

Dimeric ferrochelatase bridges ABCB7 and ABCB10 homodimers in an architecturally defined molecular complex required for heme biosynthesis

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Supplemental Information

Supplemental Methods

ShRNA- and siRNA-mediated knockdowns of ABCB7, MFRN2 and IRP2 in HEK293T, HeLa or G1E-ER4 cells

The SMARTvector Inducible Lentiviral shRNA system from Dharmacon was used to generate four HEK293T and four HeLa stable cell clones with tightly controlled expression of three individual shRNAs targeting different regions of the *ABCB7* transcript (sh_*ABCB7*-1, -2 and -3) and a scramble shRNA, used as negative control (Cat: #VSC6847). The Tet-On 3G tetracycline-inducible expression system ensured minimal leakiness and potent activation of shRNAs expression upon exposure to doxycycline (0.1µg/ml). The presence of the TurboGFP reporter enabled to visually track expression of shRNAs. The shRNA sequences targeting the *ABCB7* transcript were:

Sh_*ABCB7*-1: V3SH11252-225074323 5'-GAATTCTGGGCTACCTTGC-3' (targets ORF);

Sh_*ABCB7*-2: V3SH11252-227670730 5'-ATAGCCTTAGATAAAGCTC-3' (targets ORF);

Sh_*ABCB7*-3: V3SH11252-230375344 5'-TACCTTACCCTGATCCAAG-3' (targets ORF)

The Trans-Lentiviral shRNA packaging kit (Dharmacon, Cat: #TLP5912) was used for packaging the lentiviral constructs, according to the manufacturer's instructions. HeLa or HEK293T cells at a starting confluence of 7.5×10^3 cells in 96-well plates were transduced at a low MOI (0.5 TU/cell) to generate a cell population with one integration event per cell. Puromycin (at a final concentration of 2µg/ml) was added 48 h after transduction to generate stable cell lines and the selection was carried out for 15 days.

The same protocol was followed in order to generate three HEK293T stable inducible cell lines which expressed shRNAs against the *MFRN2* transcript. The three *ABCB7*/*MFRN2* double knockdown cell lines were generated by transducing the HEK293T sh_*ABCB7*-1 stable cell line with one of the three pools of lentiviral particles carrying the constructs targeting the *MFRN2* transcript. The three shRNA sequences targeting the *MFRN2* transcript were:

Sh_*MFRN2*-1: V3SH11252-226008190 5'-GAACGTTTCATGGTCAGCTG-3' (targets ORF);

Sh_*MFRN2*-2: V3SH11252-226813786 5'-GGGTGGATTACATCACTCA-3' (targets 5'UTR and ORF);

Sh_*MFRN2*-3: V3SH11252-230447350 5'-TAATGTGTGAGTTCAAAGC-3' (targets ORF).

Knockdown of *Abcb7* or *Irp2* in G1E-ER4 cells was achieved by using the Accell siRNA delivery system (Dharmacon). Briefly, G1E-ER4 cells (5×10^6 per transfection) were transfected twice with 1µM siRNAs (Accell siRNAs work at higher concentrations than conventional siRNAs and do not require transfection reagent) at a 24-hour interval. On the day of the second transfection, cells were re-plated and induced to differentiate for the time points indicated in the different experiments. A pool of four Accell siRNAs (Cat: # EU-040745-00) targeting the ORF and the 3'-UTR of the *Abcb7* transcript was used to silence the expression of *Abcb7*. A pool of four Accell siRNAs (Cat: # EU-061938-00) targeting the ORF and the 3'-UTR of the *Irp2* transcript was used to silence the expression of *Irp2*. The Accell Non-Targeting Control siRNAs 1 (Cat: # D-001910-01), 2 (Cat: # D-001910-02), 3 (Cat: # D-001910-03), and 4 (Cat: # D-001910-04) were pooled

together and used as negative control for non-sequence-specific effects, as recommended by the manufacturer's protocol.

In vitro and in vivo crosslinking and mass spectrometry

A homo-bifunctional crosslinker, BS³, was used in vitro, and DSS, which is the membrane-permeable analog of BS³, was used in vivo. BS³ and DSS react with primary amines present in lysine side chains and N termini of proteins.

Immunoprecipitation (IP) experiments

Immunoprecipitations in vivo were done on mitochondrial fractions from G1E-ER4 cells co-expressing FLAG-tagged ABCB7 and HA-tagged FECH, three days after knock down of endogenous Abcb7 and 48h after induction of differentiation. Anti-FLAG immunoprecipitations were performed using M2-FLAG beads (Sigma). Lysates from mitochondrial fractions were incubated with M2 beads for 2h at RT, and then washed extensively with lysis buffer I: 25mM Tris, 0.15M NaCl, 1mM EDTA, 1% NP-40, 5% glycerol (pH 7.4), protease and phosphatase inhibitor cocktail with no EDTA (Roche). Bound proteins were eluted with Tris-Glycine pH 2.8 for 10 min at 4°C. Aliquots corresponding to 10% of mitochondrial lysates were run alongside the IP fractions onto the gels as inputs.

IP of endogenous Abcb7 was done by covalently coupling anti-Abcb7 antibody produced in rabbit (Abcam, Cat: # ab151992) onto an amine-reactive resin (Pierce Co-IP kit, Cat. No. 26149), following the manufacturer's instructions. IP with anti-rabbit IgG was used as negative control. Lysates from crude mitochondrial fractions were incubated with the beads overnight at 4 °C, and then washed extensively with lysis buffer I. Bound proteins were eluted with Tris-Glycine pH 2.8 for 10 min at 4 °C. Aliquots corresponding to 15% of mitochondrial lysates were run alongside the IP fractions onto the gels as inputs.

In vitro coupled transcription/translation and pull-down assay of ³⁵S-labeled proteins

The TNT Quick transcription/translation system (Promega), which couples transcription/translation reactions for in vitro synthesis of eukaryotic proteins starting with plasmid DNA as a template (1µg of DNA/reaction), was used to synthesize ³⁵S-labeled proteins for pull-down assays. 20 µCi of [³⁵S]-methionine/cysteine (EasyTag™ EXPRESS35S Protein Labeling Mix stabilized aqueous solution from PerkinElmer) were added to the reaction mix and the incubation was performed at 30 °C for 3-5 hours. Pull down assays of ³⁵S-labeled FLAG-tagged ABCB7 wild type or mutated proteins were carried out in the presence of the ³⁵S-labeled FECH-HA. Binding was performed in buffer I2 (25 mM TrisHCl; 300 mM NaCl; 1 mM EDTA; 1% NP-40; 1% DTT; 1 mM PMSF; 5% glycerol) for 2h at RT, and immunoprecipitations were carried out with anti-FLAG antibody to immunocapture FLAG-tagged ABCB7. The presence of co-eluted FECH-HA was analyzed by SDS-PAGE and autoradiogram. Aliquots corresponding to 30% of the inputs were run on the gel for comparison.

DPYD activity assay

The dihydropyrimidine dehydrogenase (DPYD) activity was determined by thin layer chromatography (TLC), as previously reported(1). Briefly, cell lysates containing 150 µg of proteins isolated from control or ABCB7 HEK293T or HeLa KD cells lines, as specified in the main text and figure legends, were applied to 50 µl of a reaction mix containing 25 mM Tris-HCl

(pH 7.5), 0.1% digitonin, 2.5 mM MgCl₂, 2mM DTT, 10 μM [4- ¹⁴C]-thymine (0.1mCi/ml Moravsek Inc. CA, USA), 10 mM NADPH. After 4 hours of incubation at 32 °C, the reaction was stopped by addition of 10 μl of perchloric acid (10% v/v). Reaction mixtures were centrifuged at 20000 x g for 5 minutes and the supernatants analyzed by TLC.

Native PAGE (BN-PAGE) and native immunoblots

The NativePAGE Novex Bis-Tris gel system (Thermo Scientific) was used to analyze native membrane protein complexes, with the following modifications: only the Light Blue Cathode Buffer was used; 20 μg of membrane protein extracts were loaded per well; the electrophoresis was performed at 150 V for 1 h, followed by 3.5 h at 250 V.

For the native IB, PVDF was used as the blotting membrane. The transfer was performed at 25 V for 4 h at 4°C. After transfer, the membrane was washed with 8% acetic acid for 20 min to fix the proteins, and then rinsed with water before air-drying. The dried membrane was washed 5-6 times with methanol (to remove residual Coomassie Blue G-250), rinsed with water and then blocked for 2h at room temperature in 5% milk, before incubating overnight at 4°C with the desired antibodies diluted in 2.5% milk.

Formation of the hexameric ABCB7-FECH-ABCB10 complex with purified proteins by native PAGE was assessed by mixing 150ng of ABCB7-FLAG purified from HEK293T cells and resuspended in buffer II (50 mM BisTris, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S, 1% Lauryl maltoside, pH 7.2, protease and phosphatase inhibitors), supplemented with 5% digitonin, with equivalent amounts of purified FECH-HA and ABCB10-Myc. The mixture was incubated at 4°C for 30 minutes and subsequently loaded on Bis-Tris native gels after addition of 1% G250 sample additive.

Two-Dimensional Native/ SDS-PAGE

Two-dimensional Native/SDS-PAGE was performed by resolving the mitochondrial protein complexes in the first dimension by BN-PAGE, as previously described(2-4). Each lane of the gel was excised, equilibrated in SDS buffer supplemented with reducing agent, and then immersed in the alkylating solution for 15 min, before quenching for additional 15 min. For the second dimension, the gel strip was fixed horizontally onto the NuPAGE 4-12% Bis-Tris Zoom Gel (Thermo Fisher Scientific), and classical SDS immunoblots were performed.

Complex I, II and IV in-gel activity assays

In-gel Complex I, II and Complex IV activities were performed as previously described(2-4). For Complex I activity, after resolution of the respiratory complexes by BN-PAGE, the gel was incubated with 0.1 M TrisCl, pH 7.4, containing 1 mg/ml nitrobluetetrazolium chloride (NBT) and 0.14 mM NADH at room temperature for 30-60 min. For complex II, detection of succinate CoQ-reductase activity (SQR) (CoQ-mediated NBT reduction) was performed by incubating the gel for 30 minutes with 84 mM succinate, 2 mg/ml NBT, 4.5 mM EDTA, 10 mM KCN, 1 mM sodium azide and 10 μM ubiquinone in 50 mM PBS, pH 7.4. For complex IV, the gel was incubated in 50 mM phosphate buffer pH 7.4 containing 1mg/ml DAB (3,3'-diaminobenzidine) and 1mg/ml cytochrome c at room temperature for 30-45 min.

Iron incorporation assay

The ^{55}Fe incorporation assays were performed essentially as previously described(2, 4), with minor modifications. For ^{55}Fe incorporation into NUBP2-V5, control and ABCB7-KD HEK293T cell lines were grown in the presence of $1\ \mu\text{M}$ ^{55}Fe -Tf for five days and subsequently transfected with NUBP2-V5. Cytosolic extracts were subjected to immunoprecipitation with anti-V5 to isolate NUBP2-V5 and radioactive iron incorporation was measured by scintillation counter, after extensive washings of the beads with buffer I: 25mM Tris, 0.15M NaCl, 1mM EDTA, 1% NP-40, 5% glycerol (pH 7.4), protease and phosphatase inhibitor cocktail with no EDTA (Roche). The background, corresponding to ^{55}Fe measurements of eluates after anti-V5 immunoprecipitations on cytosolic extracts from cells transfected with the empty vector was subtracted from each reading.

For assessment of ^{55}Fe incorporated into heteropolymeric ferritin (Ft) and into hemoglobin (Hb), G1E-ER4 cells radiolabeled with $2\ \mu\text{M}$ ^{55}Fe -laden transferrin were lysed in buffer II (50 mM BisTris, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S, 1% Lauryl maltoside, pH 7.2, protease and phosphatase inhibitors). Equal amounts of proteins were separated by BN-PAGE. Gels were dried, followed by autoradiography.

Labile iron pool (L.I.P.) measurement

Labile iron pool (LIP) was measured as previously described(5) with the following modifications. Briefly, 10^6 control or Abcb7-KD G1E-ER4 cells were washed twice with PBS, and incubated with 250 nM calcein/AM in 154 mM NaCl, 20 mM HEPES, 5 mM glucose, pH 7.4 with or without 100 μM of the cell-permeable iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) for 20 min at 37°C under constant agitation. After washing twice with cold PBS, fluorescence was measured by flow cytometry on FITC channel with FACSAria flow cytometer on 100,000 counts. The LIP was calculated by subtracting the MFI (mean fluorescence intensity) of the samples without SIH from the MFI of the corresponding samples treated with SIH.

Heme content measurements in G1E-ER4 cells

Heme content in cell lysates was measured using the QuantiChrom Heme Assay Kit (BioAssay Systems) according to the manufacturer's instructions.

May-Grünwald-Giemsa staining

Control or Abcb7-KD G1E-ER4 cells (5,000 cells) before or after 72h in differentiation medium, were resuspended in sterile Dulbecco's PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free), cytospun onto a slide with a Cytospin centrifuge and stained with May-Grünwald and Giemsa buffers (Sigma), according to manufacturer's protocol.

Flow cytometry analyses and cell sorting

To assess the cell membrane expression levels of Tfrc, control and Abcb7-KD G1E-ER4 cells (10^6 cells) were washed with sterile Dulbecco's PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free) and then incubated with FITC-CD71 antibody (TFRC, BD Biosciences, Cat: #553266) and 7-AAD (7-aminoactinomycin D, BD Biosciences, Cat: #559925). A gate was applied to sort 7-AAD negative and FITC-CD71 positive cells.

In order to analyze apoptosis and mitochondrial reactive oxygen species production in control and Abcb7-KD cells, 10^6 cells were stained with PE-Mitosox (Invitrogen, Cat: #M36008), FITC-Annexin V (BD Biosciences, Cat: #556547) and 7-AAD. Cells were incubated for 15 min with PE-Mitosox at 37 °C and subsequently with 7-AAD and anti-FITC-Annexin V for 20 min at 4°C under constant agitation. Single cells were first gated into 7-AAD negative cells, and then gated into Annexin V and Mitosox positive cells. For analysis of Annexin V and Mitosox positive cell population, 7-AAD negative cells were plotted by combination of Annexin V and Mitosox.

