Repressing ABCB7 Potentiates Cisplatin Response in Pediatric Group 3 Medulloblastoma by Triggering Ferroptosis

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Abbreviations: ABC, ATP binding cassette; ANOVA, analysis of variance; BLC-2, B-cell lymphoma 2; BRCA1, breast cancer gene 1;CB, cerebellum; CD276, cluster of differentiation 276 (B7-H3); CDK6, cyclin-dependent kinase 6; c-Myc, c-myelocytomatosis oncogene; DDR, DNA damage repair; DCFDA, 2′,7′-Dichlorofluorescin diacetate; DFO, deferoxamine; FACs, fluorescence-activated cell sorting; FISH, fluorescence *in situ* hybridization; GPX4, glutathione peroxidase 4; GSH, glutathione; GSS, glutathione synthetase; IC_{50}, 50% inhibitory concentration; IHC, immunohistochemistry; i(17q), isochromosome 17q; KEGG, Kyoto encyclopedia of genes and genomes; KO, knock-out; LIP, labile iron pool; LPO, lipid peroxidation; MB, medulloblastoma; MDR, multiple drug resistance; miR, microRNA; miR-1253, microRNA 1253; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin; mtROS, mitochondrial reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control; NHA, normal human astrocytes; Non-SHH/WNT, non-Sonic Hedgehog/non-Wingless; PARP, poly ADP ribose polymerase; PCR, polymerase chain reaction; Ped, pediatric; PNET, primitive neuro-ectodermal tumor; ROS, reactive oxygen species; RSL3, RAS-selective lethal; SHH, Sonic Hedgehog; WNT, Wingless; XIAP, X-linked inhibitor of apoptosis; XTT, sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

Abstract

Medulloblastomas (MB), the most common malignant pediatric brain tumor and a leading cause of childhood mortality, are stratified into four primary subgroups. Deletions within chromosomal locus 17p13.3, which houses multiple tumor suppressor genes including miR-1253, characterize high-risk group 3 tumors. These aggressive tumors also enrich iron transport genes to satisfy their high proliferative need. MiR-1253 targets iron transport by inhibiting the mitochondrial Fe-S transporter, ABCB7. This study elucidated the impact of repressing ABCB7 on cisplatin cytotoxicity in group 3 MB and whether these effects were mediated by ferroptosis. *In silico* and *in vitro* analyses revealed specific enrichment of ABCB7 and GPX4, a critical regulator of ferroptosis, in group 3 MB cell lines and tumors. MiR-1253 overexpression (miR-1253^{OE}) resulted in downregulation of both ABCB7 and GPX4, concurrently increasing mitochondrial iron overload, mitochondrial oxidative stress, and lipid peroxidation, leading to cell death and abrogation of medullosphere formation; repressing ABCB7 (si-ABC7 and ABCB7^{KO}) recapitulated these effects and abrogated GPX4 expression. Fractionation studies confirmed the inhibitory impact of ABCB7 repression on GPX4 expression. Seahorse studies further revealed mitochondrial dysfunction with ABCB7 repression. Cisplatin, a chemotherapeutic agent used in group 3 MB treatment, induces cell death by DNA crosslinking; it also inhibits GPX4 expression and may trigger ferroptosis. In ABCB7-repressed group 3 cancer cells, cisplatin IC_{50} was reduced 2-fold. Resultantly, cisplatin treatment augmented oxidative stress and lipid peroxidation, depleted glutathione stores, and culminated in a higher index of cell death via ferroptosis. In an orthotopic group 3 tumor model, ABCB7^{KO} potentiated cisplatin, prolonging survival and reducing tumor burden. Taken together, the current study illustrates how targeting iron transport can augment ferroptosis to potentiate cisplatin cytotoxicity in group 3 MB tumors.

Key words: ABCB7; ferroptosis; GPX4; medulloblastoma; miR-1253; ROS

Introduction

Of the primitive neuro-ectodermal tumors (PNET) afflicting the posterior fossa, medulloblastoma (MB) is the most common malignant tumor of childhood, accounting for 85% of PNETs and 20% of all posterior fossa tumors.[1-3] Based on high-throughput gene profiling studies, MBs are stratified into four major molecular subgroups: WNT, SHH, group 3 and group 4.[4] Among these, patients with group 3 tumors suffer the worst prognosis (5-year overall survival <50%) due to a highly aggressive phenotype punctuated by c-Myc amplification, haploinsufficiency of chromosome 17p, presence of metastatic lesions at diagnosis, and high rates of recurrence.[4-10] Recurrence can further reduce 5-year overall survival to <10% and is partially fueled by the inability of young patients to tolerate effective chemo- and/or radiation therapy.[4,11-13] For instance, systemic toxicity is an oft-cited justification for limitations in cisplatin dosing, especially for group 3 disease, leading to <20% of treated patients receiving an effective dose.[14-16] These facts underscore the urgent need to develop new strategies for high-risk disease.

Recently, combining microRNAs with chemotherapy has garnered support in enhancing therapeutic efficacy via targeting critical regulators of DNA damage repair (DDR), apoptosis, or cell cycle.[17] In breast cancer, for example, miR-9 and miR-218 improved cisplatin responsiveness by targeting BRCA1 and impeding DNA damage repair.[18,19] The miR-15 family sensitized cisplatin-resistant cancer cells to apoptosis by targeting the G_2/M cell cycle checkpoint.[20] In gastric and lung cancer cell lines, the miRNA cluster miR-200bc/429 sensitized resistant cell lines to both vincristine and cisplatin by targeting BCL2 and XIAP.[21] In a

gemcitabine-resistant pancreatic xenograft model, miR-205 delivered with gemcitabine in conjugated micelles resulted in significant tumor growth inhibition.[22] Most notably, miR-34a replacement therapy (MRX34) has reached a phase 1 clinical trial (NCT01829971) in patients with primary unresectable or metastatic liver cancer.[23]

Group 3 tumors have amongst the highest frequency of cytogenetic aberrations targeting the short arm of chromosome 17 compared to the other subgroups, with high incidence reported on locus 17p13.3.[24-27] We recently revealed strong tumor suppressive properties for miR-212 and miR-1253, which reside on locus 17p13.3.[27,28] We showed that miR-1253 targets the cell cycle checkpoint protein, CDK6, and CD276 (B7-H3), an immune checkpoint molecule implicated in tumor aggressiveness. [27,29,30] Aside from reducing tumor cell viability, migration/invasion, and colony formation, miR-1253 arrested cells at the G_0/G_1 phase, activated apoptotic pathways, and triggered oxidative stress.[27] Numerous studies have recapitulated these strong tumor suppressive properties in various cancers, including non-small cell lung carcinoma[31], osteosarcoma[32], pancreatic cancer[33], male breast cancer[34], and colon cancer[35].

Amongst the putative targets of miR-1253 are the ATP-binding cassette (ABC) transporters that confer multi-drug resistance to various tumors, including MB.[36,37] In metastatic breast cancer, for example, miR-1253 was shown to inhibit the drug efflux pump, ABCB1, thereby potentiating the cytotoxicity of doxorubicin.[38] However, this particular ABC transporter is not amongst the deregulated ABC transporters reported in group 3 MB.[39] Another putative target of miR-1253 is ABCB7, an iron transporter residing on the inner mitochondrial membrane involved in iron homeostasis and Fe-S cluster biogenesis.[40] Deregulation of ABCB7 has been shown to help cancer cells withstand apoptosis and ferroptosis.[41] Ferroptosis is a form of cell death triggered by iron-mediated oxidative stress leading to lethal lipid peroxidation. This process is

tightly controlled by glutathione peroxidase 4 (GPX4), whose primary substrate is the anti-oxidant, glutathione (GSH).[42-44] Recent studies have identified ferroptosis mitigation in tumor progression and drug resistance.[45,46]

Of the standard chemotherapeutic agents used in the treatment of MB, cisplatin, a platinum-based alkylating agent that induces DNA damage, has been shown to trigger cell death via oxidative stress and ferroptosis.[45,47] Whether deregulation of ABCB7 leads to cisplatin resistance in group 3 tumors or if miR-1253 can sensitize cisplatin response through inhibition of ABCB7 remains unstudied. Here, we show that targeting ABCB7 can potentiate cisplatin cytotoxicity in group 3 MB by inducing iron overload, oxidative stress, and lipid peroxidation, which together trigger ferroptosis.

Materials and Methods

Patient Samples

Formalin-fixed paraffin embedded tissue blocks and frozen tissues of normal cerebellum (pediatric=12, adult=5) and pediatric MB specimens (WNT=1, SHH=9, grp 3=11, grp 4=16, unknown=7) were obtained from the Children's Hospital and Medical Center, Omaha and the University of Nebraska Medical Center after Institutional Review Board approval. Informed consent was not required since the status of the study was exempted. For expression profiles of ABCB7 and GPX4, we cross-analyzed two primary MB datasets (Kanchan *et al.*, GSE148390 and Weishaupt *et al.*, GSE124814).[48-50] For Spearman correlation, we used GSE148390. For Kaplan-Meier Survival Analysis, we used the R2 database (Cavalli *et al.*, GSE85217).[9]

Cell Lines and Cell Culture

D283 and D341 were purchased from ATCC (Manassas, Virginia); D425 and D556 were kind gifts from Darell Binger (Duke University Medical Center, Durham, NC); HDMB03 cells were a

kind gift from Till Milde (Hopp Children's Tumor Center, Heidelberg, Germany). Cell line genotyping was verified using short tandem repeat (STR) DNA profiling (UNMC). D283, D341, D425 and D556 cell line were maintained in DMEM supplemented with 10%-20% FBS and 100µg/ml penicillin/streptomycin. Normal human astrocytes (NHA) were purchased from Lonza Bioscience (Walkersville, MD) and grown in ABM basal medium supplemented with growth factors (Lonza Biosciences). All cell lines were maintained in 95% humidity, 37°C, 5% CO_2.

Transient Transfections

Cells at a density of 0.5 x 10^6 were seeded in 6-well plates for 24 h and subsequently serum starved for 4 h prior to transfection. Cells were transfected with miR-1253 mimic (miRVanaTM miRNA mimic, ThermoFisher, 100 nM) or scramble negative control (100 nM) with Lipofectamine 2000 (Invitrogen) for 24 h.

CRISPR/Cas9 Knockouts

Lentiviral particles were prepared by transfection of plasmid expressing Cas9 or sgRNA of ABCB7 (Addgene) co-transfected with pCMV-dR8.2 dvpr (Addgene) and pCMV-VSV-g (Addgene) lentiviral packaging plasmids into HEK293T cells using polyethyleneimine (PEI) transfection reagent. Virus-containing supernatant was collected and filtered 48 h after transfection. HDMB03 cells in 6-well plates were infected with Cas9 viral supernatant containing 4 µg/mL polybrene. Following blasticidin selection (10 µg/ml), the expression of Cas9 was confirmed by Western blotting. Stable HDMB03 cells expressing Cas9 expression were infected with the ABCB7 sgRNA viral supernatant containing 4 µg/ml polybrene. After 24 h of infection, cells were selected with 0.5 µg/ml puromycin. Single cell clones with Cas9 expression and ABCB7 knockout were amplified and used for subsequent experiments.

Cell Viability

After transfection, HDMB03 cells (5 x 10^3 cells/well) were re-seeded into 96-well plates and treated with defined concentrations of cisplatin (1-50 μM) for 24-72 h. Subsequently, MTT (5 mg/mL) or XTT (0.3 mg/mL) was added to each well and incubated for 2 or 6 h, respectively, at 37 °C. MTT absorbance was measured at 570 nm; XTT absorbance was measured at 440 nm. Data were analyzed using the SOFTMAX PRO software (Molecular Devices Corp., Sunnyvale, CA, USA).

Colony Formation

After transfection, HDMB03 cells (1 x 10^3 cells/well) were re-seeded in 6-well plates. Cells were treated with cisplatin (2 μM) and grown in complete medium for 9 days. Colonies were stained with 0.25% crystal violet (dissolved in 50% methanol) for 30 min. Crystal violet was dissolved in 10% acetic acid and absorbance read at 590 nm.

Luciferase Assay

Luciferase assay was performed as previously described.[27] Primers ABCB7 Forward: 5'-TAAGCCTGACATAACGAGGA-3'; ABCB7 Reverse: 5'-GCATCTCAGTATTAACTCTAGC-3') were purchased from Eurofins. 3'UTR Wild and 3'UTR-Mutant were incorporated into XbaI restriction site of PGL3-control vector (Promega) expressing firefly luciferase. Dual luciferase assay was done in HDMB03 cells (3 x 10^5cells/well) in 12-well plates. Luciferase activity was the measured using Dual-Luciferase Reporter Assay System (Promega) with a Luminometer (Biotek).

