Therefore, Yme2ΔC was expressed in wild type, Δ*phb1* and *PHB1PHB2* overexpressing cells. After addition of cycloheximide and incubation at 37^{o}C the stability of the protein was assessed by immunoblotting. Similarly to Oxa1^{ts}, overexpression of Phb1 and Phb2 did not stabilize Yme2ΔC confirming that prohibitins do not function as chaperones (Figure 9B). On the other hand, lack of prohibitins resulted in an accelerated degradation of Yme2ΔC, when

compared to wild type cells, indicating a role of prohibitins for degradation of this substrate. It is therefore unlikely that direct interactions between the IMS-domains of substrates and prohibitins play a role during proteolysis. However, binding of prohibitins to the transmembrane domains of proteolytic substrates can not be excluded. Therefore, the effect of prohibitins on the stability of peripheral membrane proteins should answer if Phb1 and Phb2 function through interactions with the substrate. However, an attempt to overexpress the peripheral membrane protein Atp7, a substrate of the *m*AAA protease (Korbel *et al.*, 2004), resulted in aggregation and inability to assess its stability.

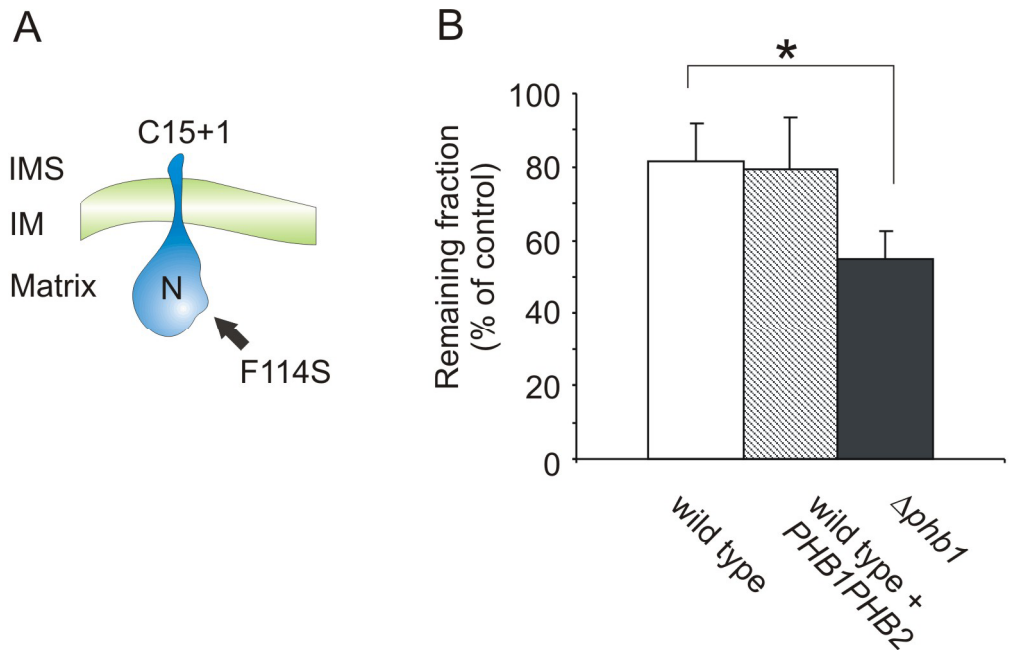

Figure 9. Topology and *in vivo* stability of Yme2ΔC. A. Yme2ΔC is an integral membrane protein of the inner mitochondrial membrane which exposes a short, 16 amino acid, C-terminal tail into the intermembrane space and is degraded only by the *m*AAA protease (Leonhard *et al.*, 2000). The N-terminus is exposed into the mitochondrial matrix. Mutational exchange of phenylalanine at position 114 to serine (F114S) was proposed to destabilize the protein via partial unfolding of the N-terminal domain (Leonhard *et al.*, 2000). **B.** Stability of Yme2ΔC in wild type, *Δphb1* and *PHB1PHB2* overexpressing cells. Induction of Yme2ΔC overexpression in galactose containing medium was followed by addition of cycloheximide and further incubation of the cells at 37°C for 120 min. The amount of Yme2ΔC in the mitochondrial fraction is presented as percentage of the protein present at time point 0. The average of three independent experiments is given. *, p≤ 0.05, IMS, intermembrane space; IM, inner membrane; WT, wild type; *PHB1PHB2*, overexpression of Phb1 and Phb2.

In summary, these experiments show that i) similarly to mitochondrially encoded proteins, proteolytic substrates of nuclear origin are also destabilized in cells lacking prohibitins; ii) overexpression of prohibitins does not stabilize model substrates arguing against a chaperone-like function of prohibitins and iii) the membrane

topology of substrates does apparently not determine whether or not prohibitins affect their stability.

1.3. Synthetic interaction of prohibitins with *oxa1^{ts}*

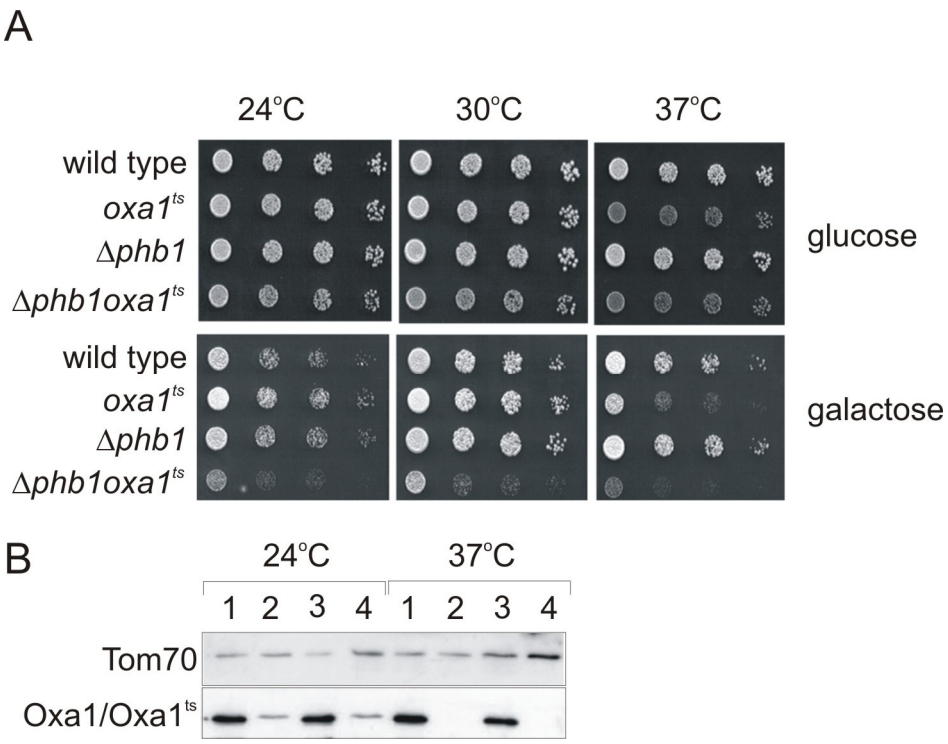

Figure 10. Synthetic growth defect of Δ*phb1oxa1^{ts}* cells. A. Growth test of wild type, *oxa1^{ts}*, Δ*phb1* and Δ*phb1oxa1^{ts}* cells. *oxa1^{ts}* cells cannot grow on glycerol and therefore serial dilutions of cells were spotted on glucose- and galactose-containing solid media and incubated at 24°C, 30°C and 37°C. **B.** Steady-state levels of Oxa1 and Oxa1^{ts} were examined by immunoblotting with polyclonal Oxa1-specific antisera (Herrmann *et al.*, 1997). Oxa1^{ts} has a slightly slower electrophoretic mobility than Oxa1 due to the L240S mutation. Gel loading was assessed by immunoblotting with polyclonal Tom70-specific antibodies. 1, wild type; 2, *oxa1^{ts}*; 3, Δ*phb1*; 4, Δ*phb1oxa1^{ts}*.

As deletion of prohibitins led to an accelerated degradation of overexpressed Oxa1^{ts} it was examined if endogenous levels of Oxa1^{ts} are also affected in the absence of prohibitins.

oxa1^{ts} cells cannot grow on glycerol and grow slowly on glucose at 37°C due to the thermo-sensitivity of Oxa1^{ts} (Bauer *et al.*, 1994; Käser *et al.*, 2003). At this temperature Oxa1^{ts} is destabilized and degraded by the *m*AAA protease (Käser *et al.*, 2003). Nevertheless, *oxa1^{ts}* cells grow as wild type at 24°C. Based on this it was hypothesized that if prohibitins affect the stability of endogenous Oxa1^{ts}, then Δ*phb1oxa1^{ts}* cells will grow slowly at 24°C due to reduced steady-state levels of

Oxa1^{ts}. Therefore, the growth of Δ*phb1oxa1^{ts}* cells was compared to wild type, *oxa1^{ts}* and Δ*phb1* cells at different temperatures and on different carbon sources.

A synthetic inhibition of growth on galactose-containing media at 24^{o}C and at 30^{o}C was observed upon deletion of the *PHB1* gene in *oxa1^{ts}* cells (Figure 10A). In contrast, *oxa1^{ts}* and Δ*phb1* cells grew indistinguishably from wild type cells at these temperatures. Interestingly, this phenotype was restricted only to galactose and was not evident on glucose. Therefore, the steady-state levels of Oxa1^{ts} were examined in cells grown on galactose at 24^{o}C and at 37^{o}C. Oxa1^{ts} was still present in Δ*phb1oxa1^{ts}* cells at steady-state levels comparable to *oxa1^{ts}* cells (Figure 10B). Thus, it is unlikely that an accelerated degradation of Oxa1^{ts} is the sole reason for the inhibited growth of Δ*phb1oxa1^{ts}* cells. In contrast, both Δ*phb1oxa1^{ts}* and *oxa1^{ts}* grew slowly on glucose and galactose at 37^{o}C. This, however, can be attributed to the instability of Oxa1^{ts} and its rapid degradation at this temperature. It is, therefore, conceivable that the synthetic interaction between prohibitins and *oxa1^{ts}* is linked to a decreased stability of some component(s) of the respiratory chain complexes as Oxa1 functions as a general machinery for insertion of mitochondrial proteins into the inner membrane (Altamura *et al.*, 1996; Hell *et al.*, 1997; Hell *et al.*, 1998; Hell *et al.*, 2001; Nargang *et al.*, 2002). Therefore, further experiments are necessary to address the stability of other inner membrane proteins in Δ*phb1oxa1^{ts}* cells.

1.4. Physical interaction between prohibitins and the *m*AAA protease in the inner mitochondrial membrane

Phb1 and Phb2 physically interact with the *m*AAA protease and form a high molecular weight complex, supercomplex, thereby determining the stability of substrates for the *m*AAA protease (Steglich *et al.*, 1999). In view of their role as negative regulators of the *m*AAA protease, prohibitins may function in two ways which are not mutually exclusive: i) as modulators of the specific activity of the *m*AAA protease and ii) effectors of the ATPase activity of the *m*AAA protease. A nucleotide dependence of the binding of the *m*AAA protease to prohibitins was examined as this can provide further insight into the mechanism by which prohibitins affect proteolysis.

1.4.1. A procedure for monitoring the assembly of the *m*AAA protease in a supercomplex with prohibitins

To assess the assembly of the supercomplex, a procedure was established for separation of the *m*AAA protease from its supercomplex with prohibitins. Glycerol gradient sedimentation was used due to the high recovery of prohibitins compared to gel filtration. Mitochondria from wild type, Δ*yta10* and Δ*phb1*Δ*phb2* cells were lysed with digitonin and the extracts were sedimented through a 10%-30% (w/v) glycerol gradient (Figure 11). Under these conditions prohibitins co-migrated with the *m*AAA protease (gradient fractions 7-8) indicating the formation of a supercomplex in wild type mitochondria. In contrast, when mitochondria from Δ*phb1*Δ*phb2* cells were used, the assembled *m*AAA protease migrated at a lower molecular weight (gradient fractions 3-5). In Δ*yta10* mitochondria, on the other hand, non assembled Yta12 did not co-migrate with the Phb1-Phb2 complex and was detectable in fractions of the gradient presumably corresponding to a molecular weight of ~250 kDa (Arlt *et al.*, 1996). No co-migration of the *m*AAA protease and the Phb1-Phb2 complex was observed when pre-lysed samples from Δ*yta10* and Δ*phb1*Δ*phb2* were pooled and incubated further at 4°C, excluding post-lysis interactions between both complexes.

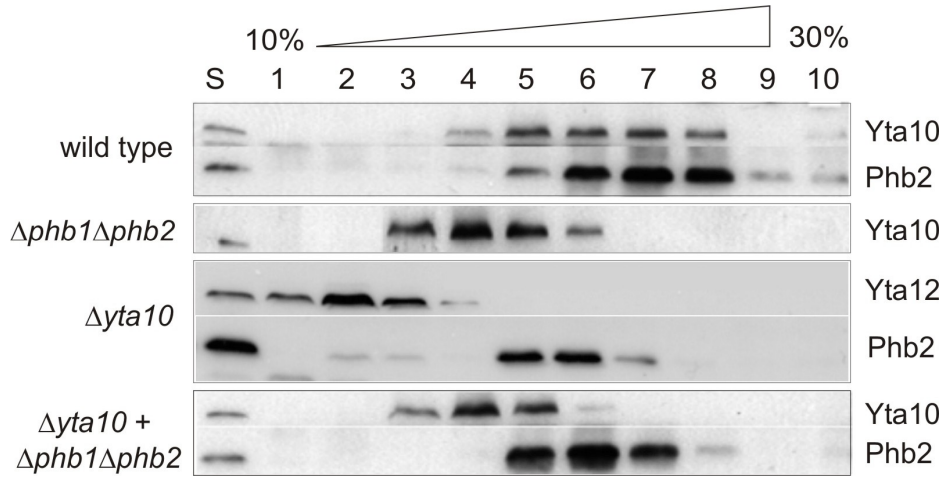

Figure 11. The supercomplex between prohibitins and the *m*AAA protease is not formed during lysis of mitochondria. Mitochondria from wild type, Δ*phb1*Δ*phb2* and Δ*yta10* were lysed in the presence of digitonin. After mixing for 20 min at 4°C, aliquots from Δ*yta10* and Δ*phb1*Δ*phb2* were pooled and further incubated for 20 min at 4°C. Samples were subjected to glycerol gradient sedimentation and 400 µl fractions were collected from the top of the gradient, TCA-precipitated and subjected to SDS-PAGE. Yta10 and Phb2, used to assess the sedimentation of the *m*AAA protease and the Phb1-Phb2 complex, were detected immunologically. S, input control corresponding to 10% of the sample.

