Supplementary Information for

Mechanistic basis for impaired ferroptosis in cells expressing the African-centric S47 variant of p53

Julia I-Ju Leu[a], Maureen E. Murphy[b,1] and Donna L. George[a,1]

[a]Department of Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA; and [b]Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA 19104, USA.

[1]To whom correspondence should be addressed: Maureen E. Murphy (mmurphy@wistar.org) and Donna L. George (georged@pennmedicine.upenn.edu)

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SI Materials and Methods

Cell Lines and Cell Culture. P47 and S47 primary MEFs, as well as immortalized clones (iMEFs), were generated and maintained as previously described (1). Human P47 and S47 LCLs were from the Coriell Institute (Camden, New Jersey) and grown as previously described (1). Human IMR90, WI38 and HT-1080 cell lines were purchased from ATCC. Human melanoma cell lines 1205Lu and WM852 were kindly provided by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). All cultured cell lines were maintained in DMEM (ThermoFisher Scientific catalog #11965084) supplemented with 10% Fetal Bovine Serum (FBS, HyClone, GE Healthcare Life Sciences) and 1X penicillin/streptomycin (ThermoFisher Scientific catalog #15140122). All cultured cell lines were seeded overnight in DMEM (ThermoFisher Scientific catalog #11965084) supplemented with 10% Fetal Bovine Serum (FBS, HyClone, GE Healthcare Life Sciences) and 1X penicillin/streptomycin (ThermoFisher Scientific catalog #15140122) at ~ 50% confluency.

For toxicity studies, primary mouse embryonic fibroblasts (MEFs), iMEFs and human LCLs were cultured in 1% FBS DMEM medium (ThermoFisher Scientific catalog #21013024 supplemented with 1% FBS, 1X penicillin/streptomycin, 0.1 mM L-Methionine, 0.25 mM L-Glutamine, 0.099 mM L-Cystine and specified factors, as indicated, in individual experiments). Toxicity studies on human cell lines IMR90, WI38, 1205Lu and WM852 were in 2% FBS DMEM medium (ThermoFisher Scientific catalog #11965084 supplemented with 2% Fetal Bovine Serum and 1X penicillin/streptomycin), and for human cell line HT-1080 the growth and test medium was 10% FBS DMEM medium (ThermoFisher Scientific catalog #11965084 supplemented with 10% Fetal

Bovine Serum and 1X penicillin/streptomycin). L-Cystine depletion studies involving iMEFs, IMR90, WI38, 1205Lu and WM852 were performed in 1% FBS DMEM medium without L-Cystine. L-Cystine depletion studies involving human cell line HT-1080 were performed in 10% FBS DMEM medium without L-Cystine (ThermoFisher Scientific catalog #21013024 supplemented with 10% FBS, 1X penicillin/streptomycin, 0.2 mM L-Methionine and 4 mM L-Glutamine).

Animal Models. P47 and S47 Hupki mice were generated by Dr. Maureen Murphy (The Wistar Institute, Philadelphia, PA). Mice were housed in plastic cages with *ad libitum* diet and raised under conditions of regulated lighting, temperature and humidity. Of note, we only included male mice in our final analyses, in part because we found that cellular response to liver damage is highly sex-dependent (1). Briefly, carbon tetrachloride (CCl_4, 1 µl/g body wt) in vegetable oil was injected into the intraperitoneal (i.p.) cavity, and the mice were then euthanized at various times after injections, as indicated. Mice with *ad libitum* access to food and water at all times were randomly assigned to control and experimental groups. Control and experimental groups were age-, sex- and genotype-matched non-littermates. All injections were performed around noon to minimize circadian differences in toxicity. For acute studies: Eight-week-old P47 and S47 male mice were injected i.p. with CCl_4, once a day for three days (referred to as 72 h). Additional cohorts of eight-week-old P47 and S47 male mice were pretreated with 50 mg/kg of CoA (Cayman Chemical, in PBS) followed by 1 µl/g of CCl_4 or co-treated with 15 mg/kg of diethyl maleate (DEM) premixed with 1 µl/g of CCl_4 in vegetable oil, once a day for three days. Animals were sacrificed 24 h after the last i.p.

injection, and livers were used for further analysis. For chronic studies: Eight-week-old P47 and S47 male mice were treated three times a week for 5 weeks. Additional cohorts of eight-week-old P47 and S47 male mice were pretreated with 50 mg/kg of CoA followed by 1 µl/g of CCl_4 or co-treated with 15 mg/kg of DEM premixed with 1 µl/g of CCl_4 in vegetable oil, three times per week for 5 weeks. Animals were sacrificed 24 h (CCl_4:5W) or 2 weeks (CCl_4:5W:O2W) after the last injection, and livers were used for further analysis. All experimental procedures involving mice were conducted in accordance with protocols approved by The Institutional Animal Care & Use Committee, Office of Animal Welfare of the Perelman School of Medicine at the University of Pennsylvania, and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

RNA Isolation and Quantitative RT-PCR. Total RNA was extracted using the Direct-zol RNA Kit (Zymo Research, Tustin, CA), according to manufacturer instructions. Equal amounts of total RNA were reverse-transcribed using the SuperScript III First-Strand Synthesis System, and the resulting cDNA was used with Fast SYBR Green Master Mix and primer sets, as listed. Quantitative RT-PCR was performed using the QuantStudio 7 Flex Real-Time PCR System (ThermoFisher Scientific). C_t values for the genes of interest were normalized to the invariant control gene IL-3. Gene expression data are expressed as fold change relative to untreated- or vehicle-treated P47 controls. All experiments were replicated in at least three independent experiments with technical replicates, as indicated, in each experiment.