Calcein AM Staining

Calcein-acetoxymethyl ester (Calcein AM) is a membrane permeable, non-fluorescent dye which emits green fluorescence once internalized into the live cells and cleaved by cytoplasmic esterase. Although calcein fluorescence is stable, it can be quenched by divalent metal ions such

as iron and cobalt. To estimate cytosolic labile iron pool (LIP), HDMB03 and D425 cells were either transfected with scramble or miR-1253 or had ABCB7 knocked down. Cells were reseeded on to glass coverslips treated with or without the iron chelator deferoxamine (DFO) for 6 h. Cells were washed twice with 0.5 ml of PBS and incubated with 0.05 μM Calcein AM for 15 min at 37 °C. Cells were analyzed under confocal microscope. Ex/Em = 488 nm/525 nm.

Detection of Cytosolic and Mitochondrial Fe^{2+}

Scramble vs. miR-1253-transfected cells or wild-type vs. $ABCB7^{KO}$ cells were seeded onto glass coverslips and were treated with or without DFO (100 μM) for 6 h. For cytosolic LIP, 1 μmol/L FerroOrange (Dojindo, Japan) and 100 nM MitoTraker™ Deep Red FM were added to each well; for mitochondrial LIP, 1 μM/L Mito-FerroGreen (Dojindo, Japan) and 100 nM MitoTraker Deep Red FM were added to each well. Cells were incubated in a 37° C incubator equilibrated with 95% air and 5% CO_2 for 30 min. After incubation cells were washed and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were observed under a confocal fluorescence microscope, $Ex/Em_{cytosolicLIP}$ = 561 nm/570-620 nm, $Ex/Em_{mitochondrialLIP}$ = 488 nm/500-550 nm.

Measurement of Intracellular Oxidative Stress

Intracellular ROS (H_2O_2) was measured using oxidation-sensitive fluorescent probe 2′,7′-Dichlorofluorescin diacetate (DCFDA) (Sigma Aldrich, USA); mitochondrial $O_2^{\bullet -}$ was measured using MitoSOX™ Red (Thermofisher, USA). Scramble vs. miR-1253-transfected cells or wild-type vs. $ABCB7^{KO}$ cells were treated with cisplatin or cisplatin and Ferrostatin-1 (Apexbio, USA) for 24 h. Cells were incubated with 10 μM DCFDA or 5 μM MitoSOX™ Red for 30 min. Oxidized DCFDA and MitoSOX™ Red were measured using at Ex/Em ~485/528 nm and Ex/Em ~510/580, respectively.[51] Images were captured using EVOS FL Auto Imaging System

(EVOS FL Auto, Life Technologies). Oxidized DCFDA and MitoSOXRed were measured using multimode plate reader at Ex/Em ~485/528 nm and Ex/Em ~510/580, respectively

Measurement of Lipid Peroxidation

The Image-iT® LPO kit was used to measure lipid ROS through oxidation of the C-11-BODIPY® 581/591 sensor according to the manufacturer's instructions. Briefly, scramble vs. miR-1253-transfected cells or wild-type vs. $ABCB7^{KO}$ cells were treated with or without cisplatin or a combination of cisplatin and Ferrostatin-1 for 24 h. Cells were then stained with Image-iT® Lipid Peroxidation Sensor (10 µM) for 30 min and counter-stained with DAPI. Images were captured by confocal microscopy.

Ferroptosis Assessment by Flow Cytometry

Scramble and miR-1253-transfected cells were treated with or without cisplatin or a combination of cisplatin and Ferrostatin-1 for 24 h. After completion of treatment, cells were incubated with 5 µM of MitoSOX™ Red for 30 min. Cells were washed and stained with Annexin-V/Cy™5(BD Biosciences). Cell populations were sorted and measured by flow cytometry.

Glutathione Estimation

Glutathione estimation was performed according to the manufacturer’s instructions using GSH-Glo™ Glutathione Assay kit (Promega, USA). Briefly, scramble vs. miR-1253-transfected cells or wild-type vs. $ABCB7^{KO}$ cells were seeded (5×10^3 cells/well) in the 96-well plates. Cells were treated with or without cisplatin or in combination with cisplatin and Ferrostatin-1 for 24 h. Plates were incubated in the dark for 30 min on a plate shaker with 100 µl of prepared 1x GSH-Glo™ Reagent. Then, 100 µl of reconstituted Luciferin Detection Reagent was added to each well and incubated in the dark for another 15 min. A standard curve was prepared using GSH standard solution to facilitate the conversion of luminescence to GSH concentration.

Immunofluorescence Imaging

In cultured cells: scramble vs. miR-1253-transfected cells or wild-type vs. ABCB7^{KO} cells were seeded onto coverslips, rinsed, and fixed using 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Cells were washed, incubated with 0.25% Triton X-100, and blocked with 3% BSA at room temperature. Cells were then incubated with primary antibody ABCB7 or COXIV overnight. Following day, cells were incubated with Alexafluor 488 and Alexaflour 547 conjugated antibodies for 1 h. Cells were washed and counter stained with DAPI. Images were captured at 63X using Carl Zeiss microscope (LSM 800 META).

In tissue: immunofluorescence was performed on surgically-resected, formalin-fixed, paraffin-embedded sections of group 3 medulloblastomas. Deparaffinized tissue sections were blocked with 5% BSA after heat induced epitope retrieval with citrate buffer (pH 6.0) and then incubated with primary antibodies, ABCB7 rabbit monoclonal antibody (1:200), GPX4 rabbit monoclonal antibody, or COXIV mouse monoclonal antibody (1:200). Following overnight incubation with primary antibody, sections were incubated for 1 h with Alexa-488 or Alexa-547-conjugated, mouse and rabbit secondary antibodies (1:200). Sections were counter-stained with DAPI. Micrographs were captured at 20X using Carl Zeiss microscope (LSM 800 META).

PCR Primers

Total RNA extraction and quantitative PCR (qPCR) were performed according to manufacturer protocol. The forward (F) and reverse (R) primer sequences were as follows: ABCB7 (F): 5'-AAGATGTGAGCCTGGAAAGC-3' (R): 5'- AGAGGACAGCATCCTGAGGT-3'; GPx4 (F): 5'-ACAAGAACGGCTGCGTGGTGAA-3' (R): 5'-GCCACACACTTGTGGAGCTAGA-3'; β-Actin (F): 5'-CACCATTGGCAATGAGCGGTTC-3'(R): 5'-AGGTCTTTGCGGATGTCCACGT-3'.

Statistical Analysis

Data are presented as mean ± SD. All experiments were conducted at least in duplicates with 3-6 replicates per experiment. Statistical analyses were performed using Prism 9.2 (GraphPad Software). Differences between groups were compared using Student's t-test or one-way analysis of variance (ANOVA). Statistical significance was established at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Statistical analyses of high-throughput sequencing data were performed using R Statistical Software v4.1.1 (R Core Team), expression values were compared using a Mann-Whitney *U* test.

Results

ABCB7, a novel target of miR-1253, is enriched in group 3 medulloblastomas.

In our previous study, we identified miR-1253 as a novel tumor suppressor gene in MB, along with its multiple oncogenic targets.[27] Here, we sought to determine other targets of miR-1253, especially those that may regulate drug sensitivity in MB. Upon restoration of miR-1253, using transient overexpression in HDMB03 cells, we observed potent downregulation of the ABC transporter superfamily by KEGG pathways analysis **(Supplementary Figures 1A)**. We wanted to then isolate specific targets of miR-1253 relevant to group 3 MB aggressiveness. We began by identifying ABC transporters enriched in group 3 MB **(Supplementary Figure 1B, column 1)**. From this list, we isolated transporters whose high expression in group 3 MB conferred a poor prognosis **(Supplementary Figure 1B, column 2, red)**. From this cohort, we selected transporters whose expression was inhibited by miR-1253 overexpression in HDMB03 cells **(Supplementary Figure 1B, column 3)**. This comparative analysis revealed ABCB7 as the best putative miR-1253 target for further study.

ABCB7 resides on the inner mitochondrial membrane and plays a key role in Fe-S cluster biosynthesis and export.[52-54] When we compared the differential gene expression of G3MB to normal cerebellum in our local cohort of MB patients, we observed enrichment of genes regulating iron homeostasis and transport, including ABCB7 **(Supplementary Figures 1C)**. Moreover, upon querying the Molecular Signature Database (MSigDB, version 7.4), we discovered an enrichment of Fe-S cluster assembly **(Supplementary Figures 1D)**. We also discovered that in multiple MB cohorts, ABCB7 is enriched **(Supplementary Figure 2A)** and that high expression is associated with a poorer prognosis **(Supplementary Figure 2B)**. We then compared subgroup-specific ABCB7 expression in 3 recent MB cohorts and identified consistent enrichment of ABCB7 in group 3 tumors **(Figure 1A; Supplementary Figures 2C and 2D)**. High ABCB7 expression in these group 3 patients was associated with dramatically poorer overall survival **(Figure 1B)**. These findings strengthened our premise to study ABCB7 enrichment as a possible mechanism for group 3 MB aggressiveness.

Now focusing on ABCB7, we first confirmed by confocal microscopy that ABCB7 co-localizes to the mitochondria, as evidenced by co-staining with COXIV, an inner mitochondrial membrane protein critical to electron transport **(Figure 1C)**. Additionally, we confirmed robust expression of ABCB7 in a panel of aggressive group 3 MB cell lines compared to normal human astrocytes **(Figure 1D)**.

To demonstrate that ABCB7 is inhibited by miR-1253, we first showed translational repression via Western blotting in 2 group 3 MB cell lines, D425 **(Figure 1E)** and HDMB03 **(Figure 1F)**. In the same cell lines, we showed co-localization of ABCB7 with COXIV to the mitochondria and visualized the effect of miR-1253 on ABCB7 expression, as illustrated by a dramatically reduced green fluorescence by confocal microscopy **(Figure 1G)**. Finally, we

showed direct binding of miR-1253 to ABCB7 via a dual-luciferase reporter assay **(Figures 1H)**. Together, these data confirmed that i) ABCB7 expression is enriched in group 3 tumors, ii) ABCB7 enrichment is a poor prognostic sign in group 3 patients, and iii) miR-1253 effectively inhibits ABCB7 expression.

ABCB7 repression attenuates G3MB cancer cell aggressiveness.

While the pathogenic role of ABCB7 has yet to be elucidated in MB, in glioma cells, ABCB7 inhibition triggered both apoptotic and non-apoptotic cell death through iron overload and ROS.[41] Given the promiscuous nature of miRs, we adopted additional strategies to repress ABCB7 expression in G3MB cancer cells by using CRISPR/Cas9 knockdown ($ABCB7^{KO}$) or small-interfering RNA (si-ABCB7) **(Figure 2A)**. Using these strategies, we observed potent inhibition of primary medullosphere formation **(Figure 2B)**, reduced cancer cell viability by MTT assay **(Figure 2C)**, and reduced calcein staining indicating loss of intracellular esterase activity due to cell turnover **(Figure 2D)**, all evidencing that ABCB7 repression attenuates aggressiveness of HDMB03 cells.

ABCB7 repression triggers iron overload.

Given its primary role as a Fe-S cluster exporter, we studied the impact of repressing ABCB7 expression on iron balance in HDMB03 cells using ferric ammonium citrate (FAC), which induces iron overload[55], as our positive control. We observed an accumulation of free iron in the mitochondria (**MitoFerroGreen**) and the cytoplasm (**FerroOrange**) in HDMB03 cells with repressed ABCB7 expression by miR-1253 and si-ABCB7; with iron chelation using deferoxamine (DFO), the phenotype reverted to wild-type **(Figures 3A and 3B)**. These data were recapitulated with $ABCB7^{KO}$ in HDMB03 cells **(Supplementary Figure 3)**. Calcein AM is a membrane-permeable dye that produces green fluorescence when internalized and can be

rapidly quenched by divalent metals like iron. Repression of ABCB7 by miR-1253 or ABCB7^{KO} led to quenching of Calcein AM in group 3 MB cell lines **(Supplementary Figure 5)**, further strengthening our observations of iron overload induced by ABCB7 inhibition.

ABCB7 repression enhances cellular ROS and induces lipid peroxidation.

Free iron generates reactive oxygen species (ROS) via the Fenton reaction and disrupts the phospholipid bilayer resulting in lethal lipid peroxidation.[56-58] We subsequently quantified total and mitochondrial ROS using DCFDA, which fluoresces green in the presence of hydrogen peroxide (H_2O_2), and Mitosox, which fluoresces red in the presence of mitochondrial superoxide (O_2^-). We also quantified lipid peroxidation through BODIPY-C11 staining, a membrane-targeted lipid ROS sensor, using an Image-iT^{TM} lipid peroxidation kit which provides a ratiometric indication of fatty acid oxidation that can be quantified by microscopy and flow cytometry (FACS). We observed that ABCB7 repression (miR, si) induced similar ROS levels to positive controls, hydrogen peroxide and erastin, a known inducer of ferroptosis[59] (ERA) **(Figures 3C and 3D)**. Repressing ABCB7 also resulted in higher lipid peroxidation than erastin **(Figure 4A)**. We showed the same impact of ABCB7 repression by miR-1253 on oxidative stress in D425 and HDMB03 **(Supplementary Figures 4A-D and 5D)**.

