Mitochondrial proteases modulate mitochondrial stress signalling and cellular homeostasis in health and disease

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ABSTRACT

Maintenance of mitochondrial homeostasis requires a plethora of coordinated quality control and adaptations' mechanisms in which mitochondrial proteases play a key role. Their activation or loss of function reverberate beyond local mitochondrial biochemical and metabolic remodelling into coordinated cellular pathways and stress responses that feedback onto the mitochondrial functionality and adaptability. Mitochondrial proteolysis modulates molecular and organellar quality control, metabolic adaptations, lipid homeostasis and regulates transcriptional stress responses. Defective mitochondrial proteolysis results in disease conditions most notably, mitochondrial diseases, neurodegeneration and cancer. Here, it will be discussed how mitochondrial proteases and mitochondria stress signalling impact cellular homeostasis and determine the cellular decision to survive or die, how these processes may impact disease etiopathology, and how modulation of proteolysis may offer novel therapeutic strategies.

1. INTRODUCTION

Mitochondria constitute a dynamic organellar network which fulfils key roles in the cellular homeostasis. Besides their role in the synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation, mitochondria regulate metabolic pathways, calcium homeostasis heme and FeS synthesis and apoptosis. Mitochondria present a sub-compartmentalised structure in which the outer membrane (OM) and inner membrane (IM) delineate the intermembrane space (IMS) and the mitochondrial matrix. All four elements, host categories of molecular complexes which fulfil specific roles to support mitochondrial functionality. These include complex systems for protein import and sorting, transport of metabolites, mitochondrial DNA (mtDNA) replication and expression, oxidative phosphorylation, metabolism and protein and organelle quality control **[1,2]**.

Mitochondria is believed to have evolved following a symbiotic process between α-proteobacteria and a pre-eukaryotic host **[3]**. In this process mitochondria have outsourced most of their genome to the nucleus. Thus, the mitochondrial proteome comprising over a thousand proteins are encoded in the nuclear DNA, synthesised in the cytoplasm and imported and processed in the mitochondria to fulfil their functions. The human mitochondrial DNA only encodes for thirteen respiratory subunits, two rRNAs and twenty-two tRNAs **[2,4]**. Therefore, at the core of the cellular bioenergetics reside chimeric enzyme complexes encoded in two different genomes. This evolutionary symbiotic organisation implies that the mitochondria work in correlation with the nucleus and other cellular components to support the overall cellular homeostasis providing

energetic and metabolic support for the cellular function and benefiting from the gene expression and protein synthesis that supply the majority of its proteome.

Given the significant roles played by the mitochondria in the cellular homeostasis its structural and functional components are subject to tight regulation and quality control mechanisms. In the process of protein import, proteases and chaperones are involved in the correct processing and folding of newly acquired components in a process termed **molecular quality control**. Mitochondria under stress from its own respiratory activity or from external stressors accumulates damage at molecular level which is also resolved through molecular quality control. When the damage accumulated cannot be resolved at this level, damaged mitochondrial components are separated via fission and dysfunctional organelles are removed through lysosomal degradation in the process of **organellar quality** control termed mitophagy.

Mitochondria under stress trigger a plethora of stress signalling mechanisms involving cellular changes that are designed to restore mitochondrial function and include changes in gene expression alongside protein synthesis and metabolic reprograming. With the significant increase in our understanding of mitochondrial stress signalling and quality control mechanisms we are now able to distinguish that mitochondrial dysfunction may lead to mitochondrial unfolded protein response (mtUPR), integrated stress response (ISR), mitochondrial import stress responses and mitophagy which were discussed in detail previously **[5]**. mtUPR and ISR are summarised in Figure 1 as they will appear more frequently in this review. Importantly, mitochondrial stress signalling may act in a cell nonautonomous and transgenerational manner **[5]**.

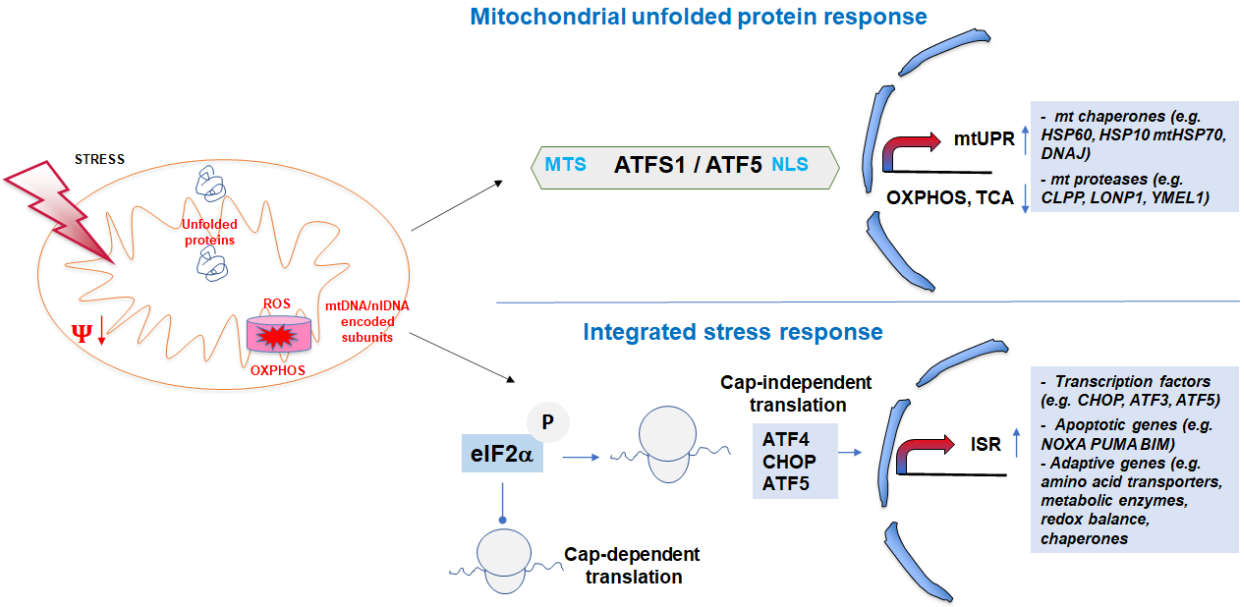

Figure 1. Mitochondrial stress signalling.

Mitochondrial stress is triggered by multiple factors including, accumulation of oxidative damage, accumulation of unfolded proteins, imbalance in the mitochondrially versus nuclear encoded respiratory subunits, loss of mitochondrial potential. The key modes of stress signalling mechanisms triggered by mitochondrial dysfunction include the **mitochondrial unfolded protein response (mtUPR)** and the **integrated stress response (ISR)**. The transduction system of these signals comprises different transcription factors and they lead to distinct transcriptional responses and cellular outcomes. The mtUPR is believed to be transduced by cytoplasmic molecules that possess both a mitochondrial targeting sequence and a nuclear targeting sequence. This is normally targeted to the mitochondria where it is rapidly degraded, while in stress it accumulates in the nucleus where triggers expression of mitochondrial proteases and chaperones that are meant to deal with the load of unfolded proteins. In *C elegans* this transcription factor has been identified as ATFS1 while in mammalian systems it is believed that this role may be played by ATF5. The ISR operates by inducing a general reduction in cap-dependent translation as a consequence of eIF2a phosphorylation, while a set of transcription factors ATF4, CHOP, ATF5 are selectively translated via cap-independent translation. Consequent activation of ISR includes transcription of factors involved in the ISR itself as well as genes that may work as pro-death or pro-survival factors.

Other quality control mechanisms of the mitochondria involve the proteasome, which supports clearance of damaged proteins in the process of outer mitochondrial membrane associated

degradation (OMMAD) and removal of proteins which fail the mitochondria import process and end up accumulating in an unfolded state in the cytoplasm **[5, 6]**.

