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AAA proteases in mitochondria: diverse functions of membrane-bound proteolytic machines

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Abstract

FtsH/AAA proteases comprise a distinct family of membrane-bound, ATP-dependent proteases present in eubacteria and eukaryotic cells, where they are confined to mitochondria and chloroplasts. Here, we will summarize versatile functions of AAA proteases within mitochondria, which ensure mitochondrial integrity and cell survival, acting both as quality control and processing enzymes.

Keywords: AAA+ proteins; ATP-dependent proteases; Mitochondria; Mitochondrial fusion; Neurodegeneration; Quality control of proteins

1. Introduction

Mitochondria evolved after the symbiotic engulfment of α-proteobacteria more than 1.5 billion years ago as essential eukaryotic organelles with multiple cellular functions. Compromised mitochondrial activities have serious and pleiotropic consequences and are associated with aging and many age-related diseases in humans [31]. The structural organization of mitochondria surrounded by two membranes, the presence of a mitochondrial genome and similarities in the protein and lipid composition between mitochondria and bacteria, illustrate the endosymbiotic origin of modern mitochondria [16]. This holds true also for ATP-dependent mitochondrial proteases, which without exception are homologous to bacterial protease families, including Lon-, Clp- and FtsH-like proteases.

Energy-dependent mitochondrial proteases are key components of an intraorganellar protein quality control system which protects mitochondrial activities against damage. At the same time, some of them have been shown to act as processing enzymes controlling crucial steps during mitochondrial biogenesis. ATP-dependent proteases are therefore crucial for the functional integrity of mitochondria. The inner membrane of mitochondria, the most protein-rich cellular membrane, houses the respiratory chain. As reactive oxygen species (ROS) are an inevitable byproduct of oxidative phosphorylation, inner membrane proteins are primary targets of oxidative damage. The integrity of the inner membrane is maintained by members of the membrane-bound FtsH/AAA protease family, which specifically degrade misfolded and non-assembled inner membrane proteins and thereby ensure inner membrane integrity. Here, we summarize the current knowledge of this versatile protease family, which exerts multiple functions, including proteolytic and non-proteolytic activities, in mitochondria.

2. Structure and assembly of AAA proteases

Mitochondrial AAA proteases build up homo- or hetero-oligomeric complexes in the inner membrane which are composed of highly conserved subunits. Members of this protein family are ubiquitously expressed in eubacteria and eukaryotic cells, where they localize to mitochondria and

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chloroplasts. As indicated by their name, each AAA protease subunit harbors a conserved P-loop ATPase domain of ~230 amino acids, which is characteristic of the AAA+ superfamily of ATPases (Fig. 1A) [18]. AAA+ proteins convert the energy derived from ATP hydrolysis to mechanical work and mediate substrate unfolding and remodelling of macromolecular structures, thereby controlling a variety of cellular processes. A conserved amino acid stretch present within the AAA domain, termed the second region of homology (SRH), defines AAA proteases as members of the classical AAA subclass of this superfamily [20]. The AAA domain of AAA protease subunits is followed by an M41 metallopeptidase domain. One or two hydrophobic transmembrane helices within the amino terminal region anchor each subunit to the inner membrane.

Crystal structures of eubacterial FtsH proteases suggest that mitochondrial AAA proteases, like other AAA+ proteins, represent hexameric, barrel-like assemblies, which are formed by identical or closely related subunits and which provide an internal cavity harboring the proteolytic sites [5,44]. Two classes of AAA protease complexes can be distinguished based on their topology in the inner membrane: *i*-AAA proteases expose their active sites to the *i*ntermembrane side, whereas *m*-AAA proteases face the *m*atrix space (Fig. 1B).