Moreover, given that ABCB7 repression induces mitochondrial iron overload, we used a Seahorse assay to study if ABCB7 repression also impacted mitochondrial function. ABCB7 repression by miR-1253^{OE} significantly reduced the oxygen consumption rate (OCR) of HMDB03 cells, a direct measure of mitochondrial ATP production. It concurrently increased the extracellular acidification rate (ECAR), a measure of ATP production under anaerobic conditions by glycolysis **(Figure 4B)**.

Ferroptosis is defined as an iron-dependent form of regulated cell death resulting from lipid peroxidation.[42] Ferrostain-1 (FER) scavenges ROS and lipid hyperoxides and prevents ferroptosis[60]. Given our observations with iron accumulation, ROS generation, and lipid peroxidation, we wanted to assess whether ABCB7 repression can, in fact, trigger ferroptosis. D425 and HDMB03 cells transfected with miR-1253 were stained with MitoSOX™ Red (for $O_2^{\bullet-}$) and Annexin-V/Cy™5 (marker of apoptotic cell death). Flow cytometry analysis revealed a significant (~3-fold) rise in dual-stained cells (Q2), indicating oxidative stress-mediated cell death **(Supplementary Figures 4E and 4F)**. Moreover, ferrostatin-1 rescued ABCB7-repressed cells from both oxidative stress **(Figures 3C and 3D)** and lipid peroxidation **(Figure 4A)**. These are the first data showing that ABCB7 repression can trigger events that culminate in ferroptosis.

ABCB7 repression inhibits G3MB tumor growth.

We next examined the impact of $ABCB7^{KO}$ on tumor burden in a validated G3MB model[28]. We generated tumors in NOD *scid* gamma (NSG) mice by orthotopically injecting wildtype or $ABCB7^{KO}$ HDMB03 cells ($1x10^5$) into the cerebellum **(Figure 5A)**. At 2 weeks, mice from the KO group had significantly smaller tumors compared to wildtype **(Figure 5B)**. Moreover, mice in the wildtype arm (5) died by day 18; in contrast, mice in the KO arm (5) survived 10 days longer **(Figure 5C)**. Histologically, we visualized reduced c-Myc expression, a defining high-risk feature of group 3 MB, in tumors procured from KO mice. These data reveal that $ABCB7^{KO}$ attenuates G3MB tumor burden.

ABCB7 repression inhibits GPX4, the primary regulator of ferroptosis.

Cancer cells have the inherent ability to activate redox buffering systems to thrive in a highly oxidative environment.[61] Glutathione (GSH) is a key player in this response and a critical cofactor for glutathione peroxidase 4 (GPX4). GPX4 uses GSH to reduce toxic lipid

hydroperoxides to non-toxic lipid alcohols and is thus a central regulator of ferroptosis, found in the nucleus, cytoplasm, and mitochondria **(Supplementary Figure 6A)**.[58] GPX4 overexpression elevates aggressiveness in multiple cancers (breast, liver, GI, pancreatic).[62] In MB, we noted significant enrichment of GPX4 in all MB tumor subtypes, with the highest expression in group 3 MB **(Supplementary Figures 6B and 6C)**; prognosis in high-expressing patients was poor **(Supplementary Figure 6D)**. In G3MB, glutathione metabolism and GPX4 are both enriched **(Supplementary Figures 1C and 1D)**. Immunofluorescence studies revealed high expression of GPX4 in group 3 MB and co-localization of GPX4 to the mitochondria **(Supplementary Figure 6E)**. In classic group 3 MB cell lines, GPX4 expression was significantly elevated compared to normal human astrocytes **(Supplementary Figure 6F)**.

Given the pivotal role of GPX4 in ferroptosis mitigation, we studied the impact of ABCB7 repression on GPX4, expecting an upregulation to counter the oxidative stress induced by iron overload. Instead, we observed the opposite. In D425 and HDMB03 cells, miR-1253^{OE} reduced GPX4 expression **(Figure 6A and 6B)**. However, ABCB7^{KO} led to a near abrogation of GPX4 expression **(Figure 6C)**. In support, we noted a positive Spearman correlation (R=0.72, p<0.001) between GPX4 and ABCB7 **(Figure 6D)**. We further explored this relationship using fractionation studies. While miR-1253^{OE} reduced GPX4 levels in the cytoplasm and mitochondria **(Figure 6F)**, ABCB7^{KO} **(Figure 6G)** and si-ABCB7 **(Figure 6H)** again showed near abrogation of GPX4 expression from the cell. GSH, a main substrate of GPX4, was concurrently reduced in miR-1253-overexpressing D425 and HDMB03 cells (**Figure 6E**). This is the <u>first time</u> a link has been proposed between ABCB7 and GPX4, providing us preliminary insight into the impact of the miR-1253/ABCB7 axis on GPX4 and the cellular response to ferroptosis.

ABCB7 repression potentiates cisplatin cytotoxicity in G3MB cells.

Cisplatin, a platinum-containing chemotherapeutic agent, induces DNA damage via various mechanisms, including (i) crosslinking DNA purine bases, (ii) inducing oxidative stress, and (iii) interfering with DNA repair machinery, that can eventually lead to cancer cell death.[63] Recent data purport that it can also inhibit GSH and GPX4.[45,47,64,65] In HDMB03 cells, we observed cisplatin to inhibit both ABCB7 and GPX4 expression **(Figure 7A)**. We next tested if ABCB7 repression potentiated cisplatin cytotoxicity. In HDMB03 cells with repressed ABCB7 expression, cisplatin elicited higher cytotoxicity compared to controls (**Figure 7B**). Moreover, in D425 **(Figure 7Ci)** and HDMB03 **(Figure 7Cii)** cells, ABCB7 repression via miR-1253^{OE} resulted in a ~2-fold reduction in the IC_{50} of cisplatin, while si-ABCB7 showed the same effect in HDMB03 cells **(Figure 7Ciii)**. Colony formation of HDMB03 cells was nearly abrogated in miR-1253^{OE} cells treated with cisplatin. **(Figure 7D)**.

We next investigated whether cisplatin treatment enhanced mitochondrial (mtROS) and cytosolic ROS in group 3 MB cells. Using prior fluorescent probes for mtROS (MitoSOX™ Red) and cytosolic ROS (DCFDA), we studied ABCB7 repression by miR-1253^{OE}-transfected cells subjected to 10 µM cisplatin in D425 and 2 µM cisplatin in HDMB03 cells at 24 h; we concurrently examined cisplatin treatment (2 µM) of ABCB7^{KO} HDMB03 cells. As prior, ABCB7 repression by these methods induced significantly higher levels of mtROS and cytosolic ROS compared to control. Moreover, in all cases, ABCB7 repression potentiated cisplatin-mediated ROS (**Figures 8A-C**). Concurrent treatment with ferrostatin-1 returned ROS levels to that of untreated miR-1253^{OE} or si-ABCB7 (**Figure 8D and 8E)**. These data suggest that a portion of cisplatin-induced cytotoxicity in group 3 MB cells is being induced by ferroptosis.

ABCB7 repression potentiates cisplatin toxicity through ferroptosis in G3MB cells.

We next wanted to confirm that ferroptosis was elicited by miR-1253 alone and potentiated by combination with cisplatin. We systematically looked at mtROS and cytosolic ROS, lipid peroxidation, GSH levels, and levels of apoptotic and ferroptotic cell death in miR-1253-transfected or ABCB7^{KO} cancer cells treated with 1) cisplatin and 2) in combination with ferrostatin-1 (a potent inhibitor of ferroptosis).

First, cisplatin alone induced oxidative stress; addition of ferrostatin-1 to cisplatin treatment led to a dramatic reduction in H_2O_2 but not $O_2^{\bullet -}$. Second, miR-1253 expression or ABCB7^{KO} led to a significant rise in oxidative stress, which was potentiated by the addition of cisplatin; treatment with ferrostatin-1 substantially reduced the generation of both H_2O_2 and $O_2^{\bullet -}$ **(Figures 9A and 9B; Supplementary Figure 7A and 7B)**. Similarly, while cisplatin alone had modest effects on lipid peroxidation, combining it with miR-1253 expression or ABCB7 knockdown resulted in the highest levels of lipid peroxidation; in turn, ferrostatin-1 significantly reduced lipid peroxidation in ABCB7-repressed HDMB03 cells **(Figure 9C and Supplementary Figures 7C and 7D)**. Given the central importance of glutathione as an antioxidant, we observed that cisplatin depleted GSH in miR-1253 transfected and ABCB7^{KO} HDMB03 cells with the addition of ferrostatin having some recovery effects. As prior, cisplatin added to cells with ABCB7 inhibition had the strongest effect in depleting GSH; ferrotstatin-1 catalyzed the recovery of reduced glutathione levels in these cells **(Figure 9D** and **Supplementary Figure 7E**).

Finally, examining O_2-mediated cell death (as a proxy for ferroptosis) at 24 h, cisplatin showed a small rise in ROS-mediated cell death and apoptosis, while miR-1253 led to a larger rise in both. Treating miR-1253-transfected HDMB03 cells with cisplatin accomplished a dramatic increase in both ROS-mediated and apoptotic cell death; ferrostatin-1 treatment rescued

the former, strongly implicating the important contribution of ferroptosis to the mechanism of miR-1253-induced cell death **(Figure 9E)**. Together these data substantiate the cardinal role of miR-1253 in inducing ferroptosis and its potentiating effects on cisplatin cytotoxicity.

Discussion

Amongst the most devastating diagnoses in a pediatric patient is a tumor of the central nervous system, with medulloblastoma being the most common malignant tumor.[3,4,66,67] Punctuated for group 3 MB, poor survival (5-year OS <50%) in these patients is attributable to a combination of young age at diagnosis (peak age 3-5), metastases at diagnosis (up to 50%), and c-Myc amplification.[9,28,68-71] Current treatment regimens have yet to impact the dismal prognosis with stagnant survival rates seen over the last decade.[72] An inability to tolerate mainstay of therapy especially for young patients fuels high relapse rates (~30%) which are universally fatal. For example, in children under the age of 4 years relapse was noted in ~60% of patients who did not receive upfront craniospinal irradiation. Moreover, relapse rates were highest in group 3 MB tumors and those with i17q and c-Myc amplification.[73] In those that do manage to survive, irreversible damage to the hypothalamic-pituitary axis is sustained from cytotoxic treatment regimens resulting in short stature, cognitive impairments, and emotional lability.[74,75] Thus, the need for novel therapeutic strategies that mitigate drug-related cytotoxicity yet accomplish widespread tumor abstraction for this subgroup is dire.

Elevated recurrence rates have often been conjectured to be linked to mechanisms for drug resistance in group 3 MB, which may include high expression of multi-drug resistance (MDR) genes belonging to the ABC transporter family.[36,37,39,76,77] Cytotoxic drugs, in combination with tumor suppressive miRNAs, have the potential for a more complex and profound effect on tumorigenesis and may possess the ability to address drug resistance patterns, especially if they

can target MDR genes.[78] Here, we demonstrated a strong negative enrichment of the ABC transporter family with miR-1253 expression restoration in group 3 MB cells. Of the multiple transporters identified via our bioinformatics approach, we isolated ABCB7, whose deregulated expression profile in group 3 tumors was strongly associated with poor prognosis, as a target of miR-1253.

ABCB7 is an iron transporter residing on the inner mitochondrial membrane and involved in Fe-S cluster biogenesis.[52,79] ABCB7 deficiency can lead to decreased expression of electron transport chain (ETC) complex proteins I, II, IV and V, which can trigger impaired oxidative phosphorylation and mitochondrial membrane integrity resulting in oxidative stress.[40,80-82] In glioma cells, inhibition of ABCB7 resulted in disruption of iron transport and ROS generation triggering apoptotic and non-apoptotic cell death.[41] Iron accumulation can trigger oxidative stress and vice versa[56,83], resulting in lethal lipid peroxidation activating ferroptosis, which is distinct from apoptosis, necrosis, autophagy, and pyroptosis.[84]

With a strong premise for exploring iron imbalance through miR-1253, our subsequent results substantiated a ferroptotic role for miR-1253 in MB. First, miR-1253 induced intracellular iron accumulation in D425 and HDMB03 cells, effectively abrogated by pre-treatment with an iron chelator, DFO. $ABCB7^{KO}$ resulted in a similar phenotype. This resulted in elevated mitochondrial ROS ($O_2^{\bullet-}$) and cytosolic ROS (H_2O_2). We also recorded a high lipid oxidation profile in these cell lines concurrent with miR-1253 expression. Finally, using AnnexinV and MitoSOX™ Red staining, we revealed a significantly elevated dual staining population of cells in miR-1253-expressing cells. Taken together, these results are highly indictive of ferroptosis induction by miR-1253 in group 3 MB cells.

