Supplemental material

Cold stress-induced ferroptosis in liver sinusoidal endothelial cells determines liver transplant injury and outcomes

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Supplemental Table 1: Discarded human livers: Donor demographic data and organ information

Case	Age (years)	Gender	BMI (kg/m^2)	Race
1	51	Female	43.3	Hispanic
2	52	Male	32.1	Hispanic
3	57	Female	26.8	African American
4	61	Female	32.3	White
5	32	Male	20.4	Hispanic
6	60	Female	30.6	African American
7	46	Female	25.8	Hispanic
8	57	Female	21.2	White

Case	DBD or DCD	Reason for discard	DM	HTN	CAD	Cold storage time (minutes)
1	DBD	Steatosis	+	+	−	538
2	DBD	Unsuitable vessel condition	+	+	+	823
3	DBD	Unsuitable vessel condition	+	+	−	402
4	DBD	Steatosis	−	+	−	1105
5	DBD	Steatosis	−	−	−	495
6	DBD	Steatosis	−	+	−	737
7	DBD	Steatosis	+	+	+	960
8	DCD	Long non-beat time	−	+	−	384

BMI; body mass index, DBD; donor after brain death, DCD; donor after circulatory death, DM; diabetes mellitus, HTN; hypertension, CAD; coronary artery disease

Supplemental Table 2: Demographic data and clinical parameters of patients classified by low vs. high liver graft NRF2 expression

A. Liver graft recipients: Demographic data and clinical parameters

Variables	Low NRF2 (n=30)	High NRF2 (n=30)	*P* value
Age (years)	59 (29-73)	59 (33-73)	0.937
Sex (M/F)	19 (48.7%) / 11 (52.4%)	20 (51.3%)/ 10 (47.6%)	> 0.999
Race			0.721
White	15 (50.0%)	18 (60.0%)	
Hispanic	13 (43.3%)	9 (30.0%)	
Black	0 (0%)	0 (0%)	
Asian	1 (3.3%)	1 (3.3%)	
Others	1 (3.3%)	2 (6.7%)	
BMI (kg/m^2)	27.8 (17.9-40.0)	24.7 (14.5-47.5)	0.476
Disease etiology			0.532
HBV	1 (3.3%)	2 (6.7%)	
HCV	14 (46.7%)	11 (36.7%)	
Alcohol	2 (6.7%)	5 (16.7%)	
NASH	3 (10.0%)	4 (13.3%)	
Malignant tumor	2 (6.7%)	0 (0%)	
Others	8 (26.7%)	8 (26.7%)	
HCC (with/without)	14 (46.7%)/ 16 (53.3%)	11 (36.7%)/ 19 (63.3%)	0.601
ABO			0.237
Identical	27 (90.0%)	30 (100.0%)	
Compatibel	3 (10.0%)	0 (0%)	
MELD score	31 (9-40)	29 (14-44)	0.970
Pretransplant AST (IU/L)	74 (26-6967)	71.5 (23-1283)	0.608
Pretransplant ALT (IU/L)	51 (13-6168)	35 (11-617)	0.297
Pretransplant T-bil (mg/dL)	3.4 (0.3-59.1)	8.1 (0.5-49.2)	0.169
Pretransplant PT-INR	1.5 (1.0-2.9)	1.7 (1.1-3.2)	0.271

BMI; body mass index, HBV; hepatitis B virus, HCV; hepatitis C virus, NASH; nonalcoholic steatohepatitis, HCC; hepatocellular carcinoma