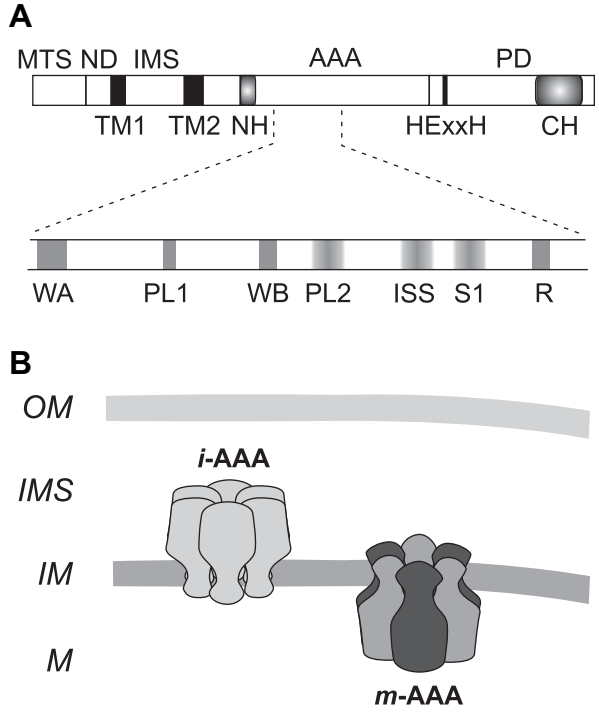

Fig. 1. AAA proteases in mitochondria. A) Domain structure and conserved elements in subunits of AAA proteases. Yeast Yta10 is depicted. MTS, mitochondrial targeting sequence; ND, N-terminal domain; TM, transmembrane helix; IMS, intermembrane space domain; AAA, AAA domain; PD, protease domain; NH, N-terminal helical substrate binding region; CH, C-terminal helical substrate binding region; WA and WB, Walker A and B motifs of P-loop ATPases; S1 and S2, sensor-1 and 2 motifs; ISS, intersubunit signalling motif; R, arginine finger; PL1 and PL2, pore loops 1 and 2. B) Topology and assembly of two distinct AAA proteases in the inner membrane of mitochondria. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; M, matrix.

The existence of two topologically distinct AAA protease complexes is conserved throughout evolution. However, the subunit composition of these complexes can vary between species and different isoenzymes of one class of AAA proteases can co-exist. Homo-oligomeric *i*-AAA protease subunits are present in yeast and most likely also in mammalian mitochondria, which are composed of Yme1 and YME1L subunits, respectively [29,49]. On the other hand, two homologous subunits, Yta10 and Yta12, assemble in hetero-oligomeric *m*-AAA protease complexes in yeast [2]. While no evidence for different assemblies exists in yeast, various isoenzymes of *m*-AAA proteases have been identified in mammalian mitochondria [26]. In humans, homo-oligomeric AFG3L2 complexes as well as hetero-oligomeric assemblies of AFG3L2 with homologous paraplegin subunits exist. The expression of a third *m*-AAA protease subunit, AFG3L1, makes the situation even more complex in mice. Both AFG3L1 and AFG3L2 can form homo-oligomeric complexes or assemble with each other or with paraplegin into hetero-oligomeric complexes. Notably, all mammalian *m*-AAA isoenzymes can functionally substitute for the yeast *m*-AAA protease upon expression in yeast, indicating that at least essential housekeeping functions of the yeast enzyme are conserved [26]. Functional differences between mammalian *m*-AAA protease isoenzymes remain to be defined. However, altering the subunit composition of *m*-AAA protease complexes may provide a means to fine-tune and adjust their activity to different physiological demands. In agreement with this possibility, tissue-specific differences in the relative expression of *m*-AAA proteases have been observed in mice [26,34].

3. Crucial roles of AAA proteases within mitochondria

The loss of AAA proteases is associated with severe and pleiotropic phenotypes (Fig. 2). Yeast cells are respiratory deficient in the absence of the *m*-AAA proteases and do not contain respiratory-chain and F_1F_O-ATP synthase complexes [1,48]. In the absence of both *m*- and *i*-AAA proteases, yeast cells are not viable [27,28]. Mutations in *m*-AAA protease subunits in mammals lead to axonal degeneration and neuronal loss [7,13,32–34]. Two distinct neurodegenerative disorders are associated with mutations in *m*-AAA protease subunits: mutations in paraplegin cause an autosomal recessive form of hereditary spastic paraplegia (HSP), while mutations of AFG3L2 have been associated recently with a dominant form of spinocerebellar ataxia (SCA28) [6–8]. Clinical phenotypes of affected patients, including deficiencies in axonal development and maintenance, are recapitulated in mouse models lacking paraplegin or AFG3L2. Loss of function of paraplegin in mice results in late-onset degeneration of long spinal and peripheral axons with accumulation of abnormal mitochondria [13]. The phenotype caused by the loss of AFG3L2 is distinct and more severe in comparison to that by the loss of paraplegin: it causes severe defects in motor axon development and premature death [32]. Phenotypic differences between mutations in paraplegin and AFG3L2 are in accordance with

