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Supplemental Information

NADPH and Glutathione Redox Link TCA Cycle Activity to Endoplasmic Reticulum Homeostasis

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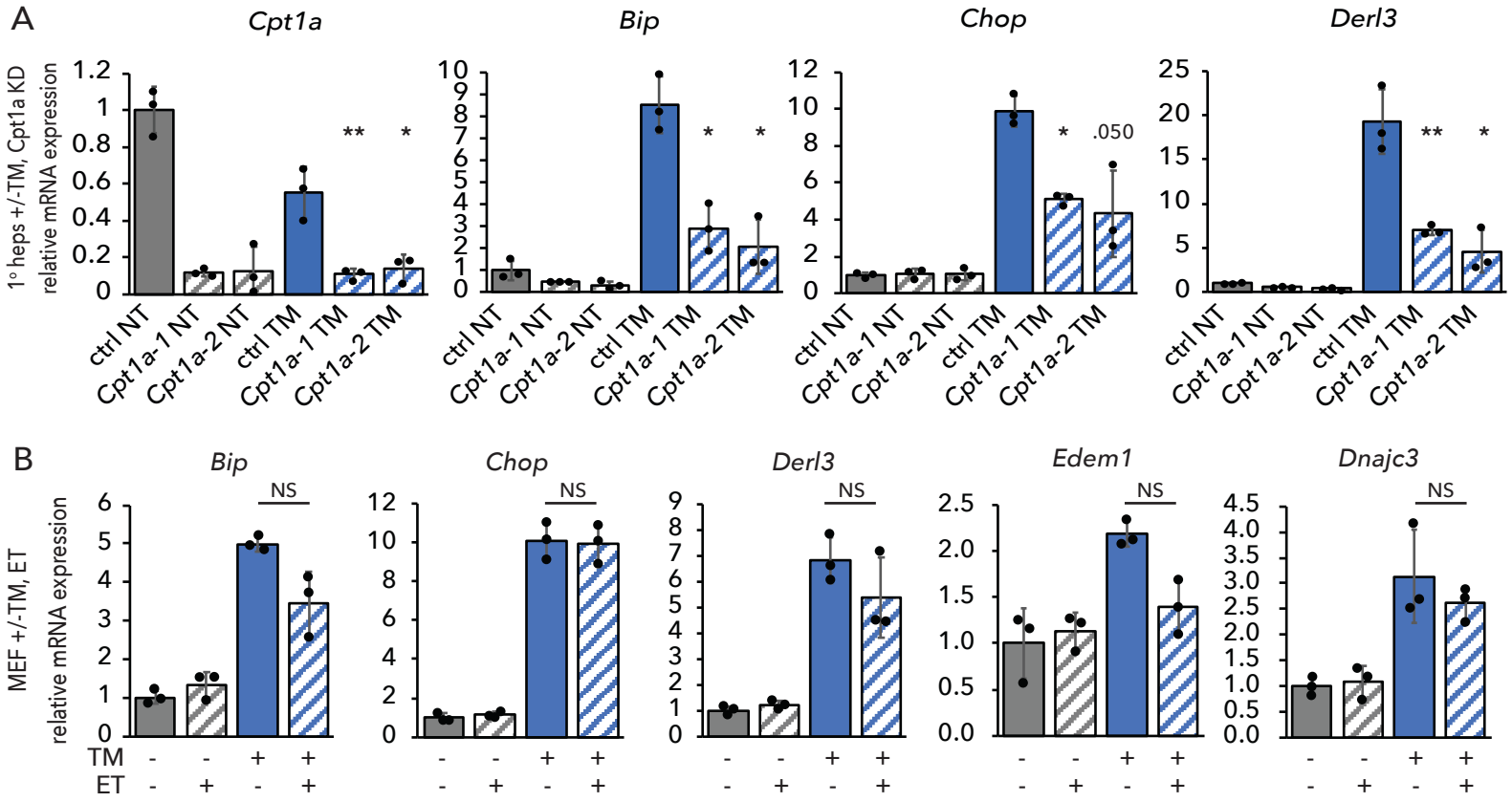

Figure S1. Additional effects of inhibition of β-oxidation, related to Figure 1. (A) Primary hepatocytes were transfected with a control dsiRNA or either of two dsiRNAs against *Cpt1a*, and then treated with vehicle or TM as in Figure 1. (B) Primary mouse embryonic fibroblasts were treated with 25 µg/ml ET and 250 ng/ml TM as indicated for 8 h. mRNA expression was assessed by qRT-PCR as in Figure 1.