Total cellular and mitochondrial iron measurements

Iron content in whole cells or in isolated mitochondria was measured by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7900 ICP-MS. 200µL of concentrated trace-metal-grade nitric acid (Fisher) were added to each sample (5×10^6 cells/sample) taken in a 15ml Falcon tube. Tubes were sealed with electrical tape to prevent evaporation, taken inside a 1L glass beaker and digested overnight in a 90 °C oven. Samples were then diluted with 4ml deionized water and analyzed by ICP-MS.

Superoxide dismutase (SOD) activity assays

SOD activity assays were carried out as previously described(6). Control or ABCB7-KD cells were lysed in 10mM TrisHCl pH 7.4, 10mM NaCl, 3mM MgCl₂, 0.5%NP-40 for 10 min on ice. Lysates were centrifuged at 21,000 x g for 10 min and the supernatants transferred to new tubes. 6µg of whole cell extracts in 50% glycerol were loaded on a 10% native gel, and SOD1/2 enzymes separated at 100 V for 60 min. The activity was developed by first soaking the gel in 5ml riboflavin/NBT solution for 15 min in the dark and subsequently in 0.1% TEMED at RT for 15 min in the dark, followed by exposure to the light until the white SOD bands became visible on the blue-purple background color of the gel (15-20 minutes).

Aconitase in-gel assay and electrophoretic mobility shift assay

Aconitase activities were assessed by a coupled assay after native PAGE separation, as described previously(7). IRPs-IRE binding activities were determined by electrophoretic mobility shift assay using a ³²P-labeled ferritin IRE probe, as described previously(7).

Metabolic labeling and pulse-chase

The main steps of the protocol for metabolic labeling of G1E-ER4 cells used in this study are as follows: cells were washed once in labeling medium (DMEM without L-cysteine and L-methionine) and incubated in labeling medium for 1 hour, followed by metabolic labeling with the EXPRESS 35S-cysteine and 35S-methionine reagent (1175 Ci/mmol; PerkinElmer) for 30 min. Cells were subsequently washed with complete IMDM medium and either immediately frozen (time 0h), or washed twice and chased in treatment medium for the indicated time points. Cell pellets were fractionated into cytosol and crude mitochondria, and the mitochondrial lysates in buffer I were subjected to immunoprecipitation essentially as previously described(4). 500 µg of mitochondrial proteins were incubated for 3h at RT with 1µg of anti-MFRN1 antibody (Proteintech, 26469-1-AP) or with 1µg of anti-FECH antibody (Proteintech, 14466-1-AP) covalently conjugated to an amine-reactive resin (Pierce Co-IP kit, Cat. No. 26149). After extensive washing of the beads with buffer I, immunoprecipitated radiolabeled Mfrn1 or Fech was

eluted under acidic conditions and visualized by SDS-PAGE, followed by autoradiography with Carestream Biomax MR single emulsion films.

Expression of human ABCB7-FLAG and FECH-HA in G1E-ER4 cells and alanine scanning mutagenesis of ABCB7-FLAG

G1E-ER4 cells were transduced with viral particles, generated with the ViraPower Lentiviral Expression System (Invitrogen), harboring the *ABCB7* ORF under the control of a CMV promoter into pLENTI6/FLAG-DEST (Invitrogen) and/or the *FECH* ORF into pLenti6/HA-DEST. Briefly, pLENTI6/ABCB7-FLAG (or pLENTI6/FECH-HA) was co-transfected with the ViraPower Packaging Mix into HEK293T cells. The lentiviral stock collected 36 hours after cotransfection was used to transduce G1E-ER4 cells.

Alanine scanning mutagenesis of the C terminus of human FLAG-tagged ABCB7 was performed using the QuikChange II site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions.

Cell extracts and fractionation

Whole cell lysates were prepared in lysis buffer I: 25 mM Tris, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol pH 7.4, protease and phosphatase inhibitors. Cellular fractionation into cytosol and intact mitochondria was done as previously described(4). Briefly, cytosolic fractions were isolated after permeabilization with a buffer containing 0.1% digitonin in 210 mM mannitol, 20 mM sucrose and 4 mM HEPES. The supernatants after the first centrifugation step at 700 x g for 5 minutes were subjected to 20000 x g for 15 minutes. The supernatants after the second centrifugation step were saved as cytosolic (soluble) fractions. The crude preparation of mitochondria isolated by differential centrifugation was lysed in lysis buffer II containing 50 mM BisTris, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S, 1% Lauryl maltoside, pH 7.2, protease and phosphatase inhibitors.

Peroxisome isolation

Peroxisome isolation of control or ABCB7-KD G1E-ER4, HEK293T or HeLa cells was performed by using the peroxisome isolation kit from Sigma (Cat: #PEROX1), following the manufacturer's instructions.

Catalase activity

Catalase activity was performed on peroxisomal lysates using the catalase assay kit from Abcam (Cat: #ab83464), following the manufacturer's instructions.

Purification of human ABCB7-FLAG, ABCB10-Myc and FECH-HA proteins from HEK293T mitochondrial lysates for in vitro binding experiments and crosslinking

HEK293T cells cultured in 150mm dishes to 70% confluence were transfected with the appropriate construct encoding human ABCB7-FLAG (in pcDNA3.1-FLAG, Thermo Scientific), human ABCB10-Myc (in pCMV6-Myc, Origene) or human FECH-HA (in pcDNA3.1-HA, Thermo Scientific). Cells were harvested 24 h after transfection by scraping with 1x PBS, and mitochondria were isolated as previously described(4). Isolated mitochondria were solubilized in

buffer A (1x PBS, 0.2% Triton) containing EDTA-free complete protease inhibitor cocktail for 30 min at 4°C under gentle shaking. Soluble and insoluble fractions were separated by ultracentrifugation (100,000 x g for 30 min at 4°C), and the supernatant was incubated with anti-M2- (FLAG), Myc- or HA- beads overnight at 4°C with gentle agitation. Beads were washed twice with Buffer A, twice with buffer B (10 mM Tris, 0.2% Triton), and 5 times with Hepes buffer (Hepes, EGTA, 1% n-octyl-beta-D-glucopyranoside). Bound proteins were eluted with elution buffer (Hepes, EGTA, 1% n-octyl-beta-D-glucopyranoside, 0.5 mg of FLAG, Myc or HA peptide). The eluted proteins were identified by using anti-ABCB7, ABCB10 or FECH specific antibodies, and by anti-FLAG, Myc or HA epitope specific antibodies.

Real-time PCR

RNA was isolated using the RNeasy kit from QIAGEN. Reverse-transcription was performed on total RNA using Superscript III (Invitrogen), and random hexamers. Real-time PCR on complementary DNA was performed with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI StepOne Plus instrument. Relative transcript abundance was calculated using the $2^{-\Delta\Delta C_t}$ method, with beta-actin (ACTB) as the internal control. The non-transformed $2^{-\Delta\Delta C_t}$ values were analyzed by one-way ANOVA with Holm-Sidak post-hoc test to determine statistical significance. Values are given as mean \pm SEM. The table lists the primers used in RT-PCR:

Gene	Forward primers	Reverse primers
<i>Abcb7</i>	CTGCTCGCGATACATTCCTG	TCAGAAGTCCGAATGCTTG
<i>Abcb10</i>	AATGGAGCAGAGCAGTTCCT	TCACAGCCCTGCTTCAGTTA
<i>Alas2</i>	ACTCAGCTCTGCTCTTCTCC	GCATGATTGCCTGCATCTGA
<i>Fech</i>	GAGACGCTCTACGAAGTGGGA	CAGGGCCTTGGAGAACAATG
<i>Hba-a2</i>	CTTCAAGGTATGCGCTGGGA	CTGCGGAGACAAAGTGGACA
<i>Hbb-b1</i>	AGGTGAACGCCGATGAAGTT	ACTTTCTTGCCATGGGCCTT
<i>Irp2</i>	CTGGAATACCGGCAATGGTG	CCACTGTGAGATCTGTCCGGA
<i>Irp1</i>	CTAGTGTCAGCGCAAAGGAC	AGCAGAATAAGGCACACCCT
<i>Tfrc</i>	GCGCTTCCTAGTACTCCCTT	CTGCAGCCAGTTTCATCTCC
<i>Slc25a37</i>	CAGCTATTGGTGCTTCTGGC	AAAGTGGGAACCTCCTCCCC
<i>Sod1</i>	GTGCAGGGAACCATCCACTT	CTGATGGACGTGGAACCCAT
<i>Sod2</i>	GTAGGGCCTGTCCGATGATG	CGCTACTGAGAAAGGTGCCA
<i>Actb</i>	GCCACTGCCGCA TCCTCTTC	AGCCTCAGGGCA TCGGAACC

RT² Profiler PCR array Gene Expression

The RT² Profiler PCR Gene Expression Arrays (QIAGEN) combine the performance of real-time PCR with a 96-well microarray format to detect the expression of 84 genes simultaneously along with several internal controls. The RNA was isolated from control and Abcb7-KD G1E-ER4 cells using the miRNeasy Mini Kit (QIAGEN, Cat: #217004), according to the manufacturer's instructions. RNA quality was determined using a spectrophotometer and on agarose gel, and was reverse transcribed using the RT² first strand kit (QIAGEN). The cDNA was used on the real-time RT² Profiler PCR Array Mouse Mitochondrial Energy Metabolism (QIAGEN, Cat: #PAMM-008Z) and on the real-time RT² Profiler PCR Array Mouse oxidative stress and antioxidant response (QIAGEN, Cat: #PAMM-065Z) in combination with RT² SYBR®

Green qPCR Mastermix (Cat: #330529) on an ABI StepOne Plus PCR system (Thermo Scientific). The data from three independent experiments were analyzed using GeneGlobe Data Analysis Center.

Holo-cytochrome c detection

Holo-cytochrome c detection was performed using the enhanced chemiluminescence (ECL) method, as previously described(8) on 10 µg of mitochondrial proteins.

Antibodies in this study

Antibodies in this study were as follows: anti-CIAO1 (sc-374498), TOM20 (sc-136211) and DPYD (sc-376681) were from Santa Cruz Biotechnology. Anti- NUBP2 (NBP1-84533) and SUCLG2 (NBP1-32521) were from Novus Biologicals. Anti- HSPA9 (HPA000898), tubulin (T9026), MFRN2 (SAB2701516), CS (SAB2701077), FLAG (F1804), HA (H9658), Ferritin (F6136) and β-actin (A2228) were from Sigma. Anti-MMS19 (16015-1-AP), POLD1 (15646-1-AP), CIAPIN1 (12638-1-AP), SOD1 (10269-1-AP), SOD2 (66474-1-Ig), CYCS (10993-1-AP), CYC1 (10242-1-AP), CAT (66765-1-Ig), PMP70 (66697-1-Ig), KDELR (27632-1-AP), HBA-A2 (14537-1-AP), HBB-B1 (16216-1-AP), MFRN1 (26469-1-AP), SUCLA2 (12627-1-AP), FECH (14466-1-AP) and GLRX3 (11254-1-AP) were from Proteintech. Anti-PPAT (PA5-12333) and TFRC (13-6800) were from Thermo Scientific. Anti-GLRX3 (H00010539-M01) was from Abnova. Anti-SDHB (ab14714), SDHA (ab14715), NDUFS1 (ab169540), NDUFS8 (ab170936), MTCO1 (ab14705), UQCRC2 (ab14745), VDAC1 (ab15895), total OXPHOS (ab110411r, Complex V, ATP5A subunit; Complex IV, COXII subunit; Complex III, UQCRC2 subunit; Complex II, SDHB; Complex I, NDUFB9 subunit), ATP5A1 (ab110273), ABCB7 (ab151992), ABCB10 (ab111483), MT-CYB (ab81215), ABCE1 (ab185548), FTH (ab65080), LIAS (ab96302), CPOX (ab169766), ALAD (ab151754), BACH1 (ab180853), ALAS2 (ab184964), CLPP (ab236064), CLPX (ab168338), GLRX5 (ab86411), FAM96B (ab166607), ELP3 (ab190907) and ERCC2 (ab54676) were from Abcam. Anti-ACO2 rabbit polyclonal antibody was raised against the synthetic peptide YDLLEKNINIVRKRLNR. Anti-lipoate rabbit polyclonal antibody was from EMD Millipore. Anti-IRP1 antibody was prepared against purified human IRP1 and used at 1:5000 dilution. Anti-IRP2 antibody was prepared against a peptide covering the amino acid residues 137–209 of human IRP2 and used at 1:2000 dilution. Anti-MFRN1/2 antibody was a kind gift of Dr. Barry Paw(9). All antibodies were diluted at 1:1000 in 2.5% milk, unless differently specified.

In vitro and in vivo crosslinking and mass spectrometry

A homo-bifunctional crosslinker, BS³, was used in vitro, and DSS, which is the membrane-permeable analog of BS³, was used in vivo. BS³ and DSS react with primary amines present in lysine side chains and N termini of proteins.

The chemical crosslinking reaction with BS³ in vitro was started by mixing 0.5mg/mL of purified wild type or mutated ABCB7-FLAG proteins with 0.5mg/mL of purified FECH-HA to analyze formation of the ABCB7/FECH complex, and by mixing 0.5mg/mL of purified ABCB10-Myc with 0.5mg/mL of purified FECH-HA to investigate formation of the ABCB10/FECH complex, in crosslinking buffer (20mM HEPES, pH 7.5) with 1mM BS³ (Thermo Scientific). The reaction was incubated for 30 min at RT and subsequently quenched by addition of 1M TrisHCl,

pH 7.5 (to achieve a final concentration of 20mM TrisHCl). The crosslinked products were analyzed by SDS-PAGE.

