Over a decade ago Polymerase δ interacting protein of 38 kDa (PDIP38) was proposed to play a role in DNA repair. Since this time, both the physiological function and subcellular location of PDIP38 has remained ambiguous and our present understanding of PDIP38 function has been hampered by a lack of detailed biochemical and structural studies. Here we show, that human PDIP38 is directed to the mitochondrion in a membrane potential dependent manner, where it resides in the matrix compartment, together with its partner protein CLPX. Our structural analysis revealed that PDIP38 is composed of two conserved domains separated by an α/β linker region. The N-terminal (YccV-like) domain of PDIP38 forms an SH3-like β-barrel, which interacts specifically with CLPX, via the adaptor docking loop within the N-terminal Zinc binding domain of CLPX. In contrast, the C-terminal (DUF525) domain forms an immunoglobulin-like β-sandwich fold, which contains a highly conserved putative substrate binding pocket. Importantly, PDIP38 modulates the substrate specificity of CLPX and protects CLPX from LONM-mediated degradation, which stabilises the cellular levels of CLPX. Collectively, our findings shed new light on the mechanism and function of mitochondrial PDIP38, demonstrating that PDIP38 is a bona fide adaptor protein for the mitochondrial protease, CLPXP.
Mitochondria are essential organelles that play crucial roles in energy transduction, haem biosynthesis, fatty acid oxidation and cell signalling. Although the vast majority of mitochondrial proteins (~99%) are encoded on nuclear DNA, human mitochondria also contain a small genome (mtDNA) that encodes 13 polypeptides. This distribution of mitochondrial genes (over two genomes) creates a number of challenges for mitochondria, most notably the maintenance of protein homeostasis (proteostasis) within the organelle. Consistent with the importance of proteostasis within this organelle, genetic mutations in components of the mitochondrial proteostasis network (PN) have been linked to mitochondrial dysfunction, disease and ageing.

A key aspect of mitochondrial proteostasis is the regulated removal of damaged or unwanted proteins. In humans, this process is performed by five different AAA+ (ATPases associated with a variety of cellular activities) proteases, three of which are located in the inner membrane (two forms of the m-AAA (matrix-AAA) protease and a single i-AAA (intermembrane space-AAA) protease) and two of which are located in the matrix, LONP (encoded by a single gene, LONPI) and CLPX (encoded by two genes, CLPX and CLPP). Although deletion of LONP is embryonically lethal and LONM is regarded as the principal AAA+ protease of the matrix compartment, genetic mutations in CLPX and CLPP are associated with severe phenotypes in mice and diseases in humans. Consistently, mammalian CLPP is reported to play a critical role in a variety of important functions, including mitoribosome assembly and haem regulation. Likewise, compounds that dysregulated mitochondrial CLPP exhibit therapeutic potential against specific cancer cells. Despite the emerging importance of this protease in human health and disease, only a handful of CLPPX substrates have been verified. Furthermore, given human CLPX exhibits a distinct substrate specificity relative to its bacterial homologues, our current understanding of this proteolytic machine is limited.

Like most AAA+ proteases, human CLPX is composed of two components: a peptidase component, CLPP and a AAA+ unfoldase component, CLPX. The peptidase component forms a barrel-shaped oligomer that is composed of two heptameric rings, stacked back-to-back. To prevent the indiscriminate turnover of proteins, the catalytic residues of CLPP are encapsulated within the barrel and access to the proteolytic chamber is limited to a narrow entry portal at either end of the cylinder. The unfoldase component, CLPX, forms a hexameric ring that sits limited to a narrow entry portal at either end of the cylinder. The unfoldase component, CLPX, forms a hexameric ring that sits limited to a narrow entry portal at either end of the cylinder. Subsequent and translocation into the proteolytic chamber of the unfoldase component CLPX, forms a hexameric ring that sits limited to a narrow entry portal at either end of the cylinder. Subsequent to its entry into the cylinder, CLPX forms an immunoglobulin-like fold. Interestingly, although CLPX has been described as a nuclear protein, it has been reported to localise to several subcellular compartments, including the mitochondrion, the cytosol and even the plasma membrane. As a result of this broad and uncertain subcellular distribution of PDIP38, its physiological function currently remains unclear. Therefore, in order to resolve this ambiguity, we examined the in vitro import of radiolabelled human preprotein (pre-PDIP38) into isolated mitochondria and performed mitochondrial fractionation experiments (Fig. 1). Consistent with previous studies from others, these in vitro import data clearly demonstrate that the PDIP38 preprotein (pre-PDIP38) is imported into isolated mitochondria in a membrane potential-dependent manner (Fig. 1a, compare lanes 11 and 12). Importantly, the processed, mature (mt) form of the protein (mt-PDIP38) was protected from cleavage by proteinase K (Prot. K). In contrast, the mt-PDIP38 (herein referred to as PDIP38) was sequestered inside mitochondria. As expected for a matrix localised protein, PDIP38 was protected from digestion by Prot. K in both intact mitochondria (Fig. 1b, lanes 1–4) and mitoplast (Fig. 1b, lanes 5–8), similar to the known matrix protein CLPP.

