SUPPLEMENTAL METHODS

Cell culture and reagents

H9c2 cardiac myoblasts were purchased from ATCC and kept in complete DMEM medium (ATCC, VA) supplemented with 10% FBS (Invitrogen, CA) and 1% penicillin-streptomycin (P/S). MEL cells were grown in suspension in complete RPMI-1640 (Cellgro, VA) medium supplemented with L-glutamine, 10% FBS and 1% P/S. Isolation and culture of neonatal rat cardiomyocytes (NRCM) was performed as described previously[1]

Adenoviral and Lentiviral Transduction of Cells

A recombinant adenoviral vector encoding GFP and human ABCB10 cDNA, separated by the polio virus internal ribosome entry site (IRES) element and under the transcriptional control of CMV promoter, was constructued. Briefly, ABCB10 cDNA was cloned into the adenovirus shuttle vector pAdCGI [2] to make the vector designed pAdCGI-ABCB10. A 25 cm_2 flask (T25; Sarstedt, Newton, NC) of CRE8 cells was cotransfected with 2.1 μg of y5 viral packaging plasmid and 2.1 μg of shuttle vector plasmid using Lipofectamine Plus (Invitrogen). Cells were incubated 5–9 days until cytopathic effects were observed. Cells and supernatant were collected and subjected to three cycles of freeze-thaw. Following centrifugation, 2 ml of the vector-containing supernatant was added to a 90% confluent T25 and returned to the incubator until cytopathic effects were observed. This procedure was repeated three to four times after which the vector was plaque purified and expanded. Large-scale vector preparations were purified on a cesium chloride gradient, as described.[3] Virus titers were determined by plaque assays. The insertion of ABCB10 cDNA was confirmed by sequencing the vector genome, and the vector was functionally validated by the expression of GFP and ABCB10. NRCMs were transduced on day 6 after isolation, and were imaged 2 days following transduction by a laser scanning confocal microscope (UltraVIEW; PerkinElmer), using a 403 water-immersion lens and

X40 optical zoom. Western blot analysis was also performed to assess gene expression. Lentivirus for shRNA-mediated knockdown of ABCB10 was purchased from Open Biosystems (cat #: V2LMM_5302) and non-silencing shRNA was used for control experiments. For ALAS2 overexpression experiments, zebrafish ALAS2 cDNA (GenBank #NM_131682) were cloned into pMMPA-HA retroviral vectors as described.[4] Viral transduction of H9c2 cells was carried out in complete medium for 48 hours.

MEL Cell Differentiation and siRNA treatment

On the day of differentiation induction 1 x 10^5 to 1 x 10^6 cells/mL were collected by centrifugation and transfected with siGENOME SMARTpool siRNA against mouse ABCB10 (Dharmacon, CO) using DharmaFECT 1 siRNA transfection reagent (Dharmacon, CO) according to the manufacturer's protocol. Cells were incubated with the transfection mix for 6 hours and differentiation was initiated by addition of complete medium containing 2% DMSO and 5mM hexamthylene bisacetamide (HMBA, Sigma-Aldrich, USA). To maintain efficient ABCB10 knockdown, the transfection was repeated on Day 3 of differentiation, and the differentiation medium was replaced. Cells were collected five days after induction of differentiation and knockdown of ABCB10 was confirmed by qRT-PCR and Western blot analyses. Differentiation was assessed by staining cells with benzidine and counting blue (differentiated) and unstained (undifferentiated) cells.

Western Blot

Fifteen-30 µg of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, CA). The membranes were probed with antibodies against ABCB10, ALAS1/2 (Abcam, MA), ferrochelatase (Proteintech,IL), GAPDH (Santa Cruz, CA), NCX (Swant, Switzerland) and tubulin (Abcam, MA). HRP-conjugated donkey anti-rabbit and donkey anti-

mouse were used as secondary antibodies (Santa Cruz, CA) and visualized by Pierce SuperSignal Chemiluminescent Substrates.

Quantitative RT-PCR

RNA was isolated with RNA STAT-60 (TEL-TEST, Inc, TX), reverse-transcribed with a Random Hexamer (Applied Biosystems, CA), and amplified on a 7500 Fast Real-Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, CA). Primers were designed using Primer3 (v. 0.4.0) software to target sequences spanning an exon-intron-exon boundary and their specificity was confirmed by running a dissociation curve. mRNA levels were calculated by the comparative threshold cycle method and normalized to β-actin gene.

Heme Content Determination

For determination of cellular heme levels, cells were lysed in 1% Triton-X100 in TBS, followed by centrifugation at 5,000 x g for 10 minutes to remove debris. For heme content in mouse hearts, ~5 mg of frozen tissue was homogenized in 1% Triton-X100 in TBS and centrifuged at 5,000 x g for 10 minutes to remove debris. For determination of mitochondrial heme levels, mitochondrial fraction was isolated using Mitochondrial Isolation Kit for Cultured Cells or Tissue (Pierce) according to the manufacturer's protocol. Protein concentration of cellular or mitochondriallysate was quantified by BCA assay (Pierce, IL) and heme was quantified as described.[5] Briefly, equal amounts of protein were mixed with 2M oxalic acid, heated to 95˚C for 30 minutes to release iron from heme and generate protoporphyrin IX. Samples were then centrifuged for 10 min at 1,000 x g at 4˚C to remove debris, the fluorescence of the supernatant was assessed at 405nm / 600nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample.