The crosslinking reaction *in vivo* using DSS (Thermo Scientific) was performed on mitochondria isolated from G1E-ER4 cells expressing human FLAG-tagged ABCB7 and human HA-tagged FECH, differentiated for 48h. Mitochondria at 5 μ g/ μ L protein concentration (total proteins 1mg) were incubated with 0.5mM DSS in crosslinking buffer (20mM HEPES, pH 7.5) for 2h at 4°C and the reaction was quenched by addition of 1M TrisHCl, pH 7.5. Mitochondria were lysed in buffer I and a Zeba column (Thermo Scientific) was used for buffer exchange with 20mM HEPES and for removal of the excess crosslinker. ABCB7-FLAG and the bound interacting partners were immunoprecipitated with anti-FLAG beads and the eluted complexes analyzed by mass spectrometry performed by Creative Proteomics (Shirley, NY). Proteins were reduced/alkylated and digested by limited proteolysis with trypsin and endoproteinase GluC, following standard protocols. The excess of proteases solution was removed and replaced with 50 mM ammonium bicarbonate solution. Samples were incubated at 37°C overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. Extracted peptides were then dried in a speed-vac (~1 hr) and stored at 4°C until analysis. On the day of analysis, samples were reconstituted in 10 μ l of 0.1% formic acid for injection onto a LTQ Orbitrap XL. The peptides were loaded onto an analytical column of 75 μ m inner diameter with 14 cm C18 resin (Thermo Scientific). Spectra were collected at a resolution of 100,000. The raw data were processed using Mascot, and the StavroX software(10) was used to generate the list of crosslinked peptides. The false discovery rate was set to 3%. Table S1 includes the scores assigned by StavroX to each crosslinked peptide based on the comparison between the theoretical fragmentation and the actual spectrum of the crosslinked peptide.

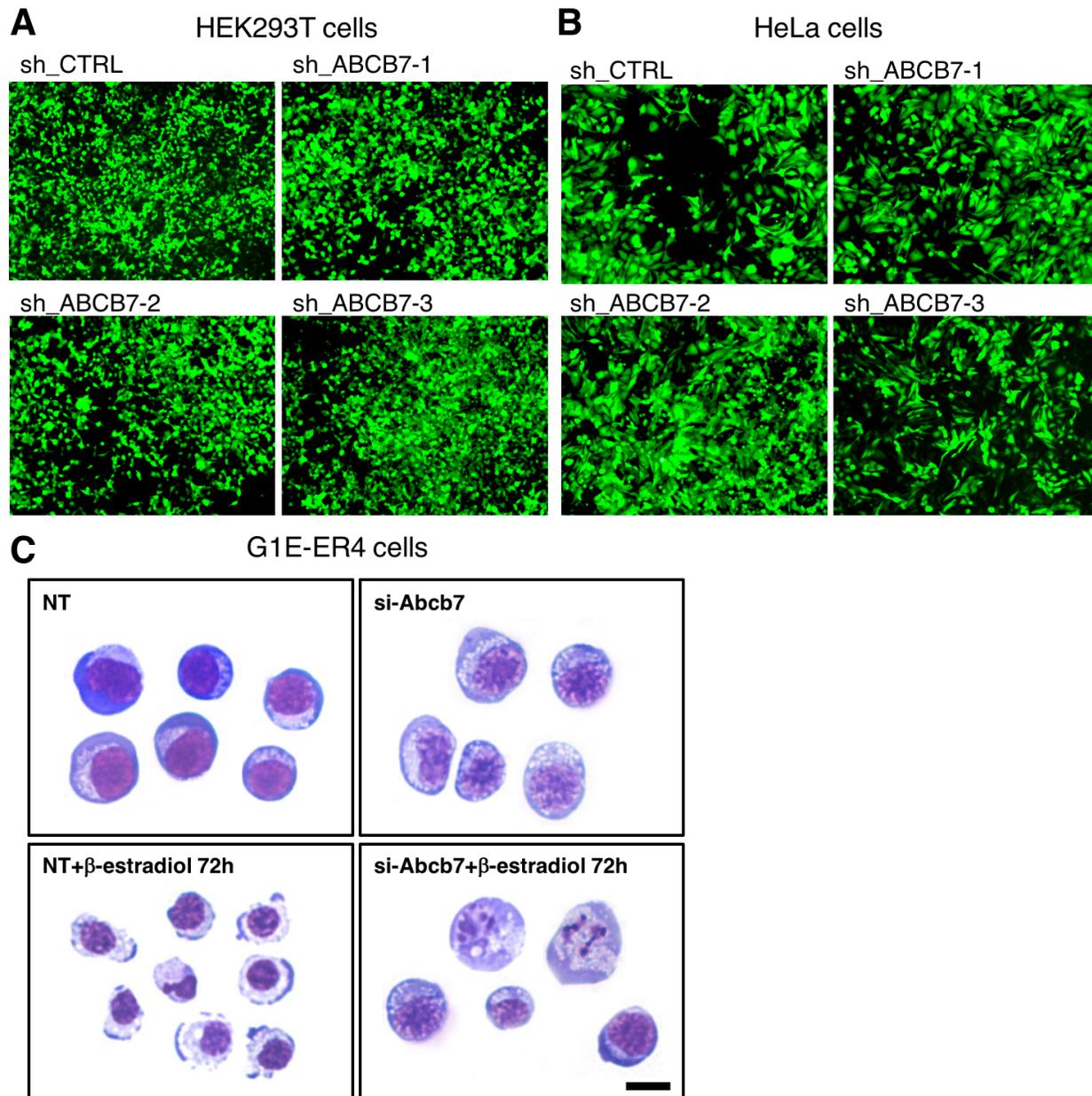
Statistical Analyses and Densitometries of immunoblots

Where applicable, data were expressed as the mean \pm SD or \pm SEM, as indicated in the figure legends. Pairwise comparisons between two groups were analyzed using the unpaired Student's t test. Analyses of multiple groups were performed using two-way ANOVA. *p* values of less than 0.05 were considered significant.

Densitometries of immunoblots were performed with ImageJ and the results were normalized against the appropriate internal controls, as indicated in the legends of Figures S13-S17. Values are means \pm SEM.

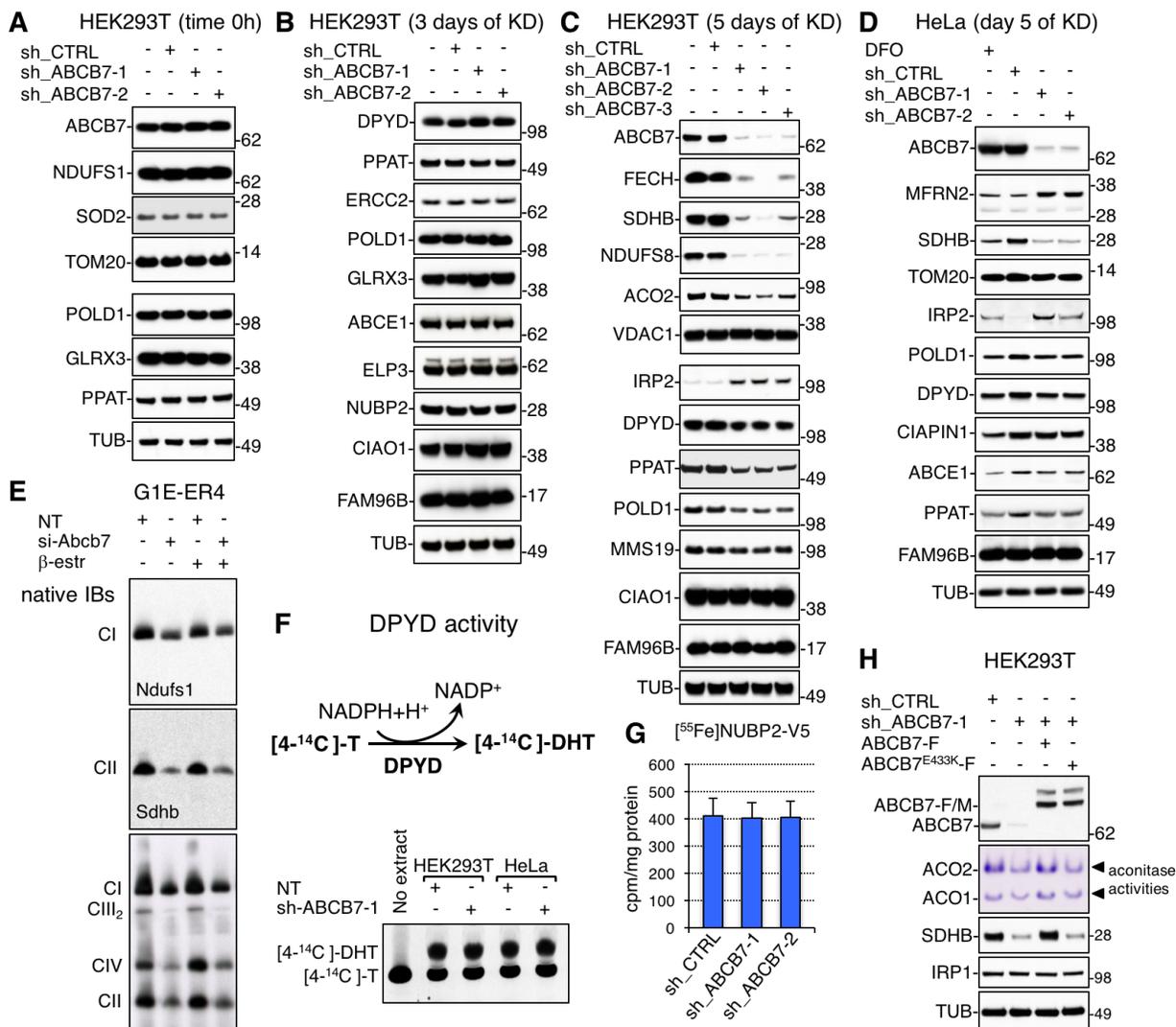
Data Sharing Contact: correspondence and requests for materials should be addressed to and will be fulfilled by the corresponding author Tracey A. Rouault (rouault@mail.nih.gov).

Supplemental Figure 1



Supplemental Figure 1. Inducible knockdown of ABCB7 mediated by short hairpin RNAs (shRNAs) in HEK293 and HeLa cells, and Abcb7 KD by si-RNAs in G1E-ER4 cells. Fluorescence pictures of HEK293T (A) and HeLa (B) cells expressing three independent shRNAs targeting different regions of the *ABCB7* mRNA (sh_ABCB7-1, sh_ABCB7-2, sh_ABCB7-3) or a scramble shRNA (sh_CTRL) 48h after induction with doxycycline. Cells show GFP fluorescence from Turbo-GFP, which was expressed upstream and in frame with the shRNAs enabling us to monitor the effective expression of shRNAs in cells. (C) Morphological changes in G1E-ER4 cells during β -estradiol-induced differentiation in control (NT) and Abcb7-KD (si-Abcb7) cells. Thin-layered cell samples prepared with a cytospin centrifuge were stained by Wright-Giemsa method. Scale bar = 10 μ m.

Supplemental Figure 2

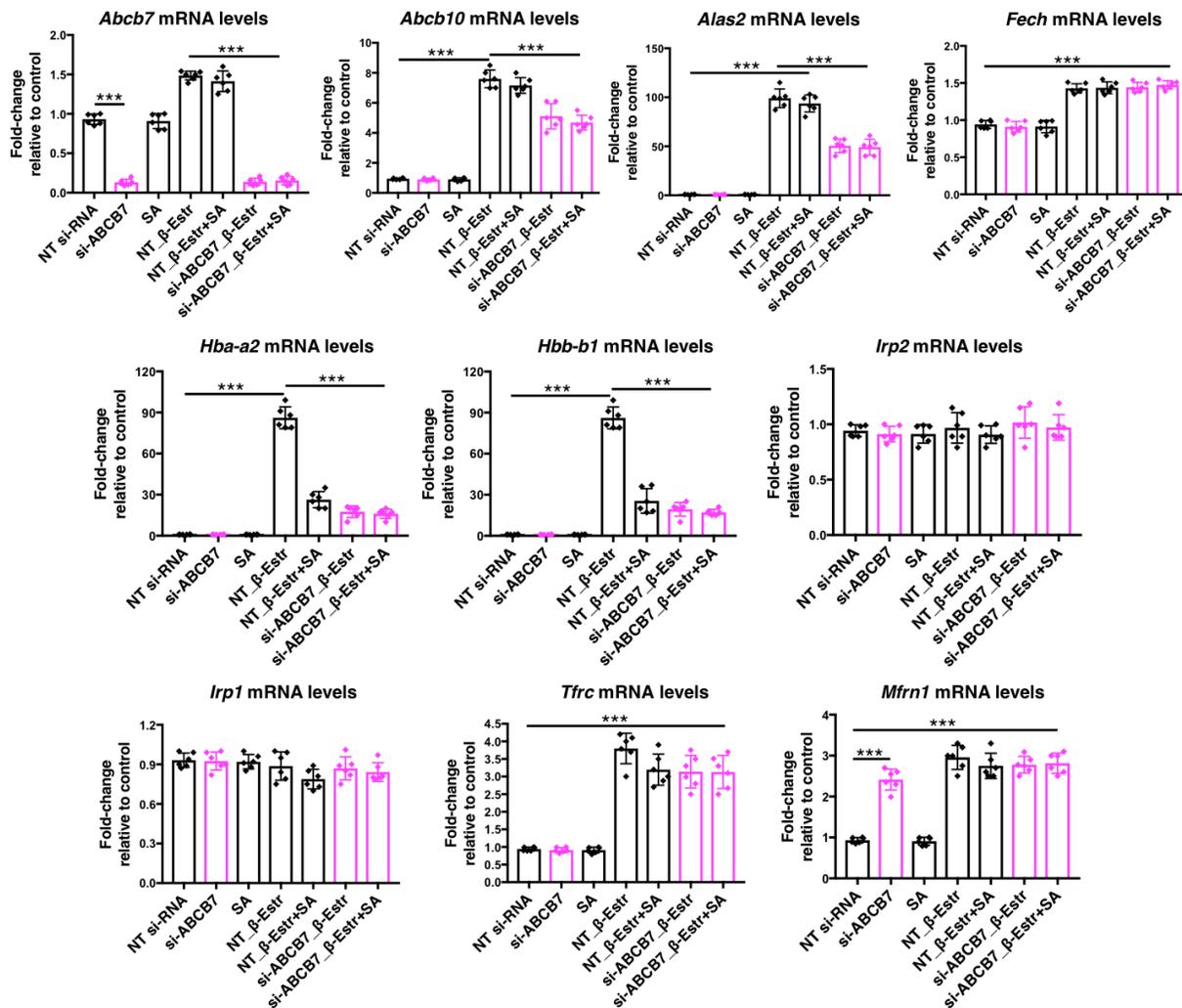


Supplemental Figure 2. In cells depleted of ABCB7, loss of mitochondrial function precedes the defect in cytosolic Fe-S proteins. (A) Immunoblots to ABCB7, NDUFS1, SOD2, POLD1, GLRX3 and PPAT in HEK293T at time 0, before induction of expression of shRNAs against ABCB7 shows tight control of shRNAs expression by the Tet3-inducible expression technology. TOM20 and α -Tubulin (TUB) were used as loading controls. (B) Immunoblots to cytosolic and nuclear Fe-S proteins DPYD, PPAT, ERCC2, POLD1, GLRX3, ABCE1, ELP3 and NUBP2 on total lysates from HEK293T cells after three days of KD of ABCB7. Westerns of the CIA components CIAO1 and FAM96B are also included. TUB was used as loading controls. (C) Immunoblots to ABCB7, and mitochondrial Fe-S proteins FECH, SDHB, NDUFS8, ACO2 in control cells (sh_CTRL) or in cells expressing 3 independent shRNAs targeting the ABCB7 transcript (sh_ABCB7-1, sh_ABCB7-2 and sh_ABCB7-3) for 5 days. VDAC1 was used as a loading control. Total lysates were analyzed by western blot (WB) to assess levels of IRP2 and of the cytosolic and nuclear Fe-S proteins DPYD, PPAT and POLD1. Immunoblots to the CIA components MMS19, CIAO1 and FAM96B are also shown. TUB was used as a loading control.

