Electronic Supplementary Material (ESI) for Metallomics.

Supplementary data

Downregulation of Hepatic Multi Drug Resistance Protein 1 (MDR1) after Copper Exposure

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SUPPLEMENTARY PROTOCOLS

HLC differentiation

iPSCs from a WD patient carrying the compound H1069Q/N1270S mutation were subjected to a hepatic differentiation protocol as described previously [1]. Briefly, iPSCs were detached using 1x Accutase solution (Sigma-Aldrich, St. Louis, MO, USA) and seeded in mTeSR-1 medium (Stemcell Technologies, Grenoble, France) into Matrigel-precoated cell culture plates (Corning, Corning, NY, USA). The next three days, cells received a treatment with DMEM/F12 medium (Gibco, Carlsbad, CA, USA) supplemented with 100 ng/ml recombinant Activin-A (R&D Systems, Minneapolis, MN, USA), 100 ng/ml fibroblast growth factor-2 (Peprotech, Rocky Hill, NJ, USA), and 50 ng/ml recombinant human Wnt3a (R&D Systems). KnockOut SR Xenofree CTS medium (Gibco) was used in increasing concentrations starting with 0% at day, 0.2% at day 2, and 2.0% at day 3. For the following eight days, cells were cultured in DMEM/F12 supplemented with 10% KSR, 1 mM NEAA (Sigma-Aldrich), 1 mM L-Glutamine (Sigma-Aldrich), 1% dimethyl sulfoxide (Sigma-Aldrich), and 100 ng/ml hepatocyte growth factor (Peprotech). Finally, cells were grown for 3 days in 10% KSR, 1 mmol/L NEAA, 1 mM L-Glutamine, and 0.1 µM dexamethasone (Sigma-Aldrich).

Immunostaining

Cells grown on tissue culture dishes were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and permeabilized with 0.5% Triton X-100 (Fisher Scientific, Boston, MA, USA). 3% BSA (Sigma-Aldrich) was used for blocking. Primary and secondary antibodies were used for staining as reported [1].

Cells were counterstained with DAPI (Sigma-Aldrich) and visualized using an Olympus CKX41-X10;

Albumin expression

Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized. After blocking, a 1:100 dilution of anti-human albumin (Abcam, Cambridge, UK; #ab2406) was added for 30 minutes. Secondary antibody staining (1:100) was performed for 30 minutes. Cells were analyzed in a Coulter Epics XL-MCL (Beckman Coulter).

DNA sequence analysis

DNA was isolated using QIAamp DNA mini kit (Qiagen, Hilden, Germany) and sequencing was performed using Big Dye Version 3.1 (Life Technologies, Carlsbad, CA, USA).

Western blot

Cells were lyzed in RIPA buffer (60 mM tris-HCl, 150 mM NaCl, 2% Na-deoxycholate, 2% Triton X-10, 0.2% SDS, and 15 mM EDTA). Protease inhibitors (Roche, Basel, Switzerland; Complete Mini, EDTA-free) were added. 10 µg protein lysate was analyzed per lane on a 10% SDS gel. Polyclonal anti-rabbit ATP7B antibody (1:1,000; kind gift of I. Sandoval, Madrid, Spain) and protein loading control was assessed by antibody against constitutive heat shock protein 70 (HSC70) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Serum parameters

Blood was taken under retrobulbar anesthesia. Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total concentration of bilirubin were analyzed using a Cobas Modular System (Roche Diagnostics, Rotkreuz, Switzerland).

Ceruloplasmin oxidase

Ceruloplasmin oxidase activity was determined using a modified protocol of Schosinsky et al. [2].

Liver histology

Liver specimens were fixed for at least 24 h in 4% paraformaldehyde, dehydrated and embedded in paraffin wax. Morphologic parameters were determined after hematoxylin and eosin staining using standard protocols and scored for polyploidy, steatosis, apoptosis, and proliferation.

