Supplementary Information:

A role for the ATP7A copper transporter in tumorigenesis and cisplatin resistance

Supplementary Figure Legends

Figure S1. H-RAS expressing MEF7A+ cells are tumorigenic in nude mice. $1x10^6$ MEF^{RAS}7A+ cells were injected subcutaneously into both flanks of nude mice and tumor volumes were measured over 38 days. Data shown is mean tumor volume (mm^3) ± S.E.M.

Figure S2. Histological analyses of the tumors reveal no differences in markers of vascular density or proliferation. Representative images of immunohistochemical detection for proliferation marker Ki67 and angiogenesis marker CD31 proteins (40X objective) are shown. Tumors were processed for paraffin embedding, sectioned and stained with anti-CD31 or anti-Ki67 antibodies (IDEXX BioResearch, Columbia, MO).

Figure S3: Loss of ATP7A confers copper-dependent hypersensitivity to hydrogen peroxide. MEF^{RAS}7A- and MEF^{RAS}7A+ cells in a 24 well plate were exposed to 40 μM H_2O_2 in either normoxia (21% O_2) or hypoxia (2% O_2) for 24 hours at 37°C. Cell viability was measured using a Crystal violet viability assay (mean ± S.E.M., *P<0.05, **P<0.01, ***P<0.001).

Figure S4. Analysis of phospho-ERK in MEF^{RAS}7A- and MEF^{RAS}7A+ cells. MEF^{RAS}7A- and MEF^{RAS}7A+ cells were grown to 80-90% confluence. Cell

lysates wereprepared and phospho-ERK was analyzed by SDS-PAGE and immunoblotting. Total ERK1/2 was detected as a loading control. Both MEF^{RAS}7A- and MEF^{RAS}7A+ cells exhibit similar levels of phosphorylated ERK proteins. ERK1/2 and Phospho-ERK1/2 antibodies were purchased from (Santa Cruz) and (Cell Signaling) respectively with 5% BSA in TBST used as blocking and antibody dilution buffers.

Supplementary Materials and Methods

Animals. All animal husbandry and euthanasia procedures were performed in accordance with and under the approval of the Animal Care and Use Committee of the University of Missouri. Male NCr nude mice were purchased from Taconic (Germantown, NY). Mice were maintained on 12hour light-dark cycle and Picolab diet 5053 (13 ppm Cu) was provided to mice (PMI International, St. Louis, MO, USA).

Reagents. All reagents were purchased from Sigma, unless otherwise indicated. Cisplatin was purchased from *TCI America* (Portland, OR) and dissolved in 0.9% NaCl (pH 7.4) at a concentration of 3.33 mM.

Cell culture. The MEF7A+ cell line was characterized as described previously. Tumorigenic MEF^{RAS}7A+ cells were generated by stable transfection of MEF7A+ cells with the oncogene H-Ras^{V12} plasmid (Addgene, plasmid 12545) [1] using LipoD293 transfection reagent (SignaGen Laboratories, Rockville, MD). Single colonies were selected in 2 μg/ml puromycin (Life Technologies, Carlsbad, CA). MEF^{RAS}7A- cells were generated by infection of MEF^{RAS}7A+ cells adenovirus expressing Cre-GFP using the manufacturer's suggested protocol (Vector Biolabs,

Philadelphia, PA). Single cells were sorted by flow cytometry into 96 wells and individual colonies were screened for ATP7A expression by PCR genotyping and immunofluorescence microscopy as described previously. Unless otherwise indicated, cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin and streptomycin (Life Technologies) in 5% CO_2 at 37 °C. Cell size determination was made using a Zeiss Axiovert 200M inverted microscope and MetaMorph analysis software.

Cell viability assays. Cell viability was measured in 96 well format using the Prestoblue™ cell viability kit (Thermofisher) according to the manufacturer's suggested protocol. Where indicated, cells were exposed to various concentrations of cisplatin for 24 hr. Fluorescence was measured using an Enspire 2300 Multilabel Reader (Perkin Elmer). Cell survival was measured in a 24 well format using the Vybrant MTT assay kit (Thermofisher) according to the manufacturer's suggested protocol. Absorbance (570nm) was measured using an Enspire 2300 Multilabel Reader (Perkin Elmer). Cell proliferation was assayed in a 96 well format using the WST-1 reagent (Roche) on days 1, 2 and 4. Absorbance at 440nm was measured using the Enspire reader. The Crystal violet cell viability assay was performed as previously described [2].