(D) Immunoblots to ABCB7, MFRN2 and SDHB on mitochondrial lysates from HeLa cells expressing a control shRNA (sh_CTRL) or 2 shRNAs targeting the *ABCB7* transcript. TOM20 was used as a loading control and treatment with the iron chelator deferoxamine (DFO) was included to compare the effect of iron deficiency to the KDs of ABCB7. Total lysates from an equivalent set of samples were analyzed by WB to assess levels of IRP2 and the cytosolic and nuclear Fe-S proteins POLD1, DPYD, CIAPIN1, ABCE1 and PPAT. Levels of FAM96B are also shown. TUB was used as a loading control. (E) Native immunoblots to Ndufs1 (CI Fe-S subunit), Sdhb (CII Fe-S subunit) and an oxidative phosphorylation cocktail on mitochondrial lysates from G1E-ER4 before ($-\beta$ -estradiol) and after 72h in differentiation medium ($+\beta$ -estradiol) and upon KD of *Abcb7* for 3 days. (F) DPYD-dependent conversion of 4- 14 C-thymine ([4- 14 C]-T) to 4- 14 C-dihydrothymine ([4- 14 C]-DHT) upon knockdown (KD) of ABCB7 (sh_ABCB7-1) for three days, assayed by thin layer chromatography (TLC) and autoradiography. DPYD activity was not decreased by KD of ABCB7 for 3 days, as the product of DPYD activity [4- 14 C]-DHT was unchanged in cells transfected with sh_ABCB7-1 compared to control. The reaction mix containing the substrate of the reaction [4- 14 C]-T without cell extract was loaded as a negative control (no extract) to visualize the substrate (4- 14 C-thymine) by TLC. (G) Levels of radiolabeled 55 Fe incorporated in the cytosolic Fe-S protein NUBP2 3 days after KD of ABCB7 with two independent shRNAs in HEK293 cells. Values are expressed as means \pm SD. (H) Complementation assay in HEK293T cells silenced for 3 days to KD the expression of ABCB7 and transfected with ABCB7-F or with the pathogenic mutant ABCB7^{E433K}-F. Levels of ABCB7, SDHB and IRP1 are presented along with the in-gel aconitase activity assays. TUB was used as a loading control. (A-H, $n=5$).

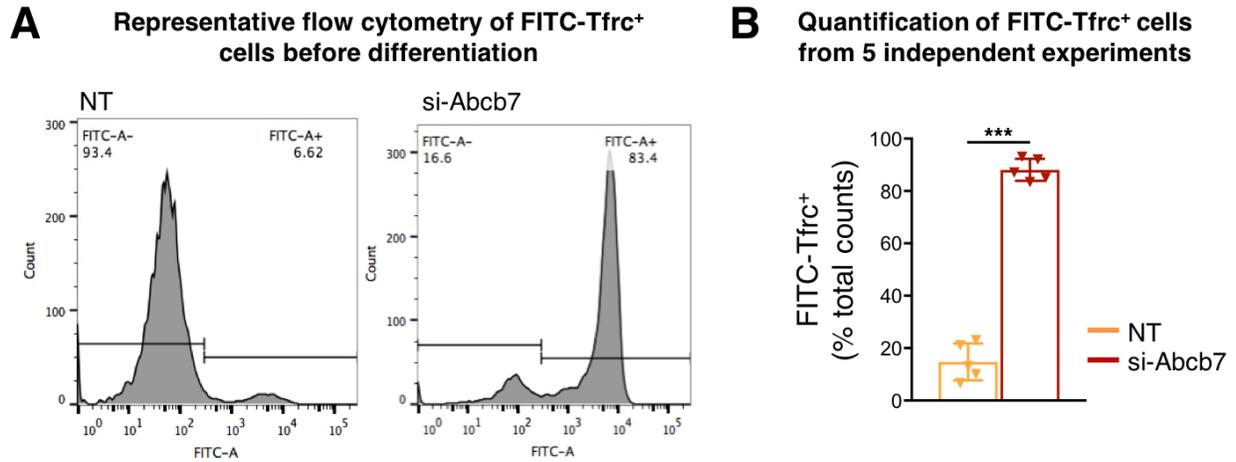
Supplemental Figure 3

Transcriptional changes in G1E-ER4 cells undergoing differentiation



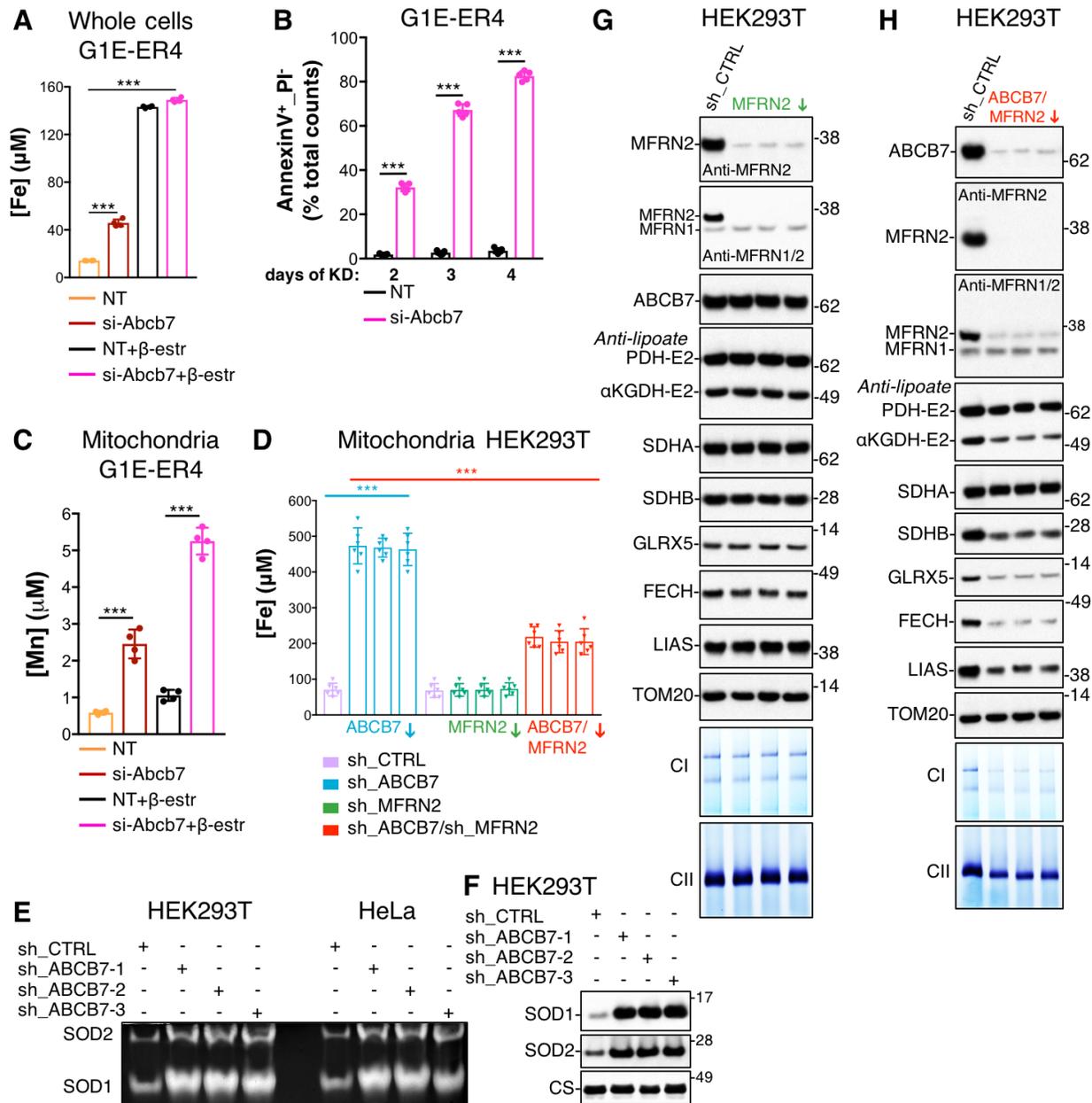
Supplemental Figure 3. Transcriptional changes in Abcb7-KD cells during differentiation. Transcript levels of *Abcb7*, *Abcb10*, *Alas2*, *Fech*, *Hba-a2*, *Hbb-b1*, *Irp2*, *Irp1*, *Tfrc* and *Slc25a37* (*Mfrn1*) in control or *Abcb7*-KD G1E-ER4 cells, before ($-\beta$ -estradiol) and 24h after differentiation in the presence of β -estradiol. Treatments of cells with the Alad inhibitor succinylacetone (SA) were included as a control for the effect of inhibition of heme biosynthesis on the levels of the transcripts analyzed. Values are expressed as means \pm SEM. ($n=6$)

Supplemental Figure 4



Supplemental Figure 4. Undifferentiated G1E-ER4 cells have increased Tfrc at the plasma membrane. (A) Representative flow cytometry results of the levels of transferrin receptor (Tfrc) at the plasma membrane of control (NT) and Abcb7-KD G1E-ER4 cells before induction of differentiation, and (B) quantification of Tfrc positive cells (% of total counts) in control and Abcb7-depleted cells from 5 independent experiments ($n=5$). Values are expressed as % of total counts \pm SD.

Supplemental Figure 5

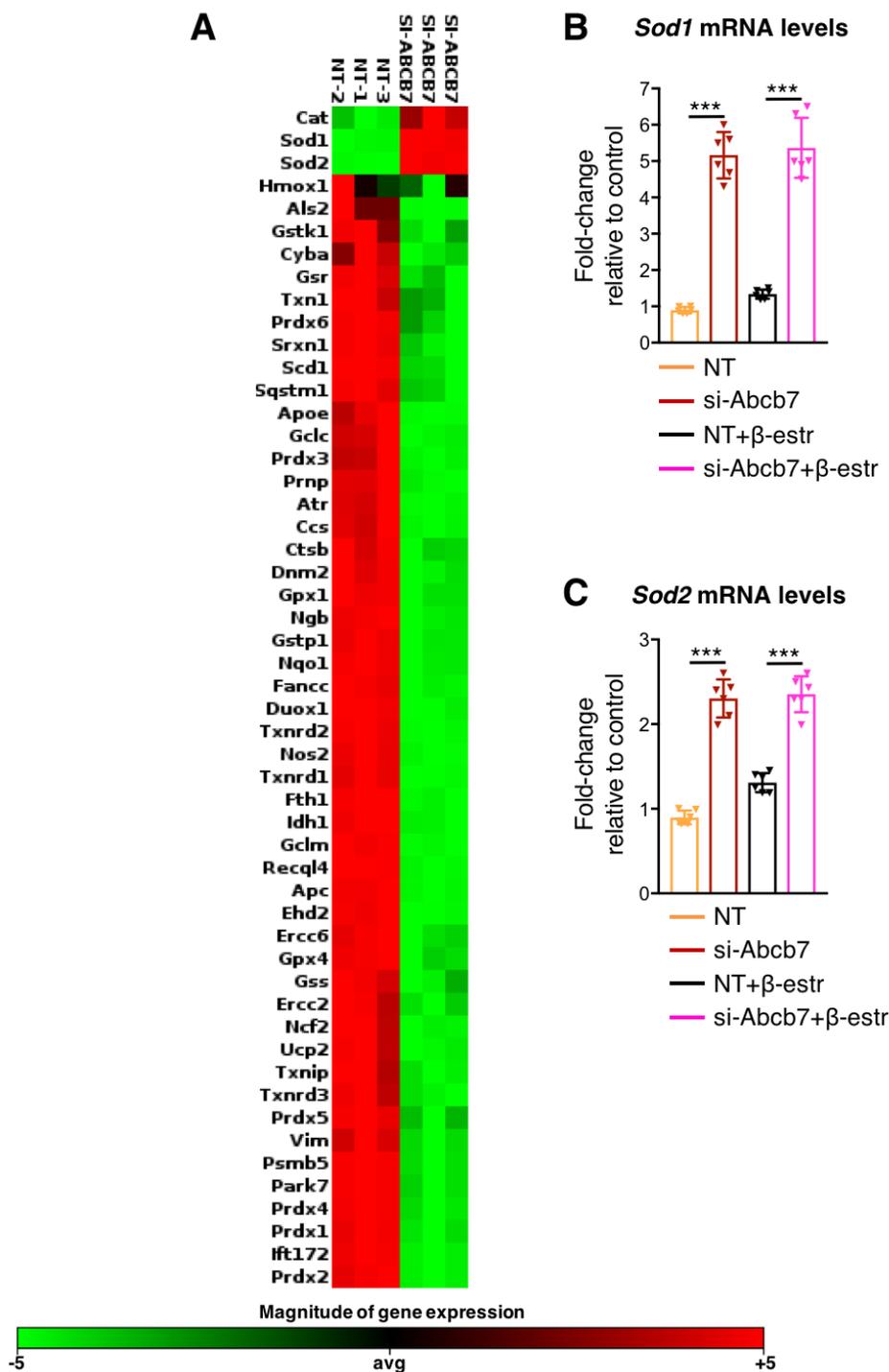


Supplemental Figure 5. Mitochondrial iron overload and oxidative stress in cells depleted of ABCB7. (A) Iron content in control or Abcb7-KD (day 3) G1E-ER4 cells before and after 72h of differentiation. (B) AnnexinV⁺/propidium iodide (PI)⁻ G1E-ER4 cells silenced for 2, 3 or 4 days to deplete levels of Abcb7. (C) Manganese content in G1E-ER4 cells treated as in (A). (D) Iron content in mitochondria isolated from HEK293T cells 5 days after KD of ABCB7, MFRN2 or of both ABCB7/MFRN2. (E) SOD1 and SOD2 in-gel activity assays show activation of SOD enzymes in HEK293T and HeLa cells after three days of KD of ABCB7. (F) Immunoblots to SOD1 and SOD2 from samples as in (E). Levels of citrate synthase (CS) were used as a loading control. (G) Immunoblots to mitoferrins, ABCB7, lipocate, SDHA, SDHB, GLRX5, FECH, LIAS and TOM20 in MFRN2-depleted cells (KD for 5 days). Respiratory complexes CI and CII were