Cell Viability Assays, GSH/GSSG Studies and CoA Analyses. Cell viability assays were performed using the MTT Kit (Sigma-Aldrich) and CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, Madison, WI), according to manufacturer instructions. Reduced GSH and GSH/GSSG ratio were quantitated using the GSH/GSSG-Glo Assay Kit (Promega Corporation), according to manufacturer instructions. CoA was measured using the Coenzyme A (CoA) Assay Kit (Sigma-Aldrich), according to manufacturer instructions.

Tissue Staining and Immunohistochemistry. Tissues were harvested and fixed in Formalde-Fresh Solution overnight at 4°C, washed with 1X PBS and transferred to 70% ethanol before paraffin embedding and sectioning. Tissue embedding and sectioning were performed by the Molecular Pathology & Imaging Core, funded by the Center for Molecular Studies in Digestive and Liver Diseases, Perelman School of Medicine, University of Pennsylvania. For immunohistochemistry (IHC) studies, tissue sections were deparaffinized in xylene, rehydrated in ethanol (100%-95%-80%-70%) and then distilled water. For Sirius Red staining, the tissue sections were immersed in Picro-sirius Red solution for 1 h at room temperature, washed three times with acidified water, dehydrated in ethanol, cleared in xylene and mounted using Cytoseal 60. For Prussian Blue staining, tissue sections were deparaffinized in xylene, rehydrated in ethanol (100%-95%-80%-70%) and then distilled water. Tissue sections were subsequently immersed in Prussian Blue Staining Solution (equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide solution) for 2-3 h at 37°C, washed with distilled water, dehydrated in ethanol, cleared in xylene and mounted using Cytoseal 60. For 4HNE

staining, antigen retrieval was performed by heating slides in 10 mM Citrate Buffer (pH 6), using the Retriever for Antigen Unmasking. After quenching endogenous peroxidase activity with 3% hydrogen peroxide, tissue sections were treated with avidin/biotin blocking reagents and StartingBlock T20 (PBS) Blocking Buffer, according to manufacturer instructions. Tissue sections were subsequently incubated with primary antibody overnight at 4°C, washed next day with 1X PBS and incubated with biotinylated secondary antibody for 30 min at 37°C. Antibody complexes were then detected using the Vectastain Elite ABC HRP Kit and DAB Peroxidase (HRP) Substrate Kit, according to manufacturer instructions. Tissue sections were then dehydrated in ethanol, cleared in xylene and mounted using Cytoseal 60. Specimens were documented photographically using a Nikon Eclipse E600 microscope and analyzed with the NIS-Elements Basic Research software.

Protein Isolation and Western Analysis. For p53 oligomerization assays: Protein lysates were prepared in 1X DPBS supplemented with 0.5% IGEPAL CA-630, 1 mM PMSF, 6 µg/ml aprotinin, and 6 µg/ml leupeptin at 4°C. Whole cell lysates (100 µg per reaction) were incubated with or without 1 mM BMH for 30 min at 30°C. The samples were quenched with an equal volume of 2X SDS-PAGE sample buffer supplemented with 10% β-Mercaptoethanol and heated for 5 min at 100°C. For PANK and GRP78 Western blot analyses: Whole cell protein extracts were prepared in Lysis Buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 2 mM EDTA, 1% IGEPAL CA-630 and 0.5% Triton X-100) supplemented with protease inhibitors at 4°C. The samples were mixed with an equal volume of 2X SDS-PAGE sample buffer supplemented with 10% β-

Mercaptoethanol and heated for 5 min at 100°C. All protein samples were size fractionated on 4-20% Tris-Glycine gradient gels (Lonza) using constant voltage at room temperature, transferred overnight onto Immuno-Blot PVDF membranes (Bio-Rad cat#1620177) at 4°C and subjected to protein blotting using the specified antibodies. Secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used at a dilution of 1:10,000.

Quantification and Statistical Analysis. All experiments were carried out in triplicate with at least three biological replicates and with at least three independent primary as well as immortalized clones of MEFs of each genotype. All mouse experiments had a minimum of $n = 7$ per genotype per experimental group. Statistical analysis was performed using Excel and GraphPad Prism. Data were analyzed using the Student's *t*-test or one-way ANOVA with Bonferroni's post-hoc test, where appropriate. Data are presented as mean ± SD. Statistical significance was annotated as follows: $*P<0.05$; $**P<0.01$.

