Supplementary Information for

Spatiotemporal regulation of NADP(H) phosphatase Nocturnin and its role in oxidative stress response

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www.pnas.org/cgi/doi/10.1073/pnas.1913712117

Detailed Methods:

Purification of NOC:
BL21(DE3) cells transformed with pHisNusA-XL::Parallel (based on pET22b plasmid) expressing NusA-(TEV-protease cleavage site)-NOC(68-431) was grown to OD600 ~0.4-0.6, induced with 100μg/mL final concentration of IPTG, and incubated overnight at 20°C in a shaker. Bacterial pellets were resuspended in lysis buffer (50mM Tris pH 8.0, 150mM KCl, 150mM Na-K tartrate, 3mM $MgCl_2$, 10% glycerol, 1mM DTT, 0.1% Tween-20, supplemented with Roche Complete EDTA-free protease inhibitor cocktail tablet) and sonicated for 5X 30-second pulses. The lysate was centrifuged at 48,000 rpm on the Beckman Type 70ti rotor for 45 minutes at 4°C. The supernatant was run through the GE HisTrap HP column, washed in 50mM Tris pH 8, 150mM KCl, 150mM Na-K tartrate, 3mM MgCl2, 1mM DTT, 10% glycerol, and 20mM imidazole, and eluted isocratically in wash buffer containing 300mM imidazole. The eluate was buffer exchanged to wash buffer containing 20mM imidazole using the GE PD-10 desalting column, and the fusion protein was cleaved with TEV protease (1:50 w/w) overnight at 4°C. The proteins were run through the HisTrap HP column to remove the NusA tag and His-TEV. The flow-through and wash fractions were concentrated to ~1.5mL and run through the HiLoad 16/60 Superdex 200pg gel filtration column in 50mM Tris pH 8.0, 150mM KCl, 150mM Na-K tartrate, 3mM MgCl2, 1mM DTT, and 10% glycerol. The protein concentration was determined by measuring absorbance at 280nm. For hNOC(68-431), concentration measurements using 1Abs=1mg/ml agree with results from the BCA assay.

Circadian tissue collection:
WT and *Noct*$^{-/-}$ animals were housed in 12:12 LD single cages, standard-diet, ad libitum. The mice were given at least 2 weeks to entrain to the light-dark cycle before they were sacrificed. A piece of fresh liver tissue was immediately fractionated and the rest were frozen in liquid nitrogen.

Subcellular Fractionation:
Liver tissue was homogenized in isotonic buffer (20mM HEPES-KOH pH 7.4, 1mM EDTA, 200mM sucrose, supplemented with 1 tablet per 10mL of Roche Complete Mini EDTA-free [Sigma Cat#11836170001] protease inhibitor and PhosSTOP [Sigma Cat#04906837001] phosphatase inhibitor tablets) using a Teflon-glass homogenizer. The lysate was centrifuged at 600g for 10 minutes to obtain the P1 fraction (nuclei and unbroken cells), and the supernatant was centrifuged again at 600g to obtain the supernatant S1 fraction. The nuclei fraction was obtained by washing the P1 fraction 3-5X times (until the pellet turns white) with 1mL of nuclei extraction buffer (10mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.05% IGEPAL CA-630) and centrifuging at 600g for 10 minutes. The S1 fraction was centrifuged at 7,000g for 10 minutes to obtain the P10 and the S10 fractions. The mitochondria-enriched P10 fraction was washed 3X times with 1mL of the isotonic buffer. The S10 fraction was centrifuged at 100,000g for 1 hour to obtain the P100 (microsome-enriched) and S100 (cytosolic) fractions.

Determination of orthophosphate production and steady-state kinetic parameters:
Rate of orthophosphate release was measured using the malachite green assay. Recombinant human NOC (0.1μM) was incubated with NADPH (0-2mM) in reaction buffer (100mM HEPES-KOH pH 7.5, 10mM KCl, 1mM $MgCl_2$, 0.005% Triton X-100) for 5 minutes at 22°C. The reaction was quenched with EDTA and assayed with the 5X malachite green detection reagent (Sigma Cat#MAK207). Detection of orthophosphate was made by absorbance measurements at 620nm in a 96-well plate reader and concentration determined from a standard curve.