Ferroptosis can also be induced by disruption of glutathione synthesis or inhibition of glutathione peroxidase 4.[85] Cancer cells have the capacity to activate redox buffering systems to survive in a highly oxidative environment resulting from deregulated cellular functions.[61] Glutathione is a key player in this response and a critical cofactor for glutathione peroxidase 4 (GPX4). GPX4, a central regulator of ferroptosis upregulated in various cancers, uses glutathione to reduce ROS and lipid hydroperoxide levels thus facilitating tumor cell survival in an environment with high oxidative stress.[86] Additionally, GSH binds Fe^{2+} to prevent iron oxidation and is thus a critical component controlling the labile iron pool.[87] Of note, ABCB7 harbors a GSH binding pocket, and GSH is a required substrate for cytosolic and nuclear Fe-S protein biogenesis and iron homeostasis.[54,88] In group 3 tumors, we not only showed deregulated GPX4 expression but also a strong positive correlation with ABCB7. Resultantly, miR-1253 expression or $ABCB7^{KO}$ strongly inhibited GPX4 expression and reduced glutathione levels. These data demonstrate the contribution of disrupting GPX4 and glutathione metabolism in miR-1253-mediated oxidative stress mechanisms resulting in ferroptosis.

MiRNA mimics have been shown to possess the capability of restoring the sensitivity of cancer cells for chemotherapeutic agents and to thus subsequently enhance their effectiveness. For example, miR-429, miR-383, miR-101-3p, miR-195, miR-634, and miR-1294 elicited a 2-5 fold reduction in the EC_{50} and IC_{50} values in combination with gemcitabine, temozolomide, and paclitaxel.[78,89-94] Of the standard chemotherapies for medulloblastoma tumors, only cisplatin has been shown to trigger both apoptosis and ferroptosis, via oxidative stress, GSH depletion, and GPX4 inactivation.[45,47,64,65] Moreover, a microarray-based study of the IC_{50} of cisplatin in 60 NCI cell lines identified multiple ABC transporters, including 3 iron transporters, i.e. ABCB6, ABCB7 and ABCB10, in conferring cisplatin drug resistance.[95]

Given the ferroptotic mechanism we elucidated for miR-1253, we studied whether miR-1253 expression can potentiate cisplatin cytotoxicity in group 3 MB cells. We report a 2-fold reduction in the IC_{50} value of cisplatin with miR-1253 induction in D425 and HDMB03 cells. Combination treatment had dramatic effects on the clonal potential of HDMB03 cells. We then demonstrated the highest induction of mitochondrial and cytosolic ROS, lipid peroxidation, and GSH depletion in miR-1253-expressing HDMB03 and $ABCB7^{KO}$ HDMB03 cells treated with cisplatin. Consequently, combination therapy resulted in the highest degree of ferroptosis in these cell lines. These effects were reversed by ferrostatin-1, a potent inhibitor of ferroptosis, lending further validity to the central role of ferroptosis in the mechanism of miR-1253-mediated effects and cisplatin potentiation.

Overall, our study has identified novel tumor suppressive properties for miR-1253. First, miR-1253 directly inhibits ABCB7 expression, thus inducing labile iron pool within MB cancer cells and stimulating ROS production. MiR-1253 can concurrently downregulate the expression of GPX4 and deplete GSH, further exacerbating ROS. Together, the generation of lipid hyperoxides progresses unabated, leading to cancer cell death via ferroptosis. We also leveraged these properties by showing potentiation of cisplatin cytotoxicity and thus enhanced therapeutic efficacy in group 3 MB cells. Together, our findings provide proof-of-concept for further exploration of tumor suppressive microRNAs as therapeutic adjuncts to standard chemotherapy. Such a strategy may mitigate the current limitations to treatment regimens in our youngest high risk patients.

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Figure Legends

Figure 1. ABCB7, a target of miR-1253, is deregulated in group 3 MB. (A) Subgroup-specific ABCB7 expression assessment by RNA sequencing (log_2 transcripts per million) of a local medulloblastoma patient cohort (Kanchan *et al.*, GSE) showing specific deregulation in group 3 tumors. *CB*, pediatric cerebellum (n=10); *SHH*, sonic hedgehog (n=6); *G3*, group 3 (n=7); *G4*, group 4 (n=12). **(B)** Poor prognostic profile demonstrable in high-expressing group 3 MB patients (Cavali *et al*. GSE85217). **(C)** Confocal microscopic images confirming high ABCB7 expression in group 3 MB tumors (n=6) compared to pediatric cerebellum (n=6) and colocalization to the mitochondria based on COXIV fluorescence. Images captured at 10X magnification. **(D)** Western blotting analysis showing high ABCB7 expression in classic MB cell lines (group 3: D341, D425, HDMB03, D556; group 3/4: D283) compared to normal human astrocytes (NHA). Western blotting analysis showing downregulation of ABCB7 with miR-1253 overexpression in **(E)** D425 and **(F)** HDMB03 cells. **(G)** Confocal microscopic images in D425 and HDMB03 cells showing co-localization of ABCB7 to the mitochondria and downregulation with miR-1253 overexpression. Images captured at 63X magnification. **(H)** Dual-luciferase assay confirming direct binding of miR-1253 to ABCB7 in HDMB03 cells. Data presented as mean ± SD from experiments done in triplicates and analyzed using Mann-Whitney U test (**A**) or Student's t-test **(H)** (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

Figure 2. ABCB7 repression attenuates G3MB cancer cell aggressiveness. (A) ABCB7 repression by miR-1253^{OE} (100 nM), si-ABCB7 (50 nM), and ABCB7^{KO} (CRISPR). ABCB7 repression attenuates medullosphere formation **(B),** cancer cell viability by MTT **(C),** and calcein staining **(D)**. ***p*<0.01, ****p*<0.001, *****p*<0.0001. *KO*, knockout; *NC*, negative control; *OE*, overexpression; *WT*, wild-type;

Figure 3. MiR-1253 triggers iron imbalance and oxidative stress in group 3 MB cells. ABCB7 repression triggers mitochondrial **(A)** and cytoplasmic **(B)** iron overload, which was rescued by iron chelation (DFO). ABCB7 repression induces oxidative stress **(C, D)**, rescued by ferrostatin-1 (FER). *ns*, not significant, **p*<0.05, ***p*<0.01, *****p*<0.0001. *DFO*, deferoxamine; *ERA*, erastin; *FAC*, ferric ammonium citrate; *FER*, ferrostatin-1; H_2O_2, hydrogen peroxide; miR, miR-1253; O_2^-, superoxide; s*i*, si-ABCB7.

Figure 4. ABCB7 repression triggers lipid peroxidation and mitochondrial dysfunction. (A) ABCB7 repression induced lipid peroxidation in HDMB03 cells, reversible by ferrostatin-1. **(B)** Seahorse assay showing that ABCB7 repression disrupts oxidative phosphorylation, shifting energy (ATP) production to the cytoplasm via glycolysis **(B)**. ***p* <0.01, ****p* <0.001, *****p* <0.0001 compared to negative control.

Figure 5. ABCB7 repression reduces G3MB tumor burden. (A) We orthotopically injected luciferase-tagged wild-type or $ABCB7^{KO}$ HDMB03 cells into NSG mice. The KO group had significantly smaller tumors **(B)**, longer survival **(C)**, and reduced c-Myc staining **(D)**. *KO*, $ABCB7^{KO}$; *WT*, wildtype.

Figure 6. ABCB7 repression inhibits GPX4 expression, a key regulator of ferroptosis. (A) ABCB7 repression by miR-1253^{OE} showing reduced GPX4 transcript levels by RT-PCR. **(B)** Western blotting analysis showing a strong inhibitory effect of miR-1253 on GPX4 expression in D425 and HDMB03 cells. **(C)** Near-abrogation of GPX4 expression with ABCB7 knockout in HDMB03 cells. **(D)** Spearman correlation showing positive correlation between ABCB7 and GPX4 expression. (Kanchan *et al.*, GSE148390). **(E)** Similar inhibitory effect noted on total glutathione (GSH) levels in miR-1253-transfected D425 and HDMB03 cells. **(F)** Fractionation studies delineating the inhibitory impact of ABCB7 repression on GPX4 expression. Data

presented as mean ± SD from experiments done in triplicates and analyzed using Student's t-test (*p <0.05, **p <0.01, ***p <0.001).

Figure 7. ABCB7 repression potentiates cisplatin toxicity in group 3 MB cells. (A) Cisplatin (2.5 µM) represses ABCB7 and GPX4 expression in HDMB03 cells. **(B)** ABCB7 repression potentiates cisplatin cytotoxicity in HDMB03 cells. **(C)** IC_{50} of cisplatin in D425 and HDMB03 cells transfected with scramble vs. miR-1253 for 24-72 h and HDMB03 cells transfected with si-ABCB7 (50 uM). Tabulated results presented as fold-change in cisplatin IC_{50} between scramble and miR-1253 in D425 and HDMB03 cells at different time points. **(D)** Colonogenic assay demonstrating inhibitory effect of cisplatin vs. miR-1253 vs. combination on colony formation in HDMB03 cells.

Figure 8. ABCB7 repression potentiates oxidative stress in cisplatin-treated G3MB cells. MiR-1253-transfected **(A)** D425 and **(B)** HDMB03, and **(C)** $ABCB7^{KO}$ HDMB03 cells treated with cisplatin (D425 24-h IC_{25} ~10 µM; HDMB03 24-h IC_{25} ~2 µM) and stained with MitoSOX™ Red (**red**) for mitochondrial superoxide anions ($O_2^{\bullet-}$) and DCFDA (**green**) for cytosolic ROS (H_2O_2). **(D** and **E)** Cisplatin potentiates ROS in HDMB03 cells with repressed ABCB7 expression; ferrostatin-1 rescues this effect. Representative images and graphs showing the potentiating effect of combining miR-1253 with cisplatin. Images captured at 20X magnification. Scale bar 400 µm. Data presented as mean ± SD from experiments done in triplicates and analyzed using one-way analysis of variance (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001). *CIS*, cisplatin; *FER*, ferrostatin-1; *miR*, miR-1253; *NC*, negative control; *si*, si-ABCB7.

Figure 9. ABCB7 repression potentiates cisplatin cytotoxicity in group 3 MB cells by triggering ferroptosis. Oxidative stress measured by quantifying **(A)** mitochondrial $O_2^{\bullet-}$

(MitoSOX™ Red, **red**) and **(B)** cytosolic H_2O_2 (DCFDA, **green**) in miR-1253-transfected HDMB03 cells showing potentiating effect of miR-1253 on cisplatin, and inhibited by a ferroptosis inhibitor, ferrostatin-1 (FER). Images captured at 20X magnification. Scale bar 200 µm. **(C)** As measured by Image-iT® Lipid Peroxidation Kit, confocal images showing the highest lipid peroxidation in combination treatment groups (miR + Cis), again rescued by ferrostatin-1, in miR-1253-transfected HDMB03 cells. Images captured at 63X magnification. **(D)** Evaluation of oxidized glutathione (GSH) showing punctuated effects in combination treatment groups (miR + Cis) with rescue in the presence of ferrostatin in miR-1253-transfected HDMB03 cells. **(E)** Analysis of cell death by flow cytometry showing significantly higher $O_2^{\bullet-}$ mediated cell death (representing ferroptosis) in miR-1253-expressing HDMB03 cells demonstrable by quantifying cells staining for both Annexin V-Cy5 (apoptosis) and for $O_2^{\bullet-}$ (Mitosox) (Q2). Data presented as mean ± SD from experiments done in triplicates and analyzed using one-way analysis of variance (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

Supplementary Figure 1. Iron homeostasis and glutathione metabolism are deregulated in G3 tumors. (A) Enrichment plot by KEGG pathways analysis demonstrating a strong negative enrichment score for the ABC transporter family with miR-1253 expression restoration in HDMB03 cells. **(B)** Deregulated ABC transporters in group 3 MB (column 1), effect of deregulated expression on survival (column 2), and transporters significantly downregulated by miR-1253 expression (column 3). This analysis revealed ABCB7 as the best putative target for miR-1253. **(C)** Key genes regulating iron homeostasis, glutathione metabolism, and ferroptosis are enriched in G3MB (GSE148390, p<0.05 for all presented gene comparisons between normal and G3MB). **(D)** Enriched pathways in G3MB include iron-sulfur cluster assembly and glutathione metabolism. *Fe-S*, iron-sulfer; *GSH*, glutathione.