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers for RT-qPCR analysis of human genes

Gene symbol	Alias	Accession #	Forward/ reverse (5’ to 3’)
AAT	Alpha 1- antitrypsin; SERPINA1	NM_000295	CATCACCAAGTTCCTGGAAAA/ CCCCATTGCTGAAGACCTTA
AFP	Alpha-fetoprotein	NM_001134	TGCAATTGAGAAACCCACTG/ CTCATGGCAAAGTTCTTCCAG
ALB	Albumin	NM_000477	GGAGATCTGCTTGAATGTGCT/ CAATGCAGTGGGATTTTTCC
APOA1	Apolipoprotein A1	NM_000039	GACCTTGGCCGTGCTCTTC/ GGGTTCATCTTGCTGCCAGAA

ATOX1	Antioxidant 1 Copper Chaperone	NM_004045	CTGTGGAGGCTGTGCTGAAG/ TGACATTGACCTGCCCAACAAGA
ATP7A	ATPase, Cu^{++} transporting, alpha polypeptide	NM_000052	AGCAATGGCTGCTTCATCTG/ GCAGGCAGTTCATAACTCTCG
ATP7B	ATPase, Cu^{++} transporting, beta polypeptide	NM_000053	TCCTCTGTGTCTGTGGTGCTC/ GTATGAGGCACAGGCGCAT
CAR	constitutive androstane receptor; NR1I3	NM_001077469	GGTCACACACTTCGCAGACA/ GTCTTCAATGGGCAGGGAAC
CCS	Copper Chaperone for Superoxide Dismutase	NM_005125	AGACCTGGGCAATGTCCGTG/ CATCCCACACCTTCAGCTGC
COX17	COX 17	NM_005694	AGGAGAAGAAGCCGCTGAAG/ TGGACATCTAATTGAGGCC
COX4I1	Cytochrome C Oxidase Subunit 4I1	NM_001861	TGGCAAGCGAGCAATTTCCAC/ GGTCACGCCGATCCATATAAGC

CTR1	Copper Transporter 1; SLC31A1	NM_001859	GTCCCAGGACCAAATGGAAC/ CTGCACATCATCCAGGTGGT
CTR2	Copper Transporter 2; SLC31A2	NM_001860	TGCAGGCTCAGATTCATTCC/ CCATGTCATCCAGGTGGTCA
DMT1	Divalent Metal Transporter 1; SLC11A2	NM_001174127	GGGTTGGCAATGTTTGATTG/ CTTCTGAACACCATGGACGC
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_002046	CCCACTCCTCCACCTTTGAC/ CCACCACCCTGTTGCTGTAG
GSS	Glutathione Synthetase	NM_000178	CAGAGCTGGGCATCTTTGGG/ CCACACCACCATCTGCATGC
HSP70	Heat Shock Protein Family A Member 1A; HSPA1A	NM_005345	AGGCGGACAAGAAGAAGGTG/ GACGAGTTTGAGCACAAGAGGA
MATE1	Multidrug And Toxin Extrusion 1; SLC47A1	NM_018242	GCAACCACACTTGGAGTGATG/ GAGCAGAATTCCCACTCCGAG

MDR1	Multi Drug Resistance Protein 1; ABCB1	NM_000927	TCGTGCCCTTGTTAGACAGC/ CCAAGAAGCCCTGGACAAAG
MRP1	Multidrug Resistance Associated Protein 1; ABCC1	NM_004996	ACTGCACCGTCCTCACCATC/ GGAGAAATCCAGGAGTACGGC
MRP2	Multidrug Resistance Associated Protein 2; ABCC2	NM_000392	CGAAGTGACAGAGGCTGGTG/ GCTCTGCTTCGGAAATCCAA
MT1	Metallothionein 1	NM_005952	CTCCTTGCCTCGAAATGGAC/ GCATTTGCACTCTTTGCATTTG
OCT1	Organic Cation Transporter 1; SLC22A1	NM_003057	CATCATAATCATGTGTGTTGGCC/ CAAACAAAATGAGGGGCAAGGCTT
OCT3	Organic Cation Transporter 3; SLC22A3	NM_021977	CGTGTGGCTAGAACTACCTCTG/ TCCACTGTCTCTGGCAAGGC
SCO1	SCO1, cytochrome c oxidase assembly protein	NM_004589	ACGAGTTATGCGGCCTCTGG/ CCTAGCAAGACTCTCGCAG