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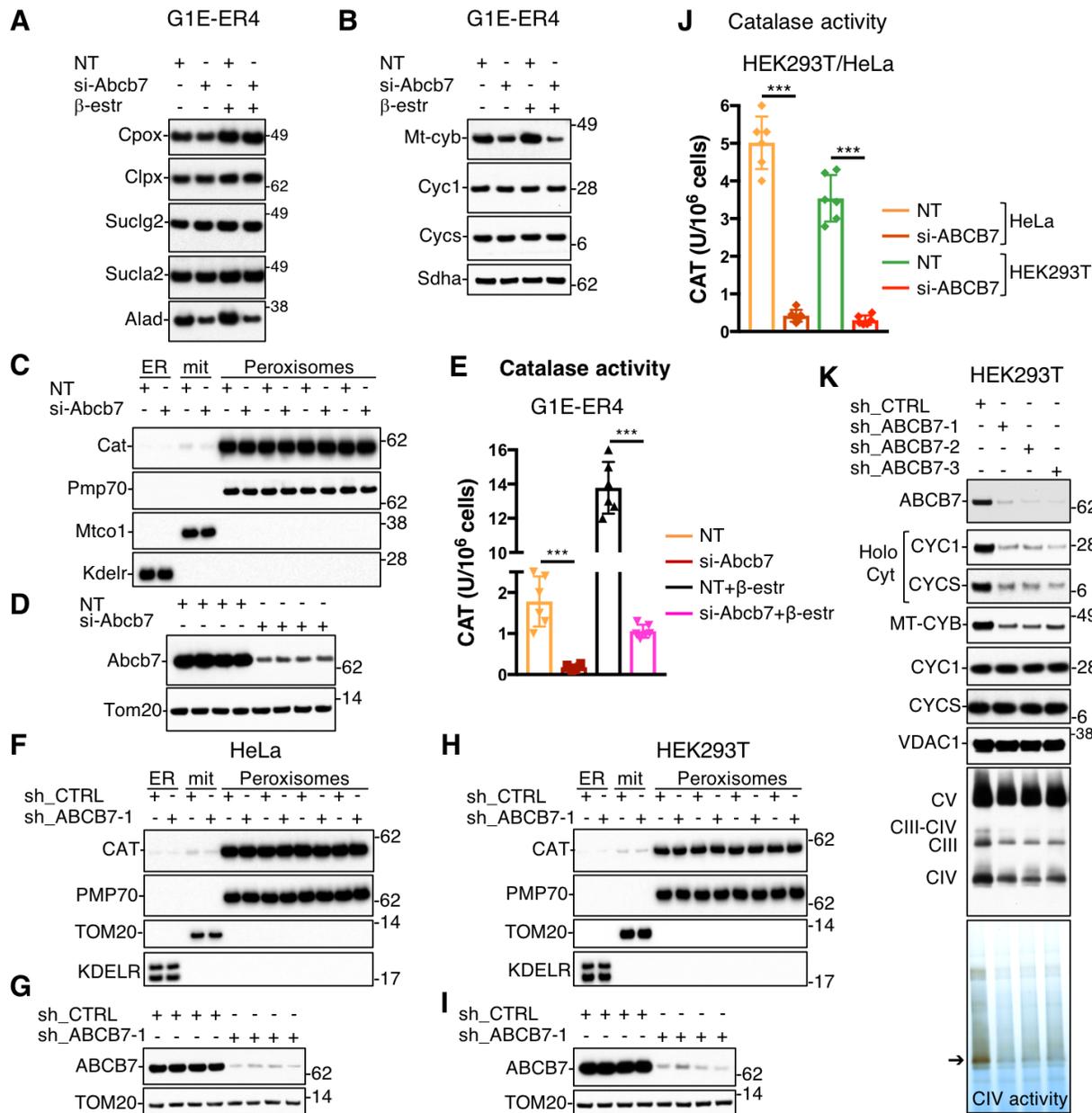
fully functional in MFRN2-KD cells. PDH-E2 and α KGDH-E2 are the lipoylated E2 subunits of pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase complexes, respectively. (H) Immunoblots to ABCB7, mitoferrins 1 and 2, lipoate, SDHA, SDHB, GLRX5, FECH and lipoic acid synthase, LIAS, in HEK293 cells silenced for ABCB7 and MFRN2 for five days. CI and CII in-gel activity assays show decreased respiration in mitochondria depleted of ABCB7 and MFRN2. (A, B, C, E, F, G, H, $n=4$; D, $n=6$).

Supplemental Figure 6



Supplemental Figure 6. Oxidative stress and antioxidant defense gene array profile in control and Abcb7-KD cells. (A) Log2fold expression of oxidative stress and antioxidant defense pathway genes which were differentially expressed in G1E-ER4 cells 48h after KD of Abcb7 (FDR<0.01, n=3 biological replicates). (B and C) Transcript levels of Sod1 and Sod2 enzymes. Values are expressed as means ± SEM (n=6).

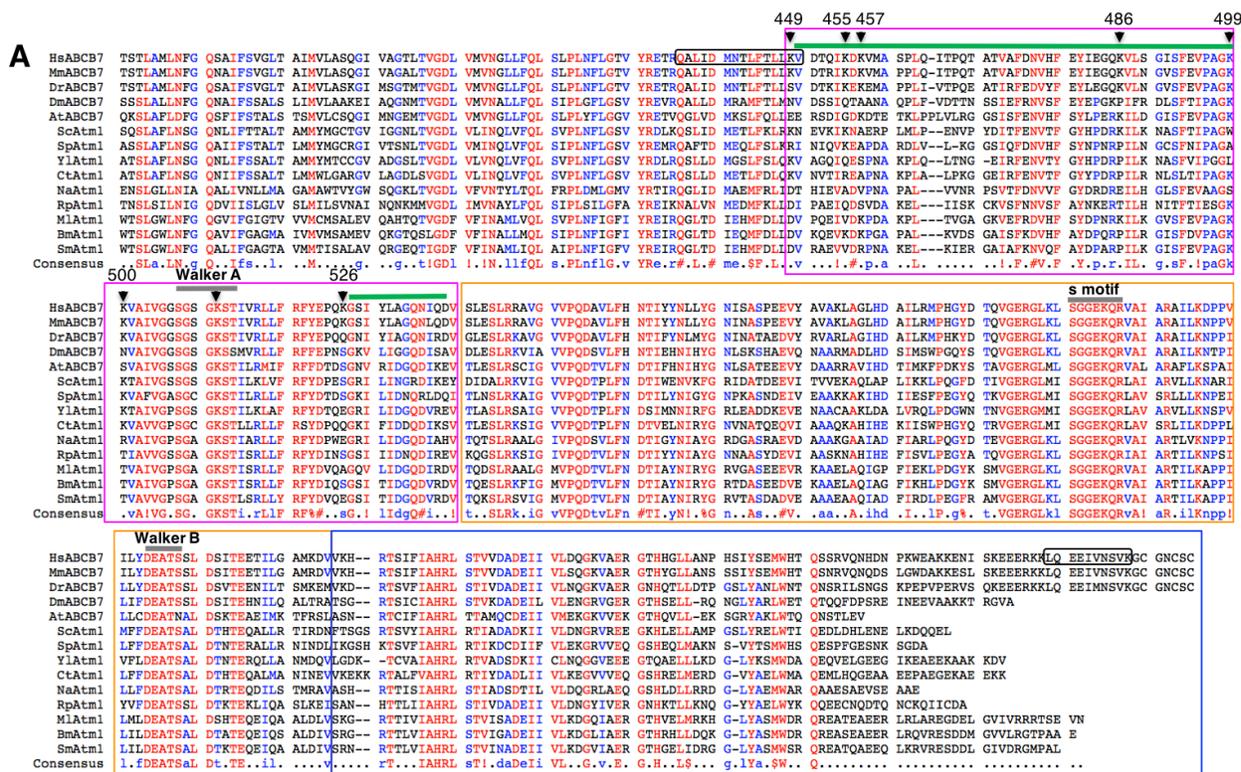
Supplemental Figure 7



Supplemental Figure 7. ABCB7 deficient cells had decreased heme levels, which caused impaired catalase and cytochrome c oxidase activities and loss of holo-cytochromes. (A) Protein blots of the heme biosynthetic enzymes, Cpx, mitochondrial unfoldase Clpx, Sucla2 and Suclg2, and cytosolic Alad in control and Abcb7-KD cells before (without β-estradiol) and after 72h of differentiation. (B) Levels of the heme containing CIII subunit cytochrome b (Mt-cyb) were decreased in Abcb7 KD cells, whereas levels of total apo-cytochromes c1 (Cyc1) and cytochrome c (Cycs) didn't change. Sdha was used as a loading control. (C) Protein blots to catalase, peroxisomal marker Pmp70, mitochondrial CIV subunit Mtco1 and endoplasmic reticulum (ER) marker Kdelr on ER, mitochondrial and peroxisomal fractions from control and Abcb7-KD G1E-

ER4 cells show efficient sucrose gradient-based purification of peroxisomes. (D) Immunoblots to *Abcb7* and loading control *Tom20* show effective KD of *Abcb7* on samples as in (C) and in Figure 3G. (E) Catalase activity in G1E-ER4 cells treated as in B and expressed as units of enzymatic activity/ 10^6 cells. Values are means \pm SD. (F and H) Protein blots to catalase, peroxisome marker PMP70, mitochondrial TOM20 and endoplasmic reticulum (ER) marker KDEL α on ER, mitochondrial and peroxisomal fractions from control and ABCB7-KD HeLa (F) or HEK293T (H) cells show efficient sucrose gradient-based purification of peroxisomes. (G and I) Immunoblots to ABCB7 and loading control TOM20 on mitochondrial fractions show effective KD of ABCB7 in HeLa (G) and HEK293T (I) cells on samples as in F and H, respectively. (J) Catalase activity in units/ 10^6 cells in HEK293T or HeLa cells after KD of ABCB7 for 3 days shows profound loss of CAT activity in cells depleted of ABCB7. (K) Heme-bound cytochrome c1 (CYC1) and c (CYCS) and cytochrome b (MT-CYB) levels were significantly decreased in HEK293T cells expressing 3 independent shRNAs targeting the *ABCB7* transcript. Levels of apocytochromes c1 and c didn't change upon KD of ABCB7. Native IB and CIV in-gel activity assay showed loss of heme-dependent CIII and CIV upon KD of ABCB7. VDAC1 was used as a loading control. (A, B, C, D, F, G, H, I and K, $n=5$; E and J, $n=6$).

Supplemental Figure 8

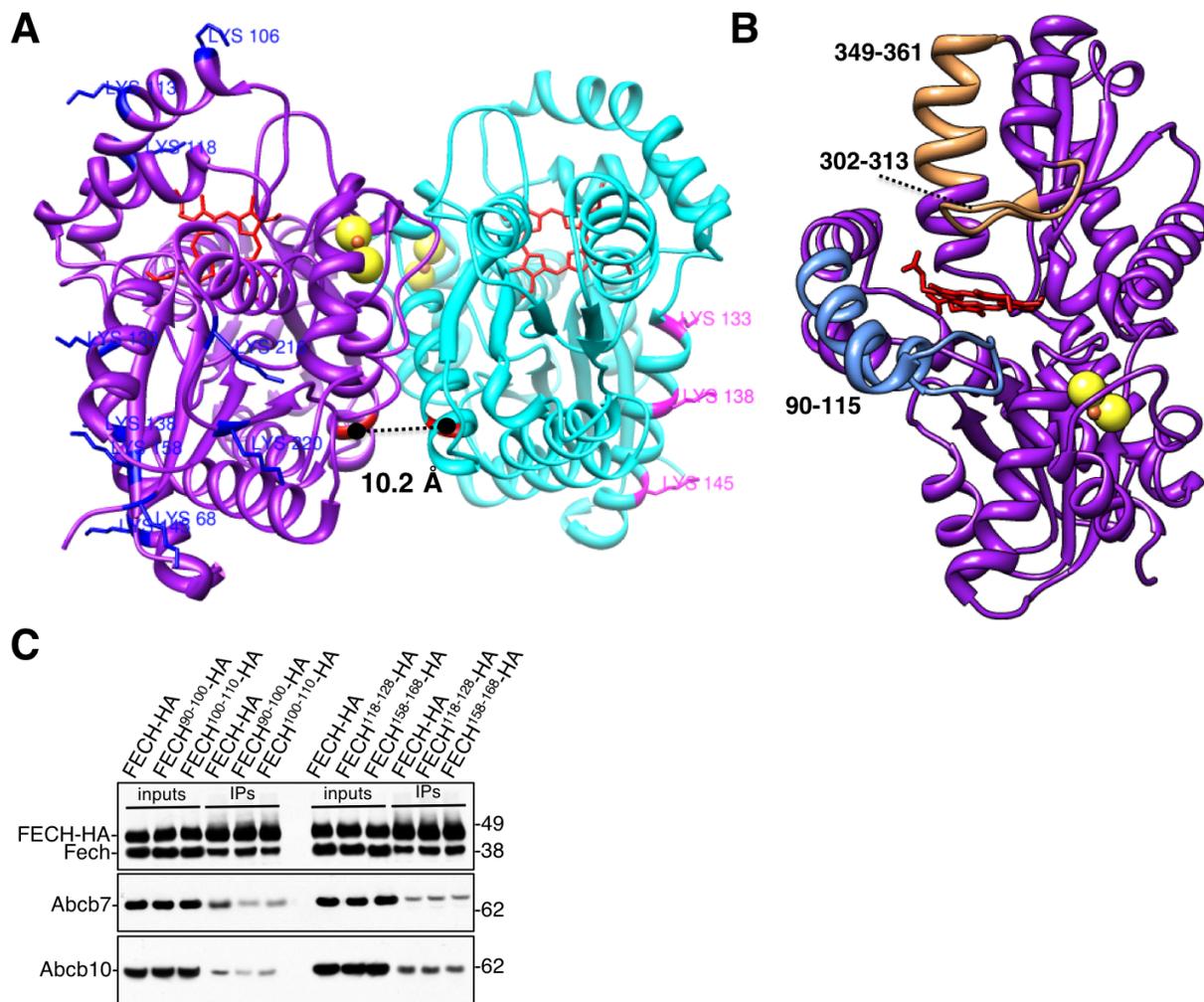


B C terminus of mouse dimeric Abcb10 modeled upon the human ABCB10 (PDB:4AYT)