Supplementary Figure 2. ABCB7 is enriched in MB and portends a poor prognosis. (A) Expression profile for ABCB7 in multiple MB cohorts. *CB*, normal cerebellum, Roth *et al.* 2008 (n=9, GSE3526); *MB 1*, Gilbertson *et al.* 2012 (n=76, GSE37418); *MB 2*, Pfister *et al.* 2017 (n=223); *MB 3*, Delattre *et al.* 2012 (n=57); *MB 4*, Kool *et al.* 2009 (n=62, GSE10327). **(B)** Poor prognostic profile demonstrable in high ABCB7-expressing medulloblastoma patients (Cavali *et al*. GSE85217). Subgroup-specific ABCB7 expression in two separate medulloblastoma patient cohorts, i.e. **(C)** Weishaupt *et al.*, GSE124814; *CB*, normal cerebellum (n=291); *WNT*, wingless (n=118); *SHH*, sonic hedgehog (n=405); *G3*, group 3 (n=233); *G4*, group 4 (n=530); and **(D)** Luo *et al.*, GSE164677; *CB*, normal cerebellum (n=4); *WNT*, wingless (n=6); *SHH*, sonic hedgehog (n=20); *G3*, group 3 (n=14); *G4*, group 4 (n=19); Data normalized via RUV method **(C)** or DeSeq2 median of ratios (MoR) method **(D)** and both analyzed using Mann-Whitney U (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

Supplementary Figure 3. ABCB7 repression triggers iron overload. Escalation in both cytosolic and mitochondrial free Fe^{2+} demonstrable with miR-1253 expression **(A and B)** and ABCB7KO **(C and D)**, abrogated by iron chelation with DFO. Mitochondria stained with MitoTracker™ Deep Red FM (**red**); cytosolic Fe^{2+} stained with FerroOrange (**orange**); mitochondrial Fe^{2+} stained with Mito-FerroGreen (**green**). Images captured at 63X.

Supplementary Figure 4. ABCB7 repression by MiR-1253 triggers oxidative stress and lipid peroxidation leading to cell death in group 3 MB cells. Confocal images showing elevated mitochondrial $O_2^{\bullet-}$ (MitoSOX™ Red, **red**) and cytosolic H_2O_2 (DCFDA, **green**) following miR-1253 expression in **(A)** D425 and **(B)** HDMB03 cells. Higher lipid peroxidation (measured by Image-iT® Lipid Peroxidation Kit) also noted in miR-1253 transfected **(C)** D425 and **(D)** HDMB03 cells. Flow cytometry analysis showing significantly higher $O_2^{\bullet-}$ mediated cell

death (representing ferroptosis) in miR-1253-expressing **(E)** D425 and **(F)** HDMB03 cells demonstrable by quantifying cells staining for both Annexin V-Cy5 (apoptosis) and for $O_2^{\bullet-}$ (Mitosox) (Q2). Data presented as mean ± SD from experiments done in triplicates and analyzed using Student's t-test (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001). Images captured either at 20X (**A** and **B**) or 63X (**C** and **D**) magnification. Scale bar 200 µm.

Supplementary Figure 5. ABCB7 repression triggers iron overload and oxidative stress. Confocal images showing Calcein AM dye quenching in miR-1253-transfected **(A)** D425 and **(B)** HDMB03 cells indicating high cytosolic labile iron compared to scramble (NC) transfected cells. **(C)** Confocal images showing Calcein AM dye quenching in $ABCB7^{KO}$ HDMB03 cells suggestive of high cytosolic labile iron compared to wild-type (WT) cells. Fe^{2+} stained with Calcein AM (**green**) and nuclei stained with DAPI (**blue**). **(D)** Elevated oxidative stress demonstrable in $ABCB7^{KO}$ HDMB03 cells as evidenced by higher mitochondrial $O_2^{\bullet-}$ (MitoSOX™ Red, **red**) and cytosolic H_2O_2 (DCFDA, **green**) compared to wild-type. Images captured at 63X (**C-E**) or 20X (**F**) magnification. Scale bar 400 µm. Data presented as mean ± SD from experiments done in triplicates and analyzed using Student's t-test (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

Supplementary Figure 6. GPX4 is a central regulator of ferroptosis that is deregulated in MB. (A) Schematic showing the important role of GPX4 in mitigating ferroptosis. Image created with Biorender.com. **(B)** Subgroup-specific GPX4 expression assessment by RNA sequencing ($\log_2$ transcripts per million) of a local medulloblastoma patient cohort (Kanchan *et al.*, GSE148390) showing deregulation in MB tumors, highest in group 3 MB. *CB*, pediatric cerebellum (n=10); *SHH*, sonic hedgehog (n=6); *G3*, group 3 (n=7); *G4*, group 4 (n=12). **(C)** Subgroup-specific GPX4 expression data from Weishaupt *et al.*, GSE124814; *CB*, normal

cerebellum (n=291); *WNT*, wingless (n=118); *SHH*, sonic hedgehog (n=405); *G3*, group 3 (n=233); *G4*, group 4 (n=530); Data normalized via RUV method and analyzed using Mann-Whitney U (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001). **(D)** Poor prognostic profile demonstrable in high-expressing MB patients (Cavali *et al*. GSE85217). **(E)** Confocal microscopic images confirming high GPX4 expression in group 3 MB tumors (n=6) compared to pediatric cerebellum (n=6) and colocalization to the mitochondria based on COXIV fluorescence. Images captured at 10X magnification. **(F)** Western blotting analysis showing high *in vitro* GPX4 expression in classic MB cell lines (group 3: D341, D425, HDMB03; group 3/4: D283) compared to normal human astrocytes (NHA).

Supplementary Figure 7. ABCB7 knockout potentiates cisplatin cytotoxicity in group 3 MB cells by ferroptosis. Oxidative stress measured by quantifying **(A)** mitochondrial $O_2^{\bullet-}$ (MitoSOX™ Red, **red**) and **(B)** cytosolic H_2O_2 (DCFDA, **green**) in miR-1253-transfected HDMB03 cells showing potentiating effect of miR-1253 on cisplatin, and inhibited by a ferroptosis inhibitor, ferrostatin-1 (FER). Images captured at 20X magnification. Scale bar 200 µm. **(C)** As measured by Image-iT® Lipid Peroxidation Kit, confocal images showing the highest lipid peroxidation in combination treatment groups ($ABCB7^{KO}$ + Cis), again rescued by ferrostatin-1, in $ABCB7^{KO}$ HDMB03 cells. Images captured at 63X magnification. **(D)** Evaluation of oxidized glutathione (GSH) showing punctuated effects in combination treatment groups ($ABCB7^{KO}$ + Cis) with rescue in the presence of ferrostatin in $ABCB7^{KO}$ HDMB03 cells. Data presented as mean ± SD from experiments done in triplicates and analyzed using one-way analysis of variance (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

Figure 1

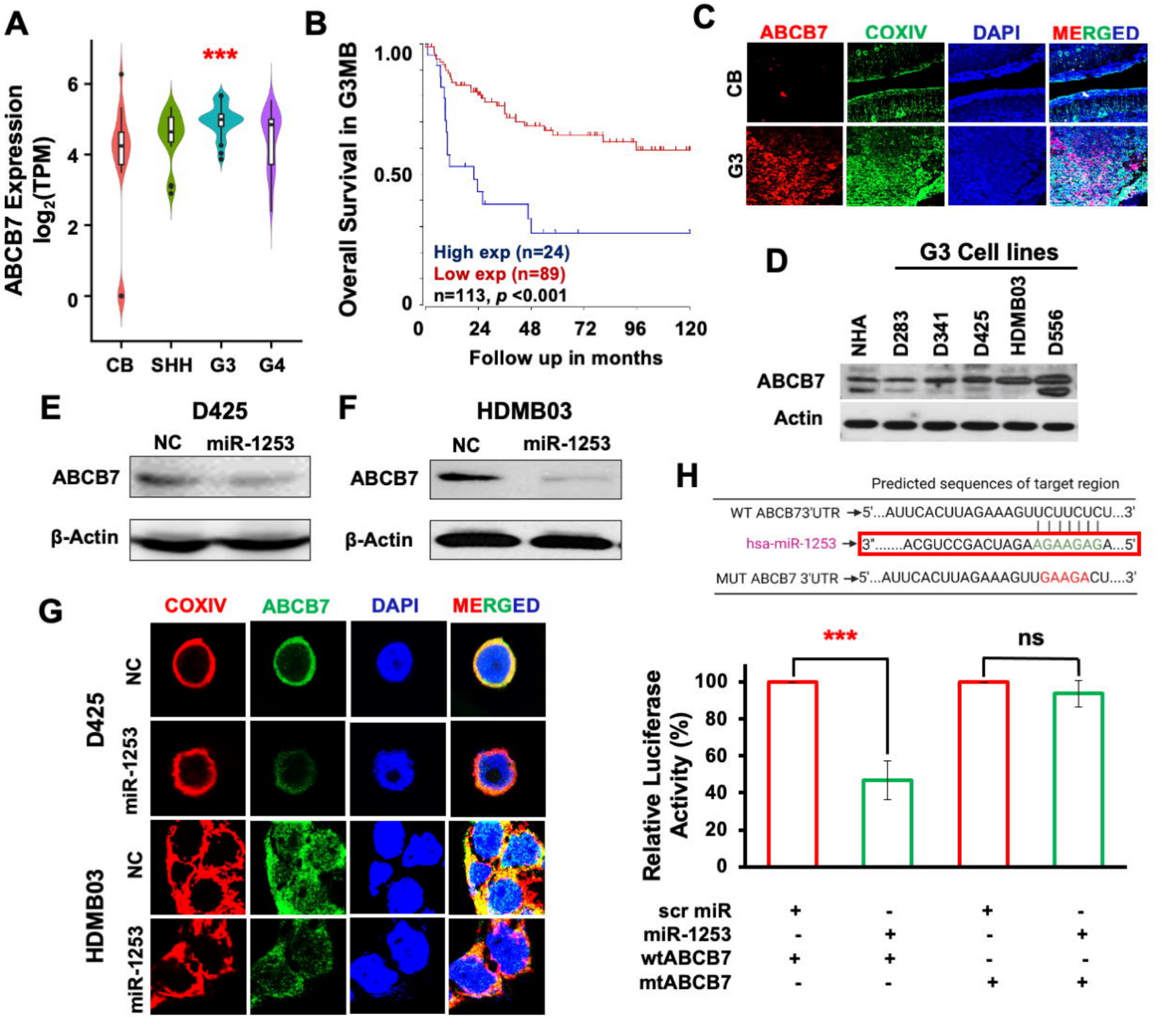

A
ABCB7 Expression
log2(TPM)
***
CB
SHH
G3
G4
B
Overall Survival in G3MB
High exp (n=24)
Low exp (n=89)
n=113, p <0.001
Follow up in months
C
ABCB7
COXIV
DAPI
MERGED
CB
G3
D
G3 Cell lines
NHA
D283
D341
D425
HDMB03
D556
ABCB7
Actin
E
D425
NC
miR-1253
ABCB7
β-Actin
F
HDMB03
NC
miR-1253
ABCB7
β-Actin
H
Predicted sequences of target region
WT ABCB73'UTR →5'...AUUCACUUAGAAAGUUCUUCUCU...3'
hsa-miR-1253 →3'.....ACGUCCGACUAGAAGAAGAGA...5'
MUT ABCB7 3'UTR →5'...AUUCACUUAGAAAGUUGAAGACU....3'
G
COXIV
ABCB7
DAPI
MERGED
D425
NC
miR-1253
HDMB03
NC
miR-1253
***
ns
Relative Luciferase Activity (%)
scr miR
miR-1253
wtABCB7
mtABCB7