SOD1	Superoxide Dismutase 1	NM_000454	ACTCTCAGGAGACCATTGCATC/ AAACGACTTCCAGCGTTTCC
TF	Transferrin	NM_001063	CCAGACTGTCCCACAGAACA/ GTACCATCAAGGCACAGCAA
TTR	Transthyretin	NM_000371	GAAAGGCTGCTGATGACACC/ TCAGTTGTGAGCCCATGCAG

Supplementary Table 2. Primers for RT-qPCR analysis of rat genes

Gene symbol	Alias	Accession #	Forward/ reverse (5’ to 3’)
Ctr1	Copper Transporter 1; Slc31a1	NM_133600.1	CACACCTGGAGAAATGGCTG/ CTTTCGAAGCAGACCCTCTCG
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_017008.4	GGACCTCATGGCCTACATGG/ TGAGGGCCTCTCTCTTGCTC
Hsp70	Heat Shock Protein Family A Member 1A; Hspa1	NM_031971.	AGGTCATCTCCTGGCTGGAC/ GCTGATGATCGGGTTGCAC
Mdr1a	Multi Drug Resistance Protein 1a; Abcb1a	NM_133401.1	CTCGTCAGACAGCCTCACATC/ GCTTTGTCCAGCGCTTCCTG
Mdr1b	Multi Drug Resistance Protein 1b; Abcb1b	NM_012623.2	CAGCCGTGTCGTGTCTCATG/ AGCTGAGTCCCTTTGTCTCCC

Mt1	Metallothionein 1	NM_138826.4	CGTTGCTCCAGATTCACCAG/ GGTGCATTTGCAGTTCTTGC

Supplementary Table 3. Disease parameters of LEC rats

		LEC1	LEC2	LEC3	LEC4	Control
Bilirubin	mg/dL	0.2	0.2	0.2	0.3	<0.5
AST	U/L	45	102	99	112	<98
ALT	U/L	55	88	60	60	<46
Liver copper	µg/g	355	254	230	383	<39

Supplementary Table 4. Antibodies

Epitop	Supplier	Catalog #	Clone #	LOT #
AFP	Santa Cruz Biotechnologies	sc-8399	Monoclonal (C3)	A0314
ALB	Abcam	ab2406	Polyclonal	GR117050
ATP7B	I. Sadoval, CMB, Madrid, Spain	NA	Polyclonal	NA
CTR1	Santa Cruz Biotechnologies	sc-66847	Polyclonal (FL-190)	E1512
HNF4α	Santa Cruz Biotechnologies	sc-6556	Polyclonal (C-19)	I3014
HSC70	Santa Cruz Biotechnologies	sc-1059	Polyclonal (K-19)	C2906
TTR	Abcam	ab75815	Monoclonal (EP2929Y)	GR129279-5

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 Downregulation of CTR1 in ATP7B KO cells.

(A) $5x10^5$ cells were transfected with antisense oligonucleotide (ASO; IONIS Pharmaceuticals; Inc., Carlsbad, CA, USA) for *CTR1* knockdown at a concentration of 1,000 nM. Scrambled oligonucleotide was used as a control. ASO was incubated with 4 µl of Lipofectamine (Invitrogen, Carlsbad, CA, USA). mRNA was determined relative to housekeeping gene GAPDH. Mean/SE are shown (n=4).

(B) Downregulation of intracellular Cu. Cells were lyzed 4 h after addition of 0.01 mM Cu. Cu concentration relative to untreated control (100%) is shown. Mean/SE are shown (n=3).

