Molecular Cell, Volume *77*

Supplemental Information

An Early mtUPR: Redistribution of the Nuclear Transcription Factor Rox1 to Mitochondria Protects against Intramitochondrial Proteotoxic Aggregates

Daniel Poveda-Huertes, Stanka Matic, Adinarayana Marada, Lukas Habernig, Mariya Licheva, Lisa Myketin, Ralf Gilsbach, Sergi Tosal-Castano, Daniel Papinski, Patrycja Mulica, Oliver Kretz, Cansu Kücükköse, Asli Aras Taskin, Lutz Hein, Claudine Kraft, Sabrina Büttner, Chris Meisinger, and F.-Nora Vögtle

Supplemental Figures and Legends

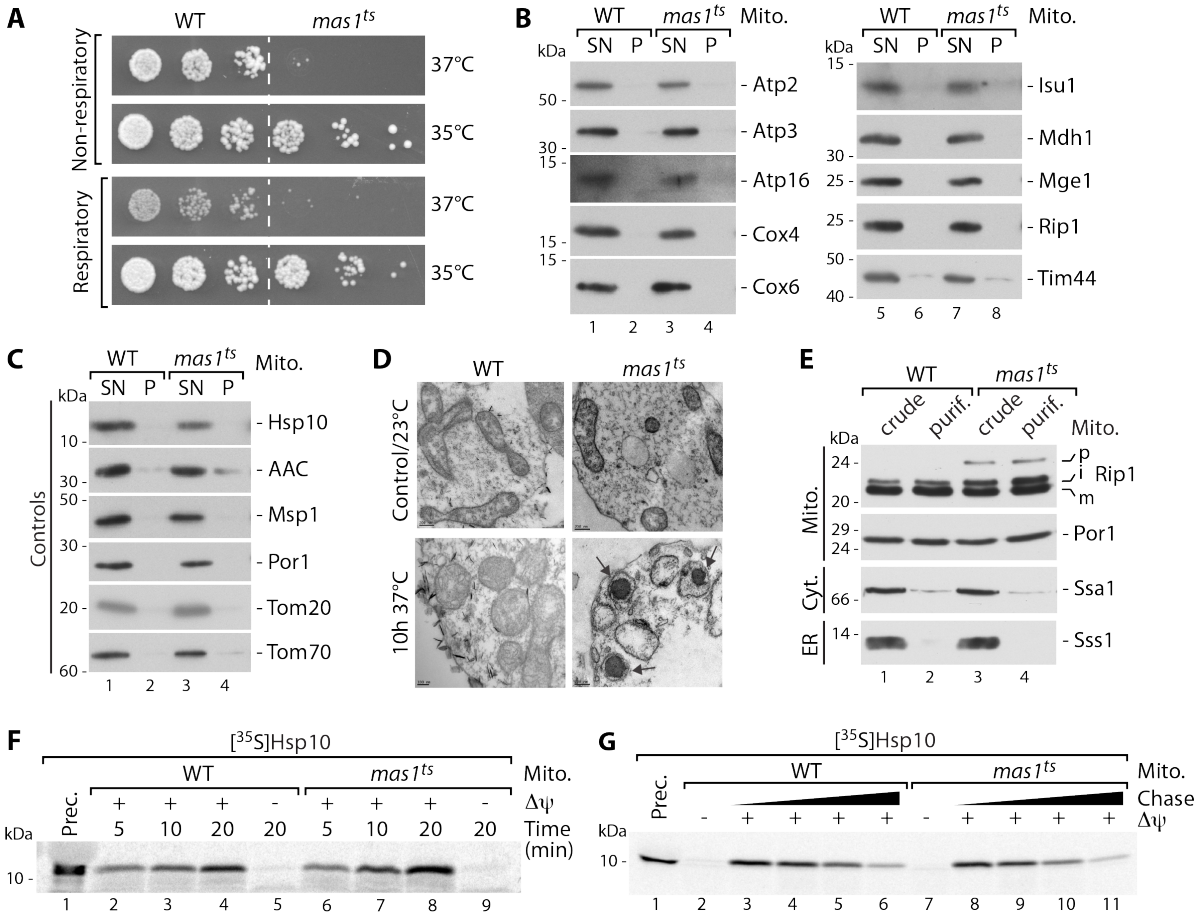

Figure S1. *mas1*^{ts} mutation does not impair presequence import pathway and degradation of mature proteins. Related to Figure 1.