Mitochondrial ^{55}Fe Analysis

^{55}Fe (Perkin-Elmer, MA) was conjugated to nitriloacetic acid (NTA, Sigma-Aldrich, USA) and dissolved to the final concentration of 150-250 nM in complete, serum-containing medium. H9c2 cells were grown until 80% confluent, followed by modulation of ABCB10 levels and incubation in ^{55}Fe containing-medium for 48 hours. Cells were then washed once with cold PBS and twice with 500 μM bathophenanthrolinedisulfonate (BPS, Sigma, USA) in PBS to remove membrane-associated radioactivity. Mitochondrial fraction was isolated using the Mitochondrial Isolation Kit for Cultured Cells (Pierce), washed once with 500 μM BPS in PBS to remove residual ^{55}Fe, and resuspended in 1% Triton-X100 in TBS. The radioactivity of mitochondrial fraction was analyzed on Beckman scintillation counter and normalized to the protein concentration of each sample determined by BCA assay.

Total Porphyrin Content Quantification

The assay was modified from Sorte et. Al.[6] Briefly, cells were resuspended in 1%Triton X-100 in TBS and centrifuged for 5 min at 5,000 x *g* to remove debris. Supernatant was collected, mixed with one part of Ehrlich's reagent and two parts of saturated sodium acetate solution. The fluorescence was measured at 405 nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample. Porphobilinogen (Sigma-Aldrich, USA) was used to generate the standard curve.

Assessment of ^{55}Fe and ^{14}C-glycine Incorporation into Heme

Following lentiviral treatment, cells were incubated with 1.2mM δ-aminolevulinic acid (ALA) and 200 nM ^{55}Fe-NTA for 8 hours, washed once with PBS and twice with 500μM BPS in PBS to remove membrane-associated ^{55}Fe, and lysed. For ^{14}C-glycine incorporation into heme, complete growth medium was supplemented with 10 miCi/mL ^{14}C-glycine followed by 3-hour incubation, three washes with PBS to remove radioactivity, and cell lysis. Protein content of each sample was determined by BCA assay. Next, one part of 0.1M HCl was added to 5 parts

of cell lysate, followed by mixing with an equal volume of ethylacetate: acetic acid (3:1) and vortexing. Fractions were separated by centrifugation at 15,000 x *g* for 5 min and the radioactivity in the organic fraction containing heme was determined by scintillation counting and normalized to protein concentration of each sample.

Enzyme Activities

Complex IV activity was measured using the Sandwich ELISA Kits – Dipsticks assay (MitoSciences) according to the manufacturer's protocol. Peroxidase activity was assessed with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, CA) as absorbance at 560nm and normalized to protein concentration of each sample. Catalase activity was determined using the Catalase Assay Kit (Abcam) in accordance with manufacturer's guidelines as absorbance at 570nm, and normalized to protein content.

Hypoxia-Reoxygenation Treatment

H9c2 cells were transfected with ABCB10 or control siRNA as described above, followed by incubation in a humidified hypoxic chamber set at 1%O_2, 5%CO_2, at 37˚C for 48 hours, and 30 minutes of reoxygenation at 21%O_2 and 5%CO_2 at 37˚C. To induce additional oxidative stress, cells were treated with 200μM H_2O_2 for 12 hours prior to reoxygenation, where indicated.

Mitochondrial ROS and Membrane Potential Quantification

MitoSox Red (Invitrogen) was used to assess mitochondrial O_2• production[7] and TMRE staining was employed as a marker of mitochondrial membrane potential. Cells were visualized by confocal microscopy and ROS levels were quantified by ImageJ software. Four fields per each sample were obtained and averaged. Nuclei were counterstained with Hoescht and subtracted from the total Mitosox fluorescence to exclude the dye which localized to the nucleus.

Assessment of Cell Death

Following hypoxia-reoxygenation, cells were collected by trypsinization and labeled with propidium iodine (Sigma-Aldrich, USA) and Alexa Fluor® 350-conjugated Annexin V (Molecular Probes, NY). Cell death was analyzed by flow cytometry in a FacsCanto flow cytometer (BD Bioscience).

Analysis of Calcium Transients

NRCM were cultured on glass cover slips followed by transfection with ABCB10 or control siRNA. Cells were loaded with 10μM fluo-4AM for 20 minutes. Intracellular Ca^{2+} transients were measured at 36^{o}C via linescan imaging using a Zeiss LSM510 confocal microscope. Fluorescence emission was measured through a 40x water immersion objective (Apochromat, NA 1.2) at wavelengths >520nm during excitation at 488nm with a 25mW argon laser (<10% laser power). Linescan imaging was recorded in spontaneously contracting cells and in cells paced at BCL=2000msec at equilibrium. Calcium transients were recorded from 10-15 cells on 4 separate coverslips in each group and F/F0, transient rise and decay times were analyzed using Zeiss LSM Examiner and ClampFit (Molecular Devices) software.

