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Substrates and interactors of the ClpP protease in the mitochondria

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Abstract

The ClpP protease is found across eukaryotic and prokaryotic organisms. It is well-characterized in bacteria where its function is important in maintaining protein homeostasis. Along with its ATPase partners, it has been shown to play critical roles in the regulation of enzymes involved in important cellular pathways. In eukaryotes, ClpP is found within cellular organelles. Proteomic studies have begun to characterize the role of this protease in the mitochondria through its interactions. Here, we discuss the proteomic techniques used to identify its interactors and present an atlas of mitochondrial ClpP substrates. The ClpP substrate pool is extensive and consists of proteins involved in essential mitochondrial processes such as the Krebs cycle, oxidative phosphorylation, translation, fatty acid metabolism, and amino acid metabolism. Discoveries of these associations have begun to illustrate the functional significance of ClpP in human health and disease.

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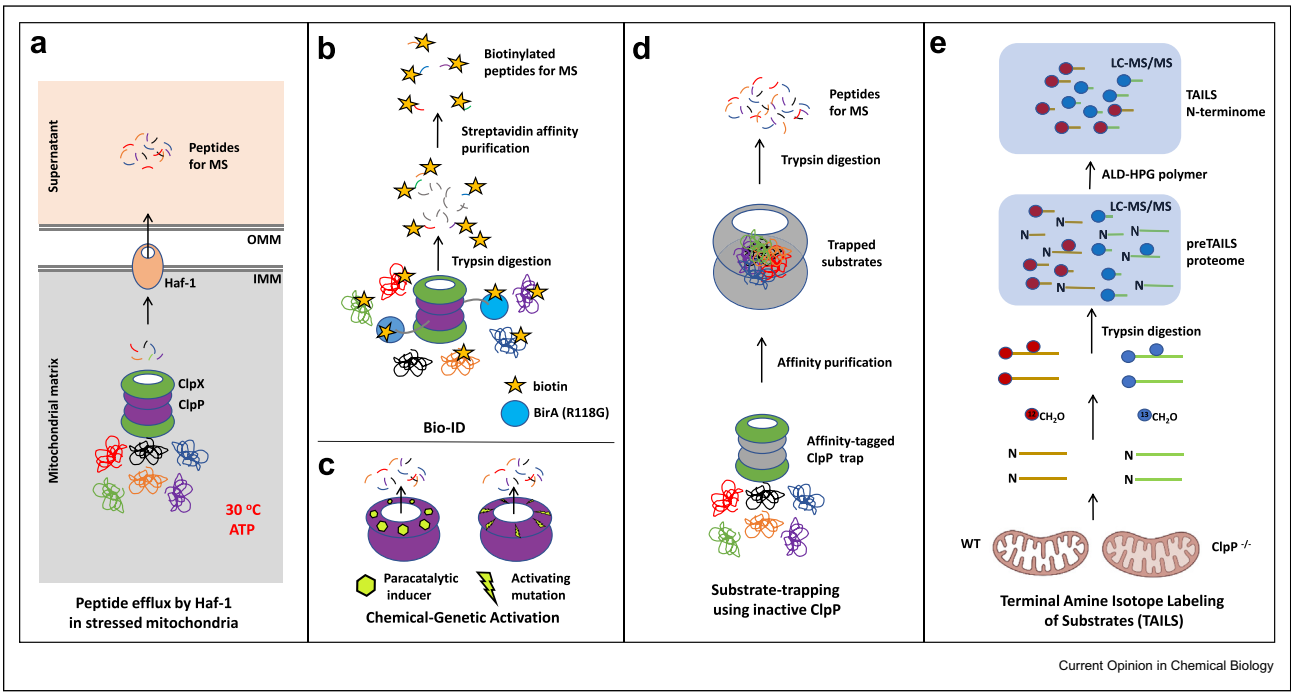
Introduction

Mitochondria are commonly referred to as the powerhouse of the eukaryotic cell. This moniker is well-deserved considering the nutrient utilization pathways localized to this organelle. Although glycolysis occurs in the cytoplasm, its metabolic products, nicotinamide adenine dinucleotide (NADH) and pyruvate, are shuttled into the mitochondria. This is where pyruvate metabolism, the Krebs cycle, chemiosmosis, and the electron transport chain (ETC) take place. Mitochondria are separated from the cytosolic environment by the bounds of their two membranes, maintain their own DNA, and can be targeted for destruction when malfunctioning (mitophagy).

To maintain the large number of proteins required for cellular sustenance and mitochondrial function, a variety of chaperones and proteases are present in the organelle [1,2]. In general, chaperones help prevent and reverse misfolding while the proteases remove unwanted and damaged proteins. The protein quality control (PQC) provided by chaperones and proteases in the mitochondria is integral to its function. As mitochondrial metabolism is closely associated with cellular health, damage to these quality control mechanisms has been associated, directly and indirectly, with disease [3–9]. Recent studies have particularly noted the importance of the ClpP serine protease in this regard. ClpP mutants are associated with Perrault syndrome, hearing loss, and infertility [4,7]. Lowered ClpP and Lon expression levels were observed in patients with hereditary spastic paraplegia [10]. ClpP has also been observed to be either overexpressed or influential in certain forms of cancer cells [11,12]. The association of ClpP mutants with disease phenotypes has thus led to an increased interest in the functional role played by this protease.

The active assembly of ClpP protease structurally resembles a double barrel of two heptameric rings. ClpP itself cannot degrade proteins effectively without coupling to the ATPase ClpX [13] (Figure 1a). The ClpX is a hexamer which belongs to the ATPases Associated with diverse cellular Activities superfamily and the HSP100 family of chaperones. Like its bacterial counterparts, mitochondrial ClpX is thought to recognize ClpP substrates, unfold them, and feed them into the interior of ClpP for degradation [13,14]. As ClpX is responsible for the interaction with substrates to be degraded by ClpP, it controls substrate specificity for the protease.

Figure 1



Shown are the proteomic methods used for the identification of mitochondrial ClpP interactors and substrates in different eukaryotic species. **(a)** Haf-1 transporter-mediated efflux of degraded peptides in stressed mitochondria. Mitochondria were isolated from *C. elegans*, then subjected to heat stress in the presence of ATP. Under these conditions, Haf-1 transports protease-degraded peptides across the inner mitochondrial membrane (IMM) into the intermembrane space. The outer mitochondrial membrane (OMM) is semiporous and allows the translocation of peptides into the supernatant. This method cannot rule out degradation by other mitochondrial proteases such as LON. **(b)** Bio-ID of ClpP interactors using a genetic fusion of affinity-tagged ClpP and the *E. coli* biotinylating enzyme, BirA* mutant (R118G). On addition of biotin, the ClpP-BirA* chimera biotinylates the surface amines of protein interactors near ClpP. Biotinylated proteins were then pulled down and identified by MS. Bio-ID *per se* cannot distinguish between bona fide substrates, mere physical interactors such as protein adapters, and 'near-neighbors.' **(c)** Bio-ID of ClpP interactors combined with chemical activation of ClpP. In the chemical activation approach, mammalian cells expressing purification tagged-ClpP-BirA* mutants were treated with imipridone ONC201 as the activator. The interactomes of activated and nonactivated ClpP were compared, and proteins of wild-type ClpP whose spectral counts decreased on chemical activation were interpreted as putative ClpP interactors or substrates. **(d)** Substrate trapping using inactive ClpP (gray double barrel). Endogenous ClpX selects, unfolds, and delivers substrates into the ClpP trap. Trapped proteins are then identified via proteomic means after trypsin digestion. Only degron-containing substrates are selected, discriminating against 'near neighbors' or transient interactors. In the trapping study in *P. anserina*, the fungal ClpP mutant did not form tetradecamers. Because the episomally introduced human ClpP reversed the longevity phenotype, suggesting functional equivalence, a human ClpP S153A mutant trap was used as a trap inside the fungal mitochondria. **(e)** Terminal amine isotope labeling of substrates (TAILS) for identifying primary and secondary ClpP substrates via N-terminome analysis. In the TAILS experiment, WT and ClpP knockout mitochondria from mice were purified and lysed; lysates were then reacted separately with either light (WT) or heavy (*CLPP*$^{-/-}$) formaldehyde to label N-termini. Proteins were digested with trypsin to produce fragments containing unlabeled neo-N-termini, followed by their covalent capture with an aldehyde-functionalized polymer. This step enriched the light- and heavy-atom labeled peptides, which were analyzed by LC-MS/MS to identify the N-terminome. Comparison of peptide abundance in WT and *CLPP* knockout N-terminomes revealed primary and secondary substrates of ClpP. Primary substrates produced accumulated levels of expected N-termini in *CLPP*$^{-/-}$ mitochondria and decreased levels in WT mitochondria owing to substrate degradation. By contrast, secondary ClpP substrates showed decreased abundance of neo-N-termini in *CLPP*$^{-/-}$ mitochondria because proteolysis was absent. Oppositely, in WT mitochondria, neo-N-termini of secondary ClpP substrates accumulated. TAILS identified secondary substrates of ClpP, placing it within a cooperative protease network that degrades a subset of mitochondrial proteins.

