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Supplemental Information

The PERK Arm of the Unfolded Protein Response Regulates Mitochondrial Morphology during Acute Endoplasmic Reticulum Stress

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and Reagents

The mitochondria GFP plasmid was constructed by incorporating GFP 3' to a CoxVII mitochondria targeting sequence and cloned between the BamHI and NotI sites in the pENTR1A Gateway entry vector. The ^{mt}GFP sequence was then incorporated into the pDEST40 Gateway destination plasmid by clonase recombination for use in our transfections. The following reagents were acquired from commercial sources and used following the manufacturer instructions: ISRIB (Sigma), Thapsigargin (AdipoGen), Tunicamycin (AG Scientific), cycloheximide (Alfa Aesar), GSK2656157 (BioVision), 4μ8c (EMD Millipore), Ceapin (a kind gift from Peter Walter), KT5720 (Santa Cruz), Tubacin (Sigma), Oligomycin (Sigma), FCCP (Sigma), Rotenone (Sigma) and Antimycin A (Sigma).

Gene	**TRC number**
SLP2 (human)	TRCN0000173985
	TRCN0000194539
Tim17a (mouse)	TRCN0000114221
Yme1l (mouse)	TRCN0000031201
Parl (mouse)	TRCN0000126236

Quantitative Polymerase Chain Reaction (qPCR)

The relative mRNA expression levels of target genes were measured using quantitative RT-PCR. Cells were treated as described, washed with DBPS, and then RNA was extracted using the Quick-RNA MiniPrep Kit (Zymo Research). qPCR reactions were performed on cDNA prepared from 500 ng of total cellular RNA using the QuantiTect Reverse Transcription Kit (QIAGEN). The FastStart Universal SYBR Green Master Mix (Roche), cDNA, and appropriate primers purchased from Integrated DNA Technologies (see table) were used for amplifications (6 min at 95°C then 45 cycles of 10 s at 95°C, 30 s at 60°C) in an ABI 7900HT Fast Real Time PCR machine. Primer integrity was assessed by a thermal melt to confirm homogeneity and the absence of primer dimers. Transcripts were normalized to the housekeeping genes Rplp2 and all measurements were performed in triplicate. Data were analyzed using the RQ Manager and DataAssist 2.0 softwares (ABI).

Primers used for qPCR

Name	**Forward**	**Reverse**
Lon (mouse)	5'-GGCAGCCTAGAGGTGACAGG-3'	5'-ATGCAGGTGGATGTGTGAGG-3'
Hspa9 (mouse)	5'-GTGCTGCTCCTGGATGTCAC-3'	5'-CATCTCTCGTTCCCCCTGAC-3'
Chop (mouse)	5'-GGAGCTGGAAGCCTGGTAG-3'	5'-TGTGCGTGTGACCTCTGTTG-3'
Rplp2 (mouse)	5'-TGTCATCGCTCAGGGTGTTG-3'	5'-AAGCCAAATCCCATGTCGTC-3'
Parl (mouse)	5'-TTCACCATTGGGTTCACAGG-3'	5'-TTTTGTGGCCGTATGCTGTC-3'
Yme1l (mouse)	5'-GGTGGAGGAAGCCAAACAAG-3'	5'-AGGGACGTCAGCTTCTCCTG-3'
SLP2 (human)	5'-AGGAGAGGCCAGTGCAGTTC-3'	5'-AGTCCTTGGCCAGTTTGGAG-3'
Rplp2 (human)	5'-CGTCGCCTCCTACCTGCT-3'	5'-CCATTCAGCTCACTGATAACCTTG-3'