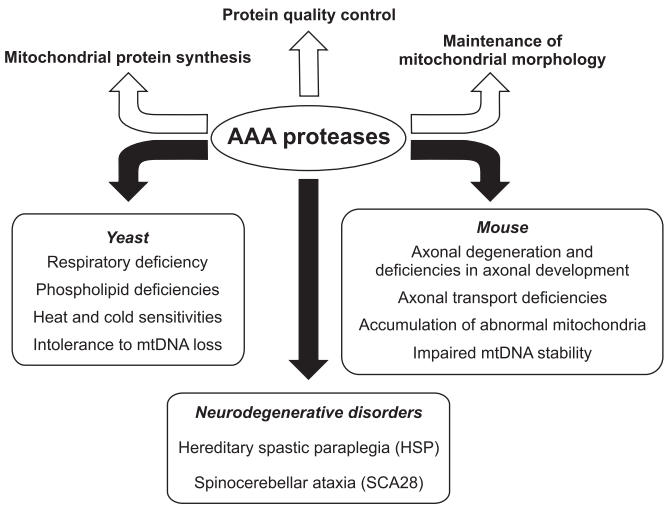

Fig. 2. Roles of AAA proteases in mitochondria. Upper panel: versatile activities of AAA proteases. Lower panel: consequences of the impaired activity of AAA proteases in yeast, humans and mice.

the presence of homo- and hetero-oligomeric *m*-AAA protease isoenzymes in brain mitochondria. Mutations in paraplegin affect only hetero-oligomeric paraplegin-AFG3L2 complexes, whereas mutations in AFG3L2 lead in addition to the loss of homo-oligomeric AFG3L2 complexes. It should also be noted that AFG3L2 is the predominant *m*-AAA protease subunit expressed in murine brain and more abundant than paraplegin and AFG3L1 [26]. Interestingly, paraplegin-deficient mice harboring reduced levels of AFG3L2 show cerebellar degeneration [34]. This suggests that functions of both *m*-AAA protease isoenzymes are at least partially redundant and that the phenotypic severity can largely be attributed to the global dosage of *m*-AAA protease activity in mitochondria.

4. Substrates of AAA proteases in mitochondria

How *m*-AAA proteases affect neuronal survival is currently not understood. Studies in yeast and mammals have linked the function of AAA proteases to an increasing number of cellular events (Fig. 2). Not surprisingly, the identification of proteolytic substrates of AAA proteases was of key importance to define these processes. Known substrates of AAA proteases, most of them identified in yeast, can be classified into three functional categories.

Non-native inner membrane proteins, which are either misfolded or non-assembled, form the first category of substrate proteins. These proteins include peripheral as well as integral inner membrane proteins, which are dislocated from the membrane bilayer by AAA proteases and selectively degraded. This group of substrate proteins includes non-assembled subunits of respiratory chain and F_1F_O-ATP synthase complexes [2,37,50]. These complexes are built up of nuclearly and mitochondrially encoded subunits; their assembly therefore depends on their coordinated expression. In case of an unbalanced expression, for instance, upon damage of mitochondrial DNA or an impaired assembly, AAA proteases degrade non-assembled subunits preventing their potentially harmful accumulation within mitochondria. However, it is presently unclear if and to what extent impaired protein quality control is responsible for pleiotropic mitochondrial defects in the absence of AAA proteases.

While misfolded or non-assembled inner membrane proteins are degraded to peptides, AAA proteases can also act as processing enzymes of specific mitochondrial proteins. Indeed, respiratory defects of *m*-AAA protease-deficient yeast cells can solely be explained by the loss of this activity [38]. The *m*-AAA protease mediates maturation of MrpL32, a subunit of mitochondrial ribosome, upon import into mitochondria. Removal of the N-terminal targeting sequence of MrpL32 by the *m*-AAA protease allows its assembly into ribosomes, a prerequisite for the synthesis of mitochondrially encoded respiratory chain subunits. Similarly, the mammalian *i*-AAA protease YME1L [17,42] and *m*-AAA isoenzymes [11,19] have been linked to the processing of the dynamin-like GTPase OPA1. Recently, AFG3L2 was shown to mediate its own maturation upon import into mitochondria in an autocatalytic manner [25]. Moreover, AFG3L2 cleaves off the N-terminal mitochondrial targeting sequence of newly imported paraplegin subunits, demonstrating that the ability to act as a processing enzyme is conserved from yeast to mammals [25].

