

Review Article

# Proteolytic regulation of mitochondrial oxidative phosphorylation components in plants

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Mitochondrial function relies on the homeostasis and quality control of their proteome, including components of the oxidative phosphorylation (OXPHOS) pathway that generates energy in form of ATP. OXPHOS subunits are under constant exposure to reactive oxygen species due to their oxidation-reduction activities, which consequently make them prone to oxidative damage, misfolding, and aggregation. As a result, quality control mechanisms through turnover and degradation are required for maintaining mitochondrial activity. Degradation of OXPHOS subunits can be achieved through proteomic turnover or modular degradation. In this review, we present multiple protein degradation pathways in plant mitochondria. Specifically, we focus on the intricate turnover of OXPHOS subunits, prior to protein import via cytosolic proteasomal degradation and post import and assembly via intra-mitochondrial proteolysis involving multiple AAA+ proteases. Together, these proteolytic pathways maintain the activity and homeostasis of OXPHOS components.

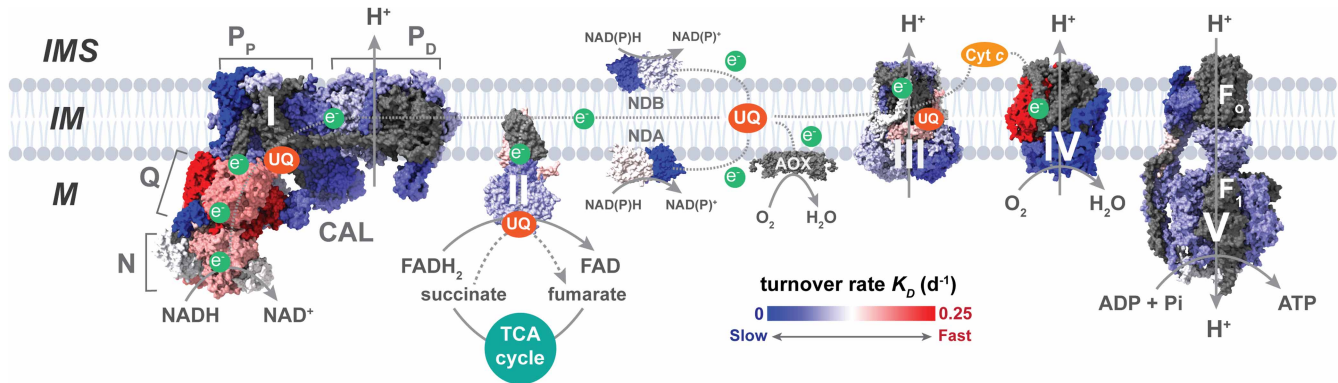
## Introduction

Mitochondria are the central hub for energy production and regulation in most eukaryotes, generating ATP as the energy source for the cell. The majority of ATPs generated by mitochondria are produced by electrochemical machinery located across the inner membrane called oxidative phosphorylation (OXPHOS). OXPHOS utilises proton gradients generated across the inner membrane to produce ATP from ADP and inorganic phosphate. The proton gradient is generated by the electron transfer chain (ETC) consisting of multi-subunit complexes: Complex I (NADH-ubiquinone oxidoreductase); Complex II (succinate-ubiquinone oxidoreductase); Complex III (ubiquinone-cytochrome *c* oxidoreductase); and Complex IV (cytochrome *c* oxidase). Apart from Complex II, which is also involved in the tricarboxylic acid (TCA) cycle, all other ETC complexes pump protons across the inner membrane (IM) from the matrix to the intermembrane space (IMS), forming a supercomplex known as a respirasome [1–3]. Electrons generated from redox reactions by ETC complexes are transferred by mobile electron carriers, such as ubiquinone (UQ) and cytochrome *c* (Cyt *c*). Electrons carried by Cyt *c* are accepted by oxygen at Complex IV, generating water in the process. Proton gradients generated by the translocation of protons powers Complex V (ATP synthase) to synthesise ATP from ADP and inorganic phosphate (Figure 1). In addition to the classical ETC pathway, plant mitochondria also possess alternative electron carrier pathways. Type II NAD(P)H dehydrogenases (NDs) such as NDA, NDB, and NDC can bypass the ETC by generating electrons from NAD(P)H to UQ, while the alternative oxidase (AOX) can transfer electrons from the UQ to oxygen, therefore bypassing Complex III and Complex IV activity (Figure 1).

As an endosymbiotic organelle, the mitochondrion contain its own genome (mitogenome), although it has limited coding capacity and most proteins needed for mitochondrial biogenesis and activity are encoded in the nucleus. In plants, such as the model flowering plant *Arabidopsis thaliana* (*Arabidopsis*), the mitogenome encodes for *in organello* protein translation machinery such as mitoribosome subunits and tRNAs, and several core OXPHOS subunits [4–6]. Therefore, the majority of

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**Figure 1. Oxidative phosphorylation (OXPHOS) pathway in plant mitochondria.**

The electron transfer chain (ETC) consisting of Complex I–IV contribute in generating electron flows ( $e^-$ ) necessary for proton ( $H^+$ ) pumping from the matrix (M) to the intermembrane space (IMS) across the inner membrane (IM). Complex I modules are indicated: NADH-binding (N), ubiquinone-binding (Q), carbonic anhydrase-like (CAL), proximal proton-pumping ( $P_p$ ), and distal proton-pumping ( $P_d$ ). Electrons are carried by mobile electron carriers such as ubiquinone (UQ) and cytochrome *c* (Cyt *c*). Plant mitochondria also contain alternative ETC components, such as type II NAD(P)H dehydrogenases NDA and NDB, and alternative oxidase (AOX). Proton gradient is then used by the ATP synthase to drive ATP synthesis. Topographical heatmap depicting turnover rate of individual subunits (per day,  $d^{-1}$ ) in *Arabidopsis thaliana* Columbia-0 ecotype (wild-type) seedlings, from relatively slow (blue) to relatively fast (red). Grey regions represent subunits that were unreliably detected. Data were retrieved from Li et al. [31].

OXPHOS components are encoded in the nucleus, translated in the cytosolic ribosome, and imported into mitochondria via various translocation pathways [7]. Consequently, the assembly of OXPHOS complexes requires intricate coordination of protein synthesis, translocation, assembly, and subsequently the maintenance of assembled subunits, a research focus that has emerged in the past 10–15 years. Previous biochemical studies and structural elucidations from various systems demonstrate that OXPHOS complexes assembly is a modular process, involving the stepwise acquisition of subunits to build modules, intermediary complexes, and the holo-complex [8–16].

The modularity of OXPHOS complexes assembly would suggest that a similar stepwise disassembly mechanism exists for quality control. Quality control and degradation of the whole mitoproteome is achieved via mitochondrial autophagy (mitophagy) [17] and to a smaller extent, the formation of mitochondria-derived vesicles (MDV) [18]. Furthermore, there are examples whereby proteins that are imported into mitochondria and misfolded, specifically IM-targeted OXPHOS subunits, can be retro-translocated back to the cytosol to be degraded by ubiquitin-proteasome system [19,20]. In most occasions however, protein homeostasis (proteostasis) and turnover of mitochondrial proteins including OXPHOS components is regulated by intra-mitochondrial proteolysis.

As endosymbiotic organelles, mitochondria contain various internal proteases derived from bacterial ancestors. Most of these proteases belong to the AAA+ (ATPases-Associated with diverse cellular Activity) protein superfamily that is highly conserved across life domains and implicated in functions, ranging from remodelling of cellular membrane and cytoskeleton through protein disassembly [21,22], DNA unwinding [23,24], to proteolysis [25–27] and chaperone [28–30] functions. Recent studies in plants has highlighted the importance of AAA+ proteases in maintaining the mitoproteome, and in particular the OXPHOS complexes. Here, we present recent studies highlighting the proteolytic regulation of OXPHOS complexes in plants, with a focus on the regulation of OXPHOS subunits by proteolytic degradation.

## Regulation of OXPHOS complexes turnover

As the primary contributor to reduction-oxidation (redox) reactions, electron transfer, and the production of reactive oxygen species (ROS), OXPHOS complexes are prone to oxidative damage. This results in a higher turnover rate of OXPHOS subunits compared with the rest of mitoproteome, including proteins involved in protein synthesis and the tricarboxylic acid (TCA) cycle [31]. Furthermore, individual subunits of OXPHOS

complexes were observed to exhibit different turnover rates, which suggests a specific degradation mechanism, especially for dysfunctional and damaged subunits.

Individual proteins in the mitoproteome have varied turnover rates depending on their intra-organellar location and function. Even amongst the OXPHOS complexes, turnover rate of individual subunits are highly varied. Protein labelling studies in Arabidopsis indicated that several OXPHOS subunits within the matrix-facing modules of Complex I: NADH-binding (N) and ubiquinone-binding (Q) modules have relatively faster turnover rates, ranging from 1.5–3-fold compared with the average turnover rate of their membrane-bound counterparts (Figure 1) [31,32]. This has similarly been observed in mouse and human cell lines, where Q-module subunits show relatively higher turnover rates compared with the rest of the complex subunits [33]. Interestingly, subunits assembled within intermediates display a higher degree of turnover compared with subunits within the holocomplex [31,34], suggesting a targeted regulation of unassembled subunits.

Regulation of protein turnover can be achieved by different spatiotemporal pathways: (a) pre-import in the cytosol, by the ubiquitin-proteasome system; (b) during import, through retro-translocation or by immediate proteolytic control; and (c) post-import, through the formation of degradation vesicles and whole mitochondrial autophagy (mitophagy), or through proteolysis by intra-mitochondrial proteases.