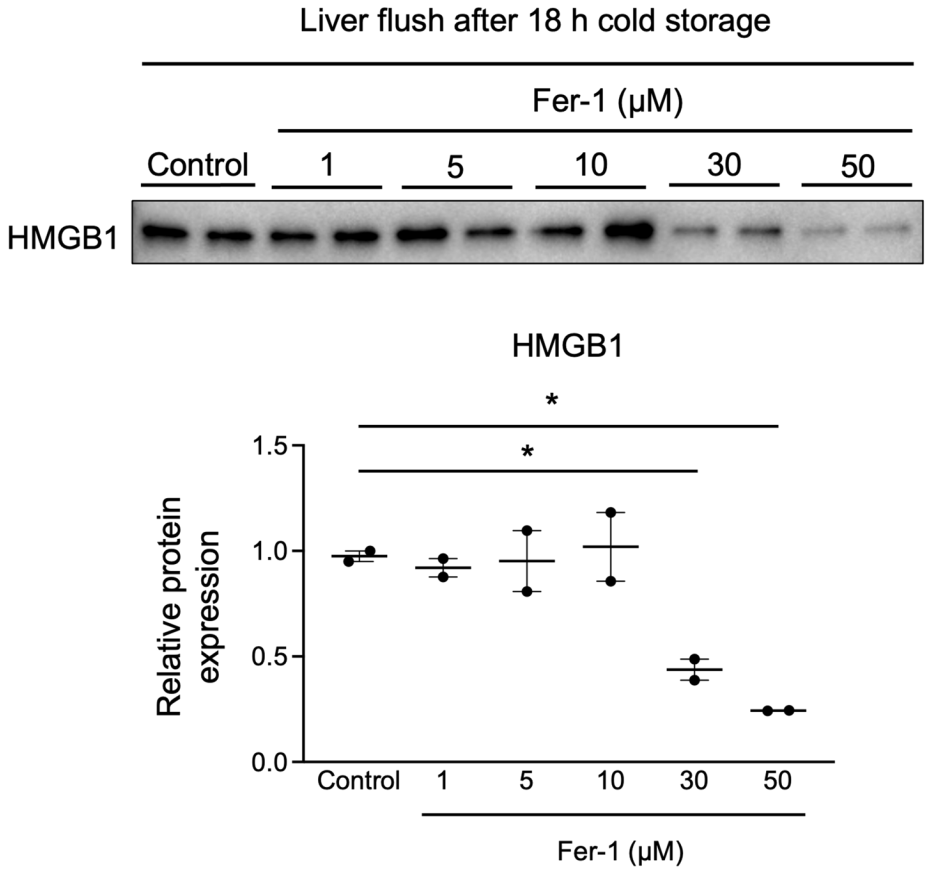
B. Liver graft donors: Demographic data and clinical parameters

Variables	Low NRF2 (n=30)	High NRF2 (n=30)	*P* value
Age (years)	47 (16-66)	42 (13-62)	0.582
Sex (M/F)	14 (46.7%)/ 16 (53.3%)	13 (43.3%)/ 17 (56.7%)	> 0.999
Race			0.512
White	17 (56.7%)	20 (66.7%)	
Hispanic	9 (30.0%)	8 (26.7%)	
Black	2 (6.7%)	0 (0%)	
Asian	2 (6.7%)	2 (6.7%)	
Others	0 (0%)	0 (0%)	
BMI (kg/m^2)	26.9 (19.7-38.4)	25.6 (13.4-42.6)	0.792
Pretransplant AST (IU/L)	35 (10-314)	34 (12-189)	0.179
Pretransplant ALT (IU/L)	28 (8-403)	23 (8-669)	0.144
Pretransplant T-bil (mg/dL)	0.8 (0.3-4.9)	0.6 (0.2-2.9)	0.054
Pretransplant PT-INR	1.3 (1.0-2.0)	1.2 (1.0-1.7)	0.619
Cold ischemic time (min)	426 (215-762)	432 (150-747)	0.945
Warm ischemic time (min)	55 (35-78)	49 (25-79)	0.653
DCD	2 (6.7%)	1 (3.3%)	> 0.999