A third and unconventional group of AAA protease substrates has been identified in yeast. The biogenesis of these substrates depends on the ATP-dependent membrane dislocation by AAA proteases only. ROS-scavenger cytochrome *c* peroxidase (Ccp1) in the intermembrane space is an example of this type of substrate [12]. The nuclear-encoded precursor form of Ccp1 is inserted into the mitochondrial inner membrane upon import and cleaved by the rhomboid protease Pcp1, an intramembrane cleaving peptidase, resulting in release of the mature form of Ccp1 into the intermembrane space. Pcp1-mediated processing depends on the *m*-AAA protease, which dislocates the precursor form of Ccp1 to the matrix side and correctly positions the processing site for Pcp1 cleavage near the membrane [45]. Membrane dislocation depends on the ATPase activity of the *m*-AAA protease, but not on its proteolytic activity [45]. Similarly, translocation across the outer membrane of mitochondria of the mammalian polynucleotide phosphorylase (PNPase) expressed in yeast requires only ATP hydrolysis but not proteolysis by the *i*-AAA protease [41].

The versatile activities of AAA proteases suggest pleiotropic effects on mitochondrial functions in AAA protease-deficient cells. Whereas the respiratory deficiency of yeast cells lacking the *m*-AAA protease can be attributed to impaired processing of MrpL32 and, concomitantly, impaired protein synthesis within mitochondria, the question of why only specific neurons are affected in mammals remains enigmatic. Specific neurons might be more sensitive to accumulating non-native polypeptides. Alternatively, processing of (a) specific substrate(s) by *m*-AAA proteases might be crucial for mitochondrial activities in these neurons. Mammalian *m*-AAA proteases cleave MrpL32 in mammalian cells, opening up the

possibility that impaired mitochondrial translation may trigger neuronal degeneration [38]. However, intrinsic activities of respiratory chain complexes are not significantly affected in *m*-AAA-deficient brain mitochondria in mice [34]. Interestingly, swollen mitochondria with aberrant cristae accumulate in affected neurons in *m*-AAA protease-deficient mouse models [13,33,34]. It is therefore conceivable that impaired processing of OPA1, regulating mitochondrial fusion and cristae morphology, leads to neurodegeneration in the absence of *m*-AAA proteases. Accordingly, *m*-AAA protease-associated diseases may represent neurological disorders caused by deficiencies in mitochondrial morphology.

5. Substrate recognition by AAA proteases

Although a number of substrates and different activities of AAA proteases have been defined, relatively little is known about how specific substrates are selected for degradation. Moreover, it is presently unclear which mechanisms ensure specific processing rather than complete proteolysis of certain proteins. Similar to other ATP-dependent proteases, no evidence for sequence-specific cleavage by AAA proteases does exist. Rather, AAA proteases have chaperone-like activities and recognize the solvent-exposed domains of membrane proteins. The chaperone activity resides within the AAA domains of AAA protease subunits, which have been demonstrated to bind unfolded model substrates in vitro [30]. N- or C-terminal tails of ~20 amino acids protruding from the membrane are sufficient to allow the proteolytic attack of a membrane protein by an AAA protease [28], which, however, can also cleave within loops of polytopic membrane proteins [21]. Interestingly, *m*- and *i*-AAA proteases have overlapping substrate specificities and it is merely the topology of a given membrane protein, i.e. the presence or absence of unfolded protein segments on one or the other membrane surface, which determines the protease(s) involved in degradation [28].

Two distinct substrate binding domains of the yeast *i*-AAA protease have been identified (Fig. 3A): C-terminal helices of the proteolytic domain (CH) and N-terminal helices of the AAA domain (NH) [15]. Whereas the NH region appears to be generally involved in proteolysis, the CH region is only required for the degradation of a subset of proteins, which initially interact with CH regions before they bind to NH regions of AAA protease subunits. The position of an unfolded domain of a substrate relative to the membrane surface is one determinant for the requirement of the CH region for proteolysis.

Substrate-specific cofactors can also affect the way substrates interact with AAA proteases. This is exemplified by yeast Cox20, which modulates degradation of non-assembled Cox2 subunits, i.e. the dependence of proteolysis on the CH region, by the *i*-AAA protease [15]. Similarly, a complex of Mgr1 and Mgr3 in the inner membrane was found to assemble with the *i*-AAA protease and to affect proteolysis of some model substrate proteins [9,10]. The Mgr1/Mgr3 complex is not required for proteolytic activity per se and can directly interact with substrate proteins independently of the *i*-AAA protease. This complex may therefore act as a substrate targeting factor. Such a function is reminiscent of adaptor proteins of prokaryotic Clp proteases which recruit specific substrates to the protease and thereby modulate substrate specificity [24]. However, Mgr1/Mgr3-specific substrates of the *i*-AAA protease in mitochondria remain to be identified.