Prospects of targeting ClpX and ClpP in anticancer treatments have been raised [11,15–17]. Yet, the pathways regulated by ClpP and the consequences of disrupting its function are poorly understood. New insights into its function across different eukaryotic species highlight the ClpXP system as an important and influential component for mitochondrial proteostasis. Greater insight into this area should be useful in gaining not only understanding of proteostasis within the mitochondria but also into the targeting of ClpP function as a defense against related human diseases. Here, we provide a summary of recent work on the identification of the mitochondrial ClpP substrates, reflect on its function, and highlight the roles it plays in human disease.

Proteomic methods used in identifying mitochondrial ClpP substrates

Clues into the role of mitochondrial ClpP were first observed in the worm species *C. elegans* [18]. In a genomic screen to identify genes linked to the mitochondrial unfolded protein response (mtUPR), ClpP

was seen to function as a regulator of the stress response. Putative ClpP substrates were identified in the form of degraded peptides trafficked across the inner mitochondrial membrane into the intermembrane space by the transporter Haf-1 under conditions of protein accumulation owing to mitochondrial stress (Figure 1a, Table S1) [18]. Although this result provided specific functional insight into the significance of ClpP in the mitochondria for *C. elegans*, a follow-up study showed that unlike in the worm species, ClpP does not appear to have a universal role in driving mtUPR in mammals [2,19].

It was the involvement of ClpP in acute myelogenous leukemia (AML) which provided the impetus for a wider assessment of mammalian ClpP substrates. AML cells often display larger mitochondria and increased reliance on oxidative phosphorylation (OXPHOS) [20,21]. The phenotype provided the rationale for pinpointing mitochondrial proteins whose inhibition might cause selective cancer cell killing. In the study by Cole et al. [9], an shRNA knockdown lentiviral screen identified that the downregulation of *CLPP* gene led to reduced viability of certain leukemic cells. To understand the mechanism, Bio-ID was used. Here, a chimera of affinity-tagged ClpP and the *E. coli* biotin protein ligase BirA* mutant (R118G) was constructed. The chimera was used to biotinylate proteins in close physical proximity to ClpP, which were subsequently pulled down and identified by mass spectrometry (MS) [22]. This led to the identification of 49 potential ClpP substrates (Figure 1b, Table S2). In a modification of the Bio-ID technique, a chemical ClpP activation approach combined with Bio-ID-MS was used to identify more than 200 ClpP interactors whose degradation in cancer cells induced apoptosis (Figure 1c, Table S2) [16].

In addition to the Bio-ID pulldowns, two techniques have been used to determine the set of ClpP proteolytic substrates: trapping and terminal amine isotope labeling of substrates (TAILS). Trapping experiments use a catalytically inactive ClpP where the active site serine is mutated to an alanine. This inactive ClpP trap retains the ability to associate with ClpX but traps the substrates that end up inside this nondegrading complex (Figure 1d). It was the first technique used for identifying ClpP substrates in *E. coli* [23]. In eukaryotes, the trapping approach was used to identify ClpP substrates in the mouse [24–26] (Table S3). Trapping experiments were also performed in the fungal aging model *P. anserina* to understand the longevity phenotype conferred by *CLPP* knockout (Table S4).

The TAILS is a novel proteomic quantification technique with the potential to identify ClpP substrates not amenable to trapping experiments [25]. As the name suggests, TAILS exploits the labeling of protein N-termini with either light or heavy carbon isotopes before tryptic digestion and the subsequent enrichment of labeled peptides by chemical means [27]. Applied to ClpP, TAILS enabled the identification of both primary and secondary substrates based on the differential accumulation and disappearance of labeled peptides from wildtype (WT) and *CLPP* knockout mitochondria (Figure 1e, Table S3).

Utilization of these techniques has provided a plethora of information about the mitochondrial ClpP and its substrates. However, the methodologies described are not without limitations. Chemical activation (i.e. paracatalytic induction [28]) is achieved by binding of specific small molecules to the apical hydrophobic pockets of ClpP that normally serve as docking sites for ClpX. This causes ClpP to retain an open conformation and degrade proteins less discriminately than the ClpXP complex [29]. Given the lesser selectivity of chemically activated ClpP, it is not surprising that combining chemical activation with Bio-ID identified a larger pool of potential substrates than Bio-ID alone (Figure 1b), such that caution must be taken in the interpretation of the results of the former.

The resulting ClpP interactome from the above-mentioned techniques portrays ClpP as having a significant influence on numerous essential cellular pathways as discussed in the following sections.

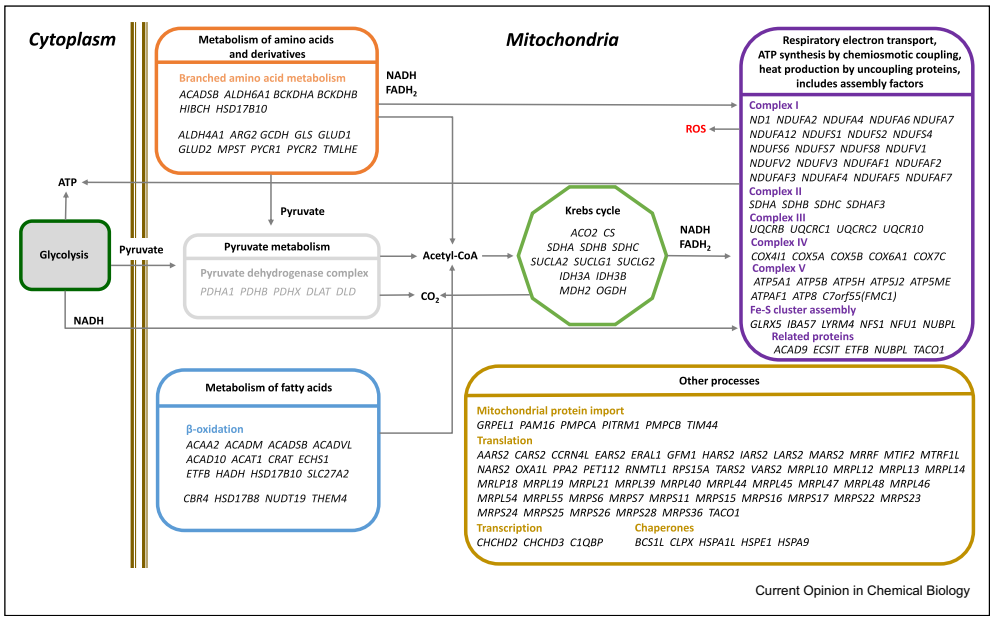
Mitochondrial ClpP interactors and associated pathways

From the results of the proteomics techniques mentioned previously, an atlas of mitochondrial ClpP interactors and substrates, from different eukaryotes, was assembled (Tables S1–S5, Figure S1**)**. The lists of ClpP interactors reflect the expansive contribution of the protease to mitochondrial protein homeostasis, influencing pathways related to the ETC and cellular respiration, metabolism, transcription and translation, protein folding and transport, redox homeostasis, and Fe–S cluster biogenesis (Figure 2).

ETC

Subunits of the ETC were among the most salient substrates of mitochondrial ClpP (Tables S1–S5, Figure S1). The study in *C. elegans* identified peptide fragments of Complex V subunits exported by Haf-1 (Table S1) [18]. The Bio-ID screen performed in a mammalian cell line identified succinate dehydrogenase (SDHA), a component of Complex II, as a top hit among 49 other proteins that were preferentially associated with ClpP (Table S2) [9]. The Bio-ID screen coupled with chemical activation studies on mammalian cell lines and mitochondrial lysates demonstrated direct degradation of ETC subunits NDUFA12 (Complex I), SDHA and SDHB (Complex II), and Ubiquinol-Cytochrome C Reductase Core Protein 2 (UQCRC2) (Complex III) (Table S2) [16]. Their degradation