Mitochondrial proteases, also known as mitoproteases, are the functional category of molecules in the mitochondria fulfilling the protein processing and molecular quality control with roles that expand beyond these aspects into organellar quality control, metabolic regulation, mitochondria--nucleus communication and cell death signalling. According to their catalytic moiety mitochondrial proteases are cysteine-, serine- and metallo- proteases. Besides 20 'intrinsic' mitochondrial proteases which perform their work in the mitochondria, additional classes of molecules termed 'pseudo- and transient – mitoproteases' have been identified. They may have similarities to proteases but lack catalytic activity and may support their function or they may transiently translocate to mitochondria to perform catalytic activities. Mitochondrial proteases are highly conserved from bacteria to eukaryotes and many of their molecular specificities and roles have been reviewed previously **[7,8]**.

Here, it will be highlighted how intrinsic mitochondrial proteases influence cellular homeostasis pathways and how their loss of function or gain of function may result in mitochondrial stress signalling and the consequences of these processes for the cellular faith in health and disease.

2. PROTEASES INVOLVED IN PROTEIN PROCESSING

Mitochondrial proteins are synthesized with a mitochondrial-targeting motif which facilitate their import into the mitochondria through the TOM/TIM complex. These pre-sequences are proteolytically removed in a process of maturation before they locate to their functional compartment. The proteolytical processing involve typically an initial cleavage by the mitochondrial processing peptidase (MMP) followed by a second cleavage by the inner mitochondrial membrane protease complex (IMMP) or the mitochondrial intermediate peptidase (MIP) **[9,10]**.

The **mitochondrial processing peptidase** (MMP) is a heterodimer protease comprising the PMPCA subunit which is involved in substrate recognition and the PMPCB proteolytic subunit. MMP dysfunction induces proteotoxic stress by accumulation of non-processed preproteins which results in an increase in mitochondrial potential and oxygen consumption **[11]**. Reduction in MMP activity leads to increased mitophagy via the reduction of PINK1 direct processing **[12]**. PMPCB loss of function is linked with mitochondrial disease associated with neurological phenotypes (Table 1). In addition, PMPCB inhibition and associated mitochondrial dysfunction have been found to increase apoptosis in hepatocellular carcinoma cells which rely on Wnt/b-catenin pathway for their survival **[13]**.

The **mitochondrial intermediate peptidase** (MIP), also termed octapeptidyl aminopeptidase 1 (Oct1), is involved in a secondary processing step, after the initial cleavage of the mitochondrial targeting motif by MMP, for a subclass of pre-proteins, and removes an octapeptide at the N terminus to provide a stable, fully mature protein **[9, 14]**. Some of the functions of MIP include maturation of mitochondrial peroxiredoxins (Prdx3 and Prdx5) which play a key role in keeping the H_2O_2 at physiological level **[15]**. MIP has also been linked to the coenzyme Q biosynthesis, a pathway essential for mitochondrial respiration **[16].** In mice, the MIP deletion leads to embryonic lethality and in humans its loss of function is linked to mitochondrial syndromes (Table 1).

The **inner mitochondrial membrane protease-like** (IMMP) is an inner membrane complex formed by the dimeric complex of IMMP1L (IMMP subunit 1) and IMMP2L (IMMP subunit 2) and is involved in processing of proteins for maturation and targeting into the inner membrane or the intermembrane space. The two subunits appear to have non-overlapping target specificities **[17]**.

The *IMMP1* gene is situated in the downstream region of the transcription factor PAX6 gene on chromosome 11p13. Genetic or epigenetic perturbations in this region have been linked to a range of disorders including aniridia, metabolic disease and mental health syndromes (Table 1).

Substrates have been identified in yeast and include cytochrome c oxidase subunit 2 (Cox2p), cytochrome b2 (Cyb2p), NADH-cytochrome b5 reductase (Mcr1p) and mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) **[18]**.

IMMP2L is required for the processing of the mitochondrial translocase TIM23, cytochrome c1, as well as the proteolytic maturation of glycerol-3-phosphate dehydrogenase 2 (GPD2) and the apoptosis inducing factor (AIF) **[19-21]**. The GPD2 processing favours the enhancement of NAD+ and favours mitochondrial phospholipid synthesis, both pathways that contribute to reducing senescence. IMMP2L contributes to the AIF processing which generates the truncated form that is able to translocate to the cytoplasm and promotes apoptotic cell death. Thus, reduced levels of IMMP2 or its inactivation, lead to metabolic reprogramming, cell cycle arrest and blockage of apoptotic cell death converging to senescence **[21]**. Studies of *Immp2l*$^{Tg(Tyr)979Ove}$ mutant mice indicate that both male and female are infertile and present early ageing phenotypes associated with mitochondrial dyshomeostasis, which include high mitochondrial superoxide accumulation, mitochondrial hyperpolarisation and higher levels of ATP. Interestingly, full *Immp2l*^{KD} knock-out mice do not recapitulate the mitochondrial and age-related phenotypes of the truncation mutant mice. In addition, the *Immp2l*^{KD} knock-out mice could not confirm IMMP2L proteolytic targets that have been verified in models other than the truncated *Immp2l*$^{Tg(Tyr)979Ove}$ mice. Thus, different mechanistic changes may be associated with the protein truncation or partial IMMP2L loss of function as compared to the full knock-out of the gene **[22-24]**.

Genetic disruptions, particularly deletions, of *IMMP2L* are associated with susceptibility for neurodevelopmental (autism, Tourette syndrome) and adult neurological disorders (schizophrenia, Alzheimer's) (Table 1).

The class of peptidases involved in processing and maturation of pre-proteins also comprises the **Met aminopeptidase 1D** (METAP1D/ MAP1D) which processes the N-terminal Met residue of proteins encoded in the mitochondria; seven of the thirteen mitochondrially encoded proteins being predicted as it substrates (ATP synthase subunit 8, NADH dehydrogenase subunits 1, 4L, and 5, cytochrome *c* oxidase subunits 2 and 3 and cytochrome *b*) **[25]**. Genetic variation of METAP1D was correlated with penetrance of Leber's Hereditary Optic Neuropathy while gain of function, through overexpression, appears to be linked to tumorigenesis ability (Table1).

The processing of pre-proteins by MPP may be followed in some cases by the removal of a single destabilizing N-terminal amino acid such as tyrosine or phenylalanine by the **X-Pro aminopeptidase 3** (XPNPEP3) **[26]**. Patients with XPNPEP3 variants present complex mitochondrial syndromes with combinations of nephronophthisis leading to severe kidney disease, neurological phenotypes and cardiomyopathies (Table 1). XPNPEP3 mutant variants leading to these phenotypes are associated with various mitochondrial function impairments including reduced respiration through Complex I and Complex IV dysfunction and increased apoptosis. There is however uncertainty as to what extent the kidney ciliary disease is determined selectively by the mitochondrial XPENP3, or the cytoplasmic isoform may pay a role **[27-29]**. Interestingly, mitochondrial XPNPEP3 has been reported to have an antiapoptotic function via the TNF-TNFR2-JNK pathway, and to support tumorigenesis being upregulated in cancer downstream of Wnt/β-catenin pathway **[30,31]**.

3. PROTEASES INVOLVED IN THE QUALITY CONTROL OF UNFOLDED, MISFOLDED AND DAMAGED PROTEINS

Protein quality control is the first step in avoiding accumulation of misfolded and/or damaged proteins in the mitochondrial compartments. Peptides resulting from protein processing and import, proteins that fail to fold properly upon import or proteins that accumulate damage during their function, or due to endogenous or exogenous stresses need to be removed through proteolytic degradation.

The **mitochondrial oligopeptidase M** (MEP) also known as neurolysin (EC3.4.24.26) resides in mitochondrial matrix and hydrolyses oligopeptides **[32]**. MEP is a zinc metallopeptidase involved in degradation of presequences, presequence fragments and hydrophobic fragments of amyloid-beta (Aβ) **[33]**. Besides the mitochondrial isoform, neurolysin gene can produce a shorter transcript, giving rise to a cytoplasmic isoform, which plays important roles in the neuroendocrine signalling **[32]**.

