Supplementary Materials for

Temporal landscape of mitochondrial proteostasis governed by the UPR\textsuperscript{mt}

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The PDF file includes:

- Figs. S1 to S8
- Tables S1 to S7
- Legends for tables S8 and S9

Other Supplementary Materials for this manuscript includes the following:

- Tables S8 and S9
Fig S1. Validation of cell lines and temporal MitoPQ analysis sample trends, related to Fig 2. (A) WT, CHOP KO, ATF4 KO, ATF5 KO and TKO cells were treated with G-TPP for 12 h and gene expression loss in each KO cell line was validated by immunoblot. (B) Principal component analysis of MitoPQ solubility values in each temporal data set, analyzed by each cell line. (C) The top 10 protein drivers from each component generated from the PCA plots in (B) for each cell line have been listed. Each data point in (B) represents one independent experimental sample.
Fig S2. Validation of protein aggregation in additional KO clones, related to Figure 2. (A-F) WT, CHOP KO, ATF4 KO and ATF5 KO cells were treated with G-TPP for 12 h (A-C) with a 48 h recovery washout period. Transcription factor levels were analysed by immunoblot (IB) to
confirm a loss of protein expression in KO cells (A–C). (D–C) IB validation of protein solubility trends identified in Figure 2.
Fig S3. Elevated protein aggregation observed in both WT and UPR^mt-deficient cells is largely comprised of select, strongly aggregating proteins, related to Figs 2 and 3. Protein aggregation trends across the mitoproteome of WT, CHOP KO, ATF4 KO, ATF5 KO and TKO cells at the indicated time points were sub-grouped into the labelled % aggregation groupings and graphed. Data represents mean data ± SD from three independent experiments.
Fig S4. Each CHOP, ATF4 and ATF5-driven signaling arm is required for UPR^mt^-mediated OXPHOS function and protein solubility protection and repair, related to Fig 4. (A – C), Violin plots of the mean % aggregation of proteins comprising complex II (A), complex III (B) and complex V (C) in WT, CHOP KO, ATF4 KO, ATF5 KO and TKO cells at the indicated timepoints. (D) Oxygen consumption rates (OCR; pmol/min) of mitochondria isolated from WT, CHOP KO, ATF5 KO, ATF4 KO and TKO samples used to calculate total respiratory capacity.
and spare respiratory capacity values in Fig 4. (E) Experimental overview of how the Galactose ATP measurements in Figure 4 were performed. Data in (A – D) represent mean data ± SD (D) from three independent experiments.
Fig S5. CHOP, ATF4 and ATF5 are required for the restoration of mitochondrial networks following proteostasis stress, related to Figure 5. Representative individual TOMM20 and SDHA immunostaining channels, related to Figure 5. Scale bars = 10 µm.
Fig S6. UPR\textsuperscript{mt} signaling regulated by CHOP, ATF4 and ATF5 drives modulation of Wnt-signaling pathway gene expression during proteostasis stress, related to Fig 6. (A) Wnt signaling pathway gene relationships were mapped and UPR\textsuperscript{mt}-related expression changes including transcription factor dependency were annotated on affected genes. (B, C) Significant gene expression changes in G-TPP treated samples relative to DMSO treated samples in WT (B) and DELE1 KO (C) samples were graphed. (D) mRNA levels relative to GAPDH in WT cells were analyzed for LONP1, CLPP, mitochondrial HSP60 and HSPE1 at the indicated treatment timepoints. (E) Selected chaperones and proteases from the transcriptomic data in Figure 6 (both cellular and mitochondrial specific) have been tabulated, including their fold change in WT 12 h G-TPP treated samples relative to DMSO levels. Data in (A – E) represents mean data from three independent experiments.
Fig S7. ~44% of the UPR\textsuperscript{mt}-regulated transcriptome is under the regulatory control of unidentified signaling elements, related to Figs 5 and 6. (A, B) UPR\textsuperscript{mt} gene expression trends across the cellular genome (A) or mitochondrial genome (B) in CHOP KO, ATF4 KO, ATF5 KO.
and TKO cells were analyzed by Principal Component Analysis (PCA). (C) Genes that did not show reduced expression in any CHOP KO, ATF4 KO, ATF5 KO or TKO transcriptome samples were classified to be under undefined regulatory control. The gene subset with undefined regulatory control was analyzed using the RegNetwork database(41) through ShinyGO v0.75(72) to identify transcription factors with enriched pathway and signaling representation in the undefined regulatory gene subset. (D) Changes in mtDNA-encoded rRNAs and mRNAs levels (expressed as log₂ fold change (FC) of counts per million mapped) were determined by RNA-seq. The expression profiles of each gene showing statistically significant increases are in red and decreases in blue relative to WT controls for the respective treatments; non-significant changing genes are in white. Data in (A), (B) and (D) represents data from three independent experiments. Data in (C) was generated using mean data from three independent experiments.
Fig S8. Proteostasis stress induces piecemeal PINK1/Parkin mitophagy, with similar levels of YFP-Parkin recruitment observed in ATF5 KO and TKO cells. (A-B) WT, ATF5 KO and TKO cells expressing YFP-Parkin were treated for 1 h with DMSO or Oligomycin/Antimycin A (OA) or for 12 h with DMSO or 9 µM G-TPP. Samples were immunostained for TOMM20 and YFP. Overview scale bars = 10 µM, zoom in scale bars = 5 µm.
| Function                                      | Relative to WT | Relative to TKO |
|-----------------------------------------------|----------------|-----------------|
|                                              | CHOP KO | ATF4 KO | ATF5 KO | TKO | CHOP KO | ATF4 KO | ATF5 KO |
| Amino acid metabolism                         | NS      | NS      | NS      | **  | NS      | NS      | NS      |
| Apoptosis                                     | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Calcium Signaling and Transport               | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Cardiolipin biosynthesis                      | NS      | NS      | **      | NS  | NS      | NS      | *       |
| Fatty Acid Biosynthesis & Elongation          | NS      | NS      | *       | *** | **      | **      | *       |
| Fatty Acid Degradation & Beta-oxidation        | NS      | NS      | NS      | **  | NS      | NS      | NS      |
| Fatty Acid Metabolism                         | *       | **      | *       | **  | NS      | NS      | NS      |
| Fe-S Cluster Biosynthesis                     | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Folate & Pterin Metabolism                    | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Glycolysis                                    | NS      | NS      | NS      | *   | NS      | **      | NS      |
| Heme Biosynthesis                             | NS      | NS      | NS      | *   | NS      | NS      | NS      |
| Import & Sorting                              | **      | **      | *       | **  | NS      | NS      | NS      |
| Metabolism of Lipids & Lipoproteins           | NS      | *       | *       | *   | NS      | NS      | NS      |
| Metabolism of Vitamins & Co-Factors           | NS      | NS      | NS      | *   | NS      | NS      | NS      |
| Mitochondrial Carrier                         | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Mitochondrial Dynamics                        | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Mitochondrial Signaling                       | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Mitophagy                                     | NS      | NS      | *       | **  | NS      | *       | NS      |
| Nucleotide Metabolism                         | NS      | NS      | NS      | **  | NS      | *       | NS      |
| Oxidative Phosphorylation                     | *       | **      | *       | *** | *       | NS      | *       |
| Pentose Phosphate Pathway                     | NS      | **      | NS      | NS  | NS      | **      | NS      |
| Protein Stability & Degradation               | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Pyruvate Metabolism                           | NS      | NS      | NS      | NS  | NS      | NS      | NS      |
| Replication & Transcription                   | **      | **      | **      | ****| **      | **      | **      |
| ROS Defence                                   | NS      | NS      | NS      | NS  | NS      | *       | NS      |
| Translation                                   | ***     | ***     | ***     | ****| *       | *       | **      |
| Transmembrane Transport                       | *       | NS      | NS      | *   | NS      | NS      | NS      |
| TCA Cycle                                     | NS      | NS      | NS      | *   | NS      | *       | NS      |
| Ubiquinone Biosynthesis                       | **      | ***     | **      | ****| ***     | **      | ***     |
| Mito-proteome                                 | *       | NS      | *       | **  | NS      | NS      | NS      |