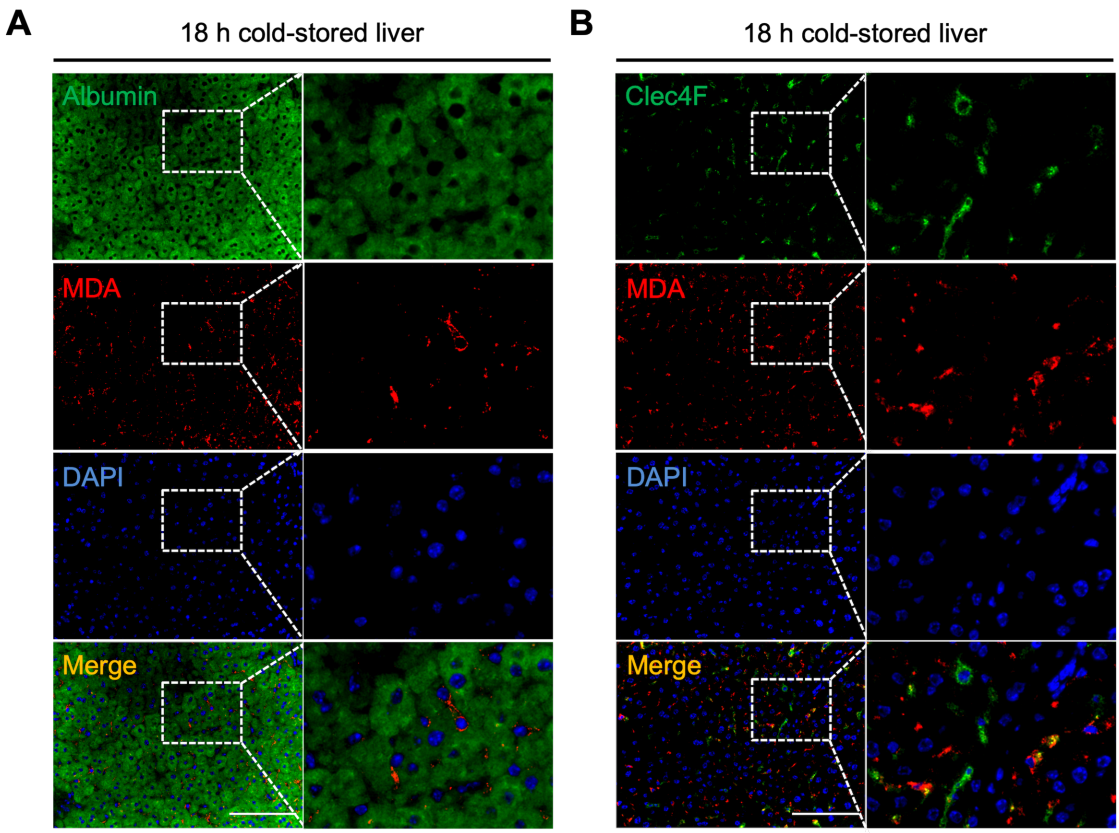
BMI; body mass index, DCD; donor after circulatory death

Supplemental Table 3: Primer sequences for real-time reverse transcription PCR (mouse study)

