Mitochondrial unfolded protein response controls matrix pre-RNA processing and translation

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The mitochondrial matrix is unique in that it must integrate the folding and assembly of proteins derived from the nuclear and mitochondrial genomes. In Caenorhabditis elegans, the mitochondrial unfolded protein response (UPRmt) senses matrix protein misfolding and induces a program of nuclear gene expression, including mitochondrial chaperonins, to promote mitochondrial proteostasis1–3. While misfolded mitochondrial-matrix-localized ornithine transcarbamylase induces chaperonin expression4–6, our understanding of mammalian UPRmt is rudimentary7, reflecting a lack of acute triggers for UPRmt activation. This limitation has prevented analysis of the cellular responses to matrix protein misfolding and the effects of UPRmt on mitochondrial translation to control protein folding loads. Here we combine pharmacological inhibitors of matrix-localized HSP90/TRAP1 (ref. 8) or LON protease9, which promote chaperonin expression, with global transcriptional and proteomic analysis to reveal an extensive and acute response of human cells to UPRmt. This response encompasses widespread induction of nuclear genes, including matrix-localized proteins involved in folding, pre-RNA processing and translation. Functional studies revealed rapid but reversible translation inhibition in mitochondria occurring concurrently with defects in pre-RNA processing caused by transcriptional repression and LON-dependent turnover of the mitochondrial pre-RNA processing nuclease MRPP3 (ref. 10). This study reveals that acute mitochondrial protein folding stress activates both increased chaperone availability within the matrix and reduced matrix-localized protein synthesis through translational inhibition, and provides a framework for further dissection of mammalian UPRmt.

Protein folding homeostasis is central to cell fitness. Protein unfolding in the endoplasmic reticulum (ER) promotes transcriptional induction of ER-associated chaperones to facilitate folding and inhibits translation to further reduce the folding load11. In contrast, mechanisms underlying the response to protein misfolding in other organelles, including mitochondria, are poorly understood. The mitochondrial matrix folding machinery consists of chaperonins HSPD1/HSP60 and HSPE1/HSP10, and chaperones including the HSP90 paralogue TRAP1 and mHSP70. This machinery assists in the folding of matrix-localized nuclear-encoded proteins, and their assembly with 13 respiratory chain proteins encoded by the mitochondrial genome (mtDNA)12. The balance between folding load and chaperone abundance is controlled, in part, by the UPRmt. In C. elegans, genetic UPRmt activation promotes nuclear localization of the ATF-1 transcription factor to induce expression of mitochondrial chaperonins, thereby enhancing matrix folding capacity1–3. While earlier work revealed that enforced expression of misfolded ornithine transcarbamylase in HeLa cells induced HSPD1 and HSPE1 expression4–6, our understanding of UPRmt in human cells is limited.

Cellular stress responses such as UPRer are typically fast acting as a result of rapid sensing of protein folding stress, but prolonged activation can produce confounding effects such as cell death12. We therefore examined whether gamitrinib-triphenylphosphonium (GTPP)—a specific inhibitor of the matrix HSP90 chaperone TRAP1 known to cause protein misfolding in this compartment6,14—would promote acute transcription of HSPD1 and HSPE1 as readout of UPRmt induction in HeLa cells. Acute GTPP treatment (6 h) induced UPRmt as assessed by quantitative PCR (qPCR) for HSPD1 and HSPE1 (Extended Data Fig. 1a) with a dynamic range (approximately twofold) similar to that seen with genetic UPRmt induction in C. elegans13. HSPE1 and HSPD1 are among the most abundant messenger RNAs (mRNAs) in untreated cells (top 2nd percentile), explaining their limited dynamic range upon UPRmt (Supplementary Table 1). GTPP treatment did not affect cell viability, mitochondrial membrane potential, ATP levels or respiratory chain architecture (Extended Data Fig. 1b–e). Longer (24 h) incubations with GTPP result in cell death15. Consistent with TRAP1 being the causal target for GTPP-dependent chaperonin induction, TRAP1 RNA interference (RNAi) also induced HSPD1 by qPCR (Extended Data Fig. 1f).

C/EBP homologous protein (CHOP), a broadly acting transcription factor, is induced via UPRer and the integrated stress response via the ATF4 transcription factor11. CHOP is also induced during UPRmt (refs 4, 5) and oxidative stress15, but the mechanisms underlying CHOP activation in UPRmt and its relationship between UPRer and integrated stress response upstream signalling remained unclear. Strikingly, we found that GTPP, but not the UPRer activator tunicamycin, respiratory chain inhibitors or mitochondrial membrane decouplers, activated HSPD1 expression (Fig. 1a and Extended Data Fig. 2a). GTPP also activated ATF4 and CHOP, but unlike tunicamycin, did not induce BIP, indicating that GTPP does not activate canonical UPRer (Fig. 1b, c and Extended Data Fig. 2a, b). We also found that individual depletion of the four known EIF2A kinases involved in integrated stress response signalling (GCN2, HRI, PERK and PKR)11 had no effect on CHOP induction by GTPP (Extended Data Fig. 2c–f), suggesting that induction of ATF4 and CHOP by UPRmt occurs through a pathway independent of individual integrated stress response kinases13 (Extended Data Fig. 2b). Taken together, these data indicate that GTPP induces UPRmt through a pathway distinct from known ER and mitochondrial stress pathways (Extended Data Fig. 2b).