Table S1. Statistical analysis of rate recovery trends across mitochondrial proteome and process groupings. Samples were analyzed relative to WT rate recovery per 24 h (‘Relative to WT’ column) or TKO rate recovery per 24 h (‘Relative to TKO’ column). ****p≤0.0001,
***p≤0.001, **p≤0.01, *p≤0.05, NS p>0.05 (one-way ANOVA). Data was generated from three independent experiments.
| Sample          | 12 h       | 24 h recovery | 48 h recovery |
|-----------------|------------|---------------|---------------|
| DMSO            |            |               |               |
| CHOP KO         | NS         | NS            | NS            |
| ATF4 KO         | NS         | NS            | NS            |
| ATF5 KO         | NS         | NS            | NS            |
| TKO             | NS         | NS            | NS            |
| ** Amino acid metabolism ** | **** | **** | **** |
| ** Apoptosis ** | NS         | NS            | NS            |
| ** Calcium Signaling and Transport ** | NS       | NS            | NS            |
| ** Cardiolipin biosynthesis ** | NS       | NS            | NS            |
| ** Fatty Acid Biosynthesis & Elongation ** | NS       | NS            | NS            |
| ** Fatty Acid Degradation & Beta-oxidation ** | NS       | NS            | NS            |
| ** Fatty Acid Metabolism ** | NS       | NS            | NS            |
| ** Fe-S Cluster Biosynthesis ** | NS       | NS            | NS            |
| ** Folate & Pterin Metabolism ** | NS       | NS            | NS            |
| ** Glycolysis ** | NS         | NS            | NS            |
| ** Heme Biosynthesis ** | NS       | NS            | NS            |
| ** Import & Sorting ** | NS       | NS            | NS            |
| ** Metabolism of Lipids & Lipoproteins ** | NS       | NS            | NS            |
| ** Metabolism of Vitamins & Co-Factors ** | NS       | NS            | NS            |
| ** Mitochondrial Carrier ** | NS       | NS            | NS            |
| ** Mitochondrial Dynamics ** | NS       | NS            | NS            |
| ** Mitochondrial Signaling ** | NS       | NS            | NS            |
| ** Mitophagy ** | ****       | ****          | ****          |
| ** Nucleotide Metabolism ** | NS       | NS            | NS            |
| ** Oxidative Phosphorylation ** | NS       | NS            | NS            |
| ** Pentose Phosphate Pathway ** | NS       | NS            | NS            |
| ** Protein Stability & Degradation ** | NS       | NS            | NS            |
| ** Pyruvate Metabolism ** | NS       | NS            | NS            |
| ** Replication & Transcription ** | NS       | NS            | NS            |
| ** ROS Defence ** | NS         | NS            | NS            |
| ** Translation ** | NS         | NS            | NS            |
| ** Transmembrane Transport ** | NS       | NS            | NS            |
| ** TCA Cycle ** | NS         | NS            | NS            |
| ** Ubiquinone Biosynthesis ** | NS       | NS            | NS            |
| ** Mito-proteome ** | NS         | NS            | NS            |