Results

Human PDIP38 is targeted to the mitochondrial matrix, where it associates with CLPX. Although PDIP38 was originally described as a nuclear protein, it has been reported to localise to several subcellular compartments, including the mitochondrion, the cytosol and even the plasma membrane. As a result of the broad and uncertain subcellular distribution of PDIP38, its physiological function currently remains unclear. Therefore, in order to resolve this ambiguity, we examined the in vitro import of radiolabelled human preprotein (pre-PDIP38) into isolated mitochondria and performed mitochondrial fractionation experiments (Fig. 1). Consistent with previous studies from others and our group, these in vitro import data clearly demonstrate that the PDIP38 preprotein (pre-PDIP38) is imported into isolated mitochondria in a membrane potential-dependent manner (Fig. 1a, compare lanes 11 and 12). Importantly, the processed, mature (mt) form of the protein (mt-PDIP38) was protected from cleavage by proteinase K (Prot. K), demonstrating that mt-PDIP38 (herein referred to as PDIP38) was sequestered inside mitochondria. As expected for a matrix localised protein, PDIP38 was protected from digestion by Prot. K in both intact mitochondria (Fig. 1b, lanes 1–4) and mitoplast (Fig. 1b, lanes 5–8), similar to the known matrix protein CLPP.
PDIP38 is neither a substrate of CLPXP nor does it trigger dissociation of the CLPXP complex. Next, we examined the consequence of PDIP38 docking to CLPX, to determine if PDIP38 is a substrate of the CLPXP protease or a regulator that triggers dissociation of the CLPXP complex? To determine if PDIP38 is a substrate of CLPXP, we monitored the stability of purified recombinant mature PDIP38 in vitro, in the presence of active CLPX (Supplementary Fig. 2). Given that substrate recognition by many Clp proteases is generally mediated by degrons located at either the N or C termini48, we generated an untagged version of PDIP38, using the Ub-fusion system49. As a control, to ensure that human CLPX was active, we monitored the turnover of casein, a model unfolded protein and well-characterised CLPXP substrate15,26. Significantly, in contrast to the rapid CLPXP-mediated turnover of fluorescein isothiocyanate (FITC)-labelled casein (FITC-casein) (Fig. 2b, middle panel), the levels of untagged PDIP38 remained unchanged throughout the time course of the experiment (Fig. 2b, upper panel). These data clearly demonstrate that mature PDIP38 is not a substrate of the CLPXP protease; however, it remained unclear if the lack of PDIP38 turnover was due to dissociation of the CLPXP complex (triggered by PDIP38 docking to CLPX). To examine this possibility, we monitored the CLPXP-mediated turnover of FITC-casein in the presence of PDIP38 (Fig. 2b, lower panel). Interestingly, the addition of PDIP38 produced distinct effects on the turnover of FITC-casein. Although the turnover of α-casein was inhibited by PDIP38 (Fig. 2b, lower panel), in a concentration-dependent manner (Supplementary Fig. 2), the turnover of αs1- and κ-casein was unaffected by the presence of PDIP38 (Fig. 2b, lower panel and Supplementary Fig. 2, black bars). Given that the CLPXP-mediated turnover of κ-casein was unchanged by PDIP38 addition, these data suggest that the CLPXP complex remains intact in the presence of PDIP38. To confirm that PDIP38 interacts not only with CLPX, but also with CLPXP, we examined complex formation using fluorescence detected analytical ultracentrifugation (FD-AUC). Specifically, we generated a GFP-PDIP38 fusion protein and examined its sedimentation (in the absence or presence of CLPX with or without the addition of CLPP). The use of GFP-PDIP38 allowed specific detection (by GFP fluorescence) of
Fig. 2 PDIP38 forms a complex with CLPX (and CLPXP), but is not a CLPXP substrate. a Coomassie Brilliant Blue (CBB) stained 16.5% Tricine-buffered SDS-PAGE of PDIP38 pull-down from E. coli lysate containing recombinant GST-PDIP38, using beads either lacking (control) or containing immobilised H6-CLPX (CLPX). T total E. coli lysate with expressed GST-PDIP38, U unbound fraction, E eluted fraction. Lane 1, SeeBlue Plus MW protein standards. b In vitro degradation assay of PDIP38 (upper panel) or FITC-labelled casein by CLPXP protease in the absence or presence of 2.4 µM untagged PDIP38 (lower panels, as indicated). Samples were separated by 16.5% Tricine-buffered SDS-PAGE and analysed by fluorescence detection (FITC-casein) or CBB staining (PDIP38). Full gels are shown in Supplementary Fig. 15.
with an estimated molecular weight of ~45 kDa. In contrast, the ZBD of CLPX (Fig. 3c, middle panel) eluted at ~15 ml, (estimated MW of ~29 kDa), which is approximately double its theoretical MW (12.1 kDa) and hence similar to the other ClpX homologues, likely forms a homodimer (ZBD2). Consistent with the pull-down (Fig. 3b and Supplementary Fig. 3), PDIP38 formed a stable complex with CLPXZBD, which is based on the elution volume of the complex (~13.6 ml, equivalent to ~60 kDa) is likely a heterodimeric complex composed of PDIP38 bound to ZBD (theoretical MW = 62.5 kDa) (Fig. 3c, lower panel). Next, to gain a better understanding of the specificity of this interaction (between PDIP38 and CLPX), we compared the ZBD of several ClpX homologues, from both pro- and eukaryotic species (Fig. 3d), focusing on a particular region within the domain that is important for adaptor docking28,60,61. Interestingly, despite broad conservation of the entire domain, there was considerable sequence divergence across the putative adaptor-docking region (Fig. 3d). Therefore, we hypothesised that this region may have co-evolved with a new adaptor protein (i.e. PDIP38). To test this idea, we compared the ability of the human CLPX ZBD and ecClpX ZBD (ecZBD) to interact with human PDIP38 (Fig. 3e).