Human Samples

Tissue samples were obtained from the tissue bank at Feinberg Cardiovascular Research Institute (Northwestern University) and consisted of samples from non-failing (n=10) and failing ischemic (n=10) human hearts. Failing ischemic tissues were obtained from the explanted hearts of cardiac transplant recipients. Non-failing heart tissue samples were obtained from unmatched organ donors whose hearts were unsuitable for transplantation but had no known cardiac disease. Explanted hearts were immediately placed in cold cardioplegic solution and subsequently frozen in liquid nitrogen. Protocols for tissue procurement were approved by the Institutional Review Board of the Northwestern University. Informed consent

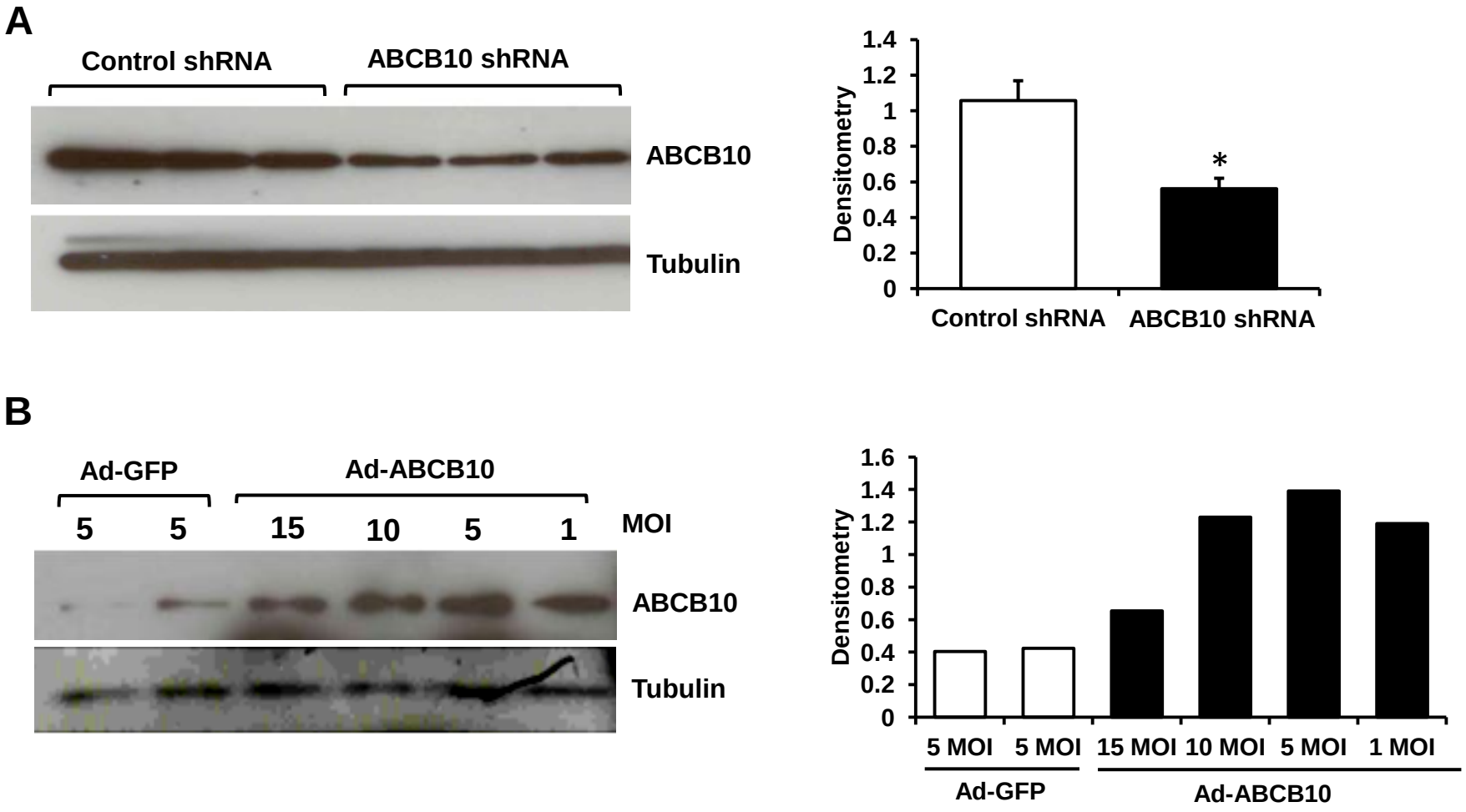
was obtained from all transplant patients and from the families of organ donors before tissue collection.

Myocardial Infarction and Tissue Harvesting

Myocardial infarction without reperfusion was induced in 10-12 week-old wild type C57 black mice by permanent ligation of the left coronary descending artery with a silk suture as previously described.[8] Anesthesia was induced with 2% isoflurane and maintained with 1.5% isolflurane throughout the procedure. Disruption of blood flow was confirmed by the pallor of left ventricle, as well as ST segment elevation and QRS complex widening on ECG, followed by chest closure in layers. In sham-operated animals the chest wall was surgically opened and closed without coronary ligation. Mice were allowed to recover for one week or one month for the MI group, and one month for the sham group. After that, mouse hearts were excised under anesthesia, washed in cold PBS and flash-frozen in liquid nitrogen. All animal studies were performed in accordance with the guidelines established by Northwestern University.