Figure 2

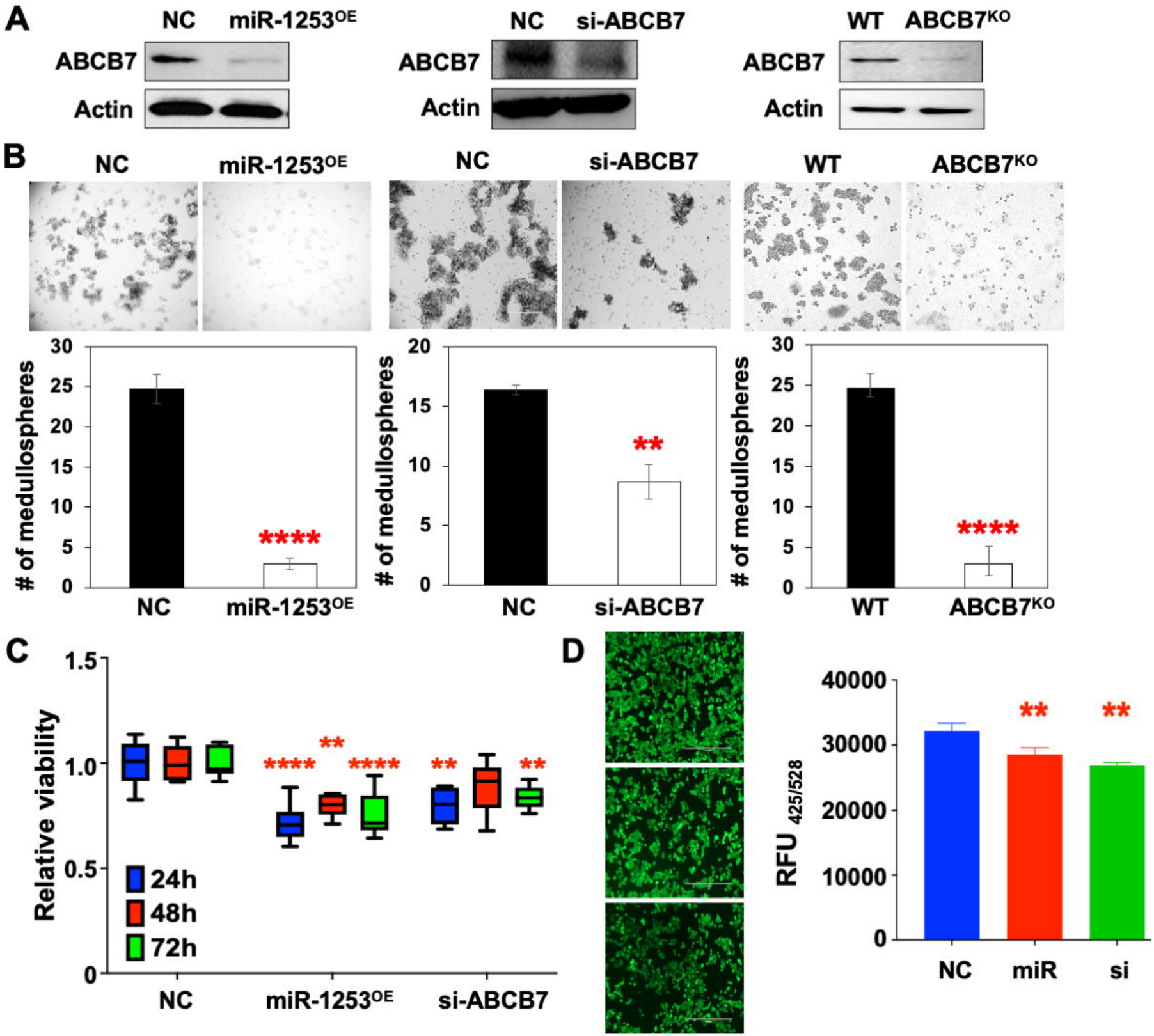
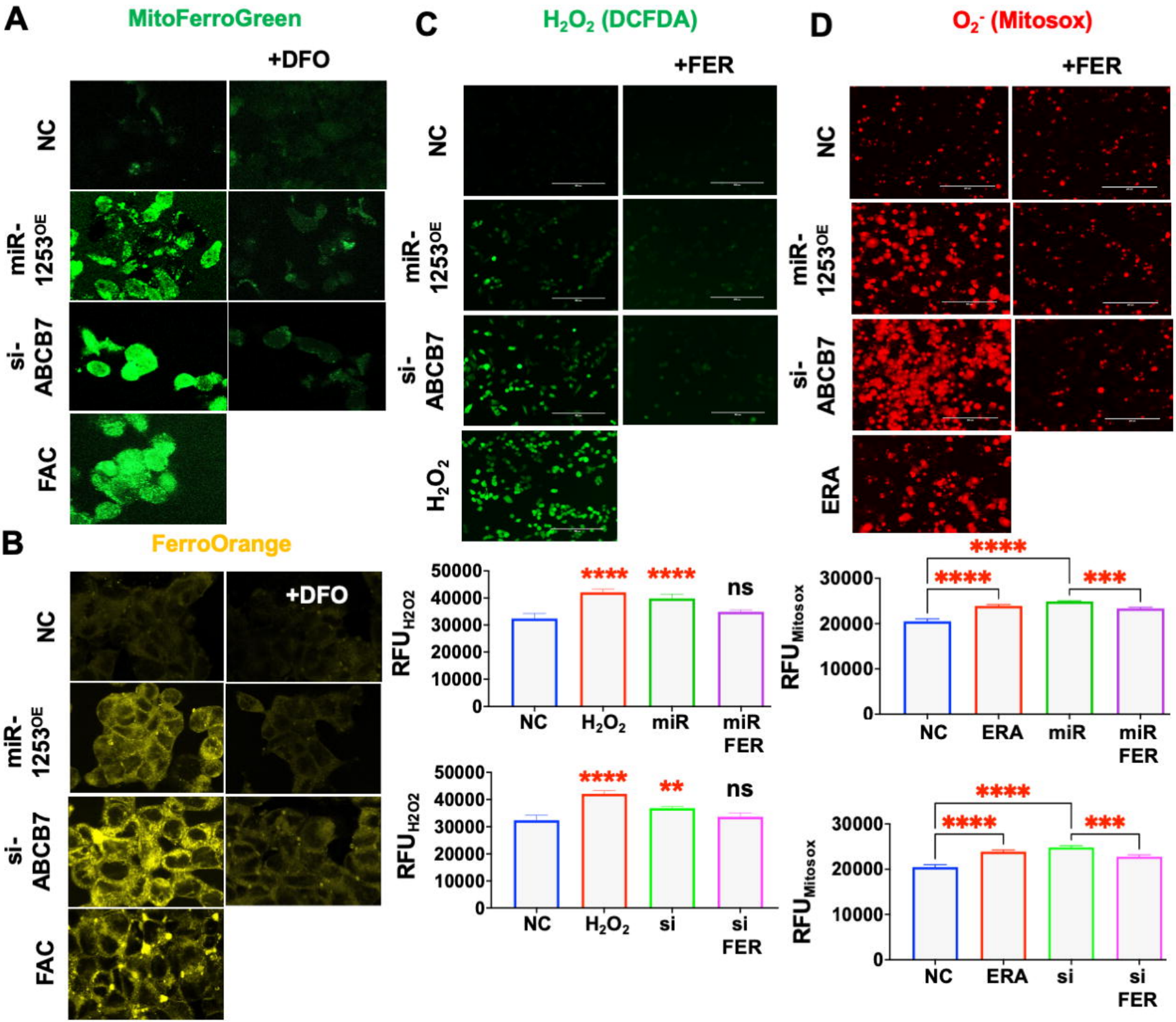

A
NC
miR-1253OE
ABCB7
Actin
NC
si-ABCB7
ABCB7
Actin
WT
ABCB7KO
ABCB7
Actin
B
NC
miR-1253OE
NC
si-ABCB7
WT
ABCB7KO
# of medullospheres
30
25
20
15
10
5
0
****
NC
miR-1253OE
# of medullospheres
20
15
10
5
0
**
NC
si-ABCB7
# of medullospheres
30
25
20
15
10
5
0
****
WT
ABCB7KO
C
Relative viability
1.5
1.0
0.5
0
24h
48h
72h
****
**
****
**
**
NC
miR-1253OE
si-ABCB7
D
RFU 425/528
40000
30000
20000
10000
0
**
**
NC
miR
si

Figure 3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+DFO
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FAC
B
FerroOrange
+DFO
NC
miR-1253OE
si-ABCB7
FAC
C
H2O2 (DCFDA)
+FER
NC
miR-1253OE
si-ABCB7
H2O2
RFUH2O2
50000
40000
30000
20000
10000
0
****
****
ns
NC
H2O2
miR
miR FER
****
**
ns
NC
H2O2
si
si FER
D
O2- (Mitosox)
+FER
NC
miR-1253OE
si-ABCB7
ERA
RFUMitosox
30000
20000
10000
0
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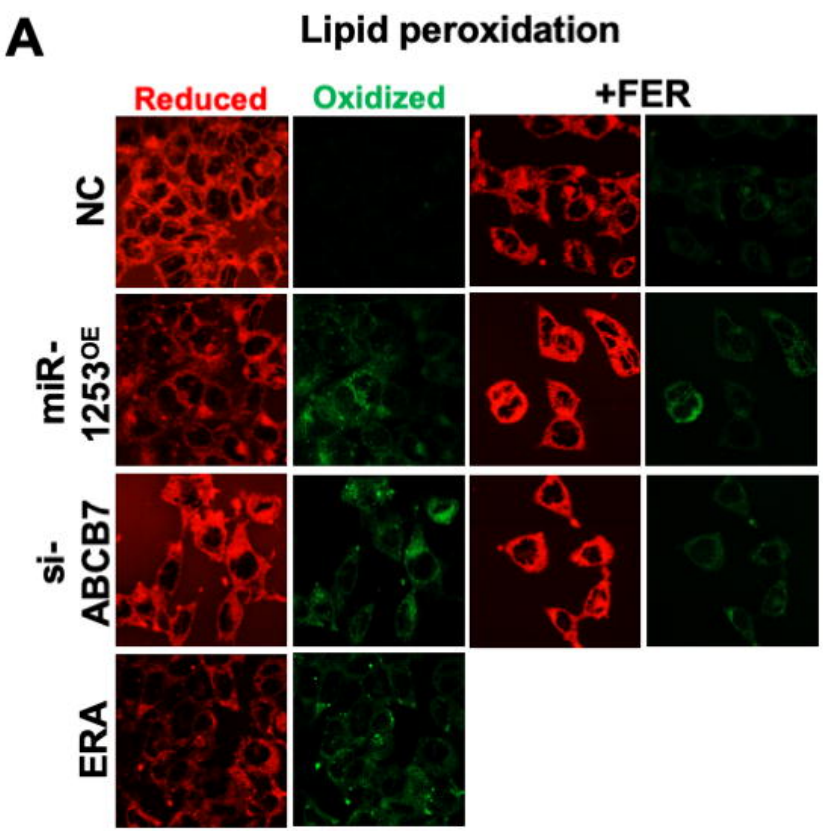
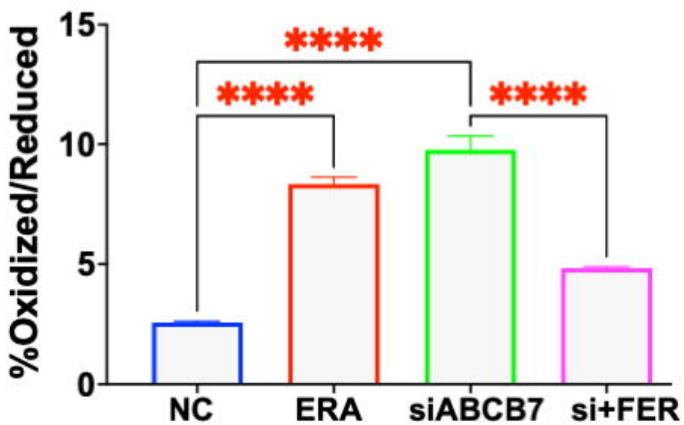
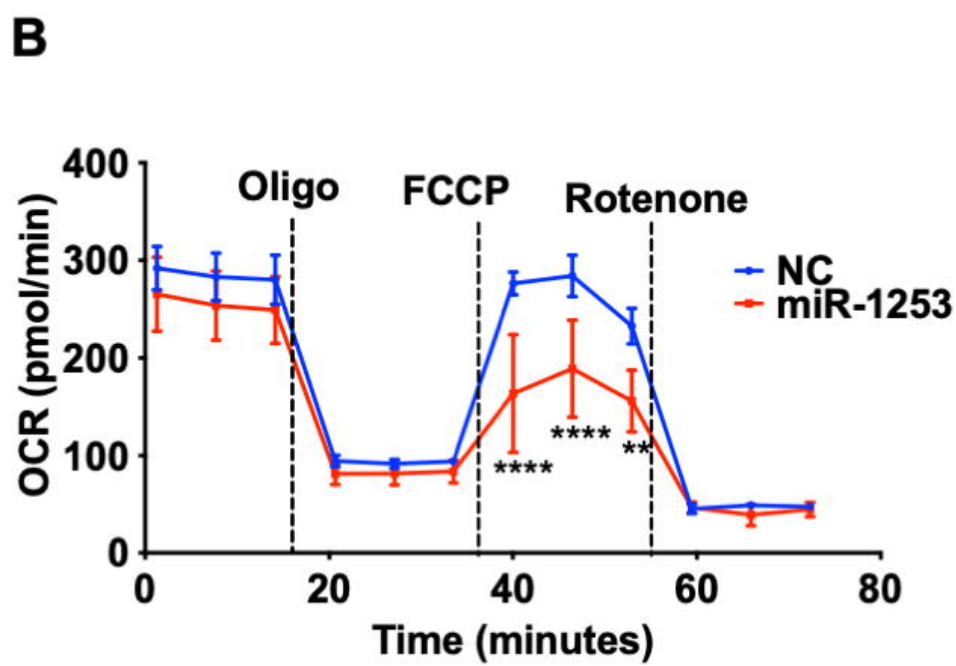
****
***
NC
ERA
miR
miR FER
****
****
***
NC
ERA
si
si FER

Figure 4


A
Lipid peroxidation
Reduced
Oxidized
+FER
NC
miR-1253OE
si-ABCB7
ERA


%Oxidized/Reduced
15
10
5
0
****
****
****
NC
ERA
siABCB7
si+FER


B
OCR (pmol/min)
400
300
200
100
0
Oligo
FCCP
Rotenone
NC
miR-1253
****
****
**
0
20
40
60
80
Time (minutes)