Figure 2



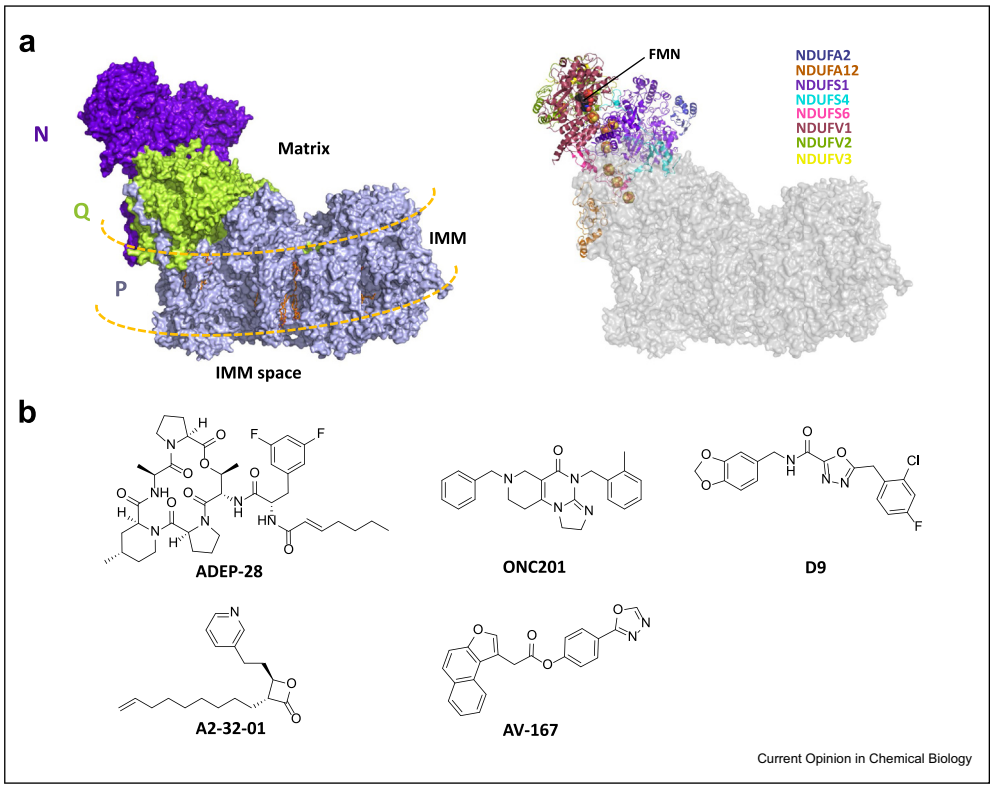
Interactors and substrates of mammalian ClpP participate in essential mitochondrial pathways. ClpP-associated proteins in human and mouse cells (summarized in Tables S2–S3) were grouped into their corresponding mitochondrial processes and pathways based on their gene ontology (GO) terms. The classification was performed using the PANTHER overrepresentation test in the PANTHER classification website (www.pantherdb.org/; Test type: Fisher's Exact; Correction: Bonferroni correction for multiple testing). Highly enriched GO terms fall under the following pathways: Krebs cycle, mitochondrial respiration (ETC, Complex assembly factors, ATP synthesis), translation, and small molecule metabolism. The relationship between these pathways within the mitochondria and glycolysis in the cytoplasm is presented. The names of protein genes associated with each pathway are italicized. Some proteins associate with more than one pathway in the cell. Only proteins participating in select mitochondrial processes are shown. Under pyruvate metabolism (gray box), proteins found as human ClpP substrates in the fungus *P. anserina* are shown, because in this species, nearly all proteins of the pyruvate dehydrogenase complex were identified in the trapping experiment. Across species, proteins related to the ETC dominate the substrate pool of mitochondrial ClpP implicating the protease in ROS-linked diseases such as cancer and Parkinson's disease. ETC, electron transfer chain.

resulted in decreased enzymatic activities for those complexes and impaired OXPHOS, which manifested morphologically as damaged mitochondrial matrix and cristae structures. Furthermore, the TAILS experiment identified the Complex III subunit UQCRC1 as a secondary substrate of ClpP, which was identified in the prior trapping and Bio-ID interaction analyses (Table S3) [9,16,25]. Based on its N-terminome profile in mouse heart mitochondria, UQCRC1 was found to be initially processed by a yet unidentified protease into intermediates containing neo-N-termini that are recognized by ClpX. The intermediates are subsequently degraded by ClpP, cleaving preferentially at sites near the C-terminus of the protein [25]. The trapping experiment in the same study, using whole cell lysates instead of purified mouse mitochondria, also identified NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1) (Complex I) as a ClpP substrate (Table S3) [25].

Among the ETC complexes, Complex I had the most subunits identified as substrates (Tables S2, S3, S4, Figure S1) [9,16,18,24,25]. Complex I is made up of a membrane-embedded (P-module) arm and a peripheral arm facing the mitochondrial matrix (Figure 3a) [30–32]. The peripheral arm, composed of 17 nuclear-encoded subunits that form interlocking N- and Q-modules, binds flavin mononucleotide (FMN) and serves as the entry site for NADH. The peripheral arm conducts electrons using eight Fe–S clusters [33,34]. As a redox center, the peripheral arm is a reactive oxygen species (ROS)-generating hotbed prone to oxidative damage [35,36].

The cascade of events that ensures Complex I maintenance by ClpXP was recently defined [37,38]. On OXPHOS stalling, ClpXP rapidly removes ROS-damaged N-module subunits that present degrons for ClpXP [38]. Based on exchange rates measured by stable isotope labeling of amino acids complexome analyses, N-module subunits were replaced more rapidly than those of the Q- and P-modules [38]. Retrograde signaling from mitochondria to the nucleus activated the transcription of nuclear-encoded Complex I components [37,38]. Newly synthesized N-module subunits were then incorporated into a 'refurbished' Complex I. This salvage pathway 'by parts' is believed to be more efficient for the cell than complete dismantling and turnover of Complex I, which necessitates P-module extraction from the membrane [38]. Provided a residual

Figure 3



ClpP participates directly in the salvage pathway for Complex I maintenance. **(a)** Complex I is embedded in the inner mitochondrial membrane (IMM, shown as two broken lines) through its peripheral arm (P) module. Bound lipids are shown as orange sticks on the left panel. The N (purple) and Q (green) modules face the mitochondrial matrix and contain eight Fe–S clusters (right panel, shown as spheres), the binding sites for FMN (right panel, black carbon spheres in N-module), and the incoming product of the Krebs cycle and glycolysis, NADH (see Figure 2). Because the N- and Q-modules are sites for electron transfer, they generate ROS and are prone to oxidative damage. ClpXP, located in the mitochondrial matrix, surveils Complex I damage and initiates the degradation of all eight N-module subunits (right panel, colored ribbons). Although the Bio-ID screens in human cells did not identify NDUFS1 as a ClpP interactor (right panel, purple ribbons), trapping and biochemical experiments confirmed it as a ClpP substrate. The surface representation of human Complex I was generated in PyMol using the cryoEM structure with PDB ID 5XTD. **(b)** Small molecules that perturb normal human ClpP function include activators such as acyldepsipeptides (ADEP-28), imipridones (ONC201), and the molecule D9, as well as inhibitors such as β-lactones (A2-32-01) and phenyl esters (AV-167). These small molecules have been used to target human ClpP *in vitro* and/or *in vivo* and are currently investigated as potential anticancer compounds. Crystal structures of human ClpP with bound activators ADEP-28 (PDB ID 6BBA), ONC201 (PDB ID 6DL7) and D9 (PDB ID 6H23) are available in the PDB.

mitochondrial membrane potential exists, it could also supersede more drastic repair mechanisms such as PTEN induced kinase (PINK1)-Parkin mediated-mitophagy [38]. This quick and efficient salvage pathway ensures a smoothly operating ETC by countervailing ROS.

Mitochondrial translation

The downregulation of ETC complexes was observed to be accompanied by upregulation of era like 12S mitochondrial rRNA chaperone 1 (ERAL1) in *CLPP* knockout mouse hearts and spurred interest in the role of ClpP in mitochondrial translation [26]. ERAL1 is a chaperone needed for 28S small ribosomal subunit assembly and whose dissociation from 28S precedes maturation of the full 55S ribosome. The trapping experiment performed in immortalized mouse embryonic fibroblasts first determined ERAL1 as a ClpP substrate, followed by Bio-ID in human cells (Tables S2 and S3, Figure S1) [26]. Gradient sedimentation analyses established that ERAL1 persistence prevented full ribosome assembly and affected the translation of ETC complexes. Normalization of ERAL1 levels reversed this translational defect as evidenced by restored levels of Complexes I and IV. Furthermore, overexpression of WT ClpP reduced levels of 28S-bound ERAL1, resulting in the maturation of the full 55S ribosome. Although the precise mechanism of ERAL1 removal from 28S by ClpXP is yet to be determined, it is believed that failure to do so might prevent recruitment of other essential translation initiation and elongation factors [26].