These findings are in agreement with previous sizing chromatography experiments (Steglich *et al.*, 1999) and show that: i) only the assembled *m*AAA protease forms a supercomplex with prohibitins, but not the non-assembled Yta10 or Yta12 (Steglich *et*

al., 1999) and that ii) the supercomplex exists *in vivo* and is not formed during lysis of mitochondria.

1.4.2. Formation of the supercomplex between prohibitins and the *m*AAA protease is nucleotide independent

The *m*AAA protease is an energy dependent enzyme i.e. proteolysis and ATP-hydrolysis are tightly coupled. Similarly to FtsH (Akiyama *et al.*, 1998; Karata *et al.*, 1999), the *m*AAA protease may undergo conformational changes upon binding of ATP and prohibitins may affect proteolysis by binding to the protease in a nucleotide dependent manner. Therefore, it was examined if a *m*AAA protease, stabilized in nucleotide-free or nucleotide-bound conformation, is able to bind prohibitins. To answer this, Yta10 and Yta12 harboring point mutations in the Walker A (*yta10*^{K334A} *and yta12*^{K394A}) and Walker B motifs (*yta10*^{E388Q} *and yta12*^{E448Q}) were generated (Steglich, 2000). By analogy to other AAA proteins (Whiteheart *et al.*, 1994; Babst *et al.*, 1998; Karata *et al.*, 1999; Krzywda *et al.*, 2002) the mutations in the Walker A motif stabilize the corresponding subunit in nucleotide free conformation by preventing binding of ATP. On the other hand, mutations within the Walker B motif are expected to prevent ATP-hydrolysis and thereby stabilize the nucleotide bound conformation of the mutated protease subunit.

The mutant Yta10 and Yta12 subunits were expressed under the control of the endogenous *YTA10* promoter and an alcohol-dehydrogenase (*ADH1*) promoter, respectively. Cells expressing the corresponding wild type subunits were used as a control. Mitochondria were isolated from these cells and subjected to glycerol gradient sedimentation (Figure 12A). Yta10 co-migrated with Phb2 (gradient fractions 7-8) during glycerol gradient sedimentation indicating assembly in a supercomplex in Δ*yta10* cells complemented with *YTA10*. In contrast, Yta10 migrated at lower molecular weights (gradient fractions 3-5) in the absence of prohibitins. Yta10^{K334A} or Yta10^{E388Q}, however, were still able to assemble with Phb1 and Phb2 into a supercomplex. Identical results were observed using mitochondrial extracts from cells expressing Yta12^{K394A} or Yta12^{E448Q} mutant subunits (not shown). It is, therefore, evident that mutations in the Walker A or B motifs of a single subunit of the *m*AAA protease do not affect its ability to assemble with prohibitins.

It should be noted that wild type subunits of the *m*AAA protease are still present in these cells. It was therefore examined if the formation of the supercomplex is affected

when both subunits of the *m*AAA protease are stabilized in ATP-bound conformation. Yta10^{E388Q} and Yta12^{E448Q} were expressed simultaneously in Δ*yta10*Δ*yta12* cells and their assembly with prohibitins was analyzed by BN-SDS PAGE (Figure 12B).

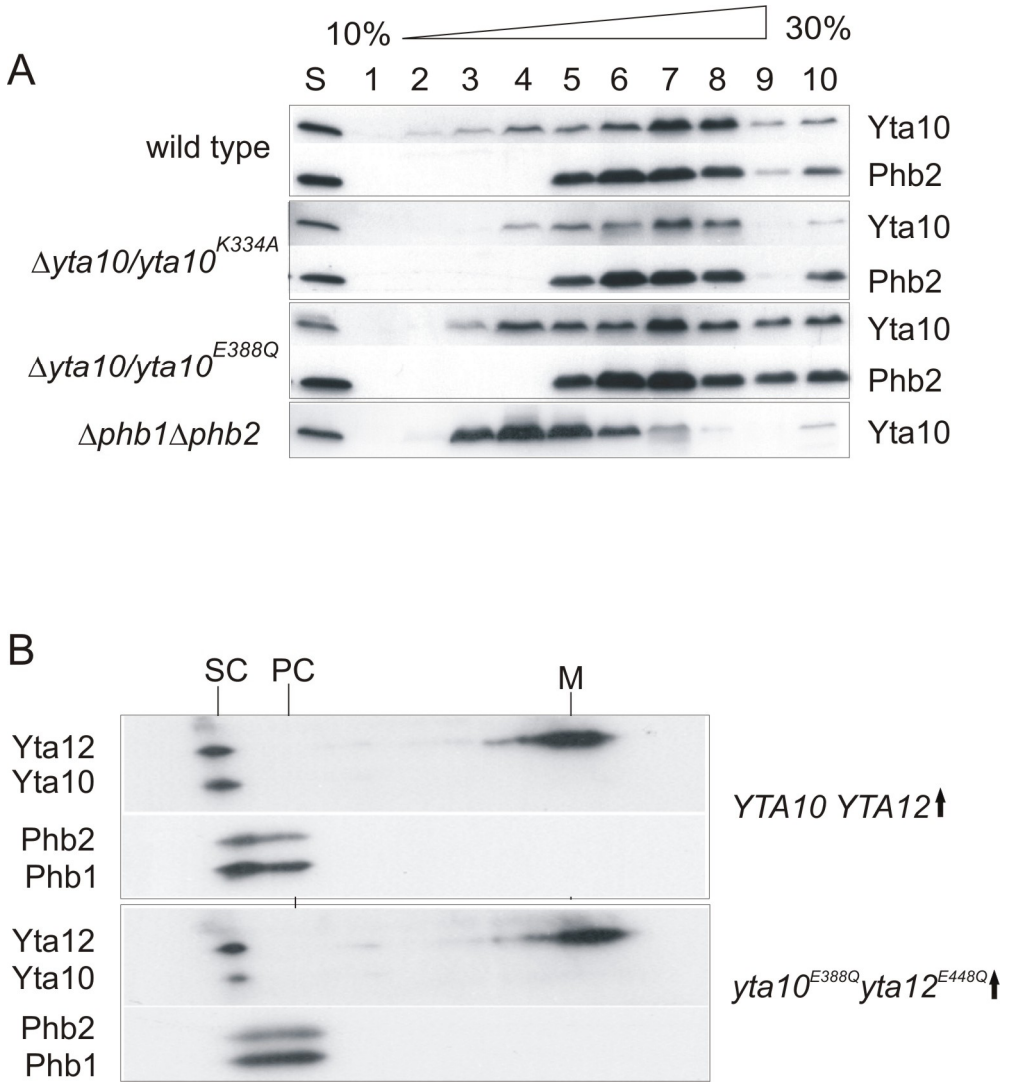

Figure 12. Glycerol gradient sedimentation and BN-SDS PAGE analyses of cells expressing ATP-binding and ATP-hydrolysis mutants of Yta10 and Yta12. A. Mitochondria (100 μg) from *YTA10, yta10*^{K334A} or *yta10*^{E388Q} expressing cells were lysed in digitonin and subjected to glycerol gradient sedimentations. 400 μl samples were collected from the top of the gradient, subjected to SDS-PAGE and analyzed by immunoblotting for the presence of Yta10 and Phb2. Δ*phb1*Δ*phb2* cells were used as negative control to detect the migration of the *m*AAA protease in absence of prohibitins. S, input control corresponding to 10% of the sample. **B.** BN-SDS PAGE analysis of mitochondrial extracts in digitonin from Δ*yta10*Δ*yta12*/*yta10*^{E388Q}*yta12*^{E448Q} cells and cells expressing wild type *YTA10* and *YTA12*. Mutant and wild type Yta10 subunits were expressed under the control of endogenous *YTA10* promoter. Wild type and mutant Yta12 subunits were expressed under the control of an *ADH1* promoter, resulting in overexpression. Samples (400 μl) were analyzed by SDS-PAGE and immunoblotting with antibodies specific for Phb1, Phb2 and Yta10, Yta12 proteins. S, supernatant; SC, supercomplex; PC, prohibitin complex. ↑, expression under the control of an *ADH1* promoter.

Similarly to the results described above, Yta10^{E388Q} and Yta12^{E448Q} assembled with Phb1 and Phb2 in a supercomplex. Consistently, the supercomplex between prohibitins and the *m*AAA protease was also not affected by depletion of ATP after apyrase treatment or by increased concentrations of ATP or ADP during lysis of mitochondria (not shown).

Taken together these data indicate that the binding of the *m*AAA protease to prohibitins is independent of nucleotide binding to its subunits and their ability to hydrolyze ATP.

1.4.3. Mutations in the Walker motifs of Yta10 and Yta12 inactivate the *m*AAA protease

AAA+ proteins, including FtsH, are inactivated by mutations of the conserved lysine and glutamate residues from the Walker motifs (Whiteheart *et al.*, 1994; Babst *et al.*, 1998; Karata *et al.*, 1999). Therefore, it was examined how mutations in the Walker motifs of Yta10 and Yta12 affect the activity of the *m*AAA protease. The assembly of respiratory chain complexes was assessed in BN-SDS PAGE together with the processing of cytochrome *c* peroxidase (Ccp1).

1.4.3.1. Impaired assembly of the respiratory chain complexes in cells expressing mutant Yta10 and Yta12 subunits

Cells expressing Yta10 or Yta12 stabilized in a nucleotide-free or nucleotide bond conformation cannot grow on glycerol (Steglich, 2000) suggesting an impaired assembly of respiratory chain complexes. To test this, mitochondria from cells expressing Yta12^{K394A} and Yta12^{E448Q} were lysed with digitonin and subjected to BN-PAGE analysis (Figure 13). Wild type and Δ*yta12* cells were used as a control. Under these conditions the ATP-synthase migrated as a monomer of ~600 kDa and a dimer of ~1.250 kDa in wild type cells consistent with previous reports (Arnold *et al.*, 1998; Schagger and Pfeiffer, 2000; Schagger, 2001). Additionally, complex III and complex IV supercomplexes were detected at the molecular weight region between the ATP-synthase dimer and monomer (Schagger and Pfeiffer, 2000; Schagger, 2001). In contrast, Δ*yta12* cells were devoid of complex III-complex IV assemblies and harbored only reduced moieties of assembled monomeric and dimeric ATP-synthase. Thus, impaired assembly of respiratory chain complexes correlates with inability of these cells to grow on glycerol. Expression of a proteolytically inactive mutant of

Yta12 (Yta12^{E614Q}), on the other hand, did not fully inhibit the activity of the *m*AAA protease. ATP-synthase monomer and dimer as well as complex III-complex IV supercomplexes were assembled in these cells as in wild type cells (Arlt *et al.*, 1996). However, cells expressing either Yta12^{K394A} or Yta12^{E448Q} were devoid of assembled complex III-complex IV supercomplexes and had decreased amounts of assembled monomeric and dimeric ATP-synthase suggesting an impaired activity of the *m*AAA protease. Similar results were obtained with mitochondrial extracts from cells expressing Yta10 mutant subunits (not shown).

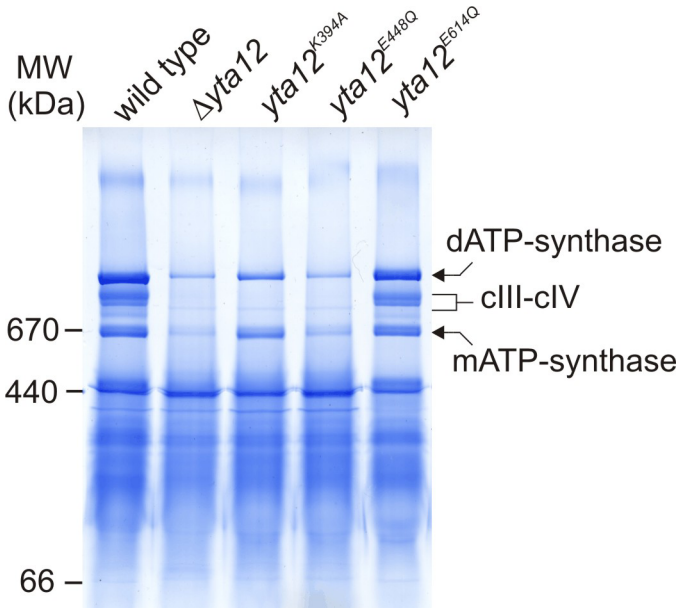

Figure 13. Assembly of respiratory chain complexes is inhibited by mutations in the Walker motifs of Yta12 subunits. Mitochondria (100 μg) from wild type, Δ*yta12* cells and cells expressing mutant Yta12 subunits were lysed in the presence of 2% (w/v) digitonin and subjected to BN-SDS PAGE. Respiratory chain complexes were visualized after colloidal staining with coomassie brilliant blue (PageBlue^{TM}, Fermentas). mATP-synthase and dATP-synthase, monomeric and dimeric ATP-synthase, respectively; cIII-cIV, complex III-complex IV supercomplexes.

1.4.3.2. Processing of Ccp1 is inhibited by mutations in the Walker motifs of Yta10 and Yta12 subunits of the *m*AAA protease

Import of cytochrome *c* peroxidase (Ccp1) into mitochondria is a two step process in which the *m*AAA protease is limiting for the generation of mature Ccp1. During import the nuclear-encoded Ccp1 precursor (p-Ccp1) is inserted into the inner mitochondrial membrane (Esser *et al.*, 2002; Michaelis *et al.*, 2005). p-Ccp1 is then processed by the *m*AAA protease and a Rhomboid protease, Pcp1, resulting in the release of mature Ccp1 (m-Ccp1) in the intermembrane space (Esser *et al.*, 2002). The first

processing step, by the *m*AAA protease, generates an intermediate form (i-Ccp1). This form, still associated with the inner mitochondrial membrane, is next processed by Pcp1 leading to the generation of the mature Ccp1 (Esser *et al.*, 2002; Michaelis *et al.*, 2005). The processing step by the *m*AAA protease is, however, limiting for the biogenesis of Ccp-1 as only the precursor form of Ccp1 is detectable in Δ*yta10* and Δ*yta12* cells (Esser *et al.*, 2002). Therefore, accumulation of the precursor form of Ccp1 is a convenient test for the activity of the *m*AAA protease.

