Scientific Report

TRANSPARENT PROCESS

EMBO reports

Loss of CLPP alleviates mitochondrial cardiomyopathy without affecting the mammalian UPR^{mt}

Dominic Seiferling[1], Karolina Szczepanowska[1], Christina Becker[1], Katharina Senft[1], Steffen Hermans[1], Priyanka Maiti[1], Tim König[2], Alexandra Kukat[1] & Aleksandra Trifunovic[1,*]

Abstract

The mitochondrial matrix protease CLPP plays a central role in the activation of the mitochondrial unfolded protein response (UPR^{mt}) in *Caenorhabditis elegans*. Far less is known about mammalian UPR^{mt} signaling, although similar roles were assumed for central players, including CLPP. To better understand the mammalian UPR^{mt} signaling, we deleted CLPP in hearts of DARS2-deficient animals that show robust induction of UPR^{mt} due to strong dysregulation of mitochondrial translation. Remarkably, our results clearly show that mammalian CLPP is neither required for, nor it regulates the UPR^{mt} in mammals. Surprisingly, we demonstrate that a strong mitochondrial cardiomyopathy and diminished respiration due to DARS2 deficiency can be alleviated by the loss of CLPP, leading to an increased *de novo* synthesis of individual OXPHOS subunits. These results question our current understanding of the UPR^{mt} signaling in mammals, while introducing CLPP as a possible novel target for therapeutic intervention in mitochondrial diseases.

Keywords cardiomyopathy; CLPP; DARS2; mitochondrial translation; mitochondrial unfolded protein response

Subject Categories Metabolism; Signal Transduction

DOI 10.15252/embr.201642077 | Received 21 January 2016 | Revised 22 March 2016 | Accepted 12 April 2016 | Published online 6 May 2016

EMBO Reports (2016) 17: 953–964

Introduction

Highly compartmentalized eukaryotic cells have evolved several pathways to recognize stress conditions, including aberrant protein folding, and in response activate programs aimed at restoring homeostasis. Fine-tuning of the protein-folding environment in mitochondria is important for cell survival and studies in mammalian cells, and especially, *Caenorhabditis elegans* has highlighted the importance of the mitochondrial unfolded protein response (UPR^{mt}) in this process. In *C. elegans*, the UPR^{mt} signaling cascade is selectively activated by defective mitochondrial protein processing, and not by heat-shock, endoplasmic reticulum stress or manipulations that exclusively impair mitochondrial ATP synthesis [1,2]. The UPR^{mt} in worms upregulates the expression of mitochondria-specific chaperones, *hsp-60* and *hsp-6*, homologs of mammalian Hsp60 and mtHsp70 [1]. A mitochondrial matrix protease, CLPP-1, seems to have a central role in mediating the UPR^{mt} signals in worms by generating peptides through proteolysis of unfolded matrix proteins, which are exported by the HAF-1 transporter into the cytosol [2]. Through a yet unknown mechanism, these peptides seem to propagate the UPR^{mt} signal by activating the transcription factor ATFS-1, which together with co-factors UBL-5 and DVE-1 stimulates expression of genes encoding mitochondrial chaperones and proteases [1–3]. In contrast, many aspects of the mammalian UPR^{mt} signaling are less well understood though it was shown that loss of mitochondrial proteostasis results in an increased expression of *Hsp60*, *Clpp,* and the transcription factor *Chop* [4,5]. The central question remains, whether CLPP is necessary for initiation and propagation of the UPR^{mt} signals in mammals.

Here, we developed mouse models that lack CLPP protease in basal and conditions of high mitochondrial proteostatic stress to answer this question. To fuel mitochondrial proteostatic stress, we used heart-specific DARS2-deficient mice that lack the mitochondrial aspartyl aminoacyl-tRNA synthetase, essential for translation [6]. These animals develop a highly progressive cardiomyopathy due to severe dysregulation of mitochondrial protein synthesis resulting in the accumulation of unfolded/unassembled respiratory chain subunits, which in turn activate the UPR^{mt} signaling cascade [6]. Remarkably, the loss of CLPP in a DARS2-deficient background does not affect UPR^{mt} signaling or activation of other cellular stress responses. In fact, we show that the absence of CLPP partly recuperates mitochondrial protein synthesis in a DARS2-deficient background, leading to attenuation of mitochondrial cardiomyopathy and longer life span.

1 Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD) and Institute for Mitochondrial Diseases and Aging, Medical Faculty, University of Cologne, Cologne, Germany
2 Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD) and Institute for Genetics, University of Cologne, Cologne, Germany
*Corresponding author. Tel: +4922147884291; E-mail: aleksandra.trifunovic@uk-koeln.de

Results and Discussion

Loss of CLPP alleviates cardiomyopathy in DARS2-deficient heart

To define the role of CLPP in proteotoxic stress, we selectively disrupted the *Clpp* gene in DARS2-deficient heart and skeletal muscle using a CRE recombinase expressed under the muscle creatine kinase promoter (*Ckmm-Cre*). To reduce the overall number of matings and animals used in the experiments, triple heterozygous animals (*Dars2*$^{+/L}$*; Clpp*$^{+/L}$*; Ckmm-Cre*$^{+/T}$) were mated with *Dars2*$^{L/L}$*; Clpp*$^{L/L}$ animals to obtain wild-type (WT), ClpP KO (*Dars2*$^{+/L}$*, Clpp*$^{L/L}$*, Ckmm-Cre*$^{+/T}$), Dars2 KO (*Dars2*$^{L/L}$*, Clpp*$^{+/L}$*, Ckmm-Cre*$^{+/T}$), and DKO (*Dars2*$^{L/L}$*, Clpp*$^{L/L}$*, Ckmm-Cre*$^{+/T}$) mice (Fig EV1A). Control experiments were performed to ensure that ClpP KO and Dars2 KO, which were also heterozygous for *Dars2* and *Clpp*, respectively, did not differ from single mutants (*Dars2*$^{L/L}$*, Clpp*$^{+/+}$*, Ckmm-Cre*$^{+/T}$ and *Dars2*$^{+/+}$*, Clpp*$^{L/L}$*, Ckmm-Cre*$^{+/T}$) in the observed phenotypes (Figs EV1B–D, EV2A and B, and EV4B and C). Since we previously did not observe activation of stress responses in the skeletal muscle due to DARS2 deficiency owing to increased proteostatic capacity and lower turnover rate of transcripts and proteins [6], the phenotypes in this tissue were not followed.

Mice of all different genotypes were born at the expected Mendelian ratios. Dars2 KO mice have severely shortened life span and the loss of CLPP resulted in a highly significant increase of both, median and maximal life span of about 35% (Fig 1A). In contrast, this early, no lethality was observed in WT and ClpP KO mice (Fig 1A). We also detected significant reduction in heart size in DKO compared to Dars2 KO animals at 6 weeks of age (Figs 1B and C, and EV1B). Moreover, DKOs had normal body weight that was significantly higher than observed in Dars2 KO mice (Figs 1D and EV1C). This was also reflected in the reduction of heart-to-body weight ratio of DKOs, when compared to Dars2 KO mice (Figs 1E and EV1D).

Based on the reduction in heart size of DKO compared to Dars2 KO, we asked whether molecular hypertrophy markers are also changed in these animals. The *Nppb* expression levels were significantly reduced upon CLPP depletion in DARS2-deficient animals, but still elevated compared to controls (Fig 1F). *Nppa* levels were normalized in DKO animals, although there was a trend toward increased expression (Fig 1F). Enzyme histochemical double staining for complex IV (COX) and complex II (SDH) activities demonstrated a milder COX deficiency in DARS2-deficient hearts when CLPP was not present, which goes in hand with reduced interstitial fibrosis shown by Masson's trichrome staining (Fig 1G and H). Very high SDH activity (blue staining), consistent with the reported increase in mitochondrial mass and hypertrophy [6], was detected only in Dars2 KO hearts (Fig 1G). These results clearly show that loss of CLPP in the condition of high mitochondrial dysfunction, as observed in DARS2-deficient hearts, ameliorates cardiomyopathy and leads to a prolonged life span.