Determination of NAD(H) production:
To measure NADH production, recombinant human NOC (0.3μM) was mixed with NADPH (50μM) in reaction buffer (20mM Tris pH 8.0, 70mM NaCl, 2mM MgCl2, 0.005% Triton X-100). The reaction was incubated at 22°C for 60 minutes, diluted 1/200 in 50μL, and 50μL 2X NAD(H) Glo assay detection reagent (Promega Cat#G9081) was added to each well. After 30 minutes of incubation, bioluminescent measurements were taking with the Synergy H1 microplate reader.

Determination of cellular NAD(H) and NADP(H) levels:
HEK 293A cells were plated at a density of 200,000 cells per well in a 6-well plate and transfected the following day with 2μg of DNA, 6μL of FuGENE 6 transfection reagent, in 100μL of OptiMEM. After 36 hours of incubation, the cells were trypsinized, resuspended in ice-cold PBS, and plated at a density of 10,000 cells in 50μL per well in a white, opaque bottom 96-well plate. The plate was incubated at 22°C for 15 minutes and then 50μL of 2X NADP(H) Glo assay detection reagent (Promega Cat#G9081) was added to each well. After 1 hour of incubation, bioluminescent measurements were taken using the BioTek Synergy H1 microplate reader. For NAD(H) measurements, metabolites were extracted from 200,000 cells in 400uL of NAD(H) extraction buffer, and colorimetric, enzymatic cycling assay was performed according to the NAD/NADH Quantification Kit (Sigma Cat#MAK037).

Co-Immunoprecipitation:
Liver tissue was taken from transgenic mice overexpressing mNoc-3XFlag in the liver, and the proteins were extracted in 6mL of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 5% glycerol, 0.5% Triton X-100, 1X protease inhibitor cocktail (Sigma Cat#P8340), and 1mM PMSF). The lysates were clarified twice by centrifugation at 4,000g for 10 minutes at 4°C. Protein concentrations were determined using the Pierce 660nm assay (ThermoFisher Scientific Cat#22660) and adjusted with the lysis buffer to 5mg/mL in 1.2mL. The diluted lysates were further clarified by centrifugation at 15,000g for 10 minutes at 4°C, 100μL of the supernatant was collected for the input fraction, and 1mL was incubated with 50μL (25μL packed volume) of Flag-M2 affinity gel (Sigma Cat#A2220) for 1 hour at 4°C. The beads were washed 5 times with 1mL of lysis buffer (without protease inhibitors), and proteins were eluted by incubating the beads with 50μL of 300ng/μL 3XFLAG (Sigma Cat#F4799) diluted in lysis buffer. After 30 minutes of incubation on ice, the eluate fraction was separated from the beads by centrifugation through a filtered column (Millipore Cat#UFC30GV). Anti-Flag HRP (Sigma Cat#A8592) and anti-NMT1 (Proteintech Cat#11546-1-AP) antibodies were used for the detection of mNoc-3XFlag and NMT1, respectively.

H_2O_2 oxidative stress assay:
HEK 293 cells were seeded in a 12-well plate at a density of 40,000 cells per well in 10% FBS, 4.5g/L glucose, 4mM L-glutamine, 1X penicillin/streptomycin, DMEM (ThermoFisher Scientific Cat#11965118). After 24 hours, each well was transfected with 1μg of DNA, 3μL FuGENE 6 transfection reagent, in 200μL of OptiMEM. The next day, H_2O_2 was added to the cells. After 24 hours of incubation, cytotoxicity was determined qualitatively using phase-contrast microscopy or quantitatively counting percentage of trypan blue-stained cells.