Supplementary Figure 2 Characterization of gene expression in copper resistant cell lines.

Gene expression of CuR (dark blue) and CuR_w cell lines (light blue) were analyzed by RT-qPCR. Fold change versus parental *ATP7B* KO cells was calculated using the $2^{-\Delta\Delta}$ method. A threshold of ±2 was set as significance level (dotted line). Genes are sorted from high modulation (left) to low (right). For primers and gene symbols see Supplementary Table 1. Note, that gene expression above threshold was only affected in CuR but not in CuR_w cell lines. Mean/SE are given (n=6).

Supplementary Figure 3 Characterization of iPSC derived HLCs.

(A) Brightfield image of HLCs derived from a WD patient (right). HLCs were from day 14 of hepatic differentiation and showed typical morphology. For comparison, iPSCs before hepatic differentiation are shown (left). Scale bars 100 µM.

(B) Flow cytometry analysis of WD HLCs showing 89.5% human albumin-positive cells. Albumin expression was absent using isotype control (data not shown).

(C) Immunofluorescence stains of WD HLCs at day 14 of differentiation. Typical hepatic markers albumin (ALB), transthyretin (TTR), α-fetoprotein (AFP), and hepatocyte nuclear factor 4 α (HNF4α) were expressed in the majority of the cell population. Scale bars, 50 µM. (D) RT-qPCR analysis of WD HLCs (grey) in comparison to HepG2 cells (white). Expression was set relative to GAPDH. Mean/SE are shown (n=3).

Supplementary Figure 4 Characterization of the WD phenotype in HLCs.

(A) Chromatograms after DNA sequencing of the HLCs. Variant nucleotides around nucleotide *ATP7B* gene position 1069 and 1270 (arrows) are shown. Chromatograms indicate a compound heterozygote mutation H1069Q/N1270S.

(B) RT-qPCR analysis of *ATP7B* mRNA expression in HLCs derived from WD patient (black) and healthy individual (grey) relative to *GAPDH.* HepG2 cells (white) are depicted for comparison. Mean/SE are shown (n=3).

(C) Western blot analysis of ATP7B from cell lysates of HLCs derived from a WD patient. For comparison, ATP7B expression of HLCs from a healthy individual and HepG2 cells are shown. Heat shock protein 70 (HSC70) is shown as a loading control. One typical of three experiments is shown.

Supplementary Figure 5 Gene expression of primary hepatic cells after copper exposure.

Gene expression in HLCs derived from a WD patient having H1069Q/N1270S compound mutation. HLCs were treated with 0.1 mM Cu for 6 days. Relative mRNA expression to untreated HLCs is given. A threshold of ±2 was set as significance level (dotted line). Mean/SE is shown (n=2).

Supplementary Figure 6 Characterization of LEC rats.

(A) Ceruloplasmin activity was determined in Long Evans Cinnamon (LEC) rats. Healthy heterozygous littermate was used as a control. Activities showed significantly reduced ceruloplasmin activity typical for WD. Mean/SE are shown (n=4).

(B) Hematoxylin and eosin staining of liver. Note, that moderate changes of liver histology in LEC rats indicating starting inflammation are observed. Liver copper and serum parameters were increased at this time point (Supplemental Table 3).

Supplementary Figure 7 Liver gene expression of LEC rats.

Livers of LEC rats were subjected to RT-qPCR analysis after sacrifice. Gene expression is given relative to healthy heterozygous littermates. Liver copper and serum parameters were increased at this time point (Supplemental Table 3). A threshold of ±2 was set as significance level (dotted line). Mean/SE of four LEC rats are reported.