As predicted, human PDIP38 was exclusively recognised by human ZBD (Fig. 3e, lane 1) as GST-PDIP38 was not recovered in the presence of ecZBD (Fig. 3e, lane 3). Next, to determine which part of the adaptor-docking region was required for interaction with PDIP38, we generated a specific mutation within this region, in which residues 120–123 (SSTR) of human CLPX were replaced with AAAA in either full-length CLPX (here referred to as CLPX4A) or the ZBD of CLPX (here referred to as ZBD4A). Consistent with the lack of binding of PDIP38 to ecZBD,
Next, we asked the question what is the consequence of the interaction (between PDIP38 and CLXP) in cells? To address this question, we knocked down PDIP38 expression in human (HeLa) cells using small interfering RNA (siRNA). Following successful knock down of PDIP38 (Fig. 4a, middle panel, compare lanes 1–3 with lanes 4–6), using the PDIP38-specific siRNA (#22994, Thermo Fisher Scientific), we analysed the steady-state levels of selected mitochondrial proteins. From this analysis, we identified that the levels of CLPX were reduced in HeLa cells transfected with the PDIP38-specific siRNA (Fig. 4a, upper panel, lanes 1–3) when compared to the levels of CLPX in HeLa cells transfected with a control siRNA (Fig. 4a, lanes 4–6). Importantly, this change was specific to CLPX as the levels of CLPP (Fig. 4a, lower panel) and the cross-reactive band recognised by the anti-PDIP38 antisera (Fig. 4a, middle panel, *) were unchanged by PDIP38 knockdown. These data suggest that PDIP38 plays an important role in maintaining the steady-state levels of CLPX in the cell. To validate this finding, we compared the steady-state levels of CLPX in cells treated with an alternative PDIP38-specific siRNA (either s25055 or s25056) with an additional control siRNA (Supplementary Fig. 6). Significantly, the loss of CLPX (as a result of PDIP38 knockdown) was specific, as the steady-state levels of two unrelated proteins (i.e. mitochondrial SDHA and the cytosolic protein, GAPDH) were not affected (Supplementary Fig. 6b, lower panels). Interestingly, these data are reminiscent of an unrelated bacterial adaptor protein—ClpS, which protects its cognate unfoldase (ClpA) from autocatalytic degradation in vivo. Therefore, in order to determine if the levels of CLPX were regulated by its autocalytic turnover, we examined the stability of CLPX in vitro, in the presence of CLPP with or without the addition of PDIP38 (Supplementary Fig. 7a).

**PDIP38 inhibits the LONM-mediated turnover of CLPX in vitro and stabilises the steady-state levels of CLPX in cells.**

Fig. 4 PDIP38 stabilises CLPX protecting it from LONM-mediated degradation. a Representative immunoblots showing the steady-state levels of CLPX (upper gel strip), PDIP38 (middle gel strip) and CLPP (lower gel strip) in PDIP38-depleted HeLa cells (lanes 1–3) relative to control HeLa cells (lanes 4–6). Samples were collected at the indicated times post transfection of either Silencer Select siRNA (22994) targeting PDIP38 or a negative control siRNA (control). Proteins were separated by 15% Tris-glycine SDS-PAGE. b Non-specific cross-reactive protein in PDIP38 antisera. Full immunoblots are shown in Supplementary Fig. 16. The lower panel shows the quantitation of CLPX levels from three independent experiments, in PDIP38-depleted HeLa cells (grey bars, each blue cross represents an individual experiment) in comparison a negative control siRNA (white bars, each red plus represents an individual experiment). Initial level of CLPX was set to 100% 24 h post transfection in control. Error bars represent the standard error of the mean (SEM), n = 3. b In vitro degradation of CLPX by LONM₆ protease (400 nM) in the absence or presence of 1 µM PDIP38. Samples were separated by 10% Tris-Tricine SDS-PAGE and analysed by CBB staining. c Quantitation of in vitro degradation of CLPX by LONM₆ (400 nM) in the absence (open symbols, blue crosses represent individual data points) or presence (closed symbols, red crosses represent individual data points) of untagged PDIP38. Samples were separated by SDS-PAGE and analysed by CBB staining. Error bars represent the SEM of three independent experiments.
However, in contrast to the idea that CLPX is degraded autocatalytically, the in vitro stability of CLPX was unchanged by the presence of CLPP (Supplementary Fig. 7a). Next, in light of recent findings\(^6,\) which suggested that CLPX is a substrate of LONM, we speculated that PDIP38 might regulate the steady-state levels of CLPX in vivo by inhibiting its LONM-mediated turnover. To explore this possibility, we initially monitored the in vitro stability of CLPX in the presence of LONM, with or without the addition of PDIP38 (Fig. 4b). Consistent with the recent findings\(^6,\) CLPX was degraded by LONM with a half-life of \(~60\) min (Fig. 4c, open circles). Crucially, the LONM-mediated turnover of CLPX was inhibited by the addition of PDIP38 (Fig. 4b, lanes 8–13; Fig. 4c, filled circles). Moreover, the PDIP38-mediated inhibition of LONM was specific to CLPX turnover, as the addition of PDIP38 had no effect on the LONM-mediated turnover of casein (Supplementary Fig. 7b). Finally, we compared the steady-state levels of CLPX in cells lacking PDIP38, either in the presence of normal or depleted levels of LONM (Supplementary Fig. 6c). Consistent with our in vitro findings, depletion of LONM (by siRNA knockdown) partially restored the steady-state levels of CLPX in cells lacking PDIP38 (Supplementary Fig. 6c, compare lanes 2 and 3). Therefore, the PDIP38-mediated inhibition of CLPX turnover is in part due to PDIP38 shielding an exposed degron within CLPX that is normally recognised by LONM. Given the location of PDIP38 docking, this suggests that the CLPX degron is most likely located within the ZBD of CLPX. Collectively, these data suggest that the cellular levels and activity of mitochondrial CLPX (P) are not only regulated by the activity of LONM, but also by the levels of mitochondrial PDIP38.