Processing of Ccp1 was examined by SDS-PAGE and immunoblotting of mitochondrial extracts from cells expressing mutant Yta10 or Yta12 subunits (Figure 14). Ccp1 was processed in wild type cells and the mature form of Ccp1 could be detected. In contrast, absence of assembled *m*AAA protease blocked the processing of Ccp-1 and results in the accumulation of p-Ccp1 in Δ*yta12* cells (Esser *et al.*, 2002). Proteolytic inactivation of Yta12 (*yta12*^{E614Q}) did not inhibit maturation of Ccp1. However, processing of Ccp1 was inhibited in cells expressing either Yta12^{K394A} or Yta12^{E448Q} mutant subunits as well as in cells expressing Yta10^{K334A} or Yta10^{E388Q} subunits suggesting that the *m*AAA protease is inactive in these cells.

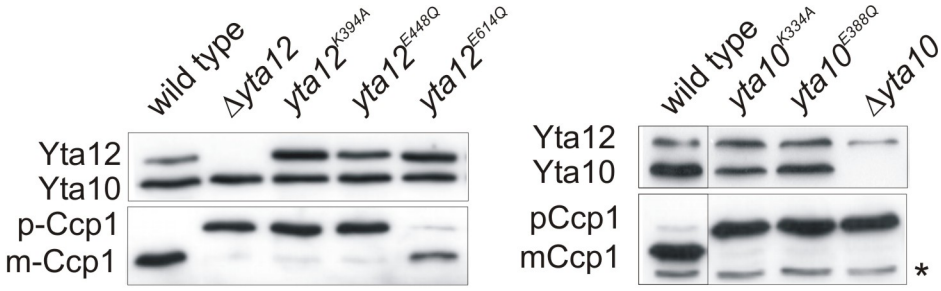

Figure 14. Maturation of Ccp1 is inhibited by mutations in the Walker motifs of *m*AAA protease subunits. Mitochondria (50 μg) isolated from wild type cells and cells expressing mutant Yta10 and Yta12, defective in nucleotide-binding or ATP-hydrolysis, as well as a proteolytic mutant of Yta12 (*yta12*^{E614Q})were subjected to SDS-PAGE and immunoblotting. Yta10, Yta12 and Ccp-1 (mature and precursor form) were by immunoblotting detected with specific polyclonal antisera. Yta12 mutants were expressed under the control of the *ADH1* promoter and Yta10 mutants under the control of endogenous *YTA10* promoter. *, cross reacting band.

Together with the impaired assembly of respiratory chain complexes, this result suggests that mutations in the conserved nucleotide binding motifs of either Yta10 or Yta12 are sufficient to inactivate the *m*AAA protease. This can be explained in view of the proposed intersubunit cooperativity of two neighboring AAA protease subunits during ATP-binding and ATP-hydrolysis (Karata *et al.*, 1999; Karata *et al.*, 2001; Krzywda *et al.*, 2002). Therefore, it is conceivable that inactivation of the ATP-binding

or ATP-hydrolysis in a single subunit can also inhibit the corresponding processes in a neighboring subunit.

2. Identification of a supercomplex containing prohibitins and *m*AAA protease subunits in murine mitochondria

In contrast to yeast, mammalian prohibitins can localize to several cellular compartments (Wang *et al.*, 2002; Fusaro *et al.*, 2003; Mengwasser *et al.*, 2004). To date, most efforts are focused on understanding the role of prohibitins as transcriptional regulators in the nucleus and little is known about their function in mitochondria. Therefore, prohibitins and their interactions were examined in murine liver mitochondria.

2.1. The Phb1-Phb2 complex in murine mitochondria

Phb1 and Phb2 form a high molecular weight complex in mammalian mitochondria (Nijtmans *et al.*, 2000; Coates *et al.*, 2001). In yeast, the Phb1-Phb2 complex has a molecular weight between ~1.000 and ~1.200 kDa (Steglich *et al.*, 1999; Tatsuta *et al.*, 2005).

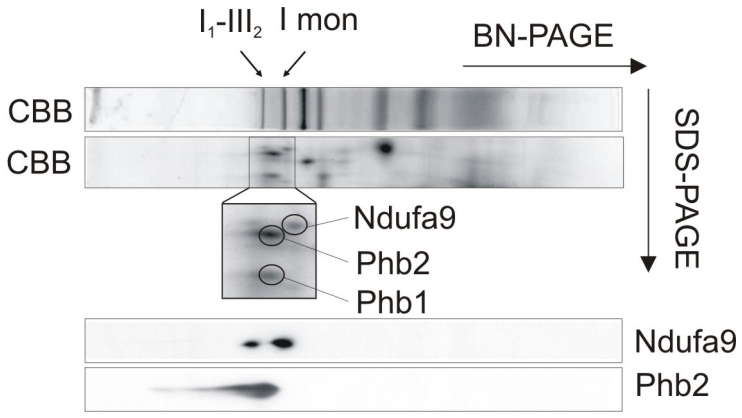

Figure 15. Determination of the approximate molecular weight of the Phb1-Phb2 complex in murine mitochondria. Mitochondria (100 μg) were lysed with 2% (w/v) digitonin and subjected to BN-SDS PAGE. Proteins were either blotted onto a nitrocellulose membrane or stained within the gel by coomassie brilliant blue G250. The 39kDa subunit of complex I (Ndufa9) and Phb2 were detected by immunoblotting with monoclonal (Ndufa9) or polyclonal (Phb2) antibodies. Phb1, Phb2 and Ndufa9 were excised from the coomassie-stained second dimension gels and identified by mass spectrometry. CBB, coomassie brilliant blue; I_1-III_2, complex I – complex III; I mon, complex I monomer.

To estimate the approximate molecular weight of the Phb1-Phb2 complex in murine mitochondria, the migration of prohibitins was examined in digitonin extracts of

murine liver mitochondria by BN-SDS PAGE (Figure 15). Respiratory chain complexes were used for calibration. The ATP synthase has been reported to migrate in two molecular weight regions corresponding to a monomer of ~750 kDa and a dimer of ~1.500 kDa (Schagger and Pfeiffer, 2000). Monomeric complex I has an apparent molecular weight of ~1.000 kDa and can form several supercomplexes with other respiratory chain complexes e.g. complex I-complex III supercomplex (~1.500 kDa, I_1III_2) (Schagger and Pfeiffer, 2000). These complexes can be detected in digitonin extracts of murine liver mitochondria by immunoblotting with antibodies raised against the 39 kDa subunit of complex I, Ndufa9. Thereby, immunoblotting with Phb2- and Ndufa9-specific antibodies revealed that Phb1 and Phb2 built up a complex with an apparent molecular weight between ~1.000 kDa and ~1.500 kDa in murine mitochondria. Hence, the mammalian Phb1-Phb2 complex has apparently a molecular weight similar to the complex in yeast and may therefore have similar subunit stoichiometry and complex dimensions.

2.2. Generation of antibodies specific for putative subunits of the murine *m*AAA protease

In view of the high sequence similarity between yeast and mammalian prohibitins it is conceivable that the Phb1-Phb2 complex assembles with the *m*AAA protease also in mammalian mitochondria. In contrast to yeast and humans, three putative *m*AAA protease subunits are present in mice: paraplegin, Afg3l2 and Afg3l1 (Kremmidiotis *et al.*, 2001). In order to analyze the subunit composition of the murine *m*AAA protease and its interaction with prohibitins, in a first step, peptide specific antibodies were generated against paraplegin, Afg3l1 and Afg3l2 and purified by immunoabsorption (*Materials and Methods*). Since paraplegin, Afg3l1 and Afg3l2 share a high degree of sequence similarity the specificity of the antibodies was examined in immunoprecipitation experiments from yeast cells expressing paraplegin, Afg3l1 or Afg3l2 (Figure 16).

In these experiments, paraplegin was precipitated with paraplegin-specific antibodies but not with Afg3l1- or Afg3l2-specific antibodies demonstrating that these antibodies do not cross-react with paraplegin. Similarly, Afg3l1 was precipitated with Afg3l1-specific antibodies but not with paraplegin- or Afg3l2-specific antibodies. Thus, antibodies directed against paraplegin and Afg3l2 do not cross-react with Afg3l1. Finally, Afg3l2 was precipitated with Afg3l2-specific antibodies. While no Afg3l2 was

precipitated with paraplegin-specific antibodies, a minute amount of Afg3l2 could be detected in precipitations carried out with Afg3l1-specific antibodies. However, Afg3l2 could also be detected in the control with preimmune antisera and therefore unspecific interactions with the protein-A sepharose matrix can not be excluded. Based on these results it can be concluded that the antibodies directed against paraplegin, Afg3l1 and Afg3l2 specifically recognize their target proteins. These antibodies were therefore used in experiments addressing the interactions between paraplegin, Afg3l1 and Afg3l2 and their interaction with prohibitins in murine liver mitochondria.

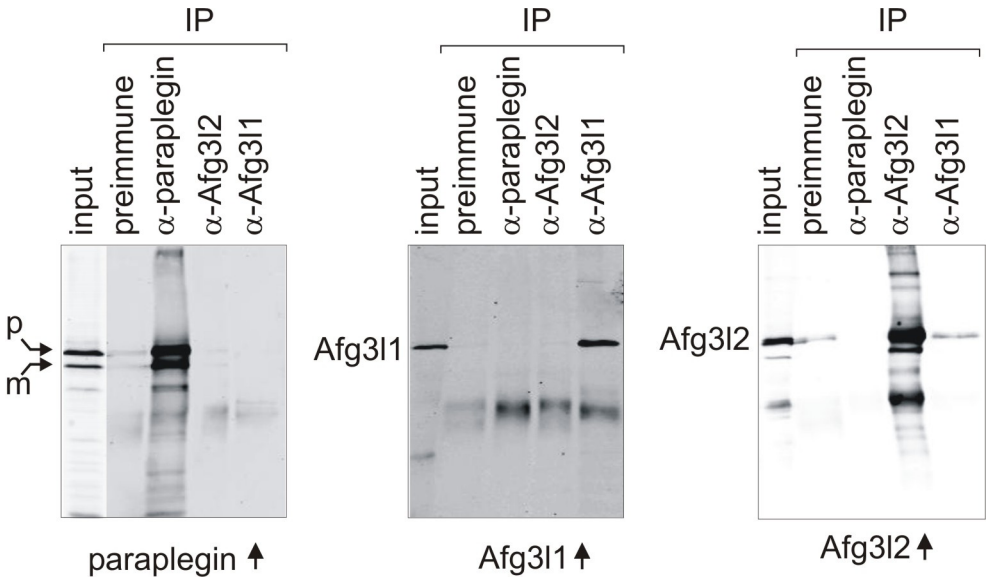

Figure 16. Specificity of affinity purified paraplegin-, Afg3l1- and Afg3l2-specific antibodies. Mitochondria (300 μg) from Δ*yta10*Δ*yta12* yeast cells, expressing paraplegin, Afg3l1 and Afg3l2 proteins fused to the mitochondrial targeting sequence of Yta10 were lysed with digitonin and used for immunoprecipitation experiments with affinity purified paraplegin-, Afg3l1- and Afg3l2-specific antibodies. Preimmune antiserum was used as a negative control. Mitochondrial lysate, corresponding to 50 μg, protein is shown as an input control. p, precursor form of paraplegin; m, mature form of paraplegin; ↑, expression of the proteins from a multi copy yeast plasmid under the control of endogenous *YTA10* promoter.

2.3. Paraplegin, Afg3l1 and Afg3l2 co-migrate with Phb1 and Phb2 in BN-SDS PAGE and sizing chromatography

To examine a potential interaction of prohibitins with paraplegin, Afg3l1 and Afg3l2, murine liver mitochondria were solubilized with digitonin and subjected to BN-SDS PAGE (Figure 17). Phb1 and Phb2 were immunologically detected at molecular weights higher than 1.500 kDa (dimeric ATP-synthase and complex I-complex III

supercomplex). Paraplegin and Afg3l2 co-migrated with at least part of Phb1 and Phb2, suggesting that paraplegin and Afg3l2 form a complex with prohibitins. Afg3l1 could not be detected in these experiments presumably due to the limited affinity of the Afg3l1-specific antibody.

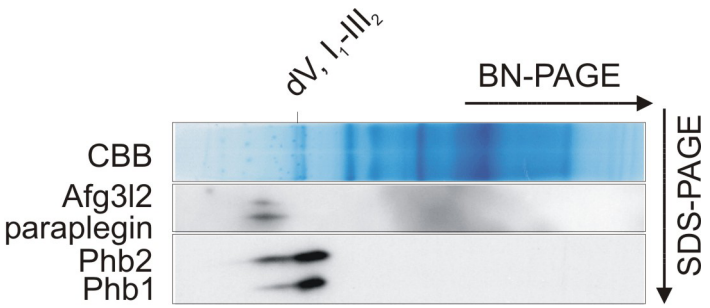

Figure 17. BN-SDS PAGE analysis of murine liver mitochondria. Mouse liver mitochondria (100 μg) were lysed with 2% (w/v) digitonin and subjected to BN-SDS PAGE. Paraplegin and Afg3l2 were immunologically detected with affinity purified paraplegin- and Afg3l2-specific antibodies. Prohibitins were detected with Phb1- and Phb2-specific antibodies. The ATP-synthase dimer and the complex I-complex III supercomplex migrate at an apparent molecular weight of ~1.500 kDa (Schagger and Pfeiffer, 2000). CBB, coomassie brilliant blue; dV, dimeric ATP-synthase; I_1-III_2, supercomplex between complex I and complex III.

In further experiments, digitonin extracts from liver mitochondria were fractionated on a Superose-6 gel filtration column. Eluted fractions were analyzed for the presence of paraplegin, Afg3l1, Afg3l2 and prohibitins by immunoblotting (Figure 18A). It was thereby estimated that Afg3l1 co-elutes with paraplegin and Afg3l2 in fractions corresponding to a molecular weight of ~1.900 kDa as estimated by calibration of the column. Phb1 and Phb2 eluted from the column in fractions apparently coinciding with paraplegin, Afg3l1 and Afg3l2. Thus, both the BN-SDS PAGE and sizing chromatography suggest that paraplegin, Afg3l1 and Afg3l2 form a high molecular weight complex with Phb1 and Phb2.