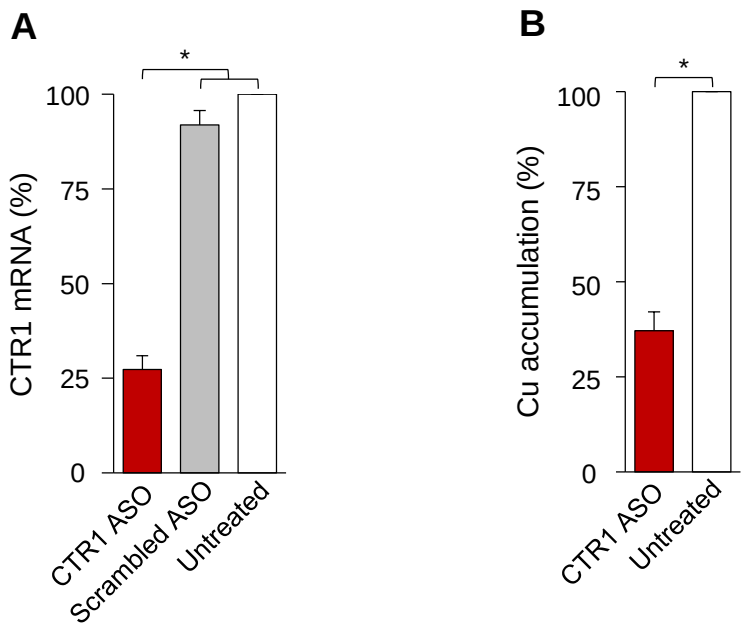
REFERENCES

1 Niemietz CJ, Sauer V, Stella J, et al. Evaluation of Therapeutic Oligonucleotides for Familial Amyloid Polyneuropathy in Patient-Derived Hepatocyte-Like Cells. *PLoS One* 2016;11(9):e0161455.

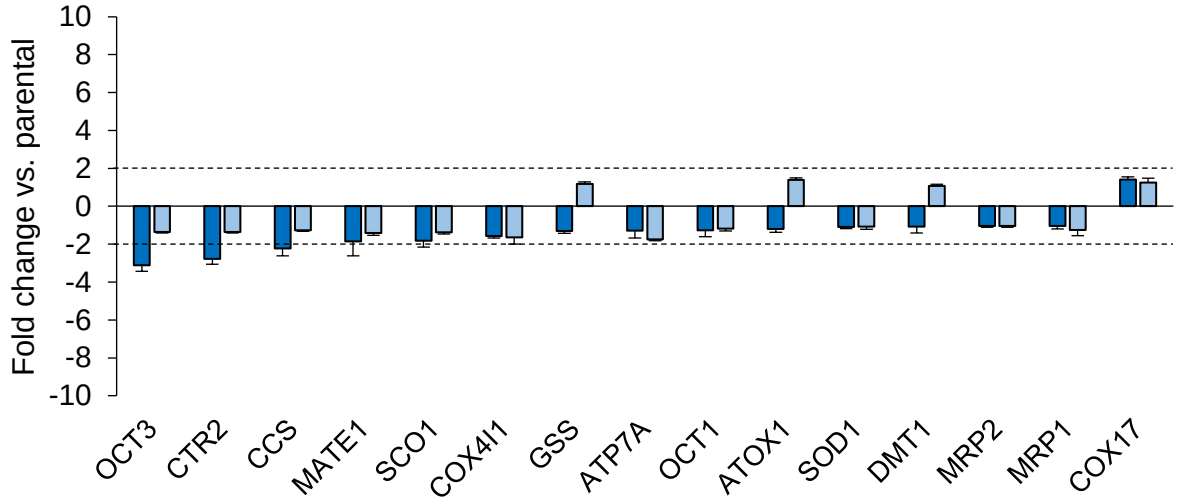
2 Siaj R, Sauer V, Stoppeler S, et al. Dietary copper triggers onset of fulminant hepatitis in the Long-Evans cinnamon rat model. *World J Gastroenterol* 2012;18(39):5542-50.