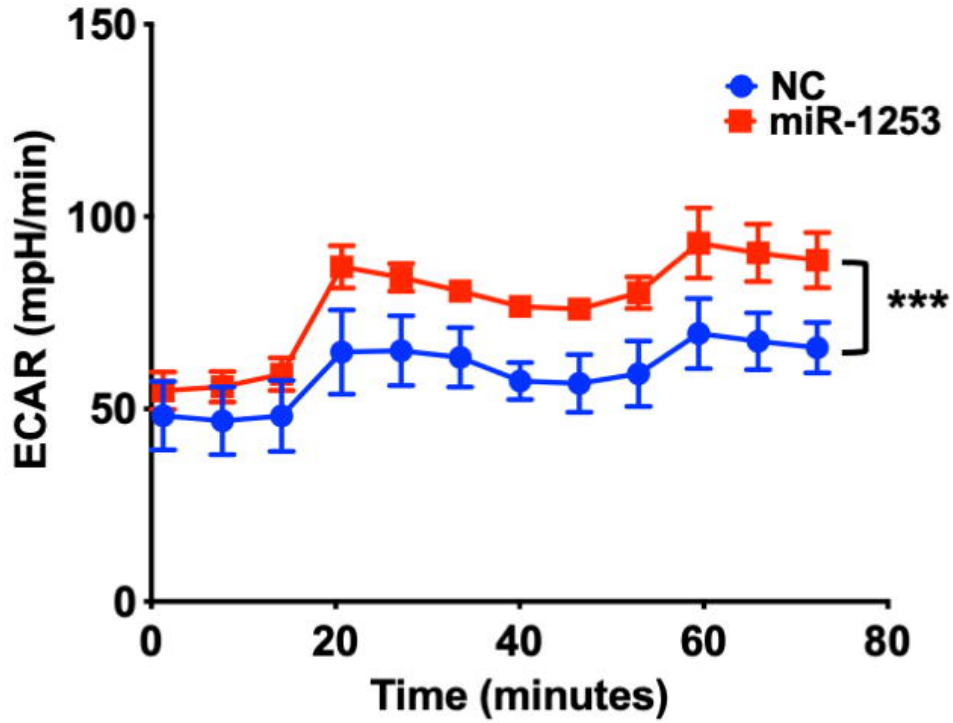

ECAR (mpH/min)
150
100
50
0
NC
miR-1253
***
0
20
40
60
80
Time (minutes)

Figure 5

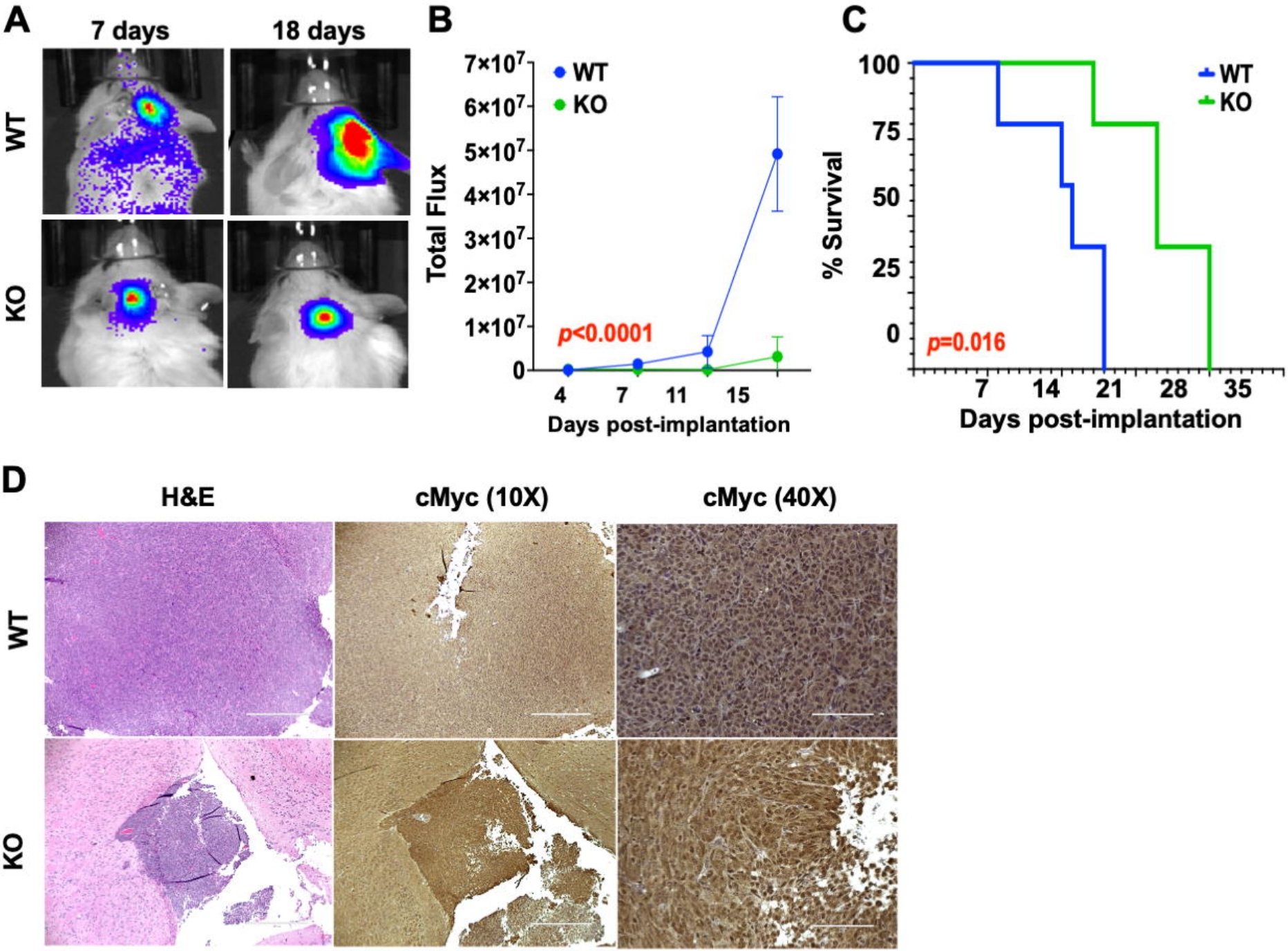
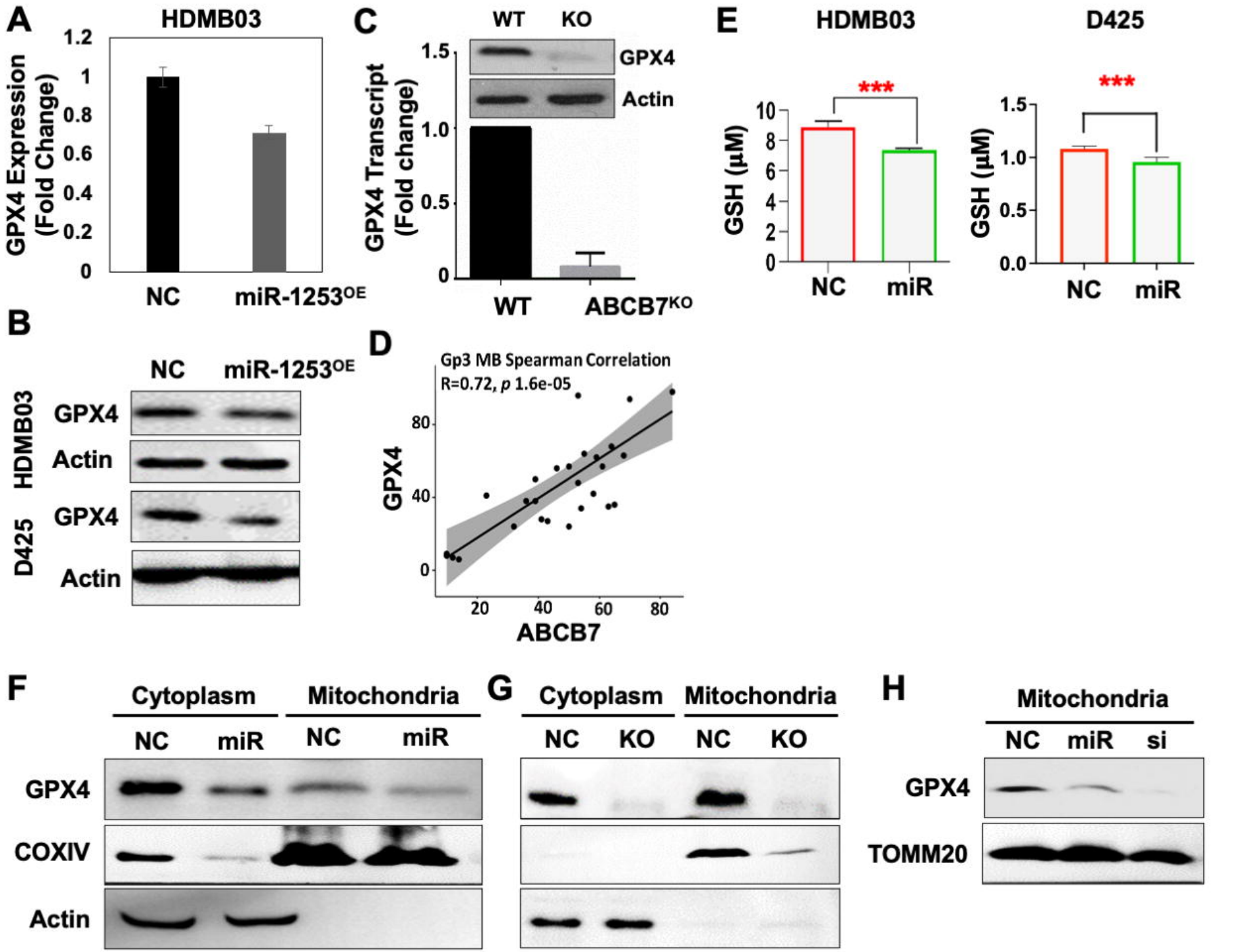

A
7 days
18 days
WT
KO
B
WT
KO
Total Flux
7×10^7
6×10^7
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
p<0.0001
4
7
11
15
Days post-implantation
C
WT
KO
% Survival
100
75
50
25
0
p=0.016
7
14
21
28
35
Days post-implantation
D
H&E
cMyc (10X)
cMyc (40X)
WT
KO

Figure 6


A
HDMB03
GPX4 Expression (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
NC
miR-1253OE
B
NC
miR-1253OE
HDMB03
GPX4
Actin
D425
GPX4
Actin
C
WT
KO
GPX4
Actin
GPX4 Transcript (Fold change)
1.5
1.0
0.5
0
WT
ABCB7KO
D
Gp3 MB Spearman Correlation
R=0.72, p 1.6e-05
GPX4
80
40
0
20
40
60
80
ABCB7
E
HDMB03
D425
***
***
GSH (μM)
10
8
6
4
2
0
1.5
1.0
0.5
0.0
NC
miR
NC
miR
F
Cytoplasm
Mitochondria
NC
miR
NC
miR
GPX4
COXIV
Actin
G
Cytoplasm
Mitochondria
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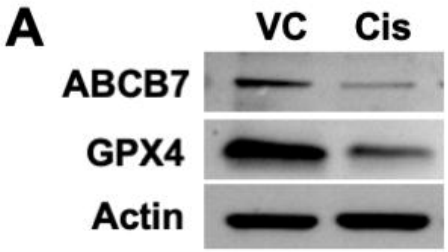
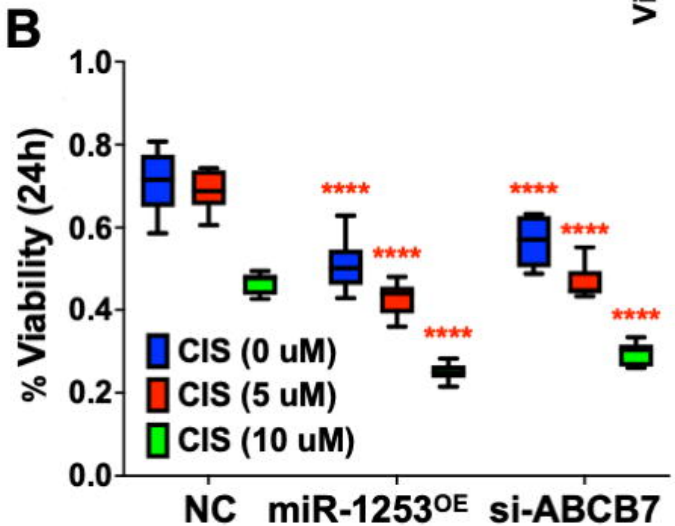
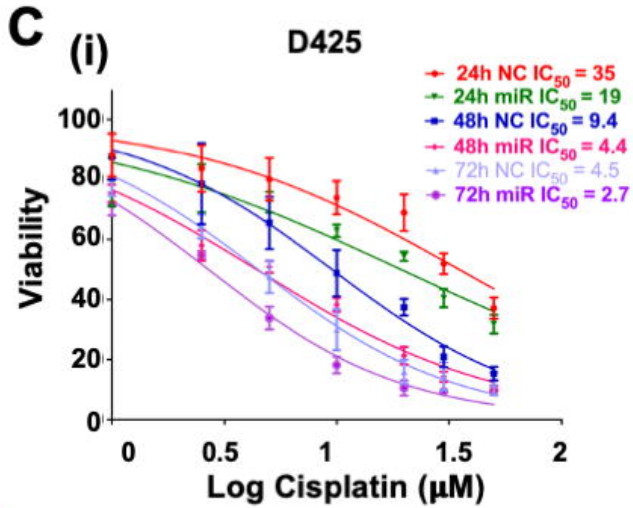
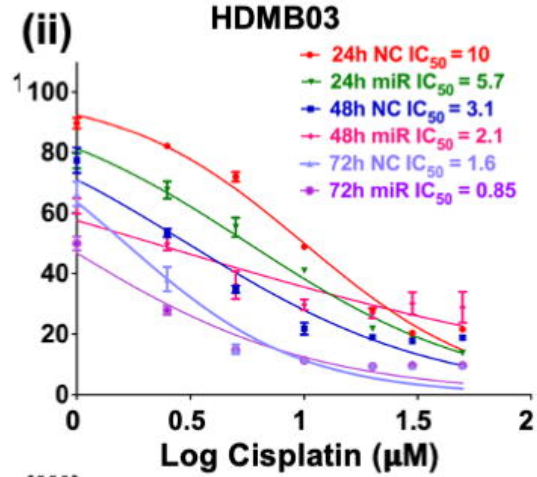
GPX4
TOMM20