2.4. Phb1 and Phb2 physically interact with paraplegin, Afg3l1 and Afg3l2

Co-immunoprecipitation experiments were next carried out to directly demonstrate a physical interaction of prohibitins with putative subunits of the *m*AAA protease. Therefore, murine liver mitochondria were solubilized with digitonin and immuno-precipitations were carried out with affinity purified paraplegin-, Afg3l1- and Afg3l2-specific antibodies (Figure 18B). Preimmune antiserum was used as a negative

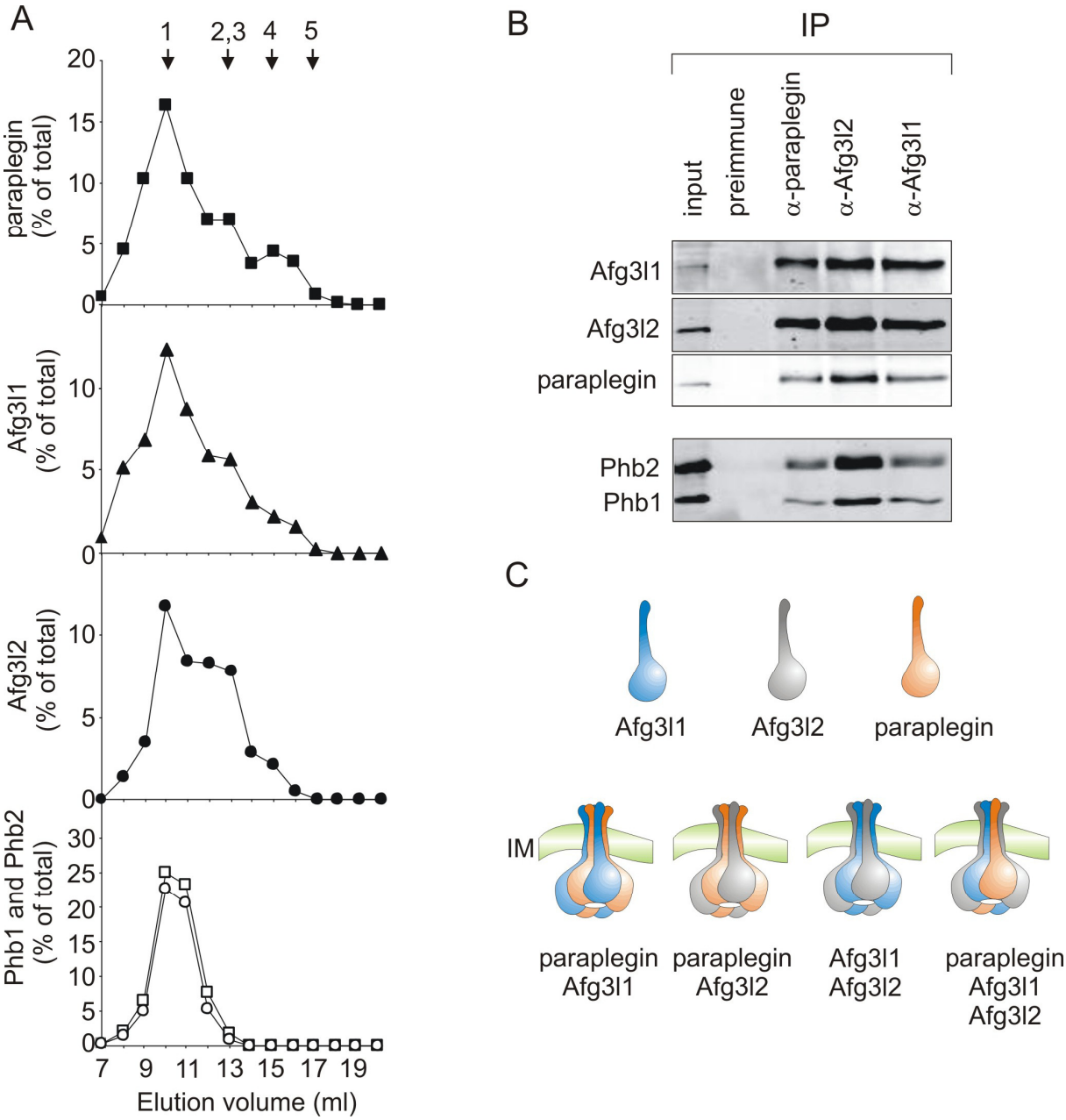

Figure 18. A Prohibitin-*m*AAA protease supercomplex in murine mitochondria. A. Murine liver mitochondria (1 mg) were lysed with 2% (w/v) digitonin and extracts were subjected to gel filtration chromatography. Eluate fractions (500 µl) were analyzed by SDS PAGE and immunoblotting using paraplegin- (filled squares), Afg3l1- (filled triangles), Afg3l2- (filled circles), Phb1- (open squares) and Phb2-specific antibodies (open circles). Proteins in the eluate were quantified by densitometry and are given as percent of total protein in the eluate (fractions of 1ml). Molecular weight sizes were estimated after calibration of the column with molecular weight markers. 1, molecular weight corresponding to ~1.900 kDa (10 ml), 2, elution of the ATP-synthase monomer (~750 kDa, 13 ml); 3, Thyroglobulin (669 kDa, 13 ml); 4, Appoferritin (440 kDa, 15 ml); 5, ADH (150 kDa, 17 ml). **B.** Physical interaction between paraplegin, Afg3l1, Afg3l2 and prohibitins in murine mitochondria. Mitochondria (400 µg) were lysed with 2% (w/v) digitonin and co-immunoprecipitations were carried out with affinity purified paraplegin-, Afg3l1- and Afg3l2-specific antibodies. Preimmune antiserum was used as negative control. Precipitate fractions were immunologically analyzed for the presence of paraplegin, Afg3l1, Afg3l2, Phb1 and Phb2. IP, immunoprecipitation. **C.** Potential *m*AAA proteases built up by paraplegin, Afg3l1 and/or Afg3l2 in murine mitochondria IM, inner mitochondrial membrane

control. Phb1 and Phb2 were thereby precipitated with paraplegin-specific antibodies indicating an interaction of prohibitins with paraplegin. Similarly, Phb1 and Phb2 were precipitated with Afg3l1- and Afg3l2-specific antibodies. These results, together with BN-SDS PAGE and sizing chromatography, show that prohibitins physically interact with paraplegin, Afg3l1 and Afg3l2 in murine mitochondria.

3. Subunit composition of murine *m*AAA proteases

3.1. Interactions between paraplegin, Afg3l1 and Afg3l2

Immunoprecipitation experiments demonstrated a physical interaction between paraplegin, Afg3l1 and Afg3l2 (Figure 18B). Afg3l2 and Afg3l1 were precipitated with paraplegin-specific antibodies. Additionally, Afg3l2 is precipitated with Afg3l1-specific antibodies and vice versa, indicating interaction between Afg3l1 and Afg3l2. Finally, paraplegin is precipitated with either Afg3l1- or Afg3l2-specific antibodies. It is therefore evident that paraplegin, Afg3l1 and Afg3l2 interact with each other and are additionally part of a high molecular weight complex with prohibitins.

These experiments, however, do not answer how the three subunits are assembled. While paraplegin, Afg3l1 and Afg3l2 may form one complex, it can not be excluded that the three proteins are part of several complexes with different subunit composition (Figure 18C). Therefore, paraplegin-Afg3l1, paraplegin-Afg3l2 and Afg3l1-Afg3l2 complexes can co-exist in murine liver mitochondria.

To distinguish between these possibilities, immunodepletion experiments were carried out using affinity purified paraplegin-, Afg3l1- and Afg3l2-specific antibodies. Therefore, mitochondria were lysed with digitonin and the extracts were incubated with saturating concentrations of the antibodies. The supernatants of the immunoprecipitates were then analyzed by immunoblotting for the presence of paraplegin, Afg3l1 and Afg3l2 (Figure 19).

Immunodepletions with paraplegin-specific antibodies failed to completely deplete paraplegin, presumably due to the limited titer of the antibody and therefore could not be used for immundepletion experiments. Using Afg3l1-specific antisera, on the other hand, resulted in apparently no detectable levels of Afg3l1 protein. The presence of paraplegin and Afg3l2 was next examined in the precipitation supernatant from immunodepletions with Afg3l1-specific antibodies. Immunoblotting showed that both paraplegin and Afg3l1 were significantly depleted (Figure 19B) indicating that Afg3l1 is indeed in a complex with paraplegin and/or Afg3l2. Nevertheless, paraplegin and

Afg3l2 were still present in the supernatant while Afg3l1 could not be detected. This suggests that paraplegin and Afg3l2 can, at least in part, form complexes independent of Afg3l1.

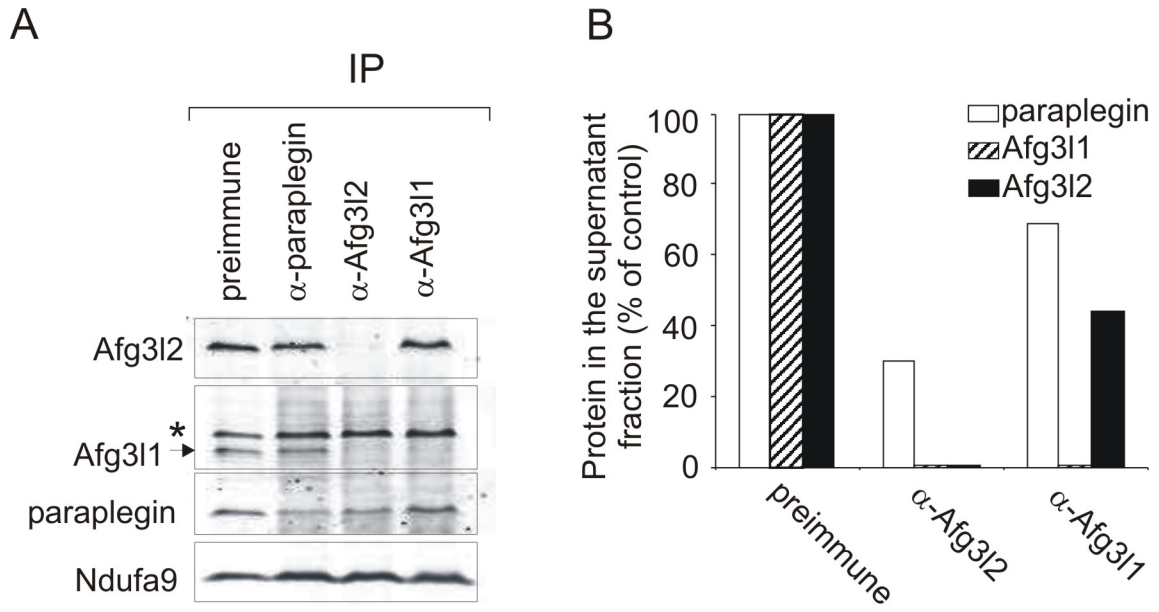

Figure 19. Immunodepletion experiments with paraplegin-, Afg3l1- and Afg3l2-specific antibodies. A. Murine liver mitochondria (150 μg) were lysed with digitonin and immunodepletions were carried out using saturating concentrations of paraplegin-, Afg3l1- and Afg3l2-specific antibodies. Preimmune antiserum was used as a control. Supernatants of the immunoprecipitates, corresponding to 75 μg mitochondrial protein, were analyzed by immunoblotting with paraplegin-, Afg3l1- and Afg3l2-specific antisera. Gel loading was assessed by immunoblotting with Ndufa9-specific antibodies. IP, immunoprecipitation; *, crossreacting band. **B.** The signals for paraplegin, Afg3l1 and Afg3l2 from immunodepletions carried out using Afg3l1- and Afg3l2-specific antibodies were quantified by densitometry, normalized to Ndufa9 and are shown as percentage of the corresponding signals in the negative control.

Precipitations with Afg3l2-specific antibodies resulted in the depletion of Afg3l2 from the supernatant, which was therefore analyzed for the presence of paraplegin and Afg3l1 (Figure 19A). Afg3l1 was not detectable in the precipitation supernatant, whereas paraplegin was strongly reduced but still present (Figure 19B). The presence of paraplegin can be explained either by its dissociation from a *m*AAA protease complex in the course of the experiment or by the presence of heterooligomeric complexes with Afg3l1 and/or Afg3l2. It should be noted that while immunodepletion with Afg3l2-specific antibodies resulted in the complete depletion of Afg3l1, Afg3l1-specific antibodies failed to completely deplete Afg3l2. This can be explained by a limited affinity of the Afg3l1-specific antibody, which does not allow

the detection of Afg3l1 in the supernatant fractions. It, therefore, remains to be determined whether Afg3l1, Afg3l2 and paraplegin assemble into one *m*AAA protease complex or whether different complexes, composed of different subunits, coexist in murine mitochondria.

To obtain further insights into the assembly of putative *m*AAA protease subunits, complex formation of Afg3l1 and Afg3l2 was analyzed in paraplegin-deficient mitochondria (Ferreirinha *et al.*, 2004).

3.2. Heterooligomeric Afg3l1-Afg3l2 complexes in mitochondria from a paraplegin-deficient mouse strain

The oligomeric state of Afg3l1 and Afg3l2 was examined in murine liver mitochondria from a paraplegin-deficient mouse strain ($SPG7^{-/-}$) (Ferreirinha *et al.*, 2004). In first experiments, mitochondria were lysed in digitonin and fractionated on a Superose-6 gel filtration column. Eluted fractions were next analyzed for Afg3l1 and Afg3l2. Both proteins co-eluted from the column in fractions corresponding to a molecular weight of about ~700-800 kDa (Figure 20A) suggesting the formation of heterooligomeric Afg3l1-Afg3l2 complexes.

Co-immunoprecipitation experiments were next carried out to directly demonstrate a complex between Afg3l1 and Afg3l2 (Figure 20B). Therefore, mitochondria from a paraplegin-deficient mouse strain were lysed with digitonin and immunoprecipitations were carried out with Afg3l2-specific antibodies. Afg3l1 was thereby immunoprecipitated with Afg3l2-specific antibodies, confirming the formation of heterooligomeric Afg3l1-Afg3l2 complexes.

