Functional characterization of the mammalian iAAA protease subunit, YME1L.

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Joanna Majczak

aus Tarnobrzeg, Polen

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Berichterstatter:

Professor Dr. Thomas Langer

Professor Dr. Jens C. Brüning

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Abstract

The iAAA protease is an ATP-dependent proteolytic complex in the mitochondrial inner membrane and belongs to the highly conserved family of AAA proteins. In the yeast *Saccharomyces cerevisiae*, the iAAA protease is a homo-oligomeric complex composed of Yme1p subunits which are active in the intermembrane space and mediate protein quality control. Yeast cells lacking Yme1p are characterized by pleiotropic phenotypes including a respiratory deficiency at elevated temperature and an aberrant mitochondrial morphology. However, the molecular basis of the different yeast *yme1* phenotypes has not been completely understood. Human YME1L was shown to be the ortholog of the yeast Yme1p, but its functions within mammalian mitochondria and specific substrate proteins have not been identified so far. In order to define the roles of the mammalian iAAA protease two main approaches were carried out: (1) down regulation studies in mammalian cells using RNA interference, and (2) inducible overexpression of YME1L and several mutant variants in the mammalian system.

The present study reports the functional conservation of the mammalian YME1L by showing its involvement in the proteolysis of prohibitin 1 whose homolog is also degraded by the iAAA protease in yeast. Furthermore, it demonstrates that YME1L has a role in the maintenance of the tubular mitochondrial morphology and in the constitutive processing of OPA1, a component of the mitochondrial fusion machinery. Moreover, the degradation of TIM23, a core subunit of the translocase of the inner membrane, in caspase-independent apoptosis depends on YME1L identifying a novel substrate for the mammalian iAAA protease and a new pathway it is involved in. The overexpression of YME1L mutants has no dominant negative effect on the iAAA protease. Its functions in mitochondrial proteolysis, import, and mitochondrial morphology remain unaltered.

Taken together, this study reveals versatile roles of mammalian YME1L in proteolytic quality control, mitochondrial morphology

and apoptosis, thus demonstrating its importance for both the cellular viability and death.

Zusammenfassung

Die iAAA-Protease ist ein ATP-abhängiger proteolytischer Komplex in der inneren Mitochondrienmembran und gehört zu der hochkonservierten Familie der AAA-Proteine. In der Hefe *Saccharomyces cerevisiae* ist die iAAA-Protease ein homooligomerer Komplex bestehend aus Yme1p-Untereinheiten, die im Intermembranraum aktiv sind und die Qualitätskontrolle von Proteinen vermitteln. Hefezellen, denen Yme1p fehlt, sind durch pleiotrope Phänotypen charakterisiert. Diese beinhalten einen Atmungsdefekt bei erhöhter Temperatur und eine gestörte mitochondriale Morphologie. Jedoch ist die molekulare Grundlage der verschiedenen *yme1*-Phänotypen in Hefe bisher nicht vollständig verstanden. Das humane YME1L wurde als Ortholog von Yme1p der Hefe beschrieben, aber seine Funktionen in Säugetier-Mitochondrien und spezifische Substratproteine sind noch nicht identifiziert worden. Um die Rolle der iAAA-Protease in Säugetieren zu bestimmen, wurden zwei experimentelle Ansätze verfolgt: (1) Depletion von YME1L in Säugetier-Zellen mittels RNA-Interferenz und (2) induzierbare Überexpression von YME1L und verschiedenen mutierten Varianten im Säugetier-System.

Die vorliegende Arbeit demonstriert die funktionelle Konservierung von YME1L in Säugetieren, indem gezeigt wird, dass es an der Proteolyse von Prohibitin 1 beteiligt ist, dessen Homolog in der Hefe ebenfalls von der iAAA-Protease abgebaut wird. Desweiteren, weist sie nach, dass YME1L eine Rolle bei der Aufrechterhaltung der tubulären mitochondrialen Morphologie und bei der konstitutiven Prozessierung von OPA1, einem Bestandteil der mitochondrialen Fusionsmaschinerie, spielt. Darüber hinaus ist der Abbau von Tim23, einer Kernuntereinheit der Translocase in der Innenmembran, während der caspaseunabhängigen Apoptose abhängig von YME1L. Dieser Befund identifiziert ein neues Substrat der iAAA-Protease in Säugetieren und einen neuen zellulären Prozess, an dem sie beteiligt ist. Die Überexpression von YME1L-Mutanten hat keinen dominant-negativen Einfluss auf die iAAA-Protease. Ihre

Funktionen in mitochondrialer Proteolyse, Morphologie und Import bleiben unverändert.

Zusammengefasst offenbart diese Arbeit vielfältige Rollen von YME1L bei der Qualitätskontrolle von Proteinen, der mitochondrialen Morphologie und der Apoptose in Säugetieren und demonstriert somit die Bedeutung von YME1L für sowohl die zelluläre Lebensfähigkeit als auch den zellulären Tod.

Contents

Chapter 1

Introduction

The mitochondrion is a complex organelle that possesses a multitude of functions, some of which are essential for the cellular survival. Beside its role in respiration and energy supply, it is involved in the apoptotic signaling, aging, synthesis of metabolites, lipid metabolism free radical production, metal ion homeostasis and the assembly of iron-sulfur (Fe-S) clusters. The latter are present in more than 120 distinct types of enzymes and proteins having function in the electron transfer, substrate binding, regulation of gene expression and enzyme activity [38]. Interestingly, synthesis of Fe-S clusters is the sole function maintained in so called "relict mitochondria" present in amitochondriate protists like *Giardia*, [156] suggesting that this function is the basis for the evolutionarily essential nature of mitochondria.

It is widely accepted that a mitochondrion has a monophyletic origin from an α - proteobacterial ancestor being an endosymbiont of eukaryotic cells, and as such it has following special features: first, it can be only inherited by the daughter cells, not synthesized *de novo*; second, it possesses its own genome and transcription and translation apparatus; and third, it is dividing and fusing. During the evolution from α - proteobacteria to eukaryotic organelle, many bacterial genes were transferred into the nuclear genome of eukaryotic cells [68]. As a consequence, targeting signals

on the nuclear encoded polypeptides as well as translocation machineries in both outer and inner membrane developed. An estimate is that the mitochondrial proteome contains around 1000 proteins [136; 151] but only 3 to 32 are mitochondrially encoded [60] depending on the organism. Those genes that resisted transfer to the nucleus encode mainly proteins incapable of translocation from the cytosol into the mitochondrion, for example extremely hydrophobic proteins like subunits of cytochrome *c* oxidase or hydrophobic segments of cytochrome *b*. The proportion of hydrophilic proteins encoded by the mitochondrial genome declined with decreasing genome complexity, but the necessity to synthesize highly hydrophobic ones in the mitochondrial matrix was probably the force that made the eukaryotic cells keep mitochondrial DNA (mtDNA).

Proteins encoded in the nuclear genome and synthesized in the cytosol are imported into one of four mitochondrial locations [42]: (1) the outer membrane (OM), (2) the intermembrane space (IMS), (3) the inner membrane (IM), and (4) the matrix (M).

Translocation through the outer membrane is performed by TOM complex. Outer membrane proteins contain targeting information within and near the transmembrane domain [85]. Insertion into the inner mitochondrial membrane proceeds in at least three different ways: (1) through the TIM23 complex and lateral insertion into the lipid bilayer; (2) through the TIM22 complex, and (3) translocation through the inner membrane protein Oxa1, which belongs to a highly conserved protein family present in mitochondria, bacteria and chloroplasts [173]. Proteins translocated in this pathway carry presequences and are completely imported into the matrix and further into the inner membrane. Matrix proteins are translocated across the inner membrane by TIM23 complex in an ATP- and transmembrane potential - dependent manner, where their presequences are processed and folding follows.

The outer mitochondrial membrane forms an envelope and presents a

barrier only for macromolecules as it contains pore forming proteins that allow the free passage of solutes up to few thousands Dalton in size [161].

Inner mitochondrial membrane encloses the matrix space. Even ions and metabolic substrates cannot pass it without the help of carrier proteins. It is the membrane, where multisubunit protein complexes reside, fulfilling various mitochondrial functions, like: oxidative phosphorylation (OXPHOS), translocation, metabolic exchange, protein assembly, Fe-S biogenesis and proteolytic degradation [161]. The inner membrane is organized in two morphologically distinct domains: (1) the inner boundary membrane, which is a second envelope, forms contact sites with the outer membrane and functionally interacts with it, and (2) cristae membrane, building up inner membrane invaginations connected to the boundary inner membrane through ring-shaped cristae junctions.

Mitochondrial cristae undergo morphological changes in response to changing metabolic requirements and/or matrix volume. An exchange of the inside content with the rest of intermembrane space was connected to metabolic and apoptotic pathways [81; 102].

1.1 Mitochondrial dynamics

Mitochondria form an interconnected tubular network, whose steady state morphology is a derivative of three different processes: First, fusion and fission which control mitochondrial shape and size; second, active transport inside a cell, controlling subcellular distribution of mitochondria; third, metabolic status of the mitochondrion itself. The balance between fusion and fission regulates mitochondrial morphology, *i.e.* shape, length and number, which affects the ability of cells to distribute the mitochondria to specific subcellular locations. Fusion and fission also allow exchange of membrane lipids as well as mitochondrial content mixing. Such a dynamic behavior is crucial for a number of cellular processes, such as apoptosis, the inheritance of mtDNA, defense against oxidative stress, and

development through spermatogenesis [26; 30; 65]. It provides one of the ways for the quality control allowing constant mixing of potentially damaged mitochondria with healthy ones [147]. Hence, mitochondrial dynamics plays and important role in both cellular survival and death [73].

Mitochondrial fusion and fission are unique in two main aspects: first, because of double membrane boundary, they have to proceed in a strictly coordinated way to ensure integrity of both membranes plus two mitochondrial subcompartments: matrix and intermembrane space; and second, there is no evidence for the involvement of SNARE proteins, having function in other cellular fusion events.

In mammalian cells fission requires dynamin-related protein 1 (Drp1) predominantly distributed in the cytosol and partially associated with the mitochondrial outer membrane [137; 175] together with hFis1, an outer membrane protein [142], endophilin B1/Bif1 [79], MTP18, GDAP1 and DAP3 [167]. A portion of cytosolic Drp1 can be recruited to mitochondria through an interaction with hFis1 [174].

Mitochondrial fission is counterbalanced by the fusion. Fzo1p was shown to be required for fusion in *Drosophila melanogaster* [65] and yeast [67]. Mammalian outer membrane proteins mitofusin 1 and mitofusin 2 are functional homologues of Fzo1p, involved in the mitochondrial fusion [27; 125; 130]. Mitofusins form homo- and hetero - oligomeric complexes and are required for adjacent mitochondria during the fusion process suggesting formation of *trans* complexes of the apposing mitochondria [89]. Another protein - yeast Mgm1, a dynamin-related GTPase, is essential for the mitochondrial inner membrane fusion, maintaining mtDNA and inner membrane structures in yeast [105; 135; 172]. Yeast outer membrane protein Ugo1 physically links Mgm1 and Fzo1, however no mammalian homologue has been discovered so far [115]. It was reported that the mammalian homologue of Mgm1, OPA1, interacts physically with Mfn1/2 [64].

OPA1 is an IMS protein with soluble or closely associated with the inner membrane pools. It is thought to form oligomers involved in the

regulation of mitochondrial cristae morphology and complete release of cytochrome *c* which is sequestered in the intra - cristae regions [9; 33; 51; 116]. **OPA**1 is a casual gene product of autosomal dominant **op**tic **a**trophy which features a progressive loss of retinal ganglion cells that leads to legal blindness [4; 40].

In human cells there are eight splice variants of OPA1 each of which is subsequently processed to form several isoforms with distinct molecular sizes [41]. The combination of long and short OPA1 isoforms is important for mitochondrial fusion activity [138]. Processing of OPA1 is activated by the transmembrane potential dissipation [44] and strongly correlates with stimulation of the mitochondrial fragmentation [73]. There have been reports linking loss of OPA1 to defects in mitochondrial respiration, increased release of cytochrome *c* [9] and susceptibility towards apoptosis [28; 92], as well as reduction of the mitochondrial transmembrane potential [116]. They point to additional functions of OPA1, independent of mitochondrial fusion. In yeast Mgm1 is required for oligomerization of F_1F_0ATP synthase, an inner membrane enzyme coupling proton pumping to ATP synthesis, essential for normal cristae structure [120]. This requirement provides a link between these two modulators of cristae structure [5].

1.2 Apoptosis and a link between apoptosis and a fusion/fission machinery

The development of cytokine - mediated apoptosis programs in higher multicellular organisms provides a crucial way to coordinate the regulation of cell numbers at the organism level in response to the environmental stimuli [39]. In *C.elegans* and mice the most frequent form of developmental cell death is apoptosis [1; 34]. It can proceed in three main pathways (summarized on Fig. 1.1): **(1)** intrinsic, **(2)** extrinsic, and **(3)** granzyme B-mediated pathway. The most noticeable and characteristic features of

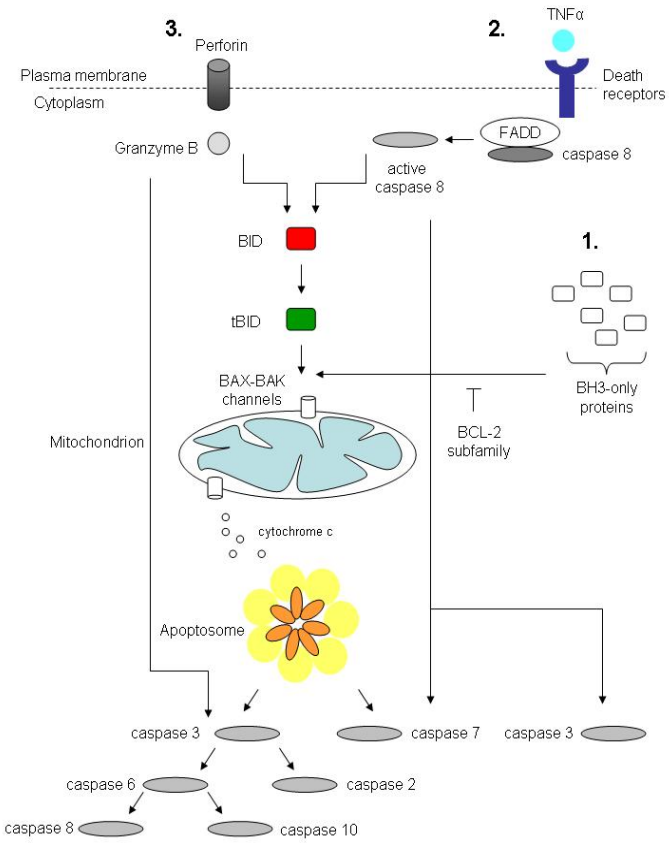

Figure 1.1: Caspase activation pathways

Intrinsic pathway **(1)**: BH3-only proteins are activated by the cell stress or damage and overcome the inhibitory effect of BCL-2 family members. BAX-BAK assembly in the mitochondrial OM permit the efflux of intermembrane space proteins, like cytochrome *c* into the cytosol. Cytochrome *c* triggers the apoptosome assembly: caspase 9 and APAF1. Conformational activation of caspase 9 propagates the proteolytic cascade of further caspase activation events. In the extrinsic pathway **(2)** caspases are activated through the binding of extracellular death ligands, like TNFα to transmembrane death receptors. It provokes recruitment of adaptor proteins, such as FADD, which in turn recruit and aggregate several molecules of caspase 8, promoting its autoprocessing and activation. Active caspase 8 proteolytically processes and activates caspase 3 and 7, provoking further caspase activation events that culminate in substrate proteolysis and cell death. In some situations, extrinsic death signals can interact with the intrinsic pathway through caspase 8-mediated proteolysis of the BH3-only protein BID (BH3-interacting domain death antagonist). Truncated BID (tBID) can promote mitochondrial cytochrome *c* release and assembly of the apoptosome. The granzyme B-dependent route to caspase activation **(3)** involves delivery of this protease into the target cell trough specialized granules that are released from cytotoxic T lymphocytes (CTL) or natural killer (NK) cells. CTL and NK granules contain numerous granzymes as well as pore-forming protein, porfirin, which oligomerizes in the membranes f target cells to permit entry of the granzymes. Granzyme B, similar to caspases also cleaves its substrates after Asp residue, and can process BID and caspase 3 and 7 to initiate apoptosis. Reprinted with changes from [149].

apoptosis are: condensation of the nucleus and its fragmentation into smaller pieces [82], hydrolysis of nuclear DNA into multiple fragments [171], fragmentation of organelle's network: Golgi, endoplasmic reticulum and mitochondria, and protein release from the mitochondrial intermembrane space [150].

During the early step of apoptosis the mitochondrial network disintegrates. Apoptotic stimuli activate mitochondrial fission and block fusion. It results in mitochondrial fragmentation and condensation [49; 81]. In this context it is closely correlated to the progression of apoptosis [92; 143]. Upon induction of apoptosis Drp1 translocates to the potential scission sites and becomes locked on the membrane during cell death in an hFis1 - independend and Bax/Bak - dependent manner [49]. It becomes stably sumoylated, which depends on the presence of Bax/Bak [167]. It occurs within the same time frame as activation of the proapoptotic Bcl-2 family member Bax and permeabilization of the mitochondrial outer membrane which in turn leads to the release of a multiple intermembrane space proteins and loss of the membrane potential [104]. Drp1 depletion blocks remodelling of the mitochondrial cristae [55] where cytochrome *c* is mostly stored [9] and delays apoptosis [46]. In yeast, fission mediated by Drp1 homolog, Dnm1, is essential for the autophagic degradation of mitochondria [113]. Proapoptotic Bax protein co - localizes also with Mfn2 at distinct foci on mitochondria [109] and is required for the regulation of Mfn2 activity and lateral assembly into foci along the mitochondrial tubules [78]. This co - localization may account for a block in fusion observed during apoptosis [80]. Recently, OPA1 has been shown to protect cells from apoptosis by controlling the remodelling of mitochondrial cristae, which is independent of its pro - fusion function [33; 51]. Out of eight OPA1 mRNA splice forms four have fusion activity. They produce a long isoform of OPA1 in addition to one or more further processed short forms [73]. It was demonstrated that those various forms are produced by processing at two distinct sites: S1 encoded in all mRNA splice variants, and S2 encoded only

in some of them [73]. There is evidence for the involvement of rhomboid protease perselin - associated rhomboid like (PARL), mAAA and iAAA Zn^{2+}-dependent metalloproteases belonging to the conserved AAA family of proteins, in the processing at those sites [61; 73; 138].

1.3 AAA$^+$ superfamily and AAA proteins

ATPases play crucial roles in transforming chemical energy into biological processes [114]. AAA$^+$ superfamily of proteins (ATPases associated with diverse cellular activities) is based on careful and multiple alignments and crystallographic studies [110]. It contains proteins having extremely various functions in cells, and sharing common features [114]. They are involved in processes ranging from thermotolerance in bacteria, fungi and plants, through membrane fusion and microtubular movement in eukaryotes, to protein degradation and DNA replication [66].

AAA$^+$ protein family is defined by the conserved AAA$^+$ domain. It consists of N- and C - terminal subdomain, whose characteristics distinguish AAA$^+$ domain from other nucleotide-binding proteins [66]. Its architecture is more conserved than the underlying sequences. It is composed of Walker A (also called a P-loop, GxxxxGKT where **x** = any amino acid residue) and Walker B (hhhhDExx where **h** = hydrophobic amino acid residue) motifs mediating ATP - binding and hydrolysis. They are followed by sensor 1, arginine fingers and sensor 2. Conserved polar residues of sensor 1 are physically located between Walker A and Walker B motifs and interact with important Walker B elements and γ - phosphate of ATP. Arginine fingers are one or two conserved arginine residues which constitute part of the nucleotide - binding site of an adjacent subunit. Sensor 2 has a role in ATP hydrolysis and substrate unfolding. Its residues participate in a nucleotide binding.