(A) Growth test of wildtype (WT) and *mas1*^{ts} strain on non-respiratory (YPglucose) or respiratory (YPglycerol) plates at indicated temperature.

(B) Immunoblot analysis of WT and *mas1*^{ts} mitochondria from cells grown at permissive (23°C) temperature. Samples were separated in supernatant (SN) and pellet (P) fractions after lysis in 1% digitonin.

(C) Immunoblots of samples as desribed in (B) analyzed with antisera against non-processed proteins.

(D) Electron micrographs from WT and *mas1*^{ts} cells grown at 23°C or shifted for 10h to 37°C. Arrows, potential protein aggregates inside mitochondria.

(E) Comparison of non-processed precursor proteins accumulating in crude and gradient-purified *mas1*^{ts} mitochondria. Ssa1, cytosolic and Sss1, ER marker.

(F) Import assay: SDS-PAGE autoradiography of radiolabelled Hsp10 precursor imported into isolated WT and *mas1*^{ts} mitochondria after cell growth at non-permissive temperature. Import was performed for indicated time points in the presence or absence of $\Delta\psi$.

(G) Degradation assay: SDS-PAGE autoradiography of pulse-chase import of radiolabelled Hsp10 precursor protein into WT and *mas1*^{ts} mitochondria isolated after growth at non-permissive temperature.

A

Transcripts	*Ctrl./23°C*	*2h 37°C*	*4h 37°C*
Up-regulated	15	180	15
Down-regulated	14	205	100

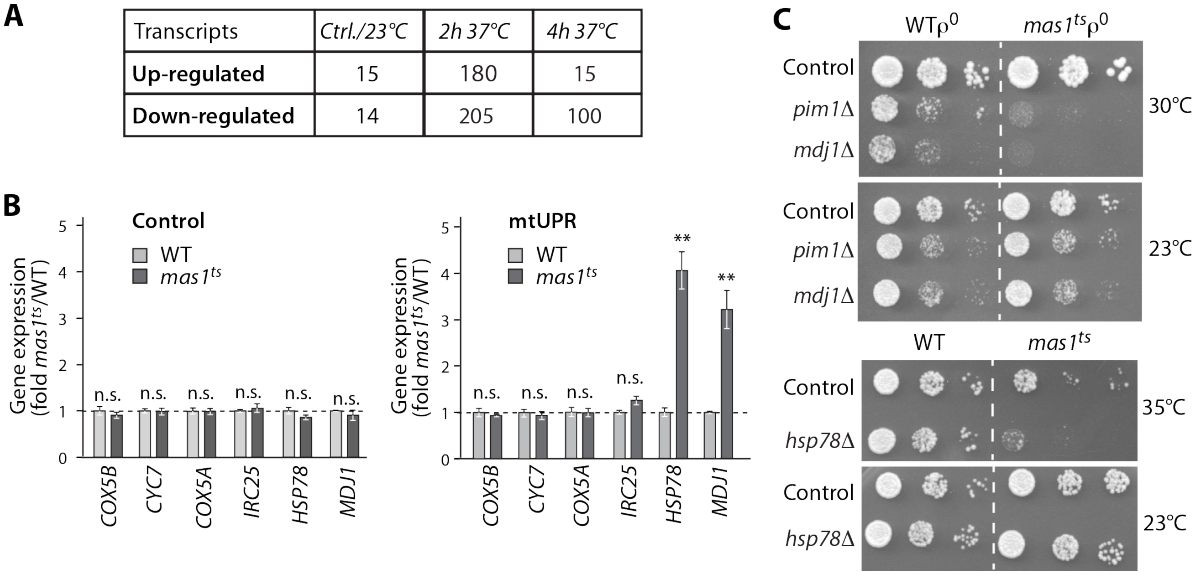

Figure S2. Aggregated non-processed precursor proteins inside mitochondria lead to an early transcriptional stress response. Related to Figure 2, Table S1 and Table S2.
(A) Summary of regulated transcripts changed in *mas1*^{ts} compared to WT cells.
(B) Gene expression analysis of representative genes by RTqPCR after growth at 23°C (Control) or for 2 hours at 37°C (mtUPR). *COX5B* and *CYC7*, Rox1 target genes; *COX5A* and *IRC25*, controls; *HSP78* and *MDJ1*, mitochondrial chaperones. Quantification for n = 3, data represent means +/- SEM, ***p* < 0.01, n.s. not significant.
(C) Growth assay to test for synthetic effects of indicated mutant strains in the *mas1*^{ts} background compared to WT. ρ^0, rho^0 background was used in case of *pim1*Δ and *mdj1*Δ that have lost mtDNA.