Expression studies in yeast revealed that Afg3l1 can form homooligomeric complexes in the absence of Afg3l2 and vice versa (Mirko Koppen, personal communication). Therefore, the existence of such complexes was examined by immunodepletion experiments with saturating amounts of Afg3l2-specific antibodies (Figure 20C). Immunoblotting revealed that Afg3l1 was no longer detectable in the supernatant fractions from immunoprecipitations with Afg3l2-specific antisera, indicating that both proteins are quantitatively assembled.

Phb1 and Phb2 were also precipitated with Afg3l2-specific antibodies suggesting that paraplegin is not essential for the interaction with prohibitins. Nevertheless, the majority of Afg3l1-Afg3l2 complexes eluted at molecular weight of ~700-800 kDa and a small fraction was detectable at ~1.900 kDa. It is therefore conceivable that

absence of paraplegin can affect the stability of the supercomplex between Afg3l1, Afg3l2 and prohibitins.This data indicates that Afg3l1 and Afg3l2 form heterooligomeric complexes in the absence of paraplegin which can interact with prohibitins.

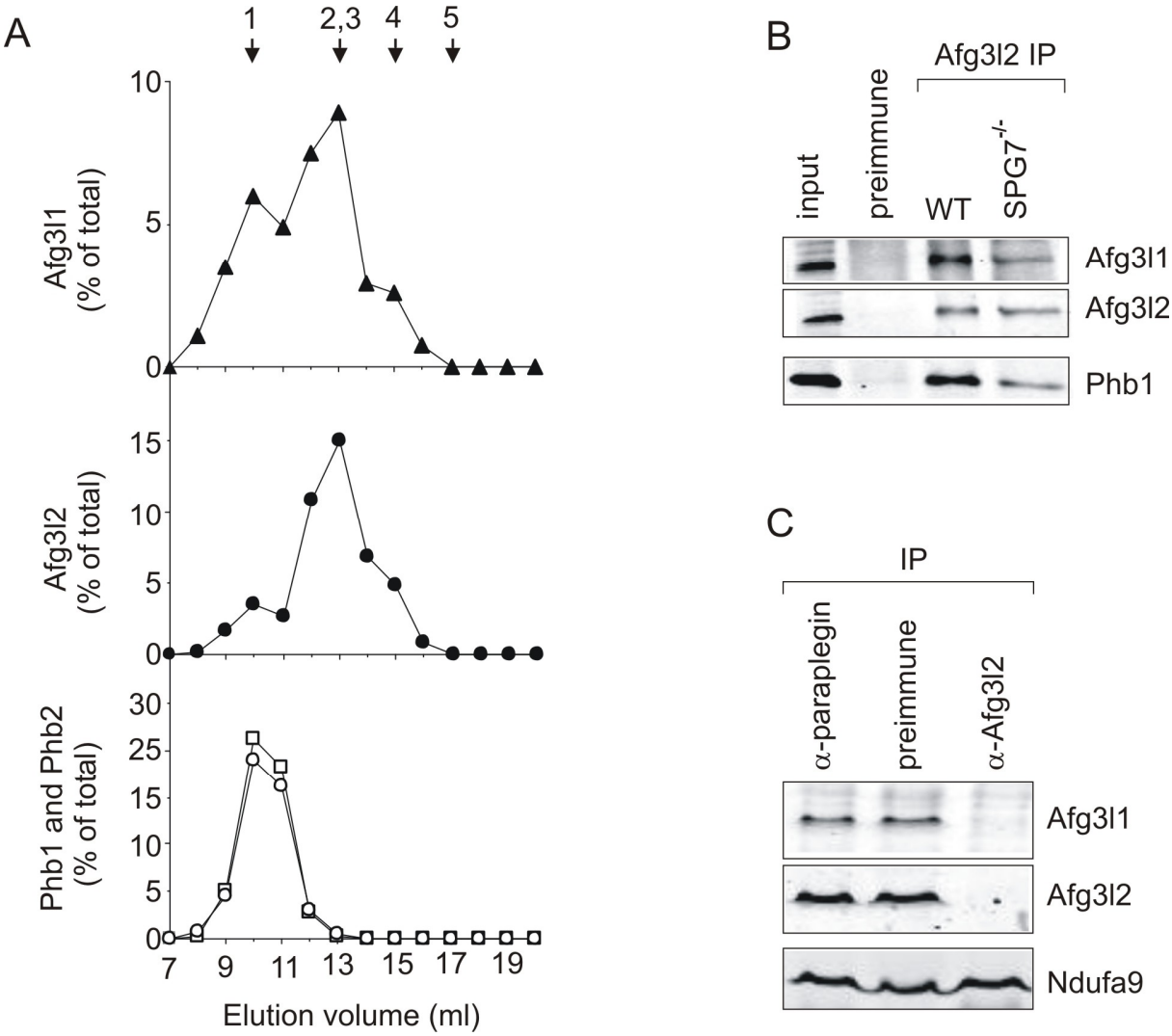

Figure 20. Afg3l1 and Afg3l2 form heterooligomeric complexes in paraplegin-deficient murine mitochondria. A. Gel filtration of mitochondria from a SPG7$^{-/-}$ mouse strain (Ferreirinha *et al.*, 2004). Mitochondria (1 mg) were solubilized with 2% (w/v) digitonin and fractionated on a Superose-6 gel filtration column. Eluate fractions (500 μl) were analyzed by immunoblotting with Afg3l1- (filled triangles), Afg3l2- (filled circles), Phb1- (open squares) and Phb2-specific (open circles) antibodies. Proteins in the eluate were quantified by densitometry and are shown as percentage of total protein (fractions of 1 ml). 1, molecular weight corresponding to ~ 1.900 kDa (10 ml), 2, elution of the ATP-synthase monomer (~750 kDa, 13 ml); 3, Thyroglobulin (669 kDa, 13 ml); 4, Appoferritin (440 kDa, 15 ml); 5, ADH (150 kDa, 17 ml). **B.** Physical interaction between Afg3l1 and Afg3l2. Wild type and SPG7$^{-/-}$ mitochondria (400 μg) were lysed with digitonin and immunoprecipitations were carried out with affinity purified Afg3l2-specific antibodies and preimmune antiserum as negative control. Afg3l1, Afg3l2 and Phb1 were detected immunologically in the precipitate. **C.** Afg3l1 and Afg3l2 are quantitatively assembled. Mitochondria from SPG7$^{-/-}$ mouse strain were lysed with digitonin and incubated with saturating amounts of affinity purified Afg3l2-specific antibodies. Paraplegin-specific antibodies and preimmune antiserum were used as negative control. Afg3l1 and Afg3l1 were detected immunologically in the supernatant fraction. Gel loading was assessed by immunoblotting with Ndufa9-specific antibodies. WT, wild type; SPG7$^{-/-}$, paraplegin-deficient; IP, immunoprecipitation.

V. DISCUSSION

1. Role of prohibitins during proteolysis in yeast

Prohibitins were proposed to function as negative regulators and/or chaperones protecting non-assembled mitochondrial proteins against proteolysis. The regulatory function of prohibitins is specifically restricted to the *m*AAA protease and its substrates since: i) prohibitins physically interact with the *m*AAA protease and ii) substrates of the *m*AAA protease but not of the *i*AAA protease are destabilized in Δ*phb1*Δ*phb2* cells (Steglich *et al.*, 1999). On the other hand, prohibitins can bind unassembled Cox2 and Cox3 (Nijtmans *et al.*, 2000). Additionally, overexpression of Phb1 and Phb2 partially stabilizes these substrates in Δ*phb1*Δ*phb2* cells (Nijtmans *et al.*, 2000). Therefore, prohibitins may function as a chaperone system for the assembly of newly synthesized subunits of the respiratory chain complexes in mitochondria (Nijtmans *et al.*, 2000). According to this model Phb1-Phb2 should function as a holdase which assists the assembly of Cox2 and Cox3 into the *COX* complex (Nijtmans *et al.*, 2000). During this process the substrates will be protected against proteolysis.

Although both possibilities are not mutually exclusive, I examined if discrimination between regulatory and chaperone function was possible by overexpression of prohibitins in wild type cells.

In first experiments the molar ratio was determined between the Phb1-Phb2 complex and the *m*AAA protease in mitochondria. It was estimated that both complexes are quantitatively assembled and are present in approximately equimolar concentrations in mitochondria. This result is in line with the exclusive co-elution of the *m*AAA protease subunits with Phb1 and Phb2 in sizing chromatography (Steglich *et al.*, 1999). Overexpression of prohibitins will therefore result in the accumulation of Phb1-Phb2 complexes which are not assembled with the *m*AAA protease and presumably function as molecular chaperones.

Chaperones often assist protein folding by repetitively binding and releasing the substrate (Fink, 1999; Hartl and Hayer-Hartl, 2002). Chaperone binding has also been demonstrated to protect the substrate against degradation. It is therefore conceivable that Phb1-Phb2 complexes, functioning as chaperones, will bind proteolytic substrates and stabilize them against proteolysis (Figure 21). Hence,

overexpression of prohibitins should result in higher substrate stability compared to cells with wild type levels of Phb1 and Phb2. This hypothesis was tested by assessing the stability of both mitochondrial translation products and overexpressed model substrates in cells harboring different levels of prohibitins.

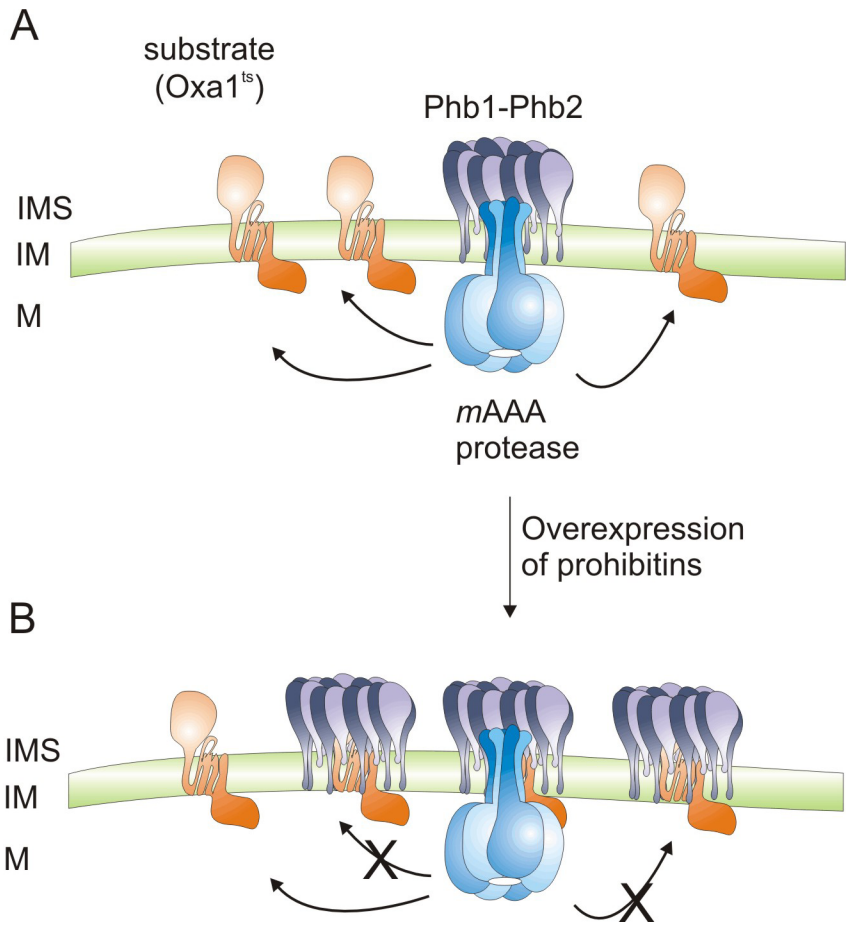

Figure 21. A potential chaperone function of the Phb1-Phb2 complex during proteolysis. A. Overexpressed Oxa1^{ts} is a proteolytic substrate of the *m*AAA protease. **B**. Overexpression of prohibitins leads to accumulation of Phb1-Phb2 complexes which are not assembled with the *m*AAA protease. These complexes, functioning as chaperones, bind Oxa1^{ts} thereby protecting it against proteolysis M, mitochondrial matrix; IM, inner mitochondrial membrane; IMS, intermembrane space.
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Pulse chase experiments with isolated mitochondria showed that mitochondrial translation products are degraded with similar kinetics irrespective of the levels of prohibitins. Similarly, cycloheximide chase experiments revealed that overexpression of prohibitins did not result in higher stability of Oxa1^{ts} and Yme2ΔC, two model substrates of the *m*AAA protease (Leonhard *et al.*, 2000; Käser *et al.*, 2003). Both proteins were degraded indistinguishably in wild type and in cells overexpressing

prohibitins. These results argue against a stable association of prohibitins and proteolytic substrates. It therefore appears unlikely that prohibitins exert a chaperone-like activity. Nevertheless, stabilization of Cox2 and Cox3 has been documented in Δ*phb1*Δ*phb2* cells overexpressing prohibitins (Nijtmans *et al.*, 2000). It should be pointed out, however, that the authors compared the stability of these substrates between cells overexpressing prohibitins and Δ*phb1*Δ*phb2* cells. It is therefore conceivable that the stabilizing effect of prohibitins in Δ*phb1*Δ*phb2* cells reflects a complementation of the negative regulation of the *m*AAA protease exerted by prohibitins in wild type cells. Moreover, a requirement of prohibitins for the assembly of respiratory chain complexes is unlikely. A direct assessment of respiratory chain complexes revealed that their assembly and activity are unchanged in the absence of prohibitins (Takashi Tatsuta, personal communication).

Similar to Cox3 (Steglich *et al.*, 1999), Oxa1^{ts} and Yme2ΔC were rapidly degraded in the absence of prohibitins. As both substrates are nuclear-encoded it can be concluded that the effect of prohibitins on proteolysis is not limited to mitochondrially encoded subunits of the COX and the ATP synthase complexes.

How do prohibitins affect substrate stability? Overexpression of prohibitins did not stabilize model substrates suggesting that only the Phb1-Phb2 complexes assembled with the *m*AAA protease function during proteolysis. It is, however, unlikely that prohibitins compete with substrates for the same recognition sites within the protease as Phb1 and Phb2 are not proteolytic substrates of the *m*AAA protease (Kambacheld *et al.*, 2005). Therefore, two mechanisms, which are not mutually exclusive and may act together, can be proposed to explain the role of prohibitins for substrate stability.