The neurolysin KO mice do not present overt striking phenotypes, but they show abnormalities in glucose metabolism, lower oxidative capacity due to lower activity or respiratory complex enzymes, and lower physical exercise endurance **[34, 35]**. Linked to these findings it has been identified that MEP is involved in formation of respiratory complexes and higher order supercomplexes in the mitochondria. At cellular pathologic level, MEP appears upregulated in acute myeloid leukemia (AML) cells alongside other mitochondrial components, where is required for the growth and viability of the cells **[36]**.

The **pitrilysin metallopeptidase** (PITRM1) is located in the mitochondrial matrix and is involved in digesting oligopeptides, including mitochondrial targeting sequences resulting from the process of precursor protein import and maturation. Loss of PITRM function has been shown to lead to severe mitochondrial dysfunction, loss of respiratory activity, accumulation of presequence peptides and consequent mitochondrial stress induced responses, mtUPR and ISR via DELE1 **[37, 38]**. Reduced PITRM1 function has been found as a risk factor for Alzheimer's disease (AD). Importantly, PITRM1 appears to have role in degrading Aβ in the mitochondria and its function appears to have a wider influence in decreasing Aβ in non-mitochondrial compartments **[39, 40]**. Besides the association with AD, the peptidase loss of function induces childhood neurological syndromes with spinocerebellar ataxia, mental retardation cerebellar atrophy (Table 1). Although this syndrome associates with Aβ accumulation there is a strong possibility that accumulation of unprocessed presequences and upregulation of stress responses may contribute to the neurological pathology.

The **High Temperature-Requirement Protein A2 (HtrA2)** is an intermembrane space serine protease which has been established as a signalling regulator in the nervous system, cancers and muscle disorders. HtrA2 has been first involved with cellular stress signalling in the context of apoptotic cell death. Under stress stimuli HtrA2 is released into the cytoplasm where it triggers caspase dependent apoptosis **[41]**. Non-apoptotic forms of cell death, necroptosis and partanathos, have also been shown to be modulated by HtrA2 **[42, 43]**. The cell death roles of HtrA2 are modulated by interactions with other mitochondrial proteins, HAX1 and PARL playing a key role in this process **[44]**.

In normal conditions HtrA2 maintains its residence in the IMS and is involved in removal of unfolded and unwanted proteins. Loss of HtrA2 function results in accumulation of unfolded proteins, defects in the electron transport chain and respiration as well as accumulation of oxidative species **[45]**. Despite these roles in maintenance of mitochondrial proteostasis and homeostasis, loss of HtrA2 does not appear to induce an upregulation of mtUPR markers Hsp60, Clpp **[45, 46]**. Instead, HtrA2 deficiency has been shown to induce cellular dyshomeostasis through mitonuclear imbalance and dysregulated mitochondrial biogenesis **[46]**. Mice lacking functional HtrA2 indicate distinct mechanisms for HtrA2 linked mitochondria dysfunction to lead to cell death in different tissues. In the brain, HtrA2 loss of function leads to the specific activation of the ISR which contributes to neurodegeneration, parkinsonism phenotypes and premature death **[45, 47, 48]**. Restoring the HtrA2 function in the brain rescues the early death phenotype and is associated with ageing phenotypes in the other organs **[49]**. Notably, mice with rescued HtrA2 in neurons present cardiac ageing characterised by heart enlargement with left ventricular atrophy, associated with decreased glucose metabolism, increased mtDNA deletions and increased autophagosome activity in heart tissues. Ultimately being less efficient in synthesizing ATP, these mitochondria can't cope with the energy demands of the cardiac tissue leading to cardiac failure and other organ abnormalities indicating early ageing **[49]**.

Novel roles have been discovered for HtrA2 in many cellular processes: i) HtrA2 is involved in supporting basic autophagy and the ability to activate autophagy in response to stresses **[50]**; ii)

HtrA2 regulates biogenesis via the GSK3b/PGC-1a pathway **[51]**; iii) HtrA2 has been shown to modulate innate immune responses by restricting the activation of ASC dependent NLRP3 and AIM2 inflammasomes, and by controlling the threshold where nuclear DNA damage starts to activate innate immunity via cGAS-STING signalling **[52, 53]**. In cancer, HtrA2 appears to be overexpressed and a tumor-promoting marker in most malignancies except for prostate and non-small lung cancer **[54]**. These indicate the involvement of HtrA2 in multiple signal transduction mechanisms which impact a range of disease conditions from cancer to neurodegeneration.

The **caseinolytic peptidase** (CLPP) is a serine peptidase, which resides in the mitochondrial matrix, highly conserved throughout bacteria and eukaryotes. In the absence of its adaptor ATPase, which in human mitochondria is CLPX (ATP-dependent Clp protease ATP-binding subunit clpX like), entry into its proteolitic chamber is restricted to a narrow portal preventing the indiscriminate degradation of mitochondrial proteins **[55]**. The human CLPP forms heptameric rings which present peptidase activity towards small peptides and assembles in the presence of the CLPX unfoldase into a tetradecamer with CLPX assembled at both ends to became a fully active protease **[56]**. Over 100 proteins have been identified as CLPP substrates and interactors in various experimental approaches **[57]**. They group in a range of functional pathways reflecting the overall importance of CLPP in mitochondrial homeostasis. These include structural components of the mitoribosome, TCA-cycle, amino acid metabolism, aminoacyl t-RNA ligase, mitochondrial import receptors as well as subunits of the respiratory complex. However, the abundance of substrates brings into question the specificity of the protease **[58]**. Despite the wide range of mitochondrial targets there is now evidence that at cellular level CLPP is involved in the surveillance and repair of complex I **[59]** and in maintenance of mitochondrial translation via mitoribosomal maturation **[60]**.
CLPP mutations have been linked to the Perrault syndrome which is characterised by sensorineural hearing loss and impaired fertility **[61]**. The CLPP KO mouse recapitulates Perrault syndrome phenotypes its loss of function increasing mtDNA instability which contributes to an innate immune response via cGAS-STING pathway **[62, 63]**.

Low levels of CLPP have also been seen in Parkinson's patients and PD murine models and CLPP dysregulation has been overall seen as important in neurological disease **[64, 65]**. CLPP has been found to be overexpressed in hematologic malignancies and solid tumours (Table 1). In this context, genetic and pharmacological inhibition and overactivation are being explored to exploit the CLPP activity modulation as an anticancer therapy **[66]**.

The involvement of CLPP in mitochondrial stress signalling has been first documented with respect to mtUPR in *C elegans* where ATFS-1 has been shown to be the signal transducing element **[67]**. However, the mtUPR in mammalian cells has been indicated to possess a higher level of redundancy with CHOP, ATF4, ATF5 being reported as key players in the signal transduction **[5]**. The CLPP involvement in mammalian mtUPR is yet to be documented as in a mouse model of mitochondrial cardiomyopathy due to deficiency in DARS2, CLPP did not appear to be involved in mtUPR **[68]**. Importantly for CLPP signalling mechanisms, CLPP overactivation with iminopridones appears to lead to an atypical ISR which results in cell death **[69]**.

