Supplemental Appendix

Functional Validation of Doxorubicin-Induced Cardiotoxicity-Related Genes

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Supplemental Methods

Variant and gene candidates from GWAS and GCAS, expressed in human heart and in hiPSC cells. We performed a comprehensive PubMed search for both original and review articles investigating genetic risk factors associated with DIC using the following search terms: doxorubicin, anthracycline, single nucleotide polymorphism, cardiotoxicity, chemotherapy, pharmacogenomics, GWAS, candidate gene studies, and gene variants. This search was completed multiple times throughout the study to confirm that all newly identified variants were included, and we terminated the search in November 2020. This search was cross-referenced with several reviews studying AIC[68-72]. The selected candidates were then tested for their expression level in hiPSC-CMs, adult human heart and fetal human heart[73]. The genes with the expression level below 10 TPM in hiPSC-CMs were removed from the study.

Human induced pluripotent stem cell culture. The hiPSC line 19c3 was previously derived from peripheral blood mononuclear cells of a healthy male using Sendai virus (Invitrogen) using chemically defined B8 as previously described[74]. Whole genome sequencing of this hiPSC line showed that it contains harbors 18 SNPs that were significant in their original studies and are associated with genes that we have studied here. Seven of these SNPs are in genes that when knocked out did not affect our *in vitro* DIC assay. Of the remaining 11, three were part of the *SLC28A3* conserved locus and there likely to be protective, and 8 were in risk genes (**Supplemental Table 9**)

Protocols were approved by the Northwestern University Institutional Review Board. This hiPSC line was modified to express an exogenous *TNNT2* promoter-driven Zeocin resistance cassette for cardiomyocyte purification. hiPSCs were passaged at a ratio of 1:15 every 4 days using 0.5 mM EDTA for 6 min at RT, achieving 80% confluence. Cells were routinely maintained in a

low-cost variant of B8 medium[74-76] on 1:800 growth factor reduced Matrigel (Corning) diluted in DMEM (Corning), except for the first 24 h after passage when B8 was supplemented with 2 μM thiazovivin (LC Labs, T-9753), hereby referred to as B8T medium. All pluripotent and reprogramming cells were maintained at 37 ºC in Heracell VIOS 160i humidified incubators (Thermo) with 5% CO_2 and 5% O_2. All cultures were routinely tested for mycoplasma using a MycoAlert PLUS Kit (Lonza) and a Varioskan LUX (Thermo Scientific) plate reader.

CRISPR/Cas9 gRNA design. To generate gene knockouts pairs of CRISPR/Cas9 guide RNAs were designed >50 bp apart to induce a large deletion within the earliest common exon possible of each gene using an online CRISPR design tool (IDT) with high predicted on-target score and minimal predicted off-target effect. DNA oligos (IDT) encoding each gRNA with BbsI ligation overhangs were annealed and inserted into the BbsI restriction site of a pSpCas9(BB)-2A-Puro (PX459, Addgene 62988) plasmid. The constructed gRNA expression plasmids were confirmed by Sanger sequencing (Eurofins) with the LKO1_5_primer (5'-GACTATCATATGCTTACCG-3'). **Supplemental Table 2-4** include all used primers for sgRNA expression vector generation, list of potential off targets and sequencing primers.

CRISPR/Cas9-mediated knockout of candidate genes. hiPSCs were cultured in B8 medium to ~80% confluence. Cells were harvested using 0.5 mM TrypLE for 6 min at RT and resuspended in B8T medium, 5 × 10^6 19c3 cells were electroporated with 5 μg of each gRNA expression vector using an Invitrogen Neon. Cells were maintained for 48 h in B8T medium supplemented with 0.5 μg/mL of puromycin (Gibco). Puromycin resistant individual colonies were picked and expanded ~10 days after electroporation. Genomic DNA was extracted from the cell pellets using a Quick-DNA Miniprep Plus kit (Zymo). Clones with indels were identified by Sanger sequencing (Eurofins) with primers outside of the targeting region. Indels were detected

using an online tool (https://benchling.com). For ABCC5-KO, which Sanger sequencing was not possible, knockout was validated using a PCR showing the lack of band in the knockout hiPSC line. A complete list of primers used for sequencing is listed in **Supplemental Table 5**.

Quantitative Real-time PCR to assess the expression of knocked out genes. RNA was isolated using TRI reagent (Zymo) and Direct-zol RNA microprep kit (Zymo) including on-column DNase digestion to remove genomic DNA. cDNA was produced from 2 μg of total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems). All PCR reactions were performed in triplicate in a 384-well plate format using TaqMan Gene Expression Master Mix in a QuantStudio 5 Real-Time PCR System (both Applied Biosystems). A list of all probes used is included in **Supplemental Table 5**. Relative quantification of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method, normalized to the reference 18S, *ACTB*, or *GAPDH* and untreated control samples as specified in the figure legends.

Cardiac differentiation. Differentiation into cardiomyocytes was performed according to previously described RBAI protocol with slight modifications[77,78]. Briefly, hiPSCs were split at a 1:15 ratio using 0.5 mM EDTA and grown in B8 medium for 4 days reaching ~75% confluence. At the start of differentiation (day 0), B8 medium was changed to R6C, consisting of RPMI 1640 (Corning, 10-040-CM), supplemented with 6 μM of glycogen synthase kinase 3-b inhibitor CHIR99021 (LC Labs, C-6556). On day 1, medium was changed to RPMI 1640 basal medium alone, and on day 2 medium was changed to RBA-C59, consisting of RPMI 1640 supplemented with 2 mg/mL fatty acid-free bovine serum albumin (GenDEPOT, A0100), 200 μg/mL L-ascorbic acid 2-phosphate (Wako, 321-44823) and 0.5 μM Wnt-C59 (Biorbyt, orb181132). Medium was then changed on day 4 and then every other day with RBAI consisting of RPMI 1640 supplemented with 500 μg/mL fatty acid-free bovine serum albumin, 200 μg/mL L-ascorbic acid 2-phosphate,

and 1 µg/mL *E. coli*-derived recombinant human insulin (Gibco, A11382IJ). Contracting cells were observed from day 7 and were treated with 25 µg/mL of Zeocin from day 10 to day 14 to purify cardiomyocytes. On day 20 of differentiation, cardiomyocytes were dissociated using DPBS for 20 min at 37 °C followed by 1:200 Liberase TH (Roche, 5401151001) diluted in DPBS for 20 min at 37 °C, centrifuged at 300 × *g* for 5 min, and filtered through a 100 µm cell strainer (Falcon). Cells were then plated in RBAI+10% Cosmic Calf Serum (Hyclone) for 2 days on 1:800 Matrigel-coated plates for each assay, media was then switched back to RBAI which was changed every 2-3 days and cells were assayed on d30. During differentiation cells were maintained at 5% CO_2 and atmospheric O_2.

Doxorubicin treatment. Doxorubicin hydrochloride (HY-15142, MedChem Express) was resuspended to 10 mM in cell culture-grade water (Corning) and aliquots were stored at -20 ºC. Day 30 hiPSC-CMs were treated for 24 h or 72 h with doxorubicin (0.01-100 µM) diluted in RPMI 1640 medium (no phenol red, Corning) supplemented with 500 µg/mL recombinant human serum albumin (Oryzogen).

384-well plate-based cell viability and reactive oxygen species (ROS) assays. hiPSC-CMs were plated at 25K cells per well in 384-well microplates (Greiner, 781098). To measure cell viability after 72 h of doxorubicin (0.01-100 µM) treatment, CellTiter-Glo 2.0 (Promega) was used per manufacturer's instructions. Luminescence was measured using a VarioSkan Lux Multi-Mode Reader (Thermo Scientific) with an integration time of 0.25 sec. 10 µM staurosporine (MedChemExpress) was used as a positive control. To measure ROS after 24 h of doxorubicin (0.01-100 µM) treatment, ROS-Glo H_2O_2 (Promega) was used according to manufacturer's instructions. 50 µM menadione (MedChemExpress) was used as a positive control. Data were analyzed using Prism 9.0 software (GraphPad) using standard dose-response guidelines.

Western Blotting. Cell lysates (25 µg) were denatured with NuPAGE LDS sample buffer (Invitrogen) containing NuPAGE sample reducing agent (Invitrogen) and protease inhibitor cocktail (Thermo Scientific) and boiled at 70 °C for 10 min. Proteins were separated by gel electrophoresis using NuPAGE Novex 4-12% Bis-Tris precast gels (Invitrogen) and transferred onto nitrocellulose membranes using the iBlot Gel Transfer Device (Invitrogen). Membranes were blocked with 1% non-fat milk, 0.2% Tween 20, in PBS (Corning) for 30 min at room temperature and then probed with appropriate primary antibodies (**Supplemental Table 6** and **7**) overnight at 4 °C. Next, membranes were incubated with IRDye 800 and IRDye 680-conjugated goat anti-rabbit (1:20,000; LI-COR Biosciences, 926-68071) or goat anti-mouse IgG antibody (1:20,000; LI-COR Biosciences, 926-68070) for 1 h at room temperature. Membranes were imaged with an Odyssey IR Imaging System (LI-COR) and immunoreactive bands were visualized using Fiji (ImageJ) software[79]. β-Tubulin antibodies (50 kDa, 1: 3000, Rabbit LI-COR, 926-42211 Biosciences and Mouse Thermo Scientific, MA5-16308) was used as positive controls in all the blots. The western blot worked for 32/36 tested proteins except for ABCC9, GPX3, ABCC4, and NOS3 despite testing two different antibodies in both hiPSCs and hiPSC-CMs. Out of 32 western blots, the correct band size was detected in all except for PRDM2, ZNF521, and SP4.