According to the first mechanism Phb1-Phb2 complexes, assembled with the *m*AAA protease, act as molecular adaptors thereby modulating the accessibility of the substrate to the *m*AAA protease. To execute this function prohibitins interact with both the substrates and the *m*AAA protease. Indeed, interaction of prohibitins with Cox3 has been demonstrated (Nijtmans *et al.*, 2000). Considering the topology of prohibitins in the inner mitochondrial membrane interactions with the substrate can occur within two regions of Phb1 and Phb2: i) the soluble domains exposed into the intermembrane space and ii) transmembrane domains into the inner mitochondrial membrane (Figure 22). These regions may interact with similarly exposed domains of the substrate. Consequently, a substrate devoid of either an IMS-domain or a TM-

domain will not be able to interact with prohibitins and will be thereforefore similalry destabilized in wild type cells and in cells lacking prohibitins. Interaction of prohibitins with IMS-domains of proteolytic substrates was tested by examining the stability of Yme2ΔC, which lacks an IMS-domain, in wild type and in Δ*phb1* cells. Similarly to Oxa1^{ts}, proteolysis of this substrate was also affected by the presence of prohibitins. It is therefore unlikely that prohibitins exert their function via an interaction with intermembrane space domains of the substrate. However, interaction of prohibitins with the TM-domains of the substrate can not be excluded at this time.

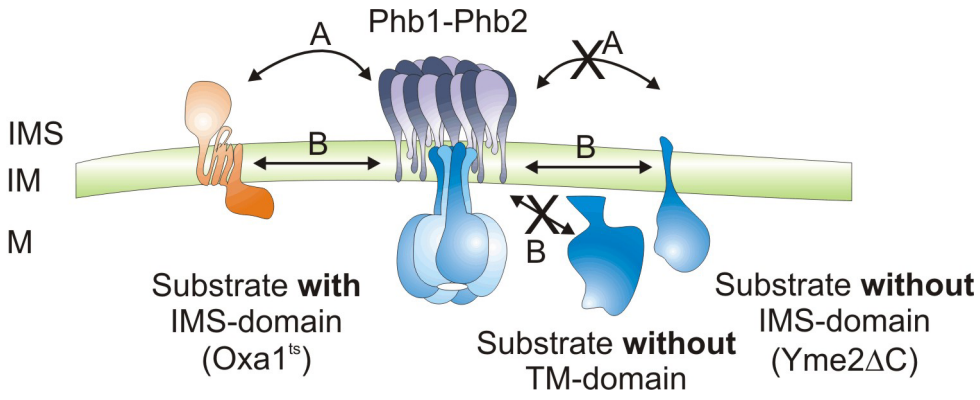

Figure 22. Possible interactions between prohibitins, assembled with the *m*AAA protease, and proteolytic substrates. A. Interaction between prohibitins and Oxa1^{ts} is mediated by their IMS-domains. Yme2ΔC does not have an IMS-domain and therefore cannot interact with prohibitins. **B**. Interaction between prohibitins and Oxa1^{ts} or Yme2ΔC is mediated by their TM-domains. A peripheral membrane protein which lacks a TM-domain cannot interact with prohibitins. IMS-domain, domain exposed to intermembrane space; TM-domain, trans-membrane domain; M, mitochondrial matrix; IM, inner mitochondrial membrane; IMS, intermembrane space.

In a second model prohibitins inhibit the ATPase or specific activity of the *m*AAA protease independently of the substrate. This model requires interaction of prohibitins only with the *m*AAA protease. Since both complexes have a different topology in the inner mitochondrial membrane it is conceivable that interactions between the *m*AAA protease and prohibitins are mediated by their transmembrane regions or via a small loop of *m*AAA protease subunits exposed into the intermembrane space. Recently, the TM-domains of the protease were shown to play a crucial role for proteolysis of integral membrane proteins (Korbel *et al.*, 2004). It is therefore possible that prohibitins exert their function during proteolysis by interaction with transmembrane domains of the protease subunits. Binding of prohibitins may induce conformational changes which are then transmitted from the N-terminal domains to the AAA-domains and/or the proteolytic domains of the *m*AAA protease subunits. Proteolysis

can thereby be inhibited, though not completely abolished, by stabilizing the *m*AAA protease in a conformation with decreased ATPase or proteolytic activity. However, binding of prohibitins is not affected by nucleotide dependent conformational changes of the *m*AAA protease subunits. A mutant *m*AAA protease whose subunits are stabilized in an either ATP-bound or nucleotide free conformation was still able to form a supercomplex with prohibitins. Moreover, substrate trap mutants of the *m*AAA protease were found to simultaneously bind the substrate and prohibitins (Nolden *et al.*, 2005a). Similarly, the non degradable substrate of FtsH, YccA11, was found bound to the FtsH-HflK/C supercomplex (Kihara *et al.*, 1998). Therefore, it is evident that the supercomplex can exist throughout the proteolytic cycle of the *m*AAA protease irrespectively of its nucleotide conformation or the binding of substrate. Further experiments addressing the ATPase and protease activities of the purified *m*AAA protease should answer whether or not these activities are affected by the absence or presence of prohibitins.

To this moment neither of both models can be excluded. However, stability tests of peripheral membrane proteins in the absence of prohibitins could provide evidence supporting either model. Decreased stability of peripheral membrane proteins under these conditions will indicate that the Phb1-Phb2 complexes function as general inhibitors of the *m*AAA protease and irrespectively of the substrate. On the other hand, peripheral membrane proteins should not be affected by the absence of prohibitins, if interaction between TM-domains of prohibitins and the substrate are involved in proteolysis. Moreover, it is conceivable that such substrates are stabilized in the absence of prohibitins by a competition mechanism similar to the one proposed for cII in *E. coli* (Kihara *et al.*, 1997). In *E. coli*, the absence of HflK/C destabilizes SecY, an integral membrane protein, and leads to its rapid degradation while the cytoplasmic cII protein is stabilized (Kihara *et al.*, 1996; Kihara *et al.*, 1997). This has been explained by the preferential degradation of integral membrane proteins in the absence of HflK/C (Kihara *et al.*, 1997). Prohibitins therefore may balance the degradation of integral and peripheral membrane proteins by the *m*AAA protease in a similar manner.

What is the function of prohibitins in yeast mitochondria? There is mounting evidence that the function of prohibitins is not limited to regulation of substrate stability in mitochondria. Synthetic lethal phenotypes with components of the mitochondrial morphology and maintenance machinery (Berger and Yaffe, 1998) and with deletions

of the subunits of the *i*AAA or the *m*AAA protease (Steglich *et al.*, 1999) are difficult to reconcile with a proteolytic function of prohibitins. Therefore, additional functions of Phb1 and Phb2 in mitochondria are likely to be discovered.

2. Role of prohibitins in murine mitochondria

In view of the high sequence similarity of prohibitins in different species it is likely that they exert conserved functions in eukaryotic cells. Nevertheless, ever since prohibitins were discovered as negative regulators of cell proliferation most studies were focused on their possible nuclear functions. Hence, little is known about prohibitins in mammalian mitochondria. Therefore, the Phb1-Phb2 complex and its molecular interactions were examined in murine liver mitochondria.

BN-SDS PAGE analysis showed that prohibitins form a similar-sized complex both in yeast and in murine liver mitochondria. Although the exact stoichiometry of the subunits in the Phb1-Phb2 complex is unknown, it was proposed that the number of alternatingly assembled Phb1 and Phb2 varies between 12 and 18 in yeast (Nijtmans *et al.*, 2000; Back *et al.*, 2002; Tatsuta *et al.*, 2005). Additionally, the yeast Phb1-Phb2 complex has a barrel-like appearance in EM analysis (Tatsuta *et al.*, 2005). It is, therefore, conceivable that prohibitins form a similarly shaped barrel-like complex in murine mitochondria.

What is the function of prohibitins in murine mitochondria? First evidence comes from biochemical experiments which show that the Phb1-Phb2 complex can physically interact with putative subunits of the murine *m*AAA protease in liver mitochondria. Phb1 and Phb2 were precipitated with paraplegin-, Afg3l1- and Afg3l2-specific antibodies indicating the formation of a supercomplex between these proteins. It is therefore conceivable that prohibitins function as regulators of the activity of the murine *m*AAA protease. To this moment, however, it is unknown if the absence of prohibitins in mammals results in decreased substrate stability. One hindrance in this direction is that in contrast to yeast deletion of Phb2 is embryonic lethal in mammals (Park *et al.*, 2005).

3. Murine *m*AAA proteases with variable subunit composition

The *m*AAA protease is crucial for both the protein quality control in mitochondria and expression of the mitochondrial genome (Arlt *et al.*, 1998; Nolden *et al.*, 2005a). In

yeast and humans the *m*AAA protease is composed of two homologous subunits - Yta10 and Yta12 or paraplegin and Afg3l2, respectively (Arlt *et al.*, 1996; Atorino *et al.*, 2003). In contrast, an additional putative *m*AAA protease subunit, termed Afg3l1, is expressed in mice (Kremmidiotis *et al.*, 2001).

To analyze the subunit composition of the murine *m*AAA protease specific antisera were generated against paraplegin, Afg3l2 and Afg3l1 and used in co-immunoprecipitation experiments. Thereby, paraplegin, Afg3l1 and Afg3l2 were found to physically interact with each other. Afg3l1 and Afg3l2 were precipitated with paraplegin-specific antibodies. Afg3l1 was precipitated with Afg3l2-specific antibodies and vice versa, and paraplegin was precipitated with either Afg3l1- or Afg3l2-specific antibodies. These findings are consistent with a complex containing three different subunits. It is however also conceivable that paraplegin, Afg3l1 and Afg3l2 assemble in several complexes with different subunit composition: paraplegin-Afg3l1, paraplegin-Afg3l2 and Afg3l1-Afg3l2 (Figure 23). To test this immunodepletion experiments were carried out using paraplegin-, Afg3l1- and Afg3l2-specific antibodies.

Immunodepletions with Afg3l1-speciffic antibodies showed that while Afg3l1 is no longer detectable, paraplegin and Afg3l2 were depleted but could still be detected in the supernatant fraction. This suggests that paraplegin and Afg3l2 can, at least in part form complexes independent of Afg3l1. The fact that both proteins were partially depleted with Afg3l1-specific antibodies also indicates that Afg3l1 is indeed in a complex with paraplegin and/or Afg3l2. On the other hand, depletion of Afg3l2 using Afg3l2-specific antibodies resulted in no detectable Afg3l1 in the supernatant fraction. In contrast, paraplegin was strongly depleted but could still be detected. Even though this can be explained by dissociation of paraplegin in the course of the experiment it is also possible that heterooligomeric complexes with Afg3l1 and/or Afg3l2 are still present in the supernatant fractions. Immunodepletion with Afg3l2-specific antibodies resulted in the complete depletion of Afg3l1 while Afg3l1-specific antibodies failed to completely deplete Afg3l2. This suggests that detection of Afg3l1 in the supernatant fractions may be limited by the affinity of the Afg3l1-specific antibody. Therefore, it remains unclear if the three putative *m*AAA protease subunits can form complexes with different subunit composition which co-exist in liver mitochondria.

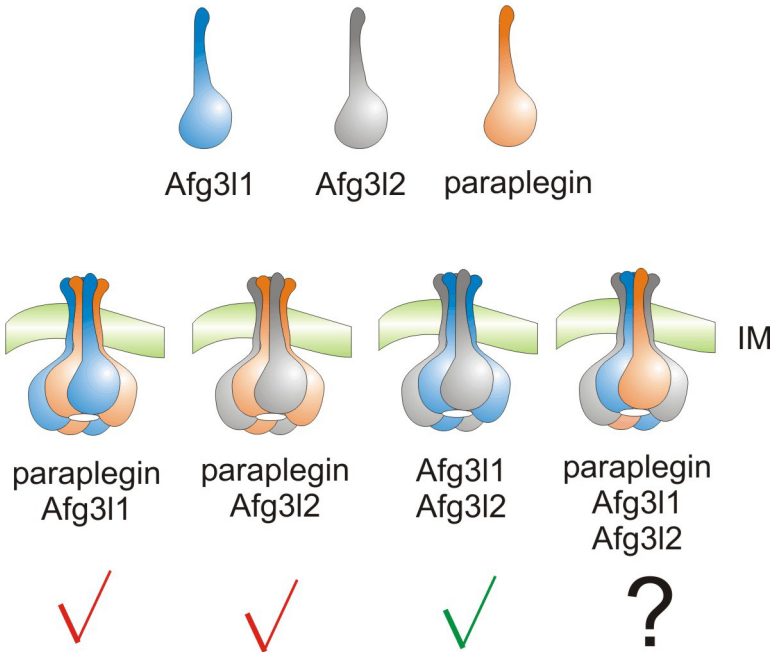

Figure 23. Putative *m*AAA protease complexes formed by paraplegin, Afg3l1 and Afg3l2. Paraplegin-Afg3l1 and paraplegin-Afg3l2 complexes have been detected after co-expression of paraplegin with Afg3l1 or Afg3l2 in *S.cerevisiae* and are shown with red tick-marks (Mirko Koppen, personal communication). Afg3l1 and Afg3l2 were shown to form heterooligomeric complexes in a paraplegin-deficient mouse strain and are shown with green tick-marks. The complex built up by three different subunits is not yet identified and is shown with a question mark.

Different assembly combinations between paraplegin, Afg3l1 and Afg3l1 can be examined in mitochondria from mouse strains deficient for either paraplegin, Afg3l1 or Afg3l2 or in yeast cells expressing these proteins. Heterologous co-expression of paraplegin with Afg3l1 or Afg3l2 in yeast showed that paraplegin-Afg3l1 or paraplegin-Afg3l2 complexes can indeed be formed (Mirko Koppen, personal communication). Notably, these complexes are active in yeast as they can complement for the absence of the yeast *m*AAA protease and can restore growth on glycerol (Mirko Koppen, personal communication). It is therefore conceivable that paraplegin-Afg3l1 or paraplegin-Afg3l2 complexes can also be formed in Afg3l2 or Afg3l1-deficient mice, respectively. On the other hand, similar experiments show that Afg3l1 and Afg3l2 but not paraplegin can also form homooligomeric complexes in yeast (Mirko Koppen, personal communication) rising the possibility that these complexes also exist in murine mitochondria. Therefore, the oligomerization of Afg3l1 and Afg3l2 was examined in liver mitochondria from a paraplegin deficient mouse strain (Ferreirinha *et al.*, 2004). Afg3l1 and Afg3l2 co-eluted at a molecular weight of ~700-800 kDa in sizing chromatography experiments, suggesting the formation of

high molecular weight complexes. The possibility that Afg3l1 and Afg3l2 are part of different complexes was then examined in co-immunoprecipitation and immunodepletion experiments. Immunoprecipitation experiments using Afg3l2-specific antibodies showed that Afg3l1 is in a complex with Afg3l2. Moreover, immunodepletion of Afg3l2 resulted in a depletion of Afg3l1 from the supernatant fractions indicating that both proteins are quantitatively assembled. Therefore, it is evident that Afg3l1 and Afg3l2 form heterooligomeric complexes in the absence of paraplegin.