Cellular Lysates and Immunoblotting

After treatments, cells were washed twice with DPBS, scraped and cell pellets were lysed with lysis buffer (20mM Hepes [pH 7.4], 100mM NaCl, 1mM EDTA, 1% Triton) containing a protease inhibitor cocktail (Roche). Total protein concentration in cellular lysates was normalized using Bio-Rad protein assay. Lysates were then denatured with 6X Laemmli buffer and boiled before being separated by SDS-PAGE. Samples were transferred onto nitrocellulose membranes (Bio-Rad) for immunoblotting and blocked with 5% milk in Tris Buffer Saline (w/v) following incubation overnight at 4°C with primary antibodies. Membranes were washed, incubated with IR-Dye conjugated secondary antibodies and analyzed using Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification was carried out with LI-COR Image Studio software. Primary antibodies were acquired from commercial sources and used in the indicated dilutions in antibody buffer (50mM Tris [pH 7.5], 150mM NaCl supplemented with 5% BSA (w/v) and 0.1% NaN_3 (w/v): Tim17A (Thermo Scientific, 1:1000), Tim23 (BD Transduction Labs, 1:1000), HSP60 [LK1] (Thermo Scientific, 1:1000), Tubulin [B-5-1-2] (Sigma, 1:5000), OPA1 (BD Transduction Labs, 1:2000), SLP2 (ProteinTech, 1:1000), PS51-eif2α (Cell Signaling, 1:1000), eiF2α (Cell Signaling, 1:1000), Lon (ProteinTech, 1:1000), P-Drp1 S637 (Cell Signagling, 1:1000), Drp-1 (BD Biosciences, 1:1000), MFN2 (Cell Signaling, 1:1000), MFN1 (Abcam, 1:1000), YME1L (Abgent, 1:1000), CHOP (Santa Cruz, 1:1000), Cleaved caspase 3 (Cell signaling, 1:1000).

[^{35}S] Metabolic labeling

Cells were metabolically labeled with 110µCi/ml EasyTag Express^{35}S Protein Labeling Mix (PerkinElmer) in DMEM without cysteine and methionine (Corning-Cellgro) supplemented with 10% dialyzed FBS, 2mM L-glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. After treatment as indicated, cells were washed and recovered in labeling media for 30min. Following labeling, whole cell lysates were prepared in RIPA buffer (50mM Tris [pH 7.5], 150mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS) containing proteases inhibitors cocktail (Roche). Samples were denatured with 6X Laemmli buffer, boiled and separated by SDS-PAGE. Gels were stained with Coomassie Blue, dried and imaged by autoradiography using a Typhoon Trio Imager (GE Healthcare).

Clonal expansion

Cells were treated for 12hrs and then washed, trypsinized and counted. Cells were seeded in three different dilutions containing 30,000, 15,000, and 10,000 cells and incubated for four days at 37°C. Crystal violet staining solution (0.5% crystal violet (w/v), 20% methanol) was added and samples were washed twice with PBS and three times with water. After drying overnight, quantification was performed by diluting crystals with 1ml sodium citrate solution (0.1M [pH 4.2], 50% methanol (v/v)) and absorbance was read at 550nm.

Mitochondrial Respiration measurements

Mitochondrial Respiration parameters were measured using a Mito Stress Test Kit and XF96 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer's protocol. In brief, 15,000 cells were plated on Cell-Tak (Corning) coated wells in their standard growing media and cultured overnight. The next day, cells were treated with drugs and incubated at 37°C, 5%CO_2 for 6 h. Seahorse media (DMEM no red phenol, no bicarbonate, 25mM glucose, 1mM pyruvate, 2mM Glutamine, MEM Non-Essential Amino Acids, pH7.4) was used to wash the cells and measure oxygen consumption rate (OCR). The mito stress test was assayed according to the manufacturer's protocol. Briefly, each parameter was measured by 4 measurements every 5 min following injections of drugs. Basal respiration measurement was followed by Oligomycin (2 µM), FCCP (0.5 µM) and Rotenone/Antimycin (1µM each) injections. Parameters are represented as average of 3 independent experiments from 4 measurements of 10 wells per conditions. Spare respiratory capacity was calculated as a % of the ratio of the maximal to basal respiration.