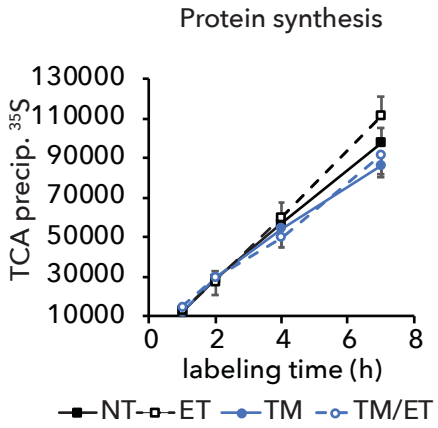

Figure S2. ET treatment does not affect protein synthesis, related to Figure 2. Primary hepatocytes were treated with 25 µg/ml ET and 250 ng/ml TM as indicated in media with 10 percent the normal amount of methionine and cysteine, and EasyTag ExpreSS^{35}S (Perkin Elmer NEG772002MC) was added to a final concentration of 50 µCi/ml. After the indicated times, cell lysates were collected in 1% SDS 100 mM Tris, pH 8.8, denatured by heating, spotted onto gridded 3MM filter paper, and air dried. Samples on the filter were then precipitated by serial incubations in ice-cold 10% trichloro-acetic acid (TCA) for 60 min., 5% TCA at room temperature for 5 min., 5% TCA preheated to 75° for 5 min., 5% TCA at room temperature for 5 min., and finally 2:1 ethanol:ether preheated to 37° for 5 min. Filter was then air dried, sliced into individual samples, and immersed in scintillation cocktail, and counted. n = 6 samples per condition. The modest effect of this dose of TM on protein synthesis is consistent with previous findings (Rutkowski et al., 2006).