Table S1: Reagents and Primers

Antibodies		
Anti-p53 (1C12) Mouse mAb	Cell Signaling Technology	Cat# 2524
Anti-4 Hydroxynonenal (4HNE) antibody	ABCAM	Cat# ab46545
Peroxidase AffiniPure F(ab')2 Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Laboratories	Cat# 715-036-150
Peroxidase AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Laboratories	Cat# 711-036-152
Biotinylated Goat Anti-Rabbit IgG Antibody	Vector Laboratories	Cat# BA-1000
Reagents and Chemicals		
2-Mercaptoethanol	Bio-Rad	Cat# 1610710
Coenzyme A	Cayman Chemical	Cat# 16147
Erastin	Cayman Chemical	Cat# 17754
Ferrostatin-1	Cayman Chemical	Cat# 17729
Lactacystin	Cayman Chemical	Cat# 70980
Nutlin-3	Cayman Chemical	Cat# 10004372
Diethyl maleate	Fisher Scientific	Cat# AC114440010
Formalde-Fresh Solution, Buffered	Fisher Scientific	Cat# SF93-4
Hyclone Fetal Bovine Serum, Characterized	Fisher Scientific	Cat# SH30396.03
Cytoseal 60	Richard-Allan Scientific	Cat# P6744
Carbon tetrachloride (CCl_4)	Sigma-Aldrich	Cat# 289116
Deferoxamine mesylate salt	Sigma-Aldrich	Cat# D9533
Direct Red 80 (Sirius Red)	Sigma-Aldrich	Cat# 365548
IGEPAL CA-630	Sigma-Aldrich	Cat# I3021
Liproxstatin-1	Sigma-Aldrich	Cat# SML1414
L-Cystine dihydrochloride	Sigma-Aldrich	Cat# C6727
L-Methionine	Sigma-Aldrich	Cat# M9625
N-Acetyl-L-cysteine	Sigma-Aldrich	Cat# A9165
L-Glutathione reduced	Sigma-Aldrich	Cat# G6013
Picric acid solution	Sigma-Aldrich	Cat# P6744
Triton X-100	Sigma-Aldrich	Cat# T8787
BMH (bismaleimidohexane)	ThermoFisher Scientific	Cat# 22330
DMEM, high glucose, no glutamine, no methionine, no cystine	ThermoFisher Scientific	Cat# 21013024
DMEM, high glucose	ThermoFisher Scientific	Cat# 11965084
Fast SYBR Green Master Mix	ThermoFisher Scientific	Cat# 4385612
L-Glutamine	ThermoFisher Scientific	Cat# 25030081
StartingBlock T20 (PBS) Blocking Buffer	ThermoFisher Scientific	Cat# 37539
UltraPure 0.5M EDTA, pH 8	ThermoFisher Scientific	Cat# 15575020
UltraPure 1M Tris-HCl Buffer, pH 7.5	ThermoFisher Scientific	Cat# 15567027
1X DPBS, no calcium, no magnesium	ThermoFisher Scientific	Cat# 14190144
UltraPure Glycerol	ThermoFisher Scientific	Cat# 15514029
Commercial Assays and Kits		
CellTiter-Glo Luminescent Cell Viability Assay	Promega	Cat# G7570
GSH/GSSG-Glo Assay Kit	Promega	Cat# V6611
Cell Proliferation Kit I (MTT)	Sigma-Aldrich	Cat# 11465007001
Coenzyme A (CoA) Assay Kit	Sigma-Aldrich	Cat# MAK034
SuperScript III First-Strand Synthesis System	ThermoFisher Scientific	Cat# 18080051