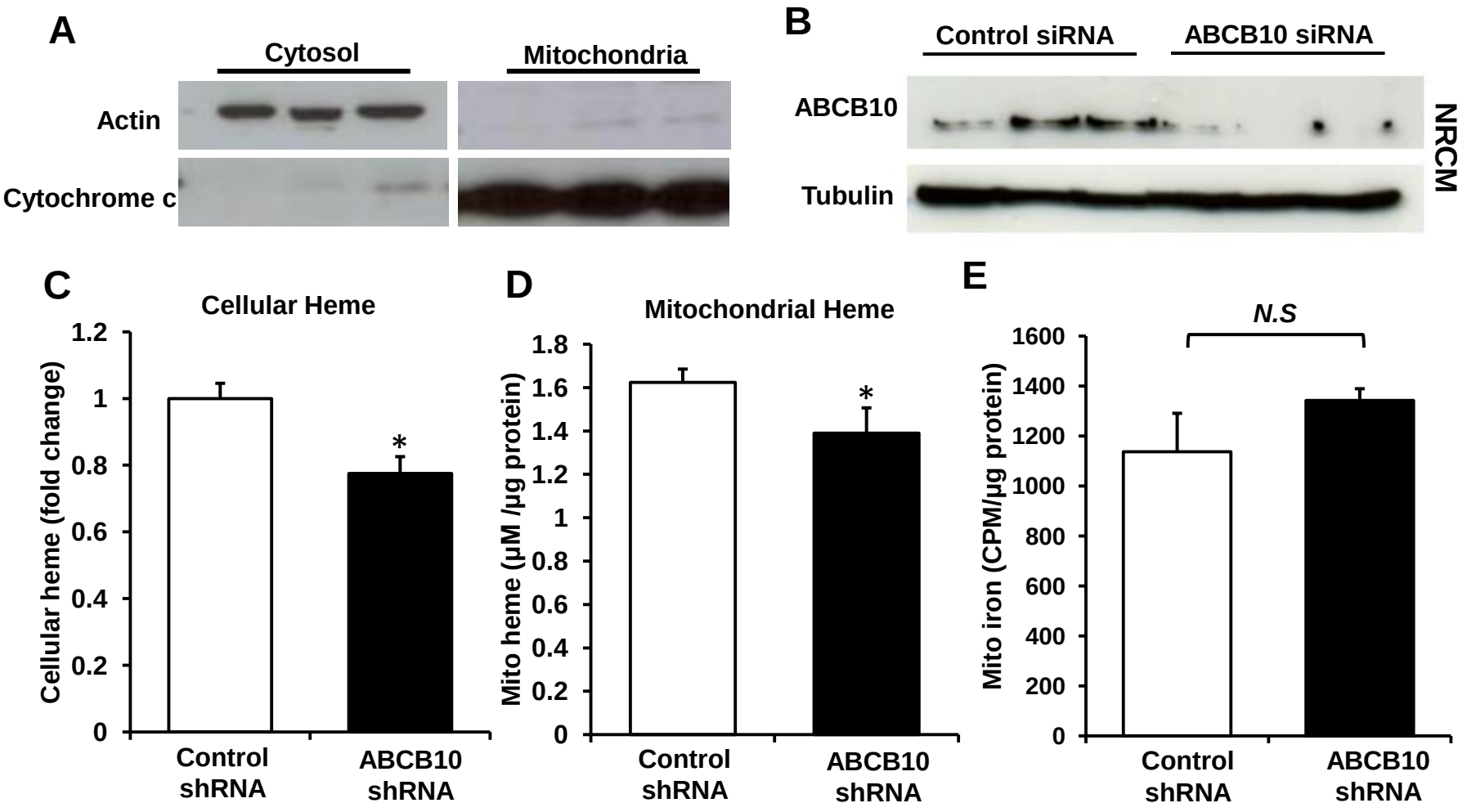
REFERENCES

1. Ardehali H, O'Rourke B, Marban E. Cardioprotective role of the mitochondrial ATP-binding cassette protein 1. *Circ Res.* 2005;97:740-742.
2. Chan CB, MacDonald PE, Saleh MC, Johns DC, Marban E, Wheeler MB. Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes.* 1999;48:1482-1486.
3. Johns DC, Nuss HB, Chiamvimonvat N, Ramza BM, Marban E, Lawrence JH. Adenovirus-mediated expression of a voltage-gated potassium channel in vitro (rat cardiac myocytes) and in vivo (rat liver). A novel strategy for modifying excitability. *J Clin Invest.* 1995;96:1152-1158.
4. Wingert RA, Galloway JL, Barut B, Foott H, Fraenkel P, Axe JL, Weber GJ, Dooley K, Davidson AJ, Schmid B, Paw BH, Shaw GC, Kingsley P, Palis J, Schubert H, Chen O, Kaplan J, Zon LI. Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature.* 2005;436:1035-1039.
5. Ward JH, Jordan I, Kushner JP, Kaplan J. Heme regulation of HeLa cell transferrin receptor number. *J Biol Chem.* 1984;259:13235-13240.
6. Sorte K PK, Goyal M, Singh AL, Basak A. Diagnosis Of Porphyria By Measuring Metabolites Of Heme Biosynthesis In Correlation With Clinical Findings. *Journal of Clinical and Diagnostic Research.* 2010;4:2013-2035.
7. Gordon LI, Burke MA, Singh AT, Prachand S, Lieberman ED, Sun L, Naik TJ, Prasad SV, Ardehali H. Blockade of the erbB2 receptor induces cardiomyocyte death through mitochondrial and reactive oxygen species-dependent pathways. *J Biol Chem.* 2009;284:2080-2087.
8. Wu R, Smeele KM, Wyatt E, Ichikawa Y, Eerbeek O, Sun L, Chawla K, Hollmann MW, Nagpal V, Heikkinen S, Laakso M, Jujo K, Wasserstrom JA, Zuurbier CJ, Ardehali H. Reduction in hexokinase II levels results in decreased cardiac function and altered remodeling after ischemia/reperfusion injury. *Circ Res.* 2011;108:60-69.