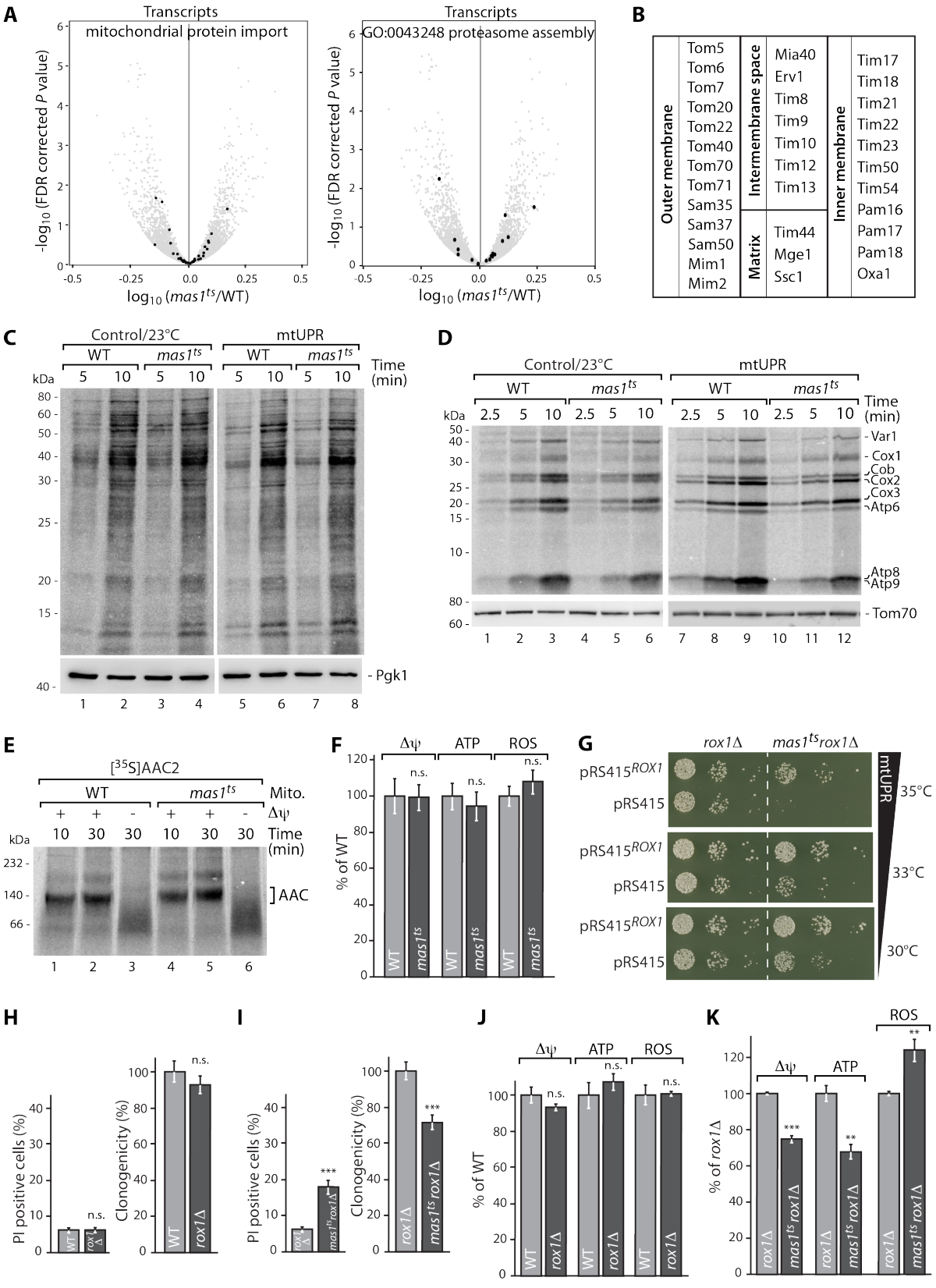

Figure S3. Rox1 rescues the typical characteristics of *mitochondrial unfolded protein response* pathways. Related to Figure 3 and Table S1.