To globally examine the mammalian UPRmt transcriptional response, we treated HeLa cells with GTPP for 6 h and performed RNA sequencing (RNA-seq) (Fig. 1d, e, Extended Data Fig. 3a–b and Supplementary Table 1). In parallel, we determined RNA-seq profiles upon treatment of cells with CDDO, an inhibitor of matrix protease LON (Fig. 1d). CDDO rapidly induces mitochondrial protein misfolding9 and induced HSPD1 expression, consistent with UPRmt induction (Extended Data Fig. 3c). From 968 (GTPP) and 1,029 (CDDO) transcripts whose abundance changed significantly (log2 ≥ 0.6), 627 were shared between individual treatments and 337 and 290 downregulated and upregulated transcripts, respectively, including HSPD1 and HSPE1, and CHOP (Fig. 1d–f and Extended Data Fig. 3d, e). Importantly, changes in transcription in GTPP treatment were distinct from changes previously reported with 17-AAG15, a derivative of GTPP that inhibits cytoplasmic and nuclear HSP90 (Extended Data Fig. 3e), indicating that inhibition of non-mitochondrial HSP90 is unlikely to account for
the transcriptional response with GTPP. Gene ontology enrichment analysis confirmed extensive overlap in the transcriptional responses, with all gene ontology clusters representing transcripts altered with both treatments (Fig. 1g and Supplementary Table 2). As expected, gene ontology terms showed enrichment for protein folding genes, consistent with UPR^{mt} induction, but also included transfer RNA (tRNA) processing and activation. Among the nuclear genes with correlated changes in transcription, 36 encode proteins known to localize in mitochondria (Fig. 1h and Supplementary Table 1). Promoter analysis of genes regulated by UPR^{mt} induction showed enrichment of CHOP and ATF4 promoter recognition sequences, as well as two ‘mitochondrial UPR Response Element’ (MURE1 and MURE2) promoter elements^6 (P < 0.0001; Extended Data Fig. 4a and Supplementary Table 3). This analysis therefore revealed a specific nuclear response to UPR^{mt} that is anticipated to promote homeostasis of protein folding within mitochondria.

We then applied MultiNotch proteomics^7 (Extended Data Fig. 5a) to purified mitochondria to quantify acute changes in the mitochondrial proteome upon GTPP treatment using untreated cells or cells treated with the mitochondrial uncoupler CCCP (carbonyl cyanide-m-chlorophenyl hydrazone) as controls (Fig. 2a and Supplementary Table 4)^7. From 606 mitochondrial proteins quantified (442 with 2 or more peptides), 61 displayed significant changes in abundance 6 h after GTPP treatment compared with control or CCCP-treated cells, including HSPD1 and HSPE1, which increased as expected (Fig. 2a, b and Extended Data Fig. 5b). Furthermore, proteins involved in respiration, transcription, tRNA processing and protein quality control, among others, were found to be regulated (Fig. 2c). In contrast, levels of the mitochondrial ribosome and respiratory chain complexes were not significantly altered (Extended Data Fig. 5c, d), consistent with their long half-lives^18. Strikingly, the abundance of the mitochondrial matrix protein MRPP3 was reduced at both the transcriptional and protein level (Fig. 1e, h and Fig. 2b–d). MRPP3 is the catalytic subunit of the RNA-free mitochondrial RNase P complex that is induced by both UPR^{mt} and CHOP (Fig. 2d), suggesting a rather specific response to GTPP or CDDO treatment. Black dots, P ≤ 0.05, changes ≥ log2 (−1) or CHOP and BIP (b) or ATF4 (c) mRNA in HeLa cells with or without the indicated treatments (mean of levels relative to untreated ± s.d. n = 3 biological replicates). d, Experimental design (top). Volcano plot showing fold changes versus P values for the analysed transcriptome of cells treated with GTPP (bottom left) or CDDO (bottom right). Transcripts significantly changing upon UPR^{mt} induction (P ≤ 0.05, changes ≥ log2 (−0.6) are represented by black dots. e, Correlation of ratios of transcripts changing upon GTPP or CDDO treatment. Black dots, P ≤ 0.05, changes ≥ log2 (−0.6); red dots, genes of interest. f, Summary of altered transcripts. g, h, Gene ontology (GO) enrichment map (g) and heat map (h) of overlapping mitochondrial transcripts altered by both GTPP and/or CDDO.