VECTASTAIN Elite ABC HRP Kit	Vector Laboratories	Cat# PK-6100
DAB Peroxidase (HRP) Substrate Kit	Vector Laboratories	Cat# SK-4100
Avidin/Biotin Blocking Kit	Vector Laboratories	Cat# SP-2001
Direct-zol RNA Kit	Zymo Research	Cat# R2071
Cell Lines		
Human: HT-1080	ATCC	Cat#ATCC CCL-121
Human: IMR90	ATCC	Cat#ATCC CCL-186
Human: WI38	ATCC	Cat#ATCC CCL-75
Human: 1205Lu	The Wistar Institute	N/A
Human: WM852	The Wistar Institute	N/A
P47 and S47 Primary MEFs	This paper	N/A
P47, S47 and p53-/- iMEFs	Jennis et al., 2016 (Ref. 1)	PMID:27034505
Human P47 and S47 LCLs	Jennis et al., 2016 (Ref. 1)	PMID:27034505
Others		
Retriever for Antigen Unmasking	Electron Microscopy Sciences	Cat# 62700-10
PAGEr Gold Gels, 4-20%, 10-well, Tris-Glycine	Lonza	Cat# 59511
Primers for qRT-PCR Analysis of Mouse Transcripts		
Primers for *ALOX12* Fwd: GATCACTGAAGTGGGGCTGT Rev: CACACATGGTGAGGAAATGG	This paper	N/A
Primers for *ALOX15* Fwd: CGGTCTACTTGTCTCCCTGC Rev: ATCCGCTTCAAACAGAGTGC	This paper	N/A
Primers for *CARS* Fwd: CAGAACCACTGGAACAGGCT Rev: GCCAGTCAGAGAGCAAGTCC	This paper	N/A
Primers for *CHAC1* Fwd: GTGGTGACCCTCCTTGAAGA Rev: AGGTACTTCAGGGCCTCGTT	This paper	N/A
Primers for *MDM2* Fwd: TGTGAAGGAGCACAGGAAAA Rev: TCCTTCAGATCACTCCCACC	This paper	N/A
Primers for *p21/CDKN1A* Fwd: GGCCCGGAACATCTCAGG Rev: AAATCTGTCAGGCTGGTCTGC	This paper	N/A
Primers for *PanK1α* Fwd: GGGTCCACCGAGGTTCCC Rev: CACGTCTTCGGCGAGCTG	This paper	N/A
Primers for *PanK1β* Fwd: TGAAGCTTGTAAATGGCAGA Rev: CTTAACCAGGGTTCCACCGA	This paper	N/A
Primers for *PanK2* Fwd: AGGATCTTGCCAGAGCAACT Rev: CCGCATAGCAATCGTGTTGA	This paper	N/A
Primers for *PanK3* Fwd: GCTACAGGAGGTGGTGCTTA Rev: AGCCCTTTACAAGGCAGTCA	This paper	N/A
Primers for *PTGS2* Fwd: ATGAGCACAGGATTTGACCA Rev: TGGGCTTCAGCAGTAATTTG	This paper	N/A

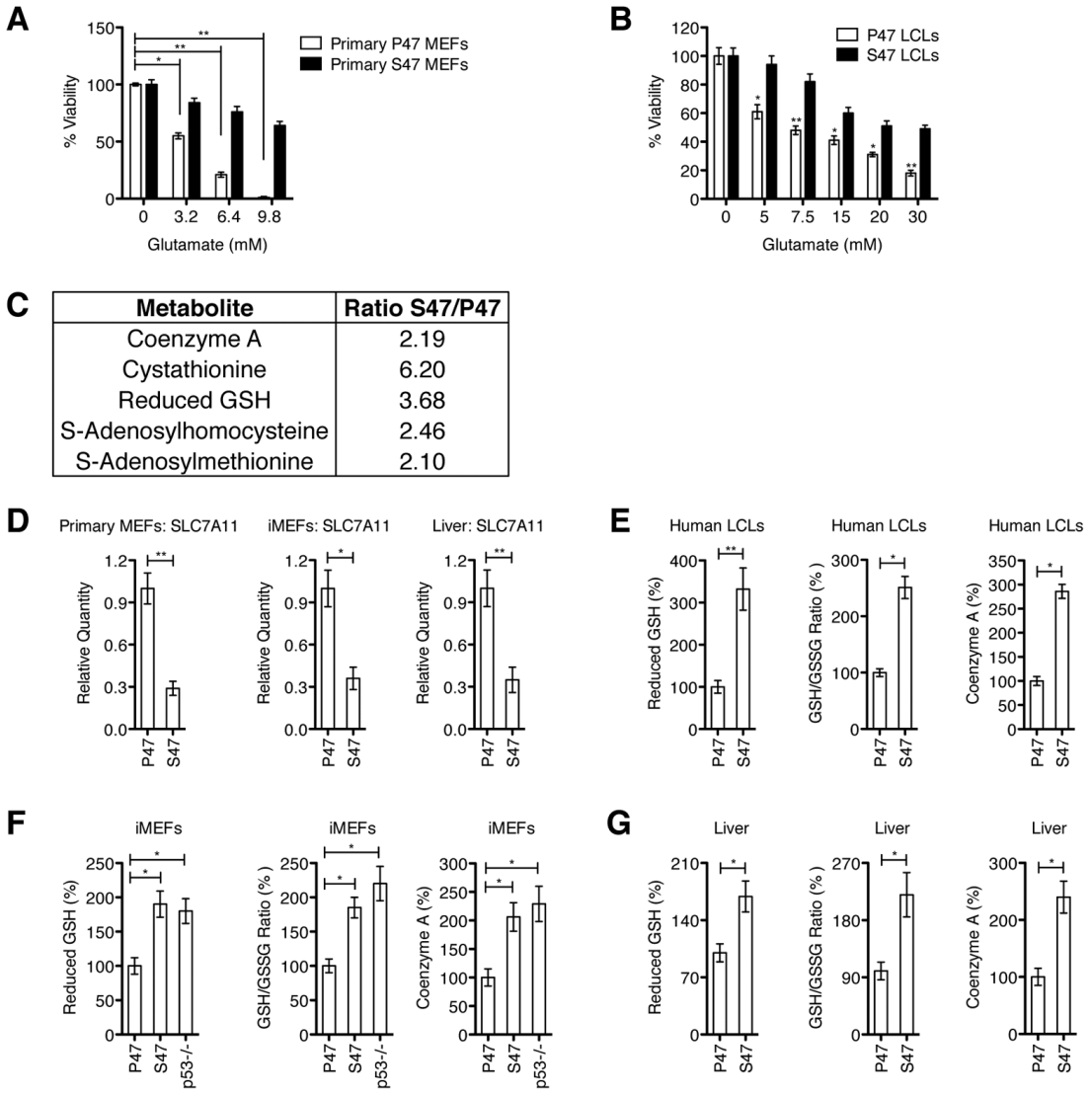
Primers for *SAT1* Fwd: GGCTAAATTTAAGATCCGTCCA Rev: CATGTATTCATATTTAGCCAGTTCCTT	Ou et al., 2016 (Ref. 2)	PMID:27698118
Primers for *SLC7A11* Fwd: CCTCTGCCAGCTGTTATTGTT Rev: CCTGGCAAAACTGAGGAAAT	Jiang et al., 2015 (Ref. 3)	PMID:25799988
Primers for *SLC7A11* Fwd: TGGGTGGAACTGCTCGTAAT Rev: AGGATGTAGCGTCCAAATGC	Jiang et al., 2015 (Ref. 3)	PMID:25799988