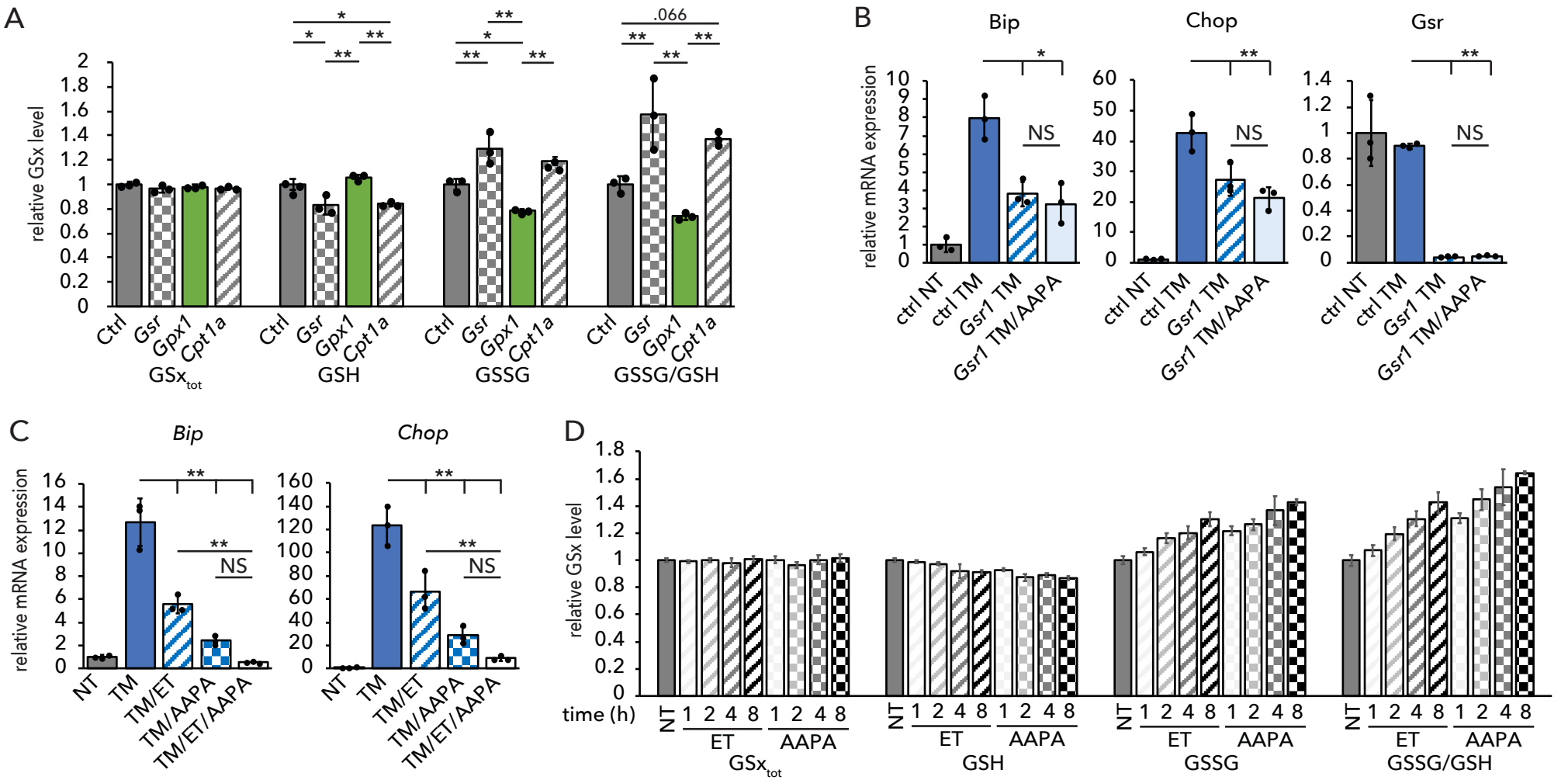

Figure S3. Manipulation of GSSG and GSH levels by knockdown, related to Figure 4. (A) Knockdown of *Gsr*, *Gpx1*, and *Cpt1a* produces the expected effects on GSSG and GSH. (B, C) Gene expression was assesed in cells treated with the indicated combinations of inhibitors (TM, ET, AAPA) or dsiRNAs (*Gsr*) for 8 h. For all knockdown experiments, non-targeting siRNA was used for control treatments. (D) AAPA elicits more rapid changes in the GSSG/GSH ratio than does ET.

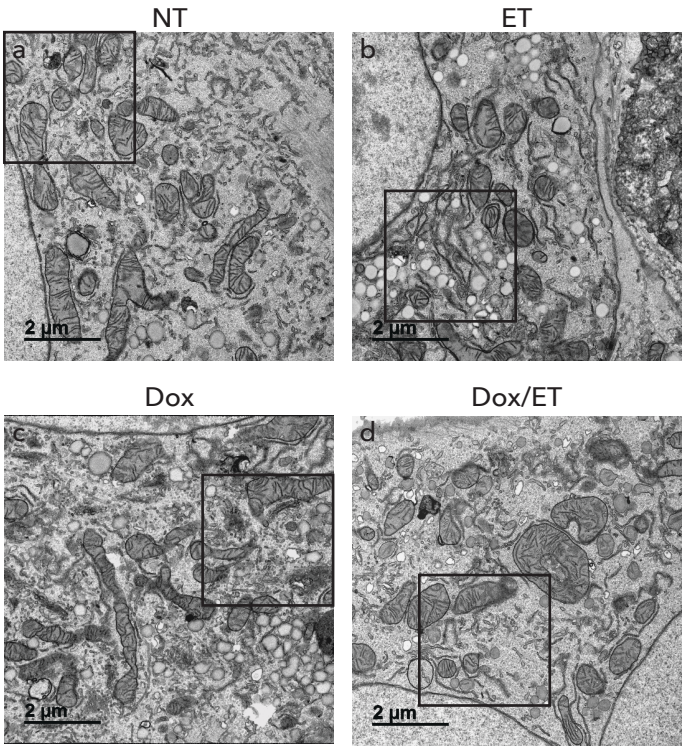

Figure S4. Improved ER structure by ET in NHK-expressing cells, related to Figure 6. Detailed EM images shown in Figure 6B are taken from the images shown above.