Immunofluorescent staining. hiPSC-CMs were plated at 50,000 cells per well in 96-well microplates (Greiner Bio, 655209). Cells were fixed with 4% paraformaldehyde (Electron Microscopy Services) in DPBS for 15 min at RT, permeabilized with 1% saponin (Sigma) in DPBS for 15 min at RT, blocked with 3% bovine serum albumin (BSA, Sigma) in DPBS for 30 min at RT, and stained overnight in 3% BSA/1% saponin/DPBS at 4 °C with 1:200 polyclonal rabbit IgG anti-TNNT2 (Abcam, ab45932) and 1:200 monoclonal mouse IgG_1 γH2AX (Sigma, 05-636). Cells were washed and then stained with secondary antibodies 1:1000 Alexa Fluor 594 goat anti-rabbit

IgG and Alexa Fluor 488 goat anti-mouse IgG_1, (all Invitrogen) in 3% BSA/1% saponin/DPBS for 1 hr at RT in the dark. Cells were washed three times. NucBlue (Invitrogen) was added during the last wash. Slides were imaged with a Ti-E inverted fluorescent microscope (Nikon Instruments) and a Zyla sCMOS camera (Andor) using NIS-Elements 4.4 Advanced software.

Flow cytometry. For staining of intracellular markers, day 30 dissociated hiPSC-CMs were fixed with 4% paraformaldehyde (Electron Microscopy Services) in DPBS for 15 min at RT, permeabilized with 1% saponin (Sigma) in DPBS for 15 min at RT, washed with DPBS, and stained for 45 min in in 3% BSA/1% saponin/DPBS at RT with 1:500 mouse monoclonal IgG_1 TNNT2-647 (BD Biosciences, 565744) and washed again with DPBS. Isotype controls mouse IgG_1-647 (BD Biosciences, 565571) was used to establish gating. Human dermal fibroblasts showed no positive staining under these conditions. All cells were analyzed using a CytoFLEX (Beckman Coulter) with CytExpert 2.0 software.

DNA damage assay. After 24 h of doxorubicin treatment, hiPSC-CMs were dissociated, processed with BD Cytofix/Cytoperm fixation/permeabilization kit per manufacturer's instructions, and stained with 1:20 mouse IgG_1 γH2AX-647 (BD Biosciences, 560447) at 4 °C for 30 min in the dark and washed again with DPBS. Isotype control mouse IgG_1-647 (BD Biosciences, 565571) was used to establish gating. Cells were analyzed using a CytoFLEX (Beckman Coulter) with CytExpert 2.0 software and Prism 9.0 software (GraphPad).

Doxorubicin uptake quantification. hiPSC-CMs were plated on 12-well plates (2 $x10^6$ /well). On day 30, cells were treated for 24 h with doxorubicin or RPMI 1640 medium (no phenol red, Corning) supplemented with 500 µg/mL recombinant human serum albumin (Oryzogen) as negative control. Cells were then treated with doxorubicin (1 and 3 µM). Cellular autofluorescence was assayed before doxorubicin treatment and serves as baseline fluorescence. Doxorubicin

intrinsic fluorescence-PE was measured 1 h and 3 h post doxorubicin treatment and normalized to baseline fluorescence. All cells were stained with NucRed Live ReadyProbes Reagent (ThermoFisher) to monitor cell viability. Cells were analyzed using a CytoFLEX (Beckman Coulter) with CytExpert 2.0 software and Prism 9.0 software (GraphPad).

Iron uptake quantification. On day 30, hiPSC-CMs were treated for 24 h with 100 μM of iron sulfate hydrate (CAS 7782-63-0, GTI laboratories) or RPMI 1640 medium (no phenol red, Corning) supplemented with 500 μg/mL recombinant human serum albumin (Oryzogen) and transferrin (T3705-1G, Sigma) as negative control. The following day, cells were dissociated using Liberase, and CellTrace calcein red-orange AM (C34851, ThermoFisher) was used according to manufacturer instructions to quantify iron uptake by the cells. All cells were stained with NucBlue Live ReadyProbes Reagent (ThermoFisher) to monitor cell viability. Cells were analyzed using a CytoFLEX (Beckman Coulter) with CytExpert 2.0 software and Prism 9.0 software (GraphPad). The iron uptake was inversely correlated with CellTrace calcein red-orange AM staining.

Ca^{+2} handling properties. We examined Ca^{2+} handling properties using Vala Sciences KIC Imager. Briefly, hiPSC-CMs were dissociated at day 30 of differentiation, plated at 75,000 in 96-well plates, and left for one week to recover. On the day of the experiment, cells were incubated with 1 mM of Cal-520 AM Ca^{2+} reporter (AAT Bioquest, 21130) and DAPI for nucleus staining for 1 hr., the reporter was then washed, and cells were imaged. Data was analyzed using CyteSeer Analysis software (Vala Sciences). Ca^{2+} transients were analyzed as the average of all Ca^{2+} transients collected from all single cells within each well. We quantified Ca^{+2} full width at half maximum (msec), calcium transient duration at 75% (msec) and decay time (msec).

Impedance measurements. hiPSC-CMs were dissociated using Liberase TH as described in cardiac differentiation section. 65,000 cells were plated in 96-well MEA plates (CardioExcyte

96, Nanion). A week after dissociation, impedance was measured at 37 °C in a 5% CO_2 environment. Data acquisition was performed using AxIS Navigator software (Axion Biosystem). Data analysis was achieved using the built-in functions in the Cardiac Analysis Tool (Axion Biosystem) and presented as beat rate (beat/min), Pulse width 50% (sec), upstroke velocity (ohm/sec) and relaxation velocity (ohm/sec).

Statistical methods. Data were presented as mean ± standard error of mean (SEM). Comparisons among more than groups were conducted using one- or two-way analysis of variance (ANOVA), and between two groups using an unpaired two-tailed Student's t-test or Mann-Whitney U test, depending in data distribution. The normal distribution was tested using GraphPad Prism 9. The *P*-values are adjusted based on the number of the multiple testing and significant differences defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

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Supplemental Table 1: Genes and variants associated with DIC. Provided as an .xlsx file

Supplemental Table 2: gRNAs sequences used for CRISPR/Cas9 directed knockout of AIC-related genes.

Gene	Primer	Sequence
ABCC1-KO	P1-F	5'-TGGGCTGACCAGAAACACTG-3'
	P1-R	5'-CAGTGTTTCTGGTCAGCCCAC-3'
	P2-F	5'-ATCCACAGCAAAAATCCCA-3'
	P2-R	5'-TGGGATTTTTGCTGTGGATC-3'
ABCB4-KO	P1-F	5'-GAAGTCGCCCTCCGCGCTCGT-3'
	P1-R	5'-ACGAGCGCGGAGGGCGACTTC-3'
ABCC2-KO	P1-F	5'-GCACGTGGAGAAGCTGCCAG-3'
	P1-R	5'-CCTGGCAGCTTCTCCACGTG-3'
	P2-F	5'-CTCATTCCTGGACAGTCCG-3'
	P2-R	5'-CCGGACTGTCCAGGAATGAG-3'
ABCC5-KO	P1-F	5'-GAAGATGAAGGATATCGACAT-3'
	P1-R	5'-ATGTCGATATCCTTCATCTTC-3'
	P2-F	5'-GGGTCAGCTTGGGCGAGTTC-3'
	P2-R	5'-GAACTCGCCCAAGCTGACCC-3'
ABCC9-KO	P1-F	5'-CCATGGGGGAGATGACTCTG-3'
	P1-R	5'-CAGAGTCATCTCCCCCATGGC-3'
ABCC10-KO	P1-F	5'-CTTGTGACAGAGCTGCTGAG-3'
	P1-R	5'-CTCAGCAGCTCTGTCACAAGC-3'
ABCC2-KO	P1-F	5'-CACGTGGAGAAGCTGCCAGG-3'
	P1-R	5'-CCTGGCAGCTTCTCCACGTGC-3'
	P2-F	5'-CTCATTCCTGGACAGTCCGG-3'
	P2-R	5'-CCGGACTGTCCAGGAATGAGC-3'
ATP2B1-KO	P1-F	5'-AATTACGCTCGCAGAGCTG-3'
	P1-R	5'-CAGCTCTGCGAGCGTAATTC-3'
	P2-F	5'-AACAACTCAGTTGCTTACAG-3'
	P2-R	5'-CTGTAAGCAACTGAGTTGTTC-3'
CAT-KO	P1-F	5'-GTCTGGTCGCTGGCGGGATCC-3'
	P1-R	5'-GGATCCCGCCAGCGACCAGAC-3'
CBR1-KO	P1-F	5'-CCGTACTCCTTGCGCAGGAAC-3'
	P1-R	5'-CCTGGCAGCTTCTCCACGTGC-3'
	P2-F	5'-TACAGCAGCTGCAGGCGGA-3'
	P2-R	5'-TCCGCCTGCAGCTGCTGTAC-3'
CBR3-KO	P1-F	5'-CAACGTACTGGTCAACAACG-3'
	P1-R	5'-CGTTGTTGACCAGTACGTTGC-3'
	P2-F	5'-AGTCGTCGATGTCCAGTTGG-3'
	P2-R	5'-CCAACTGGACATCGACGACTC-3'
CELF4-KO	P1-F	5'-GGCCACGTTAGCAAACGGAC-3'
	P1-R	5'-GTCCGTTTGCTAACGTGGCC-3'
CYBA-KO	P1-F	5'-TAGGCACCAAAGTACCACT-3'
	P1-R	5'-AGTGGTACTTTGGTGCCTAC-3'
COL1A2-KO	P1-F	5'-TGCTCAGCTTTGTGGATACG-3'
	P1-R	5'-CGTATCCACAAAGCTGAGCAC-3'
CYP2J2-KO	P1-F	5'-CCCAAAGAACTACCCGCCG-3'
	P1-R	5'-CGGCGGGTAGTTCTTTGGGC-3'
	P2-F	5'-AGGATGGACCACTGCCCAG-3'
	P2-R	5'-CTGGGCAGTGGTCCATCCTC-3'