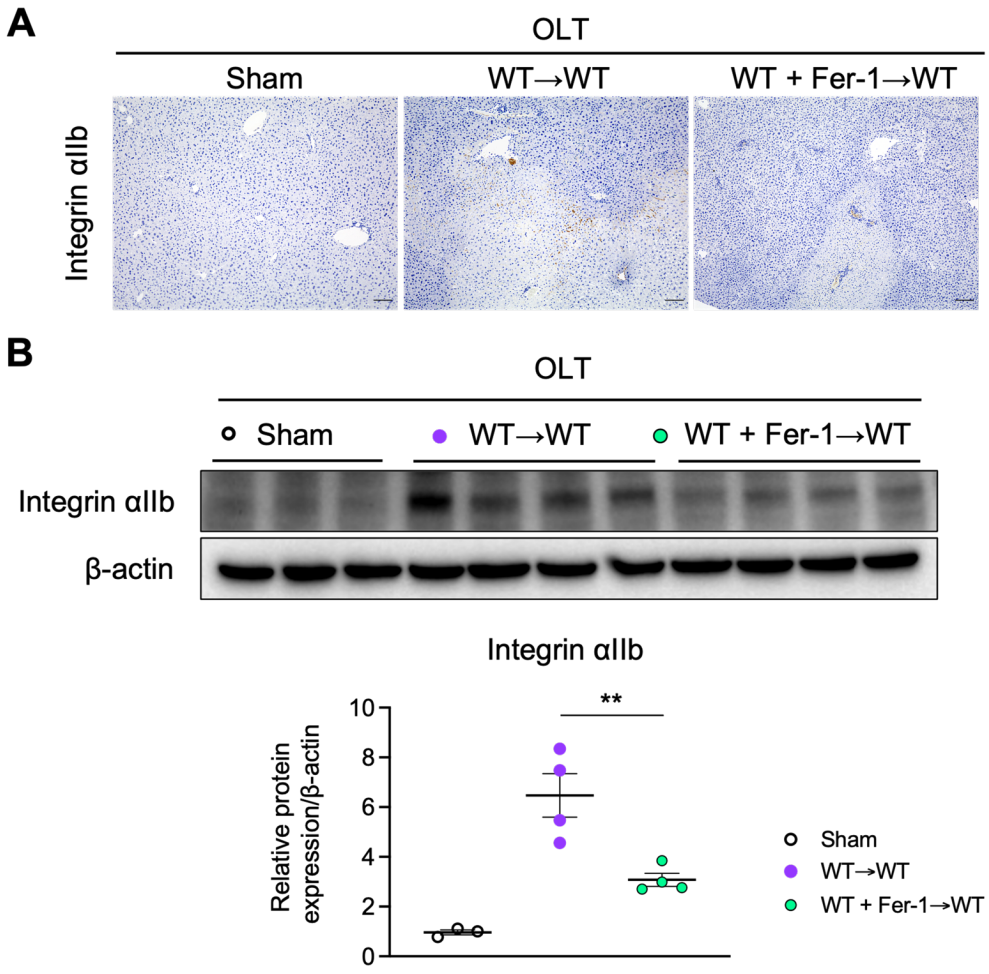
Gene	Forward	Reverse
MCP1	5'-CATCCACGTGTTGGCTCA-3'	5'-GATCATCTTGCTGGTGAATGAGT-3'
CXCL1	5'-ACCCAAACCGAAGTCATAG-3'	5'-TTGTATAGTGTTGTCAGAAGC-3'
CXCL2	5'-ACTTCAAGAACATCCAGAG-3'	5'-CTTTCCAGGTCAGTTAGC-3'
CXCL10	5'-GCTGCCGTCATTTTCTGC-3'	5'-TCTCACTGGCCCGTCATC-3'
IL-6	5'-GTACCATAGCTACCTGGAGT-3'	5'-GGAAATTGGGGTAGGAAGGA-3'
TNF-α	5'-CCTATGTCTCAGCCTCTTCT-3'	5'-TTGGGAACTTCTCATCCCTT-3'
CHOP	5'-CTGCCTTTCACCTTGGAGAC-3'	5'-CGTTTCCTGGGGATGAGATA-3'
HIF-1α	5'-AGGAGCCTGATGCTCTCACTCT-3'	5'-TGTGTCATCGCTGCCAAAAT-3'
HRPT	5'-GATTAGCGATGATGAACCAGGTT-3'	5'-CCTCCCATCTCCTTCATGACA-3'