Mitochondrial and cellular stress responses do not depend on CLPP

CLPP was shown to be essential for the UPR^{mt} signaling in worms and to be upregulated in mammalian mitochondria in conditions of increased stress [2,4]. Therefore, we set out to understand how the loss of CLPP partially rescues the mitochondrial cardiomyopathy of Dars2 KO hearts, if essential for the mammalian UPR^{mt}. First, we analyzed the levels of mitochondrial chaperones and proteases that are markers of increased proteostatic stress. A clear upregulation of mitochondrial chaperones, HSP60, mtHSP70, and TRAP1, was detected in both, Dars2 KO and DKO hearts (Fig 2A and B). We also observed a comparable increase in the steady-state levels of mtHSP70 and TRAP1 in the ClpP KO mice (Fig 2A and B), indicating that CLPP deficiency on its own induces mitochondrial stress, in agreement with previous results [7]. Mitochondrial proteases, LONP1 and AFG3L2 were upregulated exclusively in Dars2 KO and DKO hearts (Fig 2A and B). As mentioned before, Dars2 KO and ClpP KO animals are heterozygous for the *Clpp* and *Dars2* allele, respectively; nevertheless, identical changes were found when single mutants were analyzed (Fig EV2A and B). To exclude that a general mitochondrial biogenesis leads to upregulation of chaperones and proteases, we measured the steady-state levels of chaperones and proteases in isolated mitochondria and found comparable levels of upregulation (Fig EV2C).

The UPR^{mt} is a response that is primarily characterized by increased expression of its target genes. Target genes for mammalian UPR^{mt} are so far defined by the presence of a mitochondrial stress-responsive element that corresponds to the CHOP (CCAAT/enhancer-binding protein (C/EBP)-homologous protein) binding site flanked by two novel mitochondrial unfolded protein response elements (MURE1/2) [8]. We have analyzed a number of genes containing only the CHOP binding site (*Hsp60*) or the MURE1-CHOP-MURE2 motif (*Dnaja3, Yme1l1, Pmpcb, Txn2*) in DARS2-deficient hearts and observed general upregulation (except for *Yme1l1*) at 3 weeks of age, independent of the presence of CLPP (Fig 2C). The activation of UPR^{mt} was stronger at 3 weeks than at 6 weeks of age, when only increased levels of *Hsp60* and *Chop* transcripts were still present (Fig EV3A), in agreement with our previous results showing that activation of stress responses in Dars2 KO hearts precedes OXPHOS deficiency [6]. Interestingly, levels of transcription factor A (TFAM), a marker of mitochondrial biogenesis, were only increased at 3 weeks of age (Figs 2D and EV3B). To promote efficient protein folding and prevent accumulation of misfolded and misassembled proteins, mitochondria have developed a number of molecular chaperones and proteases. Here, we show that *mtHsp70*, *Lonp1,* and *Afg3l2* transcript levels are highly and persistently elevated in Dars2 KO and DKO hearts at 3 and 6 weeks of age (Figs 2D and EV3B). Collectively, these data demonstrate that CLPP is not required for the activation of mammalian UPR^{mt} in cardiomyocytes *in vivo* under conditions of high proteotoxic stress.

To test whether the CLPP presence is required for the activation of UPR^{mt} in a different model, we established CLPP-deficient HEK293T cells using the CRISPR/Cas9 technology. We activated the UPR^{mt} in these cells by knocking down paraplegin/SPG7 commonly used in *C. elegans* for the same purpose [2,9]. Indeed, also in human cells, knockdown of *paraplegin/SPG7* leads to high upregulation in the expression of the UPR^{mt} markers, including *DNAJA3, YME1L1, PMPCB,* and *TXN2* that was fully independent of CLPP (Fig EV3C). The activation of *bona fide* UPR^{mt} markers is accompanied by upregulation of *mtHSP70, LONP1*, and *AFG3L2* (Fig EV3D), as previously observed in Dars2 KO and DKO knockout hearts (Fig 2C and D). Remarkably, the levels of *HSP60* were only mildly increased in CLPP-deficient cells, in agreement with our *in vivo* data

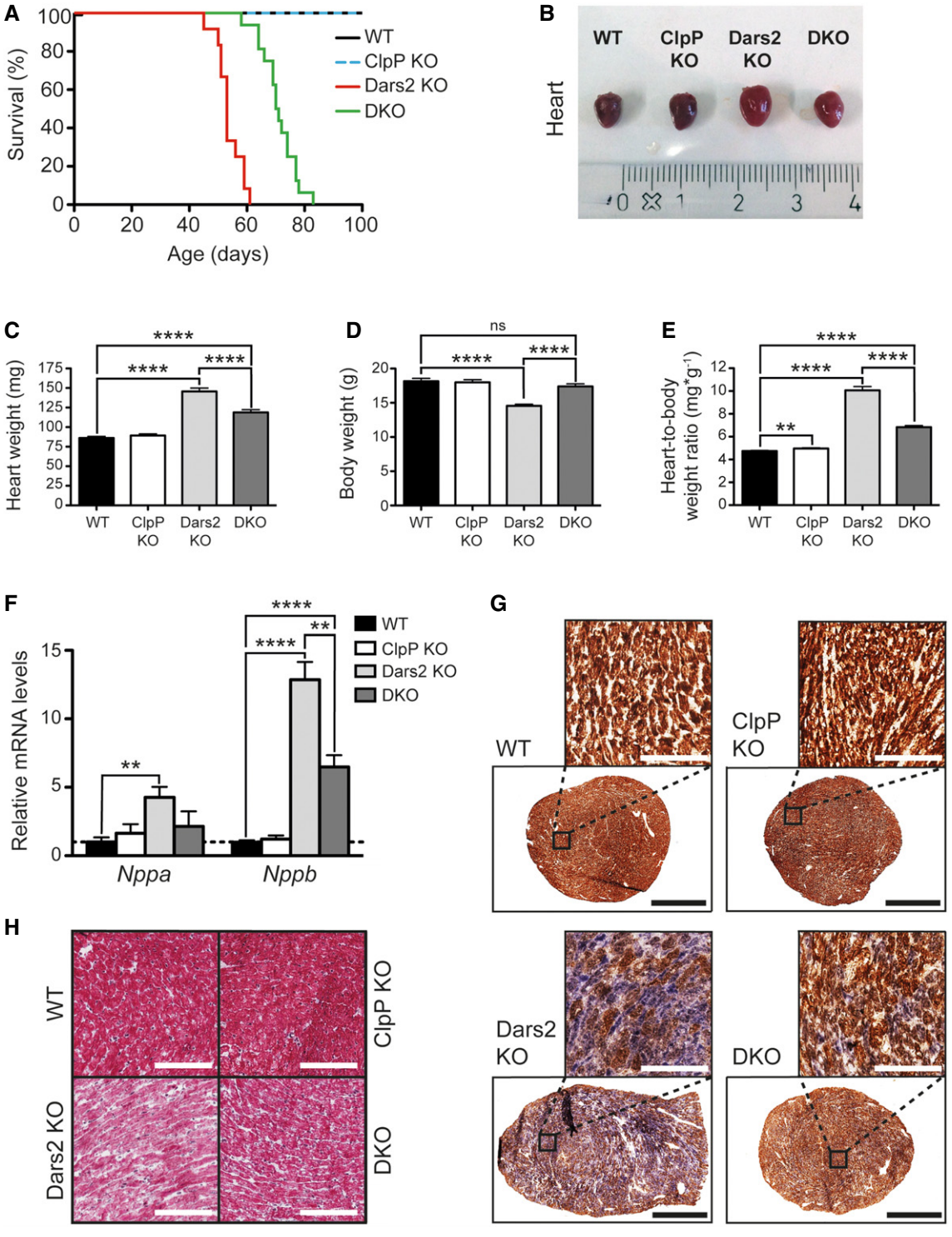

Figure 1. Loss of CLPP alleviates cardiomyopathy in DARS2-deficient heart.

A Kaplan–Meier survival curves (n = 12–16).

B Heart gross morphology.

C–E (C) Body weight, (D) heart weight, and (E) heart-to-body weight ratio (n = 28–40).

F Relative expression levels of cardiac hypertrophy markers (*Nppa* and *Nppb*) (n = 5).