SUPPLEMENTAL FIGURES

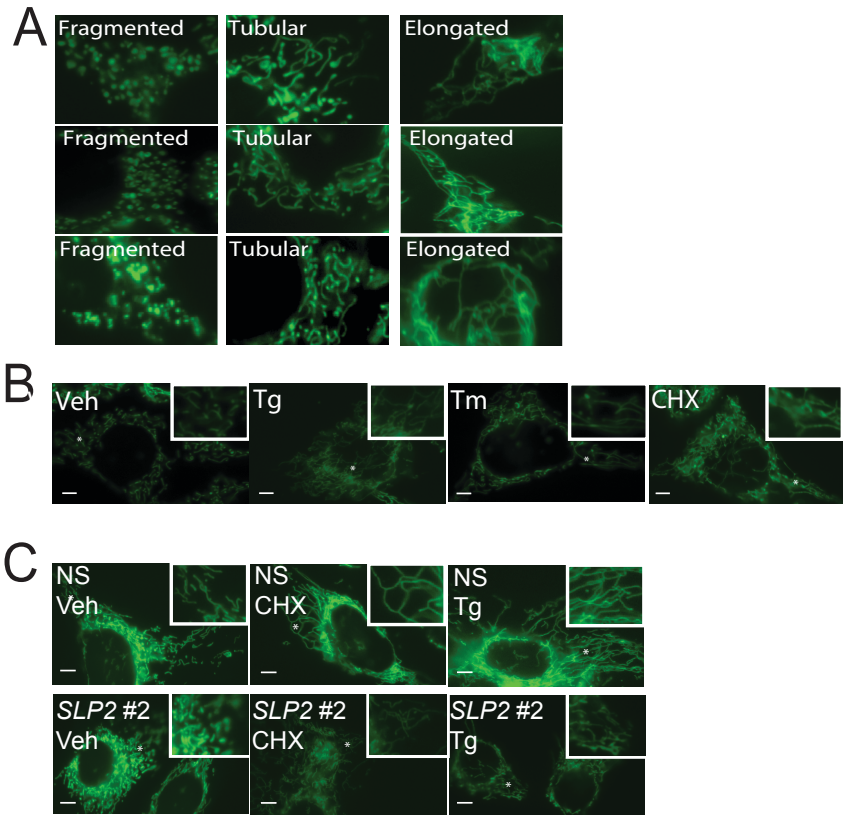

Figure S1 (Supplement to Figure 1)

- **A.** Representative images showing the fragmented, tubular, and elongated morphologies of MEF^{mtGFP} cells.
- **B.** Representative images of MEF^{mtGFP} cells treated for 6 h with thapsigargin (Tg; 500 nM), tunicamycin (Tm; 1 µM), or cycloheximide (CHX; 50 µg/mL). The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 µm.
- **C.** Representative images of HeLa cells stably expressing non-silencing (NS) shRNA or *SLP2* shRNA #2 and transfected with mitochondria-targeted GFP. Cells were treated for 6 h with thapsigargin (Tg; 500 nM) or cycloheximide (CHX; 50 µg/mL), as indicated. The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 µm.
- **D.** Graph showing the fraction of HeLa cells stably expressing non-silencing (NS) shRNA or *SLP2* shRNA #2 and transfected with mitochondria-targeted GFP containing fragmented (red), tubular (yellow), or elongated (green) mitochondria following treatment for 6 h with thapsigargin (Tg; 500 nM), cycloheximide (CHX; 50 µg/mL) or ISRIB (200 nM), as indicated. Error bars show SEM for two independent experiments. Representative images from these cells are shown in **Fig. S1C**.
- **E.** Graph showing the relative mRNA levels of *SLP2* in HeLa cells expressing non-silencing (NS) shRNA, *SLP2* shRNA #1 or *SLP2* shRNA #2, confirming depletion of *SLP2* in these cells. Data are normalized to cells expressing NS shRNA. Error bars show 95% confidence interval.