SUPPLEMENTARY FIGURES

Supplementary Figure 1



Supplementary Figure 2



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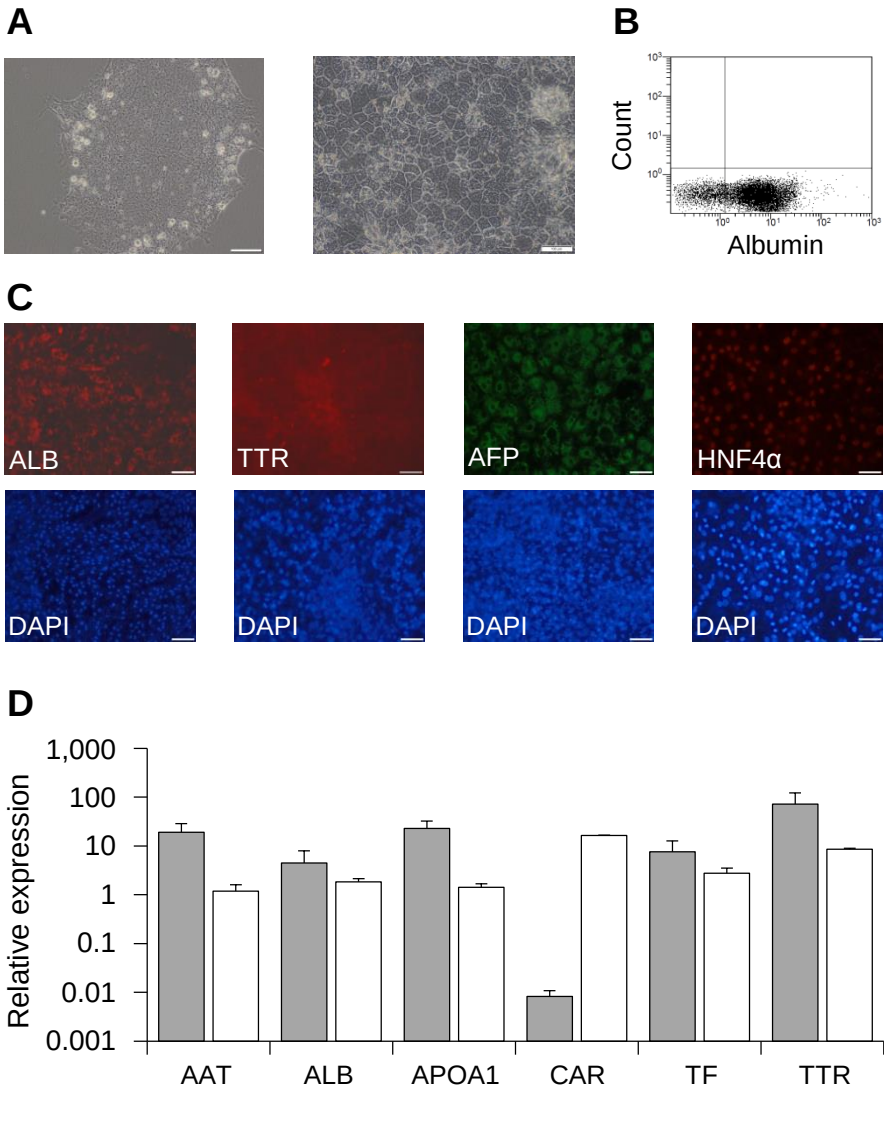