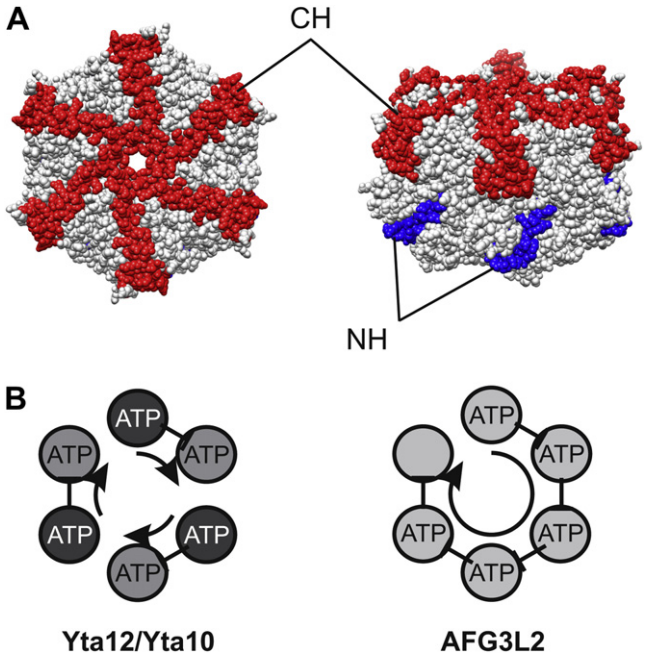

Fig. 3. A) Substrate binding domains of the *i*-AAA protease. N-terminal (NH) and C-terminal (CH) helical domains are highlighted in blue and red, respectively. Structure model of *i*-AAA protease is based on the crystal structure of *T. maritima* FtsH. Left panel: top view of the proteolytic domain; right panel, side view. NH domains are in close proximity to the membrane surface. B) Intersubunit regulation of ATPase activity by ATP binding in hetero-oligomeric yeast *m*-AAA protease complexes (left) or in homo-oligomeric human AFG3L2 complexes (right). A dimer of Yta10 and Yta12 is the functional unit in the yeast enzyme. In the AFG3L2 homoligomer, the nucleotide occupancy at a given time will determine how many subunits hydrolyze ATP coordinately. The number of ATP molecules bound to an AAA ring is speculative.

6. Substrate handling by AAA proteases

Based on the crystal structure of bacterial FtsH, both CH and NH regions are located at the surface of catalytic domains of AAA proteases in a lattice-like arrangement (Fig. 3A). Thus, substrates appear to initially interact at the outer surface of a ring-like structure before they are inserted into the central pore formed by the AAA domains and degraded within the proteolytic chamber of AAA proteases.

Substrate translocation into the proteolytic chamber of AAA proteases and proteolysis is accompanied by the extraction of membrane proteins from the hydrophobic membrane bilayer [28]. Direct evidence for this energy-dependent dislocation step mediated by an AAA protease was obtained for yeast Ccp1 [45]. It is currently poorly understood how the energy derived from ATP hydrolysis by AAA protease subunits is coupled to membrane dislocation of substrates.

However, in analogy to other AAA+ proteins, it is likely that loop regions of the AAA domain which protrude into the central pore of the predicted AAA ring complexes play a pivotal role in this process [4]. Consistently, mutations in a conserved hydrophobic amino acid residue within pore loops-1 of yeast *i*- and *m*-AAA proteases abolish proteolysis, but not substrate binding to NH and CH regions at the surface [14]. The functionality of this loop depends on its hydrophobicity, suggesting hydrophobic interactions with unfolded polypeptides in transit [14,51]. Moreover, crystal structures of bacterial FtsH suggest large conformational changes of the pore loop-1 upon ATP hydrolysis, consistent with the model that substrate translocation into the proteolytic chamber is driven by ATP-dependent conformational changes in pore loops [5,44].