A conserved region that is positioned C - terminally from the Walker B motif named a **S**econd **R**egion of **H**omology (SRH) is a characteristic for a subgroup of AAA$^+$ superfamily, called AAA proteins. Its sequence is not strictly conserved, but the proteins share comparable structural features of SRH [74].

1.3.1 AAA$^+$ proteases

AAA$^+$ enzymes assemble mostly into hexameric complexes which is their biologically active form [66]. There has been a common mechanism proposed for ATP - dependent proteolysis by proteases belonging to the AAA$^+$ superfamily. First, they oligomerize into barrel - shaped micro compartments allowing sequestered proteolysis [133], and second, ATP-dependent proteases need the energy from ATP hydrolysis to regulate the accessibility of the proteolytic sites and to unfold substrates to drag them into the proteolytic chamber of the protease [8; 71]. The unfolding process is the rate - limiting step in proteolysis [84]. The initial substrate binding step does not require ATP hydrolysis, but for many AAA$^+$ proteins ATP binding is required to generate the active, oligomeric form of the enzyme [114].

The mechanism of ATP hydrolysis involves the nucleophilic attack of an activated water molecule at the γ - phosphate of the ATP and the formation of a penta - coordinate transition - state [114]. Negative charge accumulating at the γ - phosphate is stabilized by the Mg^{2+} and by surrounding of positively charged groups and/or hydrogen bond donors. Hence, the active sites of ATPases should contain a catalytic base able to activate nucleophilic water, and electrophilic groups able to stabilize the negatively charged transition state, plus groups coordinating the magnesium ion which is an essential co - factor. The active sites contain "sensors" which function is to "sense" the γ - phosphate and mediate the conformational changes that relay this information to remote sites. ATP binding pocket is located in between pairs of the hexamer subunits. Conserved residues that are functionally important for ATP binding and hydrolysis are: (1) in the Walker A motif: lysine (Lys, K) which forms ionic interactions with β- and γ - phosphate oxygens, and threonine (Thr, T) which provides a metal ligand; (2) in the Walker B motif: aspartate (Asp, D) which is involved in the Mg^{2+} coordination sphere and glutamate (Glu, E) which is a catalytic residue important for ATP hydrolysis; (3) asparagine (Asn, N),

serine (Ser, S), threonine (Thr, T) or histidine (His, H) located at the N - terminal end of SRH in AAA proteases forming a polar contact with γ - phosphate of ATP and functioning as a sensor (sensor 1 [77]); (4) arginine (Arg, R) residue at the beginning of the third α-helix in the C-domain of the AAA^+ module which in AAA proteins corresponds to the C - terminal part of SRH. It mediates relative movement of the C - domain to the N - domain during the ATP hydrolysis cycle. There are two conserved Arg residues in AAA proteins and one in the other AAA^+ family proteins. They function as an arginine finger that transduces the chemical event of ATP hydrolysis into the conformational changes of the neighboring subunit of a hexamer. Such a coordinated cooperation of subunits greatly enhances ATPase activity of a complex comparing to the singular ATPase activity of each subunit [98].

As a consequence of oligomerization, AAA proteases form a central cavity/pore lined with residues from each subunit [66]. Most of the pore surface is provided by the loop with three conserved residues (aromatic - hydrophobic - glycine [163]) which have a role in a function of several AAA proteins.

In the mitochondrial compartment both soluble and membrane ATP - dependent proteases have been found (Fig. 1.2).

1.4 Mitochondrial proteolytic quality control

A highly conserved proteolytic system conducts the surveillance of protein quality control within mitochondria, which has to cope with the diverse challenges imposed on mitochondrial integrity [147]. Molecular chaperones and energy - dependent proteases monitor the folding and assembly of mitochondrial proteins and selectively remove excess and damaged proteins from the organelle [87]. Key components are ATP - dependent proteases, which are derived from bacterial proteases and highly conserved in eukaryotes [87]. ATP - dependent proteases sense the folding

state of substrates by exerting chaperone - like properties and trigger the proteolysis of non - native proteins [147]. A central role in this process is exerted by conserved ATPase modules, which are characteristic of the AAA^+ family of ATPases and present in all ATP - dependent proteases [147].

1.5 Mitochondrial ATP - dependent proteases

1.5.1 ATP - dependent proteases in the mitochondrial matrix

Two mitochondrial matrix proteases have been identified in various organisms: Lon and ClpXP. Lon proteases have been identified in the mitochondrial matrix of yeast and mammals [145; 159; 166; 165; 168]. They harbor a catalytic serine - lysine dyad and as such are classified as serine proteases [21; 126]. Lon forms presumably homooligomeric, ring - shaped complexes with hexameric or heptameric structure [140]. In most cases impaired folding appears to trigger protein degradation by Lon protease [159]. The yeast Lon ortholog, PIM1 has been shown to degrade various misfolded and non - assembled polypeptides, like thermally denatured and aggregated or oxidatively damaged proteins, such as aconitase [20; 100].

Molecular chaperones of the Hsp70 and Hsp100 family co - operate with Lon protease during proteolysis by stabilizing misfolded proteins against aggregation or by dissolving already aggregated proteins [14; 124; 162]. Yeast Δ*pim1* cells show inhibited growth on glycerol. Electron dense inclusions in the mitochondrial matrix could be observed presumably as a result of accumulation of non - degraded mitochondrial matrix proteins [145]. Similarly, down regulation of the human Lon protease leads to accumulation of protein inclusions, impaired mitochondrial function and apoptotic cell death [19]. It was demonstrated that Pim1p was

required for the maintenance of mtDNA in yeast and present in the mitochondrial nucleoids, and that it was able to bind GT - rich DNA sequences [31; 53; 52; 97]. Cellular effects of Lon deletion as well as known physiologic functions of this protease suggest its regulatory function, but the substrates and probable mechanisms it could be involved in are still unknown.

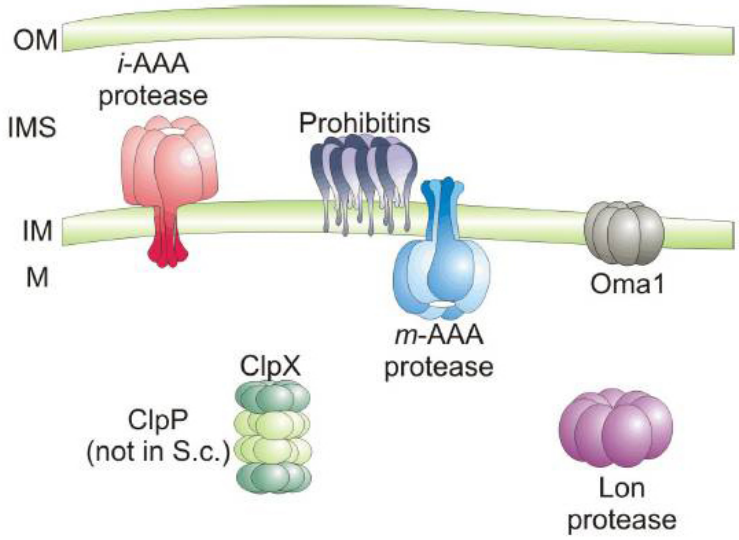

Figure 1.2: ATP-dependent proteases in the mitochondrial matrix and in the inner mitochondrial membrane. The Lon - protease and ClpXP (ClpP not present in yeast) are responsible for proteolytic breakdown of misfolded polypeptides in the mitochondrial matrix. Integral membrane and peripherally associated proteins are degraded by proteases of the inner mitochondrial membrane, the iAAA protease, active on the intermembrane side of the inner membrane; the mAAA protease, active in the mitochondrial matrix and the ATP - independent metallopeptidase Oma1, which is thought to possess catalytic domains at the side of the inner membrane. Prohibitins built up a supercomplex with the mAAA protease. **OM** - outer membrane; **IMS** - intermembrane space; **IM** - inner membrane; **M** - mitochondrial matrix. Reprinted with modifications from ([112]

The other mitochondrial matrix protease ClpX (caseino-lytic protease) was found in all organisms whereas ClpP only in mammals and plants, but not yeast [37; 76; 160]. 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. The ATPase domains determine the substrate specificity of the Clp protease and also exert regulatory functions during proteolysis [17; 117; 133; 169].

1.5.2 ATP dependent proteases in the mitochondrial membranes

Two membrane bound AAA proteolytic complexes have been identified in mitochondria. They are integrated into the inner mitochondrial membrane and build up of orthologs of FtsH - membrane bound AAA protease essential for cell viability in *Escherichia coli* [2; 154; 155].

In yeast there are three FtsH orthologues: Yta10p and Yta12p forming a heterohexameric mAAA protease complex active at the matrix side, and Yme1p which is the only enzymatic component of iAAA protease active in the intermembrane space. They share several common features: N - terminal targeting signal followed by the AAA consensus and HEXXH motif characteristic for metallopeptidases of the thermolysin family M41 [123]. Mutation of the glutamate residue within the proteolytic centre inhibits protein degradation by AAA proteases [7]. Opposite orientation of catalytic domains of iAAA and mAAA proteases is a consequence of having one or two transmembrane domains respectively (Fig. 1.3). AAA protease - mediated degradation of membrane proteins involves dislocation or extraction of the substrate from the membrane [95]. This process requires around 20 residues to protrude from the membrane surface [32] probably to reach deep into the ATPase domain and establish productive binding. Mutational analysis of a conserved loop motif YVG (aromatic - hydrophobic - glycine) present in the central pore of hexameric AAA^+ ring complexes [164], indicates substrate translocation into the proteolytic chamber through the central pore of mitochondrial AAA proteases [59]. Recently,

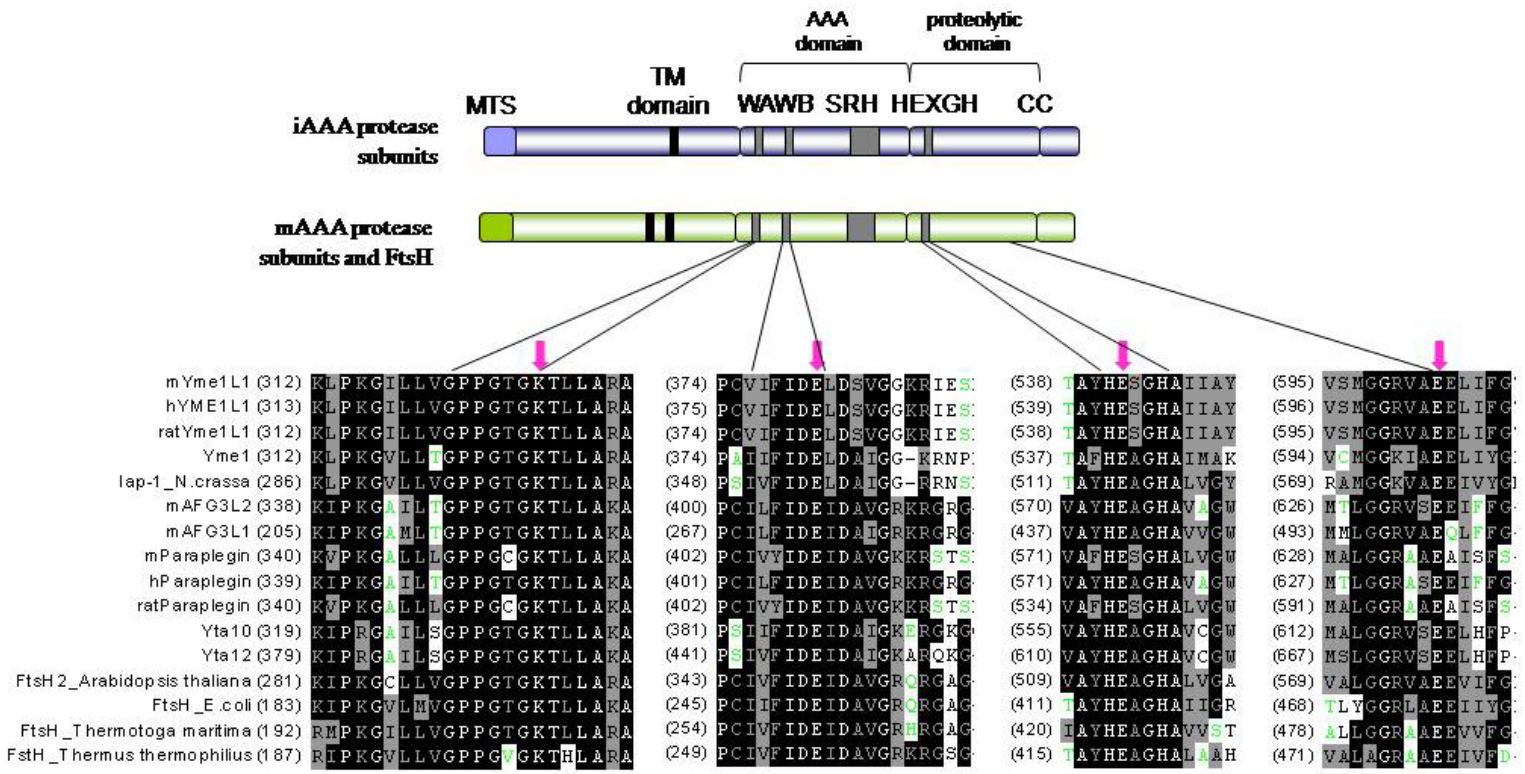

Figure 1.3: Domain structure of the mAAA and iAAA protease subunits

A. The nuclear encoded subunits are imported into mitochondria via an N-terminal mitochondrial targeting sequence (MTS) which is removed after import. One (Yme1p) or two (Yta10p, Yta12p, 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 second region of homology (SRH). The AAA domains are followed by proteolytic domains harboring the HEXXH metal binding motif. At the most C-terminal end is the coiled-coil region (CC,(Reprinted with modifications from [112]). **B.** A multi sequence alignment of the amino acid regions containing the conserved motifs from yeast mammalian and bacterial AAA protease subunits. Sequence coordinates of the amino acids are indicated. The color code as well as the whole sequence alignment is presented in the Appendix 1 on page 81

Tatsuta *et. al.* has shown the ability of the mAAA protease to mediate vectorial membrane dislocation of proteins in an ATP - dependent reaction [146]. This membrane extraction of substrate proteins is likely to be facilitated by the membrane - embedded parts of AAA protease subunits which might form a pore - like structure or provide at least a more hydrophilic environment [88].

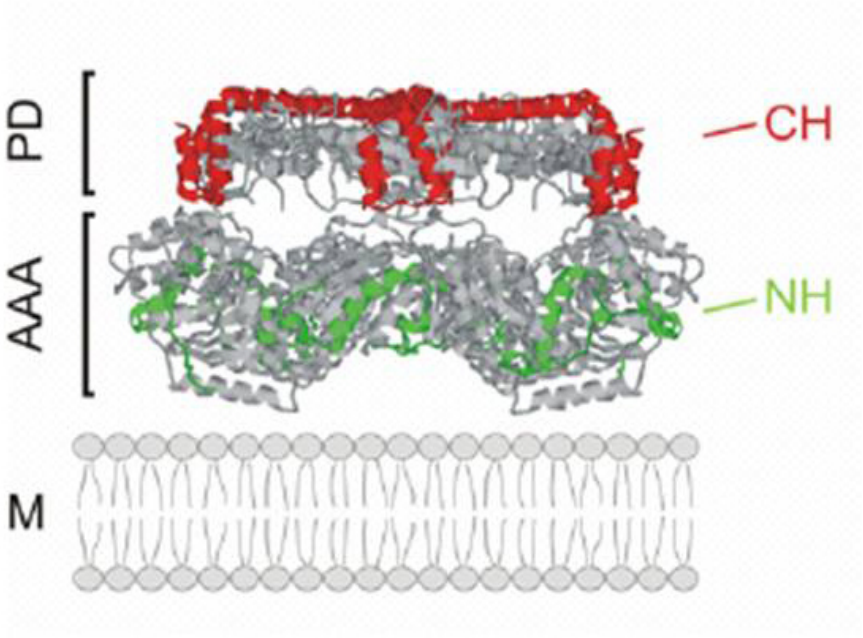

Figure 1.4: Folding of the yeast iAAA protease and substrate engagement
Side view of crystal structure of Thermotoga maritima FtsH [15] showing three subunits was used as a model for mapping the substrate binding regions of the yeast iAAA. Two identified regions CH and NH (red and green helices respectively) of the yeast Yme1p responsible for substrate engagement are located on the surface of proteolytic cylinder (CH helices marked on PD) and AAA domain (NH helices marked on AAA). AAA, AAA domain; PD, proteolytic domain; IM, inner membrane. Reprinted with modifications from [87].

Two FtsH structures from two eubacterial organisms have been solved recently [15; 90; 144] which facilitated studies on the yeast iAAA protease subunit Yme1p. It allowed the localization of two helical binding regions. The C - terminal helices of the proteolytic domain (CH - region) and the

N-terminal helix (NH-region) in the AAA domain form a lattice-like structure at the surface of the proteolytic cylinder and mediate the initial encounter of substrate proteins with the protease (Fig. 1.4, [58; 87; 94]). The NH - region is located in a close proximity to the membrane surfaces and highly negatively charged. Thus, substrate proteins initially interact with the iAAA protease at the outer surface of the proteolytic cylinder, before they enter the proteolytic chamber. Interestingly, the binding properties of the surface - exposed interaction sites vary suggesting that alternative pathways for substrate entry into the proteolytic chamber of iAAA proteases exist. The CH - region is only required for the binding and degradation of a subset of proteins. The distance of an unfolded domain from the membrane surface, might be one parameter determining the involvement of the CH - region for substrate binding. In case of a non - assembled subunit 2 of the cytochrome *c* oxidase, Cox2p, destabilization of the solvent - exposed Cox2 domain at high temperatures renders CH - dependent proteolysis by the iAAA protease [58]. In contrast to the CH - region, the NH - region appears to be generally involved in proteolysis. It is therefore likely that CH - dependent substrates bind in a sequential manner at CH- and NH - regions before they enter the proteolytic chamber through the central pore formed by the AAA domains [87].

Growing evidence suggests that substrate binding and proteolysis by AAA proteases is modulated by additional factors within mitochondria [87]. It can appear in a substrate - specific manner. Like Cox20p which affects proteolysis of the Cox2p by the iAAA protease modulating recognition of a substrate [58]. Proteolysis of the non - assembled Cox2p was demonstrated to be strictly dependent on the CH - region in Cox20 - deficient mitochondria, but not in the presence of Cox20p [58] indicating overlapping functions of Cox20 and CH - region of Yme1p. Another co - factor for iAAA protease is also not essential for proteolysis [42]. Together with Yme1p, Mgr1 is part of the iAAA proteolytic complex in yeast [42]. It was

suggested to act as an adaptor - like protein which targets specific substrates in the IMS for degradation by iAAA protease [87].

No substrate-specific co-factors of mAAA protease have been identified so far. However, the yeast mAAA protease is part of a large supercomplex with prohibitins, which modulate proteolysis [141]. Prohibitin 1 (Phb1p) and prohibitin 2 (Phb2p) are ubiquitous and highly conserved subunits of those supercomplexes. They assemble in a multimeric complex which is exposed in the intermembrane space and anchored N-terminally to the inner membrane [11; 111; 141]. Deletion of prohibitins results in accelerated protein degradation by the mAAA protease [141]. However it is not yet understood how they affect the proteolysis by the mAAA protease.

Both the m- and the iAAA protease recognize and degrade non-native and non-assembled polypeptides to peptides [7; 96]. These proteases exhibit degenerate substrate specificity and, similar to molecular chaperone proteins, recognize the folding state of solvent-exposed domains of membrane proteins [94]. mAAA and iAAA proteases are the main components of mitochondrial quality control. They degrade proteins to peptides, which are subsequently either exported from the organelle or degraded further to amino acids by various oligopeptidases [147].

Yeast cells lacking both m- and iAAA proteases are not viable demonstrating the crucial function of this proteolytic system for cellular homeostasis [93; 95]. Their depletion or inactivation causes severe pleiotropic phenotypes in various organisms, which are best characterized in the yeast *Saccharomyces cerevisiae*. Here, all observed defects can be attributed to a loss of the proteolytic activity as identical phenotypes were observed after deletion of an AAA protease subunit or after inactivation of the proteolytic sites of all subunits of AAA protease complexes [6; 96; 170]. Both proteases exert overlapping functions within mitochondria, having overlapping substrates; however different phenotypes resulting from mutations indicate substantial differences.