In mice and humans, absence of paraplegin results in the development of a neuronal degeneration called HSP, which affects the longest motor axons (Casari *et al.*, 1998; Ferreirinha *et al.*, 2004). This peculiar tissue specificity can be accommodated with the existence of *m*AAA proteases with different substrate specificity and/or tissue distribution. As SPG7$^{-/-}$ mice develop HSP phenotype, *m*AAA protease complexes built up by Afg3l1 and Afg3l2 are not functionally identical to paraplegin-containig *m*AAA protease(s) complexes. Notably, paraplegin-deficient mice are phenotipically different than mouse strains lacking Afg3l2 (Giorgio Casari, personal communication). While SPG7$^{-/-}$ mice are viable and can live up to adulthood, Afg3l2-deficient mice suffer massive paresis and die soon after birth. Although the molecular mechanism behind the phenotype of Afg3l2-deficient mice is not yet clear, it is conceivable that an axonal degeneration is also involved. The different phenotype of paraplegin- and Afg3l2-deficient mice also suggests that paraplegin and Afg3l2, and presumably Afg3l1, are not functionally equivalent. Therefore, it is conceivable that different assembly combinations between paraplegin, Afg3l1 and Afg3l2 will result in complexes which differ in their specific activity or tissue distribution.

What is the physiological significance of *m*AAA complexes with different subunit composition? Although distantly related, bacterial Clp proteases may be helpful in exemplifying the advantage of co-existence of different subunit combinations. ClpP, ClpX and ClpA form cytoplasmic complexes composed of proteolytic (ClpP) and chaperone (ClpX and ClpA) subunits. The assembled ClpAP and ClpXP complexes are functionally not identical and differ in their substrate specificity (Flynn *et al.*, 2001). Nevertheless, a recent report suggests ClpXP and ClpAP complexes can co-exist together with ClpXAP complexes *in vivo* (Ortega *et al.*, 2004). ClpXAP complexes possess the substrate specificities of both ClpXP and ClpAP complexes and have higher activity than the single ClpXP or ClpAP complexes. Thus, the

substrate range is efficiently increased as well as the activity of the complex (Ortega *et al.*, 2004). In analogy, it is conceivable that different combinations of paraplegin, Afg3l1 and Afg3l2 assemblies co-exist in mammalian mitochondria and may differ in their substrate specificity and tissue distribution. Analyses of substrate stability *in vitro* combined with proteomic approaches *in vivo* can reveal if the complexes differ in their substrate specificity.

As previously mentioned, paraplegin, Afg3l1 and Afg3l2 physically interact with prohibitins and are contained within a high molecular weight complex in mitochondria from a wild type mouse strain. Interestingly, the Afg3l1-Afg3l2 complex could also interact with prohibitins. Phb1 and Phb2 were precipitated with Afg3l2-specific antibodies from SPG7$^{-/-}$ mitochondria suggesting that paraplegin is not essential for the interaction with prohibitins. Therefore, it is conceivable that paraplegin-Afg3l1 and paraplegin-Afg3l1 complexes also be able to interact with prohibitins. This, however, can be experimentally confirmed only in mitochondria from Afg3l1- or Afg3l2-deficient mice.

VI. APPENDIXES

Appendix I

A multisequence alignment of AAA-protease subunits from bacteria, yeast, mouse and human. Alignment was done using the multisequence alignment software AlignX with default setting and visualized with GeneDoc. Identical amino acids are also shown on a black background. Blocks of conserved amino acids between several AAA protease subunits are shown in grey. Walker A, Walker B and the HEG*X*H motifs are shown with red, yellow and green lines, respectively. Protein accession numbers used for the alignment are provided in the rable bellow. Sequences were extracted from the national center for biotechnology information (NCBI).

PROTEIN/ORGANISM	ACCESSION NUMBER
Afg3l2 Mouse	AAH43056.1
Afg3l2 Human	NP_006787.1
Afg3l1 Mouse	NP_473411.2
Yta10 *S. cerevisiae*	YER017C [1]
Yta12 *S. cerevisiae*	YMR089C [1]
Paraplegin Mouse	AAN03852.1
paraplegin Human	CAA76314.1
Yme1l Mouse	AAC35558.1
YME1L Human	AAQ88848.1
Yme1 *S.cerevisiae*	YPR024W [1]
FtsH *E. coli*	AAA97508.1