In human mitochondria, the **mitochondrial ATP-dependent protease LON** (LONP1) is a hexameric complex, which resides in the mitochondrial matrix where it has been shown to perform protein quality control, support mitochondrial gene expression and to be involved in mitochondrial stress signalling. LONP1 is involved in protein quality control both through its proteolytic activity by cleaving unfolded and damaged proteins and by acting as a chaperone, which is critical for the assembly of the mitochondrial respiratory system and regulation of its function. Its levels are increased in response to ER stress, oxidative stress and hypoxia, therefore LONP1 has been designated as a stress responsive protein **[58, 70]**. LONP1 directly interacts with HSP60 and mtHSP70 (mortalin) and regulates their stability under oxidative stress **[71]**. LONP1 has targets involved in metabolic pathways namely aconitase (ACO2) and glutaminase C (GAC), in the TCA cycle, and in mitochondrial cholesterol transfer by regulating the turnover of steroidogenic acute regulatory protein (StAR) **[58, 70]**. LONP1 is also important for regulation of pyruvate dehydrogenase (PDH) activity by selectively degrading modified PDH subunits, as well as isoforms of PDH kinase and/or phosphatases **[72]**. LONP1 has been shown to directly be

involved in reducing ROS production by degrading the Complex I ROS generating domain **[73]** and it is required in hypoxic conditions to minimize ROS production and to sustain the cellular adaptation to a hypoxic environment **[70]**. In addition, it has been implicated in removal of oxidized proteins which has been specifically evidenced for aconitase. LONP1 shows the ability to degrade the oxidized aconitase early in the oxidation process while it does not appear to be able to degrade aggregates of the severely oxidatively damaged protein **[74]**. The role of LONP1 in stresses associated with an enhanced oxidative stress lies in reducing the toxicity of the oxidative environment and the probability of protein aggregates accumulation. Loss of LONP1 function is leading to the perturbation of mitochondrial homeostasis at multiple endpoints including protein quality control, mitochondrial stress signalling and mitochondrial bioenergetics **[75]**. Identification of additional directly oxidized protein substrates would strengthen the evidence for LONP1 as a being a protease sensing and degrading oxidatively damaged components in the mitochondria in the wider sense.

LONP1 has a recognised role in maintenance of mtDNA. It is a component of the mitochondrial nucleoids where it has the particular ability to bind to DNA and to interact with other protein nucleoid components implicated in mitochondrial transcription. Among the nucleoid components, LONP1 can degrade the free, phosphorylated form of TFAM **[76]**. LONP1 loss of function results in partial loss of mtDNA, blocks mitochondrial translation, leads to accumulation of its unprocessed targets and triggers the integrated stress response **[77]**.

In mitochondrial stress signalling and organellar quality control LONP1 has been shown to intervene by rapidly degrading ATFS1 the signal transducer of mtUPR in *C elegans* and PINK1 preventing its accumulation on the outer membrane which is the initial step in the PINK1/Parkin mitophagy process **[12, 78]**. In *Drosophila*, loss of Lon function, (LONP1 homolog) alters the respiratory system activity, and leads to accumulation of unfolded proteins inducing the mtUPR. Interestingly, overexpression of ClpP rescues the Lon protease inactivation in flies **[79]**.

Mutations in LONP1 are associated with CODAS syndrome and its upregulation has been seen in multiple malignancies (Table 1).

The **ATP23** is an intermembrane space metallopeptidase which has been mainly studied in yeast. The yeast work has identified a dual role for ATP23 in the assembly of the ATP synthase F1/F0 working both as a peptidase and a chaperone in interaction with the chaperone ATP10 and prohibitins. ATP23 has also been shown to work in a pathway that regulates the mitochondrial phospholipids. While we may hypothesise that some of these functions would be evolutionarily conserved, more work would be needed to establish ATP23 functions in mammalian systems **[80-82]**.

4. MEMBRANE INTEGRATED ATP-ases ASSOCIATED WITH DIVERSE CELLULAR ACTIVITIES

Membrane integrated ATP-ases associated with diverse cellular activities, comprise inner membrane protease complexes with their active sites facing the matrix (mAAA) and the intermembrane space (iAAA), respectively. The human mAAA can be either a hetero-oligomer of **SPG7/ paraplegin** and **AFG3-like protein 2** (AFG3L2) or a homo-oligomer comprising only AFG3L2 subunits. The subunit composition of the mAAA appears to be important for the substrate specificity and efficacy of proteolysis **[83]**.

SPG7 encoded by the spastic -paraplegia-gene 7 has been found to present loss of function mutations associated with hereditary spastic paraplegia (HSP) which may be complicated by optic atrophy, peripheral neuropathy and cerebellar and cortical atrophy **[84]**. SPG7-KO mouse models present long spinal tracts axonal degeneration and cerebellar degeneration phenotypes which were attributed principally to mitochondrial abnormalities **[85, 86]**.

In the study of cellular and animal models of SPG loss of function, it has become apparent that the mAAA protease is involved in the quality control of the respiratory complexes, removing damaged or misfolded proteins in the inner membrane, its loss of function leading to impaired assembly of the respiratory system and defective respiration **[87]**. An additional processing function of newly imported proteins has been identified in yeast, as a step required in the assembly of mitochondrial ribosomes and consequently influencing mitochondrial translation **[88]**. The defective processing and synthesis of respiratory complex subunits may lead to imbalance in the mitochondrial versus nuclear encoded respiratory subunits which may be reflected in the complex I deficiency observed in HSP and the mitochondrial stress signalling associated with SPG-7 loss of function **[89, 90]**. Consequently, upregulation of mtUPR has been reported in *C. elegans* upon downregulation of spg-7 and oxidative stress and senescence pathways have been observed in SPG7-mutant iPSC derived neurons **[91, 92]**. Interestingly, the decrease in mitoribosomal translation, has been indicated to lead to remodelling of mitochondrial solute carriers and transporters **[86]**. The SPG7 mutations lead to mtDNA deletions, increased mitochondrial mass and mitochondrial hyperfusion **[93]**. The role of SPG7 in regulating cell death has been attributed to its ability to modulate the mitochondrial transition pore via the regulation of the mitochondrial calcium uniporter (MCU) regulator 1 (MCU-R1) and MCU complex assembly which leads to lower basal mitochondrial calcium concentration **[94]**.

The AFG3L2 has protein degradation and protein processing attributes as part of the m-AAA complex, being involved in nascent protein maturation, self-processing through an autocatalytic function and contributing to the maturation of the SPG7 subunit **[95, 96]**. Mutations disrupting AFG3L2 activity in human have been associated with the degeneration of Purkinje cells in autosomal dominant spinocerebellar ataxia (SCA) type 28 (SCA28) and spastic ataxia type 5 (SPAX5) and progressive external ophtalmoplegia **[97, 98]** (Table 1).

The neurological phenotypes linked to AFG3L2 deficiency have been attributed to a combination of cellular dysfunctions that occur in neuronal cells and glial cells which have been uncovered in human cells from AFG3L2 mutations patients, murine and *Drosophila* loss of function models. Interestingly, a wide range of stress responses have been identified in AFG3L2 impairment. In neurons from murine models and in human fibroblasts, AFG3L2 loss of function leads to mitochondrial dysfunction and activation of OMA1 a generating stress sensing mechanism feeding into the integrated stress response which is protective for SPAX5 **[99, 100]**. OMA1 activation dependent on AFG3L2 mutation appears to be linked to a mitochondrial protein synthesis stress response resulting in loss of mitochondrial ribosomes and consequent remodelling of membrane ultrastructure **[101]**. The neuronal cell death related to AFG3L2 deficiency has also been related to the increased neuronal vulnerability to Ca^{2+} induced MPTP opening by modulating the assembly of the MCU complex and its key subunit EMRE **[102]**.

In glia, astrocytes with AFG3L2 impairment show an abnormal mitochondrial network, although mitochondrial respiration is not affected, and activation of inflammation signalling and a metabolic stress response. These have been also associated with accumulation of necroptotic markers which may be due to both neuronal and non-neuronal cell death **[103]**. In oligodendrocytes, AFG3L2 impairment triggers mitochondrial abnormalities, however only the full ablation of the m-AAA complex leads to cell death indicating that the sensitivity to m-AAA protease deficiency varies between cell types **[104]**.

AFG3L2 has been found to be transcriptionally upregulated, alongside SPG7, LONP1 and YME1L1 as part of a selective transcriptional response to accumulation of its proteolytic target StAR during steroidogenesis termed ‘StAR overload response’. In this selective transcriptional process, the elements of putative mtUPR, CLPP and HSP60 are not affected **[105]**. Despite this, in *Drosophila* the mitochondrial dysfunction due to AFG3L2 deficiency leads to mtUPR and autophagy/mitophagy **[106]**.