SI References

1. Jennis M, et al. (2016) An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. *Genes Dev* 30:918-930.

2. Ou Y, Wang SJ, Li D, Chu B, Gu W (2016) Activation of SAT1 engages polyamine metabolism with p53-mediated ferroptotic responses. *Proc Natl Acad Sci USA* 113:E6806-E6812.

3. Jiang L, et al. (2015) Ferroptosis as a p53-mediated activity during tumour suppression. *Nature* 520:57-62.



Metabolite	Ratio S47/P47
Coenzyme A	2.19
Cystathionine	6.20
Reduced GSH	3.68
S-Adenosylhomocysteine	2.46
S-Adenosylmethionine	2.10

Fig. S1. Effect of codon 47 polymorphism of p53 on sensitivity to glutamate toxicity, and correlation with GSH and CoA abundance. (*A* and *B*) Cells, as indicated, were treated for 24 h (*A*) or 72 h (*B*) with indicated concentrations of glutamate, and cell viability was assayed. Data represent the mean ± SD of multiple experiments (**P*<0.05, ***P*<0.01). (*C*) Metabolomics analyses of P47 and S47 cells (*n* = 4 per genotype). Relative change is presented as the ratio between the mean metabolite abundance in the S47 cells relative to the P47 cells (S47/P47). (*D*) qRT-PCR analyses of *SLC7A11* in primary MEFs, iMEFs and livers, as indicated. Relative expression data are plotted as the mean ± SD of multiple experiments (**P*<0.05, ***P*<0.01). (*E*) Human P47 and S47 LCLs were collected, washed with PBS and analyzed for reduced GSH, GSH/GSSG ratio and CoA abundance. Means and SD are shown (*n* = 4, **P*<0.05, ***P*<0.01). (*F*) iMEFs, as indicated, were collected, washed with PBS and analyzed for reduced GSH, GSH/GSSG ratio and CoA abundance. Means and SD are shown (*n* = 4, **P*<0.05). (*G*) P47 and S47 livers were analyzed for reduced GSH, GSH/GSSG ratio and CoA abundance. Means and SD are shown (*n* = 7, **P*<0.05).