ERBB2-KO	P1-F	5'-CTGGCATTGGTGGGCAGGT-3'
	P1-R	5'-ACCTGCCCACCAATGCCAGC-3'
	P2-F	5'-CCGGCACAGACATGAAGCTG-3'
	P2-R	5'-CAGCTTCATGTCTGTGCCGGC-3'
ERCC2-KO	P1-F	5'-GCGGGAGCTCAAACGCACGC-3'
	P1-R	5'-GCGTGCGTTTGAGCTCCCGC-3'
GSTP1-KO	P1-F	5'-GGGAAATAGACCACGGTGTA-3'
	P1-R	5'-TACACCGTGGTCTATTTCCC-3'
GPX3-KO	P1-F	5'-CAGGAGCAGGGAAAGCAGGC-3'
	P1-R	5'-GCCTGCTTTCCCTGCTCCTGC-3'
GSTM1-KO	P1-F	5'-GCGGGAGCTCAAACGCACGC-3'
	P1-R	5'-CCATCGTGTACTTCTTTTCC-3'
HAS3-KO	P1-F	5'-GAACTGGTAGCCCGTCACAT-3'
	P1-R	5'-ATGTGACGGGCTACCAGTTC-3'
HFE-KO	P1-F	5'-GCCGCGGTCTGCAAAAGCATC-3'
	P1-R	5'-GATGCTTTTGCAGACCGCGGC-3'
HNMT-KO	P1-F	5'-GCTGCATGCACTGGTGTTCCG-3'
	P1-R	5'-CGGAACACCAGTGCATGCAGC-3'
MLH1-KO	P1-F	5'-TGACTGCAGCTTGTACCCCC-3'
	P1-R	5'-GGGGGTACAAGCTGCAGTCAC-3'
MYH7-KO	P1-F	5'-GTTCTCTGACTTGCGCAGGTA-3'
	P1-R	5'-TACCTGCGCAAGTCAGAGAAC-3'
NOS3-KO	P1-F	5'-GCCTTGGGCTGTGCGGCAAGC-3'
	P1-R	5'-GCTTGCCGCACAGCCCAAGGC-3'
POR-KO	P1-F	5'-GTACTTCTTCGGCCACCGCCT-3'
	P1-R	5'-AGGCGGTGGCCGAAGAAGTAC-3'
PRDM2-KO	P1-F	5'-GTACCCGAACATGTGCTGCG-3'
	P1-R	5'-CGCAGCACATGTTCGGGTAC-3'
	P2-F	5'-GCCGCTTCTAATCGCTCGTC-3'
	P2-R	5'-GACGAGCGATTAGAAGCGGC-3'
PLCE1-KO	P1-F	5'-GTTTCAGCATCATTCGTCCAG-3'
	P1-R	5'-TGGACGAATGATGCTGAAAC-3'
	P2-F	5'-AGTATCGCGCCACCCTCCAA-3'
	P2-R	5'-TTGGAGGGTGGCGCGATACT-3'
RAC2-KO	P1-F	5'-GCTCTCCGGGAAAGGCGTTGG-3'
	P1-R	5'-CCAACGCCTTTCCCGGAGAGC-3'
RARG-KO	P1-F	5'-GACTTTTGGAGGCCCAGTGG-3'
	P1-R	5'-GAGGCCATCTCCTTGGGGA-3'
RIN3-KO	P1-F	5'-AATGGTGTATTCGAGCACCT-3'
	P1-R	5'-AGGTGCTCGAATACACCATTC-3'
SLC22A17-KO	P1-F	5'-GCCCGTAGTGGCAATGCAGCG-3'
	P1-R	5'-CGCTGCATTGCCACTACGGGC-3'
	P2-F	5'-GCTGAGCGAAACCGGCCCCAT-3'
	P2-R	5'-ATGGGGCCGGTTTCGCTCAGC-3'
SLC28A1-KO	P1-F	5'-ATGGACTCTCTTCGTCTCGA-3'
	P1-R	5'-TCGAGACGAAGAGAGTCCATC-3'
SLC28A3-KO	P1-F	5'-GCTGAGGGCTACAGCAACGT-3'
	P1-R	5'-ACGTTGCTGTAGCCCTCAGC-3'
SP4-KO	P1-F	5'-TAATGGAATGCAGAATGCAC-3'
	P1-R	5'-GTGCATTCTGCATTCCATTAC-3'
SPG7-KO	P1-F	5'-GCCGGGTCCTCGGCCGCTGTG-3'
	P1-R	5'-CACAGCGGCCGAGGACCCGGC-3'

WDR4-KO	P1-F	5'-GTCGGCAGTGAGGATGAAG-3'
	P1-R	5'-CTTCATCCTCACTGCCGACC-3'
ZNF521-KO	P1-F	5'-CGAGGGACCAGTCATGTCAG-3'
	P1-R	5'-CTGACATGACTGGTCCCTCGC-3'

Supplemental Table 3: Potential off targets of each gRNA used for CRISPR/Cas9 directed knockout of AIC-related genes. Provided as an .xlsx file

Supplemental Table 4: Confirmation of the knockout clones in isogenic hiPSC using Sanger sequencing. The codon and protein changes introduced by CRISPR/Cas9 at target loci.

Gene knockout line	Codon changes	Protein changes
RARG-KO	heterozygote, one allele insertion 1 bp (T), one allele deletion 8 bp	allele with insertion 1 bp (T): frameshift premature stop codon, WT 443 aa, KO 39 aa allele with deletion 8 bp: frameshift premature stop codon, WT 443 aa, KO 35 aa
PRDM2-KO	homozygote insertion 1 bp (T)	frameshift premature stop codon, WT 1718 aa, KO 37 aa
WDR4-KO	homozygote deletion 3 bp in frame (TTC)	new protein of 411 aa instead of 412 aa, RNA expression decreased by 87%
ZNF521-KO	homozygote insertion 1 bp (A)	frameshift premature stop codon, WT 1311 aa, KO 398 aa
SP4-KO	homozygote insertion 52 bp	frameshift premature stop codon, WT 784 aa, KO 573 aa
RIN3-KO	heterozygote deletion 61 bp	frameshift premature stop codon, WT 985 aa, KO 118 aa
SLC28A3-KO	homozygote deletion 8 bp	frameshift premature stop codon, WT 691aa, KO 18aa
ABCC9-KO	heterozygote insertion 1 bp (T)	frameshift premature stop codon, WT 1549aa, KO 419 aa
HNMT-KO	homozygote deletion 10 bp	frameshift premature stop codon, WT 292aa, KO 39a
SLC22A17-KO	homozygote insertion 1 bp (T)	frameshift premature stop codon, WT 631 aa, KO 133 aa
GPX3-KO	homozygote deletion 14 bp	frameshift premature stop codon, WT 226 aa, KO 11 aa
SLC28A1-KO	heterozygote deletion 9 bp	different amino acid sequence starting from aa 7
MYH7-KO	heterozygote deletion 38 bp	frameshift premature stop codon, WT 1935 aa, KO 18 aa
CYP2J2-KO	homozygote deletion 94 bp	frameshift premature stop codon, WT 502 aa, KO 40 aa
CBR3-KO	homozygote insertion 27 bp	no protein due to removal of ATG
COL1A2-KO	heterozygote, one allele insertion 1 bp (T), one allele deletion 8 bp	allele with insertion 1 bp (T): frameshift premature stop codon, WT 1366 aa, KO 37 aa allele with deletion 8 bp: frameshift premature stop codon, WT 1366 aa, KO 34 aa
SPG7-KO	homozygote insertion 1 bp (T)	frameshift premature stop codon, WT 794 aa, KO 72 aa
HFE-KO	homozygote deletion 18 bp	loss of 6 aa
ABCC10-KO	heterozygote, one allele deletion 1 bp (C), one allele insertion 1 bp (T)	allele with deletion 1bp (C): frameshift premature stop codon, WT 1492 aa, KO 465 aa allele with 1bp insertion (T): frameshift premature stop codon, WT 1492 aa, KO 690 aa
ABCB4-KO	heterozygote, one allele insertion 1 bp (A), one allele deletion 18 bp	allele with insertion 1 bp (A): frameshift premature stop codon, WT 1279 aa, KO 21 aa allele with deletion 18 bp: a shorter protein, WT 1279 aa, KO 1273 aa
GSTP1-KO	heterozygote, one allele deletion 1bp (C), one allele insertion 1bp (A)	allele with deletion 1 bp (C): frameshift premature stop codon, WT 210 aa, KO 33 aa allele with insertion 1bp (A): frameshift premature stop codon, WT 210 aa, KO 99 aa
PLCE1-KO	homozygote deletion 104 bp	frameshift premature stop codon, WT 2302 aa, KO 459 aa

GSTM1-KO	homozygote insertion 1 bp (A)	frameshift premature stop codon, WT 218 aa, KO 39 aa
CELF4-KO	homozygote deletion 8 bp	frameshift premature stop codon, WT 486 aa, KO 10 aa
CYBA-KO	homozygote deletion 1 bp (G)	frameshift premature stop codon, WT 195 aa, KO 72 aa
HAS3-KO	homozygote deletion 1 bp (C)	frameshift premature stop codon, WT 553 aa, KO 76 aa
MLH1-KO	homozygote deletion 22 bp	frameshift premature stop codon, WT 756 aa, KO 499 aa
POR-KO	homozygote deletion 1bp (C)	frameshift premature stop codon, WT 677 aa, KO 24 aa
RAC2-KO	homozygote 1 bp deletion (A)	frameshift premature stop codon, WT 192 aa, KO 43 aa
CAT-KO	heterozygote deletion 12 bp plus 1 bp insertion	frameshift premature stop codon, WT 527 aa, KO 20 aa
NOS3-KO	homozygote insertion 1 bp (A)	frameshift premature stop codon, WT 1203 aa, KO 36 aa
ABCC2-KO	heterozygote, one allele deletion 89 bp, one allele deletion 81 bp	allele with deletion 89 bp: frameshift premature stop codon, WT 1545 aa, KO 22 aa allele with deletion 81 bp: a different protein, WT 1545 aa, KO 1518 aa
ATP2B1-KO	heterozygote deletion 67 bp	frameshift premature stop codon, WT 1220 aa, KO 40 aa
CBR1-KO	homozygote deletion 86 bp	frameshift premature stop codon, WT 277 aa, KO 69 aa
ERBB2-KO	homozygote deletion 97 bp	frameshift premature stop codon, WT 1255 aa, KO 58 aa

Supplemental Table 5: Sequencing primers used to verify knockouts of AIC-related genes in hiPSCs.