Redox indicator of cytotoxicity assay:
Twenty-four hours after transfection, HEK 293 cells were trypsinized, resuspended in fresh media, and plated on a 96-well plate at a density of approximately 10,000 cells in 100μL per well. After 24 hours, 50μL of H_2O_2 – diluted to 3X concentration in complete media – was added to each well. After 24 hours of incubation, 50μL of 4X AquaBluer™ detection reagent (MoBiTec Cat#6015MT) was added to each well. After 4 hours of incubation, the fluorescence signal (540ex/590em) was measured using a microplate reader.

SDS-PAGE and Western blot of NOC:
Protein concentrations for various liver subcellular fractions were determined using the BCA assay (ThermoFisher Scientific Cat#23225), then the lysates were diluted to 2.5mg/mL with RIPA buffer and adjusted a final concentration of 2mg/mL with 5X Laemmli sample buffer. Each well on a 4-20% Tris-glycine (Bio-Rad Cat#4568096) gel was loaded with 20μg of total protein and SDS-PAGE was performed at 80V for 2 hours. Proteins were transferred to a PVDF membrane at 100V for 1 hour using wet transfer. Blocking was done in 5% milk/TBST for 1 hour at room temperature. For NOC blots, membranes were incubated in rabbit polyclonal anti-NOC primary antibody, diluted in 3% BSA/TBST. For secondary antibody incubation, membranes were incubated with anti-rabbit HRP (Cell Signaling Cat#7074S) at 1:2,000 dilution in 5% milk/TBST for 1 hour at room temperature. ECL

reaction was performed using Bio-Rad's Clarity Max (Bio-Rad Cat#1705062) for 5 minutes, and the chemiluminescence signal was measured on the ChemiDoc MP imager. For WCL and S100 fractions, alpha-tubulin loading controls were probed with anti-alpha-tubulin antibody (Sigma Cat#T6199). For mitochondrial fraction, loading controls were probed with anti-HSP60 antibody (Santa Cruz Cat#SC-1052). For P100 fraction, Ponceau S staining was used as loading control.

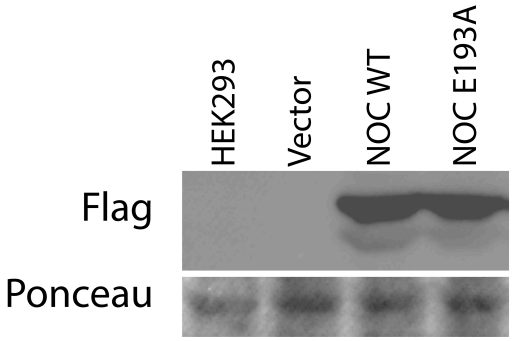
Detection of glutathionylation:
Fractionation of liver tissue was performed as described above, using buffers that were supplemented with 25mM of N-ethylmaleimide to alkylate free sulfhydryl groups. Total protein amount of 20μg were resolved on a 4-20% gradient gel at 80V for 2 hours, blocked in 5% milk/TBST, and probed with mouse anti-glutathione antibody (Virogen Cat#101-A) overnight at 4°C. Negative control samples were reduced with TCEP, and Ponceau S staining was used as loading control.

Live-cell imaging:
HeLa cells, cultured in minimal essential medium supplemented with 10% FBS (Hyclone), and penicillin and streptomycin solution - were seeded on 8-well Lab-Tek chambered cover glass (Nunc Cat#155383) at low density 24 hours prior to transfection. DNA plasmids (20-40ng/well in 8-well Lab Tek chambered cover glass) were transfected into HeLa cells with TransIT-LT1 reagent (Muris Bio). At 16 hours post-transfection, cells were washed once with extracellular buffer (ECB, 125mM NaCl, 5mM KCl, 1.5mM $MgCl_2$, 20mM HEPES, 10mM glucose, and 1.5mM $CaCl_2$ [pH 7.4]) and imaged in ECB. Live-cell confocal imaging experiments were performed at room temperature with a CFI Apo 100× objective (1.49 NA) and a custom spinning-disk confocal system built around an Eclipse T*i* microscope (Nikon) with an EM camera (Hamamatsu Photonics). The microscope was controlled by Micro-Manager software(36). Excitation of mCherry and YFP fusion proteins was achieved using a Sapphire 561 (561nm, Coherent Inc., Santa Clara, CA) and an LS200 Argon (514nm, Dynamic Laser, Salt Lake City, UT) laser, respectively.