G Enzyme histochemical double staining for COX and SDH activities (n = 4).

H Assessment of cardiac fibrosis by Masson's trichrome staining (n = 4). White scale bars, 100 μm; black scale bars, 1 mm.

Data information: (C–F) Bars represent mean ± SEM (Student's t-test, $^{**}P < 0.01$, $^{****}P < 0.0001$).

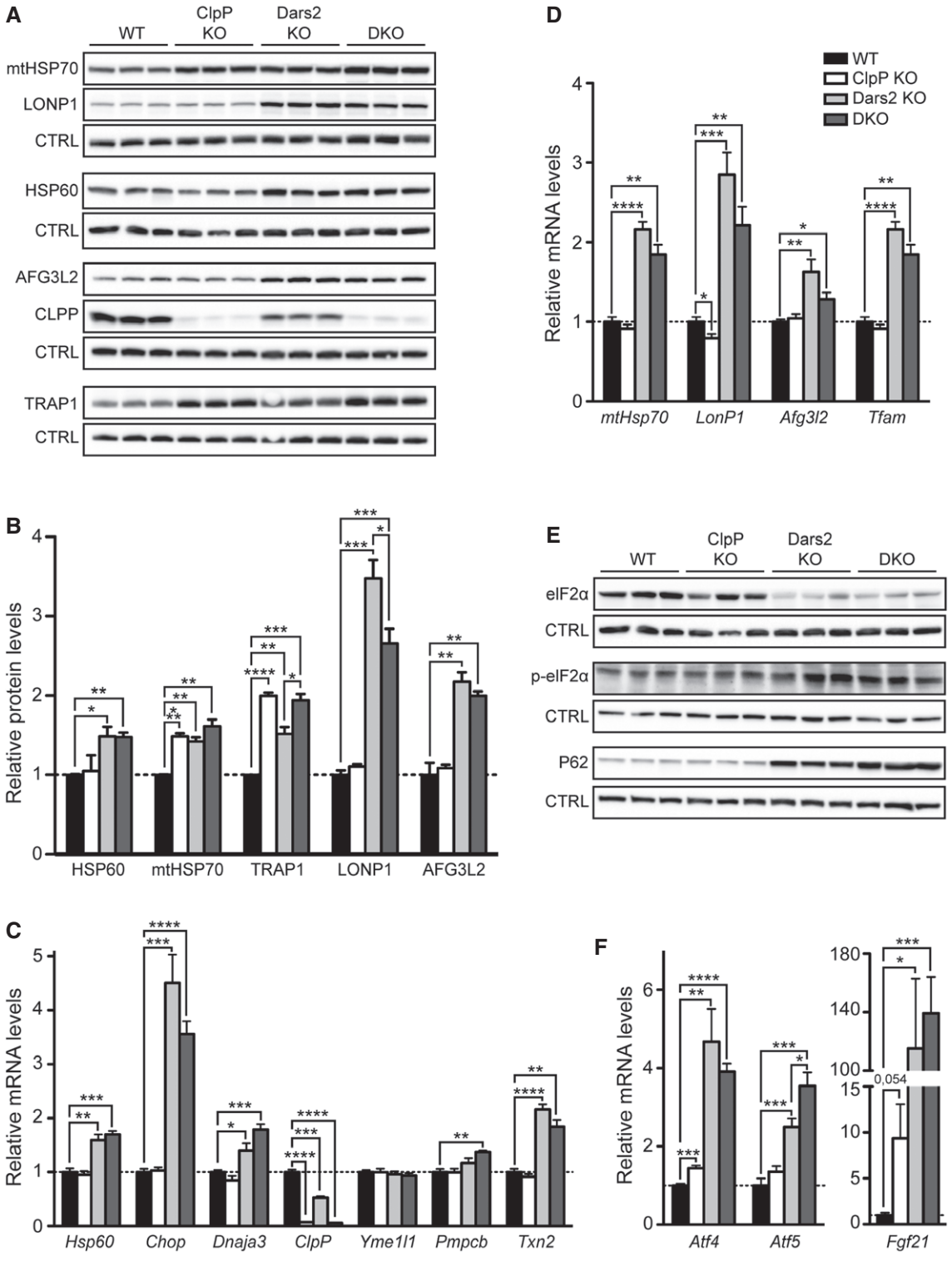

Figure 2. Mitochondrial and cellular stress responses do not depend on CLPP.

A, B (A) Western blot analysis and (B) relative quantification of UPR^{mt} markers in heart extracts. HSC70 is used as a loading control (CTRL) (n = 3).

C, D Relative expression levels normalized to WT of (C) established UPR^{mt} markers and (D) mitochondrial chaperones, proteases, and TFAM in 3- to 4-week-old mice (n = 3–4).

E Western blot analysis of cellular stress markers. HSC70 is used as a loading control (CTRL) (n = 3).

F Relative expression levels of transcription factors involved in UPR^{mt} and ISR (left) and *Fgf21* (right) (n = 5).

Data information: (B–D, F) Bars represent mean ± SEM (Student's t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

on DARS2-deficient hearts (Figs 2C and EV3A and C). Similarly, upregulation of mtHSP70, but not HSP60 levels, is observed in mammalian cells depleted of LRPPRC, a key posttranscriptional regulator of mtDNA expression [10]. The knockdown of *LRPPRC* and its *C. elegans* homolog *mma-1* induces mito-nuclear imbalance and UPR^{mt} in both model organisms [10].

Our results provide the first evidence that CLPP is neither required for, nor it regulates the UPR^{mt} signaling in mammals. Although we cannot exclude that CLPP might have a role in activating UPR^{mt} in other mammalian/murine tissues and under different conditions, our results argue against a general role for CLPP in this process supported by our strong data in two different model systems. In the pioneering work that described the existence of UPR^{mt} in mammalian cells, increased *Clpp* expression was detected upon overexpression of a folding-impaired protein (ΔOTC) in the mitochondrial matrix [4]. More recently, it was shown that treatment of hepatocytes with rapamycin, which induces mito-nuclear protein imbalance and activates UPR^{mt}, also increases *Clpp* expression [5]. Therefore, unlike in *C. elegans*, previous data in mammals suggest that CLPP is a target, not the driving force of UPR^{mt} [4,5]. In agreement with that and our study, recently it was shown that LONP1, not CLPP, degrades misfolded proteins inside mitochondrial matrix, including ΔOTC [11], and therefore, CLPP could not produce an efflux of peptides needed to activate UPR^{mt} as described in *C. elegans* [12]. Furthermore, in contrast to the UPR^{mt} in *C. elegans*, knockdown of *CLPP* did not prevent PINK1 accumulation induced by ΔOTC expression that was rather dependent on the presence of LONP1 [13].

We previously observed that loss of DARS2 in heart causes strong dysregulation of mitochondrial protein synthesis leading to high upregulation of mitochondrial chaperones and proteases, accompanied with an early increase in CLPP levels, suggesting activation of UPR^{mt} [6]. Conversely, here, we show that CLPP is dispensable for the UPR^{mt} activation in the DARS2-deficient heart and upon knockdown of *paraplegin/SPG7*, as we detected an unaffected increase of both, protein and transcript levels of UPR^{mt} markers and various other mitochondrial chaperones and proteases, in the absence of CLPP. The expression of CHOP and ATF5 (mammalian homolog of *C. elegans* ATFS-1), two transcription factors proposed to play a central role in the UPR^{mt} signaling [4,14], was highly elevated in Dars2 KO hearts and not attenuated by the loss of CLPP (Fig 2C and F). Interestingly, both CHOP and ATF5 are also involved in the integrated stress response (ISR) [15] that in mammals could be activated by mitochondrial dysfunction [16,17]. ISR, characterized by a phosphorylation of the translation initiation factor eIF2α and upregulated expression of *Atf4* transcription factor, was induced by DARS2 depletion in hearts and was not dependent on the presence of CLPP (Figs 2E and F, and EV3E).