YTA10 and *YTA12* yeast mutants exhibit respiratory incompetence

[62; 148] and lack assembled respiratory chain and ATP-synthase complexes in the inner membrane [54; 118; 158]. Yeast mAAA protease was found to degrade non assembled mitochondrialy encoded respiratory chain subunits. Also peripheral membrane protein such as Atp7 was identified as a mAAA substrate [88]. Recently, a subset of specific substrates was identified proving a regulatory, housekeeping function of mAAA protease. That includes proteolytic cleavage of a conserved, nuclearly encoded subunit of mitochondrial ribosomes MrpL32 [112] and cytochrome *c* peroxidase (Ccp1), a heme-binding ROS scavenger in the intermembrane space [45; 146]. Maturation of Ccp1 within mitochondria represents the first non-proteolytic function of the mAAA protease in the mitochondrial biogenesis, and rather severe mutant phenotypes of *YTA10/YTA12* could be explained not only by loss of respiration but also by the overall mitochondrial malfunction.

SPG7 - a mammalian homologue of the yeast mAAA protease subunits was identified in the genetic screen of patients affected with an autosomal, recessive, neurodegenerative disorder called **H**ereditary **S**pastic **P**araplegia (HSP) [24]. Further identification of mammalian AAA proteases homologues followed. Complementation studies identified a complex of mammalian paraplegin and AFG3L2 as the functional orthologue of the yeast mAAA protease [12; 112] and a knock-out mouse model was generated [47] where the human phenotype of the disease was reproduced. It has been proposed that mAAA mutations mapped in the HSP patients led to the defects in an axonal transport of mitochondria, resulting in the energy insufficiency and a neuronal decay [47]. AFG3L2 was shown to homooligomerize, which was proposed to be responsible for increased severity of the Afg3l2 mutants [87; 101].

The mammalian mAAA protease was linked to the transmembrane potential-dependent proteolysis of OPA1 [43; 73] together with a mammalian rhomboid protease PARL [33] and prohibitin 2 (PHB2) - a part

of a prohibitin-mAAA supercomplex, is involved in the OPA1-dependent cristae remodeling in mitochondria [106].

Yeast lacking *YME1* lose mtDNA at an accelerated rate forming petite negative colonies, *i.e.* unable to grow on glucose in the absence of mtDNA. Δ*yme1* cells fail to grow on a non fermentable carbon source at 37°C , as well as on glucose at 14°C [23; 152]. The *yme1* mutant cells show deficient oxygen consumption and mitochondrial morphological abnormalities: from abbreviated branched structures to swollen forms, together with alterations in protein/lipid composition. Mitochondrial abnormalities could be partially reversed in *S.cerevisiae* by mutations of *YNT1* gene encoding a 26S protease subunit suggesting similar functions [23]. The Yme1p is immunologically detectable as an 80 kDa protein present in the mitochondria [153]. It has a role in the degradation of unassembled inner membrane protein complexes, like cytochrome *c* oxidase subunit 2 [121] and Phb1p and Phb2p [75] as well as external NADH dehydrogenase (Nde1) [13]. Rainey *et al.* reported crucial non-proteolytic function of Yme1p in the import of an exogenously expressed human PNPase [122] - mitochondrial intermembrane space protein required for the maintenance of mitochondrial homeostasis [29]. Selective removal of malfunctional mitochondria in the autophagic process, named mitophagy [83] suppresses apoptosis and has a cytoprotective function [99]. Interestingly, recent reports suggest a new function of Yme1p, namely the proteolytic turnover of a mitochondrial inner membrane protein phosphatidylserine decarboxylase 1 (Psd1p) responsible for the synthesis of phosphatidylethanolamine - an essential mitochondrial component, having function in the autophagy [108].

Human ortholog of Yme1p has been identified in the complementation studies [134]. It has been expressed in the Δ*yme1* yeast cells rescuing the temperature-sensitive phenotype, and demonstrating functional complementation and conservation of the iAAA complex [134]. Human YME1L1 (Yme1-like 1, further related to as YME1L) and paraplegin have

42% and 33% homology with the yeast Yme1p respectively. Expression and immunofluorescence studies revealed that YME1L and paraplegin share similar expression pattern and the same subcellular localization [36]. Two transcript variants encoding different isoforms have been found for the human gene. Transcript variant 3 lacks an internal coding exon 3 compared to transcript variant 1. However, it maintains the same reading frame, and encodes an isoform 3 (716 aa; 80kDa) that is missing a 57 aa segment compared to isoform 1 (773 aa; 86.5kDa). The exon 3 encodes an N-terminal part of the protein, which is presumably processed off upon import of YME1L into the mitochondria. There is only one transcript variant annotated for the murine *Yme1l1* encoding only one form of YME1L: 715 aa, 80kDa. Because of the high conservation and homology (see alignment and homology tree in the Appendix 1 on page 81) possible involvement in spastic-like disorders has been suggested for the mammalian iAAA protease [36; 134]. However, Coenen *et al.* [35] failed to identify YME1L mutations in patients with combined defects in the oxidative phosphorylation system. Only recently, it has been suggested that YME1L may have a function in a novel apoptotic pathway independent of caspases [57]. Thus, the mammalian iAAA protease is believed to have an additional role in the regulation of mitochondrial dynamics and morphology, independent of its proteolytic quality controlling in the mitochondria.

Chapter 2

Aim of the thesis

Identification of the YME1L as an ortholog of the yeast iAAA protease subunit opened a question about its action and its importance for the mammalian cell. Little is known about the mammalian iAAA protease complex as no functional studies were done on that protein. The question was, whether evolutionary conservation of the protein results in a functional conservation within the mitochondria. To address it and to examine the relevance of the mammalian iAAA protease for mitochondrial morphology, proteolysis and function two approaches were carried out:

- YME1L down regulation studies in the mammalian cells and
- overexpression studies of the wild-type and various mutant variants of the YME1L protein in the mammalian inducible system.

Chapter 3

Results

3.1 Down regulation of YME1L using small interfering RNAs (siRNAs)

3.1.1 Specific antibody against YME1L

The proper recognition of the protein by the antibodies used throughout the study was verified prior to the verification of the efficiency and specificity of YME1L down regulation and its overexpression in mammalian cells. As a reference for the running behavior on SDS-PAGE of the mature YME1L data were used from the *in vitro* processing of the human YME1L by the **m**atrix **p**rocessing **p**rotease (**MPP**; Figure 3.1A; courtesy of Dr Mirko Koppen). The cloned construct was encoding human isoform 3, *i.e.* having 716 amino acids in length (80kDa) which was also used by Shah *et al.* in complementation studies [134]. The hYME1L was transcribed and radiolabeled in a cell-free system and incubated at 30°C for 20 min with the MPP purified from *E.coli*. In the next experiments three different antibodies were checked for the recognition specificity:

- polyclonal antiserum raised against hYME1L protein (Figure 3.1B and C, kind gift of Dr Carla Koehler);

- affinity purified polyclonal antiserum SPY531 raised against two peptides common for the murine and human protein sequence: (152-166aa) TLKSRTRRLQSTSER and (517-531aa) DKILMGPERRS-VEID (Fig. 3.1D);
- commercial polyclonal antibody (ptglab; Fig. 3.1E).

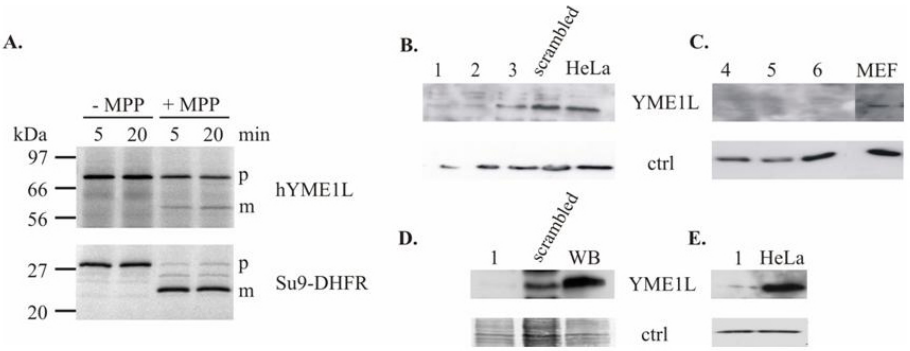

Figure 3.1: Recognition of the mature YME1L by different antibodies used in the present work

A. Human YME1L processing *in vitro* by the matrix MPP protease. Mature form of the hYME1L runs at the size of ∼60kDa. Efficiency of the processing was controlled by Su9-DHFR, a construct cleaved by MPP (courtesy of Dr Mirko Koppen). Four different antibodies used in the present work were checked for specific recognition of YME1L by comparison of the size of bands recognized in Western blotting. As a reference for the proper band size, *in vitro* processing of hYME1L was used **(A.)**. With numbers 1-3 are marked cell lysates from HeLa cells transfected with siRNA1-3; and 4-6 show lysates form MEF cells transfected with siRNA4-6. Extracts from the scrambled negative siRNA control and non-transfected cells were used as protein down regulation reference. WB refers to cell extract from HEK293 Flp-In clone expressing mYME1L^{E381Q} induced with 1μg/ml tetracycline for 24h. β-actin was used as a loading control (anti β-actin 1:5000, Sigma, clone AC15); **B.-C.** polyclonal antiserum raised against hYME1L protein (kind gift of C.Koehler) specifically recognized both human **(B.)** and murine **(C.)** YME1L; **D.** polyclonal antiserum SPY531 raised in the present study against two peptides from the murine YME1L recognized specifically both human (scrambled) and murine (WB) YME1L E. commercial polyclonal antibody (ptglab, 1:500 dilution) recognized human YME1L protein.

In all of the cases the antibodies recognized specifically a band running on SDS-PAGE at the size of ∼60kDa, which disappeared upon siRNA transfection *i.e.* down regulation of YME1L protein in all of the used cell lines: HeLa (Fig. 3.1B and E) MEF (Fig. 3.1C) and HEK293 (Fig. 3.1D). As reference cell extracts were used from cells transfected with a scrambled

negative siRNA control (Invitrogen) as well as from non-transfected cells. The size of the protein running at that position is in agreement with the size of the mature hYME1L processed *in vitro* (Fig. 3.1A.). The exclusive disappearance of the 60 kDa band was observed only upon siRNA1-6 transfection which demonstrates specificity of the antibody recognition.

3.1.2 Depletion of YME1L in mammalian cells

Three different siRNAs were used to down regulate the YME1L expression on the mRNA level. For human YME1L (hYME1L) that were siRNA1, siRNA2 and siRNA3, whereas siRNA4, siRNA5 and siRNA6 were designed against murine YME1L (mYME1L). All sequences hybridize within the coding sequence. Scrambled negative control (Invitrogen) *i.e.* siRNA duplexes with no match to the mRNA of YME1L, was used as a control. It allowed verification of a knock down specificity on YME1L. Western blot analysis revealed the most efficient down regulation in the case of siRNA1 and 2 for hYME1L (Fig. 3.1B lane 1 and 2) and siRNA4 and 6 for mYME1L (Fig. 3.1C and not shown data). The protein was reduced to a level which was not detectable by immunoblotting with any of the used α YME1L antibodies. siRNA 1, 2 and siRNA 4 and 6 were chosen for further experiments.

3.1.3 Mammalian YME1L role in proteolysis

Proteolysis of PHB1 upon depletion of its assembly partner PHB2

Depletion of PHB2 in mammalian cells is accompanied by the loss of its assembly partner PHB1; however, transcription of *Phb1* was shown not to change [106]. This suggests proteolysis of PHB1 while the PHB2/PHB1 complex is destabilized upon absence of PHB2; an effect that was observed previously in yeast [75]. Therefore, the PHB1 steady-state level was as-

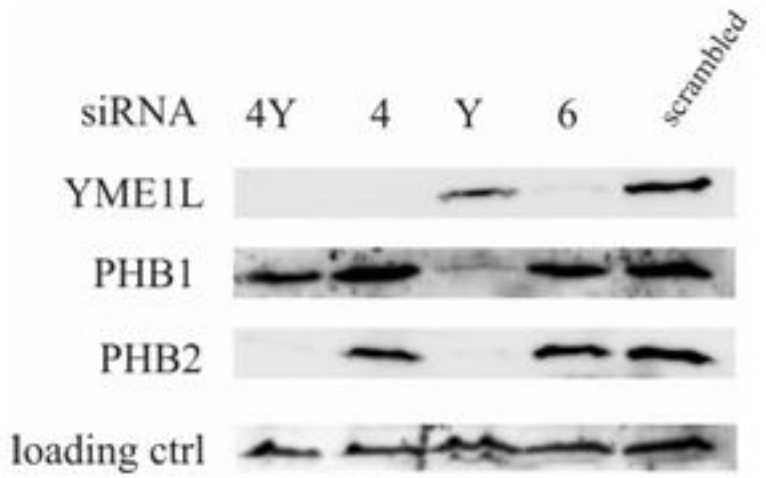

Figure 3.2: YME1L degrades PHB1 upon destabilization of the prohibitin complex

Cell extracts from MEFs transfected with siRNA designed against mYME1L (**4** and **6**) and against mPHB2 **(Y)** or both of them **(4Y)** were analyzed after 48-60h post transfection by immunoblotting with α YME1L (ptglab), α PHB1 (NeoMarkers) and α PHB2 (BioLegend). Cells transfected with scrambled siRNA were used as reference control. Immunoblotting with α complexII antibody (Molecular Probes) was used as a loading control.

sessed in PHB2 deficient MEF cells by single (siRNA4 and 6 against *Yme1l*, siRNAY against *Phb2*) or double siRNA transfection (siRNA4 and Y; Fig. 3.2) to verify the potential role of YME1L in PHB1 degradation. In the case of single transfections Western blotting showed an efficient and specific down regulation of YME1L (Fig. 3.2, lane 2 and 4) while neither the level

of PHB1 and PHB2, nor complex II used as a loading control, changed. Similarly, efficient depletion of PHB2 (Fig. 3.2, lane 3) did not lead to depletion of YME1L or complex II. It led however to destabilization of PHB1. Simultaneous knock down of PHB2 and YME1L (Fig. 3.2, lane 1) stabilized PHB1 to the wild-type level (scrambled control). This indicates a role of the YME1L protein in the degradation of the unassembled prohibitin complex subunit - PHB1. This finding clearly shows that structural conservation of Yme1p throughout the evolution (compare with Fig. 1.3) results also in the functional conservation of the protein, as Phb1p was shown before to be a specific substrate for the yeast iAAA protease [75].

YME1L role in the new apoptotic pathway

The molecular bases of phenotypes associated with a deletion of *YME1* in yeast as well as the phenotypes observed upon down regulation of YME1L in mammalian cells are poorly understood. It appears likely that they are connected with the diverse roles of the so far undiscovered substrates of the iAAA protease. Given those pleiotropic functions, we wanted to examine further effects of YME1L down regulation in the mammalian cells. Recently, Goemans *et al.* have reported a new apoptotic pathway activated by mitochondrial outer membrane permeabilization and inhibition of caspases [57]. It involves a specific TIM23 degradation which impairs mitochondrial import and inhibits cell proliferation. Tim23p is a central component of the TIM23 translocase complex in the inner mitochondrial membrane, crucial for the pore formation [107]. It was further shown that TIM23 degradation depends on factors residing in the mitochondria. The authors proposed this novel apoptotic pathway as an "emergency exit" for the apoptosis to occur upon inhibition of caspases. To investigate the specific function of YME1L in TIM23 degradation, apoptosis was induced with 200μM or 50μM etoposide (Eto, Fig. 3.3A, B) in the cells transfected with siRNA against YME1L and scrambled negative control (scr). Cells were treated with 10μM caspase inhibitor Q-VD-OPH

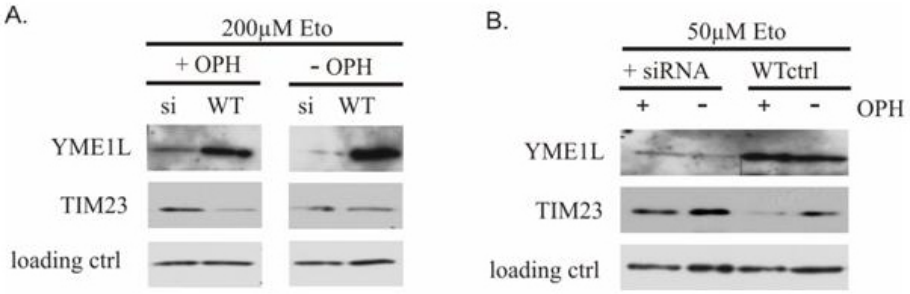

Figure 3.3: Stabilization of TIM23 upon YME1L down regulation
HeLa cells transfected with siRNA and scrambled negative control (scr) were treated with 200 μM **(A.)** and 50 μM **(B.)** of an apoptotic stimulus (Eto) in the presence or absence of caspases inhibitor (OPH) for 24h and 48h respectively. Destabilization of TIM23 (α TIM23, BD Biosciences) observed upon simultaneous apoptosis stimulation and inhibition of caspases can be rescued by siRNA knock down of YME1L (α YME1L, ptglab). Immunoblotting with α complexII antibody (Molecular Probes) served as a loading control.

(OPH). Inhibition of caspases simultaneous with the apoptosis induction resulted in TIM23 destabilization as shown on Fig. 3.3A lane 2, and 3.2B lane 3. Knock down of YME1L stabilized the protein, revealing YME1L role in the apoptosis- and caspases inhibition-induced specific degradation of TIM23. The effect was stronger with 50μM etoposide treatment for 48h, than 200μM etoposide for 24h, suggesting that this pathway is rather late reacting.