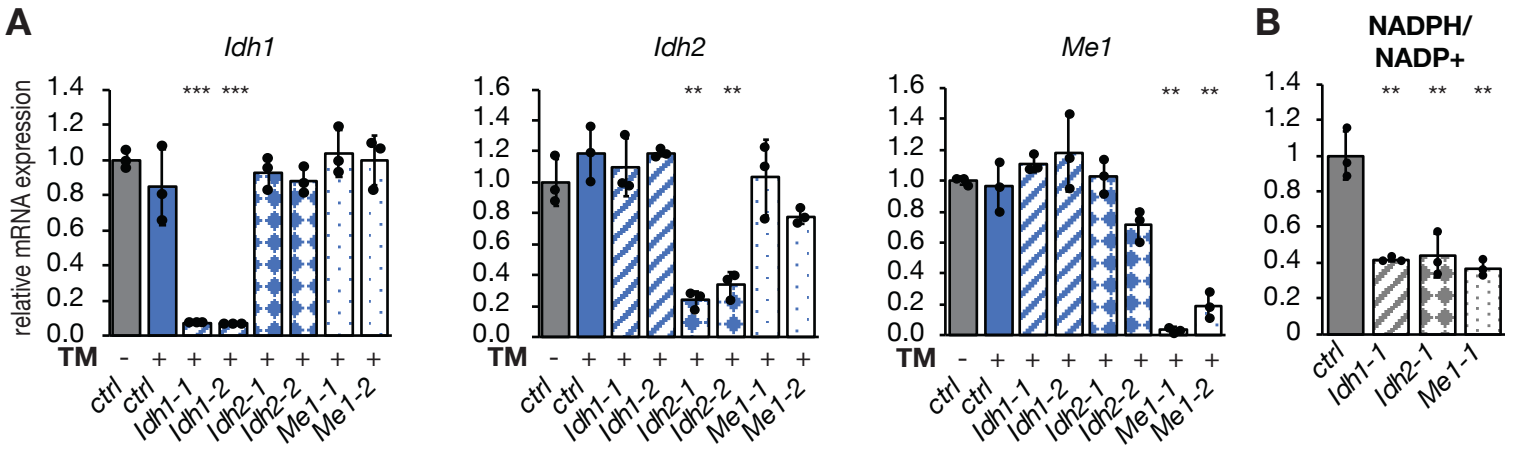

Figure S5. Effective and specific knockdown of TCA-dependent NADPH-producing enzymes, related to Figure 7. (A) qRT-PCR confirmed specific decreases in mRNA expression of *Idh1*, *Idh2*, and *Me1* using two independent pairs of siRNAs for each gene, compared to cells transfected with a control siRNA. (B) Knockdown of any of these enzymes diminished the relative NADPH/NADP+ ratio. As with changes in the UPR-dependent gene expression and the GSSG/GSH ratio, effect sizes varied from one experiment to another but the trends remained consistent.

Transparent Methods

Cell culture and drug treatments

Primary hepatocytes were isolated from C57BL6/J mice of both sexes between 6-12 weeks of age. Mice were anesthetized with isoflurane for the duration of the isolation. All animal experiments were approved by the University of Iowa Institutional Animal Care and Use Committee. The liver was perfused through the portal vein with freshly prepared Perfusion Medium followed by digestion with Liver Digest Medium. Media formulas were as follows: Liver Perfusion Medium: HBSS, no calcium, no magnesium, no phenol red (Life Technologies, Carlsbad, CA), 0.5 mM EDTA, 0.5 mM EGTA, 25 mM HEPES, and penicillin-streptomycin (10,000 U/mL); Liver Digest Medium: HBSS, calcium, magnesium, no phenol red, 25 mM HEPES, penicillin-streptomycin (10,000 U/mL), 72 μg/ml Trypsin Inhibitor (Sigma, St. Louis, MO), 500 μg/ml Collagenase Type IV (210 U/mg) (Worthington Biochemical Corp., Lakewood, NJ). Flow rates were 4 ml/min for 5 min for perfusion, and 8 min for digestion. The liver was quickly excised, placed in cold Wash Medium (DMEM, 10 mM HEPES, 5% FBS, 100 μg/mL penicillin-streptomycin), dispersed by tearing Glisson's capsule, and filtered through a sterile 70 μm cell strainer. Hepatocyte suspensions were centrifuged at 500 rpm for 3 min and resuspended in 30 mL of Wash Medium with 35% Percoll. Cells were centrifuged for 5 min at 1000 rpm, followed by resuspension in Wash Medium for a final wash with centrifugation for 3 min at 500 rpm. Viable hepatocytes were resuspended in Hepatocyte Medium (William's E, 5% FBS, 10 nM insulin, 100 nM dexamethasone, 100 nM triiodothyronine, and 100 μg/mL penicillin-streptomycin), or, for *Mpc*$^{fl/fl}$ and liver-specific knockout (*Mpc2*^{LKO}) hepatocytes, in high glucose DMEM, 10% FBS, penicillin-streptomycin, and 0.5 μg/ml amphotericin B, and plated on collagen-coated tissue culture plates. Media was changed 4 h after plating to remove any non-adherent cells. MPC2-deficient hepatocytes were isolated from *Mpc2*$^{fl/fl}$ animals bred into the Albumin-CRE line. MPC1-deficient C2C12 cells were generated by CRISPR and cultured as described (Oonthonpan et al., 2019). gRNA sequences were 5′-GCGCTCCTACCGGTGCCCGA-3′ and 5′-GCCAACGGCACGGCCATGGC-3′.