As mitochondria are an important hub for many cellular functions, mitochondrial dysfunction also affects other adaptive responses including autophagy. We showed that this stress pathway is affected by the DARS2 depletion in murine hearts [6], and now, we asked whether their activation depends on the presence of CLPP. Increased accumulation of P62 that suggests a suppression of autophagy was detected in both Dars2 KO and DKO, but not in WT and ClpP KO hearts (Figs 2E and EV3E). DARS2 depletion in the heart also results in a vast increase of transcripts for fibroblast growth factor 21 (FGF21) [6], a stress marker that serves as a "mitokine" upon mitochondrial dysfunction, which was not affected by the loss of CLPP (Fig 2F). Therefore, our findings demonstrate that, unlike in *C. elegans*, mammalian mitochondrial and cellular stress responses are not influenced by CLPP deficiency in conditions of increased proteostatic stress in the heart. Taken together, these data also suggest that mammalian UPR^{mt} might be intimately linked with ISR, yet our current results refute a role of CLPP in this process.

Our study also highlights a clear need for novel models systems and redefinition of UPR^{mt} markers in order to better understand the mammalian UPR^{mt} signaling. Although it has been instrumental for the discovery of the UPR^{mt} in mammals, cell culture-based systems in which a truncated version of the rat OTC gene (ΔOTC) is expressed in simian cells (COS7) and subsequently the activation of targets was measured using reporter plasmids containing either rat (*Hsp60*) or human (all other genes) promoters [4,8] do not reflect the most physiological state to dissect the UPR^{mt} in mammals. Furthermore, the relevance of the MURE1-CHOP-MURE2 element [8] has been questioned as this motif is widespread in the genome and not restricted to the UPR^{mt}-responsive genes. Besides, no new data since the initial study were published confirming the importance of these elements *in vivo* as well as ligands putatively associated with these two regulatory elements have not yet been identified [18]. Our results also suggest that mitochondrial chaperones and proteases including mtHSP70, TRAP1, AFG3L2, and LONP1 might be strong candidates as novel mammalian UPR^{mt} markers, not only because of their obvious involvement in proteostasis control in mitochondria, but also due to their strong increase in conditions when UPR^{mt} is upregulated. Therefore, we think that further research and understanding of endogenous targets and the signaling cascade of UPR^{mt} in mammals is clearly needed.

Lack of CLPP in DARS2-deficient hearts increases mitochondrial respiratory activity

Since loss of CLPP in DARS2-deficient animals significantly increases a very short life span and recuperates cardiac dysfunction (Fig 1), we asked what is the molecular basis behind this. We initially analyzed steady-state levels of individual OXPHOS subunits to determine their levels upon the loss of CLPP. We found a general improvement in the level of individual complex I, III, and IV subunits with loss of CLPP in DARS2-deficient hearts, including an increase in the steady states of COXI/MT-CO1, a mtDNA-encoded subunit of complex IV (Figs 3A and EV4A). This finding indicates that steady-state levels of complex I, III, and IV nDNA-encoded subunits depend on the availability of mtDNA-encoded polypeptides in order to be assembled in correct stoichiometry, as previously observed in mtDNA mutator mice [19]. In contrast, complex II and V subunits were not affected by depletion of either DARS2 or CLPP in heart mitochondria (Figs 3A and EV4A).

We next analyzed the level of assembled OXPHOS complexes by BN-PAGE and subsequent Western blot analysis. As seen for the single subunits, loss of CLPP in the DARS2-deficient hearts primarily elevated the level of complexes I, III, and IV (Figs 3B and EV4B and C). Remarkably, even loss of one CLPP allele in the DARS2 background mildly elevated the levels of OXPHOS complexes that were even more increased upon complete loss of CLPP (Fig EV4B). The observed change in the steady-state level was clearly mirrored by the increased complex I and IV in-gel activity in DKO hearts (Fig 3C

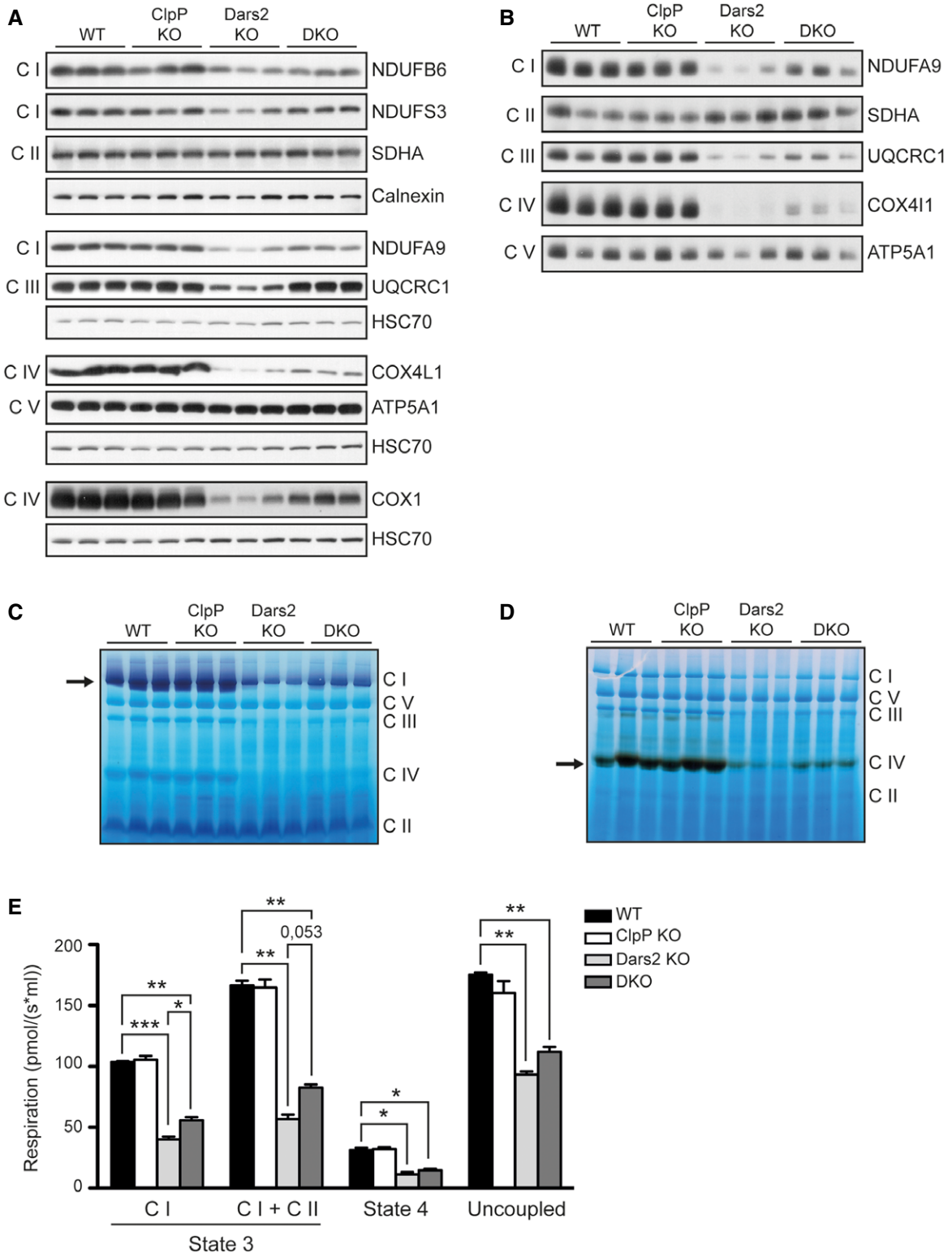

Figure 3. Lack of CLPP in DARS2-deficient hearts increases mitochondrial respiratory activity.

A, B (A) Western blot analysis of individual OXPHOS subunits in heart extracts. HSC70 and calnexin are used as loading controls (n = 3). (B) BN-PAGE and subsequent Western blot analysis for OXPHOS complexes. Antibodies against individual OXPHOS subunits (on the right) were used to detect OXPHOS complexes (on the left).

C, D In-gel activity of complexes I (C) and IV (D) performed after BN-PAGE.