Table S2. Statistical analysis of AI trends across mitochondrial proteome and process groupings. Samples were analyzed relative to WT aggregation index (AI) levels at each time
point. ****p≤0.0001, ***p≤0.001, **p≤0.01, *p≤0.05, NS p>0.05 (two-way ANOVA). Data was generated from three independent experiments.
Table S3. CRISPR and TALEN sequences and genotyping results of the knockout cell lines generated in this study. The indels for the targeted genes detected in the indicated knockout cell lines (“Mutation” column) and their translated proteins (“Protein impact” column) are formatted according to Human Genome Variation Society (HGVS; [http://varnomen.hgvs.org/](http://varnomen.hgvs.org/)). Mutation positions within genes which have multiple splice variants are determined using the variant indicated as the canonical isoform in Uniprot. del = deletion; ins = insertion; c. = coding DNA; p. = protein; fs = frame shift; * = stop codon; [?] = affecting splicing; N= any nucleotide (A, C, T and G); X denotes one of the three amino acids A, T or S. The numbers following the asterisks denote the numbers of amino acids between the first amino acid changed after the mutation(s) and the first subsequent stop codon encountered.
| Gene  | Exon | Primer | Primer sequence (5’ to 3’)                                      | PCR product (bp) |
|-------|------|--------|---------------------------------------------------------------|-----------------|
| CHOP  | 1    | Forward| GACCATCCTCTTAGACTGCCGATCCGGATGGCAGCTGAGTCATTG                | 650             |
|       |      | Reverse| GCGAGATTATAGAGATCCCAAGCTTTTCATGCTTTCGAAGCCATGTCAGAAGCACC    |                 |
|       |      |        |                                                               |                 |
| ATF4  | 2    | Forward| ATGCCTGAGATCCGATCCAGCAACAGACACATGTCAGAAGCACC                 | 742             |
|       |      | Reverse| ATGCAAGCTTTGAGATAGTCTGGCTTC                                |                 |
|       |      | Sequencing | TGAGTGGGCCCACCACACATC                                      | N/A             |
| ATF5  | 1    | Forward| GCGCGGATCCGATAGTCTTTCCACATGTCAGAAGCACC                      | 338             |
|       |      | Reverse| GCGGACAGTTCTTTCCACCTGCCCATTACCT                            |                 |
| DELE1 | 2    | Forward| GGAGTGGCATAGCCATGAGCTGAGAAGACAGACCCATGTCAGAAGCACC            | 664             |
|       |      | Reverse| TAGCAGGCTTTGAGATAGTCTGGCTTC                                  |                 |
|       |      | Sequencing | CAGCAGAATCCACATGGGC                                       | N/A             |