(A) Distribution of transcripts quantified by RNA-seq in wildtype and *mas1*^{ts} cells after 2 h mtUPR induction. Displayed are Benjamini-Hochberg adjusted *P*-values. GO terms provided by *Saccharomyces genome database*. Increased transcript in left panel is *SSC1*, a mtHsp70 member and also included in Figure 2E. FDR, false discovery rate.

(B) List of transcripts for term *mitochondrial protein import* included in the analysis in (A).
(C and D) Cytosolic (C) and mitochondrial (D) translation activities assayed in WT and *mas1*^{ts} cells under non-induced (23°C) condition and after mtUPR induction (4 h 37°C).
(E) Radiolabelled Aac2 precursor was imported into isolated WT and *mas1*^{ts} mitochondria. Assembled AAC complex was analyzed via autoradiography after Blue Native PAGE.
(F) Measurement of $\Delta\psi$, ATP levels and ROS in WT and *mas1*^{ts} mitochondria (10 h ($\Delta\psi$, ATP) and 4 h (ROS) mtUPR induction). n = 3, data represent means +/- SEM. n.s., not significant.
(G) Re-expression of Rox1 from the pRS415 plasmid in *rox1Δ* and *mas1*^{ts}*rox1Δ* cells. Empty plasmid served as control. Growth was tested on YPglycerol plates at indicated temperatures. Strains were used for the functional analysis in Figure 3 and 5.
(H and I) Determination of cell death via PI staining and of clonogenic survival via survival plating in WT and *rox1*Δ cells (H) and *rox1*Δ and *mas1*^{ts}*rox1*Δ cells (I) (12 h induction). n = 4 for PI staining and n = 6 for clonogenic survival, data represent means +/- SEM. ***$p < 0.001$, **$p < 0.01$.
(J and K) Measurements of $\Delta\psi$, ATP levels and ROS in WT and *rox1*Δ (J) and *rox1*Δ and *mas1*^{ts}*rox1*Δ (K) mitochondria ($\Delta\psi$, ATP levels 10 h, ROS 4 h induction). n = 3, data represent means +/- SEM.