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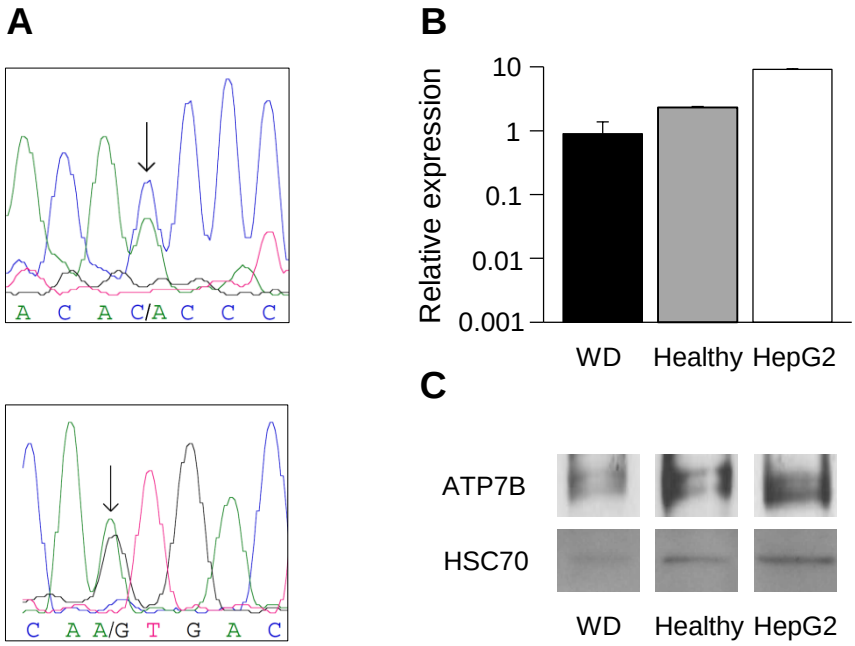

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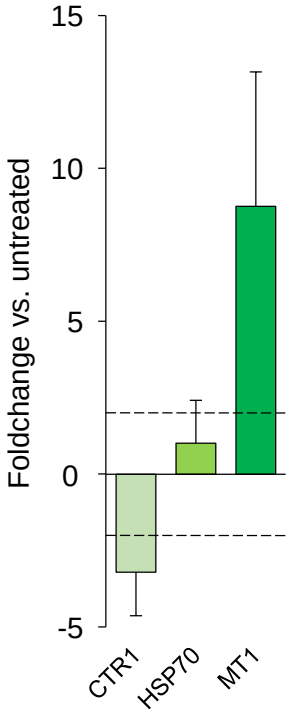

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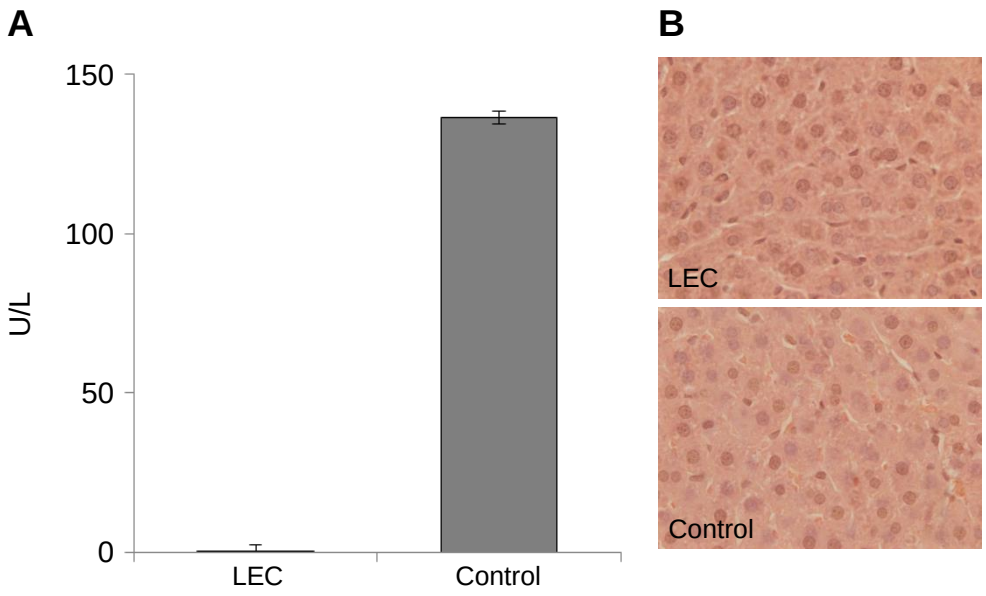

Supplementary Figure 7