Online Figure I. Overexpression and Downregulation of ABCB10 in H9c2 cells

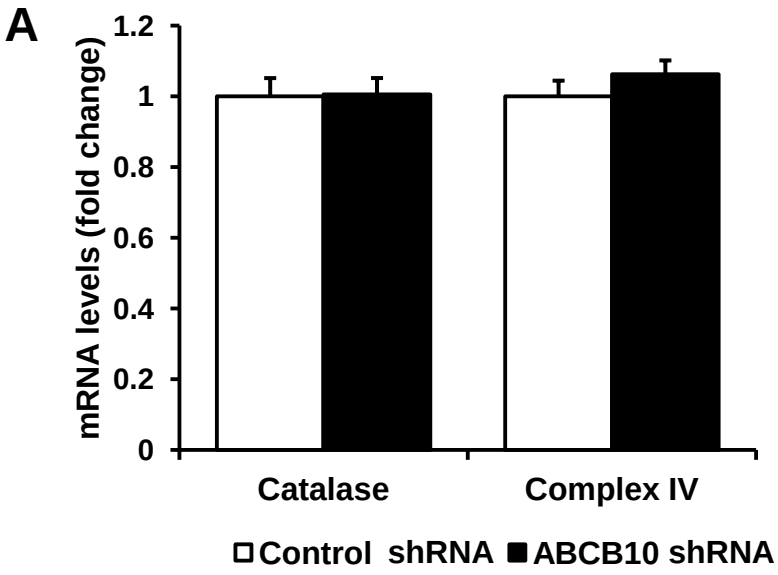
A, ABCB10 protein in H9c2 cells transduced with control non-silencing shRNA or ABCB10 shRNA lentivirus. Densitometry analysis of the Western blot is presented on the right. **B**, Adenoviral overexpression of GFP and ABCB10 using various multiplicities of infection (MOI). Densitometry analysis of the Western blot is presented on the right. 5MOI dose of each virus was used in all subsequent overexpression studies. Data are presented as mean ± SEM. * $p<0.05$ vs. control.

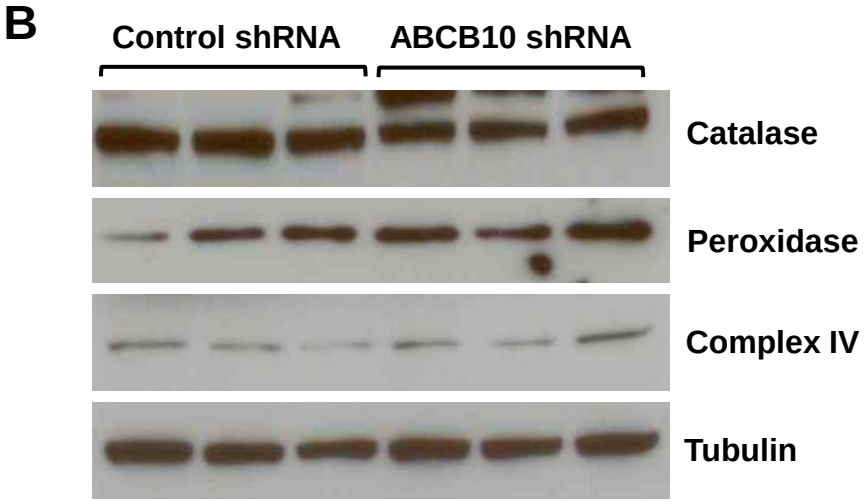


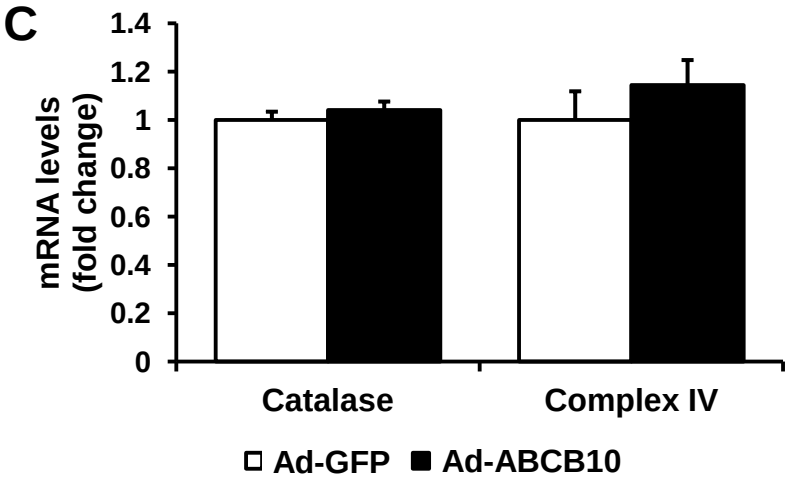
Online Figure II. ABCB10 knockdown reduces heme levels in NRCM

A, Enrichment of mitochondrial and cytosolic fractions isolated from H9c2 cells was evaluated by Western blot analysis of mitochondria- and cytosol-specific proteins, cytochrome c and actin, respectively. **B**, Western blot analysis of ABCB10 knockdown in NRCM with using shRNA-encoding lentivirus (n=3). **C,D**, Cellular (**C**) and mitochondrial (**D**) heme levels in NRCM with shRNA-mediated knockdown of ABCB10 (n=6). **E**, Mitochondrial ^{55}Fe content in H9c2 with ABCB10 knockdown (n=6). Mito, mitochondrial. Data are presented as mean ± SEM. * p≤0.05 vs. control.