Figure 7

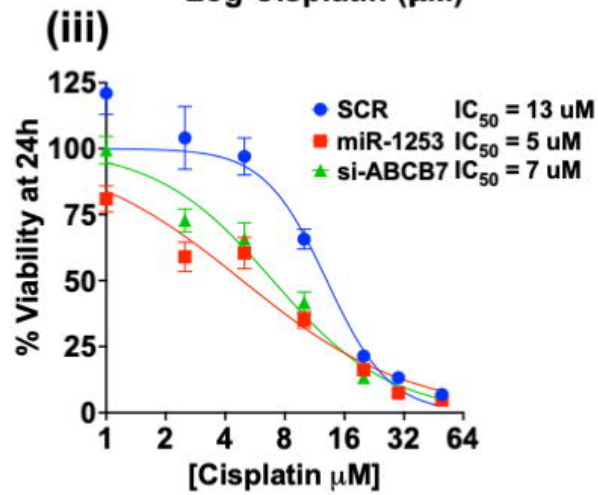


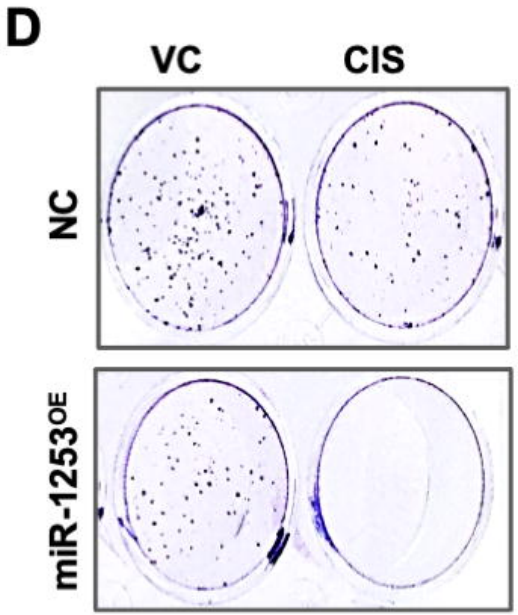






Fold Reduction (IC_{50})		D425	HDMB03
	24h	1.8	1.8
	48h	2.1	1.5
	72h	1.7	1.9





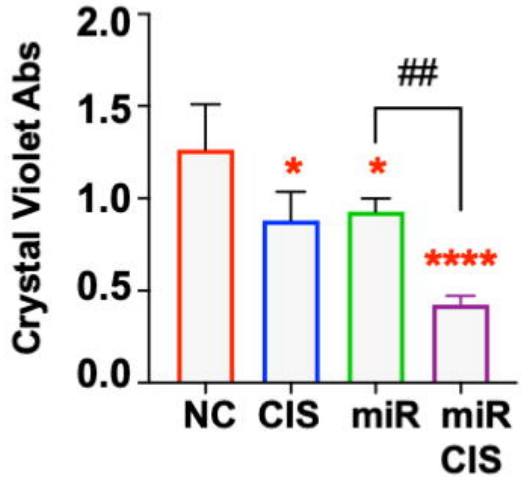


Figure 8

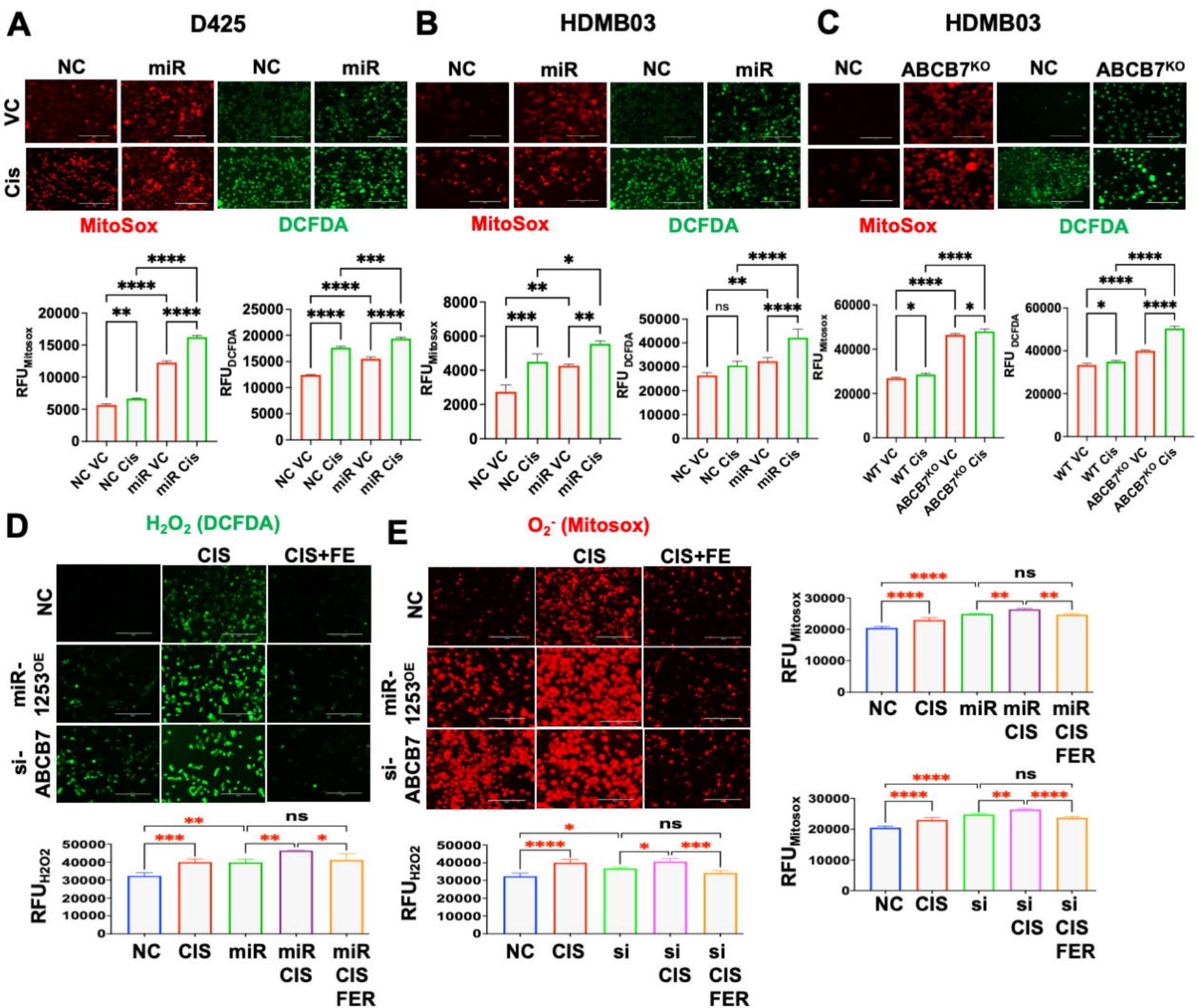
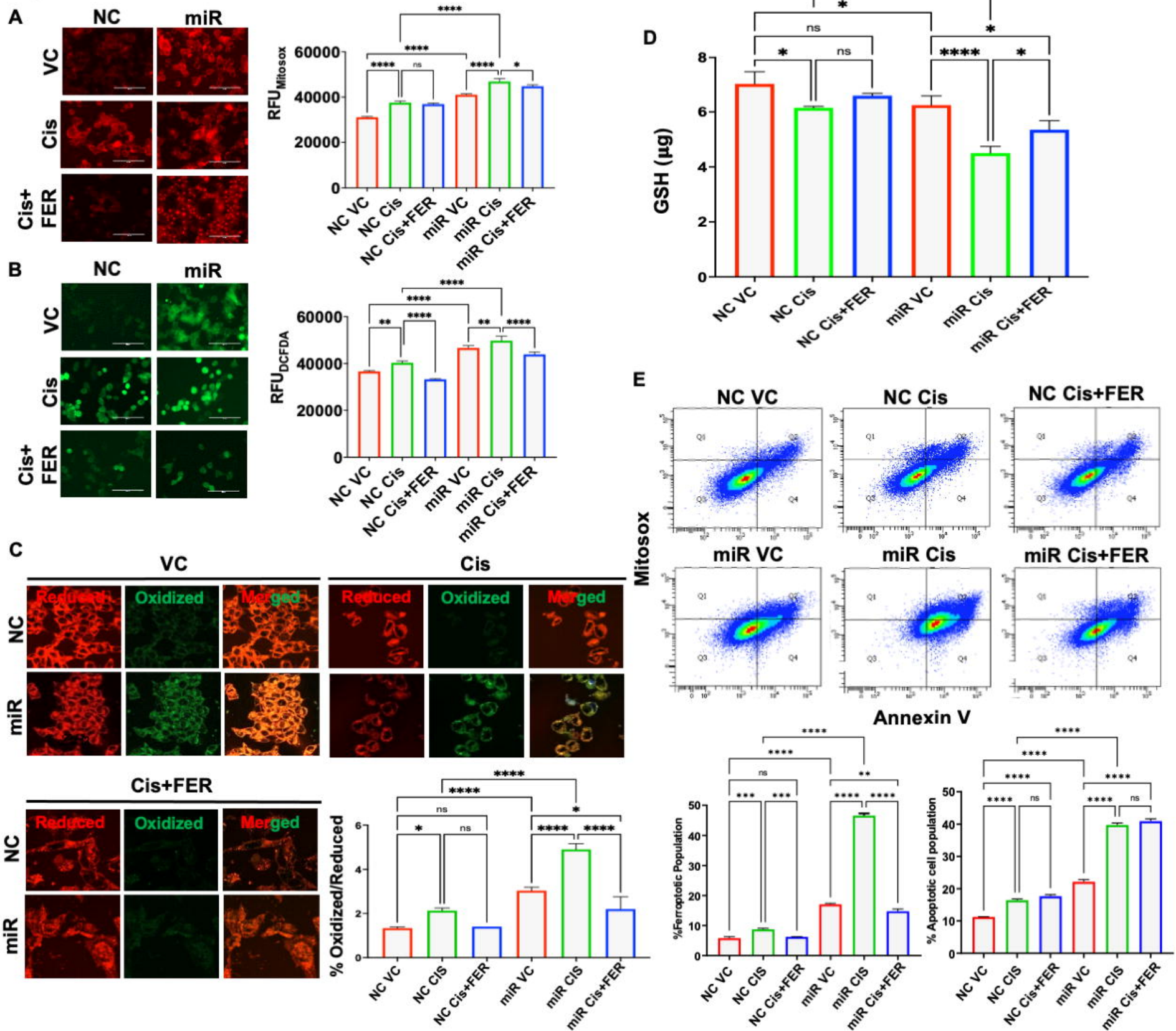

A
D425
B
HDMB03
C
HDMB03
NC
miR
ABCB7KO
VC
Cis
MitoSox
DCFDA
RFUMitosox
RFUDCFDA
NC VC
NC Cis
miR VC
miR Cis
WT VC
WT Cis
ABCB7KO VC
ABCB7KO Cis
D
H2O2 (DCFDA)
CIS
CIS+FE
miR-1253OE
si-ABCB7
RFUH2O2
NC
CIS
miR
miR CIS
miR CIS FER
E
O2- (Mitosox)
si
si CIS
si CIS FER
ns

Figure 9


A
NC
miR
VC
Cis
Cis+ FER
RFU Mitosox
60000
40000
20000
0
NC VC
NC Cis
NC Cis+FER
miR VC
miR Cis
miR Cis+FER
B
RFU DCFDA
C
Reduced
Oxidized
Merged
% Oxidized/Reduced
D
GSH (µg)
E
Mitosox
Annexin V
%Ferroptotic Population
% Apoptotic cell population