The N-terminal domain (NTD) of PDIP38 is essential for interaction with CLPX. Next, to better understand the molecular basis of the interaction between CLPX and PDIP38, we examined the domain structure of PDIP38 and determined which domain (or domains) is required for docking to the ZBD of CLPX. To identify domain boundaries of PDIP38, we performed limited proteolysis (using thermolysin) of the mature protein (Supplementary Fig. 8). This approach demonstrated that PDIP38 is composed of two stable structural domains, which, based on the transient appearance of two intermediate fragments \((f_1'\text{ and } f_2')\) are likely to be joined by an exposed linker. To identify the boundary of these two domains, we performed six rounds of Edman degradation on the \(f_1\) fragment, revealing the sequence FLANHD. Based on this analysis, we defined fragment \(f_1\) as the C-terminal DUF525 domain and generated two GST-fusion proteins (Fig. 5a), the NTD of PDIP38 (residues 52–153) fused to the C terminus of glutathione S-transferase (GST) (GST-PDIP38\(_N\)) and the C-terminal domain of PDIP38 (residues 157–368) fused to the C terminus of GST (GST-PDIP38\(_C\)). To determine which domain was required for docking to CLPX, we performed a series of pull-down assays, in which \(H_2\)O-CLPX was immobilised to Ni-NTA agarose beads and then incubated with a bacterial cell lysate containing either overexpressed GST-PDIP38, GST-PDIP38\(_N\) or GST-PDIP38\(_C\) (Fig. 5b, lanes 2, 4 and 6, respectively). As a control, the different GST-PDIP38 fusion proteins were also incubated with Ni-NTA agarose beads lacking immobilised protein (Fig. 5b, lanes 3, 5 and 7, respectively). As expected, and consistent with Fig. 2a, full-length GST-PDIP38 was specifically eluted from the column containing immobilised \(H_2\)O-CLPX (Fig. 5b, lane 2). Importantly, deletion of the NTD of PDIP38 (GST-PDIP38\(_N\)) was sufficient to prevent any specific interaction between the two proteins (Fig. 5b, compare lanes 6 and 7). Consistent with these results, the NTD of PDIP38 alone was sufficient for interaction with CLPX as GST-PDIP38\(_N\) was specifically recovered from Ni-NTA agarose beads containing immobilised CLPX (Fig. 5b, lane 4) and not from beads lacking immobilised protein (Fig. 5b, lane 5). Taken together, these data clearly demonstrate that the NTD of PDIP38 docks to the ZBD of CLPX. Next, in order to better understand PDIP38 function, we crystallised mature PDIP38 (residues 52–368) and solved its structure by X-ray crystallography to 3.4 Å resolution (see Table 1 for statistics). Consistent with our biochemical analysis, the structure of PDIP38 is composed of two domains, an N-terminal YccV-like domain (residues 64–186) and a C-terminal DUF525 domain (residues 231–368), which are separated by a short linker region (Fig. 5c). The N-terminal YccV-like domain forms an antiparallel \(\beta\)-sheet structure composed of six \(\beta\)-strands (\(\beta_0–\beta_5\), \(\beta_1–\beta_2\), \(\beta_3–\beta_4\)), in which strands \(\beta_0\) to \(\beta_4\) are connected by loops and \(\beta_5\) is connected by a short \(\alpha_1\text{ helix}\) (Fig. 5c and Supplementary Fig. 9). Interestingly, in contrast to bacterial YccV (HspQ) homologues, the YccV-like domain of PDIP38 contains a large insertion between \(\beta_2\) and \(\beta_3\), which forms an extended structural element that contacts the C-terminal DUF525 domain. Specifically, \(\beta_2/\beta_3\) extension forms an antiparallel \(\beta\)-sheet with \(\beta_8\) and the proximal sheet of the C-terminal DUF525 domain. Not surprisingly, the \(\beta_8\)-strand of PDIP38 is also absent from bacterial ApaG homologues, suggesting that both of these regions have evolved in order to stabilise the interaction of the two PDIP38 domains. In addition to the extended \(\beta_2/\beta_3\)-sheet, the NTD of PDIP38 orthologues also contain an additional insertion, located between \(\beta_3\) and \(\beta_4\) (residues 143–166). This insertion is not only exposed in human PDIP38 (as it was susceptible to partial proteolysis), but is also highly flexible as it was not visible in the structure, presumably due to disorder. Based on the expected location of this loop, suspended over the putative substrate-binding pocket (see later) of the C-terminal DUF525 domain, we speculate that the L4 loop could play a role in regulating substrate-binding to the CTD. The linker (or intermediate) region, which connects the N- and C-terminal domains, is formed by a small N-terminal \(\alpha\)-helix (a1), a two-stranded antiparallel \(\beta\)-sheet (\(\beta_6\) and \(\beta_7\)) and a C-terminal \(\alpha\)-helix (a2). This domain makes extensive contact to the NTD, wrapping around the domain, which appears to form a hinge point for movement of the CTD and potential delivery of its “cargo” to the associated ATPase component, CLPX. The CTD of PDIP38 (residues 231–368) forms an immunoglobulin-like fold. However, in contrast to its bacterial homologues (i.e. ApaG), which form a seven-stranded \(\beta\)-sandwich, PDIP38 contains an additional strand (\(\beta_8\)), forming an eight-stranded \(\beta\)-sandwich composed of two four-stranded antiparallel \(\beta\)-sheets. The proximal sheet is composed of \(\beta_8–\beta_9–\beta_{10}–\beta_{13}\), while the distal sheet is composed of strands \(\beta_{12}–\beta_{11}–\beta_{14}–\beta_{15}\). Interestingly, although this domain exhibits only weak amino acid identity (~30%) with bacterial ApaG proteins and select eukaryotic F-box proteins with “other” domains (i.e. Fbox3), all of these proteins share considerable structural homology (root-mean-squared deviation of ~1.5 Å for superposition of Ca atoms). Notably, the DUF525 domain of human Fbox3 is essential for substrate recognition by the SCF- Fbox3 ubiquitin ligase\(^{64,65}\). Therefore, to gain a better understanding of PDIP38 function, we compared the known structure of human Fbox3 DUF525 domain with our structure of human DUF525 domain and several other bacterial ApaG proteins (Supplementary Fig. 10). From this analysis, we identified a conserved hydrophobic groove, located between the two \(\beta\)-sheets of the C-terminal domain (Fig. 5d and Supplementary Fig. 10). Significantly, all but one of the nine hydrophobic residues are absolutely conserved from bacteria to humans (see Supplementary Table 1 and Supplementary Fig. 11), hence we propose that this groove plays an important role in substrate recognition. Consistently, the Fbox3 substrate antagonist BC-1215 is proposed to dock into this conserved hydrophobic groove\(^{66,67}\). Based on its
similarity to the substrate-binding domain of Fbxo3, we speculate that the CTD of PDIP38 is responsible for the recognition of as yet undefined proteins, and hence their delivery to CLPX, for removal by the CLPXP protease. Taken together, these data indicate that PDIP38 possesses key structural elements required to function as an adaptor of a AAA+ protein, specifically mitochondrial CLPX (Fig. 5e). PDIP38 contains a distinct NTD that docks to the accessory ZBD of CLPX (a well-established adaptor protein binding platform in bacterial homologues) and a distinct C-terminal domain, with likely capacity to support protein–protein interaction, to either, directly facilitate substrate delivery to CLPXP or indirectly regulate the CLPXP protease function.