Figure 2 | Changes in the mitochondrial proteome upon UPR^mt induction. a, Volcano plot showing fold changes versus P values for total quantified and quantified mitochondrial proteins. b, Volcano plot showing fold changes versus P values for quantified mitochondrial proteins. Proteins significantly changing are indicated by green dots. c, Heatmap organized by gene ontology groups of mitochondrial protein level changes. Proteins that did not change significantly are indicated in grey. d, Histogram of protein (b) and/or mRNA (Fig. 1) abundance for chaperonin and mitochondrial RNase P subunits. Two-tailed P values *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, mean of n = 3 (RNA) or n = 2 (protein) biological replicates. NS, not significant. #P = 0.06.
Previous studies have shown that alterations in the abundance of mitochondrial RNase P components can alter pre-RNA processing in unanticipated ways, making interpretation of effects of MRPP3 over-expression difficult. Similarly, we found that elevated MRPP3 levels (~11-fold) altered steady-state processing efficiencies, with enhanced tRNA\textsuperscript{Met} processing and tRNA\textsuperscript{Lys} displaying enhanced 3' processing and decreased 5' processing (Extended Data Fig. 7a, b). While MRPP3 levels were still reduced upon GTPP treatment, consistent with LON activity not being limiting, residual MRPP3 remained approximately fivefold higher than in untreated cells (Extended Data Fig. 7a). Importantly, residual MRPP3 partly rescued tRNA\textsuperscript{Met} and tRNA\textsuperscript{Lys} processing (Extended Data Fig. 7c), consistent with the notion that loss of MRPP3 during UPR\textsuperscript{mt} contributes to pre-RNA processing defects.

UPR\textsuperscript{mt} inhibits cytosolic translation through phosphorylation of eIF2\(\alpha\) and local degradation of mRNAs by IRE1 (ref. 11). The alterations in genes linked with mitochondrial protein synthesis (Figs 1 and 2) together with the finding that mitochondrial pre-RNA processing is deficient during UPR\textsuperscript{mt} led us to examine whether UPR\textsuperscript{mt} affects translation of mRNAs derived from mtDNA (Fig. 4a). Indeed, GTPP treatment (6 h) strongly inhibited \(^{15}\)S-methionine incorporation into newly synthesized respiratory chain components in a concentration-dependent manner (Fig. 4b, c and Extended Data Fig. 8a) without affecting cytoplasmic translation rates (Extended Data Fig. 8b). To further validate the inhibitory effect of UPR\textsuperscript{mt} on mitochondrial translation, we used stable isotope labelling by amino acids in culture (SILAC) and mass spectrometry to quantify the ratio of newly synthesized (K8-Lys) to pre-existing (K0-Lys) protein for mitochondrially encoded proteins (Fig. 4d). Translational inhibition was confirmed for ND5, COI, ATP6 and ATP8 (Fig. 4d and Extended Data Figs 8c and 9a, b), with peptide coverage comparable to previous deep proteome studies in HeLa cells\(^{20}\). Translational inhibition by GTPP, as well as pre-RNA processing, was largely recovered within 4 h of GTPP wash-out (Fig. 4e and Extended Data Fig. 10a, b), indicating that UPR\textsuperscript{mt} is rapidly reversible.

We find that acute mitochondrial folding stress promotes a multifaceted response involving (1) altered expression of nuclear genes, including mitochondrial chaperons, to increase matrix protein folding capacity, (2) transcriptional repression and LON-dependent degradation of MRPP3 to reduce pre-RNA processing and (3) induction of rapid but reversible translational inhibition of mtDNA-encoded proteins, thereby reducing matrix folding load (Fig. 4f). As with pre-RNA processing (Extended Data Fig. 7b), cells overexpressing MRPP3 display altered translation of mtDNA-encoded proteins, with ND5, COI, ND2 and COIII showing decreased translation relative to control cells (Extended Data Fig. 10c), which complicates interpretation. However, residual MRPP3 post-GTPP treatment did not rescue bulk mitochondrial translation (Extended Data Fig. 10c). This could reflect sub-threshold levels of tRNA processing despite partial rescue (Extended Data Fig. 7) or redundancy in the UPR\textsuperscript{mt} pathway, thereby affecting other steps in the translation pathway (Fig. 4f), as is the case with UPR\textsuperscript{eIF2}\(\alpha\) (ref. 11). Alternatively, because MRPP1-dependent tRNA methylation critical for tRNA maturation requires assembly with MRPP3 (ref. 21), MRPP3 overexpression may uncouple pre-RNA processing from tRNA methylation, resulting in translational defects despite the presence of MRPP3. While the TFBB1M methyltransferase responsible for mitochondrial 12S rRNA methylation is reduced transcriptionally (Fig. 1h), its protein abundance is unchanged at 6 h post-GTPP (Extended Data Fig. 10d), indicating that defects in tRNA methylation do not underlie translational inhibition. Thus, further studies are required to understand the regulation of mitochondrial translation with and without mitochondrial stress. In keeping with the transient nature of stress responses\(^{13}\), our work has focused on acute effects of UPR\textsuperscript{mt}. Components linking mitochondrial protein misfolding to the nucleus remain to be identified, as ATFS-1 orthologues are lacking in mammals. Interestingly, the stress-inducible protein ATF3 (ref. 22), which contains a basic leucine zipper-like domain similar...
to ATFS-1, and which function with CHOP, is also induced 1.5- to 4-fold by UPR^mt, (Supplementary Table 1), suggesting a possible role in UPR^mt signalling. Prolonged UPR^mt and concomitant translational inhibition probably leads to confounding effects that would be detrimental to mitochondrial health, consistent with the application of GTPP to cancer therapeutics\(^1\). The transcriptional and proteomic data reported here provide a framework for the further elucidation of circuits that contribute to protein homeostasis within mitochondria, and for the development of approaches that can manipulate the response of cells to mitochondrial folding stress, as might occur in pathological conditions including cancer and neurodegenerative diseases.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y. & Ron, D. ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. *Dev. Cell* **13**, 467–480 (2007).
2. Haynes, C. M., Yang, Y., Blais, S. P., Neubert, T. A. & Ron, D. The matrix peptide exporter HAF-1 signals a mitochondrial UPR by activating the transcription factor 2C367.6 in C. elegans. *Mol. Cell* **37**, 529–540 (2010).
3. Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M. & Haynes, C. M. Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science* **337**, 587–590 (2012).
4. Zhao, Q. et al. A mitochondrial specific stress response in mammalian cells. *EMBO J.* **21**, 4411–4419 (2002).
5. Horibe, T. & Hoogenraad, N. J. The chop gene contains an element for the positive regulation of the mitochondrial unfolded protein response. *PloS ONE* **2**, e835 (2007).
6. Alldredge, J. E., Horibe, T. & Hoogenraad, N. J. Discovery of genes activated by the mitochondrial unfolded protein response (mUPR) and cognate promoter elements. *PloS ONE* **2**, e874 (2007).
7. Haynes, C. M., Fiorese, C. J. & Lin, Y. F. Evaluating and responding to mitochondrial dysfunction: the mitochondrial unfolded-protein response and beyond. *Trends Cell Biol.* **23**, 311–318 (2013).
8. Kang, B. H. et al. Combinatorial drug design targeting multiple cancer signaling networks controlled by mitochondrial Hsp90. *J. Clin. Invest.* **119**, 454–464 (2009).
9. Bernstein, S. H. et al. The mitochondrial ATP-dependent Lon protease: a novel target in lymphoma death mediated by the synthetic triterpenoid CDDO and its derivatives. *Blood* **119**, 3231–3239 (2012).
10. Holzmann, J. et al. RNase P without RNA: identification and functional reconstitution of the human mitochondrial RNA processing enzyme. *Cell* **135**, 462–474 (2008).
11. Walter, P. & Ron, D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334**, 1081–1086 (2011).