The iAAA protease is formed from subunits of the ATP-dependent protease **yeast mitochondrial DNA escape 1-like** (YME1L1, also known as YME1L) whose key function is the processing of the dynamin-like GTPase, OPA1. OPA1 mediates inner membrane fusion and controls cristae morphogenesis thus playing a critical role in regulation of respiratory capacity and apoptosis.

OPA1 regulatory processing is undertaken by YME1L1 alongside the OMA1 protease to regulate mitochondrial fission and fusion **[107]**. Specifically, YME1L1 loss of function leads to increased fragmentation as a result of increased mitochondrial fission **[108]**. Besides OPA1, YME1L1 targets include subunits of protein translocases, mitochondrial lipid transfer proteins, and proteins functionally linked to mitochondrial metabolism. Its proteolytic function is regulated by mTORC1 and the phosphatidylethanolamine levels in the mitochondrial membranes **[109]**. YME1L1-mediated proteolysis limits mitochondrial biogenesis upon mTORC1 inhibition by degrading protein translocases and lipid transfer proteins and metabolically rewires mitochondria. Thus YME1L1-mediated proteolysis has been indicated to broadly preserve mitochondrial proteostasis under normoxia and to reshape the mitochondrial proteome in response to hypoxia **[109]**.

YME1L1 is an oxidative stress responsive protease, changing its conformation in response to oxidative stress in vitro and being degraded under oxidative stress in vivo, which removes its protective regulation of proteostasis and enhances cellular sensitivity to death **[110, 111]**.

5. PROTEASES INVOLVED IN MITOCHONDRIAL STUCTURE, REMODELING AND DYNAMICS

The **o**verlapping with **m**-**A**AA protease (OMA1) inner membrane metalloprotease has emerged as a critical sensor of mitochondrial stress and key initiator of mitochondrial stress response mechanisms. Mechanistically, the C-terminus of the protein, oriented towards the intermembrane space performs the catalytical activity while the N-terminus domain residing in the mitochondrial matrix acts as a mitochondrial potential sensor **[112]**. The proteolytic function of OMA1 has been proposed to be regulated by reduction-oxidation modifications of its two evolutionarily conserved cysteines **[113]**. OMA1 activation through loss of mitochondria potential also leads to self-cleavage and degradation **[114, 115]**.

Activation of OMA1 may be initiated by a variety of mitochondrial stressors inducing loss of mitochondrial potential, perturbation in mitochondrial import, perturbation in OXPHOS activity, as well as stresses initiated outside the mitochondria like DNA damage **[116, 117]**. Interestingly, the response to stress can manifest at subdomain levels with OMA1 detecting local fluctuations in mitochondrial potential, thus being able to work on restoring homeostasis while the mitochondria is still able to perform its functions **[118]**. However, a threshold has been identified for the decrease of mitochondrial potential before OMA1 becomes activated **[119]**. Whether such threshold operated for localised activation of OMA1, remains to be seen. The proteolytic function of OMA1 is the key process that involves this protein in cellular responses to stress which may progress towards cell death or cell survival depending on the specific stress context and in a cell type specific manner. These pathways include apoptosis, development and cellular differentiation.

OMA1 is involved in processing OPA1 from its fusion active L-OPA1 into its fusion inactive S-OPA1 form, which results in remodelling of mitochondria to present a fragmented morphology, and impaired mitochondrial bioenergetics **[120]**. This enhances the cell's susceptibility for increased stress response via apoptosis, autophagy, and unfolded protein response. In this context, decreased expression of OMA1 in murine models of neurodegeneration acts as a prosurvival mechanisms, stabilizing the mitochondrial network against fragmentation, preventing loss of mtDNA and delaying neuronal death **[121]**.

OMA1-initatiated stress response is perhaps one of the best characterised for mitochondrial proteases. Upon loss of mitochondrial potential, activated OMA1 cleaves DELE1, a protein previously linked to apoptosis. Cleaved DELE1 migrates to the cytosol where it interacts with HRI and activates eIF2α kinase activity and its downstream ATF4 and CHOP dependent ISR signalling **[122, 123]**. Activation of this pathway may be pro-survival or pro-death depending on the type of mitochondrial stress and the persistence of the stress response activation. OMA1-DELE1-ATF4 ISR activation protects against ferroptosis in OXHOPS deficient cardiac tissue and protects against cardiomyopathy **[124]**. A stress signalling mechanism mediated by OMA1

independently of OPA1 and DELE1 is induced in response to DNA damage and comprises an OMA1 metabolic shift towards glycolysis which acts as a pro-survival mechanism **[117]**. This may potentially remove the need for mitochondrial OXPHOS based ATP production which competes for metabolic support with DNA repair mechanisms **[5]**. Although the exact mechanism of how OMA1 mediates this metabolic shift is not clear, this finding strengthens the argument that OMA1 may play an important role in tumorigenesis and the cellular response to cancer therapy.

Presenilin associated rhomboid like (PARL), also named 'PINK1/PGAM5 associated rhomboid like protease' **[125]**, is an evolutionarily conserved protease located in the inner mitochondrial membrane with fundamental roles in maintaining mitochondrial and consequently cellular homeostasis. Investigations of the PARL KO mice have indicated that PARL loss of function in the nervous system, whether in tissues specific or systemic KO, leads to respiratory chain defects at the level of complex II and coenzyme Q, and is associated with calcium dyshomeostasis and cristae remodelling. Ultimately, PARL loss of function leads to a Leigh-like syndrome with neuronal loss through a necrotic cell death process **[126]**. PARL deficiency has also been demonstrated to lead to infertility by arrest of spermatogenesis and ferroptosis **[127]**.

Key roles in maintenance of mitochondria homeostasis, cellular health and cell death mechanisms are achieved through its main downstream effectors/interactors and proteolytical targets. PARL has been identified to control mitochondrial morphology and integrity upstream of OPA1 through genetic interaction and potentially direct cleavage, which in mammalian systems may occur in parallel with redundant mechanisms controlling mitochondrial cristae remodelling and homeostasis **[128,129]**.

Genetic interactions have initially shown that rhomboid-7 the Drosophila homolog of PARL operates upstream of *pink1* and *parkin* to modulate mitophagy **[130]**. This has been confirmed by evidence that in mammalian systems PARL cleaves PINK1 in healthy mitochondria thus controlling the level of PINK1 and mitophagy **[12, 131]**. When mitochondria are under stress and lose membrane potential, PINK1 accumulates on the outer membrane and initiates PINK1/Parkin dependent mitophagy. Under mitochondrial stress PARL cleaves preferentially another target, PGAM5 which may work as a pro-survival or pro-death mechanism depending on the stress level. In mild stress, PGAM5 maintains mitochondrial homeostasis inducing mitochondrial biogenesis and mitophagy. In severe stress, PGAM5 induce cell death enhancing mitochondrial fission and regulating multiple death signals **[132-134]**. Thus, PARL processes PINK1 and PGAM5 depending on the stress level and in an inversely correlated manner; this was attributed to the differences in import and processing of these proteins in the different mitochondrial states **[135]**.

PARL undergoes constitutive and regulated cleavage processes that result in the release of a 25 ammino acid peptide which has a strong nuclear localisation sequence and is able to translocate to the nucleus **[136]**. This process appears to be relevant in development and is cell type specific. Although it was hypothesised that this peptide may have roles in mitochondria nucleus communication, there are no further studies to analyse this possibility in more depth **[137]**.

Together these findings indicate that PARL-dependent cleavage of substrates and the downstream events act as a signalling hub for mitochondria stress and quality control. Differences in the way PARL influences mitochondrial ultrastructure and processes substrates have been evidenced between yeast, *Drosophila* and mammalian systems indicating that PARL may have a more complex processing and level of redundancy in mammalian cells **[125]**.