Discussion

We report the structural and functional characterisation of PDIP38, a component of the PN in mammalian mitochondria. Our biochemical analysis of PDIP38 shows that it is imported into the mitochondrion where it interacts specifically with the AAA+ unfoldase, CLPX (Fig. 1). PDIP38, however, is neither a substrate of CLPXP nor does it cause dissociation of the CLPXP complex (Fig. 2). We propose that PDIP38 is a modulator of the CLPXP protease. This proposal is based on our biochemical and cellular analysis of PDIP38, which demonstrate that PDIP38 not only fine-tunes the proteolytic activity of CLPXP in vitro, but also stabilises the cellular levels of CLPX by protecting CLPX from LONM-mediated degradation (Fig. 4). These features are commonly found in several bacterial AAA+ adaptor proteins including the bacterial N-recognin, ClpS, which modulates both the specificity and the stability of its cognate unfoldase (ClpA) both in vitro and in vivo.80,52,68–70. Our structural and biochemical studies demonstrate that PDIP38 is composed of two domains that are joined by a hinge region. The N-terminal yccV-like (NDTDU525) domain (orange) and the C-terminal DUF525 (CTDDUF525) domain (blue) are separated by a hinge or linker region (red). Two unstructured loops (L3 and L4) within the NTDYccV-like domain are illustrated by dotted lines.

Fig. 5 PDIP38 structure and proposed substrate delivery. a Schematic representation of GST-PDIP38 fusion constructs (preprotein numbering is used). b In vitro pull-down using Ni-NTA agarose with (lanes 2, 4 and 6) or without (lanes 3, 5 and 7) purified immobilised H10CLPX, incubated with E. coli lysate expressing GST-PDIP38 (lanes 2 and 3), GST-PDIP38N (lanes 4 and 5) or GST-PDIP38C (lanes 6 and 7). Eluted fractions are shown with samples analysed by Coomassie Brilliant Blue (CBB) staining or immunoblotting (with anti-GST) following separation by SDS-PAGE. As a control, purified H10CLPX is shown in lane 1. Full gels and immunoblots are shown in Supplementary Fig. 17. c Ribbon representation of human PDIP38 highlighting its two domains. The N-terminal yccV-like (NDTDU525) domain (orange), highlighting the conserved hydrophobic residues (sidechains shown) that line the proposed substrate-binding groove of Fbxo3 (as described in Supplementary Table 1 and Supplementary Figs. 11 and 12). The docking site for interaction with the ZBD of CLPX is also indicated. d Cartoon representation of the interaction between mitochondrial PDIP38 and CLPXP(P), illustrating the proposed recognition of a substrate via the CTD, for its delivery to CLPX, either for disassembly (via CLPX) or degradation (via CLPXP).
Table 1 Data collection and refinement statistics.

| PDIP38 |
|--------|

- **Data collection**
  - Space group: P2₁
  - Cell dimensions: 120.1, 120.1, 48.6 Å
  - Resolution (Å): 39.31-3.39 (3.60-3.39)
  - CC² in outermost shell: 73.1
  - Completeness (%): 99.8 (99)
  - Redundancy: 10.6 (10.6)

- **Refinement**
  - Program: PHENIX
  - Resolution (Å): 40-3.39 (4.27-3.39)
  - No. of reflections: 5690
  - Rwork/Rfree: 0.25/0.29 (0.29/0.33)
  - No. of atoms: 2001

- **Ramachandran statistics**
  - Residues in favoured region, no. (%): 92.8
  - Residues in allowed region, no. (%): 6.8
  - Residues in outlier region, no. (%): 0.4
  - PDB entry: 6ZLX

*Values within parentheses are for highest-resolution shell.*

revealed by our data. Given the obvious evolutionary relationship of these proteolytic systems, it will be fascinating to see if PDIP38 plays any role in regulating a potential N-degron pathway in human mitochondria.