## Cytosolic degradation of mitochondrial proteins

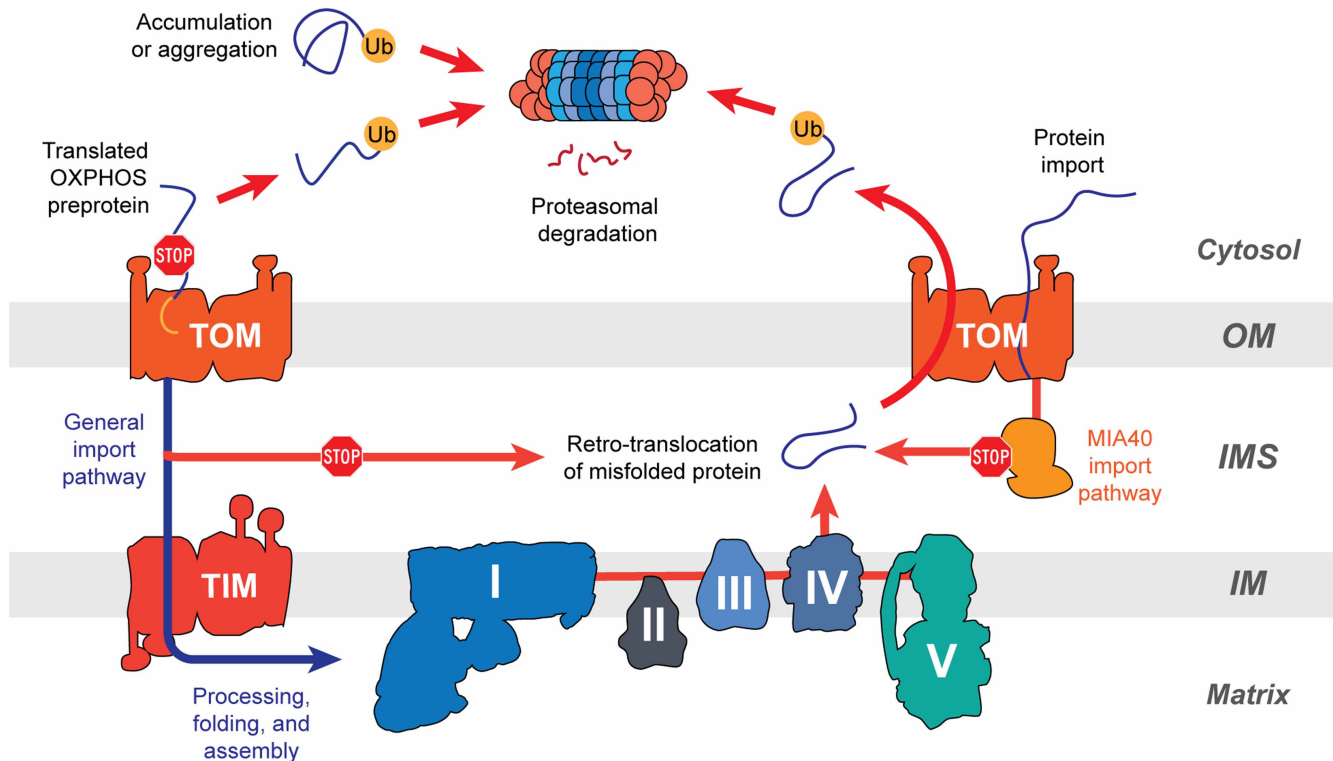
Nuclear-encoded mitochondrial proteins can be degraded by cytosolic proteasomes prior to import into the organelle. The degradation is mainly following protein import failure, resulting from a decrease in mitochondrial membrane potential, low ATP levels, or the misfolding of mitochondrial precursor proteins [35,36]. Protein import failure can also result from the blockage and disruption of the translocase of outer membrane (TOM) complex, the main protein import pore of the outer membrane [35,37,38]. Failure of protein import into mitochondria can lead to protein accumulation that triggers aggregation in the cytosol [39], therefore degradation of misfolded and aggregated proteins is essential to maintain cellular homeostasis [20,40,41]. However, this mechanism is not restricted to OXPHOS components but applies to most mitochondrial proteins, triggered by protein import failure rather than dysfunctional OXPHOS components.

Retro-translocation of inner membrane imported proteins and degradation by the cytosolic ubiquitin-proteasome system has been observed for uncoupling proteins (UCP) [42] (Figure 2). Studies in murine cells showed that knockout of ubiquitylation and inhibition of cytosolic proteasome resulted in accumulation and slower turnover rate of OM-located UCP2 and UCP3, indicating that these proteins are subjected to proteasomal degradation [42–44]. Further analysis proposed that a linker protein recruits and attaches proteasomes to the membrane surface, which recognises ubiquitylated proteins for degradation [42].

Protein retro-translocation followed by proteasomal degradation has also been observed for proteins translocated via mitochondrial intermembrane space import and assembly (MIA40) pathway [45]. The MIA40 protein import pathway assists the import of IMS-located twin-cysteine containing proteins [7]. In mammals, IM-located OXPHOS subunits have been characterised as MIA40 substrates, including the Complex I subunits NDUFB10 (PDSW) [46], NDUFA8 (PGIV) [47], NDUFS5 (15 kD), and NAD9 [48], in addition to a variety of Complex IV subunits and assembly factors [49–51]. However, since MIA40 import pathway has limited substrates, the cytosolic degradation of OXPHOS subunits may only play a minor role in overall OXPHOS subunit turnover.

## Whole proteomic degradation: mitochondria-derived vesicles and mitophagy

Mitochondria are capable of forming cargo vesicles dedicated to exporting damaged proteins to the peroxisomes and lysosomes (extensively reviewed in [18]). This mechanism is generally used as the last resort in degrading proteins that are unable to be degraded by mitochondrial proteases, such as heavily misfolded and aggregated proteins. In plants, the formation of mitochondria-derived vesicles (MDV) increased following enhanced oxidative stresses, such as prolonged dark photoperiod and senescence [52]. In mammals, MDV has also been implicated in the specific degradation of OXPHOS complex proteins. Inhibition of Complex III with antimycin A (AA) triggered ROS production and the formation of MDV enriched with the Complex III subunit UQCRC2 [53] for degradation by lysosomes [54]. ROS accumulation can also trigger the oxidation and curvature of the mitochondrial membrane cardiolipin, leading to the formation of MDV [18]. MDV is also required to degrade severely aggregated proteins that simply cannot be degraded by mitochondrial AAA+



**Figure 2. Cytosolic degradation of nuclear-encoded OXPHOS proteins.**

Failure of OXPHOS components to import is caused by protein accumulation and aggregation in the cytosol or by disruption and blockage of translocase of outer membrane (TOM) transporter which triggers ubiquitination (Ub), leading to proteasomal degradation in the cytosol. Cytosolic degradation may also occur to retro-translocated OXPHOS subunits. Defects in the import and processing of proteins by the intermembrane space MIA40 pathway triggers the retro-translocation of substrates back to the cytosol, rendering the protein prone to ubiquitination and proteasomal degradation.

proteases such as LON and CLPXP [55,56]. This suggests that MDV formation is essential for maintaining quality of mitochondrial proteins in a specific and a smaller scale, particularly under oxidative stress exposure.

In contrast with MDV, mitophagy is a process involving the degradation of whole mitochondria. Mitophagy selectively occurs against severely damaged mitochondria to salvage mitochondrial components in response to severe stress conditions [17]. In plants, mitophagy was widely reported to involve autophagy-related (ATG) proteins, where stress signals received by the Target of Rapamycin (TOR) pathway triggers post-translational modifications (PTMs) such as ubiquitylation, acetylation, and phosphorylation to ATG proteins [57,58]. These PTMs recruit ATG proteins to the damaged mitochondria to form autophagosomes, for transport to the vacuole for degradation [17]. Similar to MDV, mitophagy also appears to be induced with increased oxidative stress in mitochondria. Inhibition of Complex III by AA and myxothiazol or Complex I inhibition by potassium cyanide (KCN) triggered mitophagy [59]. However, in these cases it is likely that mitophagy was triggered by ROS accumulation, rather than in response to OXPHOS inhibition or damaged subunits [17,60].

## Rapid degradation of nascent subunits

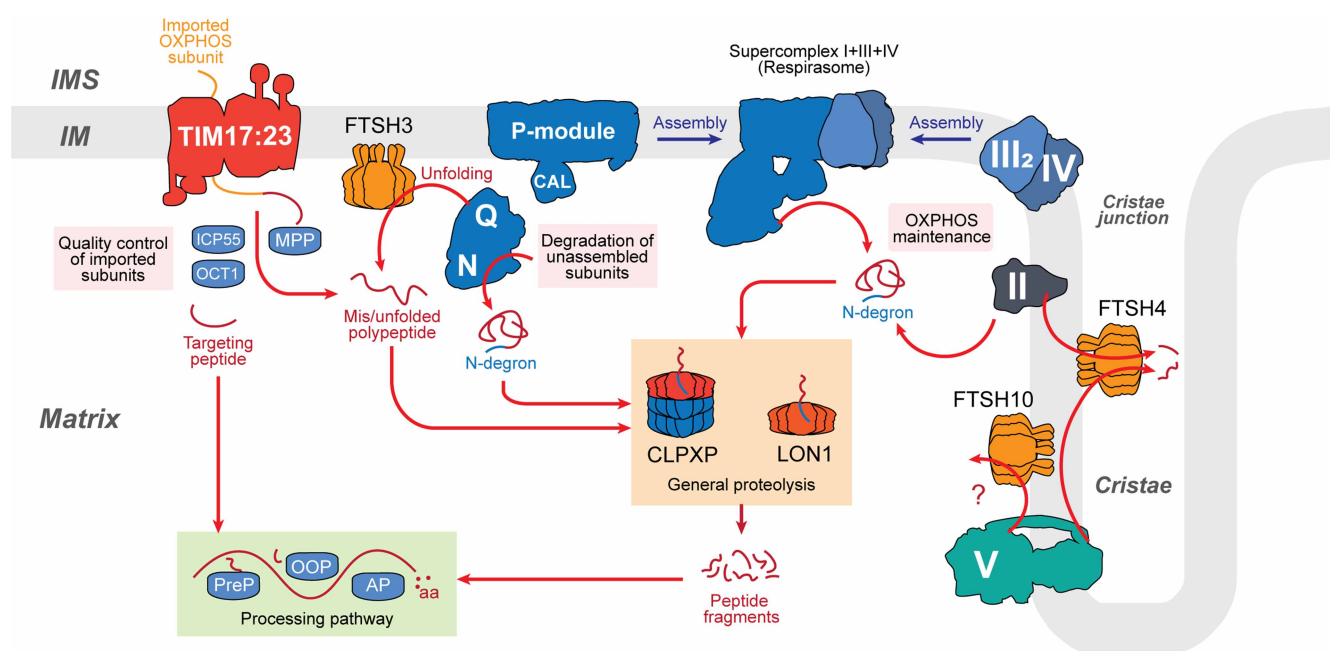
Following successful import into mitochondria, cytosolically synthesised OXPHOS precursor proteins undergo additional processing, mainly to remove N-terminal targeting peptide and unstable residues. Targeting peptides are initially cleaved by the mitochondrial processing peptidase (MPP), which in plants, constitutes the cytochrome *bc<sub>1</sub>* complex (Complex III) [61–63]. Additional removal of unstable residue(s) is carried out by the intermediate cleavage peptidase (ICP55) and octapeptidyl peptidase (OCT1) [64–68]. Failure of correct processing results in misfolding and degradation following the N-end rule, whereby certain N-terminal amino acids destabilise the protein and trigger protein degradation [69]. In animals, degradation of misfolded precursors is assisted by the intra-mitochondrial AAA+ proteases such as LONP1 and CLPXP systems [70].