A similar mode of substrate handling has been proposed for many AAA+ proteins, raising the intriguing question as to how the energy derived from ATP hydrolysis is converted into mechanical action. To address this issue, we have recently exploited the hetero-oligomeric nature of the yeast *m*-AAA protease, allowing independent mutagenesis of Yta10 and Yta12 subunits [3]. These experiments provided first evidence for an intersubunit communication between neighboring AAA protease subunits which at least partially coordinates ATP hydrolysis within the AAA ring complex (Fig. 3B). Mutations in the P-loop (Walker A motif) of Yta10 and Yta12 prevent ATP binding to the mutated subunit, but do not abolish ATP hydrolysis by the assembled AAA protease. This indicates that an individual subunit can hydrolyze ATP and function independently. Mutations in the Walker B motif of Yta12, on the other hand, trap ATP at Yta12 and block ATP hydrolysis by neighboring Yta10 subunits, demonstrating coordinated ATP hydrolysis. Intersubunit communication is conserved and occurs in a similar manner in human homo-oligomeric AFG3L2 and hetero-oligomeric AFG3L2-paraplegin complexes.

Unbiased genetic *gain-of-function* suppressor screens led to defining an intersubunit signalling pathway involving conserved AAA motifs and revealed an intimate coupling of ATPase activities to central AAA pore loops [3]. An impaired intersubunit signalling had substrate-specific consequences on proteolysis and specifically affected the turnover of substrate proteins with a higher energy demand for proteolysis, i.e. whose proteolysis involves ATP-dependent membrane dislocation. This indicates that coordinated ATP hydrolysis within the AAA ring is crucial for optimal handling of substrates, i.e. an efficient power stroke by the *m*-AAA protease. Since some of these motifs are well conserved within AAA+ proteins, intersubunit coordination within AAA+ rings might be generally required to efficiently trigger ATP-dependent conformational changes in substrate proteins.

7. Regulation of *m*-AAA protease activity by prohibitins

m-AAA proteases are present in large assemblies with prohibitins in the inner membrane of yeast mitochondria [43]. Prohibitins comprise a highly conserved protein family with two homologues, Phb1 and Phb2, ubiquitously expressed in eukaryotes [36]. Prohibitins are not required for protein degradation by AAA proteases, but appear to affect their activity. Degradation of non-assembled respiratory chain subunits by the yeast *m*-AAA protease is accelerated in the absence of prohibitins [43]. Interestingly, the bacterial AAA protease FtsH forms a similar-sized supercomplex with HflK and HflC, which are distantly related to prohibitins and affect proteolytic activity of FtsH in a similar manner [22,23]. Prohibitins may also regulate the activity of *m*-AAA protease in mammalian mitochondria. In mammalian cells, prohibitins are essential for cell proliferation and associated with multiple processes in mitochondria, including mtDNA maintenance, biogenesis of the respiratory chain and maintenance of mitochondrial morphology [36]. Depletion of prohibitins induces processing of OPA1 and leads to mitochondrial fragmentation in mammalian cells [35]. Since *m*-AAA protease is also associated with the processing of OPA1, it is conceivable that prohibitins regulate the activity of mammalian *m*-AAA proteases.

Several models currently revolve around how prohibitins regulate proteolysis by *m*-AAA proteases. Prohibitins form large ring-like complexes in the inner membrane with an outer diameter of 20–25 nm which is composed of multiple copies of Phb1 and Phb2 [46]. The formation of ring complexes is reminiscent of molecular chaperone proteins and has stimulated proposals of a chaperone activity of prohibitins. An alternative but not mutually exclusive model assigns a scaffolding function to prohibitin complexes in the inner membrane. Such a view is supported by similarities between prohibitins and a large family of distantly related membrane proteins, the SPFH family, which also includes bacterial HflK and HflC [47]. A scaffolding function of prohibitins would also be consistent with the genetic interactome of prohibitins in yeast [39]. In contrast to mammalian cells, prohibitins are dispensable for the growth of yeast cells. The systematic identification of genes, which are essential for the survival of prohibitin-deficient cells, revealed a deleterious effect of reduced levels of the non-bilayer lipids phosphatidyl ethanolamine (PE) and cardiolipin (CL) in the absence of prohibitins. Prohibitins acting as membrane scaffolds for both proteins and lipids, perhaps specifically for PE and CL, may define functional domains within the inner membrane, which are crucial for the maintenance of mitochondrial activities [40]. According to this scenario, prohibitins may recruit *m*-AAA proteases to specific membrane domains, which are characterized by a specific lipid composition and may facilitate substrate handling. While this model is consistent with the available evidence, clearly, additional experiments are required to define the regulatory role of prohibitins for the activity of *m*-AAA proteases. These studies may also shed new light on molecular mechanisms of AAA protease function and cellular processes controlled by these versatile proteolytic machines.

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