Mitochondrial metabolism and other processes

From the same trapping experiment that identified ERAL1 as a ClpP substrate, and from Bio-ID screening in human cells, the enzyme Acyl-CoA Dehydrogenase

Very Long Chain (ACADVL) was also identified (Tables S2 and S3, Figure S1) [26]. ACADVL catalyzes the first rate-limiting step in fatty acid β-oxidation (FAO) that supplies key metabolites (acetyl-CoA and NADH) to the Krebs cycle (Figure 2). By knocking down *CLPP* in mouse mitochondria, Becker et al. [39] showed the effects of ACADVL accumulation and highlighted the opposing, tissue-dependent metabolic outcomes of ClpP ablation. In the liver, skeletal muscle, and brown adipose tissues, ACADVL accumulation was compensated for by the downregulation of carnitine palmitoyltransferase II, an inner membrane enzyme also involved in FAO in the mitochondrial matrix. The net result was reduction of FAO. In parallel, there was increased glucose uptake through upregulation of GLUT4 insulin receptor in the skeletal muscle, resulting in robust glucose metabolism as the cells turned to glycolysis for energy in response to diminished FAO (Figure 2). Systemic loss of ClpP in mice therefore resulted in a lean phenotype with resistance to diet-induced obesity owing to improved glucose metabolism. Reduced FAO also caused the 'whitening' of brown adipose tissues, making *CLPP* knockout mice less able to cope with cold stress. By contrast, in white adipose tissues, ClpP ablation resulted in ACADVL accumulation without downregulation of carnitine palmitoyltransferase II, resulting in increased FAO and enhanced energy metabolism. Thus, ClpP depletion in mice conferred a systemic benefit by ameliorating energy metabolism at the expense of adaptive thermogenesis [39].

Using a human ClpP trap in *P. anserina*, the substrates identified had human orthologs functioning in various mitochondrial processes (Table S4) [24]. These include three subunits of the translocase of the outer membrane (TOM) complex (TOMM20/40/70A), indicating a possible role for ClpXP in protein import similar to the function of the orthologous ClpCP complex in plant chloroplasts [40,41]. Other substrates include proteins involved in fatty acid and amino acid degradative pathways and nearly all subunits of the pyruvate dehydrogenase complex (PDC). The PDC is central to pyruvate metabolism, and in degrading PDC subunits, ClpP links glycolysis in the cytoplasm to the Krebs cycle in the mitochondria (Figure 2). Other identified substrates are Krebs cycle enzymes and Complex I subunits, pathways downstream to amino acid and fatty acid catabolism (Figure 2), as well as proteins that contain, bind to, or partake in Fe−S cluster biogenesis (aconitase, biotin synthase, NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1), cysteine desulfurase NFS1, HSPA9 chaperone, glutaredoxin-related protein 5, GDR5) (Table S4).

Indeed, ClpP is a major player in the control of organismal aging in *P. anserina* through the regulation of proteins involved in interlinking mitochondrial pathways, especially those related to metabolism and energetics (Table S4) [42]. In this light, the longevity phenotype conferred by the absence of ClpP might seem counter-intuitive, especially given the deleterious effect of certain ClpP mutations or knockout in humans and mice. It seems reasonable to assume that in more complex organisms, ClpP dysregulation is more consequential to different tissues and organs whose metabolism and energetics have been fine-tuned in evolution. In simpler eukaryotes such as *P. anserina*, alternative quality control mechanisms such as autophagy might be sufficient to compensate for the lack of functional ClpP [24]. In fact, mitophagy is known to increase the lifespan of *P. anserina* under conditions of nutrient starvation [43].

Finally, in the fungal species *Aspergillus flavus*, a chemical activation strategy using the natural product dioctatin as a paracatalytic inducer identified a few proteins involved in mitochondrial energy metabolism as potential ClpP substrates (Table S5) [44]. Their degradation by activated ClpP caused metabolic disturbances in *A. flavus* that impaired its production of aflatoxin, a known human carcinogen [45]. Of note, this study was the first to use the endogenous ClpP to identify fungal substrates and underscored ClpP's conserved role in mitochondrial metabolism and energetics, despite the fact that the protease is not found in most fungal species [46].

Mitochondrial ClpP in human health and disease

The current atlas of ClpP substrates and interactors (Tables S1−S5, Figure S1) enables us to discern the molecular link between ClpP and various human ailments of mitochondrial etiology. As many substrates of mitochondrial ClpP are ETC and OXPHOS components, this points to a potential role of ClpP in cancer pathology. There is mounting evidence that increased intracellular ROS produced by the ETC is critical to sustain oncogenesis [47].

Moreover, in many types of solid cancers, ClpP overexpression has been observed and is moderately to highly elevated compared with normal tissues. For example, in prostate cancer cells, ClpP overexpression is required for proliferation and metastasis [48−50]. However, in breast cancer cells, ClpP overexpression appears to be less important, emphasizing the role of ClpP expression to be dependent on cancer cell type [49].

Recent studies highlight the importance of intact mitochondrial function in the growth and viability of leukemia cells, with the loss of ClpP leading to decreased OXPHOS and damaged Complex II [9]. Interestingly, ClpP upregulation also correlates with increased expression of mtUPR genes in primary AML patient samples [9]. It is suggested that ClpP overexpression in cancer cells serves to counteract the

effects of high ROS levels, allowing cancer cells to survive under the increased presence of protein damage. The accompanying expression of PQC proteins such as heat shock proteins and chaperones augments the protection against ROS-induced oncogenic mutations and cancer cell death [51]. ClpX upregulation has also been observed to result in the upregulation of PQC components in myoblasts, which suggests that ClpX/ClpP can serve as a mitochondrial stress marker [52].

Current efforts in this area now aim to develop ClpP-targeting compounds as novel cancer therapies by disrupting mitochondrial function, including activators or paracatalytic inducers (e.g. acyldepsipeptides and imipridones), and small molecule inhibitors (e.g. β-lactones and phenyl esters) that covalently bind the active site serine or that occlude the active site (Figure 3b) [9,16,17,28,53–56]. Although ClpP inhibition results in accumulation of a subset of respiratory chain protein substrates, ClpP activation leads to their uncontrolled degradation. Both strategies have been shown to impair cellular respiration and OXPHOS and lead to cancer cell death *in vitro* and *in vivo* [9,16]. ClpP activation induces lethality in leukemias and lymphomas owing to selective proteolysis of substrates involved in mitochondrial respiration and OXPHOS [16]. Given that cancer stem cells and chemo-resistant cells are highly reliant on OXPHOS, these small molecule modulators of ClpP function may be promising candidates for eliminating chemo-resistant cancer populations and prevent relapse. The observation that patient samples with low ClpP expression are less sensitive to ClpP activation also suggests the feasibility of using ClpP levels as a biomarker in predicting response to chemotherapy [16].

Apart from links to cancer epidemiology, the widespread influence of ClpP on mitochondrial proteins has linked it to a few human diseases. The identification of various ClpP substrates containing Fe–S clusters, or related to their assembly, has pointed to ClpP as a contributing factor in Friedreich ataxia [24]. This autosomal-recessive genetic disease is caused primarily by *FXN* gene mutations that lower the mitochondrial levels of the Fe–S assembly protein frataxin, leading to nerve tissue degeneration in the spinal cord that manifests as ataxia [57]. In addition, in deciphering the control exerted by ClpP on ERAL1 levels, ClpP's precise role in Perrault syndrome is now better understood. ClpP is among five, Perrault syndrome-implicated mitochondrial proteins (along with HARS2, LARS2, Twinkle, and ERAL1) whose mutations have direct effects in this disease [58–61]. Recent findings on the systemic effect of ACADVL accumulation in mice highlight the importance of ClpP in the physiological response to high-fat diet or cold stress and can guide the treatment of metabolic disorders [39]. The same findings have brought into question mitochondrial dysfunction as a major cause of insulin resistance in type 2 diabetes, as deletion of mitochondrial *CLPP* in mice has been found to enhance glucose metabolism [39,62].

Finally, given the direct role of ClpP in Complex I maintenance and mitochondrial energy metabolism as a whole, a potential role of ClpP in Parkinson's disease (PD) has been put forth [63]. Mitochondrial dysfunction is characteristic of most genetic variants of PD [63]. The brains of patients with sporadic PD also manifest deficiency in Complex I activity and cellular damage by oxidative stress [63]. As the largest component of the respirasome and the main driving force of ATP synthesis, Complex I is crucial in PD pathogenesis. PD pathogenesis also includes Lewy body formation, implicating PQC components such as ClpXP, as their malfunction can lead to protein aggregation [63]. Still, the precise role of Complex I deficiency and mitochondrial dysfunction in PD pathogenesis is only beginning to be unraveled.

Conclusions and future directions

We have provided an atlas of mitochondrial ClpP interactors based on proteomic studies (Tables S1–S5) reflecting the expansive contribution of ClpP to mitochondrial protein homeostasis. Several protein interactors of ClpP, such as those identified by trapping experiments, have been validated as ClpP substrates. Others, such as those identified by Bio-ID, still require characterization. With this atlas, the molecular basis of mitochondrial diseases in humans can be better appreciated in the context of failure in their quality control by ClpP. More work needs to be performed to establish the direct causation of mitochondrial diseases owing to mutations or change in protein levels of specific ClpP substrates. Recent studies that target ClpP function using small molecules to develop novel compounds against certain cancers or other mitochondrial diseases appear encouraging. Therefore, it is very likely that ClpP-targeting compounds will be used to treat patients in the coming years.