METHODS

Chemicals and antibodies. LysC (VWR catalogue number 100369-822), CDDO (Cayman Chemicals catalogue number 81035), emetine (Sigma catalogue number E2375), CCP (Sigma catalogue number C2759), rotenone (Sigma catalogue number R8875), paraquat (Sigma catalogue number 36541), TFFA (Sigma catalogue number T20706), 3-Nitropropionic acid (Sigma catalogue number N5636), a-Antitrypsin (Sigma catalogue number A8674), myxothiazole (Sigma catalogue number T5580), potassium cyanide (Sigma catalogue number 60178), v-mycin (Sigma catalogue number V0627) and 85S virus (presumably C167-1001) are anisotopes. An original aliquot of GTPP was a gift from D. C. Aller; a second aliquot was custom synthesized by Shanghai ChemPartner Co. Antibodies were used anti-MRP3 (LSBio catalogue number LS-C33251, western blot: 1:500), anti-TOM20 (Santa Cruz catalogue number sc-11415, western blot: 1:500), anti-LON (Sigma catalogue number HPA002192, western blot: 1:500), anti-ACTIN (Santa Cruz catalogue number sc69879, western blot: 1:500), anti-TF61M (Abcam, catalogue number 69871, western blot: 1:200), anti-NDUFA9 (Abcam catalogue number ab14713, blue 1:n:1,000), anti-SHDA (Abcam catalogue number ab4715, blue 1:n:1,000) and anti-UQ2ARC (Abcam catalogue number ab14745, blue 1:n:1,000).

Cell culture and assays for cytotoxicity. Mitochondrial membrane potential and cellular ATP levels. HeLa cells were purchased from the American Type Culture Collection (ATCC) and not further authenticated. They were confirmed to be mycoplasma negative, and grown in RPMI medium supplemented with 1× glutamax (Inviotiregt; catalogue number 61870-127) and 10% fetal bovine serum. For all experiments, cells were treated with DMSO and 10 μM GTPP (or concentration as indicated) and/or 2.5 μM CDDO for 6 h. For CCK8 cytotoxicity assays, cells were plated in clear bottom 96-well plates, processed according to the manufacturer’s instructions (CCK8 Dodoing CK04-05) and quantified on a VersaMax microplate reader (Molecular Devices). For mitochondrial membrane potential determination, cells were treated with 1 μg/ml JC-1 (Life Technologies catalogue number, T3168) according to the manufacturer’s instructions. Cells were harvested and analysed by fluorescence-activated cell sorting on a BD FACScalibur. To assess cellular ATP levels, cells were plated on 96-well clear-bottom plates and treated with DMSO, GTPP or 10 μM antimycin A. ATP levels were measured with the Mitochondrial ToxGlo assay (Promega G8000) and analysed on a Molecular Devices SpectraMax M5 multi-mode plate reader.