3.1.4 YME1L role in mitochondrial biogenesis

Mitochondrial morphology of the mammalian cells

Next, the effects of YME1L depletion on the mitochondrial morphology were addresses in human HeLa cells and murine MEFs. To this end both types of cells were transfected with siRNA against YME1L to knock down the protein. Subsequently, cells were transfected with a plasmid encoding mitochondrialy targeted red fluorescent protein (pDsRed2-Mito) for the visualization of mitochondria by fluorescent microscopy. We used cells transfected with the scrambled negative control as controls (Fig. 3.4B)

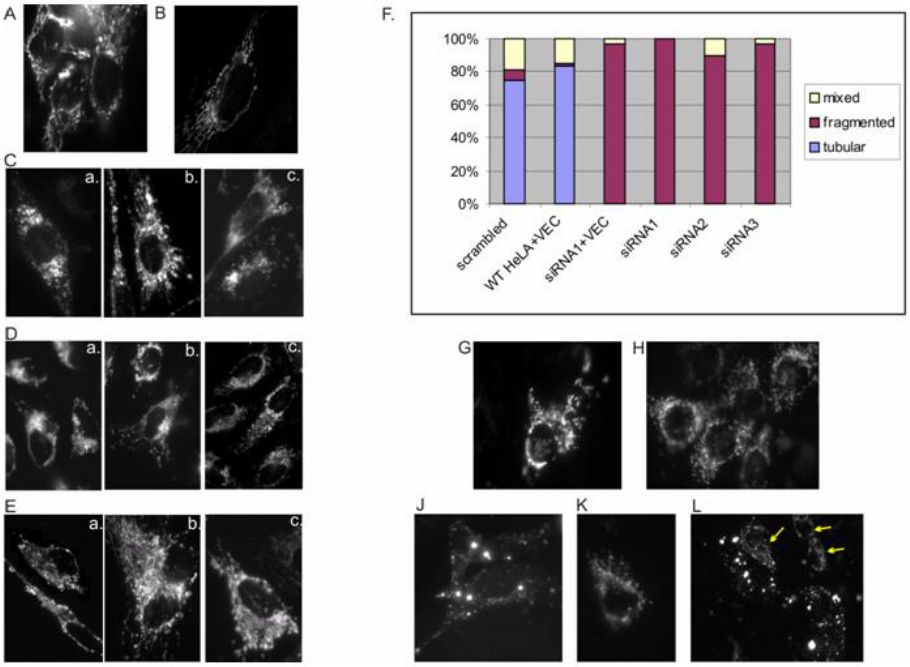

Figure 3.4: Aberrant mitochondrial morphology in the absence of YME1L
A. - E. HeLa cells were transfected with: siRNA1 (**C. a, b, c**) siRNA2 (**D. a, b, c**) siRNA3 (**E. a, b, c**) designed against human YME1L mRNA, and scrambled negative control **(B.)** or no siRNA **(A.)** followed by pDsRed-Mito (1 μg) transfection and analyzed by fluorescence microscopy after 48-60 h post-transfection. Different morphological types of mitochondria were quantified **(F.)** and compared to controls: HeLa transfected with scrambled negative control siRNA (scrambled) and pDsRed-Mito (1 μg), HeLa cotransfected with pcDNA3.1Hygro empty plasmid and pDsRed-Mito (4:1, 1 μg of total plasmid DNA) (WT+VEC), HeLa transfected with siRNA1 and co-transfected with pcDNA3.1Hygro:pDsRed-Mito (4:1, 1 μg of total plasmid DNA) (siRNA1+VEC). 100 cells were calculated for each sample. **G. - L.** MEF cells transfected as **A. - E.** with no siRNA (WT control; **G.**); scrambled negative control **(H.)** and siRNAs designed against murine YME1L mRNA: siRNA4 **(J.)** siRNA5 **(K.)** and siRNA6 **(L.)**. Yellow arrows point to the non affected cells with mitochondrial morphology as in the control cells.

as well as cells not transfected with siRNA (WT, Fig. 3.4A). In all of target siRNA-transfected cells we could see aberrant mitochondria. From swollen but still interconnected branches (Fig. 3.4Cb, Ea-c) to the complete lack of the network (Fig. 3.4Ca, Dc, J, K, L). HeLa cells were quantified: 100% cells transfected with siRNA1 had fragmented/aggregated mitochondria; 90% transfected with siRNA2 and 97% with siRNA3. None of the transfected HeLa cells had wild-type mitochondrial tubular net-

work. Only 6% of the cells transfected with scrambled control had fragmented mitochondria. WT and siRNA1 transfected cells were additionally co-transfected with an empty pcDNA3.1Hygro vector (VEC) to exclude deleterious effect of plasmid transfection. They had 2% and 97% of fragmented mitochondria respectively. Even in the populations, where Western blotting analysis still revealed residual levels of the YME1L protein (Fig.3.1B lane siRNA3) almost all of the cells showed abnormal mitochondrial morphology. That could indicate the primary effect of down regulation of YME1L on mitochondrial network not tightly related to its high enzymatic/proteolytic activity. Similar mitochondrial phenotypes in the MEFs transfected with siRNA4-6 were observed (Fig. 3.4J-L). The fragmentation/aggregation was even more striking than in HeLa cells (Fig. 3.4J, K). However, the transfection efficiency was lower as mixed populations of cells having aberrant and wild-type morphology could be seen (Fig. 3.4L).

OPA1 processing in the YME1L knocked down cells

The dynamin-related GTPase OPA1 is essential for the maintenance of cristae in the mitochondrial inner membrane and for mitochondrial fusion [116; 138]. There are eight OPA1 splice variants which are proteolytically processed to two long isoforms (L1 and L2) which can be in turn processed to three short isoforms (S3-S5). S4 is a form that appears to be generated by YME1L [61; 73; 138]. In further experiments a relation of mitochondrial abnormalities in MEFs (Fig. 3.5A) to accumulation of the different OPA1 forms (Fig. 3.5B) was examined. Immunoblotting of the MEF cells transfected with siRNA4 revealed alterations in OPA1 cleavage (Fig. 3.5B, lane 2). Longer isoforms are absent (L1) or present in the smaller amount (L2) and the shorter isoforms accumulate. This effect is in line with the aberrant morphology visible by fluorescent microscopy (Fig. 3.5A). Noteworthy is the difference in mitochondrial morphology of cells transfected with siRNA against PHB2 (siRNA Y), and YME1L (siRNA 4). Mitochondria in

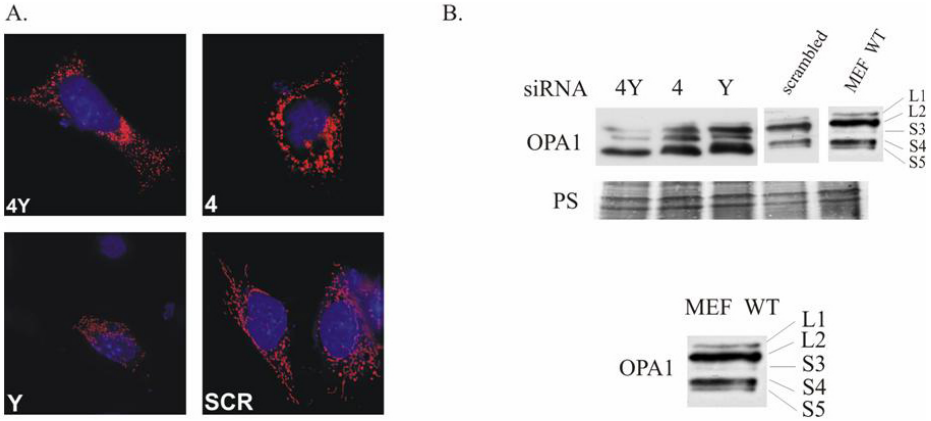

Figure 3.5: Processing of OPA1 depends on the presence of YME1L

Lysates from the MEF cells transfected with siRNA against murine YME1L **(4)**, murine PHB2 **(Y)** or both of them **(4Y)** were checked for OPA1 expression pattern by immunoblotting with the anti-OPA1 antibody (BD Biosciences). Ponceau S staining (PS) serves as a loading control. Fluorecence microscopy **(A.)** of indicated cell samples shows mitochondrial morphology effect in relation to OPA1 processing **(B.)**. Scrambled transfection control (scrambled, SCR) and wild-type cells (MEF WT) were used as reference controls.

cells lacking PHB2 are small and dispersed, no elongated forms are visible. No interconnections are present. Mitochondria from cells lacking YME1L are bigger, swollen with visible interconnections. Therefore they resemble more aggregated than fragmented tubular network. Recently, PHB2 was shown to influence the OPA1 processing and cristae remodeling, with the mitochondrial fragmentation as PHB2-depletion effect [106]. Apparently, PHB2 down regulation has an epistatic effect over YME1L down regulation, since double knock down of the proteins (siRNA 4 and Y) results in the morphology resembling single PHB2 depletion (Fig. 3.5A).

Mitochondrial transmembrane potential maintenance in the YME1L knocked down cells

Mitochondrial dysfunction and dissipation of the membrane potential across the inner membrane can induce OPA1 processing and lead to mitochondrial fragmentation [44; 73; 106]. The YME1L knock down could also

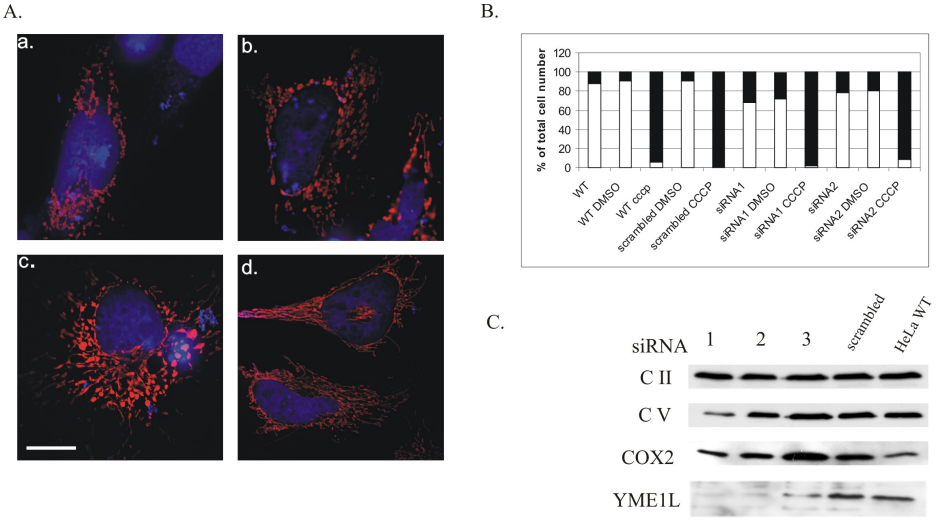

Figure 3.6: YME1L affects mitochondrial morphology but not respiration
A. Aberration in the mitochondrial morphology upon down regulation of YME1L by 3 different siRNAs: 1 **(a.)** 2 **(b.)** and 3 **(c.)** in HeLa cells transfected with pDsRed-Mito and analyzed after 48-60 h by fluorescence microscopy. Scale bar 2 μm. **B.** Maintenance of mitochondrial transmembrane potential in YME1L-deficient HeLa cells. Indicated cell samples were stained with the fluorescent dye JC1 and analyzed by flow cytometry at 590 nm. Dissipation of the membrane potential with CCCP both in wild-type HeLa cells and transfected with siRNAs 1 and 2 or scrambled control, together with DMSO treated cells were used as controls. White bars represent the percentage of cells giving red and green signal whereas black bars show percentage of cells giving only green signal, e.g. with the dissipated membrane potential. **C.** Immunoblot analysis of siRNA 1-3 transfected HeLa cells. Steady state levels of respiratory chain proteins: complex II (Molecular Probes), complex V (Molecular Probes) and complex IV subunit 2 (COX2; Molecular Probes) were analyzed by SDS-PAGE of total cell lysates and immunoblotting with the specific antibodies. Scrambled siRNA transfected and non transfected cells serve as reference controls. Immunoblotting with an anti-YME1L antibody (Dr. C. Koehler) shows YME1L-specific siRNA down regulation efficiency.

impair the transmembrane potential. That would explain induced OPA1 processing and mitochondrial morphology abnormalities (Fig. 3.6A). To examine this possibility, siRNA transfection was repeated. Subsequently cells were stained with JC1 (Molecular Probes). This is a fluorescent dye,

whose oligomerization in the living cell takes place in mitochondria and depends on the presence of the mitochondrial potential. JC1 aggregates in the mitochondria emitting a red fluorescence, or/and stays in the cytosol as monomer, emitting a green signal. Thus, in normal cells with transmembrane potential present, both red and green signals are present, while no red signal can be detected upon dissipation of the membrane potential. siRNA1 and siRNA2 were used, as the most efficient in down regulating YME1L levels. Wild-type HeLa cells and the negative siRNA control were used to exclude deleterious effect of the transfection. CCCP (carbonyl cyanide m-chloro-phenyl-hydrazone) treatment was used as a positive control for the transmembrane potential dissipation. CCCP is an uncoupling agent which abolishes the obligatory linkage between the respiratory chain and the phosphorylation system observed with intact mitochondria. Cells were subjected for fluorescence-activated cell sorting (FACS) and quantified (Fig. 3.6B). The membrane potential was maintained in YME1L depleted HeLa cells. Furthermore, respiratory chain complex II, complex V and cytochrome c oxidase subunit 2 (COX2) steady state levels were not affected (Fig. 3.6C). Taken together, increased OPA1 processing and mitochondrial fragmentation in YME1L-depleted cells is not caused by an impaired transmembrane potential or respiratory activity. These findings are in agreement with the previous reports showing abnormalities in the mitochondrial network upon YME1L down regulation [61] proposing YME1L role in the processing of OPA1 in a constitutive and transmembrane potential-independent manner at the S2 processing site [63; 138].

3.2 Overexpression of YME1L in the inducible system: HEK293 T-REx Flp-In cells

3.2.1 Expression constructs

Mitochondria depend on the functional iAAA and mAAA inner membrane proteases. The mAAA protease subunits paraplegin and AFG3L2 were reported in the pathogenesis of Hereditary Spastic Paraplegia [24; 25; 127], axonal development [101], in the maintenance of mitochondrial morphology [43; 73] and in the proteolytic cleavage/maturation of other mitochondrial proteins [112; 146]. Similarly, the present study reports a role of the iAAA protease in mitochondrial morphology, apoptosis and specific degradation. However, little is known about the mechanisms that could carry out those functions. Recent mutational studies on the yeast Yme1p revealed different modes of substrate binding [58] which could participate in different effects of gene deletion or Yme1 protein mutation on its different functions. Furthermore, studies on the mAAA protease have shown that a mutation of the conserved glutamate residue in the Walker B motif, required for ATP hydrolysis has a dominant negative effect on the mAAA complex (personal communication: Ines Raschke, Florian Gerdes, Dr Takashi Tatsuta and Dr Steffen Augustin). It is therefore conceivable, that similar mutation in iAAA could lead to the similar effect. To test that possibility we constructed several expression constructs encoding different variants of murine YME1L (Fig. 3.7A). Mutations were introduced into:

- the lysine residue required for ATP binding in the Walker A motif (mYME1^{K327A}; **WA**),
- the glutamate residue of the Walker B motif required for ATP hydrolysis (mYME1L^{E381Q}; **WB**),

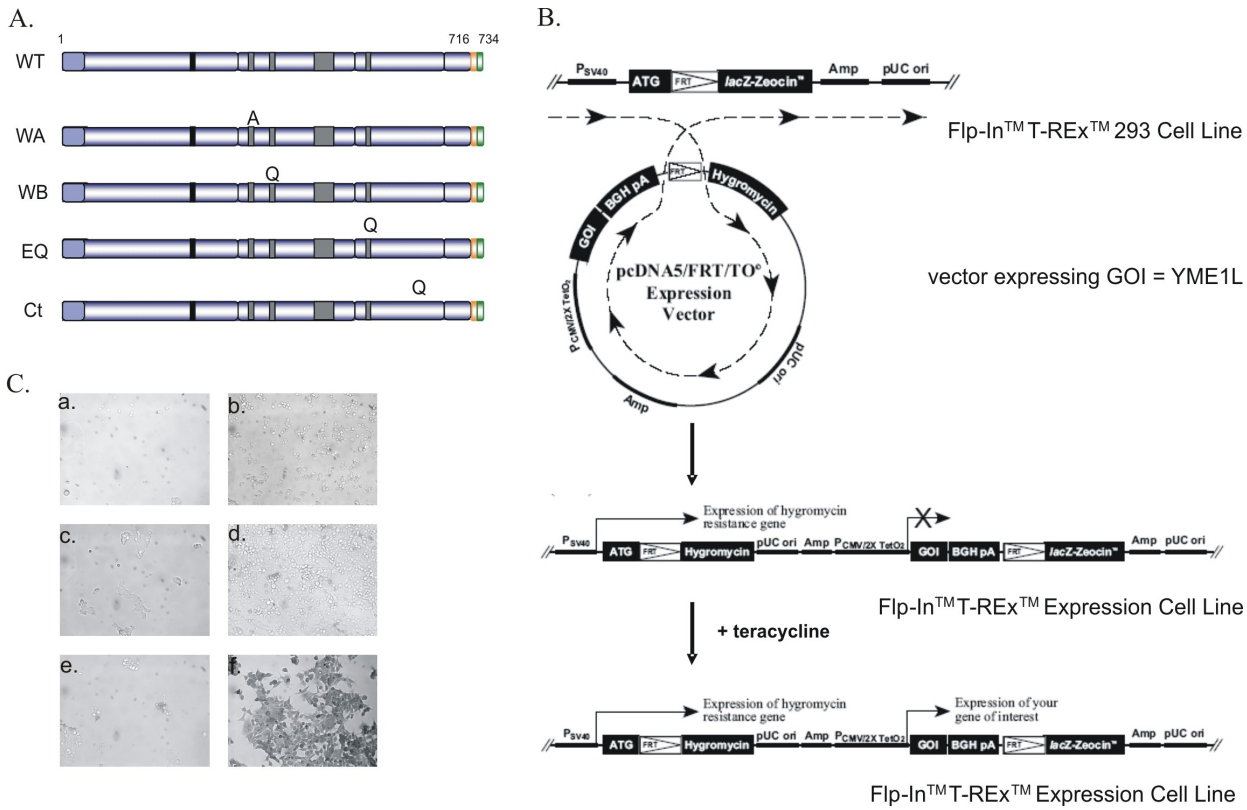

Figure 3.7: Generation of Flp-In™ T-REx™ 293 cell lines expressing mYME1L and its mutant variants.

A. Schematic representation of mYME1L constructs with C-terminal StrepII tag (orange) followed by 8 histidine residues (green) wild type (WT) and mutated in: Walker A domain K327A (WA), Walker B domain E381Q (WB), catalytic domain E542Q (EQ) and C-terminal part E603Q (Ct). **B.** Mechanism of single integration of Gene Of Interest (GOI) and repression/induction of its expression. FRT sequences provide recombination site for single genome integration. LacZ-Zeocine cassette disruption in recombinants provides the possibility for positive selection by ?-galactosidase staining of the cells and negative selection by zeocine treatment (reprinted form www.invitrogen.com). **C.** β-galactosidase staining of clones: WT4 **(a)**, WA6 **(b.)**, WB9 **(c.)**, EQ7 **(d.)**, Ct2 **(e.)** and HEK293 control cells (f.) expressing mYME1L forms represented in (A.). Blue staining occurs in the cells with LacZ expression *i.e.* not transfected HEK293 control cells and when random integration into the genome. No staining (whites) is a signal of LacZ cassette disruption *i.e.* recombination between FRT sites and correct integration into the genome.

- the glutamate residue responsible for enzymatic activity in the proteolytic domain (mYME1L^{E542Q}; **EQ**),
- highly conserved glutamate residue which has not been yet reported as essential for any of the functions of AAA proteases (mYME1L^{E603Q}; **Ct** see also Fig. 1.3 and Appendix 1 on page 81 for the sequence alignment).

An additional construct with a wild-type variant of mYME1L (mYME1L^{WT}; **WT**) was prepared for a control. The protein was C-terminally tagged with StrepII tag followed by eight histidine residues (8HIS). They were introduced to allow:

- differentiation of the endogenously and exogenously expressed proteins by immunoblotting with an antibody against the tags.
- two-step protein purification using affinity chromatography
- pull down of YME1L-substrate complexes

All the constructs were cloned into a vector allowing inducible expression (pcDNA5/FTR/TO™) and stable HEK293 lines were generated with a help of Flp-In™ system (Invitrogen).

3.2.2 Generation of stable cell lines

The Flp-In™ system allows single genome integration of the coding cassette through Flp-driven recombination between two FRT sites (Fig. 3.7B). HEK293 T-REx™ Flp-In™ cells harbor one FRT site within a LacZ-Zeocine cassette integrated into the genome. The second FRT sequence is located on the pcDNA5/FRT/TO™ vector. Sequences encoding the **G**ene **o**f **I**nterest (**GOI**, Fig. 3.7B) are cloned into the pcDNA5/FRT/-TO™ vector under a tetracycline-inducible promoter ($P_{CMV/2xTetO2}$).
The pOG44™ plasmid harbors a coding sequence for Flp recombinase. Both are co-transfected into HEK293 T-REx Flp-In™ cells. Constitutively

expressed Flp mediates trans recombination between both FRT sites. As a result, the whole pcDNA5/FRT/TO™ plasmid with GOI gets inserted into the HEK293 genome and the LacZ-Zeocine cassette gets disrupted and cells lose zeocine resistance. They gain, however, hygromycine resistance which allows positive selection. Clones isolated after ten days selection with hygromycine were verified for a correct genome insertion by β-galactosidase activity test. It is based on the presence of the functional LacZ cassette in the genome of non-transfected cells and cells where random integration occurred. In the test they are stained blue, as β-galactosidase is active. No strain reports LacZ disruption *i.e.* proper integration of the coding construct into the genome (Fig. 3.7C).