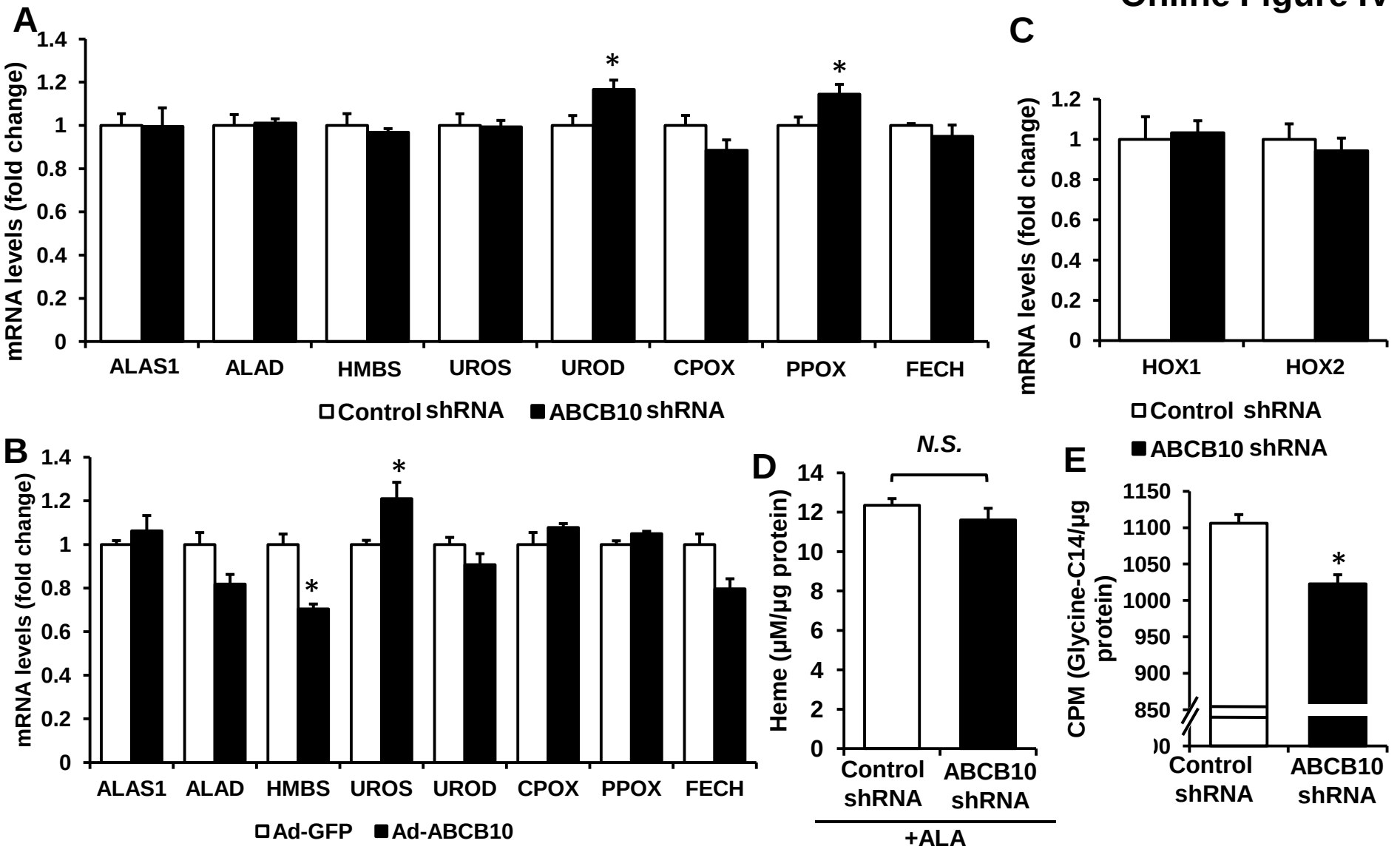
Online Figure III





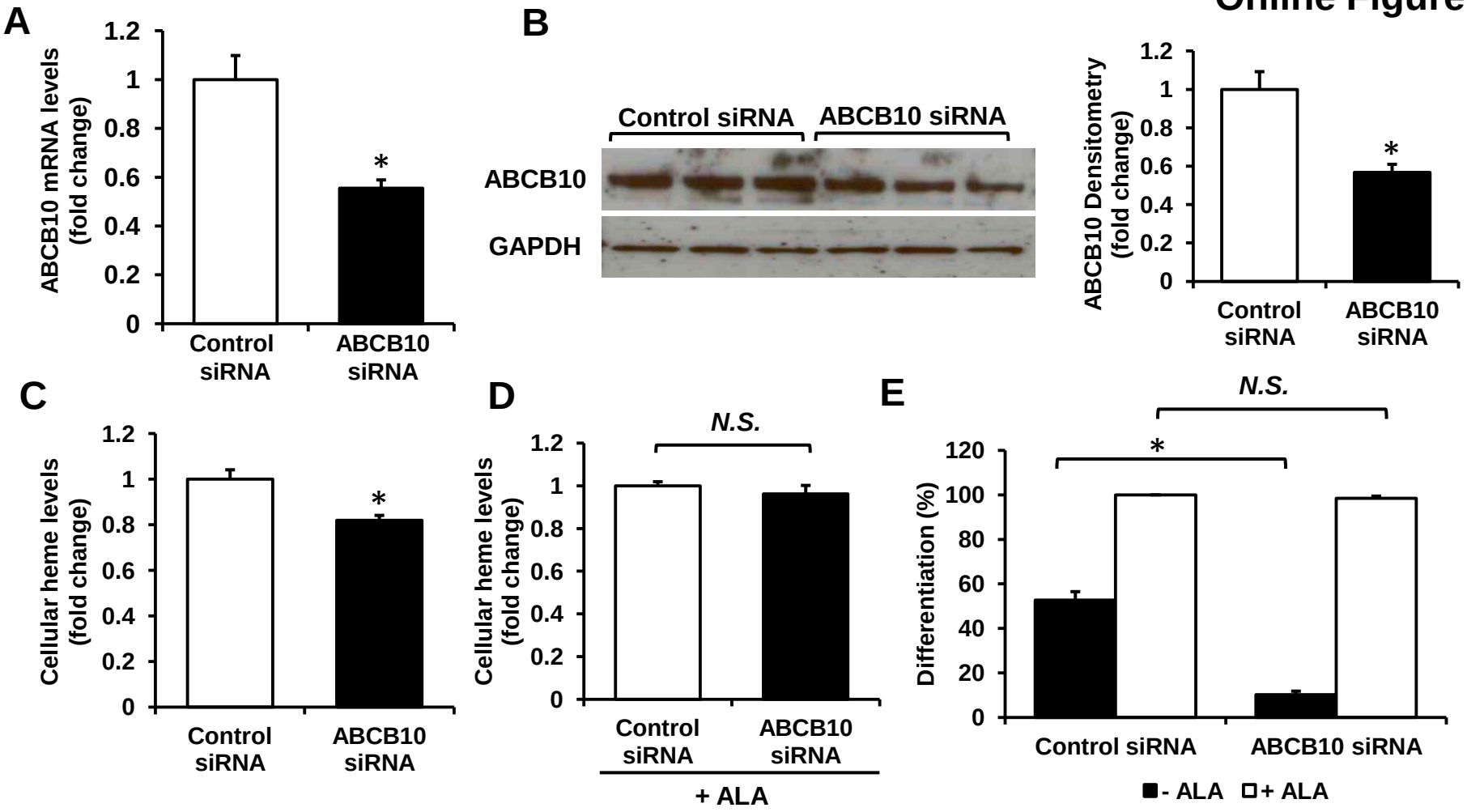


Online Figure III. Levels of heme containing enzymes are unaffected by ABCB10 modulation
A,**B** qRT-PCR analysis of mRNA levels (n=6) (**A**) and Western blot analysis of protein levels (n=3) (**B**) of heme-containing enzymes with ABCB10 knockdown. **C,** mRNA levels of heme-containing enzymes with ABCB10 overexpression (n=6). Data are presented as mean ± SEM.



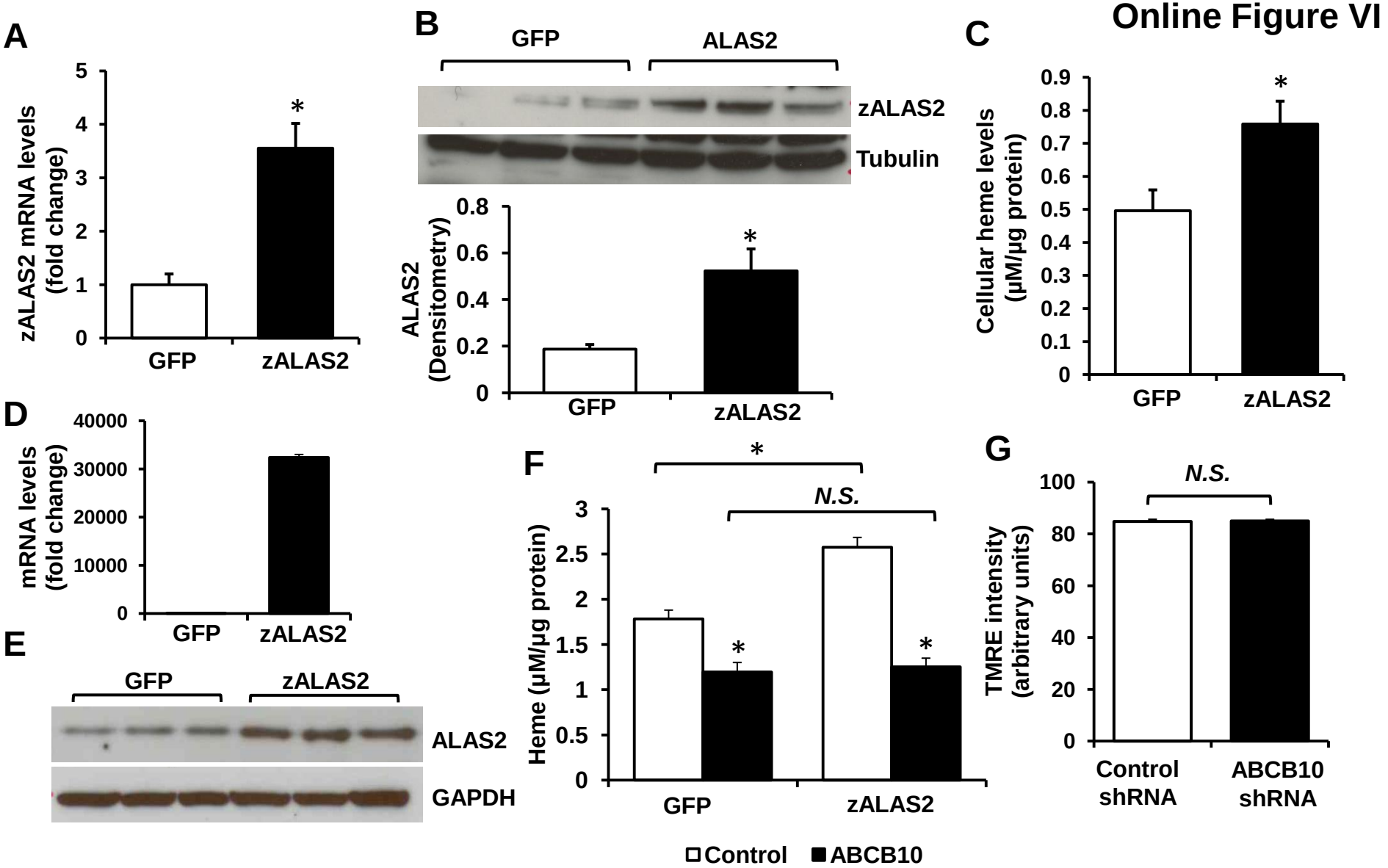
Online Figure IV. ABCB10 does not regulate heme synthesis or degradation