Author contributions

Conceptualization: MFM, VB, WAH; Data curation: MFM; Formal analysis: MFM, VB, WAH; Funding acquisition: WAH; Writing (original draft): MFM, VB; Writing (editing): MFM, VB, WAH.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.07.003.

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* of special interest
** of outstanding interest

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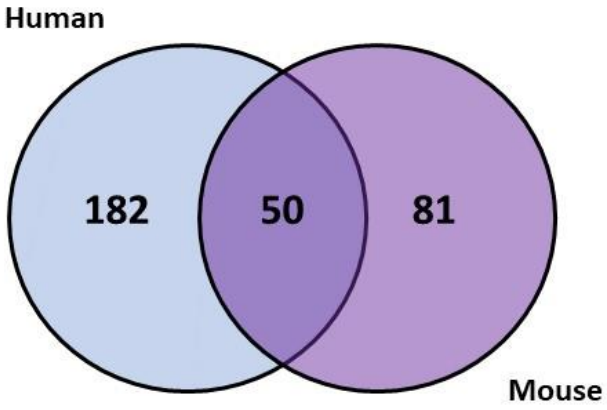
Figure S1. Venn diagram showing putative ClpP interactors and substrates common between human and mouse mitochondria.
A total of 50 proteins were identified and are listed below.



ABCB7 ACAA2 ACAD10 ACADM ACADVL ACAT1 ALDH1L2 ALDH4A1 ALDH6A1 ATPAF1 CHCHD3 CLPX COX4I1 CPOX CS ERAL1 ETFB IARS2 MRPL12 MRPL45 MRPL47 MRPS7 MRPS15 MRPS17 MTHFD1L NDUFA6 NDUFA12 NDUFAF7 NDUFS1 NDUFS2 NDUFS6 NDUFS7 NDUFS8 NDUFV2 NFS1 NME4 OGDH PCK2 PDPR PITRM1 PMPCB PNPT1 POLDIP2 SDHA SUCLG2 TIMM44 TMLHE UQCR10 UQCRB VARS2

Table S1. Cellular proteins whose peptides were found in the supernatant of purified, stressed mitochondria of *C. elegans*, suggesting putative ClpP substrates for further validation [1].

Gene Name	Protein Name
ACO2	Aconitase[1,7,8]
ALH-8	Methylmalonate semialdehyde dehydrogenase[1,8]
ATP-2	Complex V (ATP synthase) subunit β[1]
B0303.3	3-Ketoacyl-CoA thiolase, β[1]
BCAT-1	Branched chain amino acid aminotransferase[1]
C04C3.3	Pyruvate dehydrogenase β 1[1]
C05C10.3	Succinyl-CoA:α-ketoic acid-CoA transferase[1]
C05G5.4	Succinyl-CoA synthetase, α[1]
C16C10.11	Uncharacterized[1]
C34C12.8	GrpE family[1,7]
CCHL-1	Cytochrome *c* heme-lyase[3]
CTS-1	Citrate synthase[1,7,8]
CYN-1	Cyclophilin[1]
DNJ-21	Tim14 ortholog[4]
ECH-6	Enoyl-CoA hydratase[1,7]
EFT-4	Elongation factor[2,7,8]
F22D6.4	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 6, probable[1,7]
F27D4.1	Electron transfer flavoprotein α[1]
F40A3.3	Phosphatidylethanolamine-binding protein[1]
F43G9.1	Isocitrate dehydrogenase, subunit α[1,7]
F44G4.2	Complex I (NADH:ubiquinone oxidoreductase) β subcomplex subunit 2, probable[4]
F47B10.1	Succinyl-CoA synthetase, β[1]
F54D8.2	Complex IV (cytochrome c oxidase) subunit Via[4,7]
F58F12.1	Complex V (ATP synthase) subunit d[1,8]
FUM-1	Fumarate hydratase[1,8]
GEI-7	Isocitrate lyase/malate synthase[1]
GTA-1	4-Aminobutyrate aminotransferase[1,7]
H28O16.1	Complex V (ATP synthase) subunit α[1,8]
MAI-2	ATPase inhibitor[1]
MDH-1	Malate dehydrogenase[1,7]
MEL-32	Glycine/serine hydroxymethyltransferase[1]
MEV-1 (SDHC)	Complex II (succinate dehydrogenase) b560 subunit[4,7]
MMCM-1	Methylmalonyl-CoA mutase[1,8]
PCCA-1	Propionyl-CoA carboxylase α[1,8]
PHB-2	Prohibitin[1,8]
PYC-1	Pyruvate carboxylase 1[1,8]
SDHA-1 (SDHA)	Complex II (succinate dehydrogenase) subunit A[1,7,8]
SDHB-1 (SDHB)	Complex II (succinate dehydrogenase) subunit B[1,7]

SOD-2	Superoxide dismutase/SOD-3[1]
SPL-1	no human homologue[6]
T05H10.6	Pyruvate dehydrogenase α[1,8]
T08G2.3	Medium chain acyl-CoA dehydrogenase[1,8]
T22B11.5	α-Ketoglutarate dehydrogenase[1]
T22H6.2	1-Pyrroline-5-carboxylate synthetase[1]
UCR-1	Processing peptidase, β[1,7,8]
VIT-1	Vitellogenin[5]
VIT-2	Vitellogenin[5]
VIT-3	Vitellogenin[5]
VIT-6	Vitellogenin[5]
Y94H6A.8	Complex I (NADH dehydrogenase:ubiquinone oxidoreductase) α subcomplex subunit 12, probable[1,7,8]
ZC262.5 (R05D3.6)	Complex V (ATP synthase)- subunit ε [1,8]

Notes

Under conditions of cellular stress, the mtUPR is activated in *C. elegans*, allowing matrix proteases to degrade misfolded or damaged proteins in the mitochondrial matrix. The ABC transporter, Haf-1, translocates degradative peptides across the inner membrane into the intermembrane space. The semiporous outer mitochondrial membrane allows exit of peptides from the mitochondria. Peptides can then be isolated followed by MS identification. Purified mitochondria were heat stressed in the presence of ATP, activating ATP-dependent matrix proteases such as Clp(X)P.

[1]Mitochondrial matrix protein
[2]Cytoplasmic protein
[3]Protein localized in the intermembrane space
[4]Inner membrane protein
[5]Secreted protein
[6]Protein localized in the endoplasmic reticulum
[7]Human ortholog is a putative interactor or substrate of ClpP [2,3].
[8]Mouse ortholog is a putative interactor or substrate of ClpP [4,5].

Table S2. Human ClpP interactors identified by Bio-ID with or without chemical activation in human cells [2,3].

Gene Name	Protein Name
AARS2	Alanyl-tRNA synthetase 2, mitochondrial
ABAT	4-Aminobutyrate aminotransferase
ABCB10	ATP binding cassette subfamily B member 10
ABCB7	ATP binding cassette subfamily B member 7
ABHD10	Abhydrolase domain containing 10
ACAA2	Acetyl-CoA acyltransferase 2
ACAD10	Acyl-CoA dehydrogenase family member 10
ACAD9	Acyl-CoA dehydrogenase family member 9
ACADM	**Acyl-CoA dehydrogenase, C-4 to C-12 straight chain***
ACADSB	**Acyl-CoA dehydrogenase, short/branched chain***
ACADVL	Acyl-CoA dehydrogenase, very long chain
ACAT1	Acetyl-CoA acetyltransferase 1
ACO2	**Aconitase 2***
ACSS1	**Acyl-CoA synthetase short-chain family member 1***
AFG3L2	AFG3 like matrix AAA peptidase subunit 2
AIFM1	Apoptosis inducing factor mitochondria associated 1
AK3	**Adenylate kinase 3***
ALAS1	Aminolevulinate δ-synthase 1*
ALDH1L2	Aldehyde dehydrogenase 1 family member L2
ALDH2	Aldehyde dehydrogenase 2 family member
ALDH4A1	**Aldehyde dehydrogenase 4 family member A1***
ALDH6A1	Aldehyde dehydrogenase 6 family member A1
ARG2	Arginase 2
ATP8	Complex V (ATP synthase) protein 8*
ATPAF1	Complex V (ATP synthase) F1 complex assembly factor 1
ATPAF2	Complex V (ATP synthase) F1 complex assembly factor 2
BCKDHA	Branched chain keto acid dehydrogenase E1, α polypeptide
BCKDHB	Branched chain keto acid dehydrogenase E1, subunit β
BCS1L	BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone
C12orf10	Chromosome 12 open reading frame 10
C7orf55 (FMC1)	Formation of mitochondrial Complex V assembly factor 1 homolog
C8orf82	Chromosome 8 open reading frame 82
CARS2	**Cysteinyl-tRNA synthetase 2, mitochondrial***
CBR4	Carbonyl reductase 4*
CDK5RAP1	CDK5 regulatory subunit associated protein 1
CDS2	CDP-diacylglycerol synthase (phosphatidate cytidyltransferase) 2*
CHCHD3	Coiled-coil-helix-coiled-coil-helix domain containing 3
CLIC4	Chloride intracellular channel 4
CLPX	ClpX ATP-dependent selectivity component X*