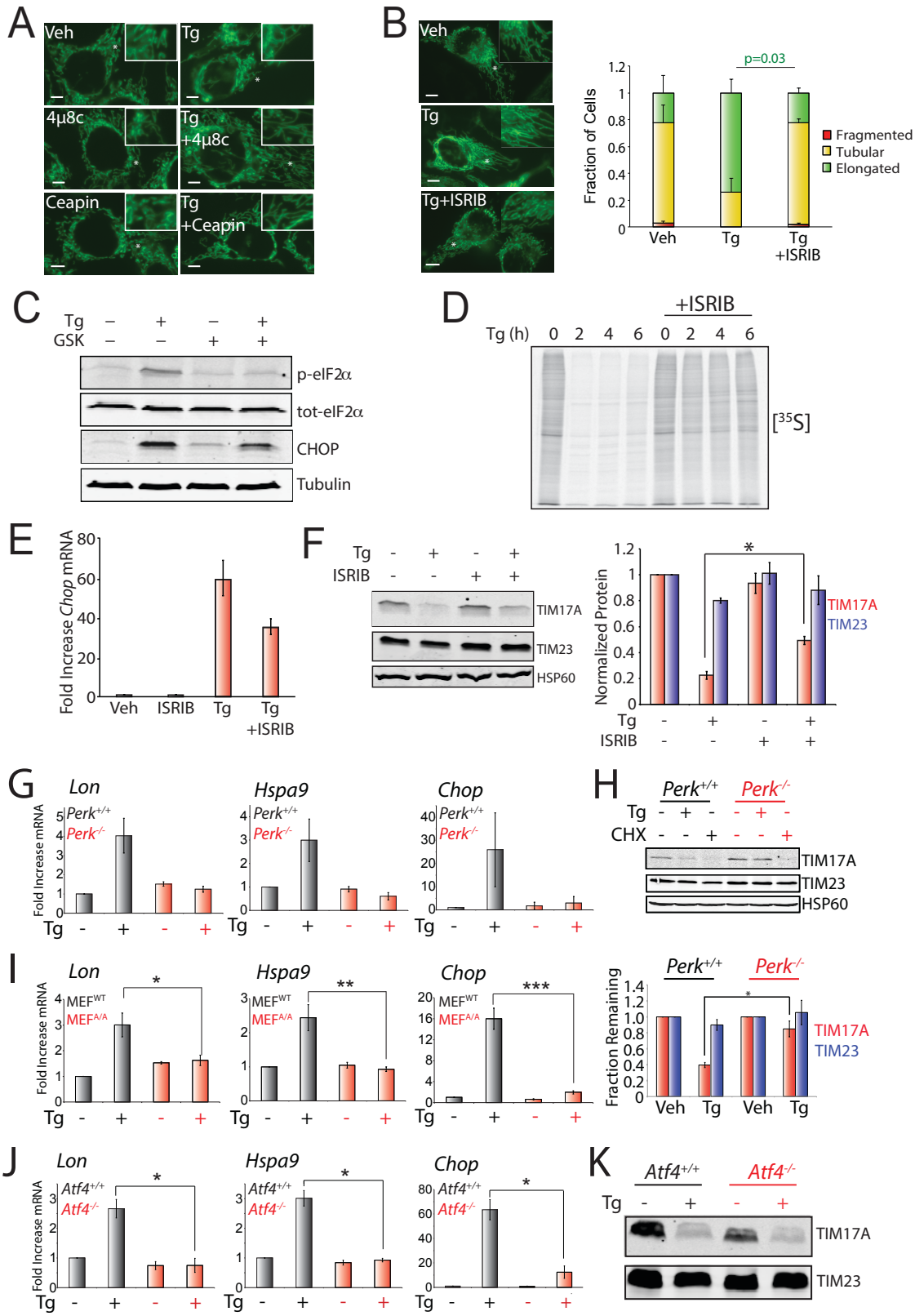

Figure S2 (Supplement to Figure 2)

A. Representative images of MEF^{mtGFP} cells treated for 6 h with thapsigargin (Tg; 500 nM), 4μ8c (an IRE1 inhibitor; 10 μM) or Ceapin (an ATF6 inhibitor; 2.5 μM), as indicated. The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 μm.

B. Representative images and quantifications of HeLa cells transiently transfected with mitochondria-targeted GFP and treated for 6 h with thapsigargin (Tg; 500 nM) and/or ISRIB (200 nM). The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 μm. Graph shows the fraction of HeLa cells containing fragmented (red), tubular (yellow), or elongated (green) mitochondria, as indicated. Error bars show SEM for three independent experiments. p-values shown for elongated (green text) mitochondria for a two-tailed paired t-test.

C. Immunoblot of MEF^{mtGFP} cells treated for 6 h with thapsigargin (Tg; 500 nM) and/or GSK2656157 (GSK; 10 μM). Treatment with GSK2656157 reduces Tg-dependent eIF2a phosphorylation and CHOP expression, confirming that GSK2656157 inhibits PERK signaling during ER stress.

D. Autoradiogram of lysates prepared from MEF^{WT} cells labeled with [^{35}S]-methionine for 15 min. Cells were pretreated with thapsigargin (Tg; 500 nM) and ISRIB (200 nM) for the indicated time prior to metabolic labeling. Treatment with ISRIB attenuates the Tg-dependent reduction in [^{35}S] incorporation demonstrating that ISRIB inhibits eIF2a-dependent translational signaling.

E. Graph showing *Chop* expression in MEF^{WT} cells treated for 6 h with thapsigargin (Tg; 500 nM) and/or ISRIB (200 nM). Error bars show 95% confidence interval. Treatment with ISRIB decreases Tg-dependent increases in *Chop* expression, confirming that this compound attenuates PERK-dependent transcriptional signaling.