Precursor processing generates free peptides that are further degraded by the downstream processing pathways (Figure 3) [71–76]. In this process, peptide fragments are sequentially degraded into smaller fragments by the presequence peptidase (PreP) [77] and organellar oligopeptidase (OOP) [71]. Single amino acids are finally recovered from oligopeptide fragments by multiple residue-specific aminopeptidases (AP) (Figure 3) [73,74,76,78].

## Intra-mitochondrial proteolytic degradation by AAA+ proteases

Plant mitochondria harbour a wide array of both ATP-dependent (AAA+) and ATP-independent proteases that mainly belong to the serine protease family such as degradation of periplasmic protein (DEG) [79] and rhomboid-like proteases (RBL) [80,81]. However, recent studies have highlighted the importance of AAA+ proteases in the disassembly, unfolding, and degradation of OXPHOS subunits. Plant mitochondrial AAA+ proteases are classified into three major classes: AAA or FTSH (Filamentous Temperature Sensitive H) proteases, LON (long filamentous phenotype) protease and CLPXP (caseinolytic protease) machinery. Substrate recognition and specificity depends on their intra-mitochondrial location with IM-bound AAA proteases being either intermembrane space (IMS)-facing (*i*-AAA) or matrix-facing (*m*-AAA). On the other hand, matrix-located proteins are degraded by the soluble matrix-located AAA+ proteases: LON and CLPXP (Figure 3).



**Figure 3. Working model of OXPHOS proteolytic regulation in plant mitochondria.**

**Quality control of imported subunits.** Nuclear-encoded OXPHOS subunits imported through translocase of inner membrane TIM17:23 complex are subjected to N-terminal processing by mitochondrial processing peptidase (MPP), octapeptidyl peptidase (OCT1), and intermediate cleavage peptidase (ICP55). Improper processing of imported subunits causes misfolding leading to immediate proteolysis. **Degradation of unassembled subunits.** Accumulation of improperly assembled or orphaned OXPHOS modules (for example Complex I NQ-modules) may lead to unfolding by *m*-AAA proteases (FTSH3 and FTSH10) or N-degradation signal (N-degron) tagging followed by proteolysis. Degradation of accumulated subunits is also necessary for proper assembly of OXPHOS complexes. **OXPHOS maintenance.** Damaged OXPHOS subunits can be degraded and replaced in a modular fashion through the disassembly and unfolding by *m*-AAA (FTSH3/FTSH10) or *i*-AAA proteases (FTSH4), followed by N-degron tagging and proteolysis, although the regulation of Complex V by FTSH10 remains unclear. **General proteolysis.** Degradation of unfolded polypeptides, damaged subunits, and N-degron tagged proteins is generally carried out by mitochondrial matrix proteases LON1 and CLPXP. **Processing pathway.** Peptides generated from preprotein processing and general proteolysis are further degraded into amino acids by a series of peptidases, including presequence peptidase (PreP), organellar oligopeptidase (OOP), and aminopeptidases (AP).

All AAA+ proteases share common structural features and mechanisms that have been reviewed extensively [24,82,83]. Recent cryogenic electron microscopy (cryo-EM) structural determinations of AAA+ proteases further expand our understanding on their mechanistic regulation [25–27,84–86]. AAA+ proteases typically consist of: (1) AAA or ATPase domain that uses ATP to mechanically unfold polypeptide substrates and translocate it to (2) the proteolytic domain, which degrade unfolded polypeptide into smaller peptide fragments (Figure 4). Substrate recognition by the ATPase domain can be mediated by a PTM N-degradation signal (N-degron) tagging and providing an accessible peptide for recognition and binding to start the unfolding process [87,88]. For substrate unfolding, the ATPase domain is mechanically active when forming a multimeric ring (typically a hexamer) with a staircase-like structure (Figure 4). The distinct structural conformation of each ATPase monomer is formed by different binding states with nucleotides (ATP/ADP) mediated by Walker A and B motifs (Figure 4) [25–27,82]. The ATP hydrolysis-driven conformational change of ATPase progressively propels the translocation of polypeptide into the multimeric proteolytic domain, which typically contains either zinc ion (Zn<sup>2+</sup>) cofactor or catalytic serine for peptide degradation [25,84]. In AAA/FTSH and LON proteases, both ATPase and protease domains are encoded by the same gene, which are active as hexamers. In contrast, CLXP proteolytic machinery consist of hexameric CLPX as ATPase and double-heptameric ring CLPP protease (Figure 4).

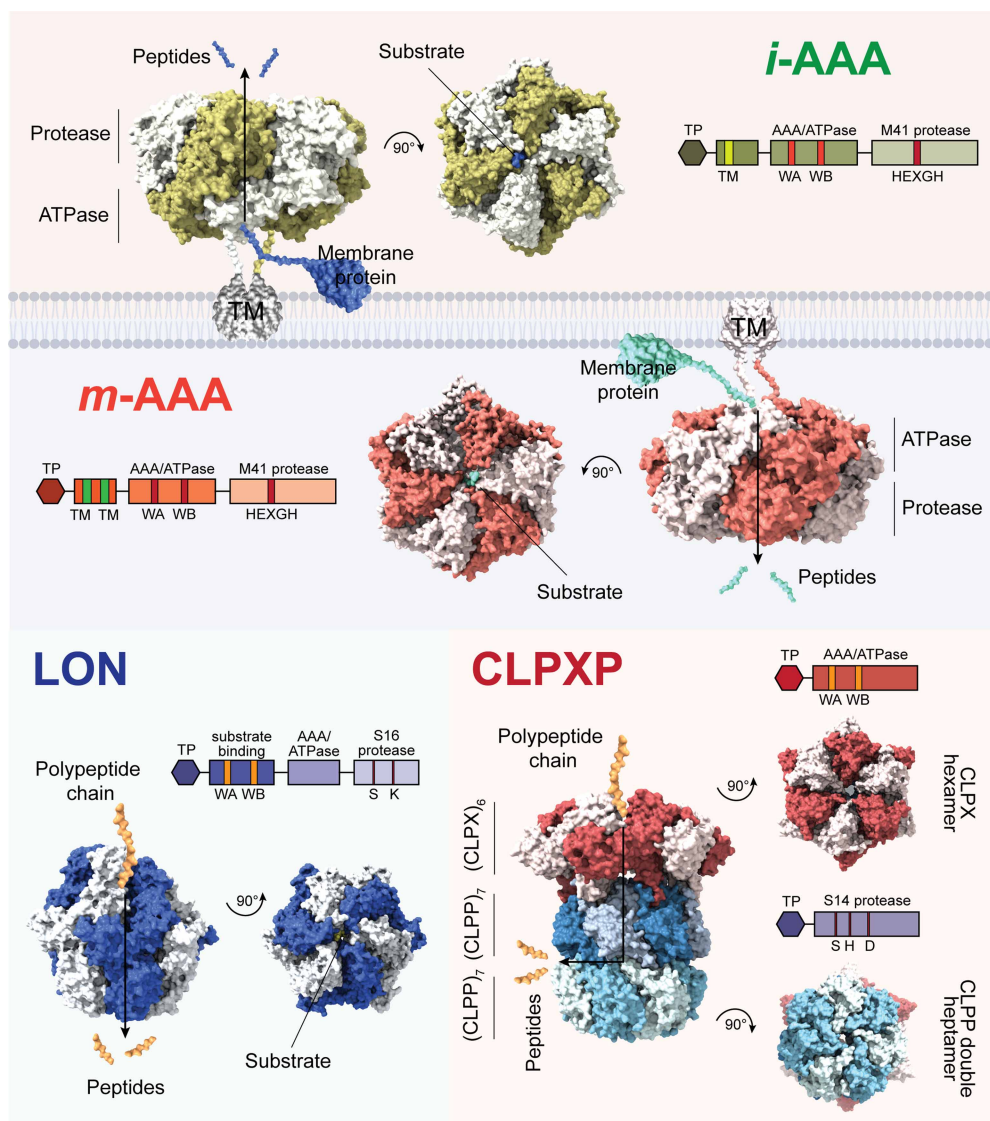
## AAA/FTSH proteases

AAA proteases are membrane-bound AAA+ proteases in which both of its ATPase and protease domains are encoded by the same gene and translated into a single polypeptide chain (Figure 4). *i*-AAA proteases typically contain one transmembrane (TM) domain homologous to yeast YME1 and human YME1L [27,75,87], whereas *m*-AAA proteases contain two TM domain homologous to human AFG3L2 [26] (Figure 4). The Arabidopsis genome encodes four mitochondrial AAA proteases: FTSH3 and FTSH10, which are *m*-AAA proteases that can form either homo-hexamer or FTSH3/10 hetero-hexamer [89,90]; and FTSH4 and FTSH11, *i*-AAA proteases found to be exclusively homo-hexameric [69]. Typically, all FTSH proteolytic domains contains a Zn-binding HEXGH conserved domain and are categorised as belonging to the M41 metalloprotease family [27,91].

Recent studies have begun to decipher the intricate role of FTSH proteases play in the proteolytic regulation of OXPHOS complexes and pathway. In Arabidopsis loss of function mutants for either *m*-AAA proteases FTSH3 or FTSH10 had no observable effect to plant growth under both normal laboratory and field conditions [91–94], although the abundance and activity of Complex I and V were reduced, which implicates their role in the assembly and homeostasis of these complexes [92]. Moreover, the effects were even more pronounced in the double knockout of *ftsh3/ftsh10*, as it displayed a decrease in the abundance and activity of Complex I, supercomplex I + III, and Complex V, which consequently resulted in delayed plant growth [94]. However, the mechanistic role of FTSH10 in the regulation of Complex I or V remains unclear and warrants further investigations (Figure 3).