The **ubiquitin specific protease 30** (USP30) has been initially identified as a mitochondrial outer membrane protease which works as a deubiquitinating enzyme and plays a role in the maintenance of mitochondrial morphology and opposes the PINK1/Parkin dependent mitophagy **[138, 139]**. Besides the mitochondria, USP30 is localised to the peroxisomes where it works to diminish the pexophagy process. USP30 appears to have a high preference for cleaving ubiquitin chains at Lys6 **[140]**.

Knockout or inhibition of USP30 have been demonstrated to protect against dopaminergic neuronal loss in a mouse model of PD suggesting that USP30 inhibition may harbour therapeutic potential in PD **[141]**. Positive effects of reducing USP30 activity have been observed in

Alzheimer's and in ischemia- reperfusion and wound healing experimental models **[142-144]**. Depletion of USP30 appears to sensitise cancer cells to apoptotic cell death via BAK/Bax pathway thus supporting a role for USP30 in cancer prognosis **[145]** (Table 1). Given the role of USP30 in mitophagy and pexophagy it has been proposed that modulating its activity may be relevant in Parkinson's disease, peroxysome biogenesis disorders and pulmonary disorders **[146]**.

Beta Lactamase-like protein (LACTB) is a mammalian mitochondrial protein residing in the intermembrane space, whose roles in cellular homeostasis are only beginning to come light **[147]**. LACTB forms filaments in the intermembrane space which are thought to play a role in preserving mitochondrial structural organisation and to be the core factor that supports LACTB proteolytic activity and physiological roles **[148]**. LACTB has been found to act as a tumour suppressor in breast cancer cells by modifying lipid metabolism and shifting the cellular fate from mitosis to a differentiated phenotype **[149]**. From the initial discovery that LACTB acts as a tumour suppressor in breast cancer, numerous studies have identified that LACTB has reduced levels in tumour cells and acts as a tumour suppressor by reducing proliferation, invasion and angiogenesis in multiple types of cancer (Table 1). The mechanism comprises a reduction of the levels of mitochondrial phosphatidylserine decarboxylase, which is involved in the synthesis of mitochondrial phosphatidylethanolamine which appears to be determined by the role of LACTB filaments in modulating the ultrastructure of the mitochondria controlling the distance between the outer membrane and inner membrane and consequently modulating the ability of phosphatidylserine decarboxylase to convert phosphatidylserine to phosphatidylethanolamine. The proteolytic activity of LACTB is modulated by the post-translational modification lysine succinylation leading to reduced proteolytic activity which favours carcinogenesis **[150]**. There is also evidence that LACTB may play the opposite role in pancreatic and nasopharyngeal cancer **[151, 152]**.

Importantly, LACTB has been found to be associated with mitochondrial supercomplexes and its level in these supercomplexes decreases with exercise **[153]**. A role for LACTB in metabolism is also supported by the fact that *Lactb* transgenic mice present an obesity phenotype **[154]**.

LACTB expression results in increase of mitochondrial reactive oxygen species, cell cycle arrest in G1 phase and increase of DNA oxidation, all converging to activation of intrinsic caspase-independent cell death pathway **[155]**. In addition, LACTB promotes autophagy and inhibits proliferation in part through the PI3K/AKT/mTOR signalling pathway **[156]**.

6. PROTEASES WITH OTHER FUNCTIONS

***O*-sialoglycoprotein endopeptidase-like protein 1** (OSGEPL1) (yeast Kae1/Qri7 homolog) was identified as the human mitochondrial enzyme which mediates the N^6-threonylcarbamoyladenosine (t^6A) modification for five mitochondrial tRNAs, mt-tRNAs for Ser, Thr, Asn, Ile, and Lys **[157, 158]**. Acetylation as posttranslational modification appears to be a rapid way to regulate OSGEPL1 activity. OSGEPL1 has an intrinsic ATPase activity which depends on Fe3+ **[159]**.

OSGEPL1 loss of function induces mitochondrial respiratory deficits, reduced ATP production, reduced mitochondrial translation mitochondrial protein synthesis. This dysfunction leads to activation of mtUPR potentially due to the imbalance between the mitochondrial encoded versus nuclear encoded subunits of the respiratory system **[5, 160]**. The absence of OSGEPL1 and t^6A37 modification has an effect on cell growth but not cell apoptosis **[160]**. Interestingly the *Osgepl1* deletion mice exhibit impaired mitochondrial translation but they develop and grow normally and do not present cardiac dysfunction.

Although direct mutation or variants of OSGEPL1 have not been linked to disease, mutations in the mitochondrial DNA that lead to inability of the tRNA to undergo OSGEPL1 mediated modifications, have been linked to myoclonus epilepsy associated with ragged red fibres (MERRF) (Table 1).

7. DISCUSSION and PROSPECTIVE

Mitochondrial proteases create an interdependent network of molecular components which cooperate to maintain mitochondrial functionality. They process each other or self-process to achieve mature active forms and they perform sequential or parallel activities on shared and or distinct targets. This offers a degree of redundancy in the activity of mitochondrial proteases, as they may have overlapping targets or may be compensating for each other. For example, it was reported that LONP1 cooperates with ClpXP in the degradation of complex I subunits in depolarized mitochondria to reduce ROS production, and CLPP overexpression may act as a compensatory mechanism in LONP1 loss of function **[161, 79, 162]**.

The cellular **homeostasis pathways in which different proteases are involved, appear to overlap**. The most common pathways that are triggered are related to organellar quality control, more specifically mitophagy, metabolic adaptation and mechanisms related to cell death or cell survival (Figure 2). Given the interconnectivity of protein processing and quality control in the mitochondria it would be difficult to attribute a single signalling mechanism to a single protease. The mitochondrial stress signalling at transcriptional level is converging to mtUPR and ISR in multiple situations. In some cases, nonautonomous signalling takes place particularly by initiation of innate immune signalling. Here, dysfunction in the mitochondrial proteolytic compartment affecting various proteases may lead to the common event of mtDNA release in the cytoplasm which triggers cGAS-STING innate immune signalling.

Interestingly there are common patterns in induction of disease, although in every case there are exceptions from these patterns. Thus, proteolytic loss of function appears to be related to mitochondrial disease, neurological phenotypes and susceptibility for age related neurodegeneration including AD and PD. Conversely, patterns of enhanced expression of mitochondrial proteases appear in various malignancies (Table 1) reinforcing the hypothesis that mitochondria in cancer cells play significant roles in supporting enhanced mitosis and bypassing apoptotic processes. Impaired mitochondrial function and proteostasis are also related to ageing, with mitoproteases dysfunction being directly linked to age-related phenotypes and disease **[7]**. A key factor in how mitochondrial stress signalling rewires the cellular homeostasis is the process termed ‘mitohormesis’ that is meant to describe the adaption of the cellular system to future stress situations **[163]**. While this process is thought to have positive effects in the context of neurodegeneration, in the context of cancer, it constitutes the basis for malignancy initiation and acquisition of drug resistance.