Quantitative PCr and RNA sequencing. Total RNA was harvested using NucleoSpin RNA or NucleoSpin miRNA for analysis of pre-RNA processing (Macherey-Nagel catalogue numbers 740955 and 740971). RNA was quantified and equal amounts were reverse transcribed into complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems catalogue number 4368814). Quantitative PCr was performed using TaqMan Fast universal PCR Master Mix (Applied Biosystems catalogue number 4366072) or Fast SYBR Green Master Mix (Life Technologies) according to the manufacturer’s instructions. Pre-amplification was performed on 12-well plates and RNAi was transfected with Lipofectamine 3000 (Life Technologies) according to the manufacturer’s instructions. RNAi used was MRP3 (Ambion, AM16708, ID 21858), and TRAPI (DE/HCC DNA Resource Core IDs: Hs00121394, Hs00121407, EFA2AK1 (Dharmacon Q-00057-00-0002), EFA2AK2 (Dharmacon Q-003527-00-0002), EFA2AK3 (Dharmacon Q-004883-40-0002) and EFA2AK4 (Dharmacon Q-005314-00-0002). Cell line generation. Human cell DNA for MRP3 was purchased from Sino Biological (HG14131-G) and transferred into a pHAGE lentiviral vector. Virus particles were produced in HEK293T cells after transfection with the lentiviral vector and helper vectors (VSVG, Tat1b, Mgp2m, CMV-Rev) and used to infect HeLa cells. Cells were selected in 1 μg/ml puromycin.

Mass spectrometry. For quantitative analysis of the mitochondrial proteome, HeLa cells were treated with 10 μM GTPP, CDDO, or DMSO for 6 h. Mitochondria were purified as previously described using Basic Protocol 1 (ref. 25). Briefly, cells were scraped into cold PBS, collected by centrifugation, resuspended in lysis buffer and sonicated. Crude mitochondria were acquired by differential centrifugation and purified mitochondria obtained by separation on a sucrose cushion. Similar amounts of mitochondria were obtained under the different treatments. Mitochondrial pellets were resuspended in lysis buffer (6 M GdnHCl, 75 mM NaCl, 50 mM Tris, pH 8.5, 1 μM PMSE, 1× OPT) and sonicated. Samples were reduced, alkylated with iodoacetamide and proteins were precipitated using chloroform/methanol. Protein pellets were resuspended in 8 M urea in 50 mM Tris, pH 8.8 and subsequently centrifuged at 100,000 g for 20 min at 4 °C. Proteins were digested with LysC overnight at 37 °C. Digestion reactions were stopped by addition of formic acid, dried and purified by C18 stage tip. Samples were taken up in 0.2 M HEPES, pH 8.5 buffer, quantified by micro BCA (Thermo Scientific catalogue number 23235) and labelled with TMT 6-plex reagents (Thermo Scientific) for 1 h at room temperature. Reaction steps were stopped by addition of 5% hydrogen peroxide for 15 min followed by addition of formic acid. Equal amounts of peptide samples were combined to a total of 10 μg and purified on a C18 stage tip. Dried peptides were resuspended in 5% acetonitrile/5% formic acid and analyzed on an Orbitrap Fusion (Thermo Scientific) running a 2 h gradient from 2% to 30% acetonitrile using a multi-notch MS3-based method27 selecting ten MS2 fragment ions for analysis by MS3 (Orbitrap, AGC 5 × 104, 60,000 resolution, maximum injection time 150 ms). Peptides were identified and quantified by a SEQUEST-based in-house tool (developed by the S. P. Gygi lab) using SEQUEST with a human UniProt database (as of 14 January 2014), and submitted to linear discriminant analysis to score peptides and proteins with protein and peptide FDR values of 2% (ref. 26). Proteins were collapsed to a protein-level FDR of 2%. Searches were run for LysC with a maximum of two missed cleavage sites and with carboxamidomethylation of cysteine residues and TMT tags on lysine residues and N termini as static modifications, and methionine oxidation as dynamic modification. TMT 6-plex quantitation was performed by TMT reporter ion analysis for all identified proteins. MS3 spectra with a summed signal-to-noise ratio of ≤ 100 were excluded and the TMT channels normalized across all TMT channels (with resulting normalization factors between 1 and 1.252). For final analysis of quantified proteins, values were transferred and analysed in Microsoft Excel and the following cut-offs were applied: minimum number of two quantified
peptides, two-tailed $P \leq 0.05$, fold change $\geq \log_2 \pm 0.35$. Quantified proteins were determined as mitochondrial if they were found in MitoCarta$^{27}$, or an IMPI score of $\geq 0.85$ (version Q1 2015, http://www.mrc-mbu.cam.ac.uk/impi). For SILAC analysis, cell culture media was replaced with lysine-free media supplemented with K8 lysine and dialysed serum, and treated with DMSO or GTPP. After 6 h, cells were harvested and mitochondria purified, lysed and either processed as for TMT experiments (experiments 1–3), or run on a NuPAGE Novex 12% bis-tris gel (Life Technologies) and cut into five fractions, in-gel reduced, alkylated, and digested by LysC (experiment 4). Digested mitochondrial extracts and gel-extracted peptides were purified on C18 stage tips and analysed by LC-MS/MS on a Q Exactive or Orbitrap Fusion (Thermo Scientific) as indicated. Q Exactive analysis used a maximum injection time of 250 ms, an AGC target of $10^6$, resolution of 70,000 and automatic dynamic exclusion settings. For SILAC analysis on the Orbitrap Fusion, maximum injection times were set at 100 ms, AGC target at $2 \times 10^5$, 120,000 resolution and a dynamic exclusion of 90 s. Experiments were processed with our in-house analysis tool and/or Maxquant (as indicated). For our in-house tool (Core), analysis was as above and quantification done by analysing peak heights for the heavy and light forms of a peptide. We performed MaxQuant analysis (version 1.5.2.8) with standard Orbitrap settings and LysC digestion mode with cysteine carbamidomethylation as static and methionine oxidation as variable modification against a UNIPROT library (as of 9 March 2015). The minimum ratio count of the protein quantification was set at 1. Results were exported into Microsoft Excel to calculate heavy-to-light ratios of peptides to determine the percentage of newly synthesized protein as a fraction of heavy peptide intensity versus total intensity. Results of the Maxquant/Core quantifications are shown in Extended Data Fig. 9c. Owing to the consistent difficulty of both analysis tools in determining heavy peptide intensities in the GTPP-treated samples, heavy and light peptide intensities were also manually determined from MS1 at the observed $m/z$ values and retention times determined by the Maxquant/Core analyses (Fig. 4d and Extended Data Fig. 10).