Detection of Xbp1 mRNA splicing by RT-PCR
Cells were treated with UPR inducers for the indicated times and lysed with TRIzol reagent (Thermofisher Scientific Cat#15596026). RNA extraction was performed using the Direct-zol RNA Miniprep Kit (Zymo Research Cat#R2051). Reverse transcription was performed on 1μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher Scientific Cat#4368814). PCR fragments were amplified with the SapphireAmp Fast PCR Master Mix (Takara Cat#RR350) using primers corresponding to nucleotides 412–431 (5'-CCTTGTAGTTGAGAACCAGG-3') and 834–853 (5'-GGGGCTTGGTATATATGTGG-3') of human XBP1 cDNA. Human GAPDH was used as internal control using the following primers: 5'-TGCACCACCAACTGCTTAGC-3' and 5'-ACAGTCTTCTGGGTGGCAGTG-3'. PCR was performed using the following conditions: 94°C for 1min, 98°C for 5s, 58°C for 5s, 72°C for 30s, and 72°C for 5min with 25 cycles of amplification for XBP1 and 20 cycles of amplification for GAPDH.

Gene Expression Analysis:
Raw reads from mRNA-seq data ((4), GEO accession GSE105413) were mapped using BioWardrobe(37, 38) and the WT and KO samples from ZT18 were analyzed for differential gene expression using DESeq2 within BioWardrobe. Those that were significantly increased in the KO ($p<0.01$; 54 genes) were analyzed using Toppgene(15).

A



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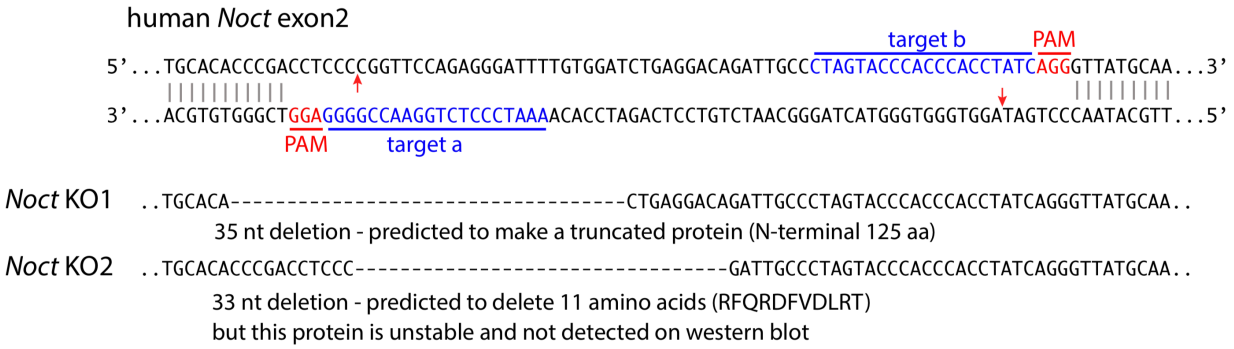

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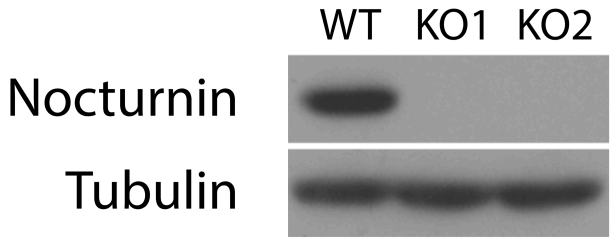


Fig. S1. NOC overexpression and knockout validation. **(A)** Western blot of NOC from lysates of HEK 293 cells overexpressing mNOC and mNOC-E193A. **(B)** Diagram of the two sgRNAs (blue) used to target spCas9n to exon 2 of *Noct* in HEK 293 cells (top). Sequence-verification of CRISPR-Cas9-mediated deletion in two independent clones (bottom). **(C)** Western blot verification of the absence of NOC in CRISPR-mediated knockout HEK 293 cell lines.