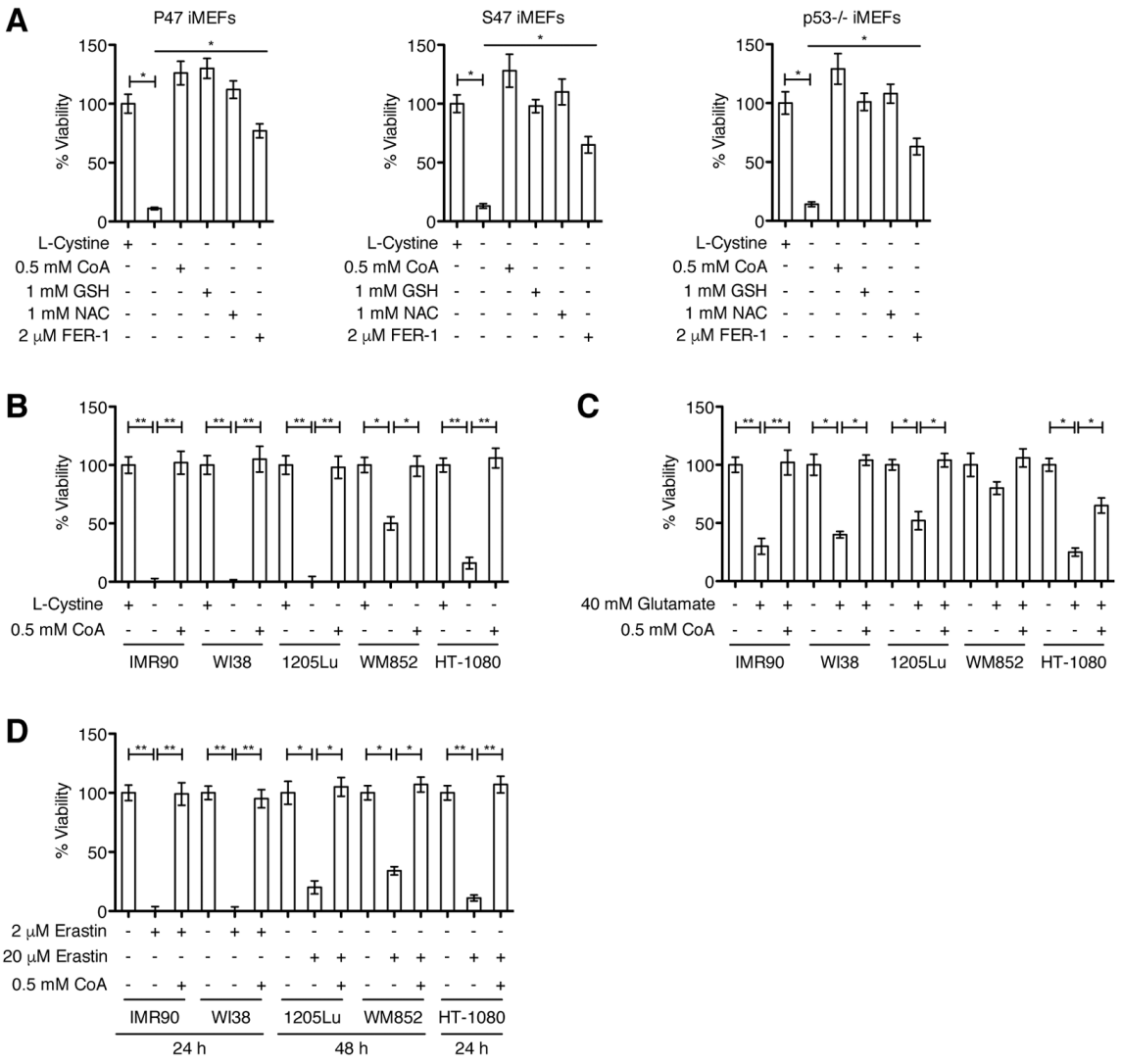


Fig. S2. Effect of CoA administration on cysteine starvation-induced cytotoxicity and oxidative stress outcomes in different cell lines. (*A*) P47 iMEFs, S47 iMEFs, and p53-null (*p53*$^{-/-}$) iMEFs were treated as indicated for 24 h and analyzed for viability. Means and SD are shown (*n* = 4, **P*<0.05). (*B*) The indicated human cell lines were cultured with L-Cystine, without L-Cystine or with 0.5 mM CoA plus no L-Cystine for 24 h. Cells were collected and analyzed for viability. Means and SD are shown (*n* = 4, **P*<0.05, ***P*<0.01). (*C*) The indicated human cell lines were treated with PBS, 40 mM glutamate or 40 mM glutamate + 0.5 mM CoA, as indicated, for 48 h and analyzed for viability. Means and SD are shown (*n* = 4, **P*<0.05, ***P*<0.01). (*D*) The indicated human cell lines were treated with DMSO, erastin or erastin + CoA for 24 h or 48 h, as indicated, and analyzed for viability. Means and SD are shown (*n* = 4, **P*<0.05, ***P*<0.01).

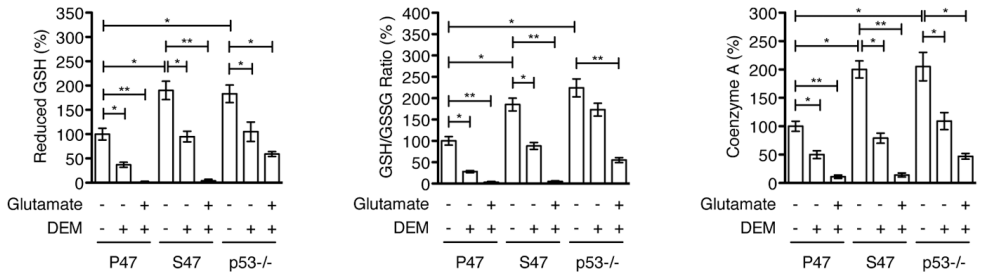


Fig. S3. Effect of DEM on CoA and GSH abundance**.** P47, S47 and p53-null (*p53*$^{-/-}$) iMEFs were treated with PBS, 50 μM DEM or 6.4 mM glutamate + 50 μM DEM, as indicated, for 24 h. Cells were collected, washed with PBS and analyzed for reduced GSH, GSH/GSSG ratio and CoA abundance. Means and SD are shown (*n* = 4, **P*<0.05, ***P*<0.01).