F. Representative immunoblot and quantifications of TIM17A and TIM23 in lysates prepared from MEF^{WT} cells treated for 6 h with thapsigargin (Tg; 500 nM) and/or ISRIB (200 nM). Graph shows normalized TIM17A (red) or TIM23 (blue protein levels relative to untreated controls. Error bars show SEM for three independent experiments. *$p<0.05$ from a two-tailed paired t-test. Treatment with ISRIB inhibits Tg-dependent TIM17A degradation induced by eIF2a-dependent translational attenuation, further confirming that this compound inhibits PERK signaling.

G. Graph showing *Lon*, *Hspa9* and *Chop* mRNA levels in $Perk^{+/+}$ (grey) or $Perk^{-/-}$ (red) cells treated for 6 h with thapsigargin (Tg; 500 nM). Error bars show 95% confidence interval. The inhibition of the Tg-dependent induction of these genes in $Perk^{-/-}$ MEFs confirms inhibition of PERK-regulated transcriptional signaling in these cells.

H. Representative immunoblot and quantification of TIM17A (red) and TIM23 (blue) in lysates prepared from $Perk^{+/+}$ and $Perk^{-/-}$ cells treated with thapsigargin (500 nM; 6 h). Lysates prepared from $Perk^{+/+}$ or $Perk^{-/-}$ cells treated with cycloheximide (CHX; 50 μg/mL, 6 h) are shown as a control in the immunoblot. Graph shows normalized TIM17A (red) or TIM23 (blue protein levels relative to untreated controls. Error bars show SEM for n=3. *$p<0.05$ for a two-tailed t-test. The inhibition of Tg-dependent Tim17A degradation in $Perk^{-/-}$ MEFs confirms that PERK-regulated translational signaling is inhibited in these cells.

I. Graph showing *Lon*, *Hspa9* and *Chop* mRNA levels in MEF^{WT} (grey) or $MEF^{A/A}$ (red) cells treated for 6 h with thapsigargin (Tg; 500 nM). Error bars show SEM for n=3 experiments. *$p<0.05$; **$p<0.01$, ***$p<0.005$ for a two-tailed paired t-test. The inhibition of the Tg-dependent induction of these genes in $MEF^{A/A}$ cells confirms inhibition of PERK-regulated transcriptional signaling in these cells.

J. Graph showing *Lon*, *Hspa9* and *Chop* mRNA in $Atf4^{+/+}$ (grey) or $Atf4^{-/-}$ (red) cells treated for 6 h with thapsigargin (Tg; 500 nM). Error bars SEM for n=3 independent experiments. *$p<0.05$ for a two-tailed paired t-test. The inhibition of the Tg-dependent induction of these genes in $Atf4^{-/-}$ MEFs confirms inhibition of PERK-regulated transcriptional signaling in these cells.

K. Representative immunoblot of TIM17A in lysates prepared from $Atf4^{+/+}$ and $Atf4^{-/-}$ MEF cells treated for 6 h with or without thapsigargin (Tg; 500 nM). The efficient Tim17A degradation observed in $Atf4^{-/-}$ MEFs confirms that PERK-regulated translational attenuation (required for Tim17A degradation) is not impaired in these cells.