Gene	Primer	Sequence
ABCC1-KO	P1-F	5’-GGGCGGTCTGTTGTAGGATA-3’
	P1-R	5’-GGTCACAGCCAGCTCCTACT-3’
	P2-F	5’-ATCCACAGCAAAAATCCCA-3’
	P2-R	5’-TGGGATTTTTGCTGTGGATC-3’
ABCB4-KO	P1-F	5’- CGAGGTTCGAGGTGAGAGAG -3’
	P1-R	5’- CCAAAAGGAGCCTCAGTGAC -3’
ABCC2-KO	P1-F	5’- GTTTTTGGAGGGTGGGTTG-3’
	P1-R	5’- ACCTGGGACAGCTGCTTAAA-3’
ABCC5-KO	P1-F	5’- TCCCTTAGAGTTGGGAGAAGG-3’
	P1-R	5’- CTCCCAAAGTGCTGGGTTTA-3’
ABCC9-KO	P1-F	5’- TGGCTGATTTAAGAAGATGATCC-3’
	P1-R	5’- GACGGGGTAGGGCAGATATT-3’
ABCC10-KO	P1-F	5’- AGGATTTGAAGGGCAGGATT-3’
	P1-R	5’- GATCCCTCCCTCCTTCTCAG-3’
ABCC2-KO	P1-F	5’-TGTGTGAAAGCAGTGGGATG-3’
	P1-R	5’-TCCACACCAGAACAGTTTGC-3’
	P2-F	5’-GCAAACTGTTCTGGTGTGGA-3’
	P2-R	5’-TGTCTCTACTGTGCACCAAGG-3’
ATP2B1-KO	P1-F	5’- AAATGTTGCTGCTGATGCTG-3’
	P1-R	5’- TCATCCCGCCAATCTAAAAC-3’
CAT-KO	P1-F	5’- TGGGTATCTCCGGTCTTCAG-3’
	P1-R	5’- CAGTTGGCAAAAGTGCAAAA-3’
CBR1-KO	P1-F	5’- CTGAGCCAGGTCTGTTCTCC-3’
	P1-R	5’- CAGCCAGGGAAACACAAAGT-3’
CBR3-KO	P1-F	5’- TTGACACTAGCTGGGCTCCT-3’
	P1-R	5’- GTTTTCCTGCACACAACAGC-3’
CELF4-KO	P1-F	5’- CGGAGAGCGAGGTGTAGAGA -3’
	P1-R	5’- GGCTTCCTCTCGCTTAGTCC -3’
CYBA-KO	P1-F	5’-ACAGTGCCTGACCCACTTCT-3’
	P1-R	5’-GGAGGCAAACAGCTCACTG-3’
COL1A2-KO	P1-F	5’- GAGGTTTCGGCTAAGTTGGA-3’
	P1-R	5’- TGACTTCCTCCACCACATTG-3’
CYP2J2-KO	P1-F	5’-CTCCTAGCCTGGCCTTTTCT-3’
	P1-R	5’-CAGCGTTAGCCACACCTCTT-3’
ERBB2-KO	P1-F	5’-GCACAGGGTGGGCCTAGTCAGA-3’
	P1-R	5’-TGACCTCGGCCAGCCACGTTAT-3’
ERCC2-KO	P1-F	5’- CTGAGGGGACGGGAACTGA -3’
	P1-R	5’- CCAGACGTCCTGCAATCTGT -3’
GSTP1-KO	P1-F	5’-TTCGCCACCAGTGAGTACG-3’
	P1-R	5’-CACACGACGGAGGGATAAGG -3’

GPX3-KO	P1-F	5’- CAGGCGACCCTGAGTGTG-3’
	P1-R	5’- TTCTTCAGGACCAGGACCAC-3’
GSTM1-KO	P1-F	5’- TAGGGACCGTTCCTCTTCAG-3’
	P1-R	5’-CAGGGTTCAGGGACAAAGAA-3’
HAS3-KO	P1-F	5’- ACCCTTCATCTCCTGCCTTC-3’
	P1-R	5’- ATGATGCACGAGAAGGTGCT-3’
HFE-KO	P1-F	5’-TTACTGGGCATCTCCTGAGC-3’
	P1-R	5’-AACTGCACAGCTGACATTGG-3’
HNMT-KO	P1-F	5’-TGGCTTTGCTGACAAAACAG-3’
	P1-R	5’-GCTGAGCGAGACCCATCTAT-3’
MLH1-KO	P1-F	5’- AGTTGCTTGCTCCTCCAAAA-3’
	P1-R	5’- GAAAATTGGTGAAATGGCTGA-3’
MYH7-KO	P1-F	5’- AGCATGGTGCTAGGTTTTGG-3’
	P1-R	5’- TGGTGAGTGACAGGGCAATA-3’
NOS3-KO	P1-F	5’- CCTCCACTGCTTTTCAGAGG-3’
	P1-R	5’- CCTGGTGGCTCTGTCTTCTC-3’
POR-KO	P1-F	5’-CCTCTGCTGACATCTGCTGT-3’
	P1-R	5’-CTGAGAGGCGGCACTTACAA-3’
PRDM2-KO	P1-F	5’- CCAGCTTCAGGTTTCGGTTA-3’
	P1-R	5’- GAGGAGGACACTCAGGCAAG-3’
	P2-F	5’- GGTACGTGGCTGGTACCCTA-3’
	P2-R	5’- TGGCTTCTCATCACACCGTA-3’
PLCE1-KO	P1-F	5’- GAGTGTTTGCACTTGGAGCA-3’
	P1-R	5’- GGGGATTTTAATAAGGGACCA-3’
RAC2-KO	P1-F	5’- TGGACCCTGAAGTCTCCACT-3’
	P1-R	5’- CTACCCCTTCCTCCATACCC-3’
RARG-KO	P1-F	5’- GCAGCACAGAGGGAGAAGAC-3’
	P1-R	5’- TGGGGTGCCAACTCTTTTAC-3’
RIN3-KO	P1-F	5’- GGGCAAATGAGAAACTGAGC-3’
	P1-R	5’- CTTCAATGTGGCCATGAGAA-3’
SLC22A17-KO	P1-F	5’- CCTGACTGCCTTCCTAGCC-3’
	P1-R	5’- GGATGTGAGAAGGGTGCAG-3’
SLC28A1-KO	P1-F	5’-GGCCTCCCTTTCAGCGTT-3’
	P1-R	5’-CAAAGAGGCTGAGGGGTCAG3’
SLC28A3-KO	P1-F	5’-AAACTGAAGCAAGCTGTGCC-3’
	P1-R	5’- TTTGTCAACCCAGAAGAGCCC-3’
SP4-KO	P1-F	5’ACTCAGGCTCAAGTTGTAACAACCC-3’
	P1-R	5’AAAGGCTGCTGCTGGATGGTCT-3’
SPG7-KO	P1-F	5’-ACGAGGTAGACGGGCTCAG-3’
	P1-R	5’-CAGACGGGTTGGGAAAGTC-3’
WDR4-KO	P1-F	5’-AGCCTGCTCTAGCACTGAGG-3’
	P1-R	5’-AGAGTGAACCCCACCCTTTC-3’
ZFN521-KO	P1-F	5’- ATTCAAGAGGGCCCAACTCT-3’
	P1-R	5’- CAAGCACTGGAGACCCAAAT-3’

Supplemental Table 6: Confirmation of the knockout clones in isogenic hiPSC using Western blotting. The table contains the antibody used to detect each target protein, the host animal, dilution of the antibody and the cell type it was tested. Ms: Mouse, Rb: Rabbit, KO: Knockout.