[1] – Protein sequence was derived by a translation from the DNA coding sequences provided at *Saccharomyces* genome database (SGD, http://www.yeastgenome.org/)

```
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      hAFG3L2 --MAHRCLRLWGRGGCWPRGLQQLLVP-GGVGPGEQPCLRTLYRFVTTQARASRNS---- :  53
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         Yta10 --MMMWQRYARGAPRSLTSLSFGKASRISTVKPVLRSRMPVHQRLQTLSGLATRN----- :  53
         Yta12 MLLLSWSRIATKVVR--RPVRFRSYYGLTHIKSLHTQYRLLNRLQENKSGNKNEDNNEDA :  58
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   hparaplegin --MAVLLLLLRALRRGPGPGPRPLWGPGPAWSPGFPARPGRGRPYMASRPPGDLAEAG-- :  56
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      hYME1L -MFSLSSTVQPQVTVPLSHLINAFHTPKNTSVSLSGVSVSQNQHRDVVPEHEAPSSEP-- :  57
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        FtsH ------------------------------------------------------------ :   -
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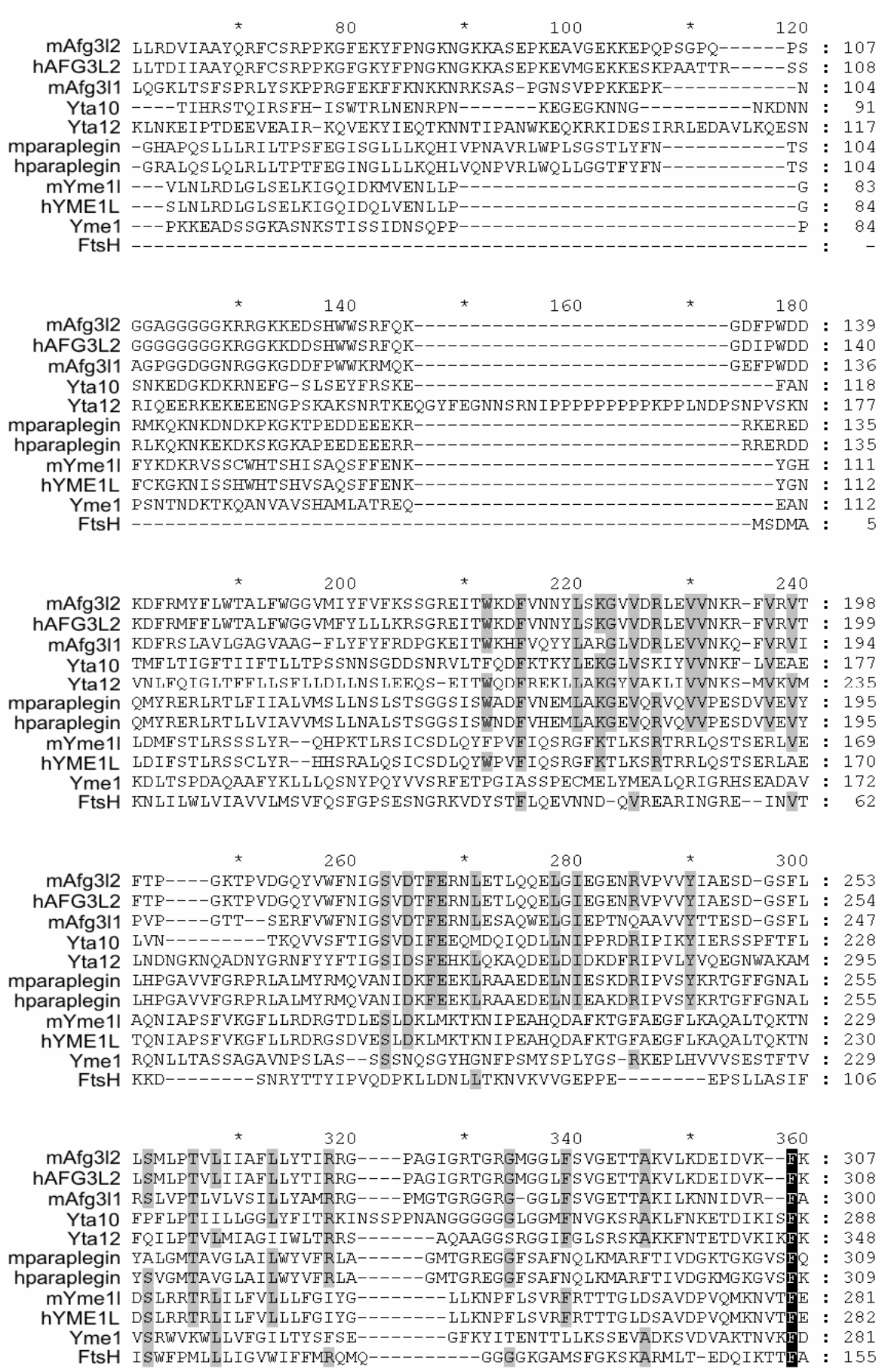
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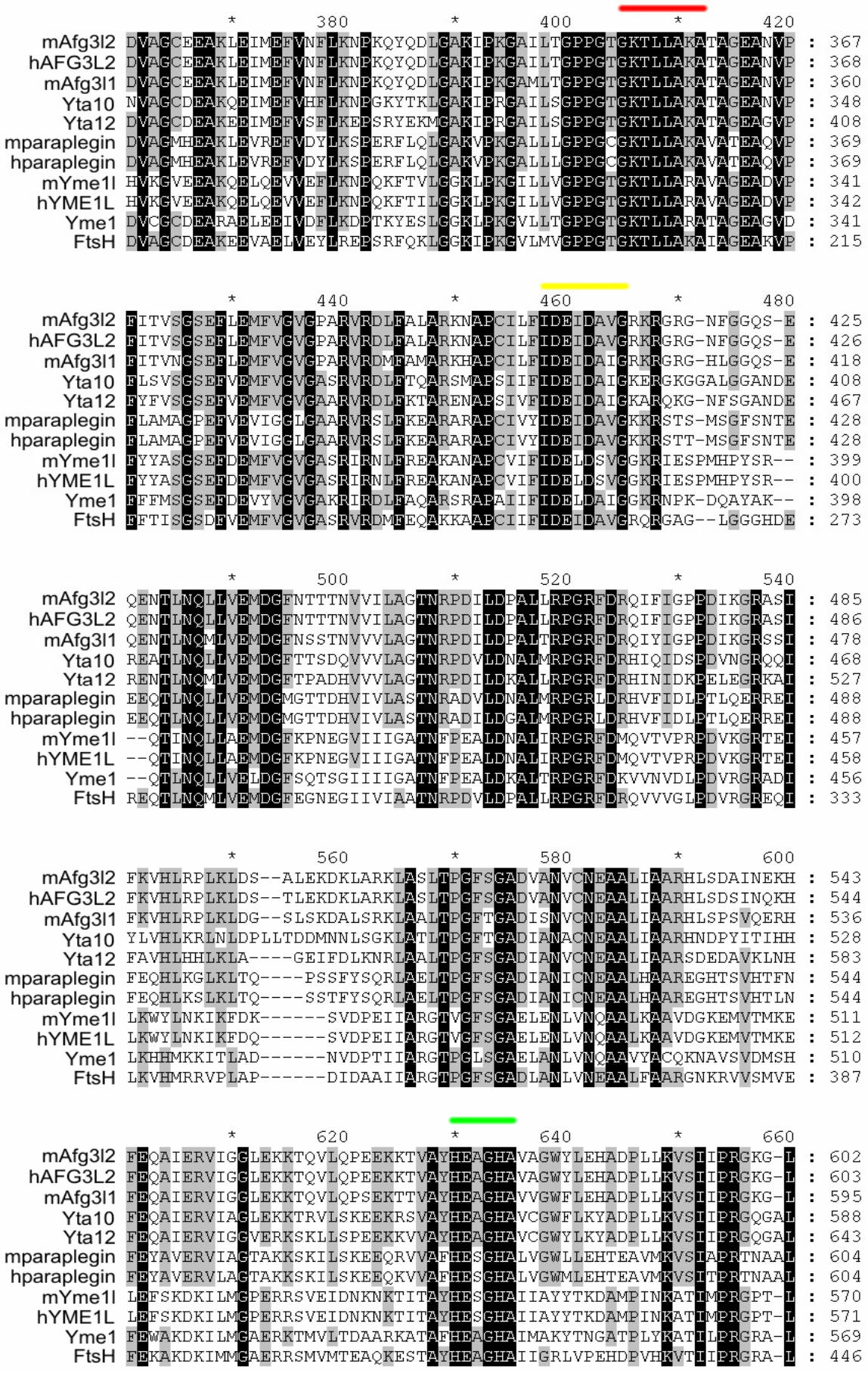
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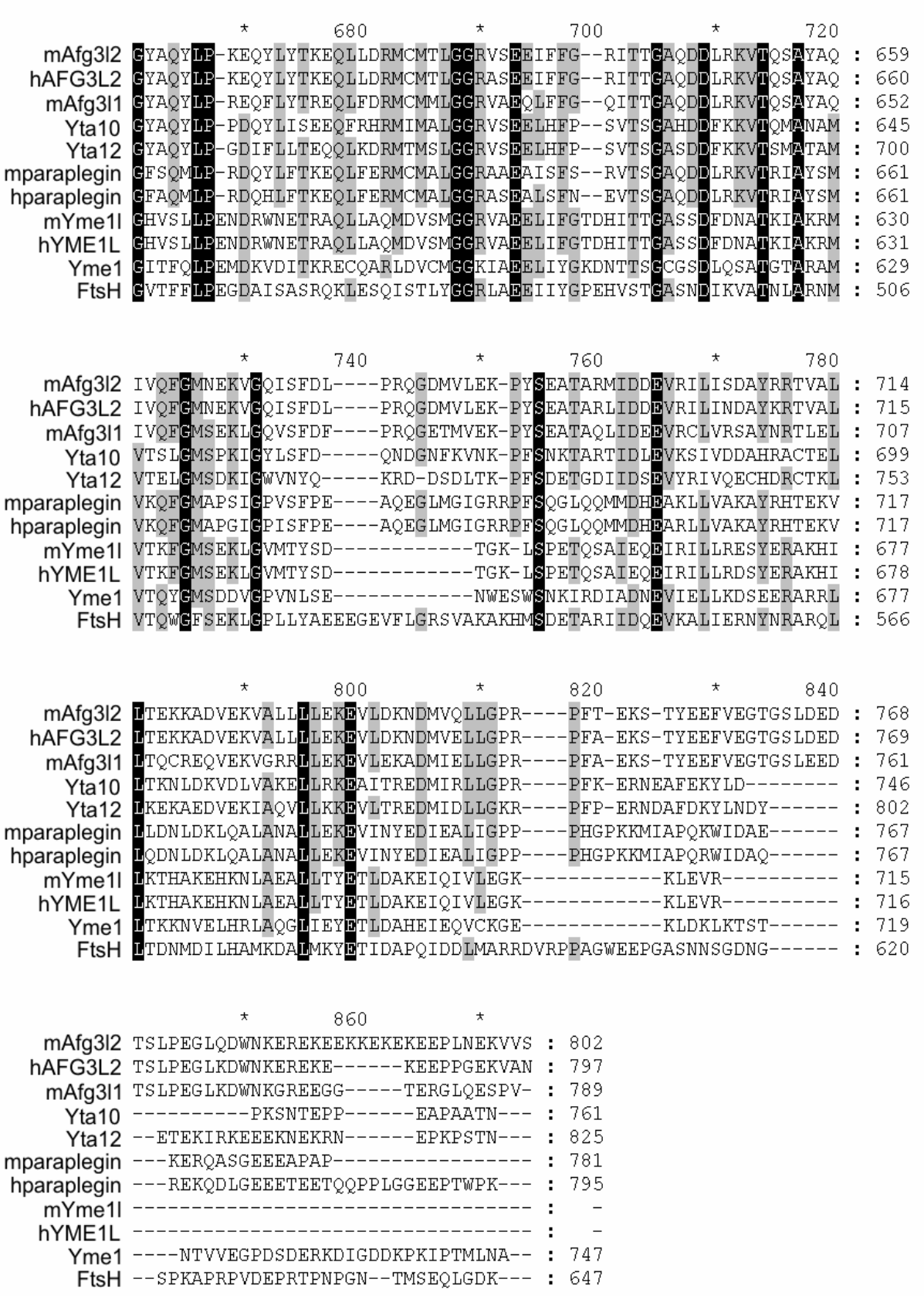
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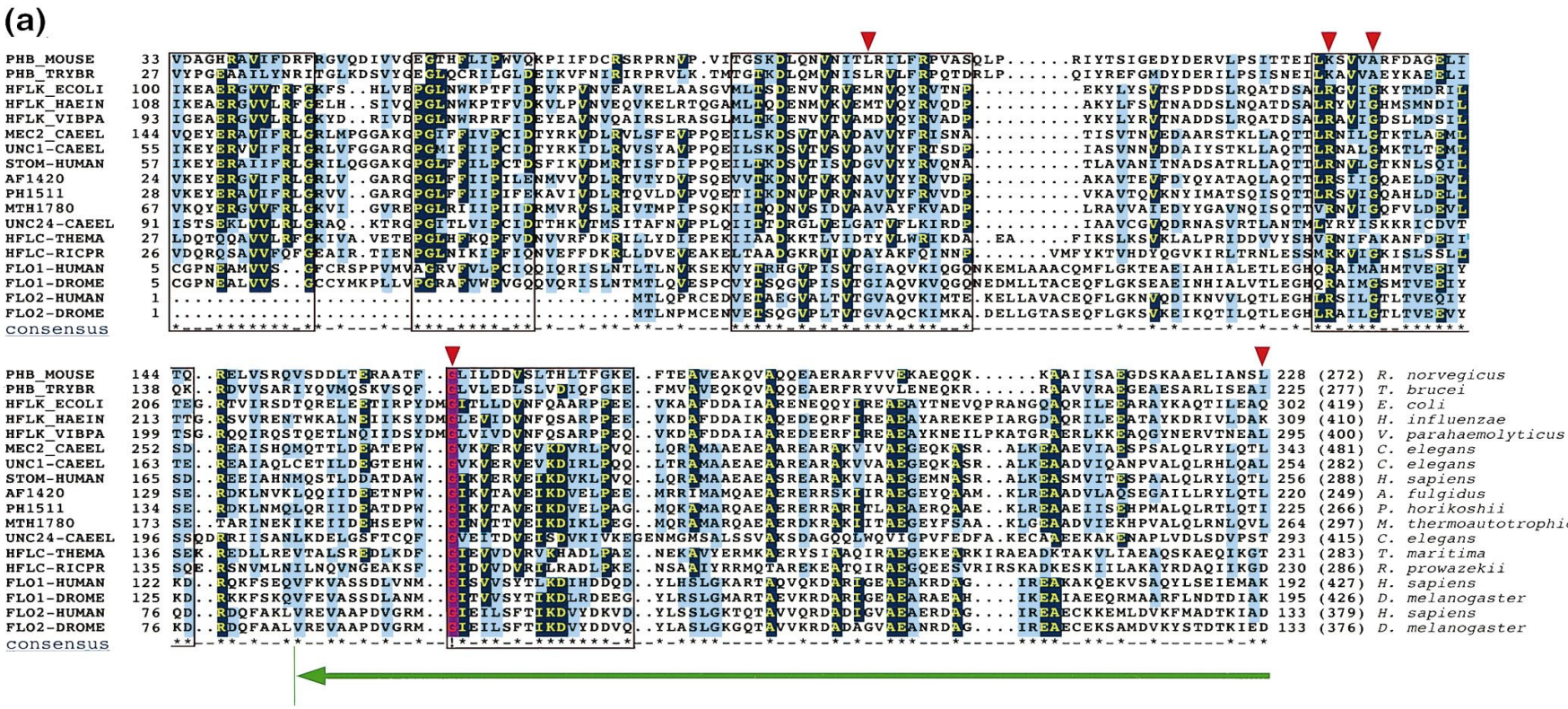
Yta12 GYAQYLP-GDIFLLTEQQLKDRMTMSLGGRVSEELHFP--SVTSGASDDFKKVTSMATAM : 700
mparaplegin GFSQMLP-RDQYLFTKEQLFERMCMALGGRAAEAISFS--RVTSGAQDDLRKVTRIAYSM : 661
hparaplegin GFAQMLP-RDQHLFTKEQLFERMCMALGGRASEALSFN--EVTSGAQDDLRKVTRIAYSM : 661
mYme1l GHVSLLPENDRWNETRAQLLAQMDVSMGGRVAEELIFGTDHITTGASSDFDNATKIAKRM : 630
hYME1L GHVSLLPENDRWNETRAQLLAQMDVSMGGRVAEELIFGTDHITTGASSDFDNATKIAKRM : 631
Yme1 GITFQLPEMDKVDITKRECQARLDVCMGGKIAEELIYGKDNTTSGCGSDLQSATGTARAM : 629
FtsH GVTFFLPEGDAISASRQKLESQISTLYGGRLAEEIIYGPEHVSTGASNDIKVATNLARNM : 506
mAfg3l2 IVQFGMNEKVGQISFDL----PRQGDMVLEK-PYSEATARMIDDEVRILISDAYRRTVAL : 714
hAFG3L2 IVQFGMNEKVGQISFDL----PRQGDMVLEK-PYSEATARLIDDEVRILINDAYKRTVAL : 715
mAfg3l1 IVQFGMSEKLGQVSFDF----PRQGETMVEK-PYSEATAQLIDEEVRCLVRSAYNRTLEL : 707
Yta10 VTSLGMSPKIGYLSFD-----QNDGNFKVNK-PFSNKTARTIDLEVKSIVDDAHRACTEL : 699
Yta12 VTELGMSDKIGWVNYQ-----KRD-DSDLTK-PFSDETGDIIDSEVYRIVQECHDRCTKL : 753
mparaplegin VKQFGMAPSIGPVSFPE----AQEGLMGIGRRPFSQGLQQMMDHEAKLLVAKAYRHTEKV : 717
hparaplegin VKQFGMAPGIGPISFPE----AQEGLMGIGRRPFSQGLQQMMDHEARLLVAKAYRHTEKV : 717
mYme1l VTKFGMSEKLGVMTYSD----------TGK-LSPETQSAIEQEIRILLRESYERAKHI : 677
hYME1L VTKFGMSEKLGVMTYSD----------TGK-LSPETQSAIEQEIRILLRDSYERAKHI : 678
Yme1 VTQYGMSDDVGPVNLSE----------NWESWSNKIRDIADNEVIELLKDSEERARRL : 677
FtsH VTQWGFSEKLGPLLYAEEEGEVFLGRSVAKAKHMSDETARIIDQEVKALIERNYNRARQL : 566
mAfg3l2 LTEKKADVEKVALLLLEKEVLDKNDMVQLLGPR----PFT-EKS-TYEEFVEGTGSLDED : 768
hAFG3L2 LTEKKADVEKVALLLLEKEVLDKNDMVELLGPR----PFA-EKS-TYEEFVEGTGSLDED : 769
mAfg3l1 LTQCREQVEKVGRRLLEKEVLEKADMIELLGPR----PFA-EKS-TYEEFVEGTGSLEED : 761
Yta10 LTKNLDKVDLVAKELLRKEAITREDMIRLLGPR----PFK-ERNEAFEKYLD-------- : 746
Yta12 LKEKAEDVEKIAQVLLKKEVLTREDMIDLLGKR----PFP-ERNDAFDKYLNDY------ : 802
mparaplegin LLDNLDKLQALANALLEKEVINYEDIEALIGPP----PHGPKKMIAPQKWIDAE------ : 767
hparaplegin LQDNLDKLQALANALLEKEVINYEDIEALIGPP----PHGPKKMIAPQRWIDAQ------ : 767
mYme1l LKTHAKEHKNLAEALLTYETLDAKEIQIVLEGK----------KLEVR---------- : 715
hYME1L LKTHAKEHKNLAEALLTYETLDAKEIQIVLEGK----------KLEVR---------- : 716
Yme1 LTKKNVELHRLAQGLIEYETLDAHEIEQVCKGE----------KLDKLKTST------ : 719
FtsH LTDNMDILHAMKDALMKYETIDAPQIDDLMARRDVRPPAGWEEPGASNNSGDNG------ : 620
mAfg3l2 TSLPEGLQDWNKEREKEEKKEKEKEEPLNEKVVS : 802
hAFG3L2 TSLPEGLKDWNKEREKE------KEEPPGEKVAN : 797
mAfg3l1 TSLPEGLKDWNKGREEGG-----TERGLQESPV- : 789
Yta10 ----------PKSNTEPP------EAPAATN--- : 761
Yta12 --ETEKIRKEEEKNEKRN------EPKPSTN--- : 825
mparaplegin ---KERQASGEEEAPAP---------- : 781
hparaplegin ---REKQDLGEEETEETQQPPLGGEEPTWPK--- : 795
mYme1l ---------- : -
hYME1L ---------- : -
Yme1 ----NTVVEGPDSDERKDIGDDKPKIPTMLNA-- : 747
FtsH --SPKAPRPVDEPRTPNPGN--TMSEQLGDK--- : 647



A Multisequence alignment generated with the ClustalW program18 and visualized with Boxshade (ISREC, http//www.isrec.isb-sib.ch,8080/software/BOX_form.html). Boxes mark the most conserved blocks of the domain, as identified with HMM searches 19. When sequence conservation is greater than 50%, identical residue positions are highlighted in dark blue and conservative substitutions in light blue. Red arrowheads denote mutations that disrupt the function of MEC-2 or UNC-1 in *Caenorhabditis elegans*. The green arrowbar denotes the region that has been shown to be important for oligomerization of vertebrate stomatin. Amino acid positions are indicated in the margins of the alignment. Numbers in parentheses at the end of the alignment denote the total length of the corresponding protein. Source organisms are also shown. Reprinted with text from (Tavernarakis *et al.*, 1999)

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VIII. ABBREVIATIONS

~	around, approximately, about, nearly
Å	Angstrom
Amp	Ampicillin
ADH	Alcochol dehydrogenase
BN-PAGE	Blue native PAGE
BN-SDS PAGE	Blue native PAGE followed by SDS PAGE
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylene-diaminetetraacetic acid
EGTA	Ethylene-glycol tetraacetic acid
ER	Estrogen receptor
g	Gram
GST	Glutathione *S*-transferase
fmol	fempto mol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HCl	Hydrochloric acid
HIS_6	hexahistidine (6xHIS, 6HIS)
IPTG	isopropyl β-D-thiogalactoside
IgG	Immunoglobulin G
KAc	Potassium acetate
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
l	Liter
LB-Medium	Luria-Bertrani-medium
LiAc	Lithium acetate
M	Molar
mM	miliMolar
µM	microMolar
MDa	Megadalton
mg	miligram
min	Minutes
ml	Milliliters
MMM-machinery	Mitochondrial morphology and maintenance machinery
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
$MnCl_2$	Manganese chloride
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
MW	Molecular weight
NaAc	Sodium actetate
NaCl	Sodium chloride

NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
ng	Nanograms
nm	Nanometers
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomol
PMSF	Phenylmethylsulphonylfluoride
RbCl	Rubidium chloride
RNAse A	Ribonuclease A
ROS	Reactive oxygen species
rpm	Rounds per minute
sec	Seconds
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEV	Tobacco etch virus protease
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiole
UTR	Untranslated Region
Δ	Deleted / mutated allele

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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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