Whilst no direct impact could be observed to plant growth under native physiological conditions, FTSH3 was reported to play a role in the restoration of plant growth in Complex I defective mutants [91]. A forward genetics approach using a knockdown of the *Complex I assembly factor-1* (*ciaf1*) generated a revertant mutant with restored plant growth and Complex I activity [8]. CIAF1 is a LYR domain-containing assembly factor proposed to be involved in iron-sulfur cluster recruitment for Complex I matrix arm domain subunits [8]. The *ciaf1* mutant was previously characterised to have a defective Complex I lacking the matrix arm domain (NQ-module), resulting in reduced overall Complex I activity and subsequently plant growth [8]. The revertant mutant of *ciaf1* contains a point mutation within the FTSH3 ATPase domain, resulting in increased Complex I activity and abundance [91]. Proteomics analyses of this line further revealed that the mutation partially restored the abundance of NQ-module subunits, suggesting the activity of FTSH3 ATPase domain in NQ-module disassembly and overall FTSH3 role in maintaining Complex I homeostasis (Figure 3) [91].

In contrast with FTSH3, a loss-of-function knockout of FTSH4 exhibits a severe growth phenotype when plants are grown under mild heat stress (30°C) conditions, exhibiting increased ROS production, subsequently accumulated carbonylated proteins and decreased ATP synthesis [95]. Furthermore, a decreased abundance of Complex V subunits was observed, indicating the role of FTSH4 in maintaining Complex V stability and activity under stress conditions [95]. FTSH4 has also been reported to be involved in Complex I maintenance under long-term heat stress. During heat stress, mitochondrial proteins are prone to misfold and aggregate, and the lack of FTSH4 reduced the capacity to degrade aggregated proteins [95]. A proteomics study on the FTSH4



**Figure 4. Schematic illustration of the activity and mechanisms of mitochondrial ATPase-associated with diverse activities (AAA+) proteases as inferred from structural studies.**

*i*-AAA (IMS-facing AAA protease): yeast YME1 (PDB: 6AZ0) [27] homologous to plant FTSH4/11 proteases; *m*-AAA (IMS-facing AAA protease): human AFG3L2 (PDB: 6NYY) [26] homologous to plant FTSH3/10 proteases; LON (long filamentous phenotype protease): *Yersinia pestis* Lon (PDB: 6ON2) [25] homologous to plant LON1; CLPXP (caseinolytic protease system): *Neisseria meningitidis* CLPXP (PDB: 6VFX) [86] homologous to plant CLPXP protease. For each AAA+ proteases, domains are coloured and indicated in linear diagram, with specific features, motifs, and catalytic residues highlighted. Generally, all AAA+ protease sequences contain cleavable targeting peptide (TP) and compose of ATPase that has Walker A and B (WA/WB) nucleotide binding motifs and proteolytic domain. Membrane-embedded *i*-AAA and *m*-AAA contain transmembrane domain (TM) and HEXGH zinc-binding motif within the catalytic site. On the other hand, LON and CLP proteases contain SK dyad and SDH triad as catalytic residues, respectively. Substrate is recognised by ATPase domain and unfolded as the multimeric structure is rearranged upon ATP binding and hydrolysis. The unfolded polypeptide is then translocated into the proteolytic chamber within protease domain for degradation into peptide fragments.

knockout indicated that Complex I matrix arm subunits such as Nad9 and PSST, and membrane-bound MNLL and B14.7 were found as aggregates, hinting their possibility as FTSH4 substrates [96]. FTSH4 activity was also reported to be involved in succinate dehydrogenase/SDH (Complex II) regulation based on multiple

reaction monitoring (MRM) of heavy nitrogen-labelled ( $^{15}\text{N}$ ) peptides [97]. Trapping approach of FTSH4 further affirms its role in Complex II regulation as two of its subunits SDH1 and SDH5 were found to be FTSH4 binding partners [98]. FTSH4 proteolytic activity was also proposed to be required for the degradation of a seed-specific SDH2 paralogue SDH2-3 (Figure 3), whilst its chaperone activity was needed for folding and assembly of the other two paralogues (SDH2-1 and 2) [97].

## LON protease

Similar to FTSH, LON proteases also contain ATPase and proteolytic domains. Cryo-EM structure of the LON protease from bacterium *Yersinia pestis* revealed a spiral staircase-like substrate translocation mechanism conserved in other AAA+ proteases (Figure 4) [25]. LON is a matrix soluble protease belonging to the S16 serine protease family, containing Ser-Lys dyad catalytic residues (Figure 4) [75,99]. In *Escherichia coli*, putative LON substrates have been identified using stable isotope labelling with amino acids in cell culture (SILAC) and protease trapping [100]. The potential substrates identified include enzymes involved in amino acid biosynthesis, sugar and fatty acid metabolism, in addition to the Complex I subunit NuoB [100], a homologue of the Arabidopsis Complex I subunit PSST [16]. In animal systems, a LON-like protease LONP1 has also been identified to play a role in the degradation of inner membrane protein import components and mitogenome regulatory modulators such as mitochondrial transcription factor A (TFAM), and OXPHOS subunits [70,101–103]. Moreover, a recent study in human cell lines highlighted the independent chaperone function of LONP1 to assist in the folding and maturation of a subset of mitochondrial proteins, including OXA1L, an OXPHOS assembly factor, and NDUFA9, a Complex I Q-module subunit [104]. Interestingly, human LONP1 was also observed to influence the availability and function of the CLPXP system, as it was required for the maturation and degradation of CLPX [70], while its knockdown triggered aggregation and reduction in CLPP abundance [105].

In plants, two LON homologues are known to be mitochondrial, LON1 which is exclusively mitochondrial-targeted [99], and LON4 which is dual-targeted to mitochondria and chloroplasts [106]. LON4 has a relatively lower expression compared with LON1, and its function is yet to be determined [107], whereas LON1 has been widely characterised in plants. Loss-of-function *lon1* mutants exhibited delayed plant growth, reduced succinate oxidation and cytochrome *c* oxidation, related to Complex II and IV activity, respectively [99]. Moreover, the activity of several TCA cycle key enzymes were also significantly reduced [99] corresponding to a decrease in the abundance of TCA cycle enzymes [108]. Similar to animal LONP1, plant LON1 acts as general protease that degrades OXPHOS aggregates, while also independently acting as chaperone to assist folding of newly imported proteins [31]. Although further experimental verification is needed, these studies suggest the plant LON1 protease may play a role in general proteolysis of matrix proteins and as a chaperone that maintains the homeostasis of matrix-facing membrane-anchored subunits (Figure 3).

## CLPXP proteolytic machinery

CLPXP is the only multi-heteromeric mitochondrial AAA+ protease that arguably has the most distinctive structural features and activity mechanisms. Consisting of stacks of multimeric complexes, the ATPase and protease domains are encoded by different genes. In plants, CLPXP is composed of a CLPX hexamer as the ATPase, and double-stacked CLPP heptamer (tetradecamer) as the proteolytic domain (Figure 4) [109,110]. Sequential hydrolysis of ATP in CLPX allows the unfolded substrate to be directionally translocated to the symmetric CLPP proteolytic chamber for degradation [84–86]. CLPP was able to form stable heptameric rings without CLPX, although it has a limited and unspecific protease and peptidase activity, with a decreased activity for longer peptides and larger unfolded proteins [111,112]. Although CLPP protease activity does not require ATP hydrolysis, the CLPX ATPase activity is required to enhance substrate access and catalytic efficiency of CLPP by unfolding the protein substrate and translocating it into the proteolytic chamber [111].

The CLPP protease is a member of S14 serine protease family that has a serine-histidine-aspartate triad as its proteolytically-active catalytic residues [75]. Arabidopsis contains three CLPX chaperones: CLPX1–CLPX3, and six CLPP protease core subunits CLPP1–CLPP6, where only CLPP2 is targeted to mitochondria and the remainder are located in the chloroplasts [69,109]. Complete loss-of-function chloroplastic CLPP resulted in embryo lethality and severe developmental phenotypes, indicating the significance in chloroplast biogenesis and functions [113–115]. In contrast, deletion of the mitochondrial CLPP2, has no obvious impact to whole plant phenotype when grown under normal condition [116]. However, steady-state proteomics revealed that soluble matrix located Complex I NQ-module subunits B14, 24 kD, 51 kD, and 75 kD accumulated in *clpp2* knockout



[116] highlighting these subunits as possible substrates. Indeed, a BN-PAGE isolation followed by mass spectrometry identification showed that a free N-module intermediate composes of 24 kD and 51 kD subunits were accumulated in *clp2* knockouts [116]. This finding was reminiscent to *lon1* knockout, where unassembled NQ-module subunits were more slowly degraded, leading to an accumulation [31]. An accumulation of potential substrates could also be observed for other matrix-facing subunits of OXPHOS complexes, including MPP that is a part of Complex III, and ATP- $\beta$  and ATP- $\gamma$  that form matrix-facing F<sub>1</sub> module of Complex V [116] suggesting that CLPXP has a role in protein degradation of membrane-embedded OXPHOS subunits that protrude the matrix, especially unassembled subunits of submodules (Figure 3).

CLPXP system is the most well-studied proteolytic system in terms of structural determination, substrate identification, and characterisation of activity from a variety of organisms including bacteria, fungi, mammals, and plants [117–119]. Furthermore it is located in multiple plant organelles such as mitochondria, chloroplasts, and peroxisomes [120,121]. A tandem affinity purification (TAP) proteomics study in the aging fungus model *Podospora anserina* revealed several TCA cycle and OXPHOS components as its putative substrates, most notably NDUFS1 and NDUFV1 [122]. Similarly, human CLPXP system has also been proposed to directly regulate disassembly and degradation of matrix-facing Complex I N-module subunits NDUFS1, NDUFV1, and NDUFV2 [33], which are orthologous to plant 75 kD, 51 kD, and 24 kD subunits, respectively. Additionally, peripheral Q-module subunits NDUFS2, NDUFS6, NDUFS7, and NDUFS8 (orthologous to plant NAD7, 13 kD, PSST, and 23 kD, respectively) were also identified as putative CLPXP substrates [33,123].