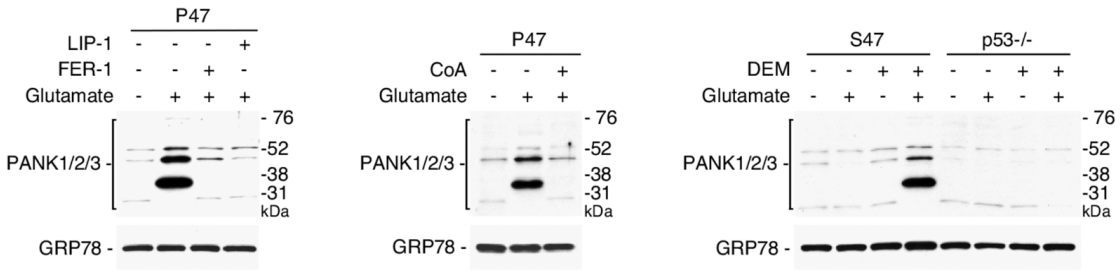


Fig. S4. Effect of redox environment on PANK protein expression**.** P47, S47 and p53-null (*p53*$^{-/-}$) iMEFs were treated with PBS or 6.4 mM glutamate, with or without 2 μM Fer-1, 2 μM Lip-1, 0.5 mM CoA or 50 μM DEM, as indicated, for 24 h. Protein lysates were analyzed by Western blot analysis for PANK isoforms; GRP78 serves as a protein loading control. Note that available antibodies do not readily distinguish among the highly homologous PANK1α, PANK1β, PANK2 and PANK3 proteins.

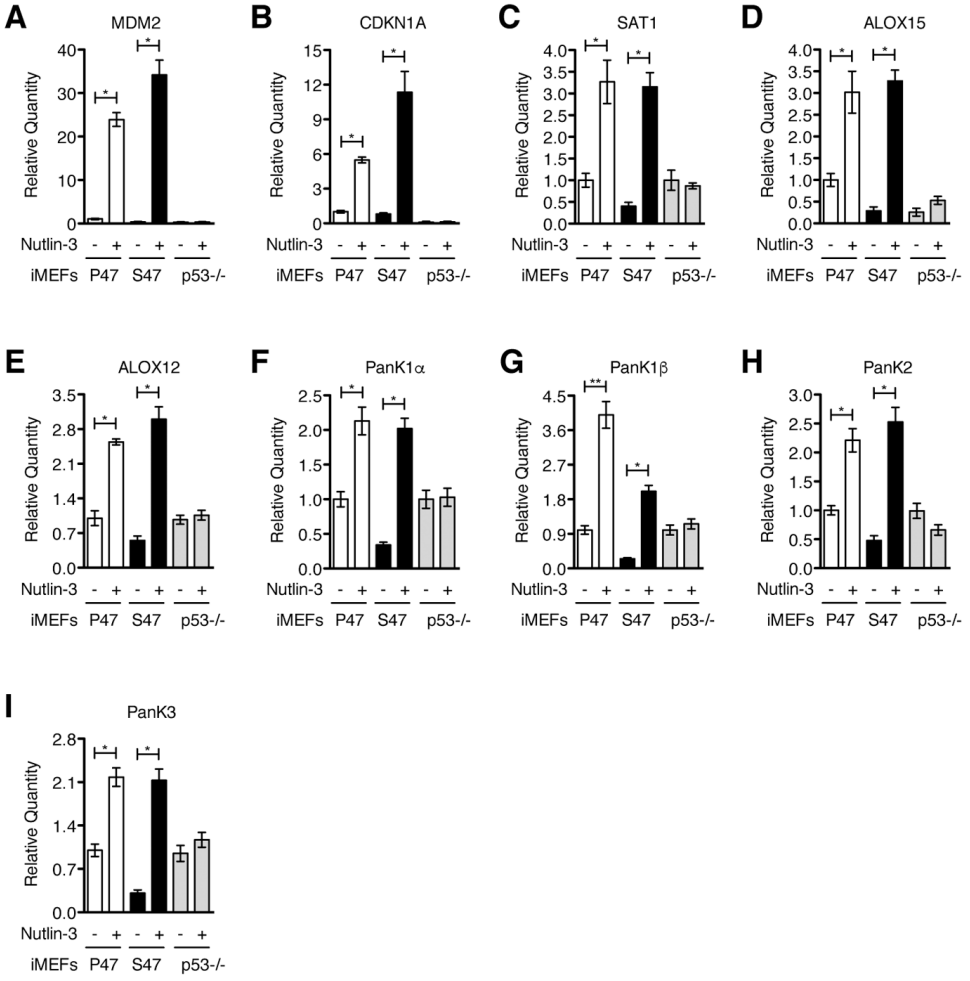


Fig. S5. Effect of Nutlin-3 on transcriptional upregulation of p53 target genes and ferroptosis markers. (*A-I*) qRT-PCR analyses of the indicated genes in P47, S47 and p53-null (*p53*$^{-/-}$) iMEFs, treated with DMSO or 10 μM Nutlin-3 for 24 h. Relative expression data are plotted as the mean ± SD (n = 4, **P*<0.05, ***P*<0.01).