For culture of primary BAT, interscapular brown adipose was removed from a litter of 4-6 day old C57BL6 pups, with white adipose from this deposit removed. The remaining BAT was digested in collagenase (1 mg/ml in HBSS with 20 mg/ml BSA) for 90 minutes, followed by dispersion by repeated pipetting. Cells were place on ice for 15-30 min, and the infranatant layer was passed through a 100 μm filter and pelleted at 700g for 5 min. iBAT stromal vascular cells (SVCs) were resuspended in preadipocyte media (high glucose DMEM supplemented with 10% FBS, HEPES, Penn/Strep, 1x non-essential amino acids, 1x GlutaMAX, and 100 nM β-mercaptoethanol) and plated on a 10 cm dish. After 3 days, cells were replated on 12-well plates. Two days after confluency, cells were differentiated for four days with differentiation media (high glucose DMEM, 10% FBS, Penn/Strep, 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM IBMX). Cells were then maintained in maintenance media (same as differentiation media without dexamethasone and IBMX) for experiments. Primary mouse embryonic fibroblasts were isolated and cultured as described. Briefly, head, limbs, and internal organs were removed from E12.5-14.5 embryos, and the remaining embryonic tissue was digested in 0.25% trypsin-EDTA for approximately 30 minutes at 37°C until cells were separated. Trypsin was inhibited by addition of complete medium, cells were pelleted at 1,000 rpm for 5 minutes, and cells were resuspended in MEF media (high glucose DMEM supplemented with sodium pyruvate, L-glutamine, 10% FBS, Penn/Strep, 0.5x MEM amino acids, and 1x non-essential amino acids). Drug treatments used the times and concentrations indicated in the figure legends. TM, auranofin, and dichloroacetate (DCA) were from Millipore Sigma; ET, 2-AAPA, and MG-132 from Cayman Chemical; stocks of each of these were stored at -20°C in DMSO, except for DCA which was made fresh for each use. Palmitic acid and oleic acid (Millipore Sigma) were diluted stepwise in DMSO to 200 mM, and then to 100 mM in 10% fatty acid-free BSA (Millipore Sigma), followed by incubation at 40°C for 90 min with gentle agitation. Uncomplexed BSA was used for controls. Doxycycline (Millipore Sigma) stocks were stored at -20°C in water. All control samples received equivalent vehicle controls.

For pulse-chase experiments, cells were labeled using an ^{35}S Met/Cys labeling mixture at 200 μCi/ml (EasyTag Express35S; Perkin Elmer) in media that contained drug treatments and that was 10 percent Hepatocyte Medium as above and 90 percent high-glucose DMEM lacking Met/Cys and with dialyzed FBS. Labeling was stopped by addition of Hepatocyte Medium with 5 mM Met/Cys, removal, and replacement of the same medium for the chase period. For immunoprecipitations, chases were stopped in ice-cold HBSS with 20 mM N-ethylmaleimide, and lysates were collected in 0.5% Triton-X100, 100 mM HEPES pH 7.5, 100mM NaCl, 1 mM EDTA, 20 mM NEM, 1 mM PMSF, and a protease inhibitor tablet (Roche). Samples were centrifuged at 12,000 rpm for 5 minutes at 4°C and endogenous α1-antitrypsin was immunoprecipitated using a commercially-available antibody (Dako A0012) that recognizes mouse α1AT by immunoprecipitation (but not by immunoblot). Immunoprecipitations were separated by non-reducing or reducing SDS-PAGE as indicated in the figure legends and dried gels were exposed to Biomax MS film (Carestream Health) using an enhancer screen.