Table S4. Genotyping primers for sequencing analysis of the generated knockout cell lines.
| Antibodies | Company            | Species | Catalog #/RRID          |
|------------|--------------------|---------|------------------------|
| CHOP       | Cell Signaling     | Mouse   | 2895/AB_2089254        |
| ATF5       | Santa Cruz         | Mouse   | sc-377168/AB_2943039   |
| ATF4       | Santa Cruz         | Mouse   | sc-390063/AB_2810998   |
| PINK1      | Cell Signaling     | Rabbit  | 6946/AB_11179069       |
| p62        | Abnova             | Mouse   | H00008878-M0/AB_4370851|
| Actin      | Cell Signaling     | Mouse   | 3700/AB_2242334        |
| PGAM5      | N/A                | Rabbit  | In house/NA             |
| LRPPRC     | Abcam              | Rabbit  | ab97505/AB_10688419    |
| AARS2      | Abcam              | Rabbit  | ab197367/AB_2943036    |
| SHMT2      | Abcam              | Rabbit  | ab180786/AB_2943037    |
| VDAC1      | Cell Signaling     | Rabbit  | 4661/AB_10557420       |
| TOMM20     | Santa Cruz         | Mouse   | sc-17764/AB_628381     |
| TOMM20     | Abcam              | Rat     | ab289670/AB_2943038    |
| Parkin     | Santa Cruz         | Mouse   | sc-32282/AB_628104     |
| VCP        | Santa Cruz         | Mouse   | sc-133212/AB_2214654   |
| ATP5A      | Abcam              | Mouse   | ab14748/AB_301447      |
| NDUFS1     | Proteintech        | Rabbit  | 12444-1-AP/AB_2282657 |
| SDHA       | Abcam              | Mouse   | ab14715/AB_301433      |
| anti-GFP   | Invitrogen         | Chicken | A10262/AB_2534023      |
| AlexaFluor-488 (Anti-Chicken) | ThermoFisher | Goat | A11039/AB_2534096     |
| AlexaFluor-488 (Anti-Rat)      | Abcam             | Goat    | ab150157/AB_2722511   |
| AlexaFluor-555 (Anti-Mouse)    | ThermoFisher      | Goat    | A21422/AB_2535844     |
| AlexaFluor-555 (Anti-Rat)      | ThermoFisher      | Goat    | A21434/AB_2535855     |
| Anti-Mouse IgG, HRP-linked      | Cell Signaling    | Horse   | 7076/AB_330924         |
| Anti-Rabbit IgG, HRP-linked     | Cell Signalling   | Goat    | 7074/AB_2099233       |
Table S5. Antibodies used in this study.
| Elution step | % ACN | % TEA (0.1%) |
|--------------|-------|-------------|
| Wash         | 5     | 95          |
| 1            | 9     | 91          |
| 2            | 13    | 87          |
| 3            | 17    | 83          |
| 4            | 21    | 79          |
| 5            | 25    | 75          |
| 6            | 29    | 71          |
| 7            | 33    | 67          |
| 8            | 37    | 63          |
| 9            | 41    | 59          |
| 10           | 45    | 55          |
| 11           | 50    | 50          |
| 12           | 55    | 45          |

Table S6. Modified high-pH elution gradient used in TMT batch sample fractionation.
| Gene | Primer | Primer sequence (5’ to 3’) |
|------|--------|-----------------------------|
| **CHOP** | Forward | AGCCAAAATCAGAGCTGGAA         |
|       | Reverse  | TGGATCAGTCTGGAAAGCA         |
| **ATF4** | Forward | CAGCAAGGAGGATGCCTTCT         |
|       | Reverse  | CCAACAGGGCATCCAAAGTC         |
| **ATF5** | Forward | AAGTCGGCGGCTCTGAGGTA         |
|       | Reverse  | GGACTCTGCCCCTTCTTCA         |
| **LONP1** | Forward | CCCGCGCTTTATCAAGATT         |
|       | Reverse  | AGAAAGACGCGACATAAGG         |
| **CLPP** | Forward | CGTATCATGATCCACCAGCCTC       |
|       | Reverse  | CCATGGCGGACTCGATCACCCTG     |
| **HSP10** | Forward | AGTAGTCGCTTGGATCCGG         |
|       | Reverse  | TGCCTCCATATTCTGGGAGA       |
| **HSP60** | Forward | CTGCCACAACCTGAAGACCAAC       |
|       | Reverse  | ATGCAATGCTCACCCTGAAAGCC     |
| **GAPDH** | Forward | TGACAACCTTGGATCTGGAGAG       |
|       | Reverse  | AGGCAGGGATGTGTCTGGAGAG       |

Table S7. qRT-PCR primers used in this study.
Table S8. TMT-batch layouts, MitoPQ data of WT 12 h G-TPP treatment samples (related to Fig 1) and WT, CHOP KO, ATF4 KO, ATF5 KO and TKO proteostasis stress and recovery samples (Related to Figs 2, 3, 4). (Provided as a separate .xlsx file)
Table S9. NGS transcriptome data of WT, CHOP KO, ATF4 KO, ATF5 KO and TKO 12 h DMSO or 12 h G-TPP treated samples (related to Figs 6, 7). (Provided as a separate .xlsx file)