Similar to the NTD, the C-terminal domain of PDIP38 has also been identified in components of mammalian degradation pathways. In this case, the CTD is composed of an immunoglobulin fold (DUF525), which is not only found in bacterial ApaG proteins of unknown function but also occurs in a subset of Fbxo3 proteins (including Fbxo3C). Significantly, Fbxo3 is the substrate recruitment component of a multidomain E3 ligase, termed the SCF complex (specifically the SCF-Fbxo3 complex). Importantly, Fbxo3 (and more specifically the DUF525 domain of Fbxo3) is essential for substrate (Fbxl2) recognition by the SCF-Fbxo3 complex. Consequently, Fbxo3 is conserved across all DUF525-containing proteins, including ApaG and mitochondrial PDIP38 (Supplementary Figs. 11 and 12).

However, based on its similarity to Fbxo3, we speculate that the DUF525 domain of PDIP38 also facilitates binding and delivery of a substrate to CLPXP (Fig. 5e). However, to date, we have yet to identify a ligand of PDIP38 that is delivered to CLPXP. Regardless of this speculative delivery function, PDIP38 clearly stabilises the cellular levels of CLPXP (inhibiting its turnover by LONM) and modulates the substrate specificity of CLPXP in vitro. As such, we propose that mitochondrial PDIP38 is a regulator of the CLPXP protease. Interestingly, PDIP38 has recently been identified as a PrimPol-interacting protein; however, the relevance of this interaction in mitochondria is currently unclear, as the N-terminal mitochondrial targeting sequence of PDIP38 is identified (by chemical cross-linking experiments) as the major site of this interaction and the mature form of PDIP38 was unable to stimulate PrimPol DNA synthesis. Therefore, many important questions about the function of mitochondrial PDIP38 still remain. What are the physiological substrates of mitochondrial PDIP38? In addition, PDIP38 is one of a growing number of mitochondrial proteins that also appears to “moonlight” in the nucleus. The link, however, between the proposed function of PDIP38 within these two compartments remains a crucial question. How is the subcellular location of PDIP38 controlled and what is the significance of its dual localisation in cells? Similarly, given PDIP38 and Fbxo3 share a conserved domain (DUF525), which is essential for Fbxo3 function, this begs the question, is there crosstalk between these two proteins, outside the mitochondria? It will be fascinating to investigate the expression and targeting of PDIP38 to mitochondria (and the nucleus) in different cell types and at different developmental stages in mammals, not only in relation to the steady-state levels of CLPXP, but also in relation to the turnover of Fbxo3 substrates. Finally, the future identification of physiological targets of the mitochondrial CLPXP/PDIP38 complex are eagerly awaited. These data will be invaluable to further develop our understanding of this system and its contribution to mitochondrial proteostasis.

**Methods**

**Plasmids.** For in vitro transcription and translation of human PDIP38, pOTB7/PDIP38 was obtained from the I.M.A.G.E. Consortium (ID 3349399). For the heterologous expression of PDIP38 in E. coli, the cdna coding for mature PDIP38 (residues 52-368) was amplified by PCR from pOTB7/PDIP38 using the appropriate primers (Supplementary Table 2) and cloned into either pHEF or SaclI and HindIII (to express untagged PDIP38), pET10N between NotI and XhoI (to express PDIP38 with an N-terminal His tag), pET10C between NdeI and NotI (to express PDIP38 as a C-terminal H10 fusion protein), pGEX-4T-1 between BamHI and XhoI (to express PDIP38 as a N-terminal GST-fusion protein) or pDD173 between NotI and HindIII (to express PDIP38 as a C-terminal GFP fusion protein with an N-terminal His tag). To generate PDIP38N (residues 52-153) and PDIP38C (residues 157-368) fused to GST, pGEX-4T-1/PDIP38 was subjected to site-directed mutagenesis using primers PDIP_bam1 and PDIP_bam2 (see Supplementary Table 2). The resulting plasmid (pDT1367, see Supplementary Table 3) contained a stop codon and an additional BamHI site (and was used directly for the expression of GST-PDIP38c). To generate GST-PDIP38c, pDT1367 was digested with BamHI, the cut vector ligated lacking the PDIP38 fragment to generate pDT1362. Plasmids for the expression of human CLPX (full-length and domain mutants) and human CLPP (either His tagged and untagged) were described previously. For expression of CLPXP and ZBD4A, pET10C/hCLPXP and pET10C/hZBD4A were generated by site-directed mutagenesis using appropriate primers (see Supplementary Table 2). For details of primer sequences and plasmid constructs, refer to Supplementary information.