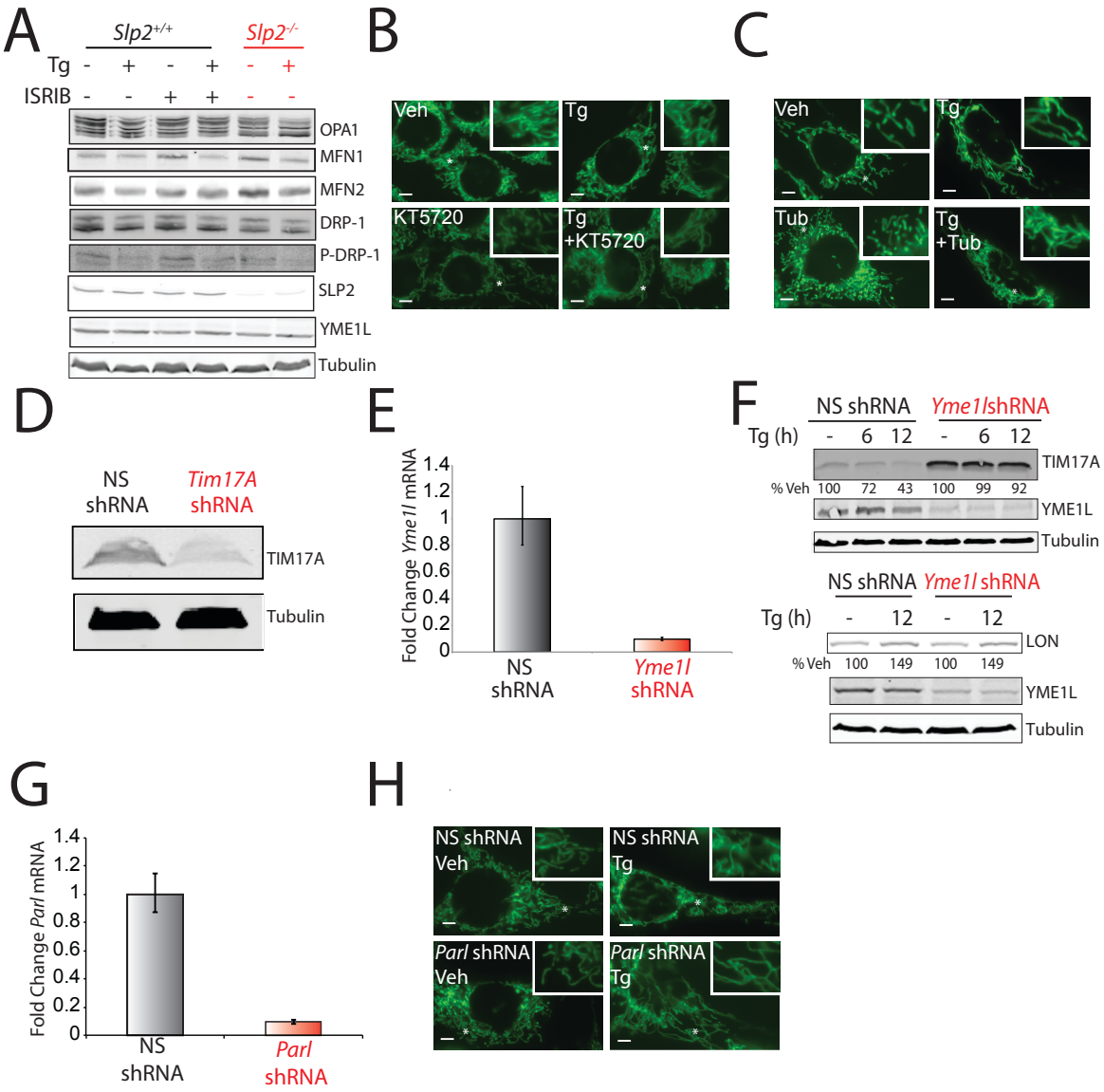

Figure S3 (Supplement to Figure 3)

- **A.** Immunoblot of lysates prepared from *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated with thapsigargin (Tg; 500 nM) and/or ISRIB (200 nM) for 6 h, as indicated. Tg treatment does not significantly change OPA1 processing, total MFN2 or MFN1 protein levels, total DRP1 protein levels, or phosphorylation of DRP1 in a ISRIB or *Slp2*-dependent manner. This suggests that ER stress induced mitochondrial elongation, which is inhibited by ISRIB or *Slp2* depletion, proceeds through a mechanism independent of these posttranslational alterations of mitochondrial GTPases.
- **B.** Representative fluorescence images of MEF^{mtGFP} cells treated with thapsigargin (Tg; 500 nM) and/or the PKA inhibitor KT5720 (1 µM) for 6h. The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 µm.
- **C.** Representative fluorescence images of MEF^{mtGFP} cells treated with thapsigargin (Tg; 500 nM) and/or the HDAC6-inhibitor Tubacin (10µM) for 6h. The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 µm.
- **D.** Representative immunoblot of TIM17A from lysates prepared from MEF^{mtGFP} cells stably expressing non-silencing (NS) or *Tim17a* shRNA, confirming depletion of *Tim17a* in these cells.
- **E.** Graph showing *Yme1l* mRNA in MEF^{mtGFP} cells stably expressing non-silencing (NS) or *Yme1l* shRNA, confirming depletion of *Yme1l* in these cells.
- **F.** Representative immunoblot of TIM17A and LON from lysates prepared from MEF^{mtGFP} cells stably expressing non-silencing (NS) shRNA or *Yme1l* shRNA, treated for 6 or 12 h with thapsigargin (Tg; 500 nM). The impaired Tg-dependent degradation of Tim17A in these cells confirms reduced YME1L proteolytic activity. In contrast, the increase in LON protein levels in Tg-treated cells depleted of *Yme1l* shows that PERK-regulated signaling is not significantly impaired in these cells.
- **G.** Graph showing *Parl* mRNA in MEF^{mtGFP} cells stably expressing non-silencing (NS) shRNA or *Parl* shRNA, confirming depletion of *Parl* in these cells.
- **H.** Representative fluorescence images of MEF^{mtGFP} cells stably expressing non-silencing (NS) or *Parl* shRNA treated with thapsigargin (Tg; 500 nM) for 6h. The inset shows a 2-fold magnification of the image centered at the *. Scale bar shows 5 µm.