**Mitochondrial translation assay.** HeLa cells were grown on a 12-well plate and treated for 6h with DMSO or different concentrations of GTPP as described. After 5.5h, media was replaced with RPMI lacking methionine and containing 10% dialysed fetal bovine serum, GTPP or DMSO (at the original concentration), and 100 $\mu$g ml$^{-1}$ emetine to block cytosolic translation. After 10min, 100 $\mu$Ci ml$^{-1}$ EasyTag $^{35}$-methionine (Perkin Elmer catalogue number NEG709A500UC) was added and incubated for another 20min, totalling 6h of GTPP treatment. Cells were washed with PBS and harvested in 1× NuPAGE LDS sample buffer (Life Technologies) containing 25 mM DTT. Samples were boiled and analysed on a NuPAGE Novex 12% bis-tris gel (Life Technologies). Gels were stained using InstantBlue (Expedeon), dried onto Whatman paper and visualized on a Bio-Rad Personal Molecular Imager for newly synthesized and radioactive proteins. An image was taken of the InstantBlue stained gel to confirm equal loading. These experiments were performed three independent times. For pulse-chase analysis, the same protocol was used with washes as indicated. This experiment was performed twice independently.

**Blue native.** Crude mitochondria were obtained as above and lysed in 1% digitonin, followed by separation on 4–16% BN-PAGE as previously described$^{28}$.

Proteins were transferred onto polyvinyldene difluoride membranes and detected using antibodies as indicated. A small aliquot was also analysed by standard western blot to confirm equal loading.

**Data reporting and statistics.** No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All quantitative experiments are presented as means $\pm$ s.d. of at least two independent biological experiments (as indicated) and were analysed by a two-tailed Student’s $t$-test (considered significant for $P \leq 0.05$).

23. Merico, D., Isserlin, R., Stueker, O., Emili, A. & Bader, G. D. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. PLoS ONE 5, e13984 (2010).

24. Grant, C. E., Bailey, T. L. & Noble, W. S. FIMO: scanning for occurrences of a given motif. Bioinformatics 27, 1017–1018 (2011).

25. Bozidis, P., Williamson, C. D. & Colberg-Poley, A. M. in Current Protocols in Cell Biology (eds Bonifacino, J. S. et al.) Ch. 3, Unit 3.27 (Wiley, 2007).

26. Huttlin, E. L. et al. A tissue-specific atlas of mouse protein phosphorylation and expression. Cell 143, 1174–1189 (2010).

27. Pagliarini, D. J. et al. A mitochondrial protein compendium elucidates complex I disease biology. Cell 134, 112–122 (2008).