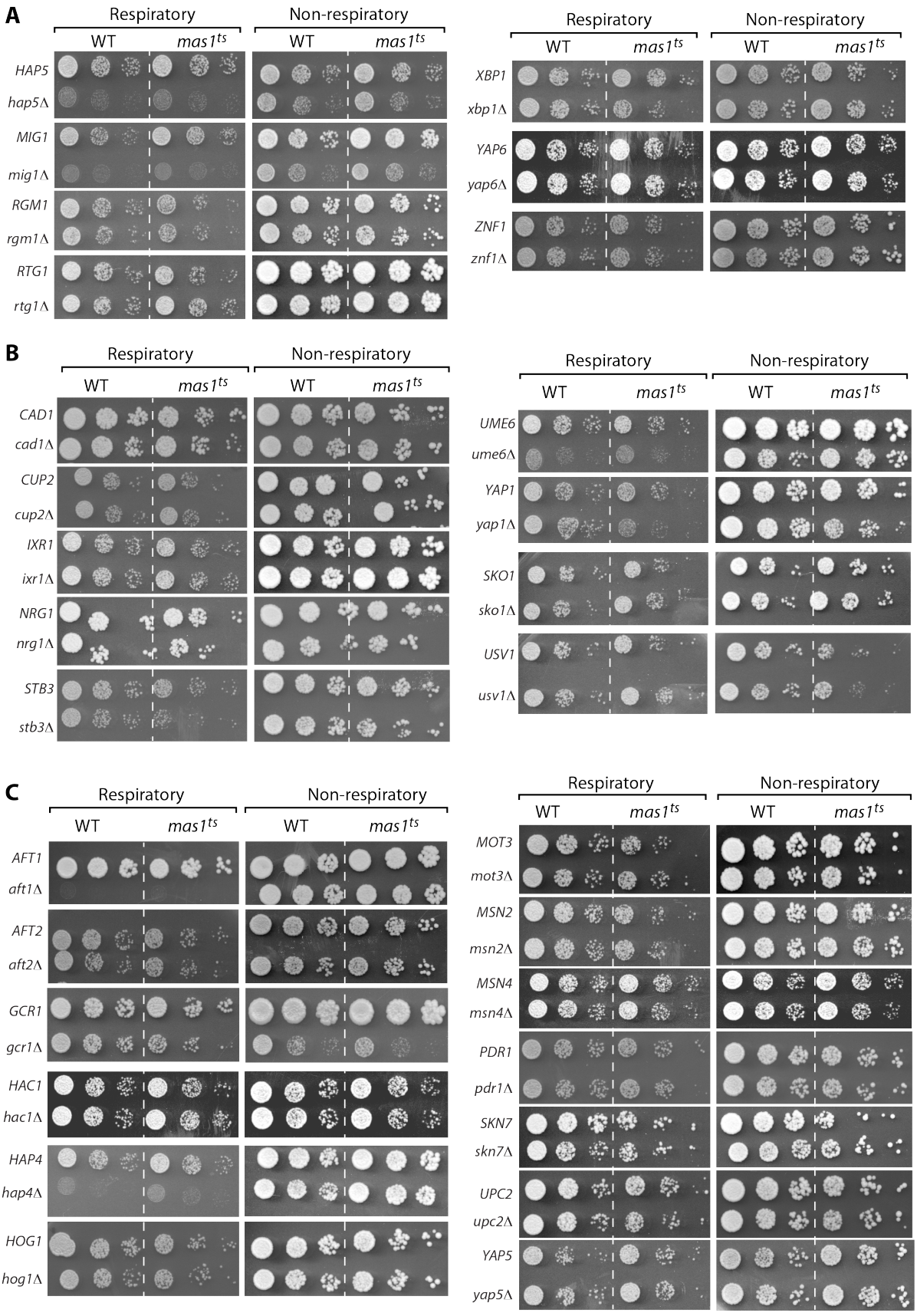

Figure S4. Screen for transcription factor mediating early mtUPR. Related to Figure 3.
Candidate genes were deleted in WT and *mas1*^{ts} background and serial dilutions tested on respiratory (YPglycerol) and non-respiratory (YPglucose) plates.
(A) Candidate genes annotated as transcription factors and regulated upon mtUPR (Table S1).

(B) Candidate genes were identified by oPOSSUM (Kwon et al., 2012) by searching for transcription factors that could target the deregulated genes identified in the transcriptomic profiling of mtUPR (Table S1).
(C) Transcription factor candidates from literature research.