E Oxygen consumption rates in intact heart mitochondria in the presence of pyruvate–glutamate–malate (C I) and pyruvate–glutamate–malate + succinate (C I + C II) as substrates. State 3 (substrates + ADP), State 4 (+oligomycin), uncoupled (+FCCP). (n = 3). Bars represent mean ± SEM (Student's t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

and D, respectively). Collectively, these data demonstrate that loss of CLPP not only increases levels of individual complexes, but also their activity in the condition of high mitochondrial stress inflicted by the loss of DARS2. We proceeded to investigate the bioenergetic consequences of the increased complex I and IV activities by assessing the oxygen consumption rates in freshly isolated cardiac mitochondria (Fig 3E). Mitochondria were incubated with respiratory substrates whose metabolism results in a delivery of electrons at the level of complex I (pyruvate, glutamate, malate) and II (succinate), and the oxygen consumption rate was recorded in the phosphorylating (state 3: ADP and Pi), non-phosphorylating (state 4: oligomycin), and uncoupled state (FCCP). Upon loss of CLPP, the respiration of DARS2-deficient mitochondria significantly increased in the phosphorylating state, although still remained lower than in WT mice (Fig 3E).

Dysregulation of mitochondrial protein synthesis is partially rescued by the loss of CLPP

Based on the observation that mitochondrial respiratory capacity was increased in DKOs compared to Dars2 KO mice, we questioned how the absence of CLPP could actually influence the load of functionally active OXPHOS complexes. Given the fact that DARS2 deficiency leads to a severe translational defect, we investigated the rates of protein synthesis and degradation in isolated cardiac mitochondria. Our analysis revealed that the overall protein synthesis rate is strongly influenced by the presence of CLPP, as its deficiency leads to a 25–30% decrease in mitochondrial translation (Figs 4A and B, and EV5A–D), not caused by lower transcript levels (Fig EV5E). We also observed higher turnover rates of newly synthesized proteins in Dars2 KO and DKO mitochondria (Fig 4A and B), probably as a consequence of the detected increase of other mitochondrial proteases (Fig 2A and B). Surprisingly, even though DKO animals have lower protein synthesis and higher turnover rates, the amount of individual OXPHOS subunits synthesized is significantly increased compared to Dars2 KO (Fig 4D). This was not due to any residual DARS2 presence, as no protein was detected in either Dars2 KO or DKO heart extracts (Fig 4C). Instead, a significant rise in the proficient vs. abortive protein synthesis upon loss of CLPP in DARS2-deficient mitochondria seems to be the major contributor (Fig 4A and E). While in Dars2 KO mitochondria, almost 50% of all newly synthesized polypeptides have low molecular mass, hence we assumed that they are aborted in the early steps of synthesis, in DKOs, the abortive translation is significantly reduced and accompanied by an increase in the synthesis rate of full-length OXPHOS subunits (Fig 4A and E). In contrast, in ClpP KO mitochondria, as in WT, only ATP8/ND4L polypeptides are present in the low molecular mass part of the gel suggesting that the loss of CLPP on its own, although significantly decreasing the overall rate of protein synthesis, does not dysregulate it (Fig 4A and E).

Mitochondria-encoded OXPHOS subunits are not predicted to be CLPP substrates, as they are embedded into inner mitochondrial membrane. Indeed, we did not see difference in COXI protein stability in wild-type and CLPP-deficient cells, using cycloheximide chase experiments to address a possible CLPP-mediated turnover (Fig EV5F). With the exception of SDHA, a matrix-soluble C II subunit that was recently identified as a possible CLPP substrate [20], the turnover of a majority of other (tested) nDNA-encoded mitochondrial OXPHOS subunits is also not dependent on the CLPP presence (Fig EV5F). Furthermore, DKO mitochondria are the only one that have increased turnover even of full-length proteins (Fig 4F). This finding implies that although synthesized in higher amounts in DARS2-deficient hearts, in the absence of CLPP, these OXPHOS subunits are less stable and therefore faster degraded.

Our results argue that a decreased rate of protein synthesis caused by the absence of CLPP allows a more proficient synthesis of OXPHOS subunits to occur inside DARS2-deficient mitochondria, leading to an increase of fully assembled OXPHOS complexes, hence partially rescuing the respiratory defect. This is not a result of an increased stability of OXPHOS subunits, as they are actually turned over more rapidly in DKO hearts. We could think of two possible explanations of how the loss of CLPP increases the level of proficient mitochondrial translation. Firstly, the aspartate-tRNA pool lasts longer, because the rate of translation is significantly reduced by CLPP depletion and hence efficient protein synthesis is prolonged. In the second scenario, slow mitochondrial translation caused by the absence of CLPP allows other tRNAs to replace $tRNA^{Asp}$ in the nascent polypeptides. It is even possible that $tRNA^{Asp}$ is mischarged with other amino acids, as we showed that $tRNA^{Asp}$ pools are stable and present in normal levels in DARS2-deficient hearts [6]. Possibly, only in conditions of slowed-down translation, these mischarged tRNAs could then be incorporated into growing polypeptides. The less-than-perfect OXPHOS subunits formed this way would be more prone to misfolding and degradation. We show that in DKO hearts, even the full-length mtDNA-encoded subunits are more rapidly turned over, providing an indirect evidence for this scenario. Similar phenotypes have been observed in mtDNA mutator mice that accumulate high levels of mtDNA mutations, leading to the expression of OXPHOS subunits with amino acid substitutions that are more rapidly degraded [19].

Besides the obvious effect on the levels of OXPHOS subunits, CLPP deficiency clearly leads to a strong decrease in the level of aborted peptides caused by the loss of DARS2. These peptides in high amounts could cause toxicity independent of the effect on the levels of OXPHOS complexes and put an additional burden to already troubled mitochondria. Indeed, it is likely that the detected solid improvement of DKO cardiomyopathy is the result of both, a mild increase in the respiratory capacity combined with a strong decrease in the amount of potentially toxic aberrant polypeptides.

Collectively, we show that loss of CLPP in the DARS2-deficient heart clearly reduces the signs of cardiomyopathy and sustains animals healthier for a longer period of time. This comes as a surprise as CLPP deficiency on its own causes Perrault syndrome characterized by sensorineural hearing loss and a premature ovarian failure in humans [21]. Although murine *Clpp* deletion represents a faithful model of Perrault syndrome [7], molecular mechanisms leading to the development of specific phenotypes are yet to be explored. Here, we provide the first evidence that loss of ClpP leads to a moderate defect in mitochondrial translation that might be the primary cause of the disease. In agreement, mutations in HARS2 [3], LARS2 [14], and TWINKLE [22], three out of four other genes causing Perrault syndrome also have a direct effect on mitochondrial protein synthesis.