Gene knockout line	Antibody	Cell type
RARG-KO	Origene, TA308949, Rb, 1:500	KO confirmed in hiPSCs
PRDM2-KO	Abcam, ab305105, Rb, 1:500	KO confirmed in hiPSCs
WDR4-KO	Abcam, ab169526, Rb, 1:500	KO confirmed in hiPSCs
ZNF521-KO	ThermoFisher Scientific, PA5-34388, Rb, 1:100	KO confirmed in hiPSCs
SP4-KO	Abcam, ab151777, Rb, 1:500	KO confirmed in hiPSCs
RIN3-KO	Abcam, ab64838, Rb, 1:500	KO confirmed in hiPSCs
SLC28A3-KO	Santa Cruz Biotechnology, sc-134529, Rb, 1:100	KO confirmed in hiPSC-CMs
ABCC5-KO	Abcam, ab180724, Rb, 1:500	KO confirmed in hiPSCs
HNMT-KO	Santa Cruz Biotechnology, sc-374306, Ms, 1:500	KO confirmed in hiPSCs
SLC22A17-KO	Abcam, ab124506, RB, 1:500	KO confirmed in hiPSC-CMs
SLC28A1-KO	Santa Cruz Biotechnology, sc-515874, Ms, 1:100	KO confirmed in hiPSCs
MYH7-KO	Santa Cruz Biotechnology, sc-53090, Ms, 1:500	KO confirmed in hiPSC-CMs
CYP2J2-KO	Abcam, ab151996, Rb, 1:500	KO confirmed in hiPSC-CMs
CBR3-KO	Santa Cruz Biotechnology, sc-374393, Ms, 1:100	KO confirmed in hiPSCs
COL1A2-KO	Abcam, ab308455, Rb, 1:500	KO confirmed in hiPSCs
SPG7-KO	Abcam, ab305255, Rb, 1:500	KO confirmed in hiPSCs
HFE-KO	Abcam, ab133639, Rn, 1:500	KO confirmed in hiPSCs
ABCC10-KO	ThermoFisher Scientific, PA5-101678, Rb, 1:500	KO confirmed in hiPSC-CMs
GSTP1-KO	Abcam, ab138491, Rb, 1:500	KO confirmed in hiPSCs
PLCE1-KO	ThermoFisher Scientific, PA5-100856, Rb, 1:500	KO confirmed in hiPSCs
GSTM1-KO	Santa Cruz Biotechnology, sc-517262, Ms, 1:100	KO confirmed in hiPSCs
CELF4-KO	Abcam, ab171740, Rb, 1:500	KO confirmed in hiPSC-CMs
CYBA-KO	Abcam, ab80896, Ms, 1:500	KO confirmed in hiPSCs
HAS3-KO	Abcam, ab170872, Rb, 1:500	KO confirmed in hiPSCs
MLH1-KO	Abcam, ab92312, Rb, 1:500	KO confirmed in hiPSCs
POR-KO	Santa Cruz Biotechnology, sc-25270, Ms, 1:500	KO confirmed in hiPSCs
RAC2-KO	Abcam, ab191527, Rb, 1:500	KO confirmed in hiPSCs
CAT-KO	Cell Signaling Technology, 12980T, Rb, 1:100	KO confirmed in hiPSCs
ABCC2-KO	Abcam, ab3373, Ms, 1:20	KO confirmed in hiPSCs
ATP2B1-KO	Abcam, ab190355, Rb, 1:500	KO confirmed in hiPSCs
CBR1-KO	Santa Cruz Bio., sc-390554, Ms, 1:100	KO confirmed in hiPSCs
ERBB2-KO	ThermoFisher Scientific, ma5-13675, Ms, 1:500	KO confirmed in hiPSCs

Supplemental Table 7: List of proteins that western blot did not work on them. The table contains the antibodies tested for each target protein, the host animal, and the dilution of the antibody. Ms: Mouse, Rb: Rabbit.

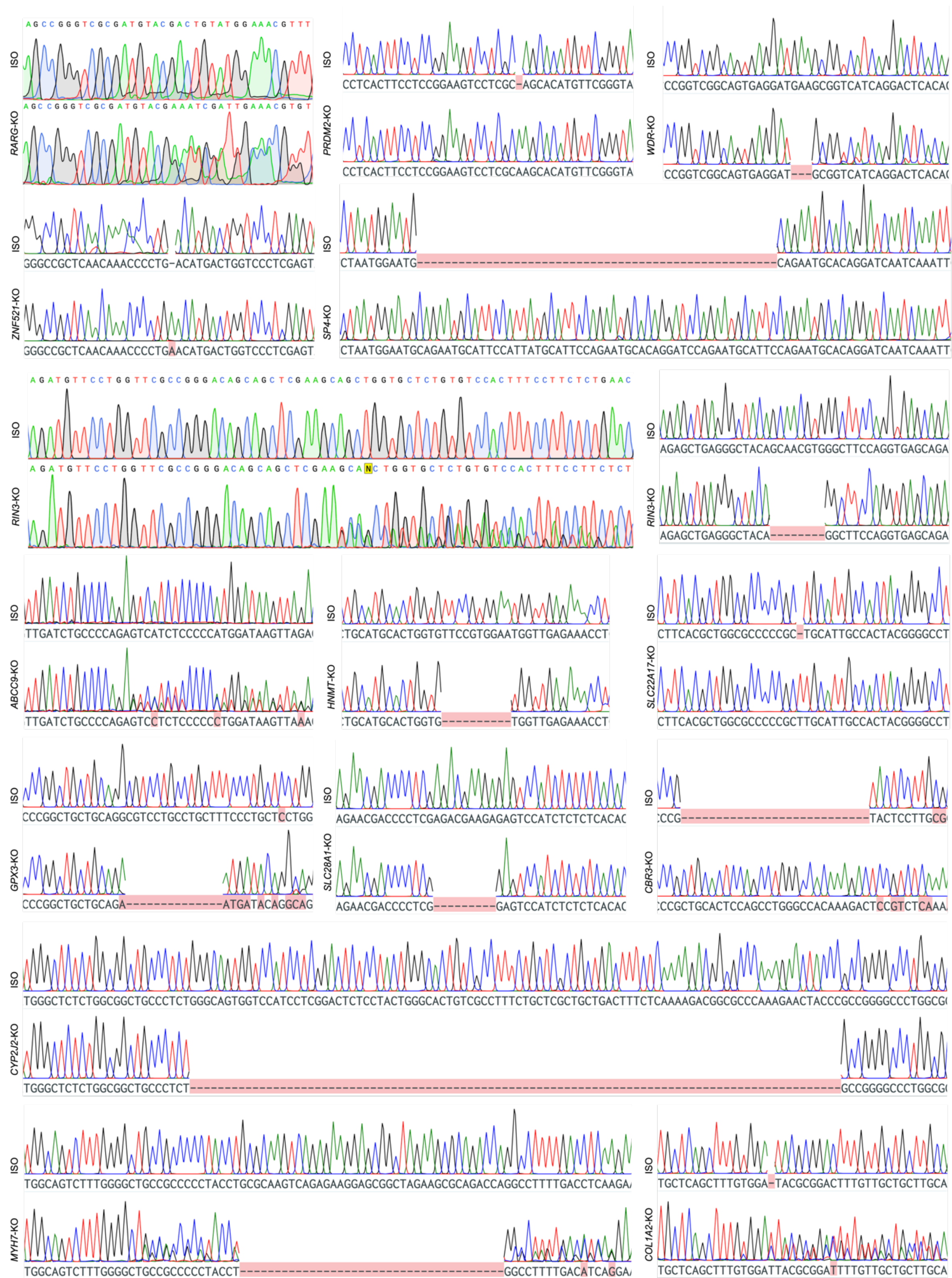
Protein	Antibody 1	Antibody 2
ABCC9	Abcam, ab174629, Ms, 1:100	BD Biosciences, 550429, Ms, 1:500
GPX3	R&D Systems, AF4199, Goat, 1:500	Abcam, ab275965, Rb, 1:500
ABCC4	Abcam, ab191058, Rb, 1:500	Santa Cruz Biotechnology, sc-58221, Ms, 1:500
NOS3	BD Biosciences, 610296, Ms, 1:100	Abcam, ab199956, Rb, 1:100

Supplemental Table 8: TaqMan probes used to quantify the mRNA expression of AIC - related genes knockouts.

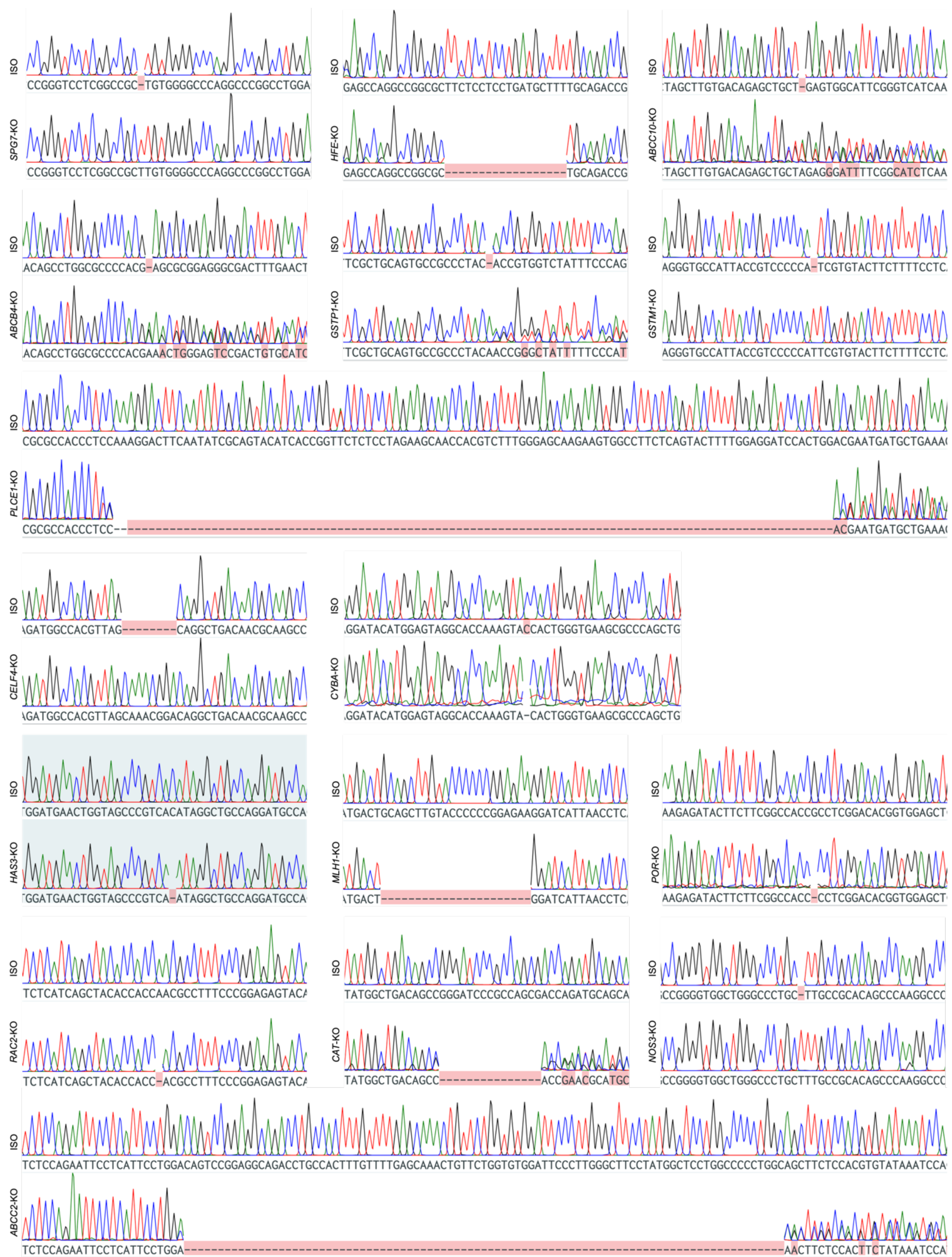
Gene	Assay ID
18S	Hs99999901_s1
ABCB4	Hs00983957_m1
ABCC10	Hs00375716_m1
ABCC2	Hs00166123_m1
ABCC5	Hs00981089_m1
ABCC9	Hs00245832_m1
ACTB	Hs01060665_g1
ATP2B1	Hs00155949_m1
CAT	Hs00156308_m1
CBR1	Hs00156323_m1
CBR3	Hs01025917_m1
CELF4	Hs00252384_m1
COL1A2	Hs01028970_m1
CYBA	Hs00609145_m1
CYP2J2	Hs00356035_m1
ERBB2	Hs01001580_m1
GAPDH	Hs02786624_g1
GPX3	Hs01078670_g1
GSTM1	Hs01683722_gH
GSTP1	Hs00943350_g1
HAS3	Hs00193436_m1
HFE	Hs05045803_s1
HNMT	Hs02759756_s1
MLH1	Hs00979919_m1
MYH7	Hs01110632_m1
NOS3	Hs01574665_m1
PLCE1	Hs00275279_m1
POR	Hs01016332_m1
PRDM2	Hs00202013_m1
RAC2	Hs00427439_g1
RARG	Hs01559234
RIN3	Hs01112079_m1
SLC22A17	Hs01033111_m1
SLC28A1	Hs00984391_m1
SLC28A3	Hs00910436_m1
SP4	Hs00162095_m1
SPG7	Hs00275795_m1
WDR4	Hs00902287_g1
ZFN521	Hs01031127_m1