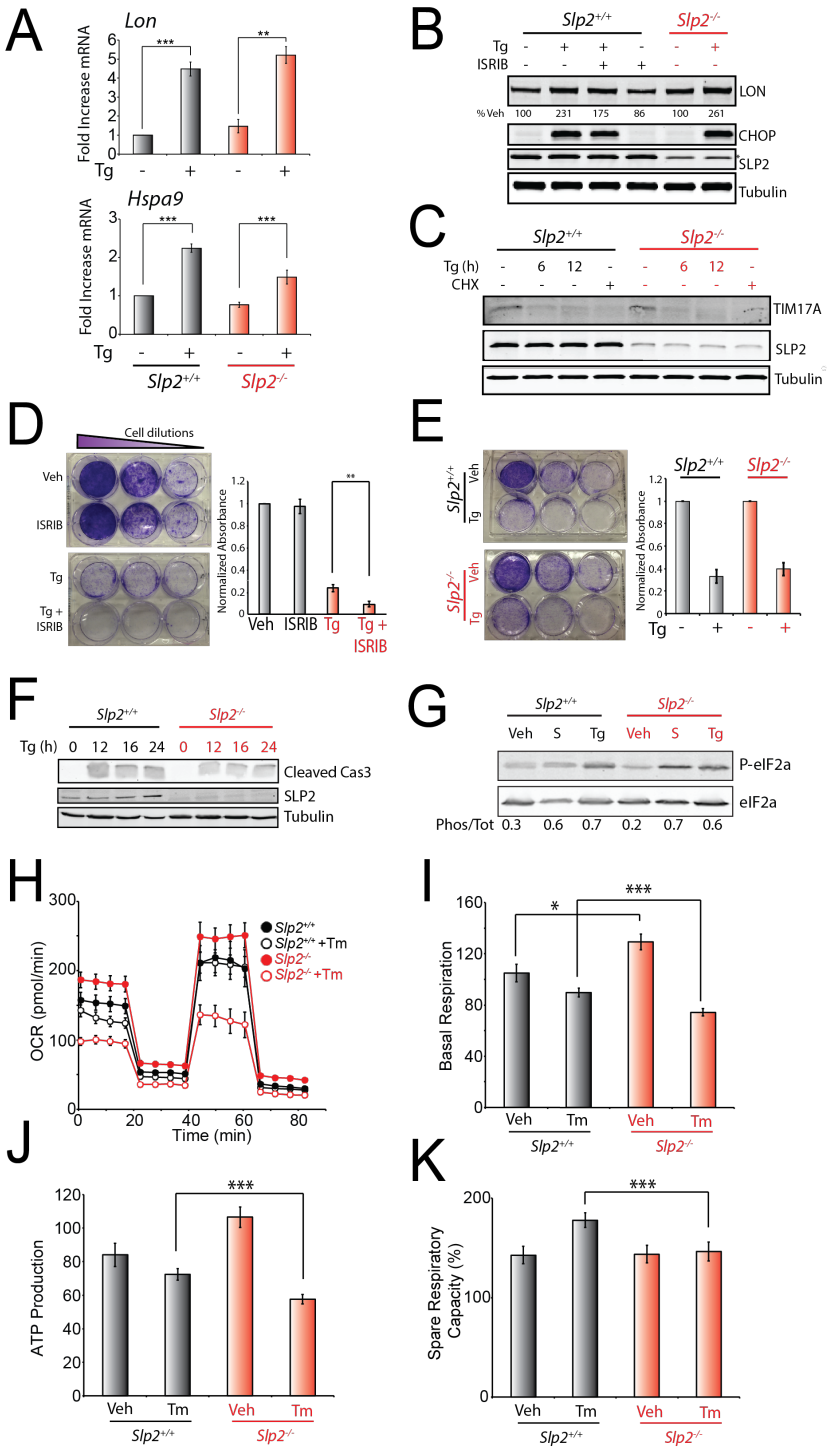

Figure S4 (Supplement to Figure 4)