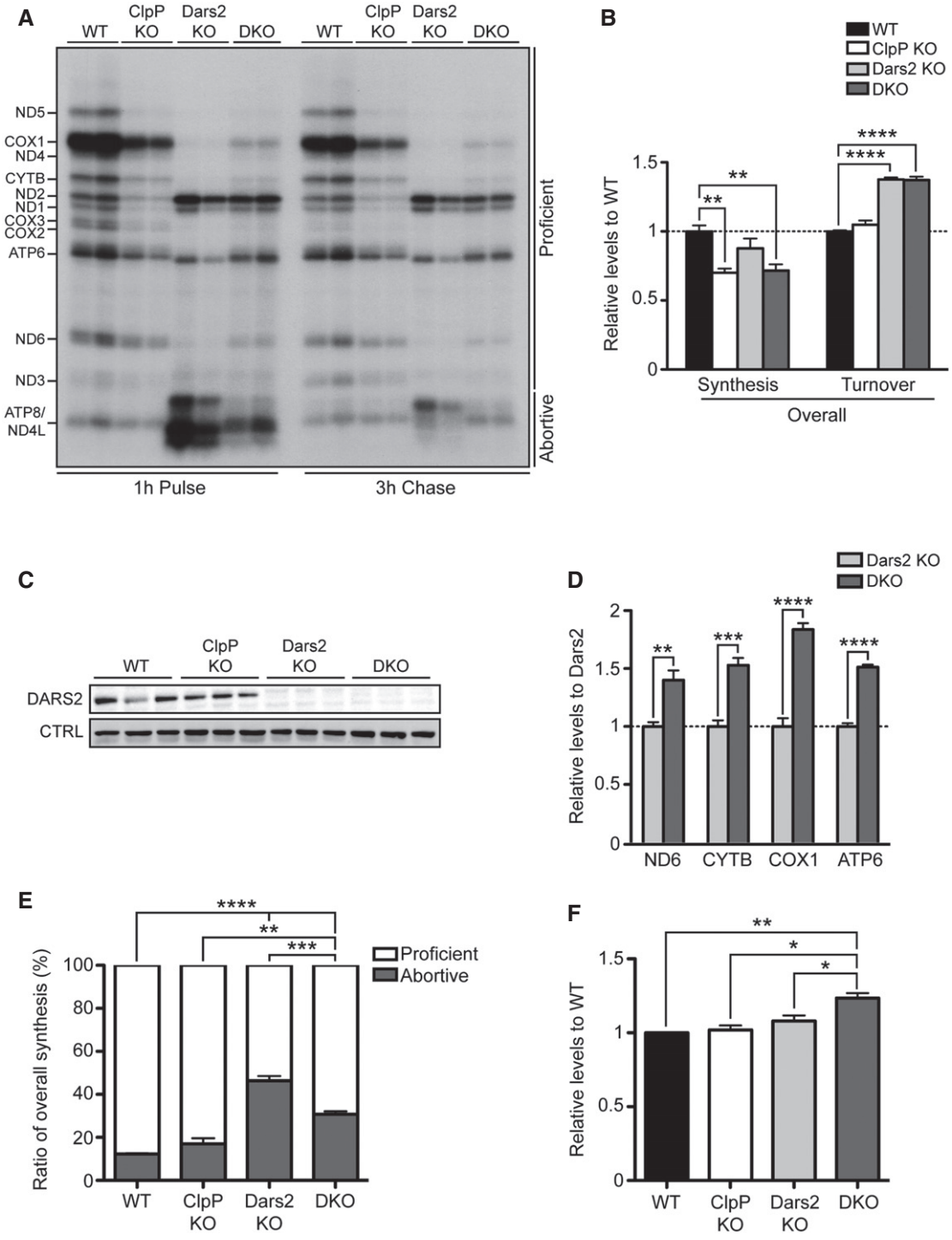

Figure 4. Dysregulation of mitochondrial protein synthesis is partially rescued by the loss of CLPP.

A Representative gel of the *in organello* translation analysis of heart mitochondria. *De novo* synthetized proteins are isolated after labeling with ^{35}S-Met (1-h pulse) or after cold chase (3-h chase). Positions of individual proteins are indicated on the left. Position of full-length proteins (proficient) and low molecular weight polypeptides (abortive) is indicated on the right. Note that in WT and ClpP KO, low molecular weight polypeptides include ATP8/ND4L proteins.

B Relative overall protein synthesis and turnover rate.

C Western blot analysis of DARS2 levels. HSC70 is used as a loading control (CTRL).

D Relative levels of the individual *de novo* synthesized OXPHOS subunits in DKO mitochondria normalized to the corresponding Dars2 KO polypeptide level.

E Relative levels of protein synthesis of proficient and abortive polypeptides.

F Quantification of the turnover rate of full-length (proficient) *de novo* synthesized proteins.

Data information: (B, D–F) Bars represent mean ± SEM (Student's *t*-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) ($n = 3$–4).

In summary, our results provide the first evidence for a role of CLPP in mitochondrial translation, while refuting its universal role in the mammalian UPR^{mt} signaling. They also open a possibility for exploration of therapeutic intervention targeting CLPP activity in the large group of mitochondrial diseases that directly affect mitochondrial protein synthesis.

Materials and Methods

Generation and genotyping of transgenic mice

Mice heterozygous for *Dars2* conditional targeting allele were created and genotyped as previously described [6]. *Clpp* gene targeting was carried out at Taconic Artemis, Germany, in Art B6/3.5 embryonic stem cell line on a C57BL/6NTac genetic background. LoxP sites flanked exons 3–5 of the *Clpp* gene; positive selection marker (Puromycin resistance, PuroR) was inserted into intron 5 and was flanked by Frt sites. The presence of Cre recombinase leads to deletion of exons 3–5 and removal of a part of the protease domain resulting in a frameshift from exons 2 to 6. The targeted locus was transmitted through germline that resulted in heterozygous $Clpp^{+/PuroR\text{-}L}$ mice that were subsequently bred to Flp deletor mice to remove the PuroR selection marker. The genotyping primers for used to determine *ClpP* alleles were ClpP^{forw} (5′-GTG GATGATGGTCAGTAGAATCC-3′) and ClpP^{rev} (5′-CCCAGACATGA TTCCTAGCAC-3′).

Conditional *Dars2*-floxed mice were ($Dars2^{L/L}$) were crossed to ClpP-floxed mice ($ClpP^{L/L}$) to obtain double-floxed animals ($Dars2^{L/L}$; $ClpP^{L/L}$) which were further mated with transgenic mice expressing Cre recombinase under the control of muscle creatine kinase promoter (*Ckmm*) to obtain triple transgenic mice ($Dars2^{+/L}$; $ClpP^{+/L}$; $Ckmm\text{-}Cre^{+/T}$). Double-floxed mice ($Dars2^{L/L}$; $ClpP^{L/L}$) were mated with triple transgenic animals ($Dars2^{+/L}$; $ClpP^{+/L}$; $Ckmm\text{-}Cre^{+/T}$) to obtain wild-type (WT), ClpP KO ($Dars2^{+/L}$, $ClpP^{L/L}$, $Ckmm\text{-}Cre^{+/T}$), Dars2 KO ($Dars2^{L/L}$, $ClpP^{+/L}$, $Ckmm\text{-}Cre^{+/T}$), and DKO ($Dars2^{L/L}$, $ClpP^{L/L}$, $Ckmm\text{-}Cre^{+/T}$) mice. For detailed mating scheme, see also Fig EV1A. Additional control animals were obtained from the following matings: (i) Dars2-deficient animals—$Dars2^{+/L}$, $Ckmm\text{-}Cre^{+/T}$ mated with $Dars2^{L/L}$, and (ii) ClpP-deficient animals—$ClpP^{+/L}$; $Ckmm\text{-}Cre^{+/T}$ mated with $ClpP^{L/L}$.

For isolation of mouse embryonic fibroblasts (MEFs), $Clpp^{+/L}$ mice were mated with mice expressing Cre recombinase under the promoter of beta-actin (*Actb-cre*). This deletion resulted in a loss of function of the *Clpp* gene, by removing a part of the protease domain and generated a frameshift from exons 2 to 6. $Clpp^{+/-}$ heterozygous mice were intercrossed to obtain the $Clpp^{-/-}$ homozygous embryos.

Unless otherwise indicated, all experiments were performed using 6-week-old mice. Animal protocols were in accordance with guidelines for humane treatment with animals and were reviewed and approved by the Animals Ethics Committee of North Rhine-Westphalia, Germany (AZ84-02.04.2012.A407).