## Concluding remarks

Although OXPHOS components can be proteolytically regulated in various ways, intra-mitochondrial proteolysis by AAA+ proteases play a substantial role in OXPHOS proteostasis. Biochemical, genetic, and structural studies have provided a deeper understanding on the mechanisms of AAA+ proteases in mitochondrial proteostasis. Historically, AAA+ proteases were considered as general proteases with relatively low selectivity and activity against any non-functional proteins. However, recent studies indicate that AAA+ proteases display unique substrate selectivity and function. AAA+ proteases also appear to modularly regulate OXPHOS components, in a similar fashion to their assembly. This way, proteostasis regulation can be energetically efficient, demanding much lower energy than de novo synthesis and assembly of the whole OXPHOS complexes, and allowing for specific regulation and maintenance.

## Perspectives

- Irregularities and disruption of mitochondrial OXPHOS have been known to be detrimental to organisms, such as fatal diseases in human and developmental delay in plants. Although some aspects of plant mitochondrial OXPHOS complexes assembly have been elucidated, the mechanism of their disassembly and degradation remains to be uncovered, which is equally important for their functions.
- While OXPHOS complexes can be regulated extensively through mitochondrial autophagy (mitophagy) and degradation of whole complexes under certain conditions, most OXPHOS components undergo intra-mitochondrial proteolysis that is modular, similar to their assembly process. Moreover, substrate identification of plant AAA+ proteases indicated that these proteases have substrate preference and specific recognition mechanism, rather than act as a general protease.
- A combination of reverse and forward genetic approach and biochemical characterisations are still warranted to elucidate the proteolytic pathway of mitochondrial OXPHOS components. In addition, structural determination and further biochemical analyses to determine activity mechanism can provide details necessary to build the proteolytic pathway model in plant mitochondria.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contributions

A.S.G. conceptualised the manuscript and prepared all figures. A.S.G. and M.W.M. drafted and revised the manuscript.

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## Abbreviations

AAA, ATPase-Associated with diverse cellular Activity; AAP, alanyl aminopeptidase; AP, aminopeptidase; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CIAF1, Complex I Assembly Factor-1; CLP, Caseinolytic protease; cryo-EM, cryogenic electron microscopy; DAP, aspartyl aminopeptidase; DIGE, difference gel electrophoresis; ETC, electron transfer chain; FTSH, Filamentous Temperature Sensitive H; HiRIEF, high-resolution isoelectric focusing; IM, inner membrane; IMS, intermembrane space; iTRAQ, isobaric tags for relative and absolute quantification; LAP, leucyl aminopeptidase; LON, Long filamentous phenotype; MDV, mitochondria-derived vesicle; MRM, multiple reaction monitoring; OOP, organellar oligopeptidase; OXPHOS, oxidative phosphorylation; PreP, presequence peptidase; redox, reduction-oxidation; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SILAC, stable isotope labelling with amino acids in cell culture; TCA, tricarboxylic acid; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

## References

- Dudkina, N.V., Eubel, H., Keegstra, W., Boekema, E.J. and Braun, H.P. (2005) Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. *Proc. Natl Acad. Sci. U.S.A.* **102**, 3225–3229 <https://doi.org/10.1073/pnas.0408870102>
- Dudkina, N.V., Heinemeyer, J., Sunderhaus, S., Boekema, E.J. and Braun, H.P. (2006) Respiratory chain supercomplexes in the plant mitochondrial membrane. *Trends Plant Sci.* **11**, 232–240 <https://doi.org/10.1016/j.tplants.2006.03.007>
- Dudkina, N.V., Kouřil, R., Peters, K., Braun, H.P. and Boekema, E.J. (2010) Structure and function of mitochondrial supercomplexes. *Biochim. Biophys. Acta Bioenerg.* **1797**, 664–670 <https://doi.org/10.1016/j.bbabi.2009.12.013>
- Michaud, M., Cognat, V., Duchêne, A.M. and Maréchal-Drouard, L. (2011) A global picture of tRNA genes in plant genomes. *Plant J.* **66**, 80–93 <https://doi.org/10.1111/j.1365-3113.2011.04490.x>
- Waltz, F., Nguyen, T.T., Arrivé, M., Bochler, A., Chicher, J., Hammann, P., et al. (2019) Small is big in Arabidopsis mitochondrial ribosome. *Nat. Plants* **5**, 106–117 <https://doi.org/10.1038/s41477-018-0339-y>
- Ghifari, A.S. and Murcha, M.W. (2020) Plant mitochondria. *eLS* **1**, 581–591 <https://doi.org/10.1002/9780470015902.a0029217>
- Ghifari, A.S., Gill-Hille, M. and Murcha, M.W. (2018) Plant mitochondrial protein import: the ins and outs. *Biochem. J.* **475**, 2191–2208 <https://doi.org/10.1042/BCJ20170521>
- Ivanova, A., Gill-Hille, M., Huang, S., Branca, R., Kmiec, B., Teixeira, P.F. et al. (2019) A mitochondrial LYR protein is required for complex I assembly. *Plant Physiol.* **181**, 1632–1650 <https://doi.org/10.1104/pp.19.00822>
- Soufari, H., Parrot, C., Kuhn, L., Waltz, F. and Hashem, Y. (2020) Specific features and assembly of the plant mitochondrial complex I revealed by cryo-EM. *Nat. Commun.* **11**, 5195 <https://doi.org/10.1038/s41467-020-18814-w>
- Schertl, P., Sunderhaus, S., Klodmann, J., Grozoff, G.E., Bartoli, C.G. and Braun, H.P. (2012) L-galactono-1,4-lactone dehydrogenase (GLDH) forms part of three subcomplexes of mitochondrial complex I in *Arabidopsis thaliana*. *J. Biol. Chem.* **287**, 14412–14419 <https://doi.org/10.1074/jbc.M111.305144>
- Klus, N., Senkler, J., Yildiz, Ö., Kühlbrandt, W. and Braun, H.-P. (2021) A ferredoxin bridge connects the two arms of plant mitochondrial complex I. *Plant Cell* **33**, 2072–2091 <https://doi.org/10.1093/plcell/koab092>
- Maldonado, M., Padavannil, A., Zhou, L., Guo, F. and Letts, J.A. (2020) Atomic structure of a mitochondrial complex I intermediate from vascular plants. *eLife* **9**, e56664 <https://doi.org/10.7554/eLife.56664>
- Maldonado, M., Guo, F. and Letts, J.A. (2021) Atomic structures of respiratory complex III2, complex IV, and supercomplex III2-IV from vascular plants. *eLife* **10**, e62047 <https://doi.org/10.7554/eLife.62047>
- Ligas, J., Pineau, E., Bock, R., Huynen, M.A. and Meyer, E.H. (2019) The assembly pathway of complex I in Arabidopsis thaliana. *Plant J.* **97**, 447–459 <https://doi.org/10.1111/tpj.14133>
- Li, L., Carrie, C., Nelson, C., Whelan, J. and Harvey Millar, A. (2012) Accumulation of newly synthesized F1 in vivo in Arabidopsis mitochondria provides evidence for modular assembly of the plant F1FoATP synthase. *J. Biol. Chem.* **287**, 25749–25757 <https://doi.org/10.1074/jbc.M112.373506>