Targeting mitoproteases in cancer. In cancer numerous mitoproteases are overexpressed as a survival strategy. Cancer cells exploit the functions of mitoproteases to combat oncogenic stressors such as hypoxia, proteotoxicity, and oxidative stress, and to reprogram energy metabolism enabling cell proliferation, chemoresistance, and metastasis. As reviewed in Table 1, multiple mitoproteases are dysregulated in various cancers which has triggered the quest for exploiting them as therapeutic targets. Significant work has been related to pharmacological targeting of CLPP and LONP1 **[164]**. Two types of approaches have been proposed for CLPP, namely activation and inhibition. The CLPP activators (e.g. acyldepsipeptides and imipridone compounds) bind to the apical part of the CLPP heptamer opening the barrel pore and transforming CLPP in an active protease by removing the need for CLPX as an adaptor. Significant differences occur in the level of activation depending on the binding sites of the activators and the level of CLPP saturation with the activators. These fine-tuning differences may shift CLPP activation from a status where it presents increased ability to degrade damaged proteins which may be beneficial in a context of proteostatic stress, to an indiscriminate protease whose overactivation contributes to cell death **[69, 165]**. CLPP inhibitors (e.g. b-lactones, phenyl esters, boronic acids) increase accumulation of ROS and damaged proteins leading to apoptosis. Importantly, these compounds are more efficient in cancer cells where CLPP is overexpressed

and appear to leave normal cells unaffected. Small molecule inhibitors (e.g Oleanane-type triterpenes, Obtusilactone A, bortezomib) target LONP1 by two distinct mechanisms, competitive inhibition of its protease activity and allosteric inhibition of its ATPase activity, both of which are crucial for its function. Mechanistically, inhibition of LONP1 leads to mitochondrial dysfunction at multiple molecular checkpoints given the wide range of functions of LONP1 in the mitochondria. Some of the compounds that have shown good LONP1 inhibitor activity have however demonstrated low specificity, for example bortezomib being able to inhibit the proteasome and CLPP as well **[166]**.

Other proteases, like HtrA2 and OMA1, are recognised for their potential for personalised therapeutics although no clear pharmacological approaches are proposed for disease treatments. Thus, HtrA2 with its complex multimodal activation mechanisms has been seen as a potential therapeutic target for both malignancies where it is mostly upregulated, and age-related diseases and neurodegeneration where it has been associated with loss of function **[167]**. In the context of cancer, OMA1 activation promotes cell death and OMA1 inhibition is protective against cell death therefore OMA1 targeted therapies have been suggested as personalised treatments as some patients might benefit from activation of the OMA1 protease, while other patients might benefit from OMA1 inhibitors depending on the type of cancer and the disease stage **[168]**.

Targeting mitochondrial quality control in neurodegeneration. Impairment in mitoproteases activity is primarily associated with mitochondrial dysfunction and mitochondrial disease, neurological and neurodegenerative conditions. Thus, improving mitochondria quality control at molecular and organellar level has long been proposed as the most obvious therapeutic strategy to alleviate these conditions. Despite the significant growth in understanding the mechanistic intricacies of these processes only limited therapeutic approaches have reached clinical trials. In neurodegenerative diseases, perhaps the most significant for therapeutical approaches using mitoproteases has been the targeting of USP30. Inhibitors of USP30 are being progressed to clinical trials given their promise to enhance mitophagy processes **[146]**. Other approaches looking to enhance mitophagy, improve mitochondrial function and reduce inflammation are looking at the mitochondria integrated in its cellular homeostatic environment and/or at exploiting naturally occurring compounds (e,g. Urolithin A, NAD+ precursors supplementation) **[169, 5]**.

Exploiting mitochondrial stress signalling pathways for therapeutic benefit. The question remains how are the signalling mechanisms generated by mitochondrial proteases influencing the general cellular homeostasis, and how are they influencing the cellular decision to survive or die? The evidence suggests thus far that moderate, transient stress signalling has a positive effect and supports the cell to resolve a temporary imbalance (Figure 2). Thus, activation of the mtUPR in the mitonuclear imbalance has been found to have positive effects on longevity **[90,5].** However, sustained stress signalling is detrimental and leads to cells death, particularly during the ISR activation via ATF4/CHOP signalling **[45]**. In some rare occasions persistent activation of integrated stress responses has been found to have a positive effect **[100]**. Evidence of mtUPR and ISR activation has been found in multiple models of neurodegenerative diseases as well as in brain samples from patients. Consequently, pharmacological approaches targeting ISR have been proposed to restore general protein translation and alleviate neurodegenerative phenotypes. These have proven successful as proof of concept in animal models for full or partial inhibition of ISR and full or partial protein translation de-repression, (e.g. GSK2606414 ISRIB and tetrazodone). However, thus far, the approaches have stumbled due to toxicity and/or efficacy concerns **[170]**.

Thus, identifying genetic and pharmacologic modulators that enhance proteolysis moderately and induce transient mtUPR/ISR and mitophagy may support therapeutic strategies in neurodegenerative diseases. Conversely either inhibition or overactivation of proteolysis may contribute to mitotic cell death and offer therapeutic opportunities in cancer. However, when it comes to proteolysis and support of cellular homeostasis perhaps the most important is the maintenance of the correct function of this complex system.

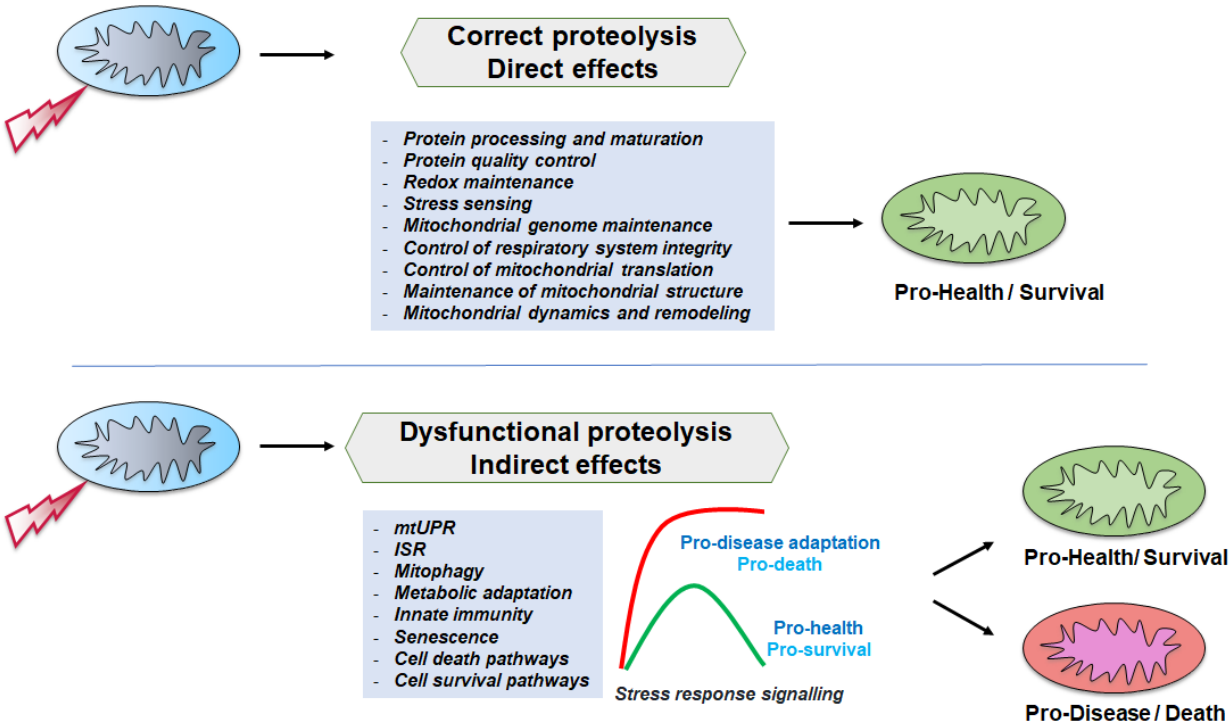

Figure 2. Effects of mitochondrial proteolysis.

Correct proteolysis supports a plethora of mitochondrial processes that ensure appropriate mitochondrial function. Dysfunction in the proteolysis capacity in normal or stress conditions triggers stress signalling mechanisms involving multiple cellular compartments. Transient, moderate stress may restore homeostasis and support survival or while prolonged stress may trigger pro-disease adaptations or lead to cell death.

Table 1. Mitochondrial proteases and their link with cellular homeostasis pathways stress signalling and disease.