Supplemental Table 9: SNPs present in hiPSC line 19-3 that were significant in their original studies and are associated with genes in this study. Grey text indicates genes that were found not to influence in vitro DIC phenotype in this study.

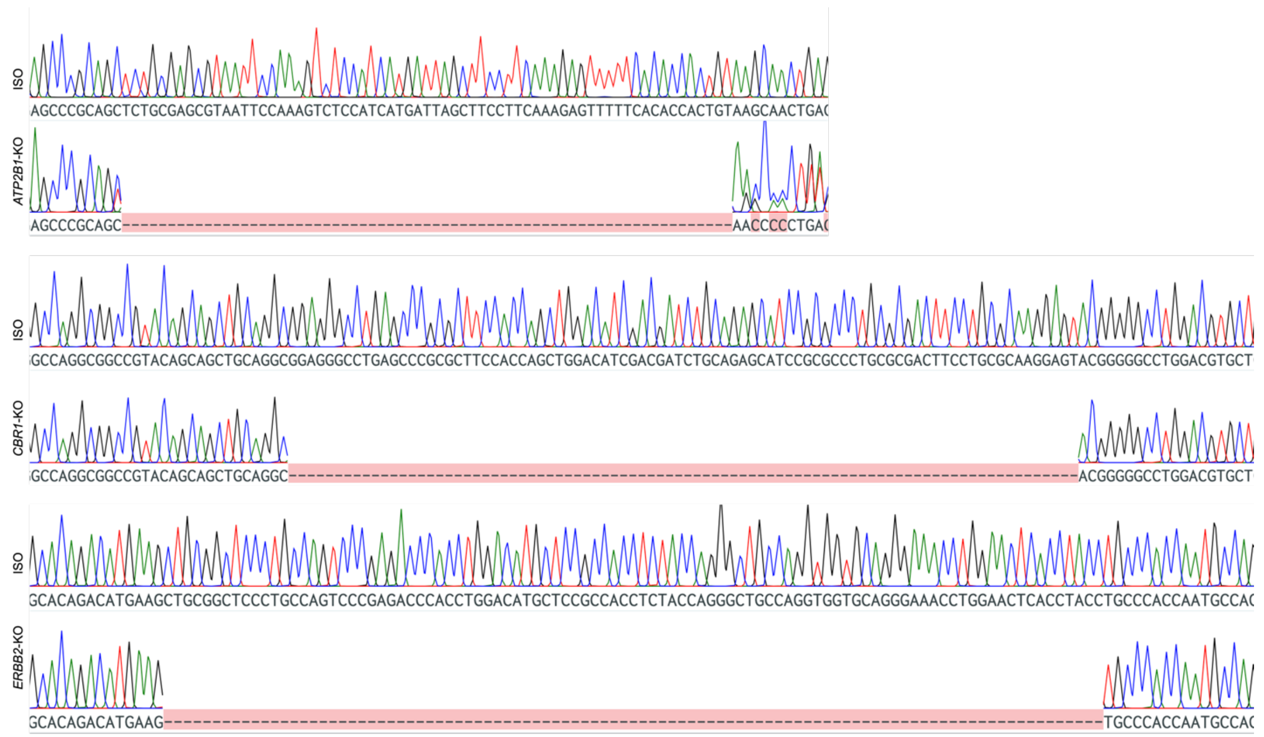
CHROM	POS	rsID	REF	ALT	19-3	Variation	Impact	GENE	MAF.gnomAD.genome.ALL	MAF.gnomAD.genome.NFE	MAF.1000 genome	CADD score	Clinvar
chr10	99781296	rs1885301	A	G	A/G	upstream gene variant		*ABCC2*	0.5905	0.5816	0.619409	.	.
chr21	36146408	rs1056892	G	A	G/A	missense variant	loss of function	*CBR3*	0.3836	0.3541	0.427117	17.19	.
chr7	94413927	rs42524	C	G	C/G	missense variant	loss of function	*COL1A2*	0.8092	0.7632	0.821685	16.99	Benign
chr16	88646828	rs4673	A	G	A/G	missense variant	loss of function	*CYBA*	0.6663	0.6728	0.664337	23.8	Benign
chr11	67585218	rs1695	A	G	A/G	missense variant	loss of function	*GSTP1*	0.3537	0.3351	0.352636	0.003	Benign
chr3	36993455	rs1800734	G	A	G/A	5 prime UTR variant		*MLH1*	0.2328	0.2231	0.320487	.	Benign
chr7	75976791	rs13240755	G	A	G/A	upstream gene variant		*POR*	0.5773	0.6704	0.492412	.	.
chr7	75960585	rs2868177	A	G	A/G	intron variant		*POR*	0.3527	0.3166	0.39996	.	.
chr7	75978290	rs4732513	C	T	C/T	upstream gene variant		*POR*	0.584	0.6704	0.495208	.	.
chr7	75971851	rs6953065	G	A	G/A	intron variant		*POR*	0.3327	0.3914	0.28155	.	.
chr22	37236730	rs13058338	T	A	T/A	downstream gene variant		*RAC2*	0.1957	0.2456	0.159944	.	.
chr14	92663465	rs9323880	C	T	C/T	intron variant		*RIN3*	0.3293	0.3652	0.295327	.	.
chr9	84286011	rs7853758	G	A	G/A	synonymous variant		*SLC28A3*	0.1878	0.1488	0.202676	.	Benign
chr9	84294635	rs885004	G	A	G/A	downstream gene variant		*SLC28A3*	0.1185	0.1326	0.131989	.	.
chr9	84331502	rs4877847	A	C	A/C	intron variant		*SLC28A3*	0.5046	0.5061	0.486022	.	.
chr21	42853748	rs15736	G	A	G/A	missense variant	loss of function	*WDR4*	0.4392	0.3824	0.34345	19.54	Benign
chr18	25132827	rs4381672	A	G	A/G	downstream gene variant		*ZNF521*	0.6456	0.6358	0.637979	.	.
chr18	25126724	rs4275929	C	A	C/A	intron variant		*ZNF521*	0.658	0.6357	0.652955	.	.



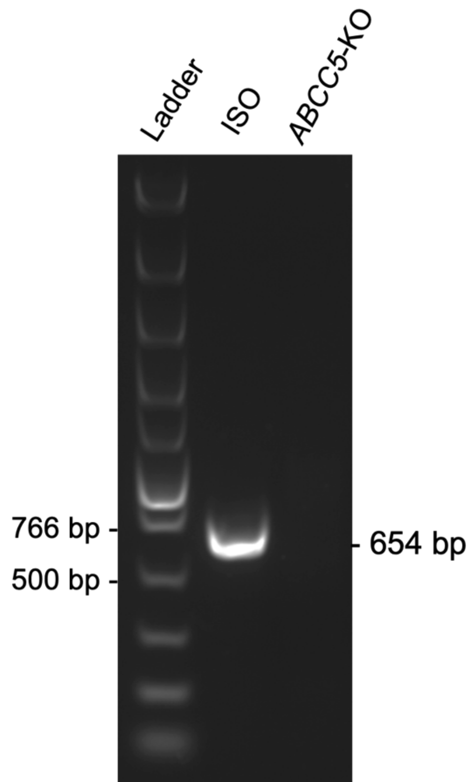
Supplemental Figure 1. Sanger sequencing of knockout lines generated for each studied gene. ISO: Isogenic control.