Adenovirus experiments

A cDNA encoding the NHK allele of α1-antitrpysin was cloned into an adenoviral shuttle vector downstream of a TRE-Tight promoter, and the shuttle was recombined with a backbone expressing rtTA under the RSV promoter. Virus was purified by the University of Iowa Viral Vector Core. Primary hepatocytes were infected with Ad-TetOn-NHK or Ad-CMV-eGFP at a multiplicity of infection of 1:1. Infection began when media was changed 4 h after cells were plated. Cells were

incubated with adenovirus for at least 12 h prior to addition of doxycycline or other treatments. NHK expression was induced with 500 ng/mL doxycycline in fresh media at the time of treatment.

dsiRNA knockdown experiments
Primary hepatocytes were cultured overnight prior to transfection. Hepatocytes were transfected with 2.75 μM dsiRNA (Integrated DNA Technologies, Coralville, IA) in nuclease-free duplex buffer using the Viromer Blue transfection kit (Origene) following the manufacturer's protocol. A non-targeting dsiRNA was used as a control, and two dsiRNAs for each gene of interest (*Idh1*, *Idh2*, *Me1, Gpx1, Gsr,* and *Cpt1*α) were used. Hepatocytes were transfected with dsiRNA for 24 h before experimental treatments. Targeting sequences were as follows: *Idh1*: GUACAACCAGGAUAAGUCAAUUGAA, GUUGAAGAAUUCAAGUUGAAACAAA; *Idh2*: AUUUAUAUUGCUCUGGAAUCACATG, AUCUUUGACAAGCACUAUAAGACTG; *Me1*: GCCAUUGUUCAAAAGAUAAAACCAA, ACCUUUCUAUCAGAUAUUAAAAUAT; *Gpx1*: GGUGGUUUCACUACUAAGAAUAAAG, GUUCGAGCCCAAUUUUACAUUGUTT; *Gsr*: GGCAUGAUAAGGUACUGAGAAAUTT, CAGAAGAACUUAUGUAUCUAAUCAG; *Cpt1a*: AUCUGUCCAUUGCAUGUAAAUACCA, GUGUGAUAUCAUCCAUGCAUACCAA;
non-targeting control: CGUUAAUCGCGUAUAAUACGCGUAT

Biochemical Assays
Levels of total (GSx), oxidized (GSSG), and reduced (GSH) glutathione were measured using a Glutathione Fluorometric Assay Kit (Biovision, Milpitas, CA) following the manufacturer's protocol. NADPH levels were measured using an NADP/NADPH Colorimetric Quantification Kit (Biovision), and acetyl-CoA levels using the PicoProbe™ Acetyl-CoA Fluorometric Assay Kit (Biovision) following the manufacturer's protocols. For all experiments, cells were lysed directly in ice-cold Glutathione Assay Buffer, NADPH Assay Buffer, or Acetyl-CoA Assay Buffer, incubated on ice with 6N perchloric acid, centrifuged to remove precipitates, and the supernatants were stored at -80°C. Perchloric acid was neutralized with potassium hydroxide prior to measurements.