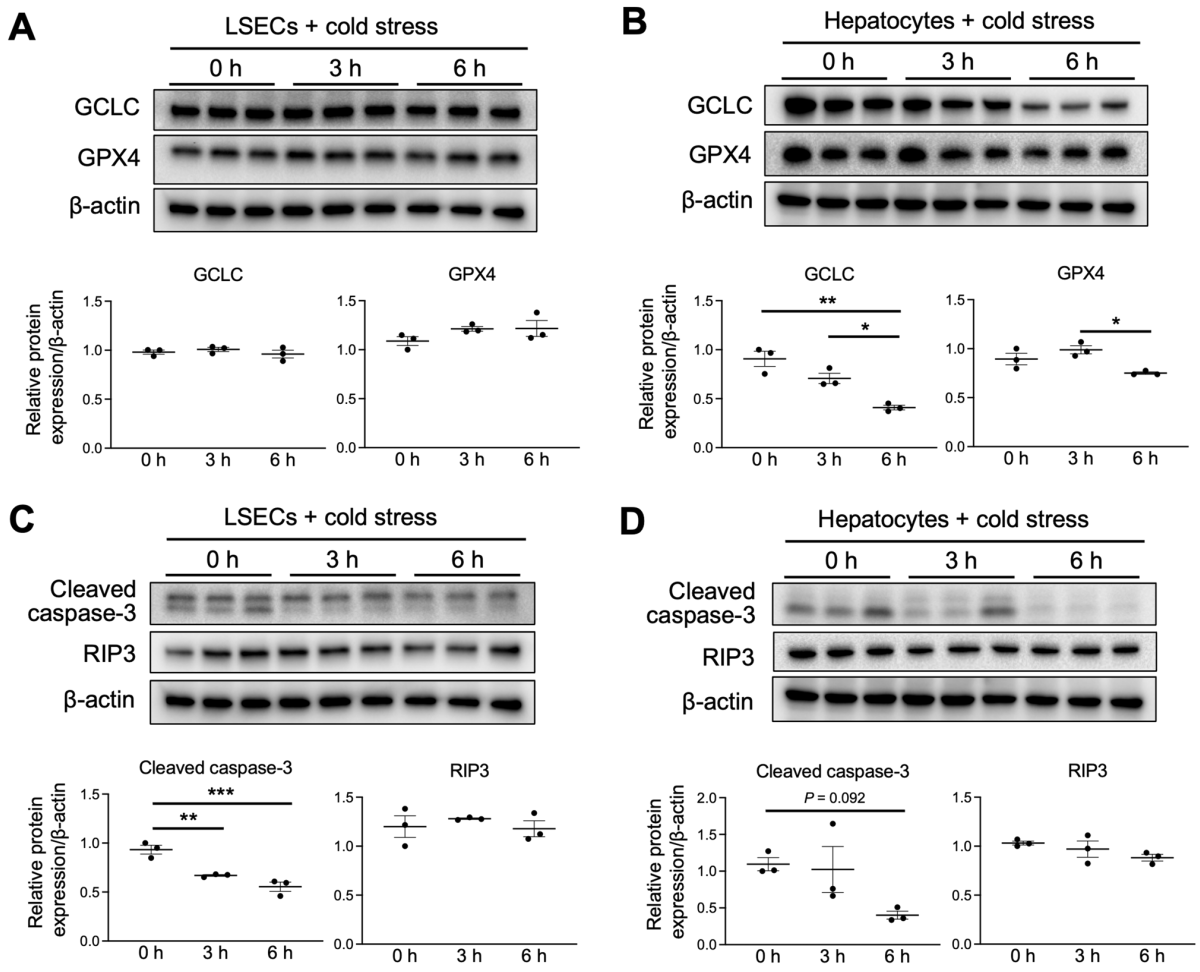
Supplemental Figure 1. Ferroptosis inhibitor (30μM) reduces HMGB1 levels in the liver flush. WT liver grafts stored in UW solution (4℃/18h) w/wo ferroptosis inhibitor (Ferostatin-1; Fer-1) were perfused with PBS (2mL) through a cuff placed at the portal vein to collect liver flush from inferior vena cava. Western blot-assisted detection of HMGB1 in the liver flush (5μL) from cold-stored liver grafts (n=2/group). Data are shown as mean±SEM. *P<0.05, one-way ANOVA followed by Tukey's HSD test.