28. McKenzie, M., Lazarou, M., Thorburn, D. R. & Ryan, M. T. Mitochondrial respiratory chain supercomplexes are destabilized in Barth syndrome patients. J. Mol. Biol. 361, 462–469 (2006).
Extended Data Figure 1 | Mitochondrial HSP90 inhibition induces UPR\textsuperscript{mt}. a, Quantitative PCR monitoring chaperonin (HSPD1 and HSPE1) mRNA levels upon treatment of cells with GTPP. Shown are means of levels relative to untreated ± s.d. (n = 3 biological replicates). b, Measurement of cell viability upon GTPP treatment with CCK8. Shown are means of levels relative to untreated ± s.d. (n = 5 biological replicates). c, Measurement of mitochondrial membrane potential upon GTPP or CCCP (mitochondrial membrane potential uncoupler) treatment, measured with JC-1 and analysed on a BD FACSCalibur. Shown are means of levels relative to untreated ± s.d. (n = 3 biological replicates). d, Measurement of cellular ATP levels upon GTPP or antimycin A (electron transport chain inhibitor) treatment. Shown are means of levels relative to untreated ± s.d. (n = 4 biological replicates) and two-tailed P values ***P ≤ 0.001; NS, not significant. e, Blue native gel analysis of mitochondrial respiratory chain complexes upon 6 h treatments of DMSO or GTPP. f, Changes in chaperonin and TRAP1 mRNA levels upon knockdown with shRNA targeting GFP or TRAP1 mRNA. Shown are means of log2 fold changes relative to control and s.d. (n = 3 biological replicates).
Extended Data Figure 2 | UPRmt signals distinctly from the integrated stress response. a, Table with summarized results of data shown in Fig. 1a–c. Induced genes are labelled green and compounds are clustered into their molecular function. GTPP induces UPRmt and tunicamycin ERUPR. All other compounds affect mitochondrial respiration and/or the mitochondrial membrane potential. b, Schematic showing how different stresses signal through the integrated stress response pathway based on the results shown in b and Fig. 1a–c. c, Quantitative PCR to assess the mRNA knockdown of the four EIF2A kinases by siRNA smart pools in biological duplicate; repl., replicate. d, Quantitative PCR monitoring CHOP mRNA levels in untreated or GTPP-treated cells with or without knockdown of the EIF2A kinases as in c. e, Quantitative PCR to monitor PERK mRNA levels upon PERK knockdown with individual siRNAs in biological duplicate. f, Quantitative PCR monitoring CHOP mRNA levels in GTPP-treated cells with or without knockdown of PERK by individual siRNAs in biological duplicate.
Extended Data Figure 3 | Global analysis of transcriptional responses to UPR\textsuperscript{mt} induction. \textbf{a,} Heatmap of measured transcript abundances of cells treated with DMSO, 10\(\mu\)M GTPP or 2.5\(\mu\)M CDDO for 6\(h\) (\(n=3\) biological replicates). Values not passing the cuffdiff threshold of FPKM abundance and read number were excluded (white). \textbf{b,} Correlation of replicates for DMSO-, GTPP- and CDDO-treated samples with \(R\) values depicting correlation value; \(\log_{10}\)-transformed FPKM values (\(\geq 0\)) are plotted. \textbf{c,} Quantitative PCR monitoring induction of mtUPR by measuring chaperonin mRNA levels upon treatment with DMSO or CDDO. Shown are means of levels relative to DMSO-treated ± s.d. (\(n=3\) biological replicates). \textbf{d,} Correlation between the abundance of transcripts significantly altered in GTPP- versus CDDO-treated cells (Fig. 1c, combined panel). \textbf{e,} Table representing changed transcripts upon GTPP or CDDO treatment (Fig. 1c) compared with the number of transcripts changed upon 17AAG previously reported\textsuperscript{16}. 
Extended Data Figure 4 | Promoter analysis of UPR^{mt} -induced transcripts encoding mitochondrial proteins. Analysis of UPR^{mt} -induced (GTPP and CDDO) transcripts encoding mitochondrial proteins for the occurrence of CHOP, MURE1, MURE2, or ATF4 promoter elements. We used FIMA version 4.11.1 with the consensus sequences shown. Cells marked in green represent the presence of the consensus sequence in the gene shown.

| Gene       | CHOP-ID'YNTGQDAHYN | MURE1-AGAAATBGCT | MURE2-GYACBCSAG | ATF4-VTKNCDHMR |
|------------|---------------------|------------------|-----------------|----------------|
| ABCB10     |                     |                  |                 |                |
| ABCD3      |                     |                  |                 |                |
| ALDH1L2    |                     |                  |                 |                |
| C22orf32   |                     |                  |                 |                |
| C0X8A      |                     |                  |                 |                |
| EAR52      |                     |                  |                 |                |
| ECST      |                     |                  |                 |                |
| FAM056A    |                     |                  |                 |                |
| FASTK02    |                     |                  |                 |                |
| GARS       |                     |                  |                 |                |
| GFER       |                     |                  |                 |                |
| GPT2       |                     |                  |                 |                |
| HSPD1      |                     |                  |                 |                |
| HSPE1      |                     |                  |                 |                |
| IDH1       |                     |                  |                 |                |
| IMP2L      |                     |                  |                 |                |
| IREB2      |                     |                  |                 |                |
| LDHAU88    |                     |                  |                 |                |
| MARS       |                     |                  |                 |                |
| MRPL18     |                     |                  |                 |                |
| MRPL22     |                     |                  |                 |                |
| MRPP3      |                     |                  |                 |                |
| MRPS31     |                     |                  |                 |                |
| MTFFD2     |                     |                  |                 |                |
| NDUFA11    |                     |                  |                 |                |
| OAT        |                     |                  |                 |                |
| PDHC       |                     |                  |                 |                |
| PDK4       |                     |                  |                 |                |
| PRELI02    |                     |                  |                 |                |
| PTGS2      |                     |                  |                 |                |
| SARS       |                     |                  |                 |                |
| SLCA22A4   |                     |                  |                 |                |
| SLCA25A12  |                     |                  |                 |                |
| SLCA25A40  |                     |                  |                 |                |
| SOCE1      |                     |                  |                 |                |
| TFB1M      |                     |                  |                 |                |
Extended Data Figure 5 | Changes in the mitochondrial proteome upon UPR\textsuperscript{mt} induction. 