RNA and Protein Analyses
Protein lysates were processed for immunoblot as described (Rutkowski et al. 2006). Primary antibodies were: CHOP (Santa Cruz sc-7351 or Proteintech 15204-1-AP), BiP (BD Biosciences 610978), PeIF2α (ThermoFisher 44-728G), MPC1 (Cell Signaling Technology 14462), calnexin (loading control; Enzo ADI-SPA-865), actin (loading control; MP Biomedicals 691001), α1-antitrypsin (Dako A0012). The oxidative state of the ER was measured by incorporation of PEG-mal (mm$(PEG)_{24}$) (ThermoFisher). Cells were rinsed twice in PBS, lysed in 2% SDS 100 mM Tris pH 6.8, heated to 100°C for 15 minutes, and equal aliquots were treated with PEG-mal or DMSO for 20 minutes at 37°C. Reaction was quenched with 100 mM DTT. Samples were run on Tris-tricine or Tris-HCl SDS-PAGE gels and transferred to 0.45 μm Immobilon-P Polyvinylidene Fluoride (PVDF) (Millipore) for Western blotting using ECL Prime substrate (GE Healthcare). Blots were imaged on Hyperfilm ECL and quantified by densitometric scanning from the images shown. qRT-PCR, including primer validation by standard curve and melt curve analysis, was as described (Rutkowski et al., 2006). Briefly, RNA was isolated following the standard Trizol protocol and RNA concentrations were obtained using the Qubit RNA Broad Range kit. Concentrations were normalized, and cDNA was synthesized using 400 ng RNA with PrimeScript RT Master Mix (Takara). PCR reactions were performed using TB Green Premix Ex Taq (Takara) in a CFX96 cycler (Bio-Rad). Oligonucleotide sequences are listed below. Gene expression was normalized against the average of two loading controls (*Btf3* and *Ppia*). Conventional RT-PCR was performed to assess splicing of *Xbp1* using Superscript III RT One-Step with Platinum Taq (ThermoFisher) according to the manufacturer's protocol. The size of the amplified product was 198 bp for unspliced *Xbp1* mRNA and 172 bp for spliced. Oligonucleotide sequences were as follows:

Gene	Sequences
Btf3 (loading)	Fwd: CCAGTTACAAGAAAGGCTGCT Rev: CTTCAACAGCTTGTCCGCT
Ppia (loading)	Fwd: AGCACTGGAGAGAAAGGATT Rev: ATTATGGCGTGTAAAGTCACCA
Bip	Fwd: CATGGTTCTCACTAAAATGAAAGG Rev: GCTGGTACAGTAACAACTG
Chop	Fwd: CTGCCTTTCACCTTGGAGAC Rev: CGTTTCCTGGGGATGAGATA
Derl3	Fwd: TGGGATTCGGCTTCTTTTTC Rev: GAACCCTCCTCCAGCAT
Edem	Fwd: CGATCTGGCGCATGTAGATG Rev: AAGTCTAGGAGCTCAGAGTCATTAA
Dnajc3	Fwd: TCCTGGTGGACCTGCAGTACG Rev: CTGCGAGTAATTTCTTCCCC
Xbp1	Fwd: TTGTGGTTGAGAACCAGG Rev: TCCATGGGAAGATGTTCTGG
Cpt1a	Fwd: GCTGGGCTACTCAGAGGATC

	Rev: CACTGTAGCCTGGTGGGTTT
Gpx1	Fwd: GGACTACACCGAGATGAACG Rev: TCGGACGTACTTGAGGGAAT
Gsr	Fwd: ATCCCTACTGTGGTCTTCAGC Rev: GGGGTAAAGGCAGTCGAGTA

Transmission Electron Microscopy

Primary hepatocytes were cultured on collagen-coated glass coverslips. Cells were washed in DPBS and fixed overnight at 4°C in 2.5% glutaraldehyde while protected from light. Cells were processed and imaged by the University of Iowa Central Microscopy Research Facility. Processing included 1% osmium fixation, incubation in 2.5% uranyl acetate, serial washes increasing from 50 to 100% ethanol, 2:1 ethanol and Epon (1 h), 1:2 ethanol and Epon (1 h), 100% Epon overnight, embedding in fresh Epon at 70°C for 24-48 h, microtomy, and uranyl/lead staining. Images were acquired on a JEOL JEM-1230 Transmission Electron Microscope. TEM images were scored blindly and binned into categories based on the percentage of ER morphology in each image that was dysmorphic. Bins were as follows: Normal (<25% of ER in an image was dysmorphic), Intermediate (25-75% dilated), or Severe (>75% dilated).

Statistical Analyses

Continuous variables were reported as the mean ± standard deviation and pairwise comparisons were analyzed using the two-tailed Student's t-test with Benjamini-Hochberg post-hoc correction for multiple comparisons (i.e., multiple genes from the same experiment). For comparisons of multiple groups, one-way ANOVA was used with Tukey's HSD post-hoc analysis. For qRT-PCR, significance was calculated prior to transformation of C_t values out of the log phase. A post-correction alpha of 0.05 was used to determine statistical significance.