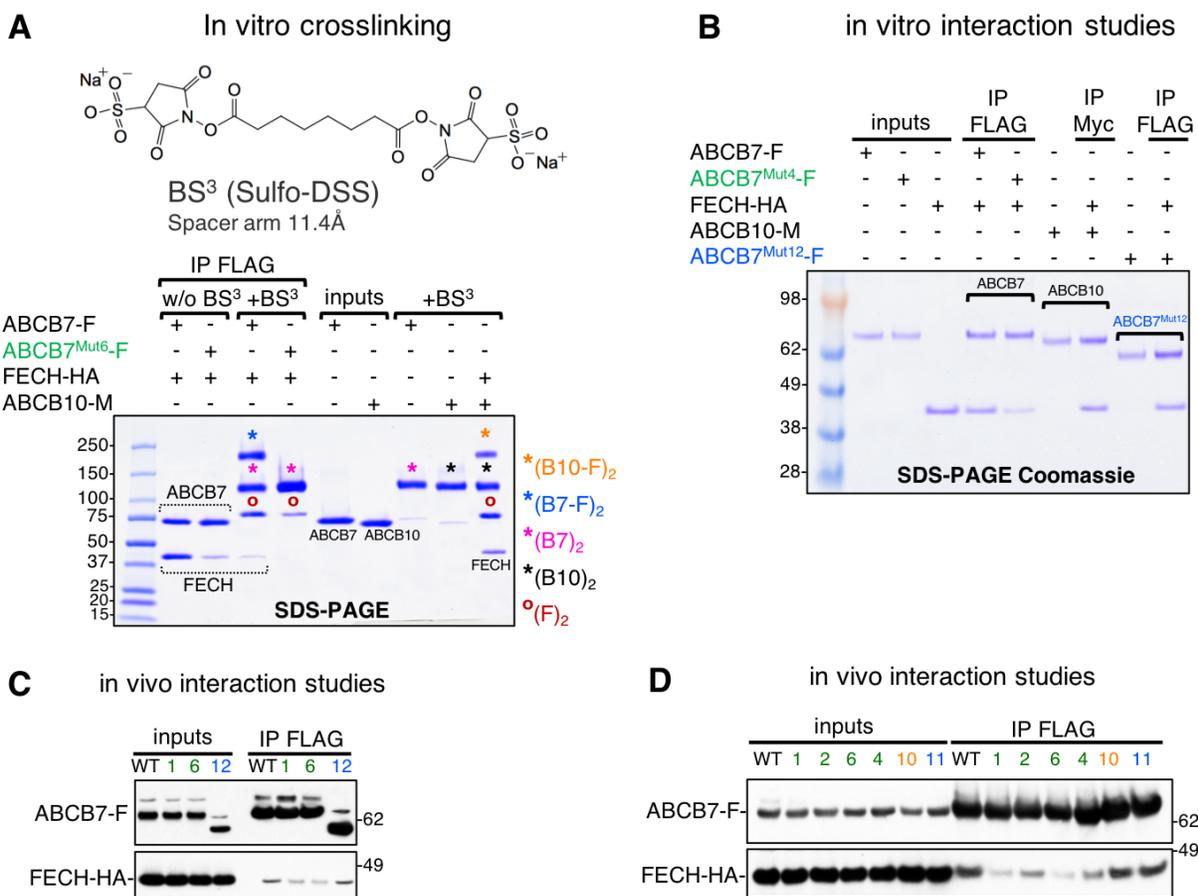
Supplemental Figure 8. Multiple sequence alignment of eukaryotic and bacterial ABCB7 orthologues and crosslinked lysine residues in the ABCB7 and Abcb10. (A) Multiple sequence alignments of eukaryotic and bacterial ABCB7 orthologues. The green lines highlight the peptide sequences involved in the interaction with FECH. The grey lines indicate the Walker A and B and the signature (s) motif. The black arrows point at the lysines that were crosslinked by DSS in the XL-MS analysis. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Yl, *Yarrowia lipolytica*; Ct, *Chaetomium thermophilum*; Na, *Novosphingobium aromaticivorans*; Rp, *Rickettsia prowazekii*; Ml, *Mesorhizobium loti*; Bm, *Brucella melitensis*; Sm, *Sinorhizobium meliloti*. (B) Three-dimensional structure of murine Abcb10 modeled upon the human ABCB10 (PDB:4AYT) using Swiss-Model(11). Lysines K451, K534 and K615 of murine endogenous Abcb10, which are conserved in the human ABCB10 and correspond to lysines 486, 569, 650, respectively, which were crosslinked with FECH, are shown in green and labeled.

Supplemental Figure 9



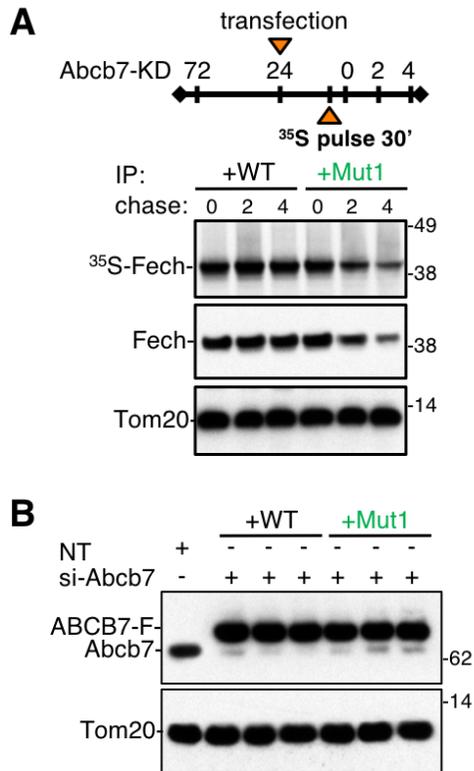
Supplemental Figure 9. Region 90-115 of human ferrochelatase, which envelops the PPIX substrate in the enzymatic active site, is required for binding to ABCB7. (A) Crystal structure of the variant E343K of human FECH (PDB:2HRE), which had a higher affinity for protoporphyrin IX in comparison to the wild-type enzyme, in its dimeric conformation. Two Fe-S clusters, each ligated by a FECH monomer, are rendered by yellow and red spheres. Two molecules of PPIX (in red) are shown per dimer of FECH. The lysines on protomer A (purple ribbon) crosslinked with lysines of ABCB7 are colored in blue. The lysines on protomer B (cyan ribbon) crosslinked with lysines of Abcb10 are in magenta. The C^α-C^α distance between the inter-subunit lysine residues 286 (shown in red) on each protomer is 10.163Å, in agreement with the length of the DSS crosslinker (11.4 Å). (B) Crystal structure of human FECH (PDB: 2HRE) in its monomeric form. The three regions that undergo significant movement in the substrate-bound form of the enzyme have been highlighted in different colors. The region 90-115 of FECH, which interacted with ABCB7, is shown in blue, whereas the regions 302-313 and 349-361 are colored in light brown. (C) Immunoprecipitation experiments of FECH-HA wild type or the mutants in which amino acid residues 90 to 100 (in FECH⁹⁰⁻¹⁰⁰-HA), 100 to 110, 118 to 128 and 158 to 168 were replaced by alanines. The region 90-115 of FECH was required for binding to Abcb7.

Supplemental Figure 10



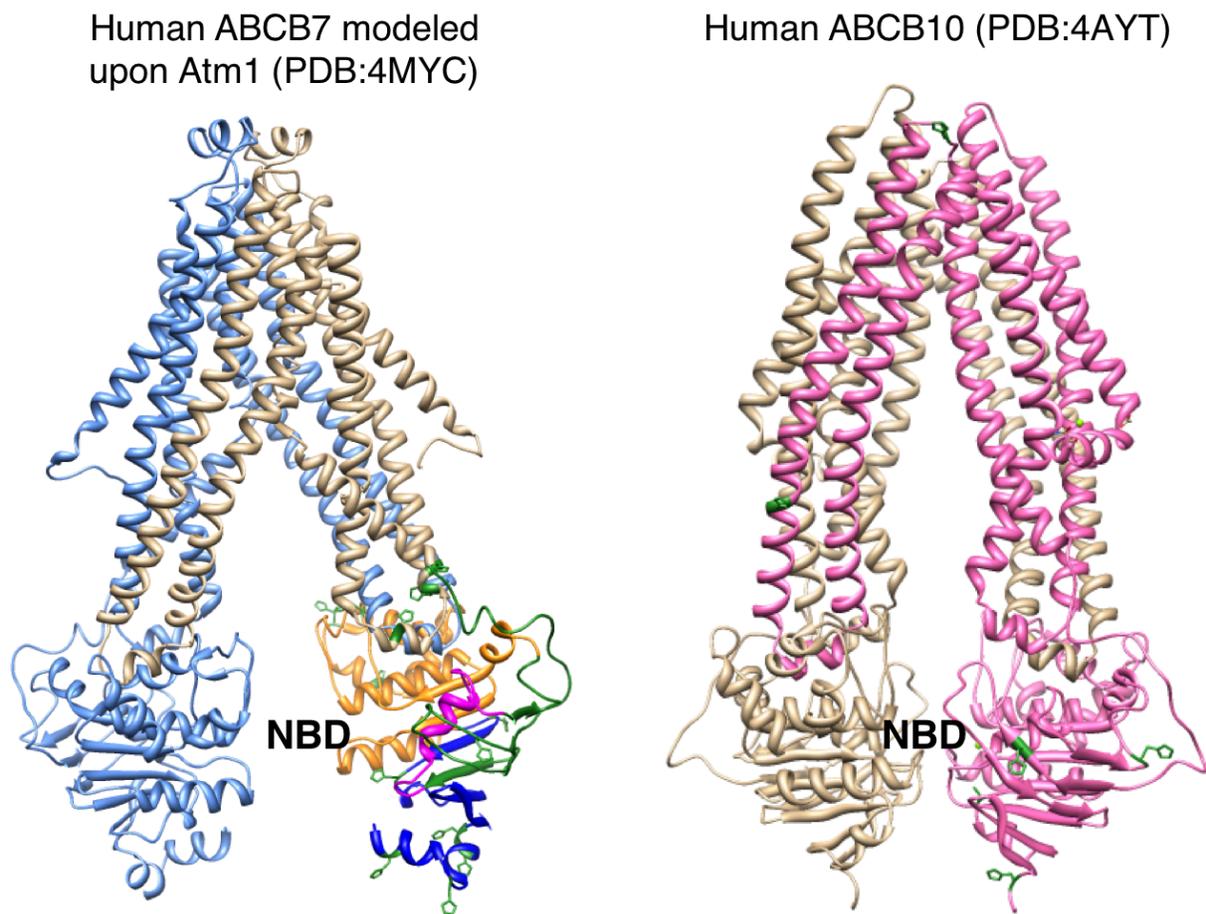
Supplemental Figure 10. Mapping the interaction of ABCB7 with ferrochelatase by crosslinking and mutational analysis. (A) Coomassie staining of FLAG IPs of ABCB7 WT or MUT6, as indicated, in the presence of FECH (samples without BS³) and in vitro crosslinked products (+BS³) on SDS-PAGE. Magenta asterisks denote dimers of ABCB7 WT or the ABCB7 mutant (Mut6) in which amino acid residues between Gly527 and Asp538, involved in binding FECH, were replaced by alanines. Blue asterisks indicate the heterotetrameric (ABCB7-FECH)₂ complex. Black asterisks denote dimers of ABCB10, orange asterisk indicates an ABCB10-FECH heterotetrameric complex, and brown circles indicate a dimer of FECH. (B) In vitro pull-down assay of purified FLAG-tagged ABCB7 wild type (B7) or mutants, as indicated, in the presence of FECH-HA, and pull-down assay of Myc-tagged ABCB10 in the presence of FECH-HA show that both ABC transporters physically interacted with FECH. ABCB7^{Mut4} had decreased binding to FECH, whereas ABCB7^{Mut12} was able to bind FECH as WT ABCB7. (C and D) IPs of recombinant FLAG-tagged ABCB7 in G1E-ER4 cells show that mutants 1, 2, 4 and 6 (green peptides and domains in Figures 6A and 6B, respectively) had impaired binding to ferrochelatase in vivo. (A, B, n=4. C, D, n=6).

Supplemental Figure 11



Supplemental Figure 11. Reduced half-life of Fech in G1E-ER4 cells expressing an ABCB7 mutant defective in binding Fech. (A) A pulse-chase experiment was performed to assess the turnover rate of Fech in cells lacking endogenous Abcb7 and transfected with either wild type recombinant ABCB7-F or with Mut1 which was defective in binding Fech. G1E-ER4 cells were silenced to knockdown the expression of endogenous Abcb7 and transfected 48h later with either WT or Mut1 human ABCB7-F. Cells were then pulsed for 30 minutes with ³⁵S-Cys/Met, followed by incubation for the indicated time points in differentiation medium. Radiolabeled Fech was visualized by autoradiography after immunoprecipitation and SDS-PAGE (top panel), whereas total protein levels were assessed by immunoblot (lower panels). (B) Immunoblot to ABCB7 shows efficiency of KD of Abcb7 and expression levels of recombinant human ABCB7-F WT or Mut1 on samples showed in panel A. A and B, *n*=4.