Real-time quantitative PCR

Isolated heart RNA was treated with DNase (DNA-free Kit, Ambion) and subsequently reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *Atf5* (Mm00459515_m1), *Atp6* (Mm03649417_g1), *Fgf21* (Mm00840165_g1), and *Hprt* (Mm00446968_m1) probes were obtained from TaqMan Assay-on-Demand kits (Applied Biosystems). Probes for *Nd1, Nd6, Cytb, Cox1, Cox2* were a kind gift from Prof. N.G. Larsson, MPI, for Biology of Aging, and their sequences are available on request [23]. All other human or mouse expression levels were determined using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) with the following primer pairs:

	FWD 5′–3′	REV 5′–3′
Mouse LonP1	ATGACCGTCCCGGATGTGT	CCTCCACGATCTTGATAAAGCG
Mouse Afg3l2	GTTGATGGGCAATACGTCTGG	GACCCGGTTCTCCCCTTCT
Mouse Hsp60	GCCTTAATGCTTCAAGGTGTAGA	CCCCATCTTTTGTTACTTTGGGA
Mouse mtHsp70	ATGGCTGGAATGGCCTTAGC	ACCCAAATCAATACCAACCACTG
Mouse Chop	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
Mouse Atf4	GCAAGGAGGATGCCTTTTC	GTTTCCAGGTCATCCATTCG
Mouse Nppa	ATGGGCTCCTTCTCCATCA	CCTGCTTCCTCAGTCTGCTC
Mouse Nppb	GGATCTCCTGAAGGTGCTGT	TTCTTTTGTGAGGCCTTGGT
Mouse Tfam	ATTCCGAAGTGTTTTTCCAGCA	TCTGAAAGTTTTGCATCTGGGT
Mouse Dnaja3	AACATCCCTGTGTCGCAAG	CACCGGGTCTCAATGTCAGC
Mouse ClpP	CAGTCTGAAAGCAACAAGAAGC	ACTGCATTGTGTCGTAGATGG
Mouse Yme1l1	ATTCTGCGGTAGACCCTGTC	CAACCACTTCCTGTAACTCTTGT
Mouse Pmpcb	GTGGGGATTTACACGAAGGCT	CACTTGTGTCTCGGGGACATT
Mouse Txn2	GGACATTGACGATCACACAGA	GTCCCCGTTCTTGATGGCT
Mouse Ppia	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
Mouse Hprt	GCCCCAAAATGGTTAAGGTT	TTGCGCTCATCTTAGGCTTT
Human LONP1	GTTCCCGCGCTTTATCAAGAT	GTAGATTTCATCCAGGCTCTC
Human AFG3L2	GACAGATTGGAAGTCGTCAACA	ACCAAACGTATTGCCCATCAA
Human HSP60	TGCCAATGCTCACCGTAAG	ACTGCCACAACCTGAAGAC
Human mtHSP70	CAGTCTTCTGGTGGATTAAGCAAA	CTTCAGCCATATTAACTGCTTCAAC
Human CHOP	ATGAGGACCTGCAAGAGGTCC	TCCTCCTCAGTCAGCCAAGC
Human TFAM	AAGATTCCAAGAAGCTAAGGGTG	AGAGTCAGACAGATTTTTCCAGTTT
Human DNAJA3	TTTGGCGAGTTCTCATCCTCT	TTGCAGCTTGATTGAATGTCAAC
Human CLPP	GCAGCTCTATAACATCTACGCC	GTGGACCAGAACCTTGTCTAAG
Human YME1L1	ATACCTGAAGCTCACCAAGATG	GAAGTCCATAAATGCCGAATAGC
Human PMPCB	TGGGGTTTCAGCGAGAGTCT	TGGGTAGCAGCCTGTGTACTT
Human TXN2	TTCAAGACCGAGTGGTCAACA	CACCTCATACTCAATGGCGAG
Human SPG7	AAGGGATCAACGGATTGTTGTT	AGCCGCTCTCGGTACATCT
Human HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

Samples were adjusted for total RNA content by *Hprt* (Taqman probes) or *Ppia* resp. *Hprt* (SYBR Green). The relative expression of mRNAs was determined with a comparative method ($2^{-\Delta\Delta C_T}$).

Western blot analysis

Protein lysates were obtained from either homogenized tissue or isolated mitochondria and subsequently subjected to Western blot analysis as described previously [24]. All antibodies were used in dilutions indicated or otherwise recommended by the

manufacturers: anti-AFG3L2 (1:1,000, polyclonal antisera, a kind gift from Prof. E.I. Rugarli, University of Cologne) [25]; anti-HSP60 (1:20,000, SMC-110A, StressMarq); HSC70 (1:2,000, sc-7298, Santa Cruz Biotechnology); TOM20 (1:1,000, sc-11415, Santa Cruz Biotechnology); p-eIF2α (1:1,000, sc-12412, Santa Cruz Biotechnology); mtHSP70 (1:1,000, ab82591, Abcam); LONP1 (1:1,000, ab103809, Abcam); eIF2α SU1 (1:1,000, ab32157, Abcam); Trap1 (1:1,000, 612344, BD Biosciences); p62/SQSTM1 (1:2,000, H00008878-M01, Abnova); DARS2 (1:1,200, 13807-1-AP, Proteintech); CLPP (1:1,000, WH0008192-M1, Sigma-Aldrich); actin (1:2,000, A5541, Sigma-Aldrich); calnexin (1:2,000, 208880, Calbiochem); MnSOD/SOD2 (1:500, 06-984, Millipore); ATP5A1 (1:1,000, MS507, MitoSciences); UQCRFS1 (1:1,000, MS305, MitoSciences); NDUFS3 (1:1,000, MS112, MitoSciences); COX4-1 (1:1,000, A21348, Invitrogen); UQCR2 (1:1,000, 459140, Invitrogen); SDHA (1:1,000, 459200, Invitrogen); NDUFA9 (1:1,000, 459100, Invitrogen); NDUFB6 (1:1,000, A21359, Invitrogen); COX1 (1:1,000, 459600, Invitrogen); and VDAC (1:1,000, 4661, Cell Signaling).

BN-PAGE and in-gel activity

BN-PAGE was carried out using the NativePAGE Novex Bis-Tris Mini Gel system (Invitrogen) according to the manufacturer's specifications. Proteins were transferred onto a nitrocellulose membrane, and immunodetection of mitochondrial OXPHOS complexes was performed. In-gel activity assays for complex I and complex IV were performed as previously described [6].

Analysis of protein synthesis and turnover rates

Quantification of *de novo* protein synthesis rate was performed by analyzing the whole range of proteins (overall rate) relative to WT (Fig 4B) or ratio between full-length polypeptides (proficient) to low molecular weight (abortive) (Fig 4E). The turnover rate of *de novo* protein synthesis was calculated as ratio of chase/pulse for the whole range of proteins (overall rate) (Fig 4B) or only full-length polypeptides (proficient) (Fig 4F). The level of *de novo* synthesis of individual DKO subunits is calculated in relation to the level of Dars2 KO (Fig 4D).

Mitochondrial isolation and oxygen consumption measurements

Mitochondria were isolated from the heart [19], and oxygen consumption rates were measured with Oxygraph-2k (Oroboros Instruments). Mitochondrial complex I respiration was measured with the addition of 2 mM ADP, 5 mM pyruvate, 2 mM malate, and 20 mM glutamate. Subsequently, 10 mM of succinate was added to determine combined complex I + complex II respiration. Next, mitochondrial coupling was evaluated by the inhibition of ATP synthase by adding 1.5 μg/ml oligomycin and uncoupling by a multiple-step carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) titration.

Enzyme histochemistry

Fresh-frozen heart tissue was sectioned in 7-μm slices and stained for COX and SDH activities or Masson's trichrome staining (Sigma-Aldrich) as previously described [6].

In organello translation

In organello translation was performed as previously described [6]. Mitochondria from two hearts of the same genotype are pulled for each sample to ensure enough material. Mitochondrial proteins are synthesized in for 1 h in the presence of ^{35}S-Met at 37°C (pulse). Subsequently, half of mitochondria were isolated using SDS–PAGE loading buffer, whereas the other half was washed and incubated for another 3 h in a translation buffer containing all amino acids, including non-radioactive methionine (chase).

Densitometry analysis

For quantification of scanned films or CCD camera obtained pictures, densitometry analysis using public domain software ImageJ (a Java image processing program inspired by National Institutes of Health (NIH) Image for Macintosh) was performed.

Generation of ClpP KO HEK293T cell line

ClpP was deleted in HEK293T cells using the double-nicking strategy plasmid (pX335) CRISPR-Cas9n system [26] targeting the first exon of ClpP (target sequences along with PAM sequence: top strand target 5′-CATGCAGGTACCCCGCGCTG-GGG-3′, bottom-strand target 5′-GGCCCCCCCTACCAATATTC-CGG-3′). Single-cell clones were isolated after serial dilution into 96-well plates. ClpP deletion was confirmed by SDS–PAGE followed by immunoblotting.

Stealth siRNA transfection

Knockdown experiments were performed using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions together with the specific stealth siRNA (Invitrogen) for 72 h: *SPG7* (5′-CCUCAAGGUUGAAGCAGAAGAAUAA-3′ [HSS110137]). After 4 h, cells were transfected a second time and the media was exchanged 24 h after transfection. The efficiency of the down-regulation was controlled by real-time quantitative qPCR.