CLYBL	Citrate lyase β like
COQ3	Coenzyme Q3 homolog, methyltransferase (S. cerevisiae)*
COQ6	Coenzyme Q6, monooxygenase
COQ8A	Atypical kinase involved in the biosynthesis of coenzyme Q
COX4I1	Complex IV (cytochrome c) oxidase subunit 4I1
COX5A	**Complex IV (cytochrome c) oxidase subunit Va***
COX5B	**Complex IV (cytochrome c) oxidase subunit Vb***
COX6A1	Complex IV (cytochrome c) oxidase subunit Via polypeptide 1*
COX7C	Complex IV (cytochrome c oxidase) subunit Viic*
CPOX	Coproporphyrinogen oxidase
CRAT	Carnitine O-acetyltransferase
CS	Citrate synthase*
DCAKD	Dephospho-CoA kinase domain containing
DHRS4	Dehydrogenase/reductase 4
DHTKD1	Dehydrogenase E1 and transketolase domain containing 1
EARS2	Glutamyl-tRNA synthetase 2, mitochondrial
ECHS1	Enoyl-CoA hydratase, short chain 1
ECSIT	ECSIT signalling integrator
EFHA1	Mitochondrial calcium uptake 2
ERAL1	Era like 12S mitochondrial rRNA chaperone 1
ETFB	Electron transfer flavoprotein β subunit
FASTKD2	FAST kinase domains 2
FASTKD5	FAST kinase domains 5
FDX1	Ferredoxin 1*
FECH	**Ferrochelatase (protoporphyria)***
FOXRED1	FAD dependent oxidoreductase domain containing 1
GATB (PET112)	**Glutamyl-tRNA amidotransferase subunit B***
GATC	Glutamyl-tRNA amidotransferase subunit C
GCDH	**Glutaryl-CoA dehydrogenase***
GFM1	G elongation factor mitochondrial 1
GLRX5	Glutaredoxin 5
GLS	Glutaminase
GLUD1	Glutamate dehydrogenase 1
GLUD2	Glutamate dehydrogenase 2*
GPT2	Glutamic-pyruvic transaminase 2
GRPEL1	GrpE like 1, mitochondrial
GRSF1	G-rich RNA sequence binding factor 1
GSTK1	Glutathione S-transferase kappa 1*
GTPBP10	GTP binding protein 10
GTPBP3	GTP binding protein 3
GUF1	GUF1 homolog, GTPase
HADH	Hydroxyacyl-CoA dehydrogenase
HARS2	Histidyl-tRNA synthetase 2, mitochondrial

HIBCH	3-Hydroxyisobutyryl-CoA hydrolase
HINT2	Histidine triad nucleotide binding protein 2
HSD17B10	Hydroxysteroid (17-β) dehydrogenase 10
HSD17B4	Hydroxysteroid (17-β) dehydrogenase 4*
HSD17B8	Hydroxysteroid (17- β) dehydrogenase 8
HSDL2	Hydroxysteroid dehydrogenase like 2
HSPA1L	Heat shock protein family A (Hsp70) member 1 like
HSPE1	Heat shock protein family E (Hsp10) member 1
IARS2	Isoleucyl-tRNA synthetase 2, mitochondrial
IBA57	IBA57, iron-sulfur cluster assembly
IDE	Insulin degrading enzyme
IDH3A	Isocitrate dehydrogenase 3 (NAD+) α
IDH3B	Isocitrate dehydrogenase 3 (NAD+) β*
IDI1	Isopentenyl-diphosphate δ isomerase 1
LARS2	Leucyl-tRNA synthetase 2, mitochondrial
LETM1	Leucine zipper and EF-hand containing transmembrane protein 1
LYRM4	LYR motif containing 4
LYRM7	LYR motif containing 7
MARS2	Methionyl-tRNA synthetase 2, mitochondrial
MDH2	Malate dehydrogenase 2
METTL17	Methyltransferase like 17
MGME1	Mitochondrial genome maintenance exonuclease 1
MICU2	Calcium uptake protein 2
MMAB	Methylmalonic aciduria (cobalamin deficiency) cblB type
MPST	Mercaptopyruvate sulfurtransferase
MRPL10	39S ribosomal protein L10
MRPL12	39S ribosomal protein L12
MRPL14	39S ribosomal protein L14
MRPL19	39S ribosomal protein L19
MRPL21	39S ribosomal protein L21
MRPL40	39S ribosomal protein L40
MRPL44	39S ribosomal protein L44
MRPL45	39S ribosomal protein L45
MRPL46	39S ribosomal protein L46
MRPL47	39S ribosomal protein L47
MRPL48	39S ribosomal protein L48
MRPL54	39S ribosomal protein L54
MRPL55	39S ribosomal protein L55
MRPS11	28S ribosomal protein S11
MRPS15	28S ribosomal protein S15
MRPS16	28S ribosomal protein S16
MRPS17	28S ribosomal protein S17
MRPS23	28S ribosomal protein S23

MRPS24	28S ribosomal protein S24
MRPS25	28S ribosomal protein S25
MRPS26	28S ribosomal protein S26
MRPS28	28S ribosomal protein S28
MRPS36	28S ribosomal protein S36
MRPS6	28S ribosomal protein S6
MRPS7	28S ribosomal protein S7
MRRF	**Mitochondrial ribosome recycling factor***
MTHFD1L	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2
MTIF2	Mitochondrial translational initiation factor 2
MTPAP	Mitochondrial poly(A) polymerase
MTRF1	**Mitochondrial translational release factor 1***
MTRF1L	Mitochondrial translational release factor 1 like
NADK2	NAD kinase 2, mitochondrial
NARS2	**Asparaginyl-tRNA synthetase 2, mitochondrial***
ND1 (MT-ND1)	Complex I (NADH-ubiquinone oxidoreductase) chain 1*
NDUFA12	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 12
NDUFA2	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 2
NDUFA4	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 4*
NDUFA6	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 6
NDUFA7	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 7
NDUFAF1	**Complex I (NADH:ubiquinone oxidoreductase) α subcomplex assembly factor 1***
NDUFAF2	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex assembly factor 2
NDUFAF3	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex assembly factor 3
NDUFAF4	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex assembly factor 4
NDUFAF5	Complex I (NADH:ubiquinone oxidoreductase) arginine-hydroxylase
NDUFAF7	Complex I (NADH:ubiquinone oxidoreductase) protein arginine-methyltransferase
NDUFS2	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 2
NDUFS4	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 4
NDUFS6	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 6
NDUFS7	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 7
NDUFS8	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 8
NDUFV2	Complex I (NADH:ubiquinone oxidoreductase) flavoprotein 2
NDUFV3	Complex I (NADH:ubiquinone oxidoreductase) flavoprotein 3
NFS1	**NFS1 nitrogen fixation 1 homolog (S. cerevisiae)***
NFU1	NFU1 iron-sulfur cluster scaffold
NIPSNAP1	**NipSnap homolog 1***
NIPSNAP2	NipSnap homolog 2
NME4	NME/NM23 nucleoside diphosphate kinase 4
NMNAT3	Nicotinamide nucleotide adenylyltransferase 3
NUBPL	Nucleotide binding protein like
NUDT1	Nudix hydrolase 1