- 16 Klodmann, J., Sunderhaus, S., Nimtz, M., Jansch, L. and Braun, H.P. (2010) Internal architecture of mitochondrial complex I from *Arabidopsis thaliana*. *Plant Cell* **22**, 797–810 <https://doi.org/10.1105/tpc.109.073726>
- 17 Broda, M., Millar, A.H. and Van Aken, O. (2018) Mitophagy: a mechanism for plant growth and survival. *Trends Plant Sci.* **23**, 434–450 <https://doi.org/10.1016/j.tplants.2018.02.010>
- 18 Sugiura, A., McLelland, G., Fon, E.A. and McBride, H.M. (2014) A new pathway for mitochondrial quality control: mitochondrial-derived vesicles. *EMBO J.* **33**, 2142–2156 <https://doi.org/10.15252/embj.201488104>
- 19 Chacinska, A., Pfannschmidt, S., Wiedemann, N., Kozjak, V., Sanjuan Skclzar, L.K., Schulze-Specking, A. et al. (2004) Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *EMBO J.* **23**, 3735–3746 <https://doi.org/10.1038/sj.emboj.7600389>
- 20 Vierstra, R.D. (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.* **10**, 385–397 <https://doi.org/10.1038/nrm2688>
- 21 Sandate, C.R., Szyk, A., Zehr, E.A., Lander, G.C. and Roll-Mecak, A. (2019) An allosteric network in spastin couples multiple activities required for microtubule severing. *Nat. Struct. Mol. Biol.* **26**, 671–678 <https://doi.org/10.1038/s41594-019-0257-3>
- 22 Han, H., Monroe, N., Sundquist, W.I., Shen, P.S. and Hill, C.P. (2017) The AAA ATPase Vps4 binds ESCRT-III substrates through a repeating array of dipeptide-binding pockets. *eLife* **6**, e31324 <https://doi.org/10.7554/eLife.31324>
- 23 Duderstadt, K.E., Chuang, K. and Berger, J.M. (2011) DNA stretching by bacterial initiators promotes replication origin opening. *Nature* **478**, 209–213 <https://doi.org/10.1038/nature10455>
- 24 Seraphim, T.V. and Houry, W.A. (2020) AAA+ proteins. *Curr. Biol.* **30**, R251–R257 <https://doi.org/10.1016/j.cub.2020.01.044>
- 25 Shin, M., Puchades, C., Asmita, A., Puri, N., Adjei, E., Wiseman, R.L. et al. (2020) Structural basis for distinct operational modes and protease activation in AAA+ protease Lon. *Sci. Adv.* **6**, eaba8404 <https://doi.org/10.1126/sciadv.aba8404>
- 26 Puchades, C., Ding, B., Song, A., Wiseman, R.L., Lander, G.C. and Glynn, S.E. (2019) Unique structural features of the mitochondrial AAA+ protease AFG3L2 reveal the molecular basis for activity in health and disease. *Mol. Cell* **75**, 1073–1085.e6 <https://doi.org/10.1016/j.molcel.2019.06.016>
- 27 Puchades, C., Rampello, A.J., Shin, M., Giuliano, C.J., Wiseman, R.L., Glynn, S.E. et al. (2017) Structure of the mitochondrial inner membrane AAA+ protease YME1 gives insight into substrate processing. *Science* **358**, eaao0464 <https://doi.org/10.1126/science.aao0464>
- 28 Yokom, A.L., Gates, S.N., Jackrel, M.E., Mack, K.L., Su, M., Shorter, J. et al. (2016) Spiral architecture of the Hsp104 disaggregase reveals the basis for polypeptide translocation. *Nat. Struct. Mol. Biol.* **23**, 830–837 <https://doi.org/10.1038/nsmb.3277>
- 29 Rizo, A.N., Lin, J.B., Gates, S.N., Tse, E., Bart, S.M., Castellano, L.M. et al. (2019) Structural basis for substrate gripping and translocation by the ClpB AAA+ disaggregase. *Nat. Commun.* **10**, 2393 <https://doi.org/10.1038/s41467-019-10150-y>
- 30 Lopez, K.E., Rizo, A.N., Tse, E., Lin, J.B., Scull, N.W., Thwin, A.C. et al. (2020) Conformational plasticity of the ClpAP AAA+ protease couples protein unfolding and proteolysis. *Nat. Struct. Mol. Biol.* **27**, 406–416 <https://doi.org/10.1038/s41594-020-0409-5>
- 31 Li, L., Nelson, C., Fenske, R., Trösch, J., Průžinská, A., Millar, A.H. et al. (2017) Changes in specific protein degradation rates in *Arabidopsis thaliana* reveal multiple roles of Lon1 in mitochondrial protein homeostasis. *Plant J.* **89**, 458–471 <https://doi.org/10.1111/tpj.13392>
- 32 Nelson, C.J., Li, L., Jacoby, R.P. and Millar, A.H. (2013) Degradation rate of mitochondrial proteins in *Arabidopsis thaliana* cells. *J. Proteome Res.* **12**, 3449–3459 <https://doi.org/10.1021/pr400304r>
- 33 Szczepanowska, K., Senft, K., Heidler, J., Herholz, M., Kukat, A., Höhne, M.N., et al. (2020) A salvage pathway maintains highly functional respiratory complex I. *Nat. Commun.* **11**, 1643 <https://doi.org/10.1038/s41467-020-15467-7>
- 34 Huang, S., Li, L., Petereit, J. and Millar, A.H. (2020) Mitochondrion protein turnover rates in plant mitochondria. *Mitochondrion* **53**, 57–65 <https://doi.org/10.1016/j.mito.2020.04.011>
- 35 Boos, F., Krämer, L., Groh, C., Jung, F., Haberkant, P., Stein, F., et al. (2019) Mitochondrial protein-induced stress triggers a global adaptive transcriptional programme. *Nat. Cell Biol.* **21**, 442–451 <https://doi.org/10.1038/s41556-019-0294-5>
- 36 Mohanraj, K., Nowicka, U. and Chacinska, A. (2020) Mitochondrial control of cellular protein homeostasis. *Biochem. J.* **477**, 3033–3054 <https://doi.org/10.1042/BCJ20190654>
- 37 Di Maio, R., Barrett, P.J., Hoffman, E.K., Barrett, C.W., Zharikov, A., Borah, A., et al. (2016)  $\alpha$ -synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease. *Sci. Transl. Med.* **8**, 342ra78 <https://doi.org/10.1126/scitranslmed.aaf3634>
- 38 Schäfer, J.A., Bozkurt, S., Michaelis, J.B., Klann, K. and Munch, C. (2022) Global mitochondrial protein import proteomics reveal distinct regulation by translation and translocation machinery. *Mol. Cell* **82**, 435–446.e7 <https://doi.org/10.1016/j.molcel.2021.11.004>
- 39 Nowicka, U., Chroscicki, P., Stroobants, K., Sładowska, M., Turek, M., Uszczyńska-Ratajczak, B., et al. (2021) Cytosolic aggregation of mitochondrial proteins disrupts cellular homeostasis by stimulating the aggregation of other proteins. *eLife* **10**, e65484 <https://doi.org/10.7554/eLife.65484>
- 40 Weidberg, H. and Amon, A. (2018) MitoCPR—A surveillance pathway that protects mitochondria in response to protein import stress. *Science* **360**, eaan4146 <https://doi.org/10.1126/science.aan4146>
- 41 Mårtensson, C.U., Priesnitz, C., Song, J., Ellenrieder, L., Doan, K.N., Boos, F., et al. (2019) Mitochondrial protein translocation-associated degradation. *Nature* **569**, 679–683 <https://doi.org/10.1038/s41586-019-1227-y>
- 42 Azzu, V. and Brand, M.D. (2010) Degradation of an intramitochondrial protein by the cytosolic proteasome. *J. Cell Sci.* **123**, 578–585 <https://doi.org/10.1242/jcs.060004>
- 43 Azzu, V., Mookerjee, S.A. and Brand, M.D. (2010) Rapid turnover of mitochondrial uncoupling protein 3. *Biochem. J.* **426**, 13–17 <https://doi.org/10.1042/BJ20091321>
- 44 Mookerjee, S.A. and Brand, M.D. (2011) Characteristics of the turnover of uncoupling protein 3 by the ubiquitin proteasome system in isolated mitochondria. *Biochim. Biophys. Acta Bioenerg.* **1807**, 1474–1481 <https://doi.org/10.1016/j.bbabi.2011.07.011>
- 45 Finger, Y., Habich, M., Gerlich, S., Urbanczyk, S., Logt, E., Koch, J., et al. (2020) Proteasomal degradation induced by DPP9-mediated processing competes with mitochondrial protein import. *EMBO J.* **39**, e103889 <https://doi.org/10.15252/embj.2019103889>
- 46 Friederich, M.W., Erdogan, A.J., Coughlin, C.R., Elos, M.T., Jiang, H., O'Rourke, C.P., et al. (2017) Mutations in the accessory subunit NDUBF10 result in isolated complex I deficiency and illustrate the critical role of intermembrane space import for complex I holoenzyme assembly. *Hum. Mol. Genet.* **26**, 702–716 <https://doi.org/10.1093/hmg/ddw431>
- 47 Fischer, M., Horn, S., Belkacemi, A., Kojer, K., Petrungraro, C., Habich, M., et al. (2013) Protein import and oxidative folding in the mitochondrial intermembrane space of intact mammalian cells. *Mol. Biol. Cell* **24**, 2160–2170 <https://doi.org/10.1091/mbc.e12-12-0862>