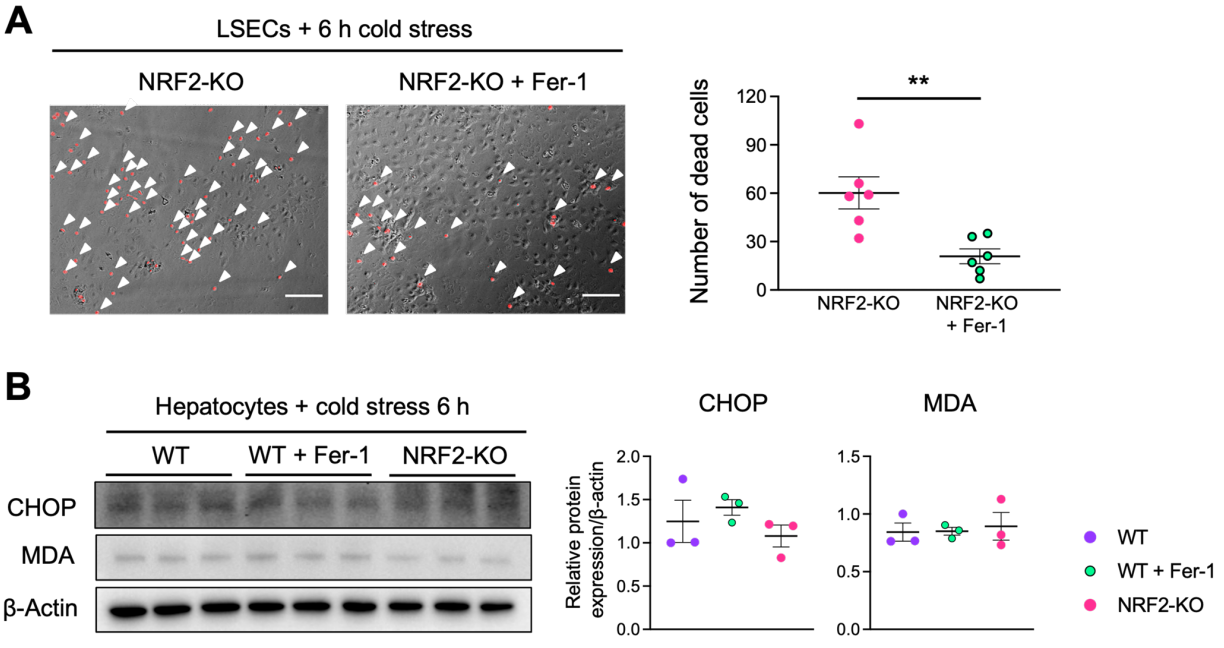
Supplemental Figure 2. Neither liver hepatocyte nor Kupffer cell represent the main source of MDA. (A, B) Representative (n=3/group) immunohistochemical staining of albumin/MDA (**A**) and Clec4F/MDA (**B**) in cold-stored mouse livers. Scale bars=100μm.



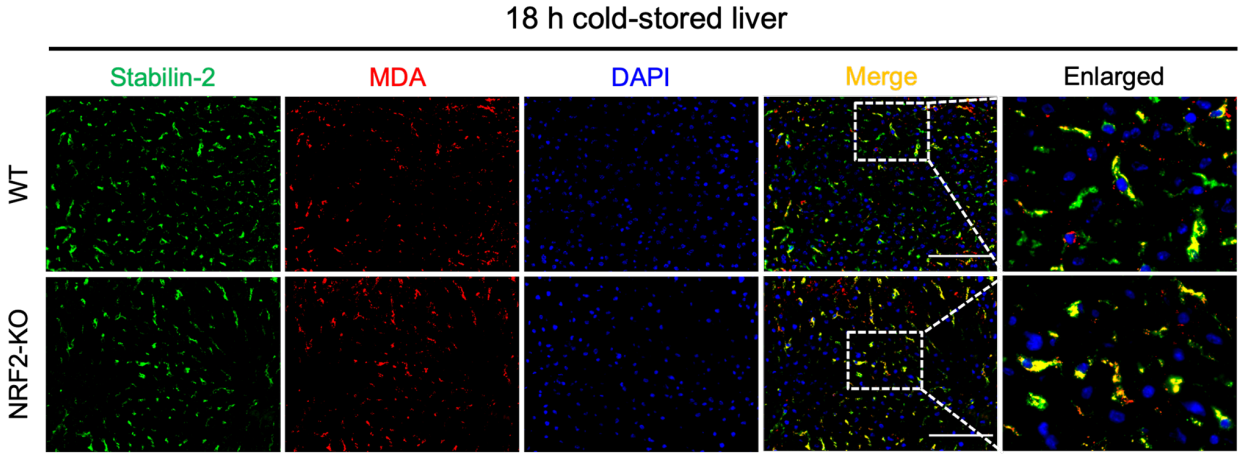
Supplemental Figure 3. Inhibition of ferroptosis during liver cold storage reduces platelet accumulation in mouse OLT. (A) Representative (n=5−6/group) integrin αIIb staining. Scale bars=100μm. **(B)** Western blot-assisted detection and relative intensity ratio of integrin αIIb in OLT. β-actin expression served as internal control and used for normalization (n=3−4/group). White circle: sham; purple circle: WT liver grafts; green circle: WT+Fer-1 liver grafts. Data are shown as mean±SEM. ***P*<0.01, Student's *t*-test.