3.2.3 Test of expression

In the following experiment, clones showing negative staining in the β-galactosidase activity test were subjected for 24h to tetracycline (tet) induction of YME1L expression. Mitochondria were isolated from non - induced and tet-induced cells to examine the relative expression level (Fig. 3.8A). Positive stable cell line clones were obtained for all of the constructed mYME1L variants. In all of the checked mitochondrial lysates an increase of the protein expression was detected in response to the tetracycline induction. As seen on Fig. 3.8A there is no band shift, despite of C-terminal double tag, 20 kDa in size, included in the expression constructs. The YME1L bands in non-induced cells represent the endogenous protein, whereas from tet-induced both: endo- and exogenously expressed protein. Batch purification of the protein from cleared cell lysates (Qiagen, according to the QIAexpressionist protocol) on Ni-agarose affinity column (Qiagen) failed, as well as a detection of the protein with α StrepII antibody (results not shown). We conclude therefore, that both StrepII and 8 HIS tags are lost, presumably cut off in proteolytic processing. It was therefore impossible to differentiate endogenous and exogenous forms of YME1L expressed in HEK293 cells.

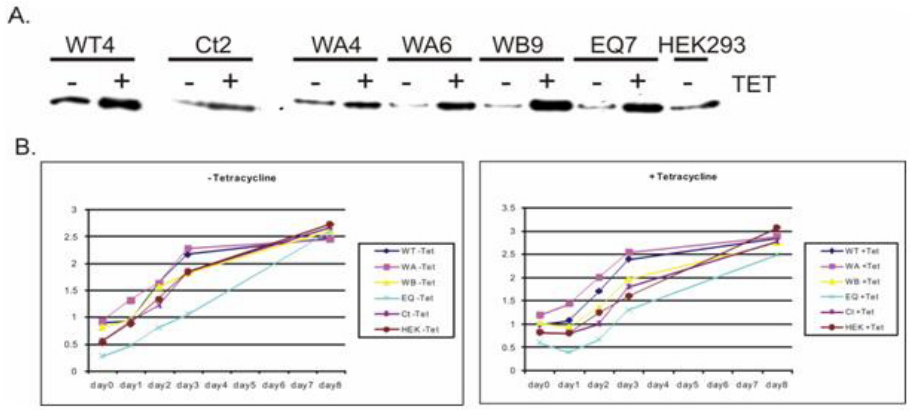

Figure 3.8: Overexpression of wild-type or mutant forms of mYME1L has no effect on the growth rate

A. Correct insertion into the genome was verified by comparison of YME1L protein level before and after induction: one part of the cells was treated with 1?g/ml of tetracycline for 24h (+Tet) and the other part was left without induction (-Tet). Both sets of cultured clones were subjected for mitochondria isolation and YME1L level was examined by immunoblotting with the commercially available YME1L-specific antibody (ptglab). Indicated clones expressed the following forms: wild-type mYME1L (WT4), mYME1L^{K327A} (WA4, WA6), mYME1L^{E381Q} (WB9), mYME1L^{E542Q} (EQ7) and mYME1L^{E603Q} (Ct2). **B.** Proliferation assay (Promega) with 1 μg/ml tetracycline induction (right panel) or without (left panel). HEK293 Flp-In™ T-REx™ control cells together with clones expressing wild-type YME11L were used as reference controls. Each clone was seeded and measured in 4 repeats.

3.2.4 Characterization of mYME1L expressing cell lines

Overexpression effect on the cellular growth

The effect of any of the introduced mutations on the cellular proliferation was examined. Deficiencies in other mitochondrial proteins, like PHB2 were shown to inhibit the proliferation of MEF cells [106] and Walker B motif mutation in the murine AFG3L2 inhibits the growth of HEK293 Flp-In™ T-REx™ cells (Ines Raschke personal communication). Therefore, the proliferation assay was performed with tetracycline induction for 8 days (Promega, Cell*Titer96* AQueous One Solution Cell Proliferation Assay). Cells were counted every 24h. Tetracycline was freshly added every 24h due to its low stability. We did not observe any inhibitory effect

of the mYME1L WT or mutant variants overexpression on the cell growth (Fig. 3.8B) when compared to the HEK293 control cells.

Subcellular localization of the overexpressed mYME1L forms

In further experiments the subcellular localization of the overexpressed protein was approached. Both StrepII and 8HIS tags could not be detected. Therefore endogenous and exogenously expressed YME1L could not be distinguished from each other. The subcellular localization of the protein was assessed by immunofluorescence with the specific polyclonal anti-YME1L antibody. To this end, transfection of the mitochondrially targeted red fluorescent protein was done with the subsequent induction of the mYME1L-variants expression and finally immunofluorescence (Fig. 3.9). Co-localization of the red fluorescent protein signal with the green signal given by the immunodetected YME1L protein was observed for all of the cell samples. Immunofluorescence also detects the endogenous YME1L whose expression is by no means inhibited. However, lack of any other specific signal pointing to the cytosolic or other localization suggests that both endogenous and construct-derived protein is targeted into the mitochondria.

Mitochondrial morphology upon overexpression of mYME1L variants

The knock down experiments have shown that functional iAAA protease is necessary for the maintenance of the mitochondrial network and this role appears to operate through the proteolytic cleavage of OPA1. Here, an effect on the mitochondrial morphology was observed upon YME1L depletion which correlates with the disappearance of the long forms of OPA1 (Fig. 3.4). To examine the effect of wild-type and mutant mYME1L overexpression on mitochondrial morphology fluorescent microscopy was performed. The cells were transfected with the mitochondrially targeted red fluorescent protein (pDsRed-Mito) and grown in the presence of tetracycline for 24h (Fig. 3.9, middle panel). No aberrations in

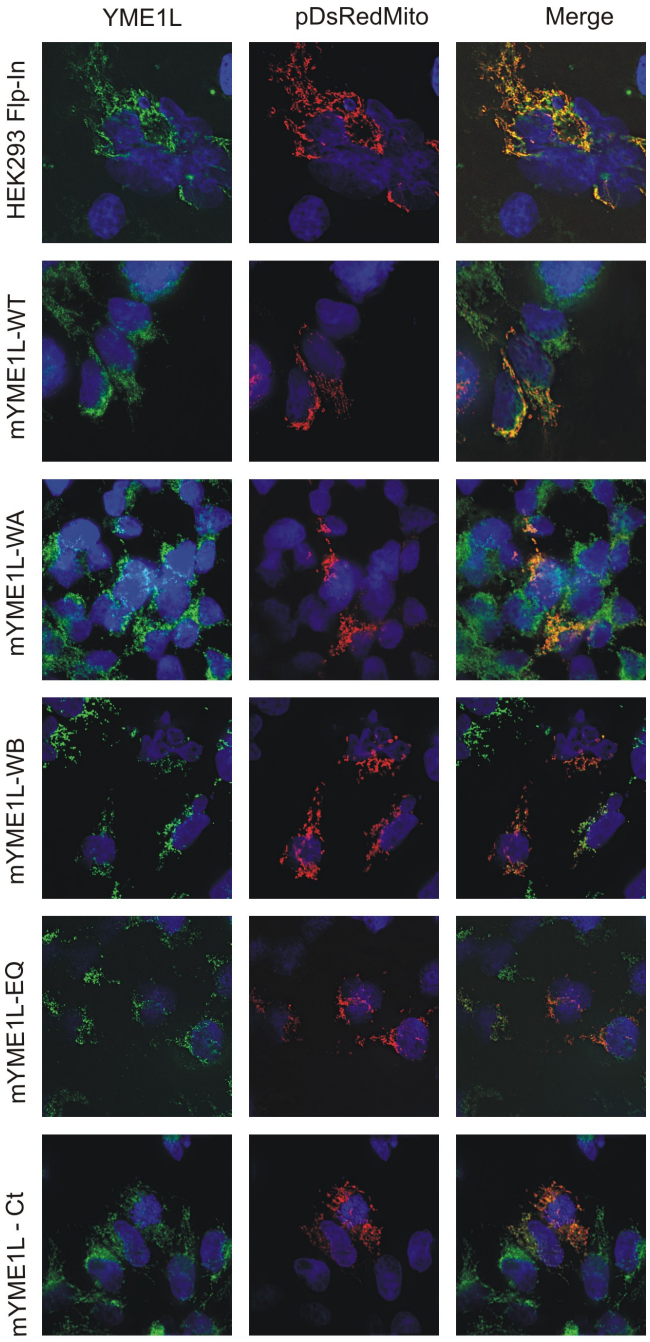

Figure 3.9: YME1L localization and mitochondrial morphology of Flp-In™ T-REx™ expression cell lines

Clones confirmed for single FRT driven integration into the genome were transfected with pDsRed-Mito (Clontech) fixed after 48 hrs post-transfection and incubated for overnight with the anti-YME1L antibody (ptglab, 1:50) and subsequently with FITC-conjugated goat anti-rabbit secondary antibody (1:1000). Mitochondrial localization of YME1L in control cells and expression cell lines can be seen by co-localization of the green signal (YME1L) with red signal (pDsRed-Mito) in merged picture.

the mitochondrial morphology were observed. This result indicates that overexpression of wild-type or mutant variants mYME1L did not significantly affect the mitochondrial network.

3.2.5 Functionality of the iAAA protease in the HEK293 cells expressing *YME1L* and YME1L

OPA1 processing at the S2 cleavage site

Given the wild-type mitochondrial morphology in the cells expressing mutant forms of mYME1L, OPA1 processing by the iAAA protease was checked. It was demonstrated that OPA1 is cleaved proteolytically in two sites: S1 and S2; where S2 is the one identified as the cleavage site for YME1L protease [138]. Hence, transiently expression of OPA1 splice variant 7 was done (sp7-OPA1). It is known to posses both of the sites, and therefore processed to one long isoform L1 and two short isoforms S4 and S5 [138]. S1 site was deleted from the constructed form giving sp7-OPA1-ΔS1 (Fig. 3.10). It resulted in the processing to only two forms: long L1 and short S4. Additionally the construct was tagged C-terminally with three Flag tags for detection independent of the endogenous OPA1. In all of the cellular lysates processing of sp7-OPA1-ΔS1 was unchanged (Fig. 3.10). This result is in agreement with the mitochondrial morphology observation, and suggests that iAAA protease is functional in all of the examined cells. An unequal loading of the protein samples is indicated by the PS loading control. However in all of the samples two forms of OPA1 are detected corresponding to the long and short isoforms detected in the wild-type HEK293 cell extracts. Different protein levels indicated by the intensity of the bands detected by Western blotting could also result from different transient transfection efficiencies.

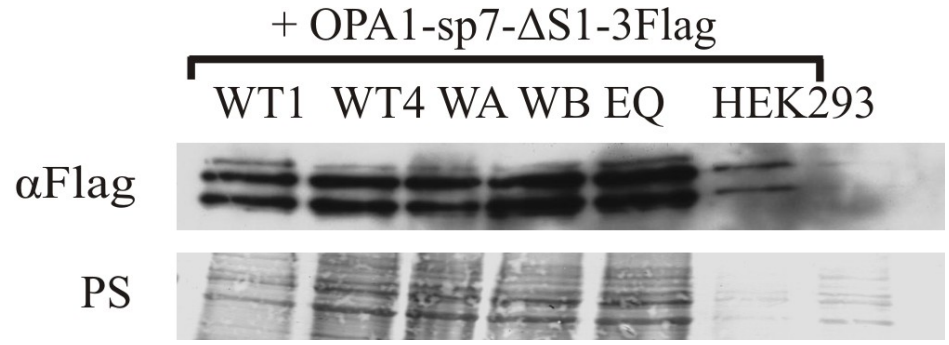

Figure 3.10: Processing of sp7-OPA1Δ S1 by various forms of mYME1L
HEK293 Flp-In™ T-REx™ control cells and expression cell lines with mYME1L wild-type (WT), mYME1L^{K327A} (WA), mYME1L^{E381Q} (WB), mYME1L^{E542Q} (EQ) and mYME1L^{E603Q} (Ct) were transfected with pCMV-sp7-OPA1ΔS1-3Flag. 24h post-transfection expression of YME1L was induced for another 24h with 1 μg/ml of tetracycline. Total cell lysates were separated on SDS-PAGE and sp7-OPA1ΔS1-3Flag processing at S2 site was analyzed by immunoblotting with the anti-Flag antibody (Sigma). Ponceau S staining (PS) shown in the lower panel serves as the loading control.

PNPase maturation

Yme1p in yeast was shown to be imported into the mitochondria via TIM23 pathway, and its assembly into the proteolytically active complex requires Tim54p belonging to the TIM22 translocon [72]. Hence, it was proposed that Tim54p links mitochondrial import and turnover pathways. Interestingly, Yme1p itself was shown to have a role independent of its proteolytic activity, in the mitochondrial translocation of an exogenously expressed human PNPase through the inner membrane [122]. It appears therefore to be an important player in both mitochondrial turnover and import and that those functions are carried out independently. The proteolytic functionality of the overexpressed YME1L variants was confirmed by showing the cleavage of OPA1 at S2 site. However other, non-proteolytic functions could be affected.

In the next experiment further YME1L function was assessed in HEK293 cells expressing mutant and WT forms of the protein. To check the maturation of the endogenous PNPase, the YME1L expression was induced with tetracycline (Fig. 3.11). All of the cells expressing mYME1L (WT, WA, WB,

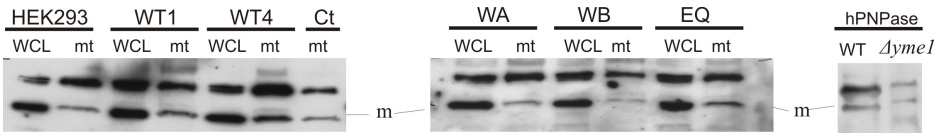

Figure 3.11: Overexpression neither of WT form of YME1L nor any of its mutant affects PNPase

Whole cell lysates (WCL) and mitochondria (mt) isolated from clones expressing wild type (WT1, WT4) or mutant murine YME1L variants: mYME1L^{K327A} (WA), mYME1^{E381Q} (WB), mYME1L^{E542Q} (EQ) and mYME1L^{E603Q} (Ct) were subjected for SDS-PAGE and analyzed for PNPase by immunoblotting with hPNPase specific antibody (Dr. C.Koehler). Yeast extracts from wild-type and ?yme1 strain expressing human PNPase were used as controls. "m" indicates the mature form of PNPase.

EQ and Ct) were divided into two fractions. One fraction was subjected for mitochondria isolation (mt). The second one was used for the preparation of protein lysates from the whole cells (WCL). As the import of PNPase into the mitochondria is coincident with its maturation, two forms of PNPase in the wild-type control should be seen (Fig. 3.11; hPNPase expressed in the yeast WT strain).And inhibition of the PNPase import should result in aberrant forms, like those in the negative control (Δ*yme1* yeast strain background). The wild-type and deletion control Δ*yme1* are consistent with the Rainey *et al.* report [122]. Whole cell lysates were checked together with the mitochondrial extracts, in case the translocation is arrested and PNPase resides in the cytosol. Still, our experiment showed the existence of both precursor and mature forms of hPNPase, similar to the WT control, and the expression of none of the mYME1L variants led to the band pattern resembling Δ*yme1* yeast control. Therefore, this indicates that iAAA function in the protein translocation is not affected by the expression of any of the mutant mYME1L proteins.

Assembly of the respiratory chain protein complexes

Mitochondrial function depends on the correct assembly of the respiratory chain complexes and mitochondrial potential. In the following experiments, other functions were examined, like respiration and ATP production. For this purpose, first the assembly of the respiratory chain com-

plexes was approached by Blue-Native PAGE (Fig. 3.12, [132]). Consistent with the wild-type tubular mitochondrial network is the assembly of respiratory chain proteins which remained unaltered. That points to the unaffected mitochondrial functionality in all of the clones.

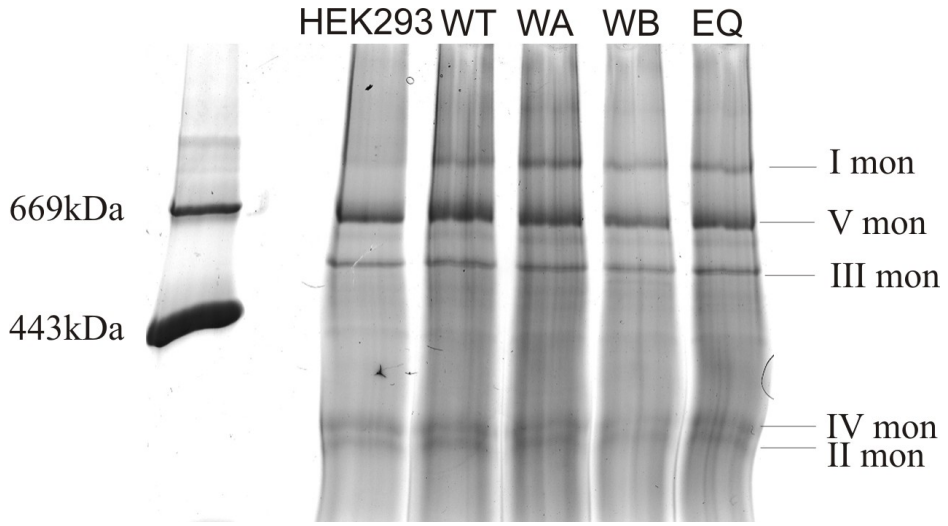

Figure 3.12: Assembly of respiratory chain complexes

Mitochondria (200 μg) isolated from HEK293 Flp-In™ T-REx™ control cells and mYME1L expression cell lines mYME1L wild-type (WT), mYME1L^{K327A} (WA), mYME1L^{E381Q} (WB), mYME1L^{E542Q} (EQ) induced with 1 μg/ml of tetracycline for 24h were solubilized in 1% (w/v) DDM. Assembly of respiratory complexes was examined by Blue-Native PAGE: samples were separated on the gradient 4-11% native gel and the complexes visualized with Coomassie brilliant blue colloidal staining. Thioglobulin (669kDa) and apoferritin (443kDa) serve as molecular mass references

Chapter 4

Discussion

Studies on the mitochondrial AAA membrane mAAA and iAAA proteases revealed their conservation and close homology to the bacterial FtsH. Experimental evidence indicated the mammalian subunits of both mAAA and iAAA proteases as functional orthologs of the yeast proteins. That revealed protein conservation from bacteria to high eukaryotes. Both mAAA and iAAA protease complexes are involved in the mitochondrial quality control, degrading non-native proteins in the ATP-dependent manner. Mutations in the mammalian mAAA protease subunits: paraplegin and AFG3L2 were identified in patients suffering of hereditary spastic paraplegia (HSP) and OXPHOS impairment. The characteristic feature of the mAAA-related HSP is an axonal decay in the motor neurons leading to the lower limbs paralysis. Intensive studies are undertaken to reveal the molecular mechanisms underlying those inheritable diseases. Close homology of the human iAAA subunit YME1L to paraplegin led to speculations about its involvement in pathogenesis of HSP. However, up to date next to nothing is known about its function in the mammalian cells and the molecular mechanisms it is involved in. Here with focus on the mammalian YME1L function in the mitochondrial proteolysis and morphology, and approach them by knock down and the over expression studies.

4.1 YME1L is specifically recognized by the antibody and efficiently down regulated with siRNAs

In vitro processing of the radiolabeled hYME1L by the MPP resulted in the 60kDa protein. That is the size of a protein recognized by all of three anti-YME1L antisera used in the present study. Immunoblotting revealed mature form of the mammalian YME1L present both in human and murine cells. With this method, no precursor form of the protein could be detected. It should be ∼80 kDa in size. Furthermore, we could detect only one isoform of the human YME1L, ∼60 kDa in size, in the human cell lysates. This suggests that the annotated isoforms 1 and 3 are only forms of the precursor protein, both efficiently maturated to the 60 kDa size.

Transfection with three different siRNAs resulted in the disappearance of the 60kDa band, which was not the case in the negative transfection control, both in MEF and HeLa cells. Taken together, we could specifically recognize the mammalian YME1L protein with three different antibodies, and efficiently and specifically down regulate the protein level using siRNAs, both in murine and human cells.

4.2 YME1L is involved in the proteolysis of proteins in the mammalian mitochondria

Destabilization of proteins leads to their degradation in the quality control process. In the case of the mitochondrial PHB1/PHB2 inner membrane complex, depletion of PHB2 in mammalian cells is accompanied by the loss of its assembly partner PHB1, with no loss of the *Phb1* transcript [106]. As reported previously, both yeast prohibitins are substrates for proteolysis by Yme1p upon destabilization of the prohibitin 1/prohibitin 2 complex [75]. Here we report a proteolytic function of the mammalian YME1L

in the degradation of PHB1, while its assembly partner PHB2 is depleted. Our result shows, that the evolutionary conservation on the sequence level - 42% homology between human and yeast Yme1p - is followed by the functional conservation of the protein.