a, Assay design. 
b, Summary of proteomic data. 
c, Analysis of changes in the average abundance of mitochondrial ribosome (left) or for individual ribosomal subunits (right). Values are mean values ± s.d. of scaled signal to noise values (that is, relative abundance) derived from the quantitative proteomics (Fig. 2) for identified mitochondrial ribosomal subunits (right, \( n = 2 \) biological replicates) and the average of all these values ± s.d. (left); repl., replicate. 
d, Analysis of the abundance of the different mitochondrial electron transport chain complexes and ATP synthase. Values are derived from quantitative proteomics (Fig. 2) and shown as mean values ± s.d. across all quantified subunits (top left) or separately per subunit for the different complexes (\( n = 2 \) biological replicates). All data depict scaled signal to noise values (that is, relative abundance).
Extended Data Figure 6 | Mitochondrial pre-RNA processing defects upon UPRmt. 
a. Primer design for monitoring pre-RNA processing. Primer pairs 1 and 3, and 2 and 4, will only produce PCR products for uncleaved mitochondrial pre-RNAs and allow quantitation of non-processed pre-RNAs. Primer pair 2 and 3 will monitor total levels for normalization.  
b. Quantitative PCR of MRPP3 mRNA levels upon knockdown with siRNA targeting a scrambled sequence or MRPP3. Shown are averages ± s.d. (n = 3 biological replicates).  
c. qPCR of mitochondrial pre-RNA at tRNA\textsubscript{Met} and tRNA\textsubscript{Lys}\textsuperscript{1\textsuperscript{th}} RNaseP processing sites upon depletion of MRPP3 by RNAi. Error bars, ± s.d. (n = 3 biological replicates).  
d. Quantitative PCR monitoring levels of non-processed pre-RNA upon treatment of cells with GTPP or the uncoupler CCCP in biological duplicate; repl., replicate e. LON protein levels as determined by quantitative proteomics (Fig. 2) in biological duplicate. Shown are scaled signal to noise values observed (that is, relative abundance).  
f. Western blot analysis of LON levels upon control or 10 μM GTPP treatment (6 h).
Extended Data Figure 7 | Rescue of UPR^{mt}-induced mitochondrial pre-RNA processing by MRPP3 overexpression. a, Western Blot analysis of MRPP3 levels upon DMSO or GTPP treatment in the context of wild-type or MRPP3-overexpressing (o/e) cells. b, Quantitative PCR analysis of non-processed mitochondrial pre-RNA levels at the tRNA^{Met} and tRNA^{Lys} cut sites in wild-type cells or cells overexpressing MRPP3. Shown are mean values ± s.d. (n = 3 biological replicates). c, Quantitative PCR analysis of non-processed mitochondrial pre-RNA levels at the tRNA^{Met} and tRNA^{Lys} cut sites in wild-type cells or cells overexpressing MRPP3 upon GTPP treatment. Shown are mean values ± s.d. (n = 3 biological replicates).
Extended Data Figure 8 | Mitochondrial translation defects upon UPR\textsuperscript{mt}. a, Coomassie gel staining as a loading control of the same experiment as in Fig. 4b. b, Analysis of cytosolic translation upon treatment with DMSO or GTPP with the same experimental procedure as in Fig. 4a without the addition of emetine. Newly synthesized proteins were monitored by phospho-imager (left) with Coomassie staining of the same gel as loading control (right). c, Table of experiment number, mass spectrometer used, analysis method, peptides sequence, protein encoded and heavy-to-light ratios (H/L) used to determine protein synthesis rates in Fig. 4d. Fusion and QE are Orbitrap Fusion or Q Exactive (Thermo Scientific), respectively; Core depicts in-house mass spectrometry analysis pipeline; #, oxidative modification on methionine; *, could not be determined by Core/Maxquant.
Extended Data Figure 9 | Proteomic determination of mitochondrial translation upon UPR<sup>mt</sup>. SILAC spectra for the data shown in Fig. 4d and Extended Data Fig. 9c for experiment 1.
Extended Data Figure 10 | Reversibility of UPR<sup>mt</sup>-induced mitochondrial pre-RNA processing and translation defects.

a, Coomassie gel staining as a loading control of the same experiment as in Fig. 4e. b, Mitochondrial pre-RNA processing was measured by qPCR in cells subjected to GTTP pulse-chase wash-out for 1–4 h. Data are averages of fold changes versus untreated ± s.d., two-tailed P values *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n = 3 biological replicates. c, Analysis of mitochondrial translation in wild-type or MRPP3 overexpressing cells with or without GTTP treatment. Newly synthesized proteins were labelled with <sup>35</sup>S and analysed by phospho-imager. d, Immunoblot of TFB1M expression with or without 6 h GTTP treatment (left). Quantification of control normalized TFB1M levels from immunoblots of two independent experiments.