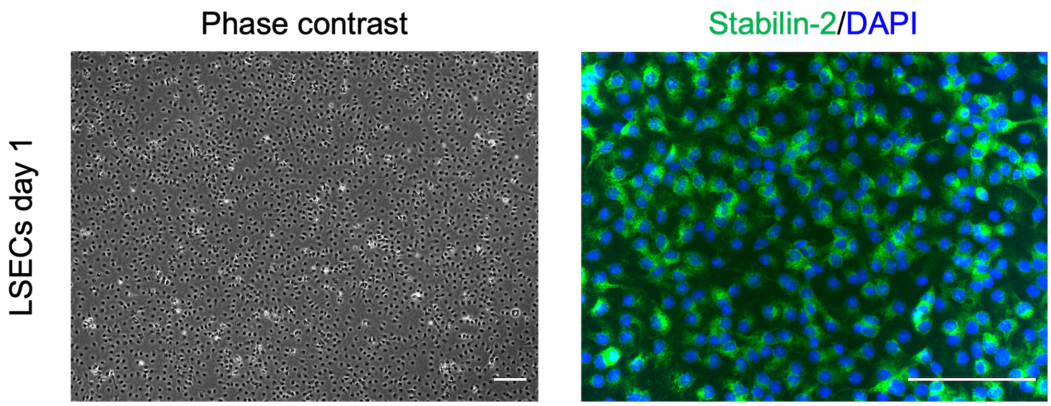
Supplemental Figure 4. Cold stress does not increase GCLC/GPX4, apoptosis, or necrosis in LSECs and hepatocytes. Western blot-assisted detection and relative intensity ratio of GCLC and GPX4 in **(A)** LSECs and **(B)** hepatocytes; cleaved caspase-3 and RIP3 in **(C)** LSECs and **(D)** hepatocytes after 6 hours of cold stress (4℃). β-actin expression served as internal control and used for normalization (n=3/group). Data are shown as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, one-way ANOVA followed by Tukey's HSD test.



Supplemental Figure 5. NRF2 deficiency does not enhance ER stress or lipid peroxidation in hepatocytes. (A) Representative (n=6/group) images and quantification of dead cells in NRF2-deficient (NRF2-KO) LSECs w/wo ferroptosis inhibitor (Ferrostatin-1; Fer-1) after 6h cold stress (4℃). Arrowheads indicate dead cells. Scale bars=100 μm. **(B)** Western blot-assisted detection and relative intensity ratio of CHOP and MDA in hepatocyte culture after 6h of cold stress. β-actin expression served as internal control and used for normalization (n=3/group). Data are shown as mean±SEM. ***P* < 0.01, Student's *t*-test.



Supplemental Figure 6. MDA expression in LSECs of NRF2-deficient and WT liver grafts. Representative (n=3/group) immunohistochemical staining of stabilin-2/MDA in WT and NRF2-KO in cold-stored liver grafts (18h/4℃).



Supplemental Figure 7. The purity of isolated and cultured murine LSECs (>85−90%). Phase contrast image and immunohistochemical staining of stabilin-2 in LSECs at day 1 after isolation. Scale bars=100μm.