- 48 Carrie, C., Giraud, E., Duncan, O., Xu, L., Wang, Y., Huang, S., et al. (2010) Conserved and novel functions for *Arabidopsis thaliana* MIA40 in assembly of proteins in mitochondria and peroxisomes. *J. Biol. Chem.* **285**, 36138–36148 <https://doi.org/10.1074/jbc.M110.121202>
- 49 Sideris, D.P., Petrakis, N., Katrakili, N., Mikropoulou, D., Gallo, A., Ciofi-Baffoni, S. et al. (2009) A novel intermembrane space-targeting signal docks cysteines onto Mia40 during mitochondrial oxidative folding. *J. Cell Biol.* **187**, 1007–1022 <https://doi.org/10.1083/jcb.200905134>
- 50 Kowalski, L., Bragoszewski, P., Khmelinskii, A., Glow, E., Knop, M. and Chacinska, A. (2018) Determinants of the cytosolic turnover of mitochondrial intermembrane space proteins. *BMC Biol.* **16**, 66 <https://doi.org/10.1186/s12915-018-0536-1>
- 51 Mohanraj, K., Wasilewski, M., Benincá, C., Cysewski, D., Poznanski, J., Sakowska, P. et al. (2019) Inhibition of proteasome rescues a pathogenic variant of respiratory chain assembly factor COA7. *EMBO Mol. Med.* **11**, e9561 <https://doi.org/10.15252/emmm.201809561>
- 52 Yamashita, A., Fujimoto, M., Katayama, K., Yamaoka, S., Tsutsumi, N. and Arimura, S.I. (2016) Formation of mitochondrial outer membrane derived protrusions and vesicles in *Arabidopsis thaliana*. *PLoS ONE* **11**, e0146717 <https://doi.org/10.1371/journal.pone.0146717>
- 53 Soubannier, V., Rippstein, P., Kaufman, B.A., Shoubridge, E.A. and McBride, H.M. (2012) Reconstitution of mitochondria derived vesicle formation demonstrates selective enrichment of oxidized cargo. *PLoS One* **7**, e52830 <https://doi.org/10.1371/journal.pone.0052830>
- 54 Todkar, K., Chikhi, L., Desjardins, V., El-Mortada, F., Pépin, G. and Germain, M. (2021) Selective packaging of mitochondrial proteins into extracellular vesicles prevents the release of mitochondrial DAMPs. *Nat. Commun.* **12**, 1971 <https://doi.org/10.1038/s41467-021-21984-w>
- 55 Bezawork-Geleta, A., Brodie, E.J., Dougan, D.A. and Truscott, K.N. (2015) LON is the master protease that protects against protein aggregation in human mitochondria through direct degradation of misfolded proteins. *Sci. Rep.* **5**, 17397 <https://doi.org/10.1038/srep17397>
- 56 Martin, A., Baker, T.A. and Sauer, R.T. (2008) Protein unfolding by a AAA+ protease is dependent on ATP-hydrolysis rates and substrate energy landscapes. *Nat. Struct. Mol. Biol.* **15**, 139–145 <https://doi.org/10.1038/nsmb.1380>
- 57 Shiba-Fukushima, K., Imai, Y., Yoshida, S., Ishihama, Y., Kanao, T., Sato, S. et al. (2012) PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci. Rep.* **2**, 1002 <https://doi.org/10.1038/srep01002>
- 58 Eiyama, A. and Okamoto, K. (2015) Protein N-terminal acetylation by the NatA complex is critical for selective mitochondrial degradation. *J. Biol. Chem.* **290**, 25034–25044 <https://doi.org/10.1074/jbc.M115.677468>
- 59 Minibayeva, F., Dmitrieva, S., Ponomareva, A. and Ryabovov, V. (2012) Oxidative stress-induced autophagy in plants: the role of mitochondria. *Plant Physiol. Biochem.* **59**, 11–19 <https://doi.org/10.1016/j.plaphy.2012.02.013>
- 60 Deffieu, M., Bhatia-Kiššová, I., Salin, B., Klionsky, D.J., Pinson, B., Manon, S. et al. (2013) Increased levels of reduced cytochrome b and mitophagy components are required to trigger nonspecific autophagy following induced mitochondrial dysfunction. *J. Cell Sci.* **126**, 415–426 <https://doi.org/10.1242/jcs.103713>
- 61 Glaser, E., Eriksson, A., Sjöling, S., Sjöling, S. and Sjöling, S. (1994) Bifunctional role of the bc<sub>1</sub> complex in plants. mitochondrial bc<sub>1</sub> complex catalyses both electron transport and protein processing. *FEBS Lett.* **346**, 83–87 [https://doi.org/10.1016/0014-5793\(94\)00312-2](https://doi.org/10.1016/0014-5793(94)00312-2)
- 62 Braun, H.P. and Schmitz, U.K. (1995) The bifunctional cytochrome c reductase/processing peptidase complex from plant mitochondria. *J. Bioenerg. Biomembr.* **27**, 423–436 <https://doi.org/10.1007/BF02110005>
- 63 Braun, H.P., Emmermann, M., Kruff, V., Bodicker, M., Schmitz, U.K., Bödicker, M. et al. (1995) The general mitochondrial processing peptidase from wheat is integrated into the cytochrome bc<sub>1</sub>-complex of the respiratory chain. *Planta* **195**, 396–402 <https://doi.org/10.1007/BF00202597>
- 64 Carrie, C., Venne, A.S., Zahedi, R.P. and Soll, J. (2015) Identification of cleavage sites and substrate proteins for two mitochondrial intermediate peptidases in *Arabidopsis thaliana*. *J. Exp. Bot.* **66**, 2691–2708 <https://doi.org/10.1093/jxb/erv064>
- 65 Huang, S., Nelson, C.J., Li, L., Taylor, N.L., Ströher, E., Peterleit, J. et al. (2015) Intermediate cleavage peptidase 55 modifies enzyme amino termini and alters protein stability in *Arabidopsis* mitochondria. *Plant Physiol.* **168**, 415–427 <https://doi.org/10.1104/pp.15.00300>
- 66 Teixeira, P.F. and Glaser, E. (2013) Processing peptidases in mitochondria and chloroplasts. *Biochim. Biophys. Acta Mol. Cell Res.* **1833**, 360–370 <https://doi.org/10.1016/j.bbamcr.2012.03.012>
- 67 Vögtle, F.N., Wortelkamp, S., Zahedi, R.P., Becker, D., Leidhold, C., Gevaert, K. et al. (2009) Global analysis of the mitochondrial N-Proteome identifies a processing peptidase critical for protein stability. *Cell* **139**, 428–439 <https://doi.org/10.1016/j.cell.2009.07.045>
- 68 Vogtle, F.-N., Prinz, C., Kellermann, J., Lottspeich, F., Pfanner, N. and Meisinger, C. (2011) Mitochondrial protein turnover: role of the precursor intermediate peptidase Oct1 in protein stabilization. *Mol. Biol. Cell* **22**, 2135–2143 <https://doi.org/10.1091/mbc.e11-02-0169>
- 69 van Wijk, K.J. (2015) Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. *Annu. Rev. Plant Biol.* **66**, 75–111 <https://doi.org/10.1146/annurev-arplant-043014-115547>
- 70 Rendón, O.Z. and Shoubridge, E.A. (2018) LONP1 is required for maturation of a subset of mitochondrial proteins, and its loss elicits an integrated stress response. *Mol. Cell. Biol.* **38**, e00412-17 <https://doi.org/10.1128/MCB.00412-17>
- 71 Kmiec, B., Teixeira, P.F., Berntsson, R.P.-A., Murcha, M.W., Branca, R.M.M., Radomiljac, J.D. et al. (2013) Organellar oligopeptidase (OOP) provides a complementary pathway for targeting peptide degradation in mitochondria and chloroplasts. *Proc. Natl Acad. Sci. U.S.A.* **110**, E3761–E3769 <https://doi.org/10.1073/pnas.1307637110>
- 72 Kmiec, B., Teixeira, P.F. and Glaser, E. (2014) Shredding the signal: targeting peptide degradation in mitochondria and chloroplasts. *Trends Plant Sci.* **19**, 771–778 <https://doi.org/10.1016/j.tplants.2014.09.004>
- 73 Teixeira, P.F., Kmiec, B., Branca, R.M.M., Murcha, M.W., Byzia, A., Ivanova, A. et al. (2017) A multi-step peptidolytic cascade for amino acid recovery in chloroplasts. *Nat. Chem. Biol.* **13**, 15–17 <https://doi.org/10.1038/nchembio.2227>
- 74 Kmiec, B., Branca, R.M.M., Murcha, M.W., Lehtiö, J., Glaser, E. and Teixeira, P.F. (2018) A common peptidolytic mechanism for targeting peptide degradation in mitochondria and chloroplasts. *Mol. Plant* **11**, 342–345 <https://doi.org/10.1016/j.molp.2017.11.008>
- 75 Ghifari, A.S., Huang, S. and Murcha, M.W. (2019) The peptidases involved in plant mitochondrial protein import. *J. Exp. Bot.* **70**, 6005–6018 <https://doi.org/10.1093/jxb/erz365>
- 76 Ghifari, A.S., Teixeira, P.F., Kmiec, B., Pružinská, A., Glaser, E. and Murcha, M.W. (2020) A mitochondrial prolyl aminopeptidase PAP2 releases N-terminal proline and regulates proline homeostasis during stress response. *Plant J.* **104**, 1182–1194 <https://doi.org/10.1111/tpj.14987>
- 77 Bhushan, S., Ståhl, A., Nilsson, S., Lefebvre, B., Seki, M., Roth, C., et al. (2005) Catalysis, subcellular localization, expression and evolution of the targeting peptides degrading protease, AtPreP2. *Plant Cell Physiol.* **46**, 985–996 <https://doi.org/10.1093/pcp/pci107>
- 78 Waditee-Sirisattha, R., Shibato, J., Rakwal, R., Sirisattha, S., Hattori, A., Nakano, T. et al. (2011) The *Arabidopsis* aminopeptidase LAP2 regulates plant growth, leaf longevity and stress response. *New Phytol.* **191**, 958–969 <https://doi.org/10.1111/j.1469-8137.2011.03758.x>