Name	**Function &** ***Main substrates (S)****	Cellular homeostasis pathways **&** **Stress Signalling**	**Disease significance**
Protein processing			
PMPCB *Metallo*	Protein maturation ***S**:* most pre-proteins, N-terminal presequence cleavage	Mitophagy Apoptosis **mtUPR**	Leigh-like neurodegeneration phenotypes [**171]** Friedrich's Ataxia **[172]** Hepatocellular carcinoma treatment sensitivity **[13]**
MIP/Oct1 *Metallo*	Protein maturation ***S**:* some pre-proteins (e.g. Prdx3, Prdx5), N-terminal octapeptide cleavage	ND	Friederich's Ataxia **[173]** Syndrome of left ventricular non-compaction, hypotonia and infantile death **[174]**
IMMP1L *Serine*	Protein maturation ***S**:* Cox2p, Cyb2p, Mcr1p, Gut2p (**)	ND	Cervical cancer **[175]** Aniridia **[176]** Diabetes, Obesity **[177]** Autism, mental retardation **[178]**
IMMP2L *Serine*	Protein maturation ***S**:* TIM23, CYC1, GPD2, AIF	Mitochondrial phospholipid metabolism Apoptosis Senescence	Autism **[179]** Schizophrenia **[180]** Tourette Syndrome **[181]** Neurodevelopmental rare diseases **[182]** Alzheimer's **[183]**
METAP1D *Metallo*	Protein maturation ***S**:* N-terminal methionine of ATP synthase subunit 8, NADH dehydrogenase subunits 1, 4L, and 5, cytochrome *c* oxidase subunits 2 and 3 and cytochrome *b*	Cell survival Regulates tumour growth	Leber's Hereditary Optic Neuropathy **[184]** Cancer **[185, 186]**
XPNPEP3 *Metallo*	Protein maturation ***S**:* N-terminal destabilising tyrosine or phenylalanine	Apoptosis	Kidney disease (nephronophthisis) **[187, 188]** Cancer **[31, 189]** Rare mitochondrial syndromes **[190]** Cardiomyopathy **[191]** Depression **[192]** Alzheimer's susceptibility **[193]**
Unfolded, misfolded and damaged proteins quality control			
MEP *Metallo*	Oligopeptide degradation Aβ degradation	Respiratory complexes and supercomplexes formation	Acute myeloid leukaemia (discovered alongside CLPP) **[194]**

	***S**: presequences, presequence fragments and hydrophobic fragments of Aβ*	Growth and viability of AML cells.	
PITRM1 *Metallo*	Oligopeptide degradation Aβ degradation ***S**: oligopeptides (mitochondrial targeting sequences resulting from the process of precursor protein import and maturation); Aβ*	**mtUPR** **Integrated stress response**	Neurologic syndrome (mental retardation, spinocerebellar ataxia, cognitive decline, psychosis) **[195]** Alzheimer's susceptibility **[196]** Friedrich's Ataxia **[197]** Chronic kidney disease **[198]**
HtrA2 *Serine*	Clears unfolded proteins in the intermembrane space Reduces ROS Preserves mitochondrial function ***S**: XIAP, unfolded proteins*	Apoptosis Non-apoptotic cell death Autophagy Mitochondrial biogenesis Innate immunity **Integrated Stress Response**	Parkinson's susceptibility **[199, 200]** Early ageing **[49]** Cancer **[54]**
CLP(X)P *Serine*	Clears unfolded and damaged proteins Complex 1 subunits recycling Mitoribosome maturation ***S**: structural components of the mitoribosome, TCA-cycle associated, amino acid metabolism, aminoacyl t-RNA ligase activity, mitochondrial import receptors, subunits of OXPHOS*	**mtUPR** **Integrated Stress Response**	Perrault Syndrome **[61]** Cancer (Acute Myeloid Leukemia **[201, 69]**, Solid tumours **[202]**
LONP1 *Serine*	Clears unfolded and damaged proteins in the matrix Mitochondrial genome maintenance ***S**: OXPHOS subunits, ACO2, GAC, PDH subunits, StAR, PINK1, TFAM, unfolded/ misfolded proteins*	Mitophagy **mtUPR** **Integrated Stress Response**	Cerebral, ocular, dental, auricular and skeletal anomalies syndrome (CODAS) **[203]** Mitochondrial disease **[204]** Cancer **[205-208]**
ATP23(**) *Metallo*	Protein processing and chaperone ***S**: Ups1, Atp6 subunit of F1F0 ATPase (**)*	Biosynthesis of F1/F0 ATPase Mitochondrial phospholipids	ND
Membrane integrated metalloproteases - ATPase associated with diverse cellular activities			
SPG7 (mAAA) *Metallo*	Protein quality control Protein processing ***S**: Substrates as part of the mAAA: respiratory chain subunits, mitochondrial ribosome subunit (MRPL32)*	Quality control of respiratory complexes Assembly of mitochondrial ribosomes and regulation of mitochondrial protein synthesis **Oxidative stress and senescence pathways** **mtUPR**	Hereditary Spastic Paraplegia **[84]** Progressive external ophthalmoplegia and spastic ataxia **[93]** Dominant Optic Atrophy **[209]** Parkinsonism **[210]** Amyotrophic lateral sclerosis **[211]** Alzheimer's susceptibility **[212]**

AFG3L2 (mAAA) *Metallo*	Protein processing ***S:*** *Substrates* as part of the mAAA: respiratory chain subunits, mitochondrial ribosome subunit (MRPL32), mitochondrial calcium uniporter subunit (EMRE), self-processing	**mtUPR** **Integrated stress response** **StAR overload response** **Metabolic stress** **Inflammation**	Spinocerebellar ataxia type 28 (SCA28) **[213, 97]** Spastic ataxia-neuropathy syndrome **[214]** Dominant Optic Atrophy **[209]** Progressive myoclonus epilepsy **[215]** Ataxia with Oculoapraxia type 2 **[216]** Parkinsonism **[217]**
YME1L1 (iAAA) *Metallo*	Protein processing Maintaining broadly proteostasis ***S:*** OPA1, YME1L1 targets include subunits of protein translocases, mitochondrial lipid transfer proteins, and proteins functionally linked to mitochondrial metabolism	Mitochondrial fission/fussion Mitochondrial biogenesis Response to hypoxia Apoptosis Cell survival	Mitochondriopathy with optic atrophy **[218]** Cancer **[219]**
Mitochondrial structure, dynamics and remodelling			
OMA1 *Metallo*	Mitochondrial stress sensor Limits fusion via OPA1 cleavage ***S:*** OPA1, DELE1	Apoptosis Development Cellular differentiation **Integrated stress response**	Heart failure prognosis **[220]** Tumorigenesis and Response to cancer treatment **[221, 222, 168]**
PARL *Serine*	Mitochondrial cristae remodelling involving OPA1 Regulates mitochondrial function during stress ***S:*** PINK1, PGAM5, OPA1, HtrA2	Apoptosis Non-apoptotic cell death Mitochondrial biogenesis Mitophagy **Mitonuclear signalling through the Pbeta peptide**	Potential roles in Parkinson's disease **[223, 224],** Alzheimer's progression **[225]**, diabetes **[226, 227]** Leber hereditary optic neuropathy (LHON) **[228]**
USP30 *Cysteine*	Deubiquitinating enzyme ***S:*** Lys6-linked ubiquitin chains	Mitochondrial morphology Mitophagy Pexophagy Apoptosis	Parkinson's disease **[157, 159, 229]** Cancer prognosis **[230, 231]**
LACTB *Serine*	Maintaining mitochondrial structure ***S:*** ND	Mitochondrial lipid metabolism Autophagy Caspase independent cell death	Cancer **[149, 151, 152, 232]**
OTHER			
OSGEPL1 *Metallo*	mt-tRNA modifications ***S:*** mt-tRNAs, modifications for Ser, Thr, Asn, Ile, and Lys	**mtUPR**	Myoclonus epilepsy associated with ragged red fibres (MERRF) **[233]**

(*) The list of proteolytic substrates may not be exhaustive; (**) studies in yeast
ND (not determined)
NB: References for the functions, substrates, and cellular homeostasis and signalling pathways are in the text.

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