A. Graph showing *Lon* and *Hspa9* and *Chop* mRNA in *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs cells treated for 6 h with thapsigargin (Tg; 500 nM). Error bars SEM for n=3 independent experiments. **p<0.01, ***p<0.005 for a two-tailed paired t-test. The efficient Tg-dependent induction of these genes in *Slp2*$^{-/-}$ MEFs confirms that PERK-regulated transcriptional signaling is not inhibited in these cells.

B. Immunoblot of LON and CHOP in lysates prepared from *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated for 12 h with thapsigargin (Tg;500 nM) and/or ISRIB (200 nM), as indicated. The relative expression of LON is quantified below the immunoblot. The efficient Tg-dependent increase in LON protein *Slp2*$^{-/-}$ MEFs confirms that PERK-regulated transcriptional signaling is not inhibited in these cells. Note we do observe a band in the SLP2 immunoblots in *Slp2*$^{-/-}$ MEFs, which likely reflects a background band recognized by the SLP2 antibody.

C. Immunoblot of TIM17A in lysates prepared from *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated for 6 or 12 h with thapsigargin (Tg;500 nM) and/or cycloheximide (CHX;50 µg/mL), as indicated. The efficient Tim17A degradation observed in *Slp2*$^{-/-}$ cells confirms that PERK-regulated translational signaling is not inhibited in these cells. Note we do observe a band in the SLP2 immunoblots in *Slp2*$^{-/-}$ MEFs, which likely reflects a background band recognized by the SLP2 antibody.

D. Representative image from a colony formation assay performed in MEF^{WT} cells pretreated for 12 h with ISRIB (200 nM) and/or thapsigargin (Tg;500 nM). Following pretreatment, cells were plated at three different concentrations in a 6-well plate to allow single colony formation. Cells were then grown for 72 h prior to visualization using crystal violet staining. Graph showing the absorbance of solubilized crystal violet. Error bars show SEM from three independent experiments. ** $p<0.01$ for two tailed paired t-test.

E. Representative image from a colony formation assay performed in *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs pretreated for 12 h with thapsigargin (Tg;500 nM). Following pretreatment, cells were plated at three different concentrations in a 6-well plate to allow single colony formation. Cells were then grown for 72 h prior to visualization using crystal violet staining. The graph shows the quantified absorbance of solubilized crystal violet from wells. Error bars show SEM from three independent experiments.

F. Immunoblot of cleaved Caspase3 in lysates prepared from *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated for 12, 16 and 24 h with thapsigargin (Tg;500 nM).

G. Immunoblot of P-eIF2α and eIF2α in lysates prepared from *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs incubated for 2 h with Seahorse media at 37°C, CO2 free incubator or treated with thapsigargin (Tg;500 nM) for 2h at 37°C, 5% CO2. The ratio of P-eIF2α relative to total eIF2α is quantified below the immunoblot.

H. Representative plot from one experiment showing OCR in *Slp2*$^{+/+}$ (black) or *Slp2*$^{-/-}$ (red) MEFs pretreated for 6 h with tunicamycin (Tm; 1ug/ml) prior to analysis by Seahorse.

I. Graph showing basal respiration of *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated with tunicamycin (Tm; 1ug/ml) for 6 h before OCR measurements. Error bars show SEM for n=20 collected over two independent experiments. *$p<0.05$; ***$p<0.005$ for a two-tailed unpaired t-test.

J. Graph showing ATP production of *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated with tunicamycin (Tm; 1ug/ml) for 6 h before OCR measurements. Error bars show SEM for n=20 collected over two independent experiments. ***$p<0.005$ for a two-tailed unpaired t-test.

K. Graph showing Spare Respiratory Capacity of *Slp2*$^{+/+}$ and *Slp2*$^{-/-}$ MEFs treated with tunicamycin (Tm; 1ug/ml) for 6 h before OCR measurements. Error bars show SEM for n=20 collected over two independent experiments. *$p<0.05$ for a two-tailed unpaired t-test.