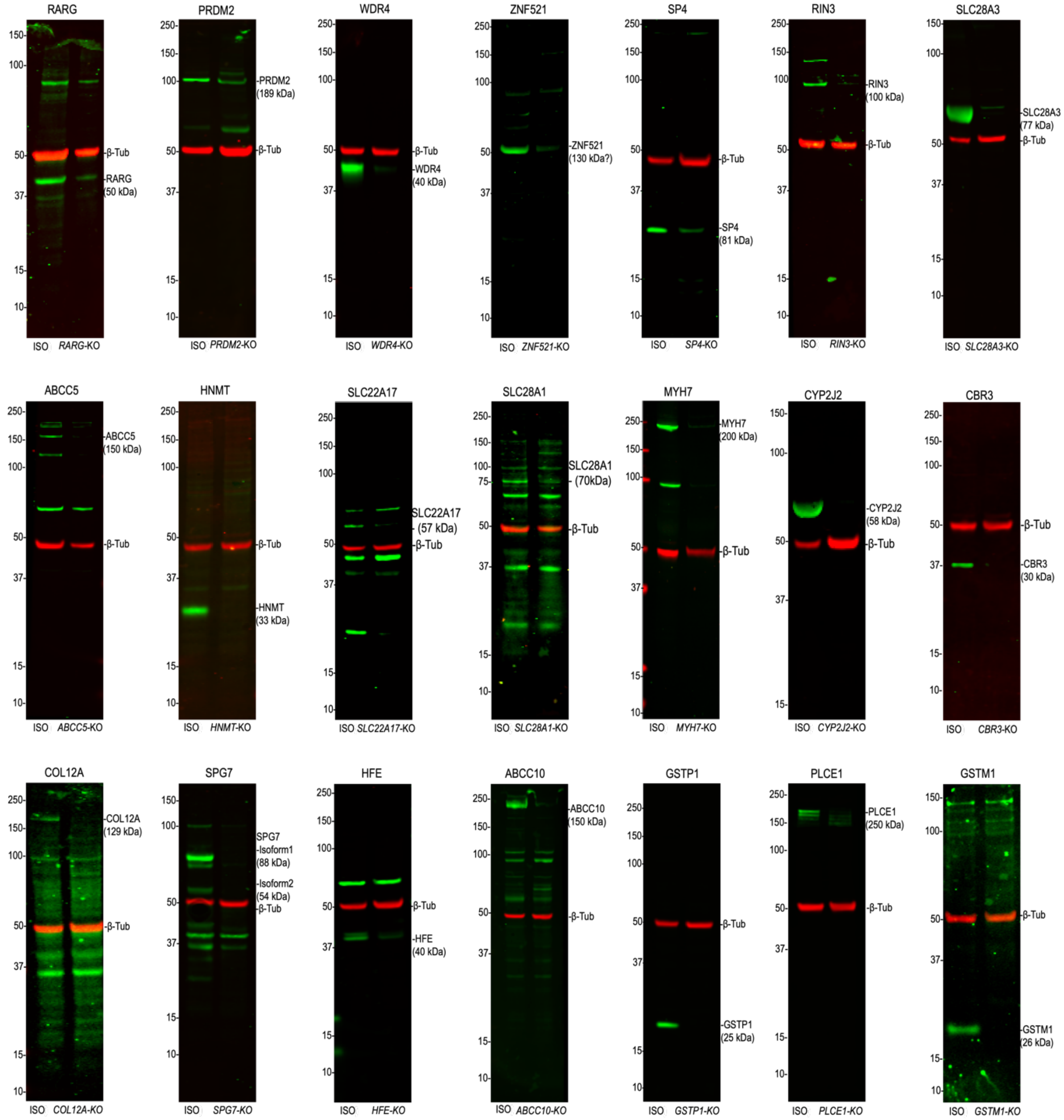
Supplemental Figure 2. Sanger sequencing of knockout lines generated for each studied gene. ISO: Isogenic control.



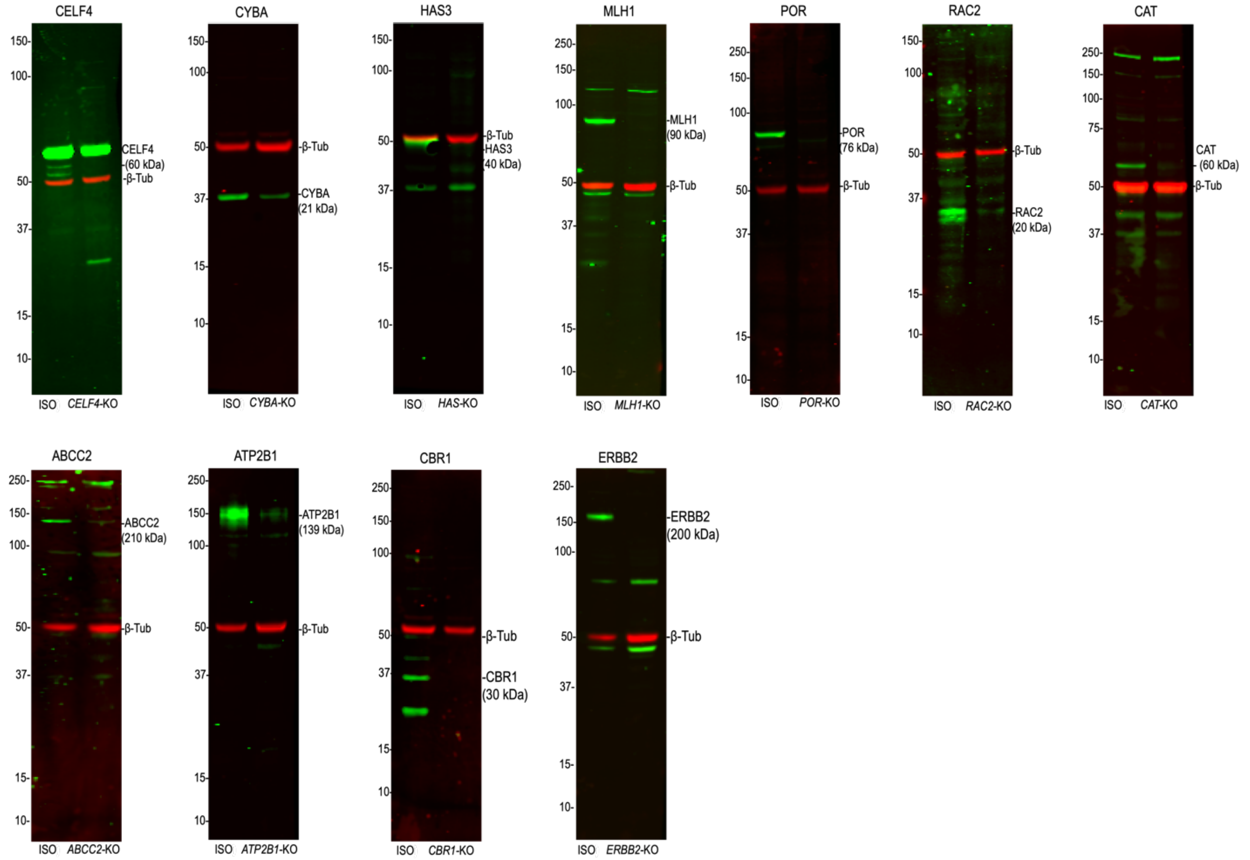
Supplemental Figure 3. Sanger sequencing of knockout lines generated for each studied gene. ISO: Isogenic control.



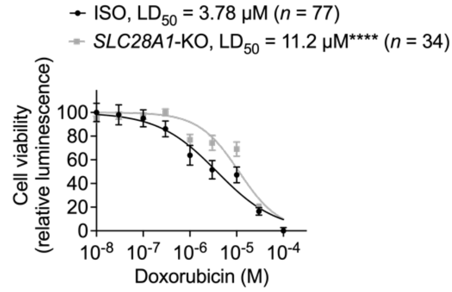
Supplemental Figure 4. Gel electrophoresis of PCR product covering the region after the guide RNA of ABBC5. ISO: Isogenic control.

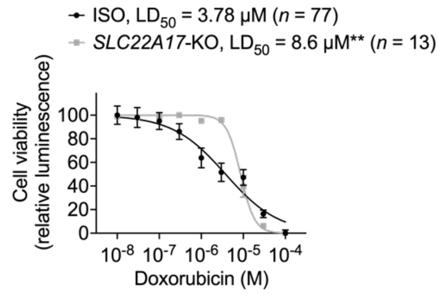


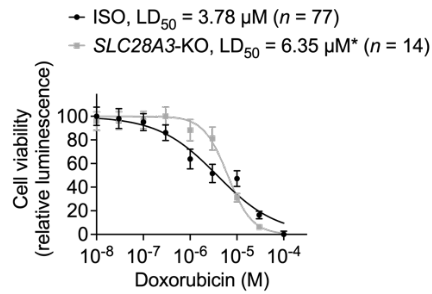
Supplemental Figure 5. Western blot analysis of knock out lines generated for each studied gene. ISO: Isogenic control. β-Tub: β-Tubulin.