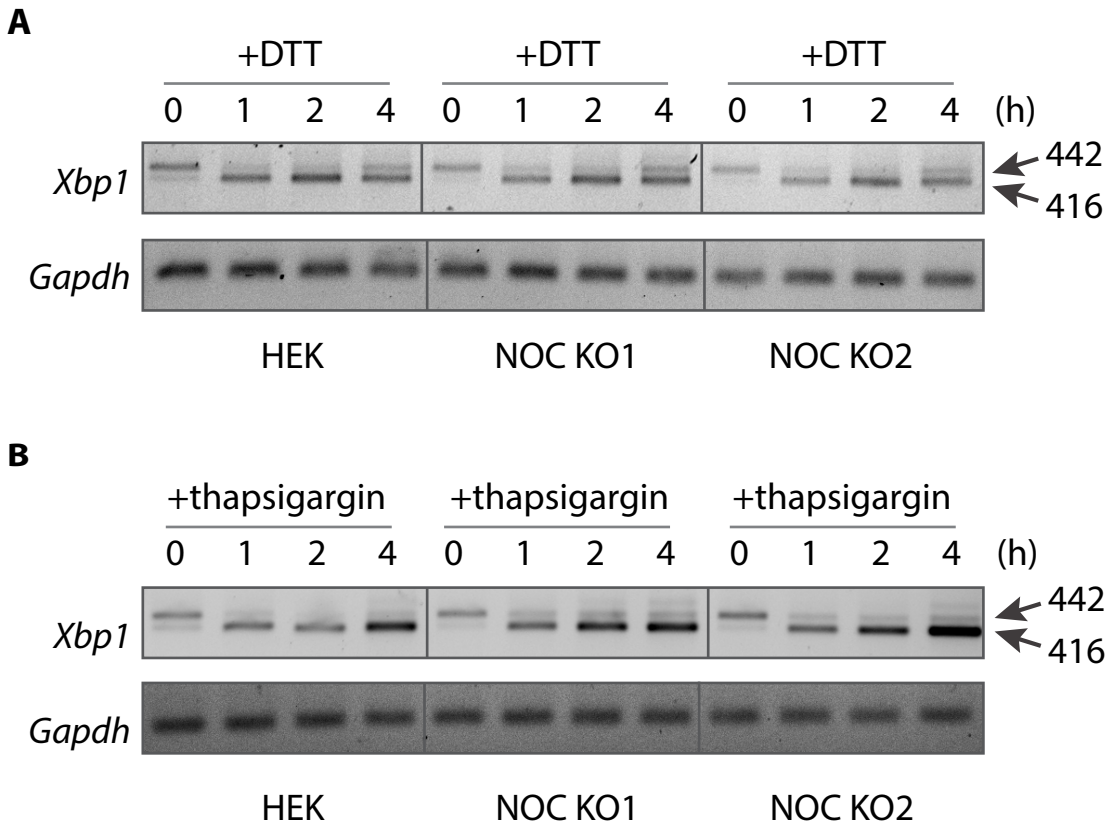


Fig. S2. NOC knockout does not affect *Xbp1* mRNA splicing in response to UPR stress. **A)** Semi-quantitative RT-PCR of *Xbp1* mRNA in NOC WT and knockout HEK cell lines that have been treated with 1mM DTT. **B)** Semi-quantitative RT-PCR of *Xbp1* mRNA in NOC WT and knockout HEK cell lines that have been treated with 1μM thapsigargin. In both cases, *Gapdh* was used as the internal control.