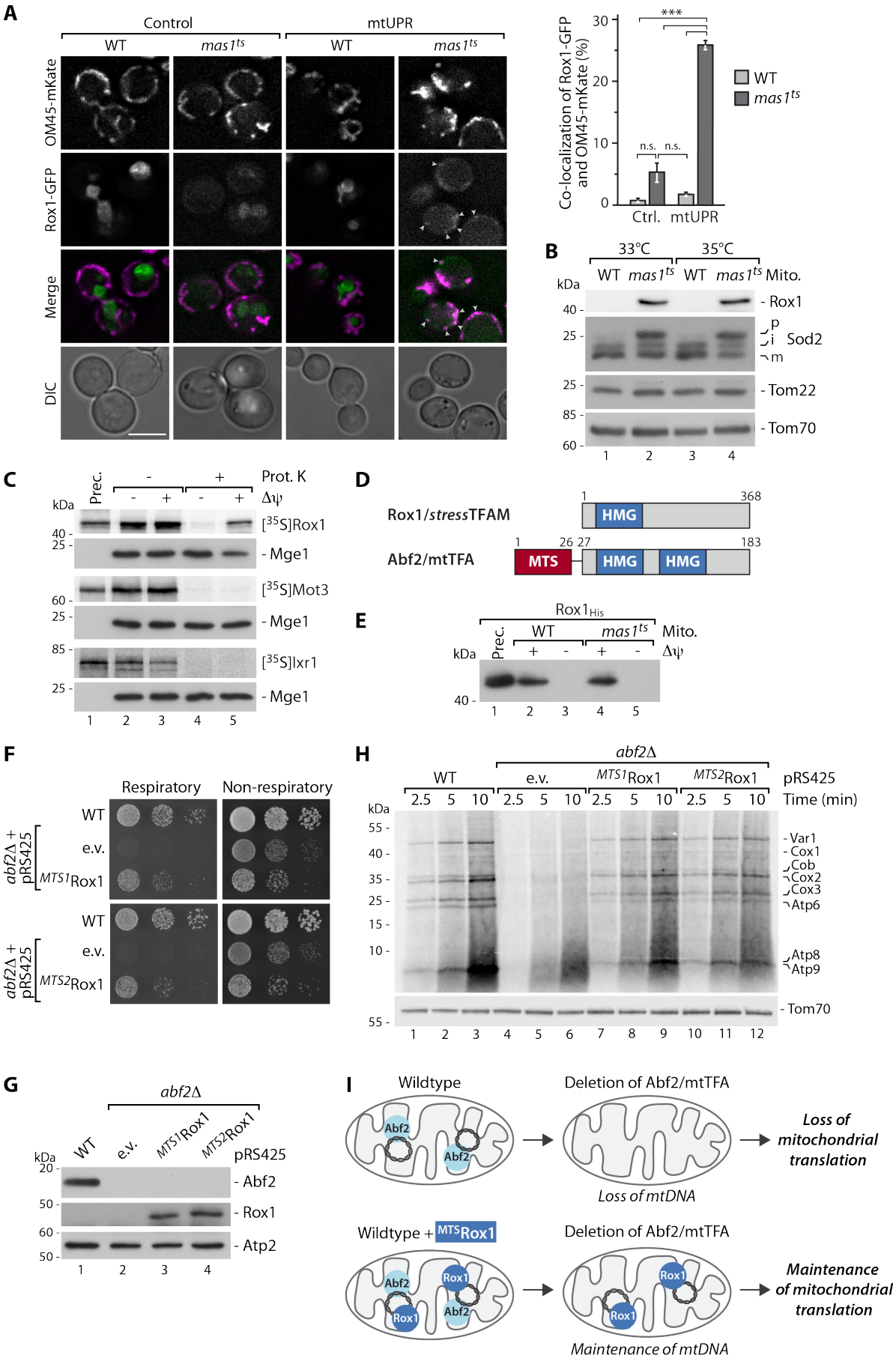
A
Control
mtUPR
WT
mas1ts
OM45-mKate
Rox1-GFP
Merge
DIC
Co-localization of Rox1-GFP and OM45-mKate (%)
***
n.s.
Ctrl.
mtUPR
B
33°C
35°C
Mito.
- Rox1
Sod2
- Tom22
- Tom70
C
Prot. K
Δψ
[35S]Rox1
- Mge1
[35S]Mot3
[35S]Ixr1
D
Rox1/stressTFAM
Abf2/mtTFA
HMG
MTS
E
Rox1His
F
Respiratory
Non-respiratory
abf2Δ + pRS425
e.v.
MTS1Rox1
MTS2Rox1
G
abf2Δ
pRS425
- Abf2
- Rox1
- Atp2
H
abf2Δ
pRS425
Time (min)
- Var1
- Cox1
Cob
Cox2
Cox3
Atp6
Atp8
Atp9
- Tom70
I
Wildtype
Deletion of Abf2/mtTFA
Loss of mitochondrial translation
Loss of mtDNA
Wildtype + MTSRox1
Maintenance of mitochondrial translation
Maintenance of mtDNA

Figure S5. Rox1 translocates to mitochondria upon mtUPR and mitochondrial Rox1 rescues the loss of yeast TFAM homolog Abf2/mtTFA. Related to Figures 4 and 5.

(A) Live cell fluorescence microscopy images of wildtype (WT) and *mas1*^{ts} cells at 23°C (Control) and after induction of mtUPR. OM45-mKate depicted in magenta; Rox1-GFP depicted in green; DIC, differential interference contrast; Arrow heads indicate co-localization of Rox1-GFP and OM45-mKate. n = 3, data represent means +/- SEM, ***p < 0.001, n.s., not significant. Scale bar, 5 μm.

(B) Immunoblot analysis of WT and *mas1*^{ts} mitochondria isolated from cells shifted to 33°C or 35°C, respectively. p, precursor; i, processing intermediate; m, mature.