Proteolysis of unassembled protein complexes is one of the quality control functions. It allows removal of potentially dangerous proteins from the mitochondria. Yme1p is the proteolytic subunit of the iAAA protease which together with mAAA protease ensures the quality control in the inner mitochondrial membrane, degrading non-native proteins. In yeast, cells deleted in either mAAA or iAAA subunits reveal similar phenotypes related to their respiratory incompetence, however the strength of those phenotypes is different. Δ*yme1* cells are only conditionally retarded in growth, *i.e.* on the non-fermentable carbon source at 37°C and on glucose at 14°C [23; 152]. Whereas *YTA10* and *YTA12* yeast mutants exhibit respiratory incompetence [62; 148] and lack of assembled respiratory chain and ATP-synthase complexes in the inner membrane and can not grow on a non-fermentable carbon source [54; 118; 158]. There were reports revealing specific substrates of the yeast mAAA protease, but not for the iAAA protease [112; 146]. Similarly, in the mammalian cells mutations in the mAAA subunits are related to the mitochondria-related disorder, HSP [24] which is related to the aberrant axonal development [101] and OXPHOS deficiencies [12]. Close homology between YME1L and paraplegin and AFG3L2, both related to HSP, raised the speculations about the involvement of YME1L in similar disorders [36]. Nevertheless, no mutations in the Yme1l gene were identified in patients with OXPHOS deficiencies [35]. It was suggested that both mAAA and iAAA proteases have an additional role in the regulation of mitochondrial dynamics and morphology, independent of their proteolytic quality controlling in the mitochondria and that those specific functions, rather than the common one which is the proteolysis of unassembled membrane proteins in mitochondria, leads to those differences.

Interestingly, the further examination of the proteolytic function of YME1L in mammalian cells revealed its involvement in the specific degradation of TIM23. Thus, it is involved in the novel cell death pathway reported recently [57]. The data show that the degradation of TIM23 accomplished by YME1L occurs upon apoptosis induction with the accompanying inhibition of caspases. Therefore, it shares similarities with necroptosis, which is also induced by the apoptotic stimulus with the simultaneous inhibition of caspases. However, the following organelle swelling, plasma membrane permeabilization and lack of nuclear fragmentation present in necroptosis point to the necrotic cell death [39]. Therefore, TIM23-dependent cell death was proposed as an emergency pathway for the apoptosis to occur under caspases inhibition [57]. In line with this, the data demonstrate that TIM23 degradation was stronger with 50μM etoposide treatment for 48h, then 200μM etoposide for 24h, indicating that this apoptotic pathway is rather late reacting. This would be in agreement with the proposed "emergency pathway" theory, as in some tissues already morphological changes occur within minutes after pro-apoptotic stimulus [131; 149]. Down regulation of YME1L inhibits TIM23 degradation, and thus cell death progress. Therefore, it might have therapeutic potential for the diseases that involve caspase-independent cell death and result in loss of irreplaceable cells, like neurons or cardiomyocytes.

4.3 Mammalian YME1L has a role in the mitochondrial morphology

OPA1 is present in the human cells in at least eight splice variants which are proteolytically processed to two long isoforms (L1 and L2) which can be further processed to three short isoforms (S3-S5) where S4 is a form that appears to be generated by YME1L [61; 73; 138]. Upon down regulation of YME1L we could observe various aberrations in the mitochondrial morphology, which resemble aggregated and fragmented tubu-

lar network. Such an effect was previously observed in yeast Δ*yme1* cells [23] therefore it further points to the conserved function of the mammalian protein. Moreover, we observed induced cleavage of OPA1. It appears that OPA1 is not degraded; rather the processing is induced, as long isoforms disappear and the short ones accumulate. Such an accelerated processing, however with an accompanying degradation is also observed upon transmembrane potential dissipation [44]. Here, YME1L down regulation did not result in the collapse of the membrane potential with neither of used siRNAs. Hence, it suggests that in both cases different mechanism are involved. This result is in agreement with the notion, that YME1L processes OPA1 at the S2 site constitutively and in the potential-independent manner, unlike PARL and mAAA protease, which require the mitochondrial potential [61; 73; 138]. Similarly, PHB2 depletion was shown to induce the cleavage of OPA1 independent of the transmembrane potential [106]. However, we observed that both PHB2 and YME1L down regulation result in different mitochondrial phenotypes. Moreover, simultaneous depletion of both proteins results in the mitochondrial morphology resembling the single PHB2 depletion. This indicates an epistatic effect of PHB2 over YME1L. It suggests also involvement of both proteins in different pathways of the mitochondrial morphology regulation. It is not known, however, which way YME1L could function in the mitochondrial fusion. We cannot exclude cristae remodeling, as YME1L cleaves OPA1. However it would presumably involve different mechanism than PHB2. Recently, F_1F_0-ATP synthase (also known as F_1F_0-ATPase) was shown to be essential for the mitochondrial fusion, cristae formation and the maintenance of the tubular morphology [10; 56; 119; 139]. It is a rotary enzyme reversibly synthesizing ATP in the coupled to the proton transport across the membrane manner. F_1F_0-ATP synthase forms dimeric and oligomeric supercomplexes. Yeast Mgm1p is required for the proper oligomerization of F_1F_0-ATP synthase [5]. Yme1p was suggested to catalyze the turnover of F_1-ATPase protein inhibitors [42; 86; 93] and influence F_1-ATPase func-

tion and activity [48]. Hence, it is conceivable, that Yme1 protein could be a link between F_1F_0-ATP synthase oligomerization or conformation and Mgm1/OPA1, and as such could influence the mitochondrial morphology. To verify that possibility the respiratory chain proteins assembly could be addressed upon the YME1L depletion. However, destabilization of the oligomerized F_1F_0-ATP synthase forms which led to the dissipation of the transmembrane potential was demonstrated not to affect other mitochondrial features, like morphology, cristae formation, mtDNA stability, enzymatic activity of cytochrome *c* oxidase, maximal respiration rate and enzymatic activity of F_1F_0-ATP synthase [18]. It appears likely therefore, that the activity of F_1F_0-ATP synthase and not its oligomeric state is important for mitochondrial function. So far no evidence has been gathered for the Yme1p - F_1F_0-ATPase direct or indirect interactions. Similarly, no clear evidence could point out and explain the influence of Yme1 on the F_1F_0-ATP synthase activity.

The double PHB2/YME1L depletion phenotype could also suggest upstream function of PHB2 to YME1L. But in such a case, one might see an effect of PHB2 down regulation on the level of YME1L, as both proteins were functionally dependent. Here, PHB2 depletion did not affect the YME1L steady-state level.

Summarizing, the present study could show that mammalian YME1L is an evolutionary conserved protein with functions in the quality control, proteolysis and mitochondrial morphology. Moreover, YME1L was demonstrated to specifically degrade TIM23 in the novel apoptotic event. Hence, YME1L appears to be an important player in both cell survival and cell death, having roles beyond its involvement in the proteolysis of non-native proteins. The results further suggest that YME1L is not essential for the cell viability, as no increase in cell death was observed upon the protein down regulation throughout the experiments. It is however noteworthy, that the cellular survival was not assessed over the longer time periods. Therefore, the long-term effects of the YME1L-absence remain an

open question. Additionally, the dividing, immortalized cells were used throughout the study, whereas the disorders YME1L was suggested to be involved in, affect neurons - the irreplaceable, finally differentiated and non-dividing cells. It is conceivable, that this kind of cells accumulate the defects over time. Malfunctions of the mitochondrial fusion/fission apparatus and lack of a fully functional proteolysis result in the decreased quality control. This, in turn, could lead to the accumulation of mtDNA mutations and reactive oxygen species (ROS) which would further damage mitochondria. In the present work, the conserved function of YME1L in the degradation of non-assembled prohibitin 1 was shown. Assuming, that its role in the proteolytic turnover of the yeast Psd1p [108] is also conserved in higher eukaryotes, its depletion might negatively influence the mitophagy. Therefore, the damaged mitochondria would not be removed efficiently, which might result in the induction of cell death. Decreased proteolytic quality control could also lead to the accumulation of the misfolded or damaged proteins within mitochondria. Furthermore, the mitochondrial transmembrane potential could collapse as a result of mtDNA mutations or deletions. This, in turn, might lead to the inhibition of the mitochondrial import, inhibition of the cell growth and/or the induction of apoptosis. The formation of intracellular and extracellular aggregates of protein fibrils are common neuropathological features of many diverse, neurodegenerative, progressive and late-onset disorders, such as sporadic and familial Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and prion encephalopathies [157]. Therefore, to further investigate YME1L function in the mitochondria, and learn about its relevance for the cell on the organism level and over the time and development, an animal deletion model might be generated.

4.4 Overexpression of YME1L in the cells

In the next experiments, the iAAA function in the mammalian cells was assessed by the expression of various forms of the murine YME1L. The goal was to examine the effects of the YME1L dominant negative mutant expression. Observations in our laboratory showed that the Walker B motif mutation in the conserved glutamate residue of AFG3L2 has a dominant negative effect on the mAAA protease (Ines Raschke and Dr Steffen Augustin personal communication). Therefore, we investigated effects of an expression of the similar mutant (mYME1L^{E381Q}) together with mutations in several other conserved residues (K327A, E542Q and E603Q).

Depletion of some mitochondrial inner membrane proteins was shown to have an effect on the cell growth. For example, PHB2 deficiency led to the inhibition of the cellular proliferation [106]. Similarly, degradation of TIM23 resulted in the cell cycle inhibition and retardation of the cell proliferation [57]. Expression of the dominant negative AFG3L2 mutant rapidly inhibited proliferation and induced cell death (Ines Raschke personal communication). Therefore, we examined effects of the YME1L expression as a mutant or wild-type protein on the cell growth. Surprisingly, none of the mutations led to the cell growth retardation. The possible reasons are that either none of the mutant variants had a dominant negative effect on the iAAA protease, or it had, but malfunctions of the iAAA complex did not lead to the cell proliferation effects. Or the overexpressed proteins were not correctly inserted into the mitochondria, and therefore had no effect on the iAAA protease, and thus on the cellular growth. But given that iAAA protease is not essential for the cell viability, it might be possible, that no growth defects would be observed even if the iAAA protease was not fully functional. Therefore next, the iAAA protease targeting and functionality were approached upon overexpression of various forms of the mYME1L.

The cleavage of both C-terminal StepII tag and 8HIS tag from the overexpressed proteins made it impossible to distinguish the exo- and endogenously expressed forms. Nevertheless, the immunofluorescence detection

revealed YME1L only in the mitochondria. No other signals were detectable which could point to other than mitochondrial localization or protein aggregation in the cytosol. Hence, this indicates that both wild-type and mutant variants of the mYME1L were efficiently targeted into the mitochondria. Having excluded the possibility of a mistargeting, the functionality of the mammalian iAAA protease was examined upon overexpression of different forms of mYME1L. The known roles of the mammalian YME1L were addressed.

The YME1L-dependent cleavage of OPA1 splice variant 7 at S2 site results in one long L1 and one short S4 isoform of OPA1. It was demonstrated that beside YME1L, some other, so far not identified protease cleaves OPA1 at this site [138]. Therefore upon YME1L depletion the long L1 form accumulates, and the short one is still present, however in lower amount [138]. Upon overexpression of mYME1L or *mYME1L* in HEK293 cells, the ratio of the long isoform to the short one did not change. The conclusion is, that neither wild-type nor mutant variant of YME1L affected proteolytic cleavage of OPA1 at the S2 site. Hence, the proteolytic activity of the iAAA complex was not affected. In line with this result is the wild-type tubular mitochondrial morphology visible in the examined cells. It points to an unaffected function of YME1L in the mitochondrial dynamics.

The morphology effect could be seen even with not complete depletion of the protein, whereas mYME1L^{WT} overexpression did not result in changes in the mitochondrial morphology. It seems therefore, that the morphology is more sensitive to the decrease, than the increase of the YME1L level. It might be possible, that also here the level of the endogenous protein is sufficient to fulfill all of the iAAA protease functions. Farther expression of the protein might have no effect on the complex, independent of its mutation. In this case, expression of those mutant forms in the *null* background would shine a light on the mammalian iAAA protease function. This was addressed by down regulation of the endogenous protein

by siRNA transient transfection with duplexes against the human YME1L. It was then followed by the murine YME1L expression (data not shown). No YME1L protein could be detected. Maybe the down regulation was as efficient as to knock down the murine protein despite of sequence mismatch between siRNA duplex and mRNA sequence? To exclude such a possibility, down regulation has to be performed with a use of siRNAs designed against the UTR fragment of mRNA, which is present only on the endogenous and not vector-derived mRNA. Unfortunately, none of the siRNAs used in the present study and shown to efficiently knock down the protein, hybridizes within the UTRs.

In yeast, Yme1p was demonstrated to have a role in the import of an exogenously expressed human PNPase [122]. It is the only known non-proteolytic function of the iAAA protease. Therefore, PNPase maturation was used to further assess functionality of the mammalian iAAA complex. No aberrant forms of PNPase were detected in the extracts from cells overexpressing YME1L variants, which could resemble those reported previously in Δ*yme1* cells [122]. Also here, iAAA protease function appears to be unaffected.

Mutations in the mammalian mAAA protease increase oxygen consumption, and decrease the maximum respiration capacity which is believed to be related to the uncoupling of the electron flow from the ATP production (Ines Raschke and Sarah Ehses personal communication). This could be related to effects on the assembly of the respiratory chain complexes. Following this line, the assembly of respiratory proteins was approached by the protein electrophoresis in native conditions. In none of the cells expressing wild-type or mutant mYME1L the assembly of those proteins was affected. It suggests functional respiratory chain, but does not exclude decreased respiration capacity. To verify it further, oxygen consumption and ATP production should be addressed.

Taken together, the collected data indicate, that none of the chosen mutations introduced into the mYME1L sequence, resulted in the dominant negative effect on the iAAA proteolytic complex.

Mammalian subunit AFG3L2 was shown to homooligomerize [87]. It was related to the increased severity of the AFG3L2 mutation when compared to paraplegin as paraplegin forms only heterooligomers with AFG3L2 [101]. So far, YME1L is the only known subunit of the mammalian iAAA complex. In yeast Yme1p was found in complexes with Mgr1p. It is however a non-enzymatic subunit, therefore it was proposed to regulate the function of the iAAA protease. In the case of mammalian iAAA no co-factors are known to modulate its activity. No homologues of Mgr1 were identified in higher eukaryotes [42]. Therefore it is assumed that only YME1L builds up the functional iAAA protease complex. Concluding from the effects of AFG3L2 mutation, dominant effect was expected for some of the mutations introduced into the sequence of mYME1L and efficiently expressed in the HEK293 cells. Contrary to this, none of the mutations inhibited the complex, nor resulted in any phenotypic effects, despite of the efficient induction of the transgene expression detected by the immunoblotting.

Then the question arises: is this possible, that YME1L is able to self - process like FtsH [3] or its overexpression triggers degradation by some not identified protease? Then, the increase of its concentration might result in the increased turnover of the protein by the endogenous YME1L or some other protease. It seems that in such a case, the induction of YME1L expression would not be detected in the extend detected here by immunoblotting. However, to further address this possibility, chase experiments should be designed to follow changes in the protein concentration over time.

The results indicate that the overexpressed protein is imported into the mitochondria. In parallel, the assembly of the iAAA protease was approached by protein electrophoresis in the native conditions (BN-PAGE).

We could observe complexes similar to those in the wild-type HEK293 cells only in the case of mYME1L^{WT} (Appendix 2, page 85). Their size is in agreement with the reported size of the yeast iAAA complex which is ~800 kDa [42]. In the cells expressing ATP-binding mutant mYME1L^{WA}, the size of the iAAA complex is shifted to bigger, as well as its assembly seems to be more sensitive towards detergent treatment. (1% DDM in all samples). Similarly, in the cells expressing ATP-hydrolysis mutant mYME1L^{WB}, the iAAA complex appears to be destabilized under the solubilization conditions used in the experiment, as a smear of not fully assembled YME1L forms appear. In the cells expressing proteolyticaly inactive mutant mYME1L^{EQ}, the iAAA complex size is shifted towards bigger. However, it seems to be stable in the used detergent concentration. This suggests, that the assembly of the YME1L subunits into the complex is not affected, and the bigger size of the complex might result from the trapping of some interaction partner/substrate by the proteolyticaly inactive subunits. Taken together, assembly of YME1L in the cells expressing mutant variants of the protein results in different than wild-type complexes. Are they less stable, or better accessible for the detergent upon membrane solubilization, which would suggest less compact folding? Regardless of it, *YME1L* protein oligomerizes into the complexes in the way, which allows fulfilling all of the examined functions of the iAAA protease, giving no phenotypic effect.

From the studies on the yeast and mammalian mAAA we know that it forms hexameric rings in the inner membrane, similar to the structures proposed for FtsH (Dr Steffen Augustin and Florian Gerdes personal communication and [15; 90]). It has been proposed that the ATPase activity of the AAA proteases is needed for the substrate unfolding and translocation, but not the proteolysis itself. Conserved loop regions within the central channel formed by the ATPase subunits have been demonstrated to be involved in a substrate binding [70]. ATP-dependent conformational changes of these loops are thought to drive the translocation and accom-

panying unfolding of associated substrates into the proteolytic chamber [58].

A mode of ATP hydrolysis proposed is a semi-sequential for yeast and sequential for mammalian mAAA where binding of ATP to one subunit blocks the ATP hydrolysis by the clockwise neighboring subunit. In such a mode of ATP hydrolysis a substrate is unfolded and pulled into the proteolytic chamber for proteolysis in an effective and coordinated manner. It was proposed that only 4 ATP molecules are bound to the complex at the same time, and ClpXP studies have shown that Walker B mutation of one subunit decreased the activity of the other to 60%, but even 1 fully active subunit in the hexameric complex could perform a substrate translocation [69; 103].

There is no experimental support for the ATPase activity followed by proteolysis mode of action for the iAAA protease, but the high homology to the mAAA suggests also conservation of common mechanisms. However, the results in the present study suggest that there could be differences in the ATP-binding and hydrolysis as well as substrate translocation mechanisms between the mammalian mAAA and iAAA protease. It could involve more independent mode of action for each subunit in the iAAA complex. Provided that each of the subunits was able to bind and hydrolyze ATP, and the block on ATP binding and/or hydrolysis by one of the subunits did not block the neighboring one, the assembly of mutated subunits would not influence the whole complex. In this hypothetical situation only the mutation of each subunit in the iAAA complex would result in its complete inhibition.

Similar situation would occur when the ATP-binding and hydrolysis were semi-sequential, like in the yeast mAAA (Dr Steffen Augustin personal communication). There, the active unit of the whole complex is a dimer of Yta12p and Yta10p. Blocked ATP-hydrolysis by Yta12p blocks its neighbouring Yta10 subunit, but not vice versa. In the case of the mammalian iAAA complex, that would be presumably homod-

imer of only YME1L protein. Therefore, no different specificities in ATP or substrate binding between all of the subunits should occur. It would be therefore hard to imagine that the ATP binding and hydrolysis were semi-sequentional, unless some up to now unidentified co-subunits of the mammalian iAAA protease existed. They could influence or regulate the manner in which the whole complex worked.

The next question is, whether the incorporation of a maximum of four non-functional subunits would still leave a functional dimer, which would keep on binding and hydrolyzing the ATP, providing the energy essential for the function of the iAAA complex? And furthermore, it is questionable that the conformational changes of the pore loop would be still possible with ATP molecules irreversibly bound to the complex.

In yeast, there is a certain threshold value for overexpression of the Yme1 protein (Tanja Engmann personal communication). In the HEK293 cells expressing the YME1L variants a mixture of an endogenous and exogenous protein is present, however we cannot conclude about the stoichiometry of both. Provided the similar threshold in the mammalian cells, it would be possible that the level of the expressed YME1L variants is not reaching the concentration needed to result in the inhibition of a whole iAAA protease complex.