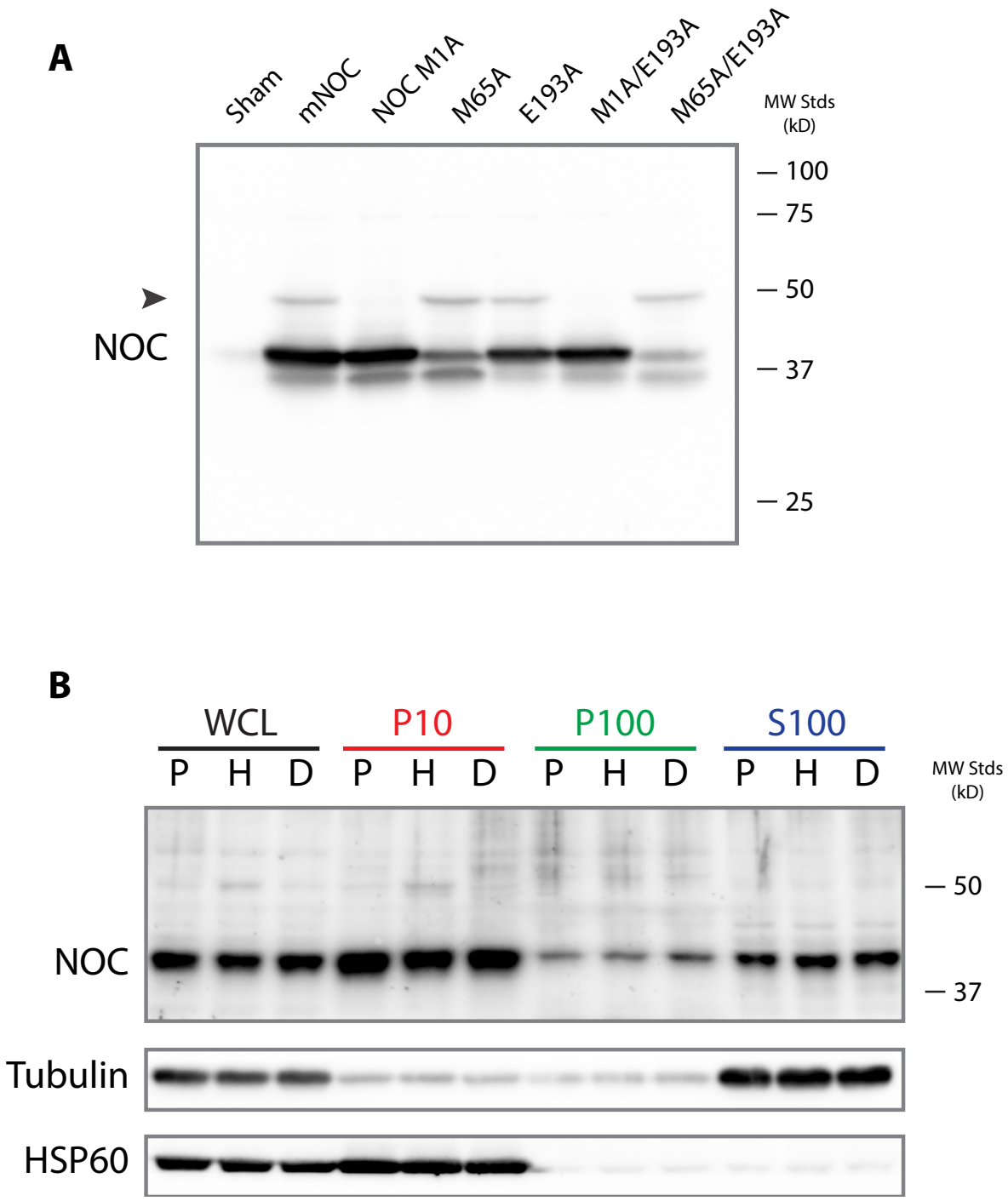


Fig. S3. The two isoforms of NOC cannot be resolved by SDS-PAGE and are not affected by cellular redox conditions. **A)** Western blot of NOC from HEK cells overexpression different mutants of mNOC. The arrow indicates the hypothesized mitochondrial isoform before MTS cleavage. **B)** Western blot of NOC from the indicated subcellular fractions of HEK cells that have been treated for 2h with PBS, 500μM H_2O_2, or 1mM DTT. α-Tubulin and HSP60 were used as loading controls and markers for cytosolic and mitochondrial fractions, respectively.