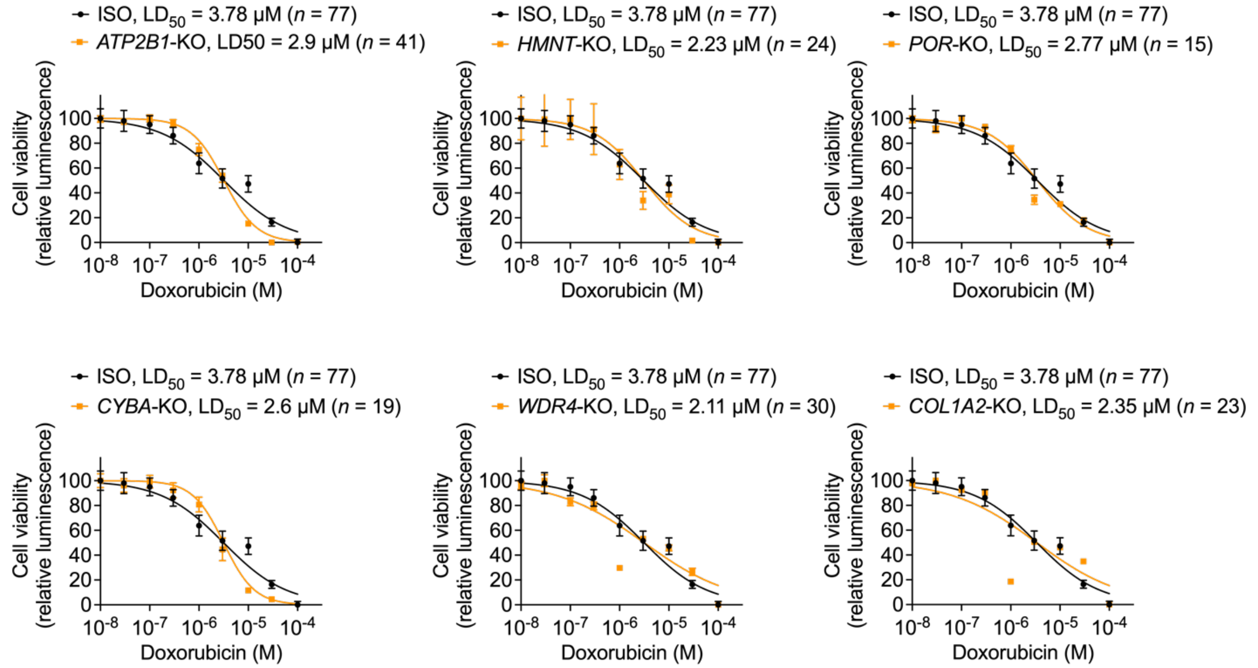
Supplemental Figure 6. Western blot analysis of knock out lines generated for each studied gene. ISO: Isogenic control. β-Tub: β-Tubulin.



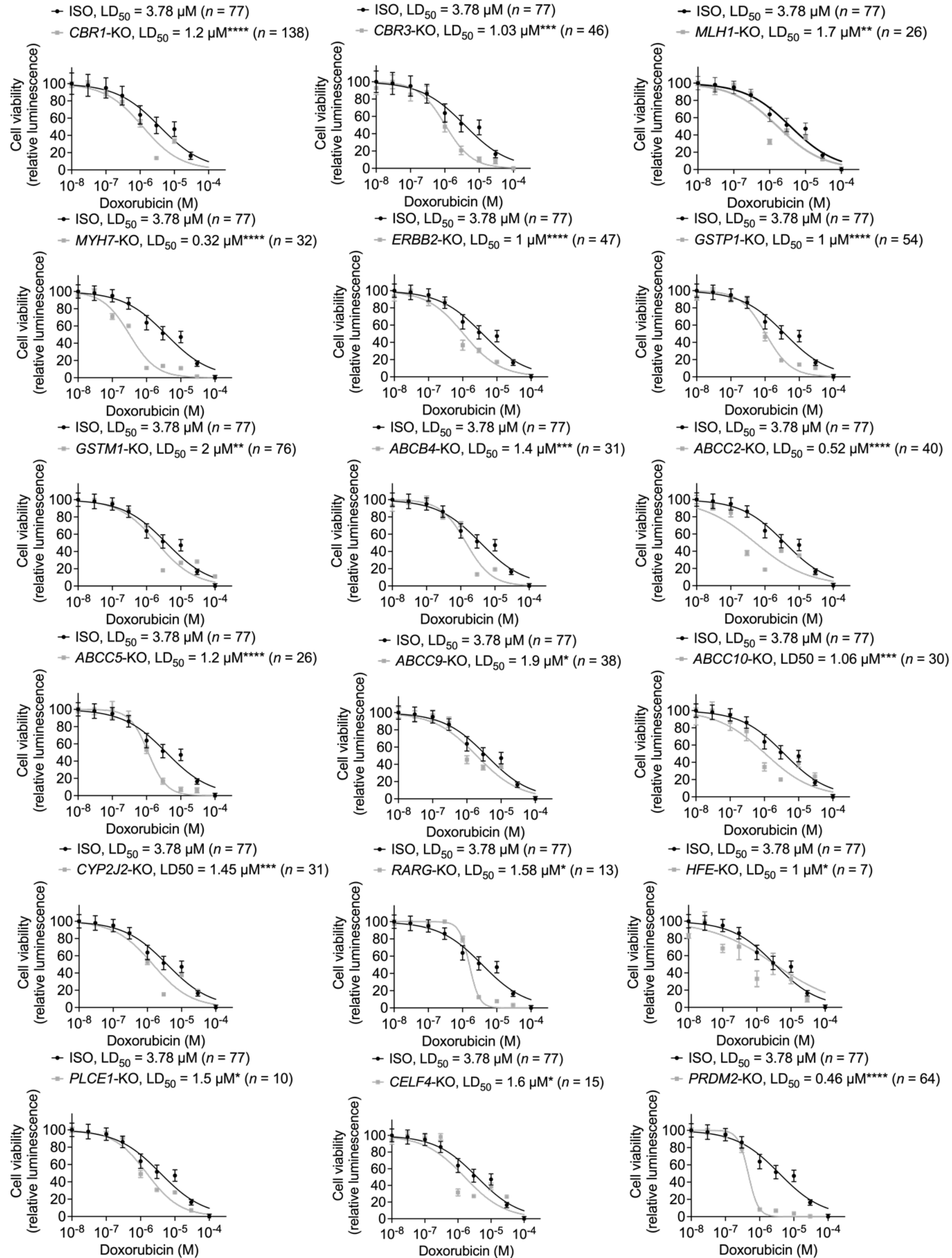




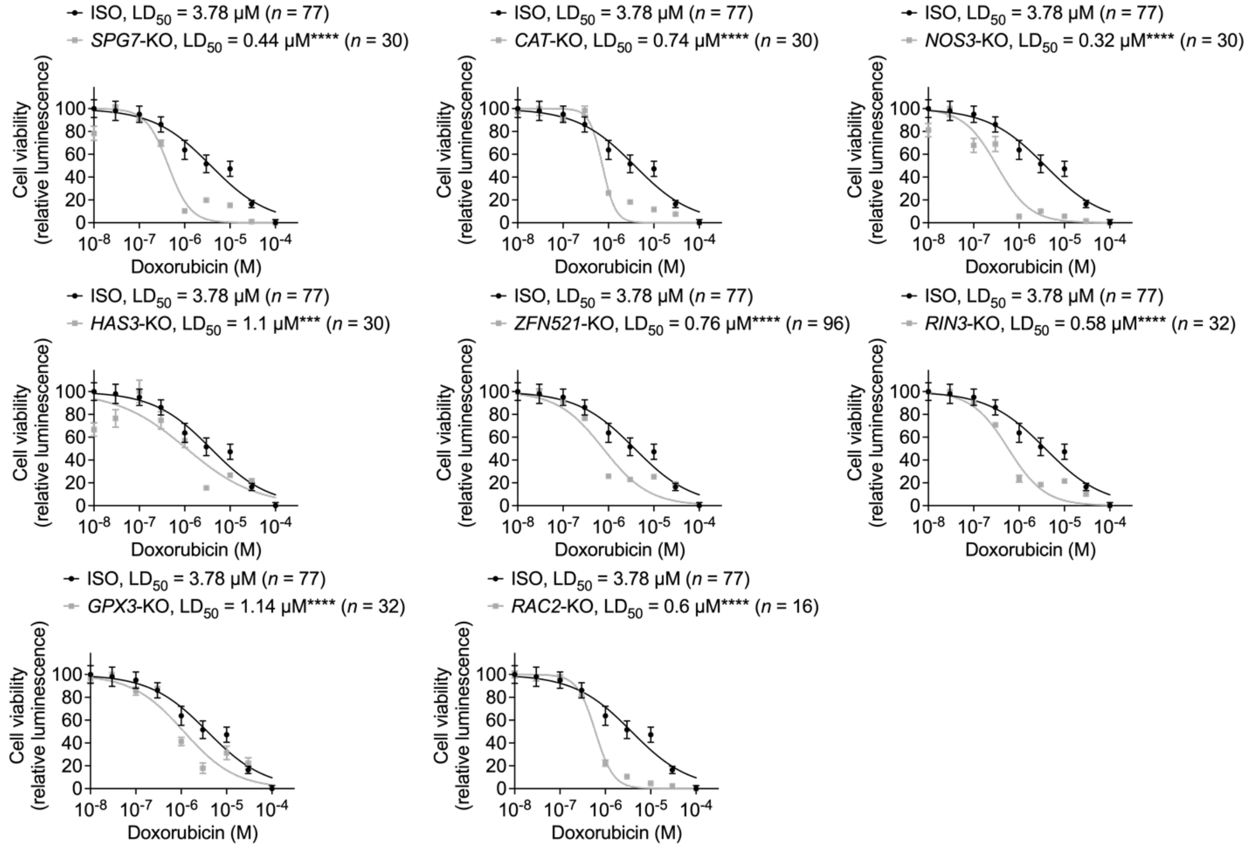
Supplemental Figure 7. Cell viability analysis of different gene knockouts using CellTiter-Glo post 72 h of doxorubicin treatment. ISO: Isogenic control, Error bars: SEM., *$P < 0.05$, **$P \leq 0.01$, ***$P < 0.001$, ****$P < 0.0001$.



Supplemental Figure 8. Cell viability analysis of different gene knockouts using CellTiter-Glo post 72 h of doxorubicin treatment. ISO: Isogenic control, Error bars: SEM., $*P < 0.05$, $**P \leq 0.01$, $***P < 0.001$, $****P < 0.0001$.



Supplemental Figure 9. Cell viability analysis of different gene knockouts using CellTiter-Glo post 72 h of doxorubicin treatment. ISO: Isogenic control, Error bars: SEM., $*P < 0.05$, $**P \leq 0.01$, $***P < 0.001$, $****P < 0.0001$.



Supplemental Figure 10. Cell viability analysis of different gene knockouts using CellTiter-Glo post 72 h of doxorubicin treatment. ISO: Isogenic control, Error bars: SEM., $*P < 0.05$, $**P \leq 0.01$, $***P < 0.001$, $****P < 0.0001$.