Tumor xenograft assays and cisplatin chemotherapy. Cell lines were injected subcutaneously into the flanks of at least 12 athymic nude mice at a concentration of 1×10^6 cells in 100 µL PBS. Tumor volume was monitored 3 times per week for a total of 4 weeks by caliper measurement of length (L) and width (W) and *calculated using the formula:* $V = (LxW^2)/2$. *Cisplatin chemotherapy was commenced at day 10* after tumor cell inoculation with

intraperitoneal injection of cisplatin (4 mg/kg in normal saline) once per week for 3 weeks. Saline vehicle was injected for the control group. *At the end of 4 weeks,* tumors were removed and either fixed with 4% paraformaldehyde. Tumors were processed for paraffin embedding, sectioned and stained with anti-CD31 or anti-Ki67 antibodies (IDEXX BioResearch, Columbia, MO).

Metal measurements. Cell lysates or frozen tissue samples were dissolved overnight in 70% nitric acid at room temperature, and elemental analysis was determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500cx coupled to a SC-DX4 autosampler (Elemental Scientific Inc.). Measurements were obtained in collision/reaction mode (He 3.5 ml/min, H2 1.5 ml/min) with 50ppb Ga as an internal standard. Values were acquired in triplicate for each sample and the results were normalized to protein concentration.

Immunoblot analysis. Cell lysates were prepared by sonicating cell pellets in RIPA lysis buffer containing Protease Inhibitor Cocktail Tablets (Roche). 40 µg of whole-cell lysates were fractionated on 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBST and incubated in blocking buffer at 4°C overnight with primary antibodies: rabbit anti-ATP7A, rabbit anti-Ctr1 [3], mouse anti-β-actin (Abcam), mouse anti-H-Ras (Millipore), rabbit anti-Ccs (Santa Cruz), mouse anti-Tubulin (Sigma). Rabbit anti-ATP7B antibodies were raised against a recombinant mouse ATP7B protein fragment corresponding to amino acid 316-491 and 1373-1460 (Primm Biotech, Cambridge, MA). ERK1/2 and Phospho-ERK1/2 antibodies were purchased from (Santa Cruz) and (Cell Signaling) respectively with 5% BSA as blocking and antibody dilution solution. Anti-mouse or anti-rabbit horseradish peroxidase conjugated secondary antibodies (Sigma) were

used, and blots were developed using the SuperSignal West Pico Substrate according to the manufacturer’s instructions (Pierce, Rockford, IL).

Statistical analyses. Values were expressed as means ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 5.0. Data were analyzed using unpaired *t*-test and considered significant when $P < 0.05$. Statistical significance representations: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

References

1. Khosravi-Far R, White MA, Westwick JK, Solski PA, Chrzanowska-Wodnicka M, Van Aelst L, et al. Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. Mol Cell Biol. 1996; 16: 3923-33.
2. Feoktistova M, Geserick P, Leverkus M. Crystal Violet Assay for Determining Viability of Cultured Cells. Cold Spring Harb Protoc. 2016; 2016: pdb prot087379.
3. Lee J, Petris MJ, Thiele DJ. Characterization of mouse embryonic cells deficient in the ctr1 high affinity copper transporter. Identification of a Ctr1-independent copper transport system. J Biol Chem. 2002; 277: 40253-9.