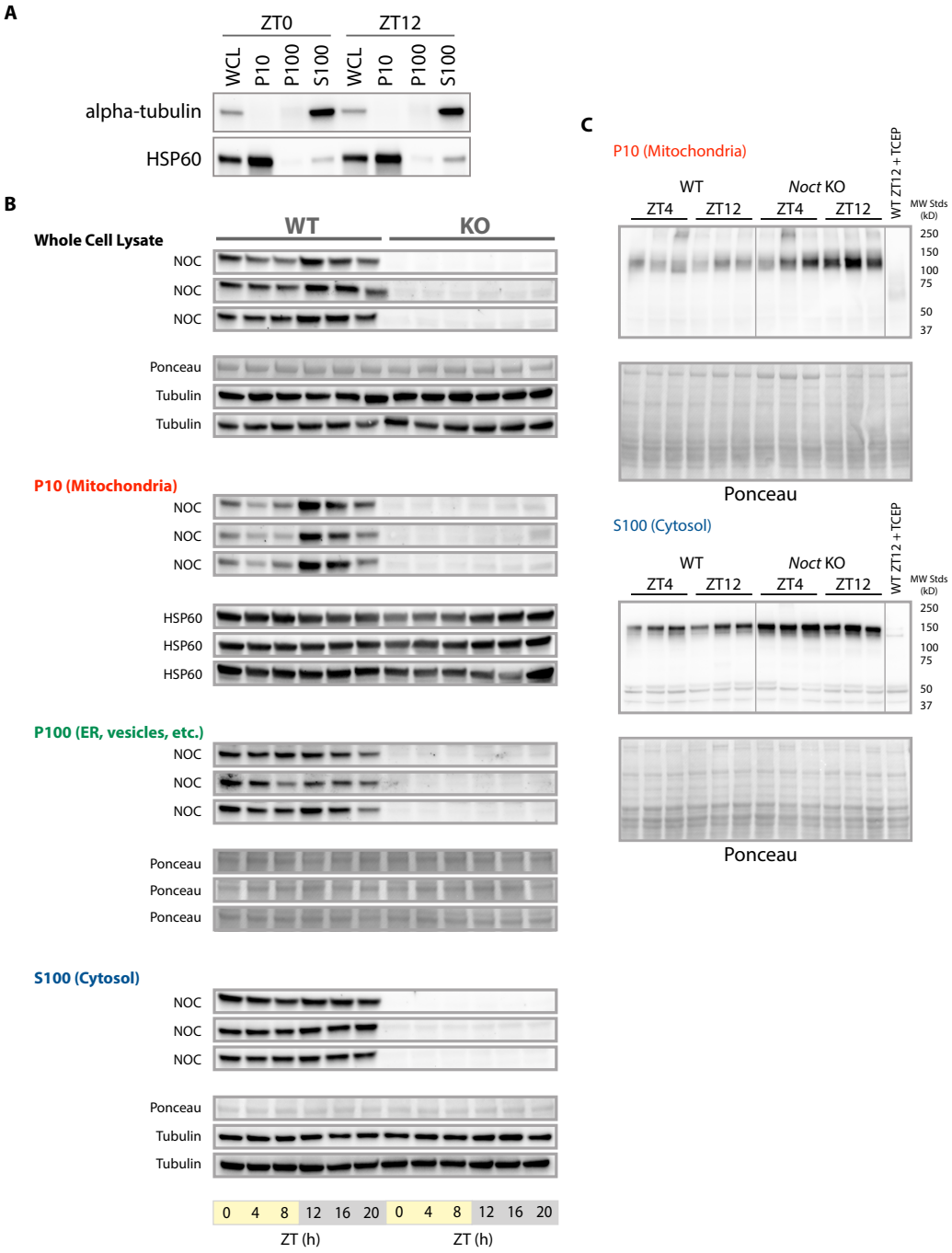


Fig. S4. Subcellular fractionation verification and replicates of NOC western blots in circadian liver fractions and demonstration that NOC knockout leads to altered redox status. **(A)** Western blots of tubulin (cytosol marker) and HSP60 (mitochondria marker) from WCL, P10, P100, and S100 fractions from ZT0 and ZT12. WCL fractions are positive for both tubulin and HSP60; P10 fractions are enriched for HSP60 and negative for tubulin; P100 fractions are negative for both markers; and S100 fractions are enriched for tubulin. **(B)** Western blots of NOC from WCL, P10, P100, and S100 fractions from circadian liver tissue and the corresponding internal controls. **(C)** Immunoblot of glutathione in the soluble S100 fraction in NOC WT and knockout liver at ZT4 and ZT12, in three replicates. Reducing agent-sensitive signal is revealed by comparison with WT ZT12 samples treated with TCEP. Ponceau stain of each gel is shown for loading control.

A

$$\frac{WCL_{ZT12}}{WCL_{ZT4}} = \frac{C + M_{ZT12}}{C + M_{ZT4}}$$

$$WCL_{(ZT12/ZT4)} = \frac{C + M_{(ZT12/ZT4)}*M_{ZT4}}{C + M_{ZT4}}$$

$$WCL_{(ZT12/ZT4)}*(C + M_{ZT4}) = C + M_{(ZT12/ZT4)}*M_{ZT4}$$