- 79 Huber, C.V., Jakobs, B.D., Mishra, L.S., Niedermaier, S., Stift, M., Winter, G. et al. (2019) DEG10 contributes to mitochondrial proteostasis, root growth, and seed yield in Arabidopsis. *J. Exp. Bot.* **70**, 5423–5436 <https://doi.org/10.1093/jxb/erz294>
- 80 Kmiec-Wisniewska, B., Krumpe, K., Urantowka, A., Sakamoto, W., Prati, E. and Janska, H. (2008) Plant mitochondrial rhomboid, AtRBL12, has different substrate specificity from its yeast counterpart. *Plant Mol. Biol.* **68**, 159–171 <https://doi.org/10.1007/s11103-008-9359-8>
- 81 García-Lorenzo, M., Sjödin, A., Jansson, S. and Funk, C. (2006) Protease gene families in *Populus* and *Arabidopsis*. *BMC Plant Biol.* **6**, 30 <https://doi.org/10.1186/1471-2229-6-30>
- 82 Puchades, C., Sandate, C.R. and Lander, G.C. (2020) The molecular principles governing the activity and functional diversity of AAA+ proteins. *Nat. Rev. Mol. Cell Biol.* **21**, 43–58 <https://doi.org/10.1038/s41580-019-0183-6>
- 83 Sauer, R.T. and Baker, T.A. (2011) AAA+ proteases: ATP-Fueled machines of protein destruction. *Annu. Rev. Biochem.* **80**, 587–612 <https://doi.org/10.1146/annurev-biochem-060408-172623>
- 84 Gatsogiannis, C., Balogh, D., Merino, F., Sieber, S.A. and Raunser, S. (2019) Cryo-EM structure of the ClpXP protein degradation machinery. *Nat. Struct. Mol. Biol.* **26**, 946–954 <https://doi.org/10.1038/s41594-019-0304-0>
- 85 Fei, X., Bell, T.A., Jenni, S., Stinson, B.M., Baker, T.A., Harrison, S.C. et al. (2020) Structures of the ATP-fueled ClpXP proteolytic machine bound to protein substrate. *eLife* **9**, e52774 <https://doi.org/10.7554/eLife.52774>
- 86 Ripstein, Z.A., Vahidi, S., Houry, W.A., Rubinstein, J.L. and Kay, L.E. (2020) A processive rotary mechanism couples substrate unfolding and proteolysis in the ClpXP degradation machinery. *eLife* **9**, e52158 <https://doi.org/10.7554/eLife.52158>
- 87 Shi, H., Rampello, A.J. and Glynn, S.E. (2016) Engineered AAA+ proteases reveal principles of proteolysis at the mitochondrial inner membrane. *Nat. Commun.* **7**, 13301 <https://doi.org/10.1038/ncomms13301>
- 88 Rampello, A.J. and Glynn, S.E. (2017) Identification of a degradation signal sequence within substrates of the mitochondrial i-AAA protease. *J. Mol. Biol.* **429**, 873–885 <https://doi.org/10.1016/j.jmb.2017.02.009>
- 89 Janska, H., Piechota, J. and Kwasniak, M. (2010) ATP-dependent proteases in biogenesis and maintenance of plant mitochondria. *Biochim. Biophys. Acta Bioenerg.* **1797**, 1071–1075 <https://doi.org/10.1016/j.bbabi.2010.02.027>
- 90 Opalińska, M. and Jańska, H. (2018) AAA proteases: guardians of mitochondrial function and homeostasis. *Cells* **7**, 163 <https://doi.org/10.3390/cells7100163>
- 91 Ivanova, A., Ghifari, A.S., Berkowitz, O., Whelan, J. and Murcha, M.W. (2021) The mitochondrial AAA protease FTSH3 regulates complex I abundance by promoting its disassembly. *Plant Physiol.* **186**, 599–610 <https://doi.org/10.1093/plphys/kiab074>
- 92 Kolodziejczak, M., Gibala, M., Urantowka, A. and Janska, H. (2007) The significance of Arabidopsis AAA proteases for activity and assembly/stability of mitochondrial OXPHOS complexes. *Physiol. Plant.* **129**, 135–142 <https://doi.org/10.1111/j.1365-3054.2006.00835.x>
- 93 Wagner, R., Aigner, H., Pružinská, A., Jänkänpää, H.J., Jansson, S. and Funk, C. (2011) Fitness analyses of Arabidopsis thaliana mutants depleted of ftsh metalloproteases and characterization of three ftsh6 deletion mutants exposed to high light stress, senescence and chilling. *New Phytol.* **191**, 449–458 <https://doi.org/10.1111/j.1469-8137.2011.03684.x>
- 94 Kolodziejczak, M., Skibiń-Błaszczak, R. and Janska, H. (2018) m-AAA complexes are not crucial for the survival of Arabidopsis under optimal growth conditions despite their importance for mitochondrial translation. *Plant Cell Physiol.* **59**, 1006–1016 <https://doi.org/10.1093/pcp/pcy041>
- 95 Smakowska, E., Skibiń-Błaszczak, R., Czarna, M., Kolodziejczak, M., Kwasniak-Owczarek, M., Parys, K. et al. (2016) Lack of FTSH4 protease affects protein carbonylation, mitochondrial morphology, and phospholipid content in mitochondria of Arabidopsis: new insights into a complex interplay. *Plant Physiol.* **171**, 2516–2535 <https://doi.org/10.1104/pp.16.00370>
- 96 Maziak, A., Heidorn-Czarna, M., Weremczuk, A. and Janska, H. (2021) FTSH4 and OMA1 mitochondrial proteases reduce moderate heat stress-induced protein aggregation. *Plant Physiol.* **187**, 1–18 <https://doi.org/10.1093/plphys/kiab296>
- 97 Heidorn-Czarna, M., Domanski, D., Kwasniak-Owczarek, M. and Janska, H. (2018) Targeted proteomics approach toward understanding the role of the mitochondrial protease FTSH4 in the biogenesis of OXPHOS during Arabidopsis seed germination. *Front. Plant Sci.* **9**, 821 <https://doi.org/10.3389/fpls.2018.00821>
- 98 Opalińska, M., Parys, K. and Jańska, H. (2017) Identification of physiological substrates and binding partners of the plant mitochondrial protease FTSH4 by the trapping approach. *Int. J. Mol. Sci.* **18**, 2455 <https://doi.org/10.3390/ijms18112455>
- 99 Rigas, S., Daras, G., Laxa, M., Marathias, N., Fasseas, C., Sweetlove, L.J. et al. (2009) Role of Lon1 protease in post-germinative growth and maintenance of mitochondrial function in *Arabidopsis thaliana*. *New Phytol.* **181**, 588–600 <https://doi.org/10.1111/j.1469-8137.2008.02701.x>
- 100 Arends, J., Griego, M., Thomanek, N., Lindemann, C., Kutscher, B., Meyer, H.E. et al. (2018) An integrated proteomic approach uncovers novel substrates and functions of the Lon protease in *Escherichia coli*. *Proteomics* **18**, 1800080 <https://doi.org/10.1002/pmic.201800080>
- 101 Matsushima, Y., Goto, Y.I. and Kaguni, L.S. (2010) Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM). *Proc. Natl Acad. Sci. U.S.A.* **107**, 18410–18415 <https://doi.org/10.1073/pnas.1008924107>
- 102 Venkatesh, S., Lee, J., Singh, K., Lee, I. and Suzuki, C.K. (2012) Multitasking in the mitochondrion by the ATP-dependent Lon protease. *Biochim. Biophys. Acta Mol. Cell Res.* **1823**, 56–66 <https://doi.org/10.1016/j.bbamcr.2011.11.003>
- 103 Cheng, C.W., Kuo, C.Y., Fan, C.C., Fang, W.C., Jiang, S.S., Lo, Y.K. et al. (2013) Overexpression of Lon contributes to survival and aggressive phenotype of cancer cells through mitochondrial complex I-mediated generation of reactive oxygen species. *Cell Death Dis.* **4**, e681 <https://doi.org/10.1038/cddis.2013.204>
- 104 Shin, C.S., Meng, S., Garbis, S.D., Moradian, A., Taylor, R.W., Sweredoski, M.J. et al. (2021) LONP1 and mtHSP70 cooperate to promote mitochondrial protein folding. *Nat. Commun.* **12**, 1–10 <https://doi.org/10.1038/s41467-020-20314-w>
- 105 Pollecker, K., Sylvester, M. and Voos, W. (2021) Proteomic analysis demonstrates the role of the quality control protease LONP1 in mitochondrial protein aggregation. *J. Biol. Chem.* **296**, 101134 <https://doi.org/10.1016/j.jbc.2021.101134>
- 106 Ostersetzer, O., Kato, Y., Adam, Z. and Sakamoto, W. (2007) Multiple intracellular locations of Lon protease in Arabidopsis: evidence for the localization of AtLon4 to chloroplasts. *Plant Cell Physiol.* **48**, 881–885 <https://doi.org/10.1093/pcp/pcm052>
- 107 Tsitsekian, D., Daras, G., Alatzas, A., Templelexis, D., Hatzopoulos, P. and Rigas, S. (2019) Comprehensive analysis of Lon proteases in plants highlights independent gene duplication events. *J. Exp. Bot.* **70**, 2185–2197 <https://doi.org/10.1093/jxb/ery440>

- 108 Solheim, C., Li, L., Hatzopoulos, P. and Harvey Millar, A. (2012) Loss of Lon1 in Arabidopsis changes the mitochondrial proteome leading to altered metabolite profiles and growth retardation without an accumulation of oxidative damage. *Plant Physiol.* **160**, 1187–1203 <https://doi.org/10.1104/pp.112.203711>
- 109 Peltier, J.B., Ripoll, D.R., Friso, G., Rudella, A., Cai, Y., Ytterberg, J. et al. (2004) Clp protease complexes from photosynthetic and non-photosynthetic plastids and mitochondria of plants, their predicted three-dimensional structures, and functional implications. *J. Biol. Chem.* **279**, 4768–4781 <https://doi.org/10.1074/jbc.M309212200>
- 110 Senkler, J., Senkler, M., Eubel, H., Hildebrandt, T., Lengwenus, C., Schertl, P. et al. (2017) The mitochondrial complexome of *Arabidopsis thaliana*. *Plant J* **89**, 1079–1092 <https://doi.org/10.1111/tpj.13448>
- 111 Kang, S.G., Dimitrova, M.N., Ortega, J., Ginsburg, A. and Maurizi, M.R. (2005) Human mitochondrial clpP is a stable heptamer that assembles into a tetradecamer in the presence of ClpX. *J. Biol. Chem.* **280**, 35424–35432 <https://doi.org/10.1074/jbc.M507240200>
- 112 Bewley, M.C., Graziano, V., Griffin, K. and Flanagan, J.M. (2009) Turned on for degradation: ATPase-independent degradation by ClpP. *J. Struct. Biol.* **165**, 118–125 <https://doi.org/10.1016/j.jsb.2008.10.005>
- 113 Kim, J., Olinares, P.D., Oh, S.H., Ghisaura, S., Poliakov, A., Ponnala, L. et al. (2013) Modified Clp protease complex in the ClpP3 null mutant and consequences for chloroplast development and function in arabidopsis. *Plant Physiol.* **162**, 157–179 <https://doi.org/10.1104/pp.113.215699>
- 114 Moreno, J.C., Tiller, N., Diez, M., Karcher, D., Tillich, M., Schöttler, M.A. et al. (2017) Generation and characterization of a collection of knock-down lines for the chloroplast Clp protease complex in tobacco. *J. Exp. Bot.* **68**, 2199–2218 <https://doi.org/10.1093/jxb/erx066>
- 115 Moreno, J.C., Martínez-Jaime, S., Schwartzmann, J., Karcher, D., Tillich, M., Graf, A. et al. (2018) Temporal proteomics of inducible RNAi lines of Clp protease subunits identifies putative protease substrates. *Plant Physiol.* **176**, 1485–1508 <https://doi.org/10.1104/pp.17.01635>
- 116 Petereit, J., Duncan, O., Murcha, M.W., Fenske, A.R., Cincu, E., Cahn, J., et al. (2020) Mitochondrial CLPP2 assists coordination and homeostasis of respiratory complexes. *Plant Physiol.* **184**, 148–164 <https://doi.org/10.1104/pp.20.00136>
- 117 Szczepanowska, K. and Trifunovic, A. (2021) Mitochondrial matrix proteases - quality control and beyond. *FEBS J*, May 2021, 1–19 <https://doi.org/10.1111/febs.15964>
- 118 Szczepanowska, K. and Trifunovic, A. (2021) Tune instead of destroy: how proteolysis keeps OXPHOS in shape. *Biochim. Biophys. Acta Bioenerg.* **1862**, 148365 <https://doi.org/10.1016/j.bbabi.2020.148365>
- 119 Liao, J.-Y.R. and van Wijk, K.J. (2019) Discovery of AAA+ protease substrates through trapping approaches. *Trends Biochem. Sci.* **44**, 528–545 <https://doi.org/10.1016/j.tibs.2018.12.006>
- 120 Bouchnak, I. and van Wijk, K.J. (2021) Structure, function, and substrates of Clp AAA+ protease systems in cyanobacteria, plastids, and apicoplasts: a comparative analysis. *J. Biol. Chem.* **296**, 100338 <https://doi.org/10.1016/j.jbc.2021.100338>
- 121 Majsec, K., Bhuiyan, N.H., Sun, Q., Kumari, S., Kumar, V., Ware, D. et al. (2017) The plastid and mitochondrial peptidase network in *Arabidopsis thaliana*: a foundation for testing genetic interactions and functions in organellar proteostasis. *Plant Cell* **29**, 2687–2710 <https://doi.org/10.1105/tpc.17.00481>
- 122 Fischer, F., Langer, J.D. and Osiewacz, H.D. (2015) Identification of potential mitochondrial CLPXP protease interactors and substrates suggests its central role in energy metabolism. *Sci. Rep.* **5**, 18375 <https://doi.org/10.1038/srep18375>
- 123 Ishizawa, J., Zarabi, S.F., Davis, R.E., Halgas, O., Nii, T., Jitkova, Y., et al. (2019) Mitochondrial ClpP-mediated proteolysis induces selective cancer cell lethality. *Cancer Cell* **35**, 721–737.e9 <https://doi.org/10.1016/j.ccell.2019.03.014>