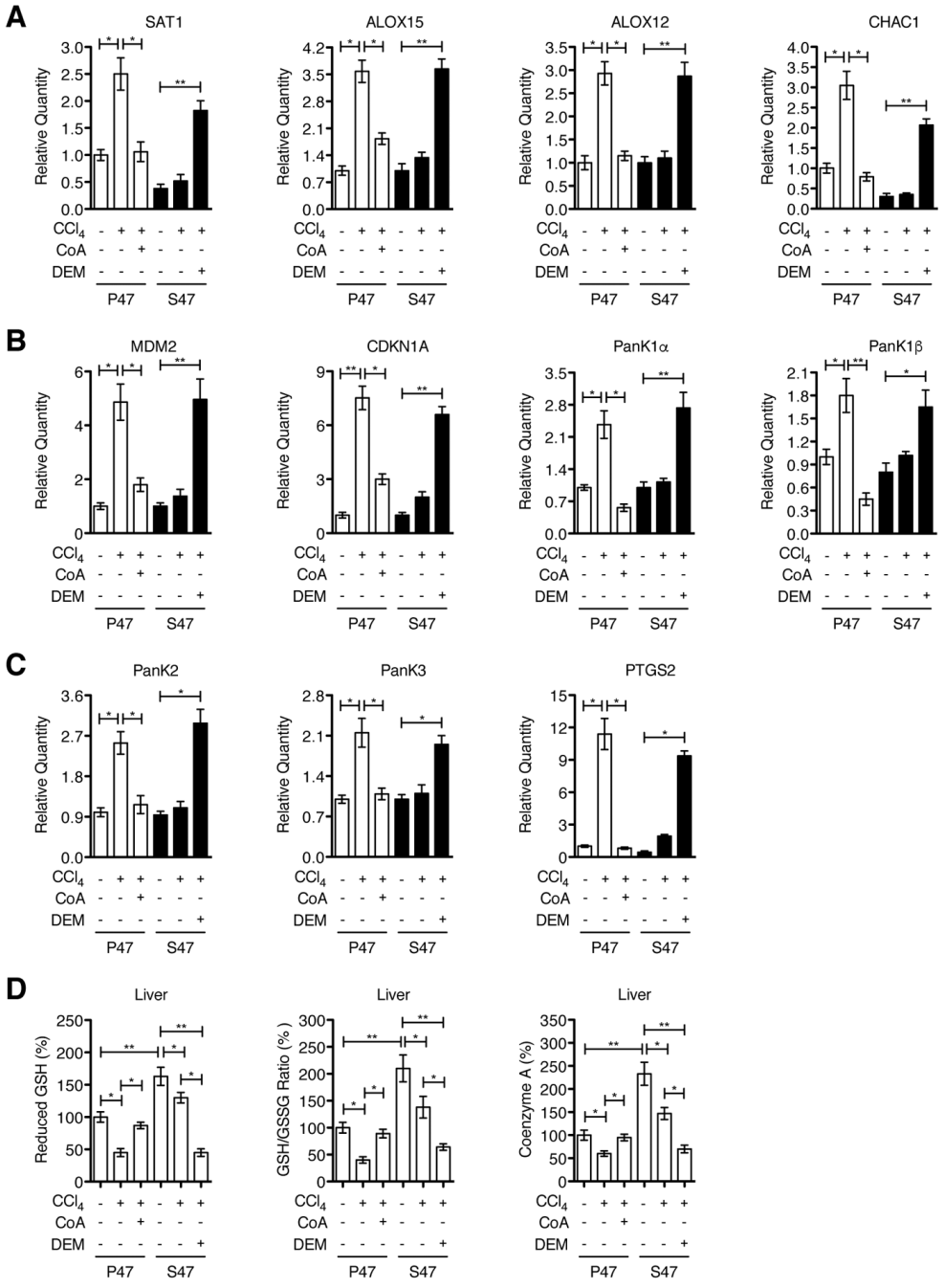


Fig. S6. Effect of CCl_4 and redox environment on liver gene expression in P47 and S47 Hupki mice. (*A-C*) P47 and S47 Hupki mice were treated as described in Figure 4. RNA was isolated from livers (*n = 7* per genotype per treatment), and qRT-PCR analyses carried out to examine expression of the indicated genes. Means and SD are shown. **P*<0.05, ***P*<0.01. (*D*) P47 and S47 Hupki mice were treated as described in Figure 4. Intracellular levels of reduced GSH, GSH/GSSG ratio and CoA abundance were determined, using liver samples (*n = 7* per genotype per treatment). Means and SD are shown. **P*<0.05, ***P*<0.01.