Supplemental Figure 12



Supplemental Figure 12. Crystal structures of human ABCB7 and ABCB10 show enrichment of histidine residues near the NBDs of each ABC transporter, in the regions that interact with FECH. Crystal structures of human ABCB7, modeled upon the solved structure of yeast Atm1 (PDB: 4MYC), and of ABCB10 show enrichment of histidines, which are potential heme ligating residues, in the regions of each ABC transporter that interact with FECH near the NBDs. The side chains of histidine residues are shown in green.

Supplemental Figure 13

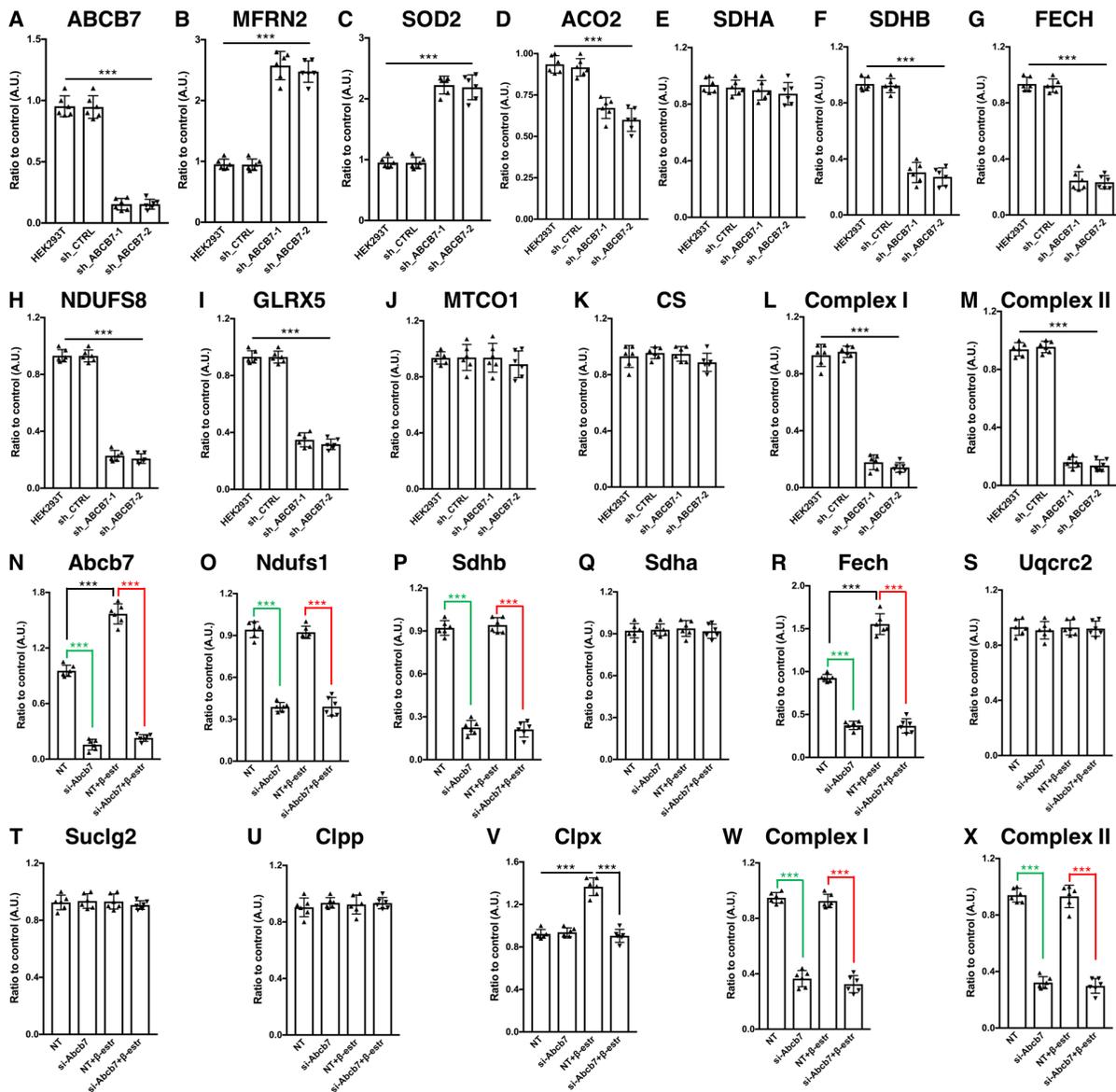


Figure S13. Densitometries of western blots and in-gel activity assays presented in Figures 1A (A-M) and 1B (N-X).

(A-K) Quantified bands from immunoblots as presented in Figure 1A were normalized to levels of VDAC1, which was used as a loading control. Values are expressed as ratios to control (HEK293T). (L, M) Quantified bands from in-gel activity assays of complexes I and II as in Figure 1A were normalized to levels of TOM20, which was used as a loading control. Values are expressed as ratios to control (HEK293T). (N-V) Quantified bands from immunoblots as in Figure 1B were normalized to levels of Vdac1, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (W, X) Quantified bands from in-gel activity assays of complexes I and II as in Figure 1B were normalized to levels of Tom20, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). Error bars are SEM. *P* values lower than 0.001 are indicated by three asterisks.

Supplemental Figure 14

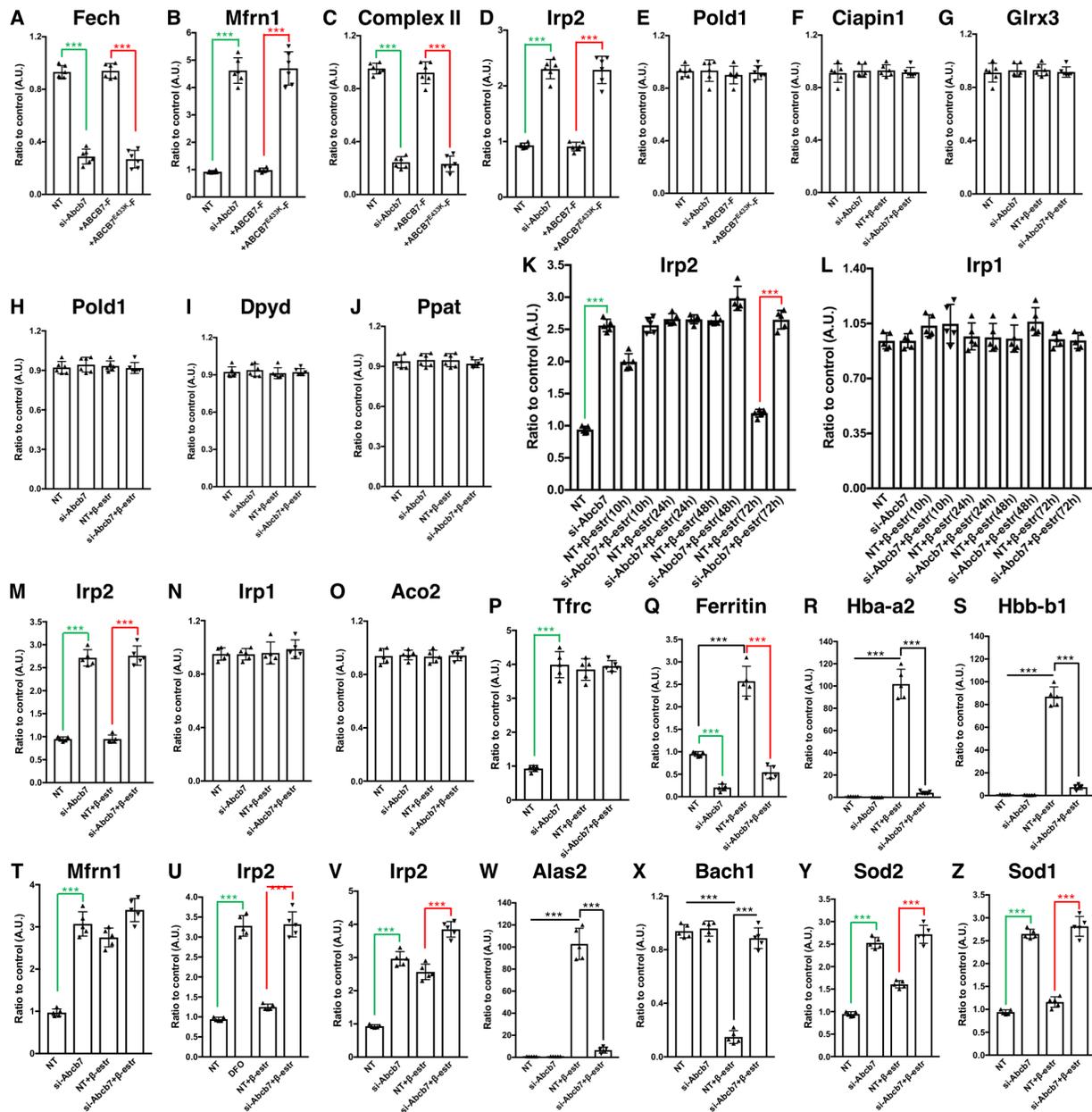


Figure S14. Densitometries of western blots and in-gel activity assays presented in Figures 1D (A-E) and 1E (F-J), and in Figure 2 (K-Z).

(A-C) Quantified bands from immunoblots as presented in Figure 1D (mitochondrial fractions) were normalized to levels of Tom20, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (D, E) Quantified bands from immunoblots as in Figure 1D (total lysates) were normalized to levels of tubulin (Tub), which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (F-J) Quantified bands from immunoblots as in Figure 1E were normalized to levels of Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting

(NT) si-RNAs). (K, L) Quantified bands from immunoblots as in Figure 2A were normalized against Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (M-T) Quantified bands from immunoblots as in Figure 2B were normalized against Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (U) Quantified bands from immunoblots as in Figure 2C were normalized against Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (V-X) Quantified bands from immunoblots as in Figure 2D were normalized against Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (Y, Z) Quantified bands from immunoblots as in Figure 2I were normalized against Tom20, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). Error bars are SEM. *P* values lower than 0.001 are indicated by three asterisks.

Supplemental Figure 15

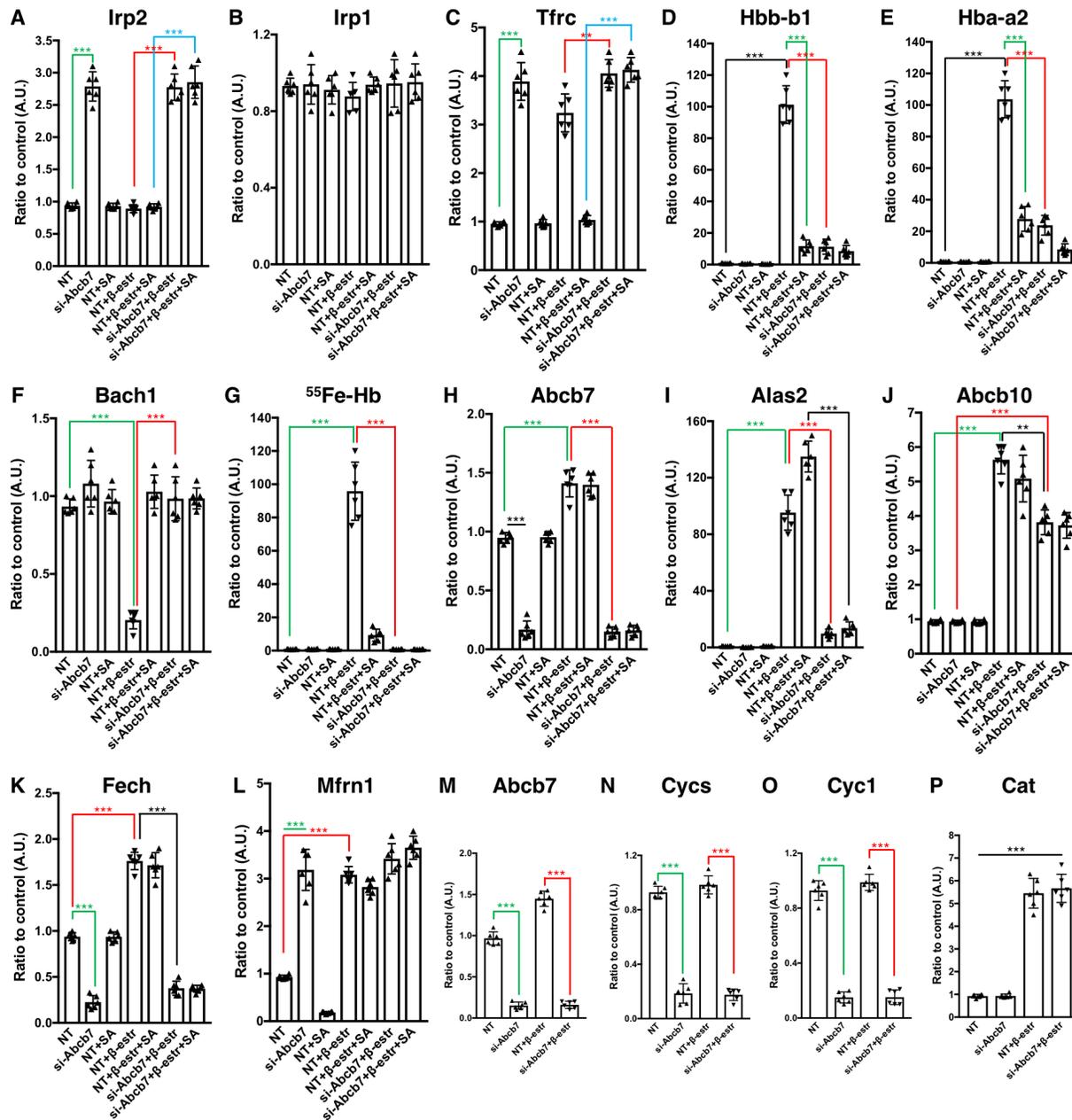


Figure S15. Densitometries of western blots and ⁵⁵Fe-autoradiograms presented in Figure 3. (A-F) Quantified bands from immunoblots as presented in Figure 3A were normalized to levels of Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (G) Levels of radiolabeled ⁵⁵Fe incorporated into hemoglobin (Hb) as presented in Figure 3A were normalized to levels of Tub and expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (H-L) Quantified bands from immunoblots as in Figure 3B were normalized to levels of Tom20, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (M-O) Quantified bands from immunoblots as in Figure 3F

were normalized to levels of Tom20, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (P) Levels of peroxisomal catalase (Cat) from immunoblots as in Figure 3F were normalized against the peroxisomal marker Pmp70, which was used as a loading control, and expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). Error bars are SEM. *P* values lower than 0.001 are indicated by three asterisks; *p* values lower than 0.01 are indicated by two asterisks.