Determination of protein turnover in mouse embryonic fibroblasts (MEFs)

Primary MEFs were isolated from E13.5 embryos obtained as a result of intercrossed *Clpp*$^{+/-}$ animals as described previously [27]. Immortalized cell lines were generated upon transformation with the SV40 T antigen [28]. Cells were grown to 80–90% confluency in a DMEM–high glucose containing media (Invitrogen). Cytoplasmic protein synthesis was blocked by addition of cycloheximide (100 μg/ml), and cells were collected at indicated time points, counted, and washed with PBS. The cell lysates were obtained in the RIPA buffer, proportional to the number of living cells, and subjected to a Western blot analysis.

Statistical analysis

All numerical data are expressed as mean ± SEM. Student's *t*-test and Kaplan–Meier distribution were used for statistical analysis. In order to detect outlier in our data sets, we have used Grubbs' test (http://graphpad.com/quickcalcs/Grubbs1.cfm). Differences were

considered statistically significant for $P < 0.05$. Most experiments were performed using at least six biological replicates. The experiments were repeated at least two times with the each set of samples.

Expanded View for this article is available online.

Acknowledgements
The work was supported by grants of the European Research Council (ERC-StG-2012-310700) and German Research Council (DFG). D.S and C.B. and P.M. received scholarships from CECAD graduate school.

Author contributions
AT conceived the project and together with DS designed the experiments, analyzed the data, and wrote the manuscript. DS, KSz, CB, KSe, SH, PM, TK, and AK performed the experiments, interpreted, and analyzed data.

Conflict of interest
The authors declare that they have no conflict of interest.

References

1. Benedetti C, Haynes CM, Yang Y, Harding HP, Ron D (2006) Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. *Genetics* 174: 229 – 239
2. Haynes CM, Petrova K, Benedetti C, Yang Y, Ron D (2007) ClpP mediates activation of a mitochondrial unfolded protein response in *C. elegans*. *Dev Cell* 13: 467 – 480
3. Pierce SB, Chisholm KM, Lynch ED, Lee MK, Walsh T, Opitz JM, Li W, Klevit RE, King MC (2011) Mutations in mitochondrial histidyl tRNA synthetase HARS2 cause ovarian dysgenesis and sensorineural hearing loss of Perrault syndrome. *Proc Natl Acad Sci USA* 108: 6543 – 6548
4. Zhao Q, Wang J, Levichkin IV, Stasinopoulos S, Ryan MT, Hoogenraad NJ (2002) A mitochondrial specific stress response in mammalian cells. *EMBO J* 21: 4411 – 4419
5. Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G, Williams RW, Auwerx J (2013) Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature* 497: 451 – 457
6. Dogan SA, Pujol C, Maiti P, Kukat A, Wang S, Hermans S, Senft K, Wibom R, Rugarli EI, Trifunovic A (2014) Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab* 19: 458 – 469
7. Gispert S, Parganlija D, Klinkenberg M, Drose S, Wittig I, Mittelbronn M, Grzmil P, Koob S, Hamann A, Walter M *et al* (2013) Loss of mitochondrial peptidase Clpp leads to infertility, hearing loss plus growth retardation via accumulation of CLPX, mtDNA and inflammatory factors. *Hum Mol Genet* 22: 4871 – 4887
8. Aldridge JE, Horibe T, Hoogenraad NJ (2007) Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. *PLoS One* 2: e874
9. Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, Ron D (2004) Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J Cell Sci* 117: 4055 – 4066
10. Kohler F, Muller-Rischart AK, Conradt B, Rolland SG (2015) The loss of LRPPRC function induces the mitochondrial unfolded protein response. *Aging (Albany NY)* 7: 701 – 717
11. Bezawork-Geleta A, Brodie EJ, Dougan DA, Truscott KN (2015) LON is the master protease that protects against protein aggregation in human mitochondria through direct degradation of misfolded proteins. *Sci Rep* 5: 17397
12. Haynes CM, Yang Y, Blais SP, Neubert TA, Ron D (2010) The matrix peptide exporter HAF-1 signals a mitochondrial UPR by activating the transcription factor ZC376.7 in *C. elegans*. *Mol Cell* 37: 529 – 540
13. Jin SM, Youle RJ (2013) The accumulation of misfolded proteins in the mitochondrial matrix is sensed by PINK1 to induce PARK2/Parkin-mediated mitophagy of polarized mitochondria. *Autophagy* 9: 1750 – 1757
14. Pierce SB, Gersak K, Michaelson-Cohen R, Walsh T, Lee MK, Malach D, Klevit RE, King MC, Levy-Lahad E (2013) Mutations in LARS2, encoding mitochondrial leucyl-tRNA synthetase, lead to premature ovarian failure and hearing loss in Perrault syndrome. *Am J Hum Genet* 92: 614 – 620
15. Teske BF, Fusakio ME, Zhou D, Shan J, McClintick JN, Kilberg MS, Wek RC (2013) CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell* 24: 2477 – 2490
16. Nakada K, Sato A, Sone H, Kasahara A, Ikeda K, Kagawa Y, Yonekawa H, Hayashi J (2004) Accumulation of pathogenic DeltamtDNA induced deafness but not diabetic phenotypes in mito-mice. *Biochem Biophys Res Commun* 323: 175 – 184
17. Silva JM, Wong A, Carelli V, Cortopassi GA (2009) Inhibition of mitochondrial function induces an integrated stress response in oligodendroglia. *Neurobiol Dis* 34: 357 – 365
18. Arnould T, Michel S, Renard P (2015) Mitochondria retrograde signaling and the UPR mt: where are we in mammals? *Int J Mol Sci* 16: 18224 – 18251
19. Edgar D, Shabalina I, Camara Y, Wredenberg A, Calvaruso MA, Nijtmans L, Nedergaard J, Cannon B, Larsson NG, Trifunovic A (2009) Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice. *Cell Metab* 10: 131 – 138
20. Cole A, Wang Z, Coyaud E, Voisin V, Gronda M, Jitkova Y, Mattson R, Hurren R, Babovic S, Maclean N *et al* (2015) Inhibition of the mitochondrial protease ClpP as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* 27: 864 – 876
21. Jenkinson EM, Rehman AU, Walsh T, Clayton-Smith J, Lee K, Morell RJ, Drummond MC, Khan SN, Naeem MA, Rauf B *et al* (2013) Perrault syndrome is caused by recessive mutations in CLPP, encoding a mitochondrial ATP-dependent chambered protease. *Am J Hum Genet* 92: 605 – 613
22. Morino H, Pierce SB, Matsuda Y, Walsh T, Ohsawa R, Newby M, Hiraki-Kamon K, Kuramochi M, Lee MK, Klevit RE *et al* (2014) Mutations in Twinkle primase-helicase cause Perrault syndrome with neurologic features. *Neurology* 83: 2054 – 2061
23. Ruzzenente B, Metodiev MD, Wredenberg A, Bratic A, Park CB, Camara Y, Milenkovic D, Zickermann V, Wibom R, Hultenby K *et al* (2012) LRPPRC is necessary for polyadenylation and coordination of translation of mitochondrial mRNAs. *EMBO J* 31: 443 – 456
24. Hance N, Ekstrand MI, Trifunovic A (2005) Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis. *Hum Mol Genet* 14: 1775 – 1783
25. Koppen M, Metodiev MD, Casari G, Rugarli EI, Langer T (2007) Variable and tissue-specific subunit composition of mitochondrial m-AAA

protease complexes linked to hereditary spastic paraplegia. *Mol Cell Biol* 27: 758 – 767

26. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8: 2281 – 2308
27. Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT, Larsson NG (2005) Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc Natl Acad Sci USA* 102: 17993 – 17998
28. Richter-Dennerlein R, Korwitz A, Haag M, Tatsuta T, Dargazanli S, Baker M, Decker T, Lamkemeyer T, Rugarli EI, Langer T (2014) DNAJC19, a mitochondrial cochaperone associated with cardiomyopathy, forms a complex with prohibitins to regulate cardiolipin remodeling. *Cell Metab* 20: 158 – 171