NUDT19	Nudix hydrolase 19
OGDH	Oxoglutarate dehydrogenase
OXA1L	OXA1L, mitochondrial inner membrane protein
OXCT1	**3-Oxoacid CoA transferase 1***
PAM16	Presequence translocase associated motor 16
PCK2	Phosphoenolpyruvate carboxykinase 2, mitochondrial
PDE12	Phosphodiesterase 12
PDIA3	Protein disulfide isomerase family A member 3
PDK3	**Pyruvate dehydrogenase kinase, isozyme 3***
PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
PITRM1	Pitrilysin metallopeptidase 1
PMPCA	Peptidase, mitochondrial processing α subunit
PMPCB	Peptidase, mitochondrial processing β subunit*
PNPT1	Polyribonucleotide nucleotidyltransferase 1
POLDIP2	Polymerase (DNA-directed), δ interacting protein 2*
POLG	DNA polymerase γ, catalytic subunit
POLG2	**Polymerase (DNA directed), γ 2, accessory subunit***
POLRMT	RNA polymerase, mitochondrial
PPA2	Pyrophosphatase (inorganic) 2
PPIF	Peptidylprolyl isomerase F
PRKCA	Protein kinase C α
PTPMT1	Protein tyrosine phosphatase, mitochondrial 1
PYCR1	Pyrroline-5-carboxylate reductase 1
PYCR2	Pyrroline-5-carboxylate reductase 2
QRSL1	**Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1***
RG9MTD1	tRNA methyltransferase 10C, mitochondrial RNase P subunit
RNMTL1	Mitochondrial rRNA methyltransferase 3
RPS15A	Ribosomal protein S15a
RTN4IP1	Reticulon 4 interacting protein 1
SDHA	**Complex II (Succinate dehydrogenase complex) subunit A, flavoprotein***
SDHAF3	Complex II (Succinate dehydrogenase complex) assembly factor 3
SDHB	Complex II (Succinate dehydrogenase complex) iron-sulfur subunit B
SDHC	Succinate dehydrogenase complex subunit C, integral membrane protein, 15kDa*
SFXN4	Sideroflexin 4
SHMT2	Serine hydroxymethyltransferase 2
SLC27A2	Solute carrier family 27 member 2
SLIRP	SRA stem-loop interacting RNA binding protein
SPRYD4	SPRY domain containing 4
SSBP1	**Single-stranded DNA binding protein 1***
SUCLA2	Succinate-CoA ligase ADP-forming β subunit
SUCLG1	Succinate-CoA ligase α subunit
SUCLG2	Succinate-CoA ligase GDP-forming β subunit

SUPV3L1	Suv3 like RNA helicase
TACO1	Translational activator of cytochrome c oxidase 1
TARS2	Threonyl-tRNA synthetase 2, mitochondrial
TBRG4	Transforming growth factor β regulator 4
TFAM	**Transcription factor A, mitochondrial***
THEM4	Thioesterase superfamily member 4
THNSL1	Threonine synthase like 1
TIMM44	Translocase of inner mitochondrial membrane 44
TMLHE	Trimethyllysine hydroxylase, ε*
TST	**Thiosulfate sulfurtransferase (rhodanese)***
UQCR10	Complex III (cytochrome b-c1 oxidase) complex, 7.2 kDa*
UQCRB	Complex III (cytochrome b-c1 oxidase) binding protein
USMG5	Upregulated during skeletal muscle growth 5 homolog (mouse)*
VARS2	Valyl-tRNA synthetase 2, mitochondrial
VWA8	Von Willebrand factor A domain containing 8
WARS2	**Tryptophanyl tRNA synthetase 2, mitochondrial***
XPNPEP3	X-prolyl aminopeptidase 3
ZADH2	Zinc binding alcohol dehydrogenase domain containing 2

Notes

Proteins in bold were identified as interactors by Bio-ID with and without chemical activation.
*Proteins identified using Bio-ID without chemical activation.

Table S3. ClpP substrates and interactors in the mouse mitochondria [4,5].
Proteins identified as potential interactors by TAILS are highlighted in blue. Proteins identified as potential substrates by trapping are in rows with no color. Proteins identified as potential interactors or substrates by both TAILS and trapping experiments are highlighted in green.

Gene Name	Protein Name
ABCB7	ATP-binding cassette sub-family B member 7, mitochondrial
AC02	Aconitase, mitochondrial
ACAA2	3-ketoacyl-CoA thiolase, mitochondrial
ACAD10	Acyl-CoA dehydrogenase family member 10
ACADL	Long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADS	Short-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial
ALDH1L2	Mitochondrial 10-formyltetrahydrofolate dehydrogenase
ALDH4A1	δ-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial
ALDH6A1	Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial
ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase
ARALAR2	Calcium-binding mitochondrial carrier protein Aralar2
ATP5A1	Complex V (ATP synthase) F1 subunit α[2]
ATP5B	Complex V (ATP synthase) F1 subunit β[2]
ATP5H	Complex V (ATP synthase) subunit d[2]
ATP5J2	Complex V (ATP synthase) subunit f
ATP5ME	Complex V (ATP synthase) subunit e/ε
ATPAF1	Complex V (ATP synthase) F1 complex assembly factor 1
BCKDK	[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial
C0Q7	5-demethoxyubiquinone hydroxylase, mitochondrial (DMQ hydroxylase)
C1QBP	Complement component 1 Q subcomponent-binding protein[2]
CCRN4L (NOCT)	Nocturnin[1]
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2[1]
CHCHD3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3
CKMT2	Creatine kinase S-type, mitochondrial
CLPX	ClpX ATP-dependent selectivity component X[1]
COII	Complex IV (Cytochrome c oxidase) subunit 2
COX4I1	Complex IV (Cytochrome c oxidase) subunit 4 isoform 1, mitochondrial
CPOX	Oxygen-dependent coproporphyrinogen-III oxidase, mitochondrial
CS	Citrate synthase, mitochondrial
CYB5B	Cytochrome b5 type B
CYC1	Complex III (Cytochrome b-c1 complex) subunit 4, heme protein, mitochondrial

D10JHU81E	ES1 protein homolog, mitochondrial
D2HGDH	D-2-hydroxyglutarate dehydrogenase, mitochondrial
DBT	Lipoamide acyltransferase component of branched-chain α-keto acid dehydrogenase complex, mitochondrial
DECR1	2,4-dienoyl-CoA reductase, mitochondrial
DLST	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial
ECH1	δ(3,5)-δ(2,4)-dienoyl-CoA isomerase, mitochondrial
EFG1	Elongation factor G, mitochondrial
ERAL1	GTPase Era, mitochondrial
ETFB	Electron transfer flavoprotein subunit β (β-ETF)
ETFDH	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial
FH	Fumarate hydratase, mitochondrial
FKBP8	Peptidyl-prolyl cis-trans isomerase FKBP8
FPGS	Folylpolyglutamate synthase[2]
FXN	Frataxin, mitochondrial
GOLPH3	Golgi phosphoprotein 3
GOT2	Aspartate aminotransferase, mitochondrial
GPD2	Glycerol-3-phosphate dehydrogenase, mitochondrial
GSTP1	Glutathione S-transferase P1[2]
HADHA	Trifunctional enzyme subunit α, mitochondrial
HK1	Hexokinase-1
HSPA9	Stress-70 protein, mitochondrial (75 kDa glucose-regulated protein) (GRP-75)[3]
IARS2	Isoleucine tRNA ligase, mitochondrial
LON1P	Lon protease homolog, mitochondrial
MCC1	Methylcrotonoyl-CoA carboxylase subunit α, mitochondrial
MCCC2	Methylcrotonoyl-CoA carboxylase β chain, mitochondrial
MRPL12	39S ribosomal protein L12[2]
MRPL13	39S ribosomal protein L13[1]
MRPL15	39S ribosomal protein L15
MRPL18	39S ribosomal protein L18[1]
MRPL39	39S ribosomal protein L39[2]
MRPL45	39S ribosomal protein L45
MRPL47	39S ribosomal protein L47
MRPP1	Mitochondrial ribonuclease P protein 1
MRPS15	28S ribosomal protein S15
MRPS17	28S ribosomal protein S17
MRPS2	28S ribosomal protein S2
MRPS22	28S ribosomal protein S22[1]

MRPS27	28S ribosomal protein S27
MRPS5	28S ribosomal protein S5
MRPS7	28S ribosomal protein S7
MTHFD1L	Monofunctional C1-tetrahydrofolate synthase[2]
MUT	Methylmalonyl-CoA mutase, mitochondrial
NDUFA12	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 12
NDUFA6	Complex I (NADH:ubiquinone oxidoreductase) α subcomplex subunit 6
NDUFAB1	Acyl carrier protein, mitochondrial
NDUFAF7	Complex I (NADH:ubiquinone oxidoreductase) complex assembly factor 7
NDUFB6	Complex I (NADH:ubiquinone oxidoreductase) β subcomplex subunit 6
NDUFS1	Complex I (NADH:ubiquinone oxidoreductase) core subunit S1
NDUFS2	Complex I (NADH:ubiquinone oxidoreductase) core subunit S2
NDUFS3	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 3
NDUFS6	Complex I (NADH:ubiquinone oxidoreductase) core subunit S6
NDUFS7	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 7
NDUFS8	Complex I (NADH:ubiquinone oxidoreductase) iron-sulfur protein 8
NDUFV1	Complex I (NADH:ubiquinone oxidoreductase) flavoprotein 1[1]
NDUFV2	Complex I (NADH:ubiquinone oxidoreductase) flavoprotein 2
NFS1	Cysteine desulfurase, mitochondrial (m-Nfs1)
NIPSNAP3B	Protein NipSnap homolog 3B
NME4	Nucleoside diphosphate kinase, mitochondrial
NNT	NAD(P) transhydrogenase, mitochondrial
OAT	Ornithine aminotransferase, mitochondrial[3]
OGDH	2-oxoglutarate dehydrogenase, mitochondrial
P32/C1QBP	Complement component 1Q subcomponent-binding protein, mitochondrial
PC	Pyruvate carboxylase, mitochondrial
PCBD2	Pterin-4-α-carbinolamine dehydratase 2
PCCA	Propionyl-CoA carboxylase α chain, mitochondrial
PCCB	Propionyl-CoA carboxylase β chain, mitochondrial
PCK2	Phosphoenolpyruvate carboxykinase (GTP), mitochondrial
PDHA1	Pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial
PDK4	Pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 4, mitochondrial
PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit, mitochondrial
PHB2	Prohibitin-2[2]
PITRM1	Presequence protease (pitrilysin metallopeptidase 1), mitochondrial
PMPCB	Mitochondrial processing peptidase subunit β
PNPT1	Polyribonucleotide nucleotidyltransferase 1, mitochondrial
POLDIP2	Polymerase (DNA-directed), δ interacting protein 2[1]
SDHA	Complex II (succinate dehydrogenase complex) subunit A, mitochondrial

SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1
SLC25A3	Phosphate carrier protein[2]
SLC25A4	ADP/ATP translocase 1
SUCLG2	Succinyl-CoA ligase (GDP-forming) subunit β, mitochondrial
TIMM44	Mitochondrial import inner membrane translocase subunit TIM44
TK2	Thymidine kinase 2, mitochondrial
TMLHE	Trimethyllysine dioxygenase, mitochondrial
TRAP1	Heat shock protein 75 kDa, mitochondrial
TYMS	Thymidylate synthase[2]
UQCR10	Complex III (cytochrome b-c1 oxidase complex) subunit 9
UQCRB	Complex III (cytochrome b-c1 oxidase complex) subunit 7
UQCRC1	Complex III (cytochrome b-c1 complex) subunit 1[1,3]
UQCRC2	Complex III (cytochrome b-c1 complex) subunit 2
UQCRFS1	Complex III (cytochrome b-c1 complex) subunit Rieske, mitochondrial
USP15	Ubiquitin carboxyl-terminal hydrolase 15[2]
VARS2	Valine tRNA ligase, mitochondrial
VDAC2	Voltage-dependent anion-selective channel protein 2 (VDAC-2) (mVDAC2)
WBSCR16	Williams-Beuren syndrome chromosomal region 16 protein homolog

Notes

[1]High confidence ClpXP substrates significantly enriched in ClpP trap over wild type ClpP [4].
[2]Putative ClpXP substrates and interactors significantly enriched in ClpP trap over negative control [4].
[3]Validated biochemically as *bona fide* ClpP substrates [4].

Table S4. Proteins copurifying with human ClpP (WT or trap) in the mitochondria of the fungal ageing model, *P. anserina* [6].
Names in bold refer to potential substrates based on exclusive purification or high enrichment in the human ClpP trap.

Gene Name	Protein Name
ACADSB	Short/branched chain specific acyl-CoA dehydrogenase
ACAT1	Acetyl-CoA acetyltransferase
ACO2	Aconitase[1]
ACU-8	Acetyl-CoA hydrolase[2]
ALDH5A1	Succinate-semialdehyde dehydrogenase[1]
ALDH6A1	Methylmalonate-semialdehyde dehydrogenase
AMT	**Aminomethyltransferase**
ATAD1	**ATPase family AAA domain-containing protein 1**
ATP5H	Complex V (ATP synthase) subunit d
AUH	Methylglutaconyl-CoA hydratase
BCAT2	Branched-chain-amino-acid aminotransferase
BIO2	**Biotin synthase**
CLPX	**ATP-dependent Clp protease ATP-binding subunit clpX-like**
CPS1	**Carbamoyl-phosphate synthetase I[1]**
CS	Citrate synthase
CYB5R1	NADH-cytochrome b5 reductase 1
CYS2	Probable serine-O-acetyltransferase cys2[2]
DECR1	2.4-Dienoyl-CoA reductase
DLAT	Pyruvate dehydrogenase E2 component[1]
DLD	**Dihydrolipoyl dehydrogenase[1]**
DLST	**2-oxoglutarate dehydrogenase E2 component**
ECHS1	Enoyl-CoA hydratase
ETHE1	Persulfide dioxygenase ETHE1
GCSH	Glycine cleavage system H protein
GFM1	**Mitochondrial elongation factor G[1]**
GLDC	Glycine cleavage system P protein
GLRX5	**Glutaredoxin-related protein 5**
GOT2	Aspartate aminotransferase
HADH	Hydroxyacyl-CoA dehydrogenase
HMGCL	**Hydroxymethylglutaryl-CoA lyase**
HNRNPA2B1	**Heterogeneous nuclear ribonucleoproteins A2/B1**
HSD17B8	Estradiol 17- β-dehydrogenase 8
HSPA9	Stress-70 protein[1]
HSPD1	60 KDa heat shock protein[1]
IDH2	Isocitrate dehydrogenase
IDH3A	Isocitrate dehydrogenase subunit α
ILV-2	Ketol-acid reductoisomerase[2]

IVD	**Isovaleryl-CoA dehydrogenase**
MCSA	2-Methylcitrate synthase[2]
MDH2	Malate dehydrogenase
NAGS	N-Acetylglutamate synthase
NDUFAB1	Acyl carrier protein
NDUFS1	**Complex I (NADH dehydrogenase:ubiquinone oxidoreductase) iron-sulfur protein 1**
NDUFS2	Complex I (NADH dehydrogenase:ubiquinone oxidoreductase) iron-sulfur protein 2
NDUFS3	Complex I (NADH dehydrogenase:ubiquinone oxidoreductase) iron-sulfur protein 3
NDUFV1	**Complex I (NADH dehydrogenase:ubiquinone oxidoreductase) flavoprotein 1**
NFS1	Cysteine desulfurase[1]
OAT	Ornithine aminotransferase[1]
OGDH	**2-Oxoglutarate dehydrogenase E1 component**
PDH1	Probable 2-methylcitrate dehydratase[2]
PDHA1	**Pyruvate dehydrogenase E1 component subunit α**
PDHB	**Pyruvate dehydrogenase E1 component subunit β**
PDHX	**Pyruvate dehydrogenase protein X component**
PDSS1	**Decaprenyl-diphosphate synthase subunit 1**
PHB	Prohibitin
PRDX5	Peroxiredoxin-5
SHMT2	Serine hydroxymethyltransferase[1]
SUCLA2	Succinyl-CoA ligase subunit β[1]
TOMM20	Mitochondrial import receptor subunit TOM20
TOMM40	Mitochondrial import receptor subunit TOM40
TOMM70A	Mitochondrial import receptor subunit TOM70
TUFM	Mitochondrial elongation factor Tu[1]
TXN	Thioredoxin
UQCRFS1	Complex III (cytochrome b-c1 complex) subunit Rieske
YMR31	**Mitochondrial 37S ribosomal protein YMR-31**

Notes

The gene names on the left column are the corresponding genes for either human proteins or, in case no human orthologs were identified, for the *P. anserina* proteins.

[1]The prokaryotic ortholog of this protein is a substrate of *E. coli* ClpXP.

[2]For this protein, no human ortholog was found. In this case, the fungal protein is listed.

Table S5. Putative substrates of ClpP in *Aspergillus flavus* identified using dioctatin in a chemical-activation strategy combined with two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [7].

Gene ID	Protein Name
AFLA_007020	Citrate synthase, putative[1,2]
AFLA_027580	Iron superoxide dismutase A, putative
AFLA_035620	Hsp70 chaperone BiP/Kar2, putative[4]
AFLA_045750	Antigenic mitochondrial protein HSP60, putative
AFLA_076680	Pyruvate dehydrogenase complex, dihydrolipoamide acetyltranferase[1]
AFLA_076710	Malate dehydrogenase[1,3]
AFLA_078520	Complex V (ATPase) subunit ATP4, putative
AFLA_085980	Regulatory protein, SUAPRGA1
AFLA_099990	Complex II (succinate dehydrogenase complex) subunit SDH1, putative[2]
AFLA_105610	Dihydroxy acid dehydratase Ilv3, putative
AFLA_119660	Complex V (ATP synthase) F1 β subunit, putative
AFLA_128580	Complex I (NADH-ubiquinone oxidoreductase) 304 kDa subunit precursor[1]
AFLA_130310	Protein disulfide isomerase Pdi1, putative[4]

Notes

Gene IDs correspond to JCVI-afl-v2.0 assembly of database version 97.2 (downloaded from http://fungi.ensemble.org/Aspergillus _flavus_Info/Index?db=core).

[1]Protein whose ortholog in *P. anserina* was identified as interactor of heterologously expressed human ClpP [6].

[2]Protein whose human ortholog was identified as an interactor of human ClpP [2,3].

[3]The human ortholog is localized in the mitochondria.

[4]Secreted protein

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