Supplemental Figure 16

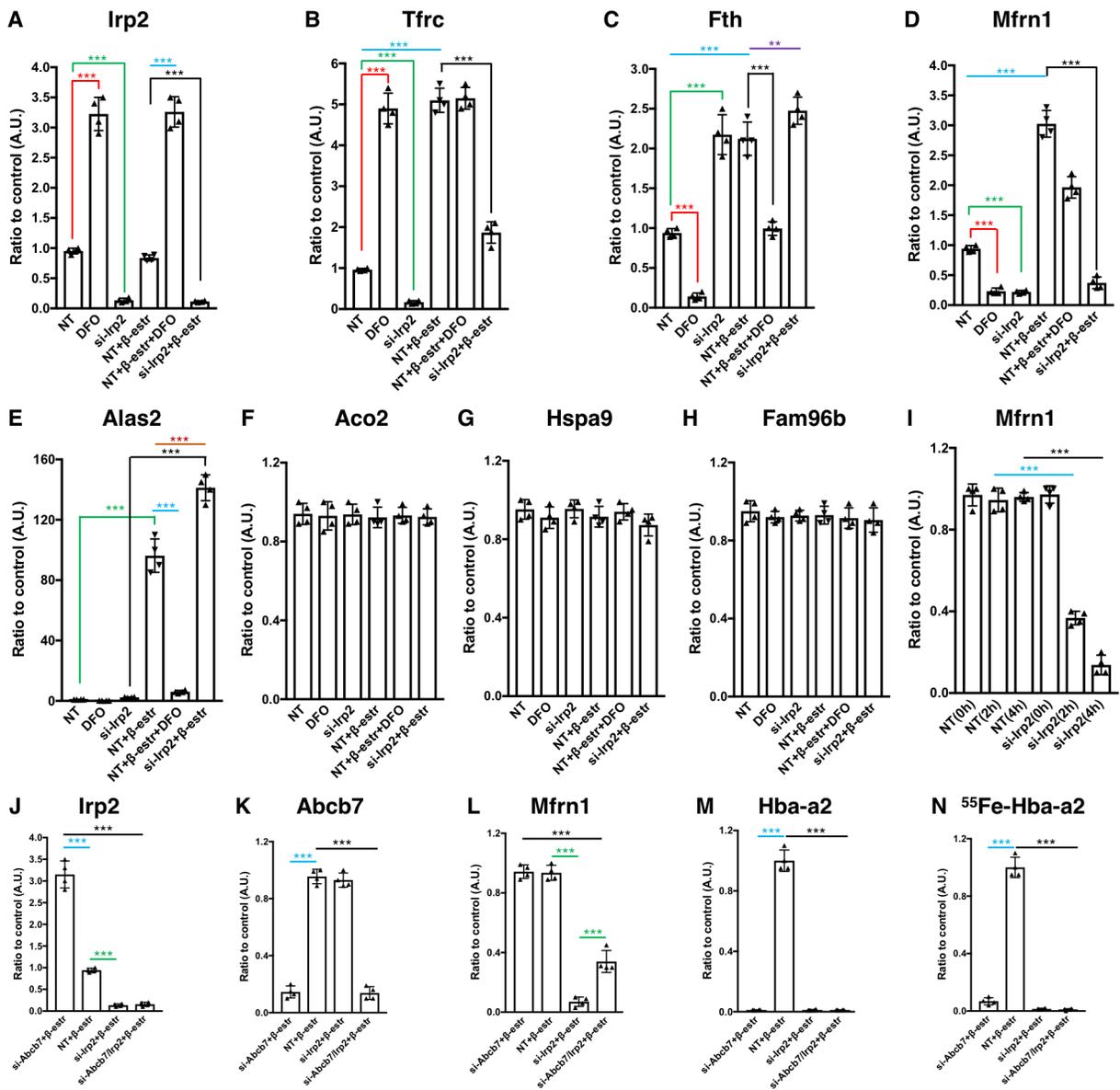


Figure S16. Densitometries of western blots and ⁵⁵Fe-autoradiograms presented in Figure 4. (A-H) Quantified bands from immunoblots as presented in Figure 4A were normalized to levels of Tub, which was used as a loading control. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (I) Levels of Mfrn1 as in Figure 4D were normalized to levels of Tom20 and expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs at time 0h chase). (J-M) Quantified bands from immunoblots as in Figure 4F were normalized to levels of Tub. Values are expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). (N) Levels of radiolabeled ⁵⁵Fe incorporated into hemoglobin (Hb) as presented in Figure 4F were normalized to levels of Tub and expressed as ratios to control (G1E-ER4 cells transfected with non-targeting (NT) si-RNAs). Error bars are SEM. *P* values lower than 0.001 are indicated by three asterisks.

Supplemental Figure 17

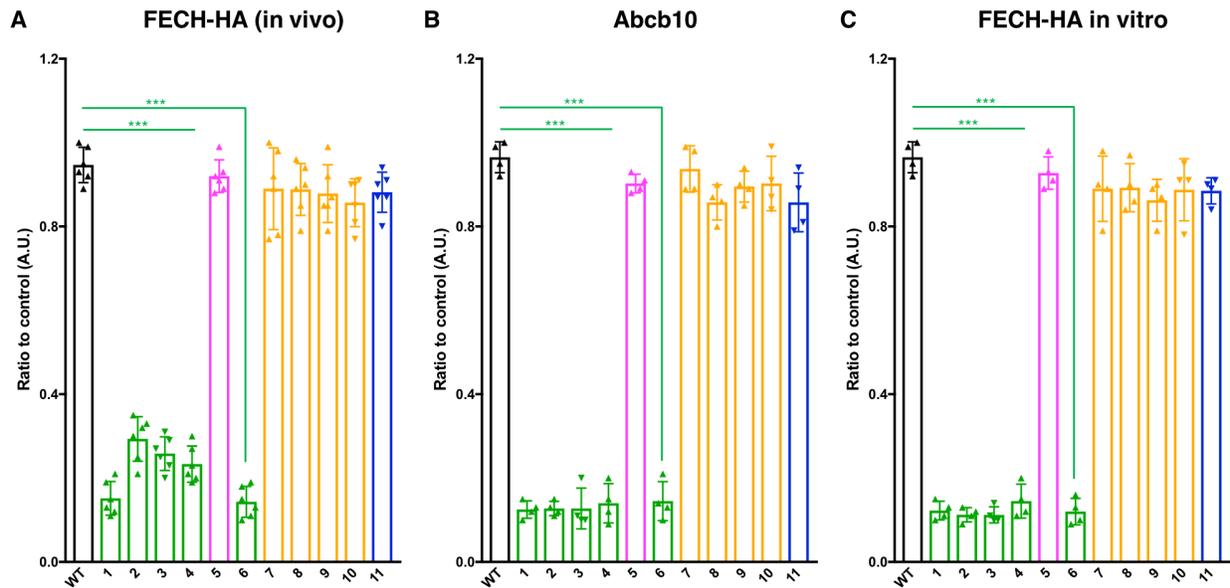


Figure S17. Densitometries of western blots and ³⁵S-autoradiograms presented in Figure 6. (A) Ratios of FECH-HA levels co-immunoprecipitated with ABCB7-F wild type (WT) or mutants as indicated, normalized to control (levels of FECH-HA co-immunoprecipitated with WT ABCB7-F), as presented in Figures 6C-E. (B) Ratios of endogenous Abcb10 levels co-immunoprecipitated with ABCB7-F wild type (WT) or mutants as indicated, normalized to control (levels of Abcb10 co-immunoprecipitated with WT ABCB7-F), as presented in Figures 6C and 6D. (C) Ratios of radiolabeled ³⁵S-FECH-HA levels co-immunoprecipitated with ³⁵S-ABCB7-F wild type (WT) or mutants as indicated in pull-down experiments in vitro, as presented in Figures 6F-H. Values were normalized to control (levels of ³⁵S-FECH-HA co-immunoprecipitated with WT ³⁵S-ABCB7-F). Values are given as mean ± SEM. *P* values lower than 0.001 are indicated by three asterisks.

Supplemental Table 1

DSS Crosslinks:	Link type	Protein a	Protein b	Sequences Proteins a	Sequences Proteins b	Position a	Position b	m/z	Highest Score	Appearance
	Intra-protein	Hs FECH	Hs FECH	LCSK 397 QLTLSCPLCVNPVCR	ETK 415 SFFTSQQL	L394	E413	1133.231	178	3
	Intra-protein	Hs FECH	Hs FECH	KPTMK 220 WSTIDR	TK 415 SFFTSQQL	K216	T414	852.6488	187	3
	Intra-protein	Hs FECH	Hs FECH	HFPLEK 252 RSE	GRK 328 NILLVPIAFTSD	H247	G326	931.18	120	4
	Inter-protein	Hs FECH	Hs FECH	VSATVQK 288 VME	VSATVQK 288 VME	V280	V280	834.1174	174	4
	Inter-protein	Hs FECH	Hs FECH	PYPQEVSATVQK 288 VMER	PYPQEVSATVQK 288 VMER	P275	P275	1243.631	167	3
	Inter-protein	Hs ABCB7	Hs FECH	MNTLFTLLK 449 VDTQIK 455 DK	LAPFIAK 113 RRTPK 118 IQEQYR	M441	L107	1067.85	195	6
	Inter-protein	Hs ABCB7	Hs FECH	QALIDMNTLFTLLK 449 VDTQIK	LAPFIAK 113 RRTPKIQE	Q436	L107	1360.113	180	6
	Inter-protein	Hs ABCB7	Hs FECH	MNTLFTLLK 449 VDTQIKD	LAPFIAK 113 RRTPK	M441	L107	1094.964	176	3
	Inter-protein	Hs ABCB7	Hs FECH	VDTQIK 455 DK	LMTLPIONK 106 LAPFIAK	V450	L98	917.1944	186	4
	Inter-protein	Hs ABCB7	Hs FECH	TOIK 455 DK 457 VMASPLQITPQTATVAFD	LAPFIAK 113 RRTPK 118 IQE	T452	L107	1467.477	194	7
	Inter-protein	Hs ABCB7	Hs FECH	VPAGK 450 VAVGGSGSGK	IGGGSPK 133 IWTSK	V495	I126	954.2185	154	3
	Inter-protein	Hs ABCB7	Hs FECH	VPAGK 449 VAVGGSGSGK	IWTSK 138 QGE	V495	I134	822.4553	181	4
	Inter-protein	Hs ABCB7	Hs FECH	GOK 486 VLSGISFE	LSPNTAPHK 158 YYIGFR	G484	L150	978.5152	160	4
	Inter-protein	Hs ABCB7	Hs FECH	GOK 486 VLSGISFE	KPK 68 TGILMLNMGGPE	G484	K66	919.1556	174	5
	Inter-protein	Hs ABCB7	Hs FECH	EYIEGOK 486 VLSGISFE	GMVK 145 LLD	E480	G142	831.0979	169	5
	Inter-protein	Hs ABCB7	Hs FECH	FYEPQK 526 GSIYLAGNIQDVSLE	KPTMK 220 WSTID	F521	K216	1091.227	146	4
	Inter-protein	Hs ABCB7	Hs FECH	POK 526 GSIYLAGNIQDVSLE	YYNQVGRK 216 PTMK	P524	Y209	1141.245	163	5
	Inter-protein	Mm ABCB10	Hs FECH	SK 534 IGTVSQE	IWTSK 138 QEGMVK	S533	I134	773.0668	238	10
	Inter-protein	Mm ABCB10	Hs FECH	QLNPWWLRSK 534 IGTVSQE	IWTSK 138 QGE	Q525	I134	970.1821	201	6
	Inter-protein	Mm ABCB10	Hs FECH	EK 451 TFQGALE	GMVK 145 LLD	E450	G142	601.6501	197	8
	Inter-protein	Mm ABCB10	Hs FECH	GMVLDEK 451 TFQGALE	GMVK 145 LLDE	G445	G142	816.4115	167	5
	Inter-protein	Mm ABCB10	Hs FECH	GMVLDEK 451 TFQGALEFR	QEGGMVK 145 LLD	G445	G142	979.1613	159	6
	Inter-protein	Mm ABCB10	Hs FECH	ALLK 615 NPVK	IGGGSPK 133 IWTSK	A612	I126	711.4246	176	9
	Inter-protein	Mm ABCB10	Hs FECH	ALLK 615 NPVKILLDE	IGGGSPK 133 IWTSKQGE	A612	I126	1048.267	171	6
	Inter-protein	Mm ABCB10	Hs FECH	IAIARALLK 615 NPVKILLD	RIGGGSPK 133 IWTSKQGE	I607	R125	1232.068	167	4
	Inter-protein	Mm ABCB10	Hs FECH	QRIAIARALLK 615 NPVKILLD	RIGGGSPK 133 IWTSKQGE	Q605	R125	1326.788	187	5
	Inter-protein	Mm ABCB10	Hs FECH	IAIARALLK 615 NPVK	QYRRIGGGSPK 133 IWTSKQGE	Q122	I126	1192.021	192	10
	Intra-protein	Mm ABCB10	Mm ABCB10	YLRK 303 L SK	QLLQLAQK 355 EALAR	Y300	Q348	798.8164	120	3
	Intra-protein	Mm ABCB10	Mm ABCB10	AFGK 334 EMTE	MTEVEK 334 YTGR	A331	M336	710.9994	100	2

Supplemental Table 1. List of peptide sequences corresponding to high confidence lysine-lysine crosslinks between ABCB7, FECH and endogenous Abcb10, obtained after in vivo crosslinking on mitochondria isolated from G1E-ER4 cells co-expressing human ABCB7-FLAG and FECH-HA.

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