Summarizing, the present study demonstrated expression of several forms of the murine YME1L which did not result in the identification of a dominant negative protein mutant. Hence, those results open many questions with regard to the molecular mechanism of the YME1L function in the mitochondria as well as long term effects of its knock-out. Further studies are needed for an identification of interaction partners and possible regulators of the mammalian iAAA protease.

Chapter 5

Materials and methods

5.1 Materials

5.1.1 Bacterial, yeast and mammalian strains and cell lines

All the bacterial and yeast strains as well as cell lines are listed in Table 5.1

Table 5.1: Strains and cell lines used in the study

Organism	Cell line/Strain	Reference
Escherichia coli	XL10 Gold	Stratagene
	XL1 Blue	Tanja Engman
Saccharomyces cerevisiae	WT + hPNPase	Dr Carla Koehler
	Δ*yme1* + hPNPase	Dr Carla Koehler
Mus musculus	Mouse Embryonic Fibroblasts/ C57BL/6	Dr Carsten Merkwirth
Homo sapiens	Human Embryonic Kidney/Flp-In™ T-REx™ HEK293	Invitrogen
	Human cervix cancer cell line/ HeLa	Dr Gerrit Praefcke

5.1.2 Oligonucleotides

The synthetic oligonucleotides used in this study are listed in Table 5.2. siRNA duplexes used in protein knock down studies are listed in Table 5.3

Table 5.2: List of oligonucleotides used in the study

Name	5‘ Sequence 3‘
TL4024	CCA AAT ATG AGT TCC TGT GCC ACT CTT CCT CCC ATG
TL4023	CAT GGG AGG AAG AGT GGC ACA GGA ACT CAT ATT TGG
TL4022	GTA ATA TGC AAT AAT AGC ATG ACC AGA TTG ATG ATA GGC TGT TAT AG
TL4021	CTA TAA CAG CCT ATC ATC AAT CTG GTC ATG CTA TTA TTG CAT ATT AC
TL4020	CCA CCA ACA GAA TCT AAT TGA TCG ATG AAT ATA ACA CAA GGA G
TL4019	CTC CTT GTG TTA TAT TCA TCG ATC AAT TAG ATT CTG TTG GTG G
TL4018	GGC AAG AAG CGT CGC CCC TGT TCC TGG TGG
TL4017	CCA CCA GGA ACA GGG GCG ACG CTT CTT GCC
TL4001	CTG CAG CTA TCA GTG ATG GTG ATG GTG ATG GTG GTG AGT ACT TTT TTC GAA
	CTG CGG GTG GCT CCA TCT CACT TCC AAT TTC TT
TL4000	GGA TCC GCC GCC ATG TTC TCC CTG TCG A
TL2677	ATA TTA CAC AAA GGA TGC AAT GCC
TL2676	GAG GCA TGA TTG TAG CTT TAT TAA TTG
TL2675	GCA GAC GAT CAA TCA GCT TCT TG
TL2674	CAT TGG GTT TGA AAC CAT CCA
TL2673	TTG ATT CTG CGG TAG ACC CTG
TL2672	ATG TTC AAA AGT GAC ATT TTT CAT CT
TL2671	GTA CAT TAC GTT CCT CTA GCT TGT ACA
TL2670	CGT AAT GTA CTG AAC ATA TCT AAG TGA CC
TL2637	CCG TCG ACT CAT CTC ACT TCC AAT TTC TTT CC
TL2636	CCG GAT CCA TGT TCT CCC TGT CGA GCA CT
TL2602	AAC AGC TAT GAC CAT GAT TAC GC
TL2601	AAT ACG ACT CAC TAT AGG GCG AAT

Table 5.3: List of siRNA duplexes used in protein knock down studies

Name	Description	5′ Sequence 3′
siRNA1	hYME1L	TTA AGG CAT TAT CTA ATG CCT CTG G
siRNA2	hYME1L	TTC GAT GGC AGA TTG GGT TTC TGG A
siRNA3	hYME1L	TTT AGT TCA GAT AAT CCA AGG TCC C
siRNA4	mYME1L	TTC AGT TCG ACC CTT CAC ATC TGG C
siRNA5	mYME1L	ATC ACT GTA GGT CAT AAC TCC AAG C
siRNA6	mYME1L	AAC AAT TTG AAT CTC TTT GGC ATC C
siRNAY	mPHB2	ATT GAA CTT GGC CAC CAC ACT CTT G
siRNAZ	mPHB2	ATT CGG TTC TGT GAT GTG GCG ATC G

5.1.3 Vectors and constructs

All the vectors and constructs used in this study are listed in table 5.4.

Table 5.4: List of plasmids used in the study

Plasmid	Reference
pGEM® -T-Easy	Promega
pcDNA™ 5/FRT/TO	Invitrogen
pDsRed2-Mito	Clontech
pcDNA5 FRT/TO-mYME1L^{WT}-StepII-8HIS	this study
pcDNA5 FRT/TO-mYME1L^{K327A}-StrepII-8HIS	this study
pcDNA5 FRT/TO-mYME1L^{E381Q}-StrepII-8HIS	this study
pcDNA5 FRT/TO-mYME1L^{E542Q}-StrepII-8HIS	this study
pcDNA5 FRT/TO-mYME1L^{E603Q}-StrepII-8HIS	this study
pCMV-sp7-OPA1™ ΔS1-3Flag	[73] #1958

5.1.4 Antibodies

The antibodies used in this work are listed in table 5.5.

Table 5.5: List of antibodies used in the study

Antibody	Dilution	Reference
α hYME1L	1:500	Dr Carla Koehler
α mYME1L	1:500	Proteintech Group, Inc. (ptglab)
α mYME1L (SPY531)	1:250 - 1:500	this study
α TIM23	1:1000	BD Biosciences
α BAP37 (PHB2)	1:500	BioLegend
α prohibitin (PHB1)	1:500	NeoMarkers
α β-actin	1:5000	Sigma, clone AC15
α complexII, 70kDa	1:1000	Molecular Probes
α complexV, su α	1:1000	Molecular Probes
α COX2	1:1000	Molecular Probes
α OPA1	1:500	BD Biosciences
α FLAG M2	1:1000	Sigma
α PNPase	1:1000	Dr Carla Kohler
Goat anti-rabbit IgG HRP - labeled secondary antibody	1:10000	BioRad
Goat anti-mouse IgG HRP - labeled secondary antibody	1:10000	BioRad

5.1.5 General reagents

All laboratory chemicals used in the present study were purchased either from Sigma or Merck unless stated otherwise. Enzymes used in this study were purchased from NEB or Invitrogen with stated exceptions.

5.2 Methods

Standard methods of molecular biology were performed according to established protocols [128].

5.2.1 Affinity purification of antisera

Rabbit SPY531 was immunized by the simultaneous injection of 2 peptides: EP071347 and EP071348. YME1L specific IgGs were purified from serum by immunoabsorption on a peptide-conjugated Sulfo-Link® (PIERCE) matrix.

Peptide coupling to the matrix

Peptides EP071347 (H_2N - TLKSRTRRLQSTSER - $CONH_2$) and EP-071348 (H_2N - DKILMGPERRSVEID - $CONH_2$) used for the immunization of rabbits and raising the polyclonal antibody against YME1L were synthesized and supplied by the Eurogentec company. For the purification, a SulfoLink® Coupling Gel (Pierce) matrix was used according to the manufacturer's protocol. 1.5 mg of each peptide was dissolved in 50μl of coupling buffer. The volume was slowly increased to obtain completely dissolved peptides. The total volume of the pooled peptides solution was adjusted to 3 ml. For each ml of peptide solution 1 ml of SulfoLink® gel was used, *i.e.* the total column volume was 3 ml. Coupling efficiency was calculated by the comparison of A_{280} nm of the non-coupled fraction (flow through) to the starting sample and was $\sim$88%.

Immunoabsorption of the antiserum

The matrix, conjugated with peptide, was equilibrated by consecutive washings with 10 ml of 10 mM Tris-HCl, pH 7,5; 100 mM glycine-HCl, pH 2,5; 10 mM Tris-HCl, pH 8,8; 100mM 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 a final volume of 10 ml, was applied on the column at a flow rate of 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 step with 10 ml buffer C. Peptide specific IgGs were eluted in three steps.

Table 5.6: Buffers used in the antiserum purification

Buffer	Composition
Coupling buffer	50 mM Tris-HCl, 5 mM EDTA-Na 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

1. 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.

2. Elution with 10 ml 100 mM glycine-HCl, pH 2,5. 1 ml fractions were collected and the pH was adjusted to 7,0 with 1M Tris-HCl, pH 8,8. The column was washed with 10 ml 10 mM Tris-HCl, pH 8,8.

3. Elution with 100 mM Na-phosphate buffer, pH 11,5. Fractions (1 ml) were collected and the 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. Elution fractions containing the majority of IgGs, were pooled and concentrated to a volume of $\sim$500 μl with Centricon YM-100 (Millipore) centrifugal filter concentrator according to manufacturer's recommendations.

5.2.2 Cell culture

Growth conditions

Mouse embryonic fibroblasts (MEFs) and HeLa cells were cultured in Dulbecco's Modified Eagle's (DMEM) GlutaMax™ -I medium (Gibco) supplemented with 10% FCS, 1x non-essential amino acids, 1 mM sodium pyruvate, and 1x penicillin/streptomycin. All the cells were kept at 37°C 99% humidity and 5% CO_2. Flp-In™ T-REx™ HEK293 cells were maintained in Dulbecco's Modified Eagle's (DMEM) Gluta-Max™ -I (Gibco) medium supplemented as above, with additional 15 μg/ml blasticidine and 100 μg/ml zeocine. Cells were maintained in culture by growing them until $\sim$80% confluency and passing in 1:50 dilutions (for MEFs) to 1:3 dilutions (for HEK293). For wild-type cell lines as well as for the stable cell lines HEK293 Flp-In™ T-REx™ clones liquid nitrogen frozen stocks were prepared.

Passing and freezing the cells

Cells grown to the desired confluency were washed twice with 1x PBS. After complete removal of the PBS, 1x trypsin solution in PBS (PAA Laboratories GmbH) was added and cell were incubated as follows: MEF and HeLa cells 5 min in the standard growth conditions, HEK293 cells not more than 3 min at room temperature. After the incubation, fresh growth medium was added, at least the same volume as the previously added trypsin. Cell suspension was pipeted up and down against the cell bottom 5-8 times and put into a fresh falcon tube. Cells were pelleted

down for 5 min, 1200 rpm, at room temperature. Liquid was removed and the cell pellet was then loosened by hitting the tube bottom a few times. Cells were passed by the addition of fresh growth medium and plating onto fresh dishes in the desired dilutions. For freezing, the cell pellet was resuspended in 10% DMSO in FBS and 1ml aliquots in cryo-tubes were prepared and put on ice. For slow freezing, isopropanol-containing freezing boxes were used. Tubes containing cells were put into the -80°C freezer and transferred to liquid nitrogen storage after 24-72h.

5.2.3 Transfection

siRNA transfection

All siRNA transfections were done twice in 6-well plate format with 6-8 hours incubation in between. For YME1L down regulation with RNAi murine - mRNA - sequence and human - mRNA - sequence - specific Stealth RNAi (Invitrogen) were purchased (listed in Table. 5.3) together with the Stealth RNAi Negative Control Duplexes. During the transfection no antibiotics were added to the medium, and Opti - MEM™ serum reduced medium (Gibco) was used. After the second transfection cells were incubated in the normal growth conditions for over night. siRNA transfections were performed using the Lipofectamine RNAiMax reagent (Invitrogen) according to the manufacturer's protocols. For single siRNA transfection 30 pmol of the siRNA duplexes [20 pmol/μl] per well were added; in double siRNA transfections the amount was doubled per well, taking 30 pmol of each siRNA duplex.

Plasmid transfection

Plasmids were transfected twice in the 6-well format with the Gene Juice reagent (Novagen) following the manufacturer's instructions, with 4-6 hours incubation in between and with 1μg of total plasmid DNA per well. After the second time, cells were incubated over night under

standard growth conditions. During the transfection DMEM GlutaMax™-I medium supplied with 1x penicillin/streptomycin and OPTI -MEM™ medium were used.

5.2.4 Protein isolation from tissue culture cells

Growth medium was removed from the cells and they were washed twice with 1x PBS which was then removed completely from the dish. Ice-cold 1x PBS was added to the cells and they were detached from the plates with a cell scraper and transferred into a cooled tube. No trypsin treatment was ever used to avoid unspecific protein degradation. The plate was washed again with fresh ice-cold PBS and the suspension was transferred into the same tube. Cells were pelleted by centrifugation: 5 min, 800 x g at 4°C , and snap-frozen in the liquid nitrogen. The pellet was resuspended in the lysis buffer and rotated for 2-3 hours at 4°C . Cell lysates were cleared by centrifugation: 10 min, at 13000 rpm at 4°C; and transferred into fresh tubes. Protein concentration was determined by the Bradford protocol [22].

5.2.5 SDS-PAGE and Western Blotting

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and semi-dry western blotting were carried out as described previously [91; 128]. Buffers and reagents used are listed in Table 5.7, page 68. The transfer was done with the use of PVDF membranes for overnight at room temperature. The BioRad mini-gel system was used.

5.2.6 Fluorescence microscopy

Cells were seeded on 6-well plates with cover slides. Dilutions depended on the examined cell line to reach the density proper for transfection reagents recommended by the manufacturers. To address the mi-

Table 5.7: Buffers and reagents used in SDS-PAGE and immunoblotting

Buffer	Composition
10xSDS running buffer	10 g/l SDS, 30,3 g/l Tris, 144,1 g/l glycine
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
Blotting buffer	0,2 l/l methanol, 0,2 g/l SDS, 2,42 g/l Tris, 11,3 g/l glycine
PonceauS solution 100 ml	2 g PonceauS, 30 g trichloroacetic acid, 30 g sulfosalicylic acid
10 x TBS	12 g/l Tris, 90 g/l NaCl, pH 7,4 adjusted with HCl
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) glycerol

tochondrial morphology in siRNA down regulation studies transfection was performed as follows: 2 times siRNA transfection with Lipofectamine RNAiMax and 2 times pDsRed-Mito transfection with Gene Juice in 4 hour intervals with the last transfection incubation lasting over night. The complexes were removed and full growth medium was added. Otherwise, only pDsRed-Mito transfection was performed according to the plasmid transfection protocol (see page 66). Cells were subjected to lysis and/or microscopy after 48h after the second plasmid transfection. Mitochondrial morphology was analyzed using the DeltaVision microscope system equipped with Softworx software (Applied Precision).

5.2.7 Transmembrane potential detection

For transmembrane potential detection JC1 (Molecular Probes) staining was performed. HeLa cells were transfected with siRNA1 and siRNA2 as well as the scrambled negative control (Invitrogen). Untransfected HeLa cells were used as a reference control. Cells were washed with 1x PBS and detached from the dishes with a scraper. The cellular suspension was transferred into a 1.5 ml tube, a small sample was used for calculation of the cell number and approximately 2 x 10^5 cells were then pelleted by centrifugation at 4°C, 800 x g, 5 min. and resuspended in 500 μl of growth medium. To the control tubes 1 μl of 50 mM CCCP in DMSO was added, to the other only 1 μl of DMSO and 15 min. of incubation at 37°C followed. JC1 fluorescent dye was added to the final concentration of 2μM to CCCP or DMSO treated or untreated samples, that were vortexed vigorously and incubated at 37°C for further 15 min. Cells were pelleted by centrifugation again, washed twice with 1xPBS and resuspended in 300 μl of 1xPBS. Fluorescence activated cell sorting (FACS) analysis at 590 nm followed. Samples were analyzed on a FACS Calibur equipped with CellQuest software (Becton Dickinson).

5.2.8 Cloning procedures

Competent cells

Competent *Escherichia coli* XL10 Gold or XL1 Blue were prepared as follows: 3 ml LB medium was inoculated with the *E.coli* strain and incubated overnight at 37°C . The whole culture was diluted into the 500 ml of SOB^{++} or LB medium and incubated at 25-30°C until the measured absorbance of A_{600} reached 0.4-0.6, then it was chilled on ice for at least 10 min. The cells were spun down for 10 min, with 1500 x g, at 4°C and gently resuspended in fresh 100 ml ice-cold TB buffer. The suspension was kept on ice for the next 10 min, and a second centrifugation followed. The pellet was then resuspended in 18.6 ml of ice-cold TB buffer and 1.4 ml of DMSO was added. After this, the whole cell suspension was incubated on wet ice for at least 10 min, and was aliquoted as 600μl per each, chilled on ice. The aliquots were frozen in liquid nitrogen and stored at -80°C .

Table 5.8: Reagents for the preparation of competent *E.coli* cells

Buffer/medium	Composition
TB buffer	10 mM HEPES pH 6.7, 15 mM $CaCl_2$, 55mM $MnCl_2$, 250mM KCl
SOB^{++} medium	20g/L bacto-tryptone, 5g/L yeast extract, 0.5g/L NaCl, 0.186g/L KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, (sterile $MgCl_2$ and $MgSO_4$ have to be added just before use)
LB medium	20g/L bacto-tryptone, 5g/L yeast extract, 5g/L NaCl

Transformation of bacteria

1 μl plasmid DNA (1ng/μl) was mixed gently with 100-200 μl of competent cells. Cells were incubated at 0°C for 15-30 min and transferred to heat-block for 90s heat shock at 42°C . They were cooled down on ice for 2 min and 1 ml LB medium without antibiotics was added. The tube lid was punched with a needle and cells were incubated at 37°C , for 30-60 min, at 800 rpm shaking. Cells were spun down for 10s, resuspended in 100 μl of the LB medium and plated on LB agar plates with according antibiotics. Plasmid DNA was isolated from the *E.coli* cells with an alkaline lysis method [16] and with NucleoSpin™ columns (Machery & Nagel) following the supplier's instructions. DNA ligations were performed using DNA T4 Ligase (NEB) following the manufacture's prescriptions.

DNA sequencing

Plasmids or DNA fragments were sequenced with the ABI Big Dye Terminator Sequencing Kit (Applied Biosystems) according to established methods [129]. The fluorescently labeled DNA fragments were analyzed with an ABI Prism 3730 DNA analyzer (Applied Biosystems).

5.2.9 Preparation of the mYME1L-StrepII-8HIS expression constructs

For expression of the full length murine YME1L and its mutants, C-terminally tagged with a StepII-8HIS double tag in the Tet-inducible T-REx™ Flp-In™ (Invitrogen) system in the mammalian HEK293 cells, High Fidelity PCR (Roche) was performed with TL4000/TL4001 primers (Table 5.2, page 60), supplied with 2%DMSO for higher PCR efficiency. The product was subcloned into pGEM-T Easy (Promega) and positive clones were confirmed by sequencing with TL2601/2602 and TL2670-2677 pairs of primers Table 5.2). Mutagenesis was done with the Quick Change® XL Site-Directed Mutagenesis Kit (Stratagene) with the following primers:

- mYME1L^{K327A}: TL4017/4018;
- mYME1L^{E381Q}: TL4019/4020;
- mYME1L^{E542Q}: TL4021/4022;
- mYME1L^{E603Q}: TL4023/4024.

When the mutation regions were sequenced and confirmed, YME1L--StrepII-8HIS and *YME1L*-StrepII-8HIS were cloned into pcDNA™ 5/FRT/TO (Invitrogen) with NotI sites. The correct insertion was checked by a HindIII digest.