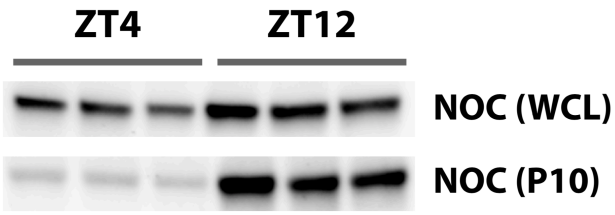
$$WCL_{(ZT12/ZT4)}*C + WCL_{(ZT12/ZT4)}*M_{ZT4} = C + M_{(ZT12/ZT4)}*M_{ZT4}$$

$$WCL_{(ZT12/ZT4)}*C - C = M_{(ZT12/ZT4)}*M_{ZT4} - WCL_{(ZT12/ZT4)}*M_{ZT4}$$

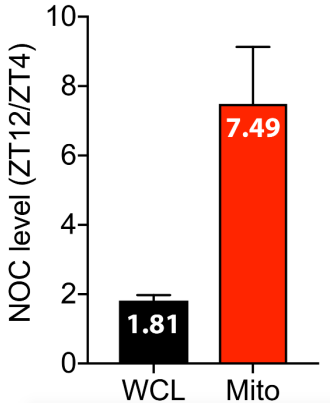
$$C*(WCL_{(ZT12/ZT4)} - 1) = M_{ZT4}*(M_{(ZT12/ZT4)} - WCL_{(ZT12/ZT4)})$$

$$C = \frac{M_{ZT4}*(M_{(ZT12/ZT4)} - WCL_{(ZT12/ZT4)})}{(WCL_{(ZT12/ZT4)} - 1)}$$

B



C



$$C = \frac{M_{ZT4}*(7.49 - 1.81)}{(1.81 - 1)} = 7.0*M_{ZT4} = 0.94*M_{ZT12}$$

Fig. S5. Approximately the same amount of NOC is present in both the mitochondria and the cytoplasm at ZT12. **(A)** Algebraic derivation of the amount of cytoplasmic NOC relative to mitochondrial NOC using the ratio of NOC at ZT12 to ZT4 in WCL and P10 fractions. WCL represents the overall amount of NOC in the whole cell lysate. C represents cytoplasmic NOC, which includes both cytosolic and membrane-bound pools. The model assumes C is constant throughout circadian times. M represents the circadian, mitochondrial NOC. **(B)** Western blot of NOC from mouse liver WCL and the mitochondria-associated P10 fraction from ZT4 and ZT12 (left). **(C)** Quantification of the relative amounts of NOC in the WCL and P10 fraction, n=3 (right).