Figure S1

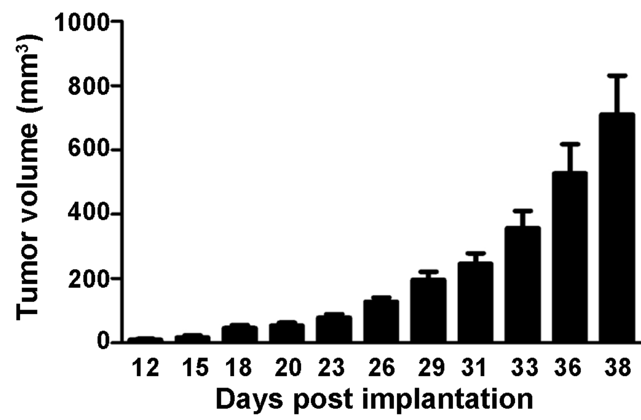

Figure S2

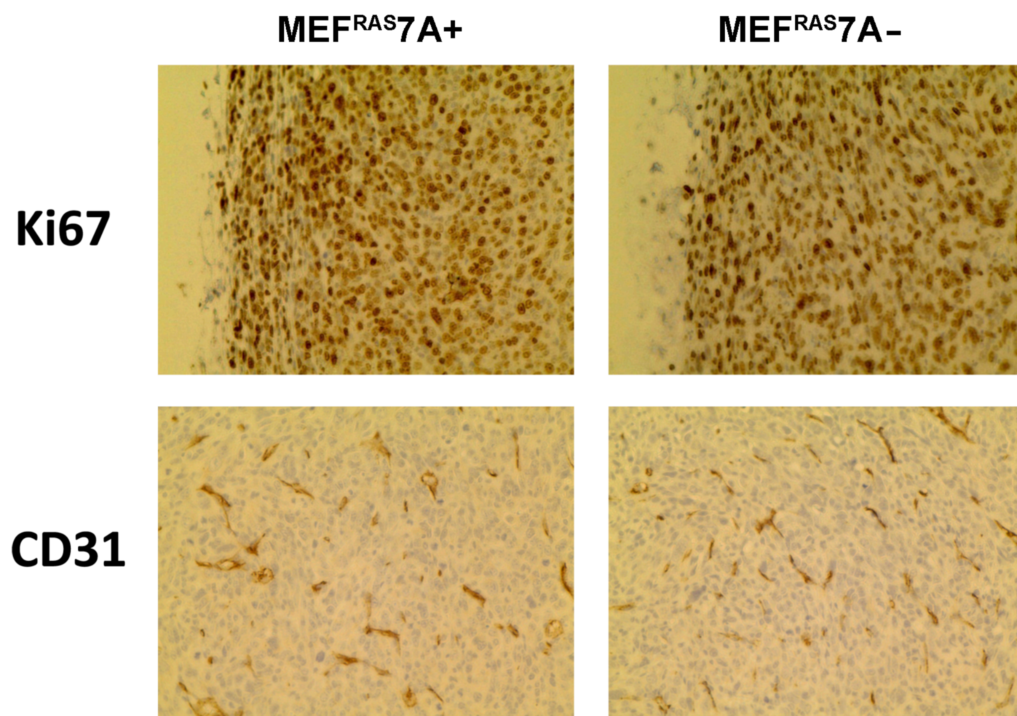

Figure S3

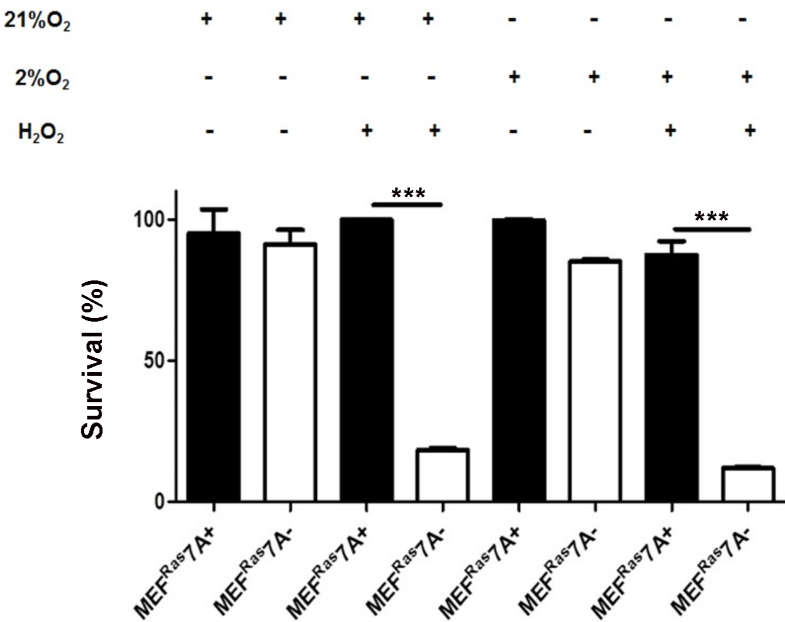

Figure S4