A,B, qRT-PCR analysis of mRNA levels of enzymes involved in heme synthesis in H9c2 with ABCB10 downregulation (**A**) and ABCB10 overexpression (**B**) (n=5-6). **C**, mRNA levels of heme oxygenases (HOX), enzymes involved in heme degradation, with ABCB10 shRNA (n=6). **D**, Cellular heme levels in NRCM supplemented with ALA with and without ABCB10 shRNA (n=6). **E,** Incorporation of C14-glycine into heme in H9c2 cells with and without ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * $p<0.05$ vs. control.



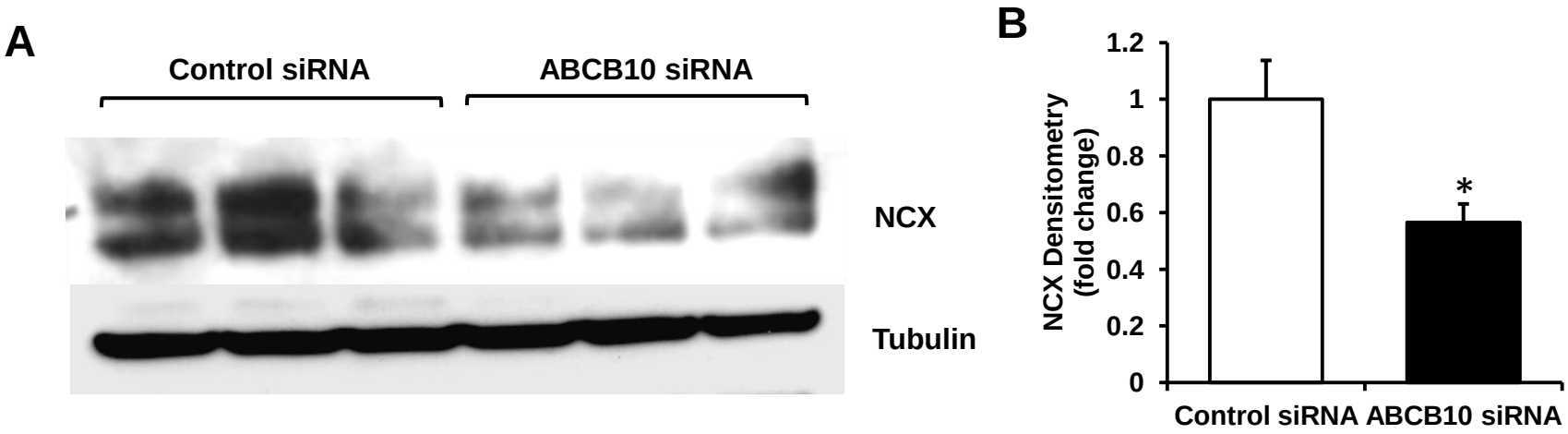
Online Figure V. ABCB10 knockdown and ALA supplementation in MEL cells

A,B, mRNA (**A**) and protein (**B**) levels of ABCB10 in MEL cells following ABCB10 knockdown at day 5 of differentiation. Densitometry analysis is shown on the right. **C**, Cellular heme levels with ABCB10 knockdown in MEL cells (n=6). **D**, Cellular heme levels in MEL cells with ABCB10 knockdown following ALA supplementation (n=6). **E**, Effects of ABCB10 knockdown with and without ALA supplementation of MEL cell differentiation (n=6). Data are presented as mean ± SEM. * $p<0.05$ vs. control.



Online Figure VI. zALAS2 overexpression increases cellular heme levels at baseline, but not with ABCB10 knockdown
A,B, mRNA (**A**) and protein (**B**) levels of zALAS2 in H9c2 cells transduced with zALAS2 or GFP lentivirus. Densitometry analysis is shown below the Western blot. **C**, Cellular heme levels with zALAS2 overexpression (n=6). **D,E,** mRNA (**D**) and protein (**E**) levels of zALAS2 in NRCM (n=3-6). **F,** Cellular heme levels with zALAS2 overexpression in NRCM with and without ABCB10 knockdown (n=6). **G,** Flow cytometry analysis of TMRE intensity as an indicator of mitochondrial membrane potential in H9c2 with ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * $p<0.05$ vs. control.

Online Figure VII



Online Figure VII. ABCB10 knockdown alters expression of a cellular calcium transporter
A, Western blot analysis of NCX expression in NRCM treated with ABCB10 or control siRNA . **B,** Densitometry analysis of the blot in A (n=3). Data are presented as mean ± SEM. * $p<0.05$ vs. control.