**Proteins.** Recombinant proteins were expressed, either in BL21-CodonPlus® (DE3)-RIL or XL1-Blue (Agilent) E. coli cells, grown in 2xTY media (containing appropriate antibiotic). Protein expression was induced with the addition of 0.5 mM isopropyl-β-D-thiogalactosidase at OD₆₀₀ ~0.8 and cultures were grown for at least 4 h at 20°C. Following expression, His-tagged (H₆ or H₁₀) recombinant proteins were purified from E. coli lysates under native or denaturing conditions by immobilised metal affinity chromatography using Ni-NTA agarose (Qiagen) essentially as described using 50 mM Tris-HCl [pH 8.0], 300 mM NaCl supplemented with an appropriate concentration of imidazole for binding (10 or 20 mM), washing (20 or 65 mM) and elution (250 or 500 mM). Purified His₆-UB-PDIP38 and His₁₀-UB-CLPP were cleaved using His₆-Usp2C and the untagged mature proteins recovered via a method outlined previously. GST-PDIP38 was purified by affinity chromatography using GSH agarose (Biorose) as outlined by the manufacturer. Radiolabelled PDIP38 preprotein was synthesised using TNT® SP6 Quick Coupled Transcription-Translation System (Promega) with undigested pOTB7/PDIP38 as template and 11 μCi of [35S]Met/CysEXPRE35S protein labelling mix (specific activity of >1000 Ci/mmol) from Perkin Elmer. Protein assay (Bio-Rad) was used to determine protein concentrations using bovine serum albumin (Thermo Scientific) as a standard. Protein concentrations refer to the protomer, unless otherwise stated. FFT-casein, thermolysin, Prot. K and hen egg white lysozyme were purchased from Sigma-Aldrich, and DNAse I was purchased from Gold Biotechnology. SeeBlue® Plus2 pre-stained and Mark127™ unstained protein standards were from Life Technologies.
Electrophoresis and protein detection. Proteins were separated using either glycerine- or Tricine-buffered sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples in 1× SDS-PAGE sample buffer (80 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 5% [v/v] glycerol, 100 mM dithiothreitol (DTT) and 0.02% [w/v] bromophenol blue) were heat treated at 95 °C for 5 min before separation. For visualisation of proteins, gels were stained with Coomassie Brilliant Blue R250 solution (CBB) or transferred to polyvinylidfluoride (PVDF) membrane using semi-dry method for immunoblotting. Primary antibodies: anti-PDIP38 (P0D1P2; abcam ab109805), anti-PDIP38 (125/88; generated in rabbit using purified recombinant PDIP38-His as antigen), affinity-purified anti-CLPX25, anti-LONM25, anti-TIM23 (BD Biosciences), anti-SDHA (Intronvent), anti-GST (GE Healthcare) and anti-eclavulinate synthetase (Life Technologies) were used at 1:10000 for Western blotting. Peroxidase-coupled secondary antibodies: anti-rabbit, anti-mouse and anti-goat IgG (Sigma- Aldrich) were used at 1:5000 for Western blotting. Antibody complexes were detected using enhanced chemiluminescence detection reagents (GE Healthcare) and digital images captured using GeneSnap (SynGene) or Image Lab™ (Bio-Rad). FITC-casein was detected by in gel fluorescence (excitation 488 nm and emission 526 nm), while radiolabelled proteins were detected by exposing dried gels to phosphor screens. Imaging was performed using a Typhoon™ Trio variable mode imager and analysed using the ImageQuant software (GE Healthcare).

Limited proteolysis. H10-PDIP38 (0.1 mg/ml) was subjected to limited proteolysis using trypsin (0.01 mg/ml) at 30 °C for 30 min. The reaction was terminated by the addition of 1× sample buffer and the proteins denatured at 95 °C for 5 min.

In vitro binding analysis. The in vitro binding analysis was adapted from the method outlined in ref. 82. Escherichia coli cells containing expressed GST-PDIP38, GST-PDIP38-H10, GST-PDIP38un, or untagged PDIP38 were resuspended (5 ml/g wet weight of cells) in binding buffer (20 mM HEPES-KOH [pH 7.5], 100 mM K(OAc), 10 mM Mg(OAc), 10% [v/v] glycerol, 65 mM imidazole) supplemented with 0.5% (v/v) Triton X-100, EDTA-free protease inhibitor cocktail (Roche), 2 mM PMSF, and DNase I (100 U/ml). Following sonication and centrifugation, the cell lysate was collected and used for analysis.

Degradation assays. The CLPX-mediated degradation of FITC-casein was performed essentially as describedref. Briefly, 0.4 µM CLPX-P38 was preincubated at (30 °C for 30 min. The reaction was stopped by the addition of 1× SDS-PAGE sample buffer. For degradation analysis, the samples were loaded onto a 10% (w/v) SDS-PAGE gel and then subjected to electrophoresis at 100 V for 90 min. The gel was then washed with 2× binding buffer volumes (BV) of binding buffer supplemented with 0.5% (v/v) Triton X-100, followed by 10 BV of wash buffer (binding buffer supplemented with 0.25% (v/v) Triton X-100). Bound proteins were eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 500 mM imidazole). For binding assays containing full-length CLPX, all buffers were supplemented with 2 mM ATP and 10 mM β-mercaptoethanol.

Cell culturing and treatment. HEAs cells (a kind gift from Prof. N. Hoogendoorn and validated by Cellbank Australia) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% [v/v] foetal calf serum at 37 °C under an atmosphere of 5% (v/v) CO2. Transfection of plasmid (10 µg) or siRNA (20 nM synthetic siRNA (Life Technologies) was performed using Lipofectamine™ plus (Life Technologies) or DharmaFECT™ (Dharmacon). Transfection efficiency was determined using the CellTiter-Glo® (Promega) kit and fluorescence counts being measured at each radial position (five averages). Data were fitted to a continuous c(s) and clm model using SEDFIT (http://www.analyticalultracentrifugation.com).