(C) SDS-PAGE autoradiography of radiolabelled Rox1, Mot3 and Ixr1 precursors imported in isolated WT mitochondria for 30 min in the presence or absence of $\Delta\psi$. Prot. K, Proteinase K. Mge1, loading control.

(D) Schematic comparison of Rox1 with the yeast mitochondrial TFAM/mtTFA Abf2. HMG, high mobility group box; MTS, mitochondrial targeting signal.

(E) Import of Rox1 precursor protein (generated by cell-free translation in wheat germ extract and containing a C-terminal His tag) in isolated WT and *mas1*^{ts} mitochondria. Non-imported precursors were removed by Proteinase K after import reaction and samples were separated by SDS-PAGE and analyzed via immunoblotting and anti-His antibody.

(F) Analysis of WT strains transformed with pRS425 empty vector (e.v.) or expressing the *ROX1* gene fused to mitochondrial targeting signals (MTS1, Aco1; MTS2, Cym1). Subsequently the *ABF2* gene was deleted where indicated and growth was tested on respiratory (YPglycerol) or non-respiratory (YPglucose) plates at 30°C.

(G) Immunoblot analysis of mitochondria isolated from strains described in (F).

(H) Mitochondrial translation in WT and *abf2*Δ cells transformed with plasmids as described in (F). Labelled mtDNA-encoded proteins visualized by incorporation of ^{35}S-methionine.

(I) The yeast homolog of TFAM, Abf2/mtTFA, is required for maintenance of mtDNA. Loss of Abf2/mtTFA can be rescued by mitochondrial-targeted Rox1 (^{MTS}Rox1), which maintains mitochondrial genome expression.

Supplemental Tables

Table S2
Oligonucleotides for RTqPCR used in this study. Related to STAR Methods. Related to Figures 5 and S2.

Name	Sequence (5' --> 3')	Source	Identifier
FwCox5a	GTGGGACTTTTTGCTGTCGT	This paper	DPH-Pr1
RvCox5a	ACCACCCCAAGGATTAGCAT	This paper	DPH-Pr2
FwCox5b	ACTAAAGGGGCACGGCTAAC	This paper	DPH-Pr3
RvCox5b	TTTCCCATCTTTCGGGCAGA	This paper	DPH-Pr4
FwCyc7	AAACGAGGTGTCAGCAGTGT	This paper	DPH-Pr5
RvCyc7	CCAACTTTGTTAGGACCACCCT	This paper	DPH-Pr6
FwHsp78	GATCCCAATCAGCAACCGGA	This paper	DPH-Pr7
RvHsp78	TTTACCGACACCAGCTCGAC	This paper	DPH-Pr8
FwIrc25	ACGATGGCCCAAGACAACAT	This paper	DPH-Pr9
RvIrc25	CGCTTTGTTCCTTCGTTGCT	This paper	DPH-Pr10
FwMdj1	TTTGGTGCTGCATTTGGTGG	This paper	DPH-Pr11
RvMdj1	GGTCCAGCGCAGAGAATCTT	This paper	DPH-Pr12
FwCox1	ATGCCTGCTTTAATTGGAGGT	This paper	DPH-Pr13
RvCox1	ACAGTTCACCCTGTACCAGC	This paper	DPH-Pr14
FwCOB	GGACAGATGTCACATTGAGGT	This paper	DPH-Pr15
RvCOB	TGTAACGCAAAGAATCTCTGGA	This paper	DPH-Pr16
FwAtp6	CATTTGCATTATCAGCTCATTTAG	This paper	DPH-Pr17
RvAtp6	GATGCTGTTAAAATAGCCCAGAC	This paper	DPH-Pr18
FwTaf10	TCCTCCTATCATTCCCGATGC	This paper	DPH-Pr19
RvTaf10	CGCTACGGAAGACCTGATCC	This paper	DPH-Pr20
FwAct1	AGAAATTGTCCGTGACAT	This paper	DPH-Pr21
RvAct1	GATTCCAAACCCAAAACAGAAG	This paper	DPH-Pr22
FwCox1	AACACTATATGCGGGAAAACC	This paper	DPH-Pr23
RvCox1	GCGGATTGTCCATACTTATACC	This paper	DPH-Pr24