Generation of stable Flp-In™ T-REx™ HEK293 cell lines expressing mYME1L-StrepII-8HIS and *mYME1L-StrepII-8HIS*

For stable cell lines Flp-In™ T-REx™ HEK293 cells were transfected with 3 μg of the total plasmid DNA per well in a 6-well plate in the ratio of 1x pOG44 : 5x pcDNA5FRT/TOmYME1L construct (listed in Table. 5.4). Transfection mixture was removed after over night incubation and fresh growth medium was poured onto the cells. After 48 hours hygromycin B (150 μg/ml) was added to the cells for selection. Visible clones were picked after another 10 days; 100 clones for each transfected construct. Genomic integration was checked by β-galactosidase staining followed by immunodetection of the YME1L and *YME1L* protein in the cell lysates from non-tetracycline-induced cells versus 1μg/ml Tetracycline-induced for 24 hours cells. Clones were cultured in DMEM Gluta-Max™ -I medium supplied with 10 μg/ml of blasticidine, 50 μg/ml of hygromycin B, 1x penicillin/ streptomycin and 7.5% Tetracycline-Reduced Fetal Bovine Serum (Biochrom AG) for keeping the expression repressed. Growing conditions: 5% CO_2, 99% humidity, 37°C .

β-galactosidase activity test

Growth medium was removed from HEK293 T-REx Flp-In™ cells and they were washed once with 1x PBS and fixed with 0.2% glutaraldehyde/ 2% formaldehyde/ PBS solution for 15 min. at 4°C . After double washing with 1x PBS, β-gal Staining Buffer (1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% SDS) was added to the cells and they were incubated at 37°C for 1 hour, washed with 1x PBS twice and pictures were taken.

Immunofluorescence of the HEK293 Flp-In™ T-REx™ clones

Cells were grown in 6-well plates on the cover slides treated with 0.1% poly-lysine solution (w/v) for better adhesion. After two washings with 1x PBS cells were fixed with 3% para-formaldehyde solution in PBS for 20 min at the room temperature. Subsequently they were washed twice with 1x PBS and permeabilized with 0.15% Triton X-100 solution in PBS for 15 min at room temperature. Blocking solution [2% BSA in PBS] was added to the cells for 1h, removed and the primary antibody was added: 100 μl of antiYME1L antibody (ptglab) 1:50 in 2% BSA/PBS. Overnight incubation in the humid and dark chambers at 4°C followed. The primary antibody was washed away with the blocking buffer 3 x 10 min. Then the Alexa Fluor 488 dye-conjugated goat anti-rabbit secondary antibody (Molecular Probes) was added (1:1000 in 3% BSA/PBS with 1 μg/ml DAPI) for 2h at room temperature. Again, 3 x 10 min. washing steps followed. Cover slides were mounted with ProLong® Antifade (Invitrogen) and sealed with a transparent nail polish.

5.2.10 Isolation of mitochondria from tissue cultures

Cells were grown in the standard conditions to ∼95% confluency. To avoid unspecific degradation of the cellular proteins no trypsin was used. Cells were washed with 1x PBS, detached form the plates with cell scrap-

ers and resuspended. The isolation of mitochondria was performed according to the previously described method [50]. Protein concentration was determined by the Bradford method [22].

5.2.11 Blue-Native polyacrylamide gel electrophoresis

The gradient gels used in this study were composed of a separation gel with a linear gradient of 3-11% polyacrylamide prepared as follows: Equal volumes of 3% and 11% gel solutions were prepared and poured into the BioRad mini-gel system plates using the gradient maker. Gels were left for polymerization at 4°C , for at least 3h. Mitochondria (200 μg) were resuspended in the solubilization buffer supplemented with 1% dodecyl-maltoside (DDM) by pipeting and kept on ice without shaking for 30 min. Final protein concentration was 2μg/μl. The non solubilized material was spun down: 30 min, 4°C , and 30000 x g. The supernatant was transferred into a fresh tube and supplemented with CBB buffer and glycerol (10% final concentration). The electrophoretic separation was carried out in Mini-Protean-3-gel chambers (Bio-Rad) at 4°C with deep blue cathode buffer, using a constant voltage of 50V and a current of 15 mA for $\sim$30 min, followed by 300V and 15 mA for $\sim$30 min. Subsequently, the deep blue cathode buffer was exchanged for a cathode buffer of identical composition but lacking Coomassie blue G-250 (colorless cathode buffer) and the separation was continued at 300V and 15mA. Thioglobulin (669 kDa) and apoferritin (443 kDa) were used for calibration. Protein complexes were detected by a Commassie Coloidal Blue stain for overnight followed by destaining in Millipore water.

Table 5.9: Buffers used in BN-PAGE

Buffer	Composition
3x gel buffer	75mM Imidazole/HCl pH 7.0, 1.5M 6-aminohexanoic acid
Acrylamide / bis-acrylamide mix	48% acrylamide, 1.5% bis-acrylamide
Blue-Native sample buffer (CBB)	1% Coomassie® Brilliant Blue G250 in solubilization buffer
Solubilization buffer	30mM Tris/HCl pH 7.4, 50mM NaCl, 4mM Mg-Acetate, 5mM 6-aminohexanoic acid, 1mM ATP, 1mM PMSF, 1x Complete® protease inhibitor (Roche)
Anode buffer	25mM Imidazole/ HCl pH 7.0
Colorless cathode buffer	50mM Tricine, 7.5mM Imidazole
Deep blue cathode buffer	50mM Tricine, 7.5mM Imidazole, 0.02% Coomassie® Brilliant Blue G250 (SERVA)
3% gel solution	3% acrylamide/0.09% bis-acrylamide, 1x gel buffer, 2% glycerol, 0.003% APS, 0.0003% TEMED
11% gel solution	11% acrylamide/0.35%, 1x gel buffer, 30% glycerol, 0.003% APS, 0.0003% TEMED

Chapter 6

List of abbreviations

- AAA - ATPases associated with a variety of cellular activities
- APS - ammoniumperoxo disulfate
- ATP - adenosine triphosphate
- bp - base pairs
- CCCP - carbonyl cyanide m-chlorophenylhydrazone
- cDNA - complementary DNA
- C-terminal - carboxyterminal
- C-terminus - carboxy terminus
- DMSO - dimethyl sulfoxide
- DNA - deoxyribonucleic acid
- EDTA - ethylene diamine tetraacetic acid
- Fig. - Figure
- g - standard gravity
- GTP - guanosine triphosphate

- h - hour(s)
- HCl - hydrochloric acid
- HEPES - N-2-hydroxyethylpiperazine-N?-2-ethanesulfonic-acid
- HSP - hereditary spastic paraplegia
- K - potassium
- kb - kilobase pairs
- KCl - potassium chloride
- kDa - kilodalton
- KOH - potassium hydroxide
- m - meter
- M - molarity (mole per liter)
- MDa - megadalton
- MEF - mouse embryonic fibroblast
- μg - microgram
- μl - microliter
- mg - milligram
- ml - milliliter
- Mg - magnesium
- min - minute(s)
- mM - millimolar
- mRNA - messenger RNA

- MPP - mitochondrial processing peptidase
- mt - mitochondrial
- mtDNA - mitochondrial DNA
- MTS - mitochondrial targeting sequence
- NaCl - sodium chloride
- NADH - nicotinamide adenine dinucleotide (reduced form)
- NaOH - sodium hydroxide
- NP-40 - Nonidet P-40
- N-terminal - aminoterminal
- N-terminus - amino terminus
- OXPHOS - oxidative phosphorylation
- PAGE - polyacrylamide gel electrophoresis
- PBS - phosphate buffered saline
- PCR - polymerase chain reaction
- PMSF - phenylmethylsulphonyl fluoride
- RNA - ribonucleic acid
- RNAi - RNA interference
- ROS - reactive oxygen species
- rpm - rounds per minute
- RT - room temperature
- s - second(s)

- SDS - sodium dodecyl sulfate
- SPG - spastic paraplegia gene
- SRH - second region of homology
- TBS - Tris buffered saline
- TCA - trichloracetic acid
- TEMED - N,N,N′,N′-Tetramethylethylenediamine
- TIM - translocase of the inner membrane
- TM - transmembrane domain
- TOM - translocase of the outer membrane
- Tris - 2-amino-2-(hydroxymethyl)-1,3-propandiole
- U - unit(s)
- UPS - ubiquitin-proteasome system
- v/v - volume per volume
- V - volt
- wt - wild-type
- w/v - weight per volume

Appendixes

Appendix 1

A multisequence alignment of AAA protease subunits from bacteria, plant, yeast, mouse, rat and human is presented on Fig. 1, 2, 3 pages 82, 83, 84.

Alignment was done using the multisequence alignment software AlignX with default setting and visualized with GeneDoc. Sequences were extracted from the national center for biotechnology information (NCBI). The color code used in alignment is presented in the table below.

Table 1:

Residues	Foreground/Background
non similar	black/white
conservative	grey/black
block of similar	black/grey
identical	white/black
weakly similar	green/white

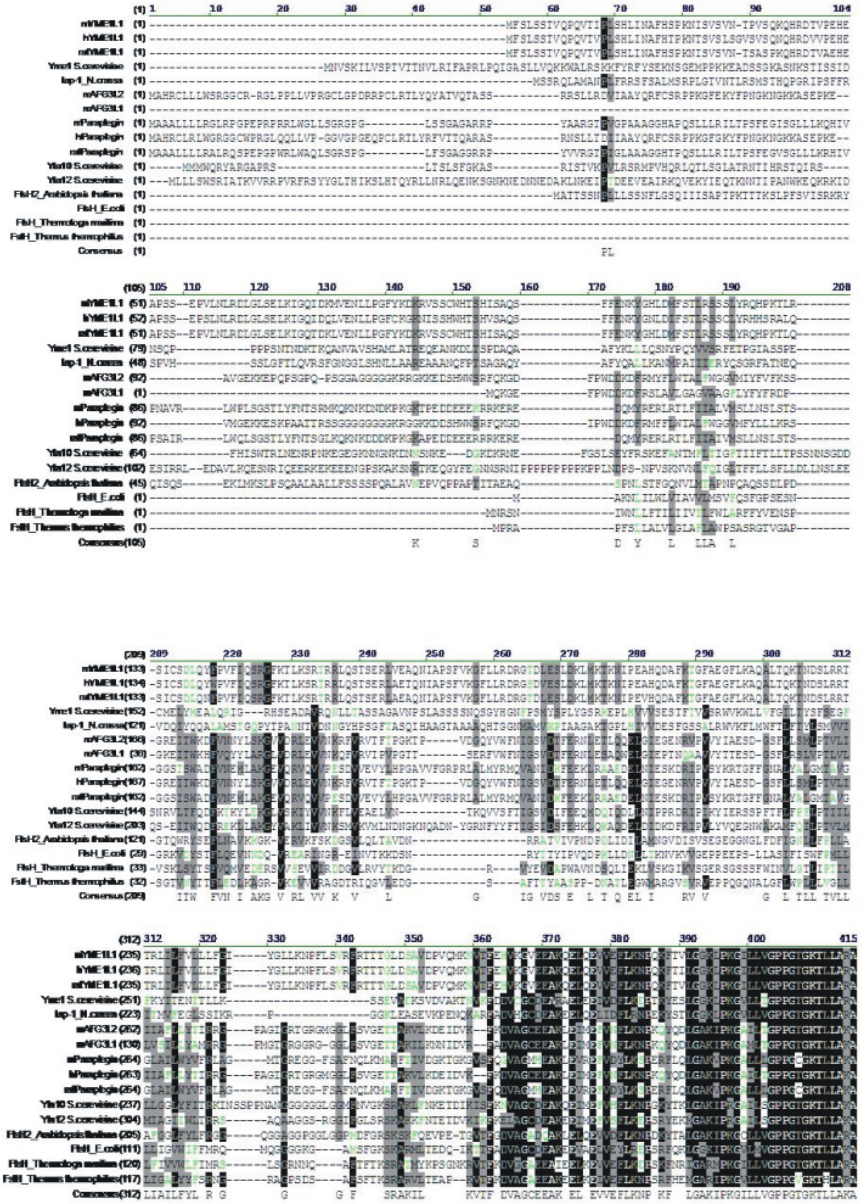

Figure 1:

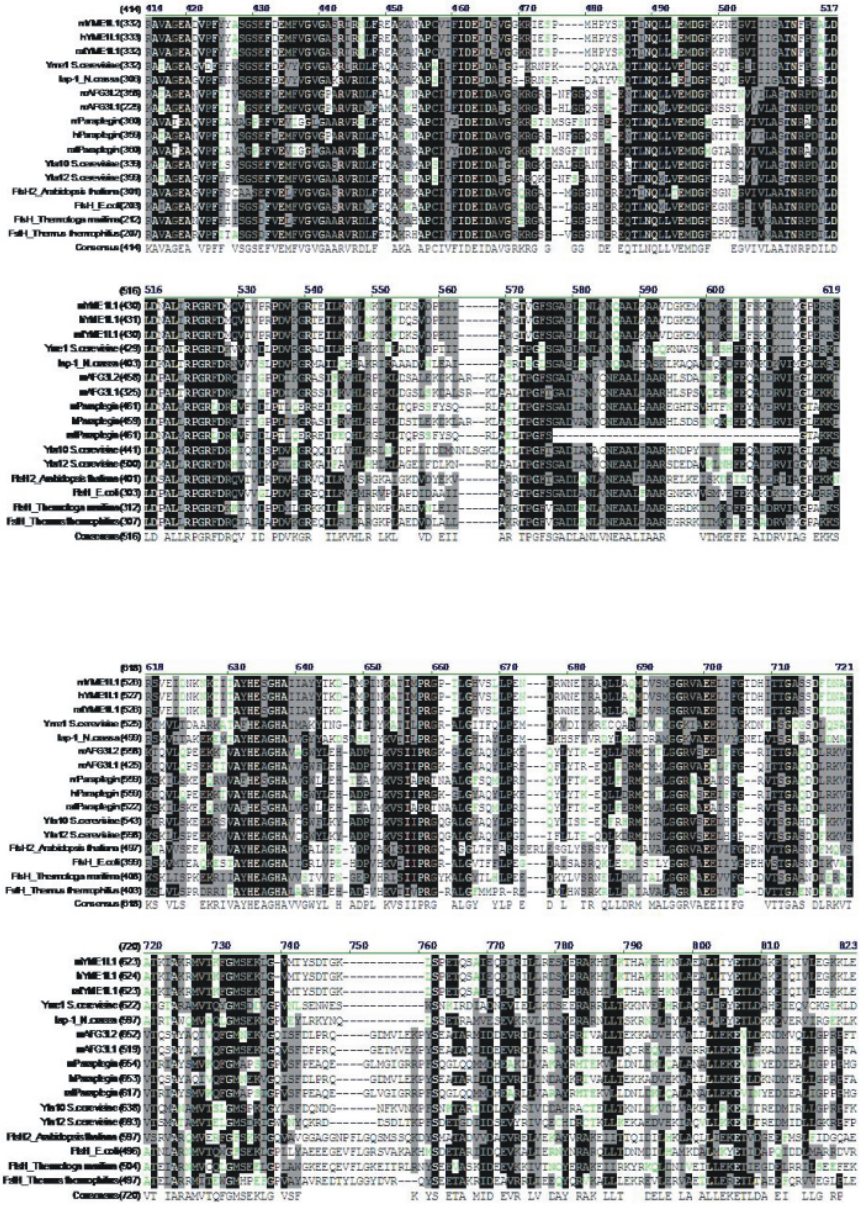

Figure 2: Continued from page 82 multisequence alignment of AAA protease subunits from bacteria, plant, yeast, mouse, rat and human

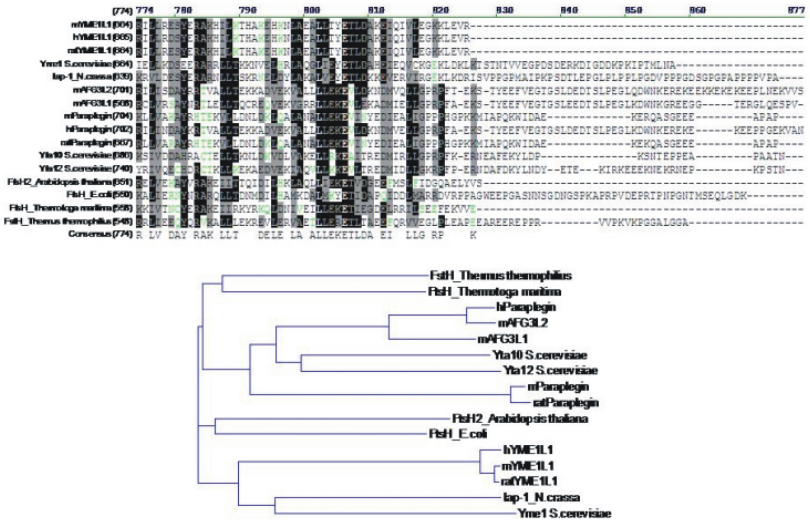

Figure 3: Continued from page 83 multisequence alignment of AAA protease subunits from bacteria, plant, yeast, mouse, rat and human

Appendix 2

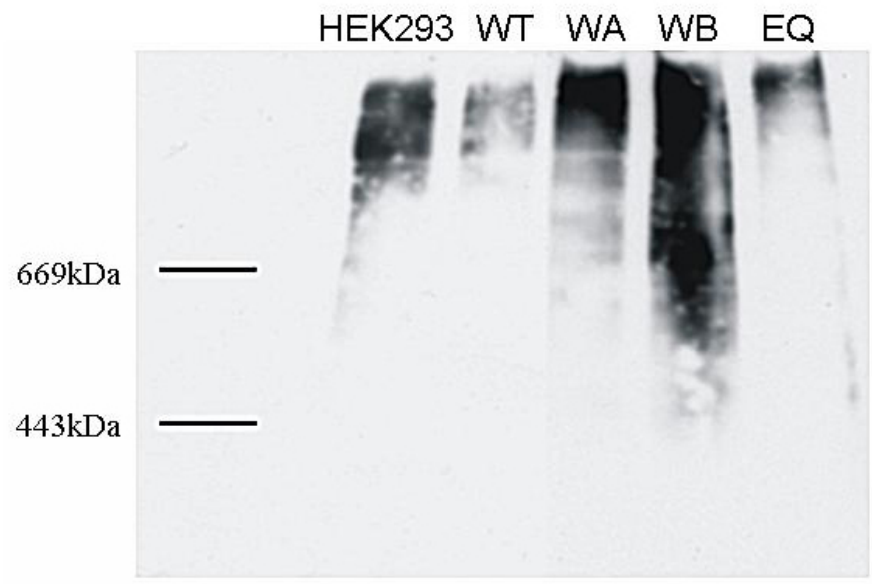

Figure 4: Assembly of the iAAA protease complexes

Mitochondria (200 μg) isolated from HEK293 Flp-In™ T-REx cells™ (lane 1) and mYME1L expression cell lines: mYME1L wild-type (WT, lane 2), mYME1L^{K327A} (WA, lane 3), mYME1L^{E381Q} (WB, lane 4), mYME1L^{E542Q} (EQ, lane 5), induced with 1 μg/ml of tetracycline for 24h were solubilized in 1% (w/v) DDM. Assembly of the YME1L into complexes was examined by Blue-Native PAGE: samples were separated on the gradient 4-11% native gel and blotted onto PVDF membrane for overnight at room temperature. Complexes were immunodetected with the specific anti-YME1L antibody (ptglab, 1:500). Thioglobulin (669kDa) and apoferritin (443kDa) serve as molecular mass references.

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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 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.

Joanna Majczak

Köln, im August 2008

Kasernenstr 5
Köln, Deutschland
+49 15114423397
+49 2212946014
j.majczak@gmx.de
polnisch
Staatsangehörigkeit

Joanna Majczak

Lebenslauf

Hochschulausbildung

2004–2008 **Doktorandin**, *International Graduate School in Genetics and Functional Genomics, Institut für Genetik der Universität zu Köln, Deutschland*.
Doktorarbeit: "Functional characterization of the mammalian iAAA protease subunit, YME1L".
Doktorvater: Prof. Dr. Thomas Langer

1999–2004 **M. Sc.**, *University of Gdańsk and Medical Academy of Gdańsk, Intercollegiate Faculty of Biotechnology*.
Vorgesetzer: prof. dr hab. Jarosław Marszałek Abschluss als Magister 26.06.2001

Schulausbildung

1987–1995 **Grundschule Tarnobrzeg, Polen**.

1995–1999 **Liceum, Tarnobrzeg, Polen**.

Sprachen

Polnish **Muttersprache**

English **Sehr gut**

Deutsch **Gut**

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