Immunoprecipitation. Mitochondrial lysate in IB buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 10 mM Mg(OAc), 5% [v/v] glycerol) containing 0.5% (v/v) Triton X-100, 10 mM ATP and 2 mM PMSF was mixed with PAS covalently attached to antibodies (anti-PDIP38 or anti-CLPX) by end-over-end rotation for 1 h at 4 °C. Beads were washed with 3 BV of IB buffer containing 0.25% (v/v) Triton X-100, 10 mM ATP and 2 mM PMSF and antibody bound protein eluted using 1 BV of 50 mM glycine (pH 2.5).

Peptide library. To examine the peptide binding specificity of human CLXLZB, peptide libraries (IPT Peptide Technologies) composed of 13-mer peptides (overlapping by 10 residues) derived from mitOSS and EFTu were immobilised to a cellulose membrane (see Supplementary Figs. 4 and 5, respectively, for peptide sequences) and panned with either ZBD or ZBDmut, essentially as described previously with minor modifications. Each peptide library was incubated either with ZBD or ZBDmut (2.5 µM) in MP2 buffer (15.7 mM Tris-HCl [pH 7.6], 100 mM KCl, 20 mM MgCl2, 5% [v/v] sucrose, 0.05% (v/v) Tween-20) for 30 min with gentle shaking at room temperature. Following transfer to a PVDF membrane as described in ref. 37, the bound proteins were detected by immunodetection using anti-human IgM antibodies (1:10 000 dilution in 3% [w/v] skim milk powder/TBS-T and -treated) and visualised using GelDoc™ XR+ (Bio-Rad) imaging system and images captured using QuantityOne (Bio-Rad). Figures were generated by overlaying 4–5 membrane images with adjusted transparency (100%, 50%, 33%, 25%, 20%) to ensure that all images had equal contribution to the overall result.

Fluorescent detected analytical ultracentrifugation. The interaction of PDIP38 (GFP-PDIP38) with CLPX (CLPXun, CLPXmut) was analysed via AF-DUC, essentially as described by ref. 85. GFP-PDIP38 (50 nM), CLPX (500 nM) and CLPP (2.8 µM) were preincubated in 350 µl of XP-AUC buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 20 mM MgCl2, 0.02% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM DTT, 5 mM ATP) and loaded into chilled aluminium cells fitted with a two-channel channel/epoon centriphere and sapphire windows (adjusted to 125 Psi) containing 50 µl of FC-43 heavy oil (3M, ID no. 98-2004-0101-8). Loading holes were sealed with thin plastic covers and screws, and cells were loaded into a prechilled An-50 Ti rotor (Beckman Coulter). The density and viscosity (poise) of buffer was experimentally determined to be 1.0394 and 1.902 × 10−2, respectively, in a DMA 4100 densitometer and Anton Paar AMVn automated microviscometer (MEP instruments) fitted with a 1.6 mm capillary tube and 1.5 mm ball. Sedimentation velocity experiments were performed at 10 °C using an XL-A ana- lytical ultracentrifuge (Beckman Coulter). The instrument was equipped with a fluorescence detector. Samples were centrifuged at 72,500 × g to optimise gain settings, and radial scans were collected at 72,500 × g continuously between 5.8 and 7.3 cm using 2 × 10−4 cm increments, with fluorescence counts being measured at each radial position (five averages). Data were fitted to a continuous c(s) and clm model using SEDFIT (http://www.analyticalultracentrifugation.com).

Crystallisation, X-ray diffraction and structure determination. To investigate the structure of PDIP38, crystal screening was performed and crystals were obtained using 20% (v/v) PEG 8000, 100 mM HEPES, pH 7.5. Crystals were frozen using the crystallisation condition containing 15% (v/v) glycerol. Derivatives were prepared after the transfer of a native crystal into a drop solution containing the crystallisation buffer and a final concentration of Pt salts (1 mM Pt solution from Merck). Crystal ‘traps’ purchased from Hampton Research. Crystals were incubated for 1 h in these drops, transferred into cryo solution (see above), flash frozen in liquid nitrogen and data collected at 100 K at the Swiss Light Source (Villigen, Switzerland; beamline PXI). Data were recorded on a PILATUS 6M detector (Dectris, Baden-Daettwil, Switzerland) and data reduction was performed using the program Xds (ref. 87). The structure of PDIP38 was solved to 3.4 Å by a single anomalous dispersion techniques using one Pt derivative for phasing. The model was refined using PHENIX88. Most of the structure was unambiguously assigned in the electron density map, for residues 52–62 at the N terminus and the loop regions (I3 between residues 108–126 and I4 between residues 144–167), due to poor density. Table 1 presents statistics for the X-ray data collection and refined model. Structural figures were generated using ChimeraX Daily.

Statistics and reproducibility. For degradation assays, the reported means values and standard error of the mean was calculated from three independent experiments.
Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Atomic coordinates for the PDIP38 structure have been deposited in wwPDB under accession code PDB 6ZLX.

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Author contributions

Conceptualization, D.A.D. and K.N.T.; methodology, D.A.D., K.N.T., M.A.P. and K.Z.; investigation, P.R.S., E.J.B., H.Z., V.J.S., L.B.R., L.M.A. and K.Z.; writing—original draft, D.A.D., K.N.T. and K.Z.; supervision, project administration and funding acquisition, D.A.D. and K.N.T.

Competing interests

The authors declare no competing interests.

Additional information

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