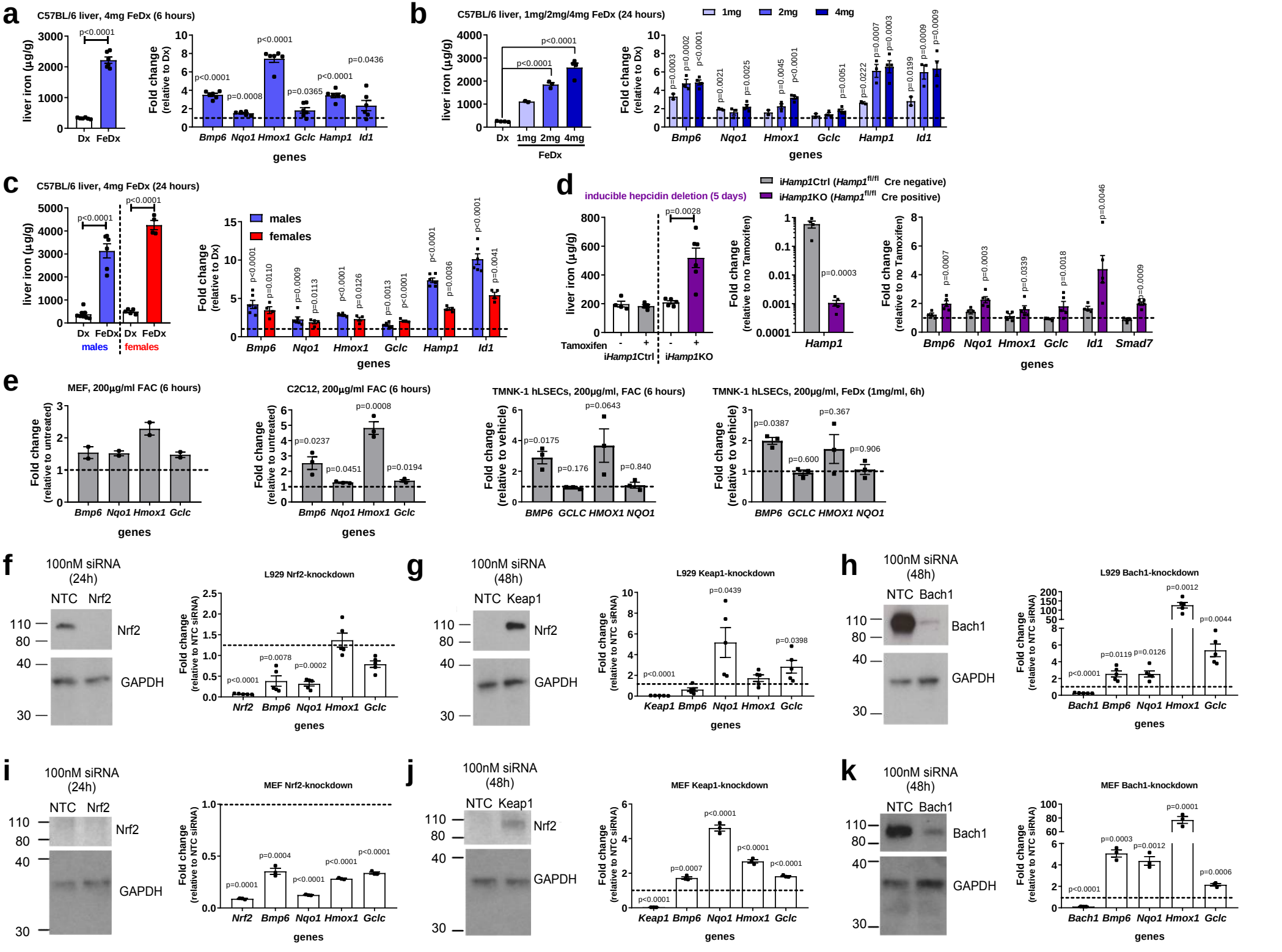
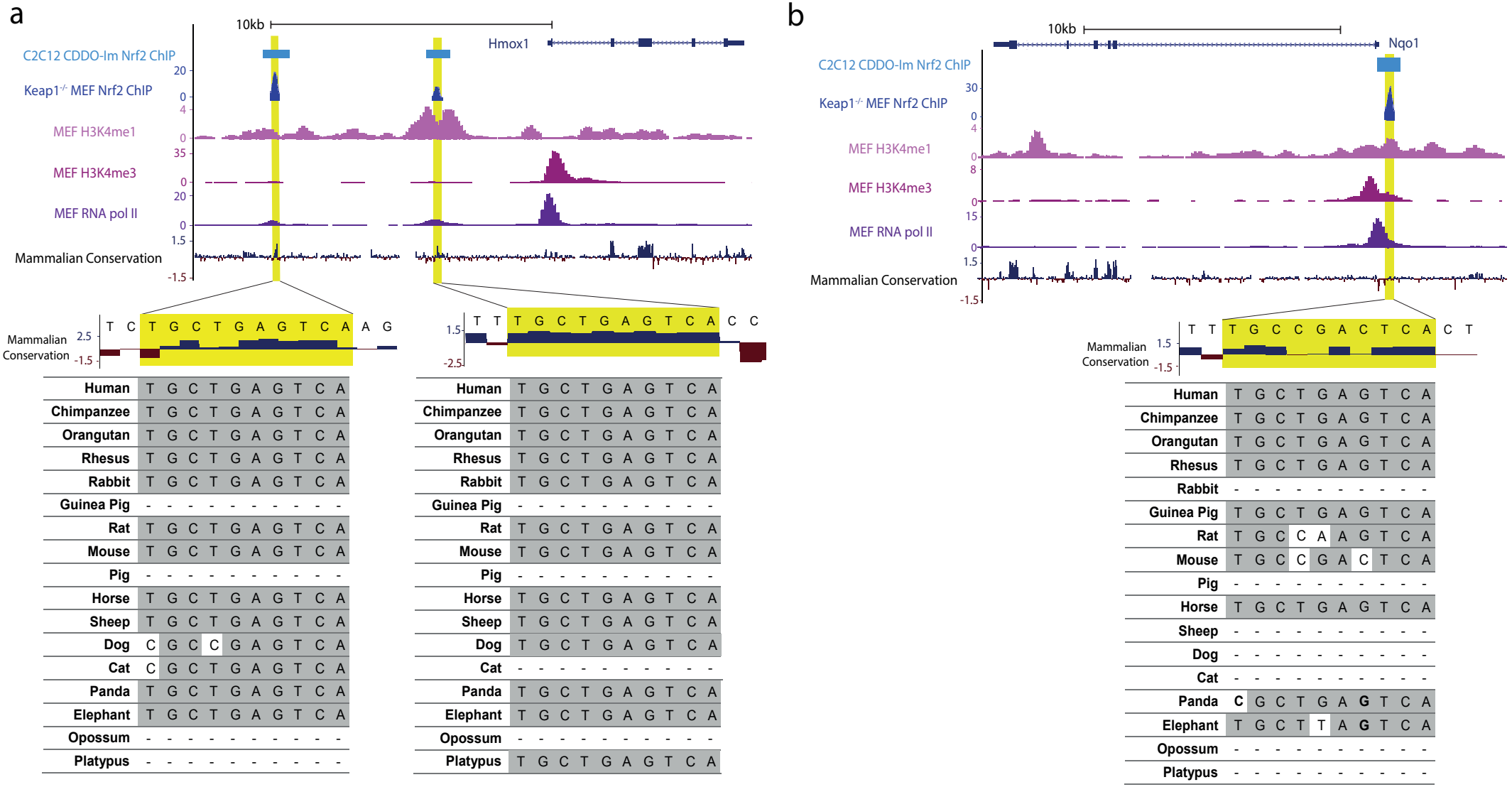
Supplementary Figure 1 (legend overleaf)



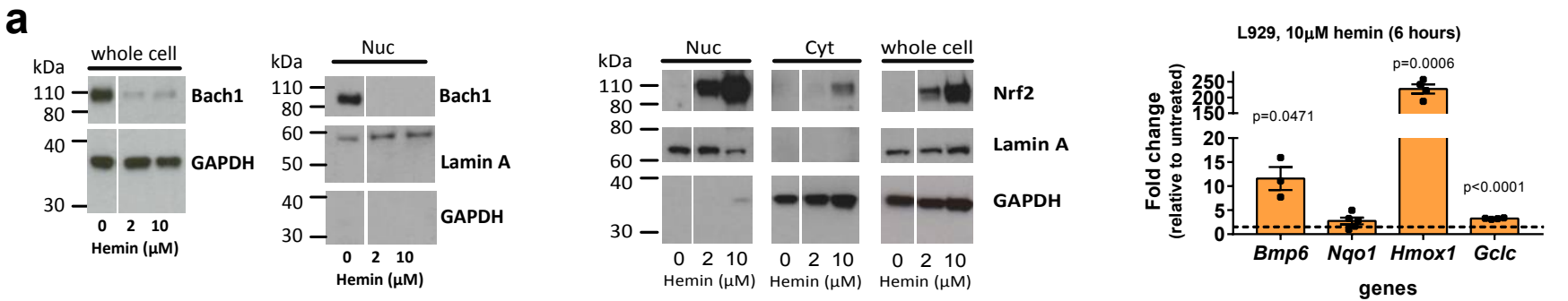
Supplementary Figure 1 *Bmp6* is upregulated concurrently with Nrf2 activity by iron and regulated by Nrf2/Keap1/Bach1. **(a)** 6-week old C57BL/6 male mice were injected with 4mg FeDx or Dx control intraperitoneally. Liver non-heme iron, *Bmp6*, *Nqo1*, *Hmox1*, *Gclc*, *Hamp1* and *Id1* expression were quantified after 6h (n=6 mice per group). **(b)** 6-week old C57BL/6 male mice were injected intraperitoneally with Dx (n=4) or 1mg (n=2), 2mg (n=3) or 4mg FeDx (n=4 mice). Liver non-heme iron, *Bmp6*, *Nqo1*, *Hmox1*, *Gclc*, *Hamp1* and *Id1* expression were quantified after 24h. **(c)** 6-week old C57BL/6 mice were injected with 4mg FeDx (males blue, females red) or Dx intraperitoneally. Liver non-heme iron, *Bmp6*, *Nqo1*, *Hmox1*, *Gclc*, *Hamp1* and *Id1* expression were quantified after 24h (n=8 (male Dx), n=6 (male FeDx), n=5 (female Dx), n=4 (female FeDx) mice per group). **(d)** Hepcidin was deleted in i*Hamp1*-knockout mice by daily tamoxifen administration for 4 days (n=4 (iHamp1-Ctrl Veh), n=4 (iHamp1-Ctrl Tam), n=5 (iHamp1-KO Veh), n=6 (iHamp1-KO Tam) mice per group). Liver non-heme iron, *Bmp6*, *Nqo1*, *Hmox1*, *Gclc*, *Hamp1* and *Id1* expression were quantified 24h after last tamoxifen dose. **(e)** MEF cells and C2C12 cells were treated with ferric ammonium citrate (FAC) for 6h. Gene expression of *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* was quantified (n=2 (MEF) and n=3 (C2C12) biologically independent experiments). Human TMNK-1 cells were treated with FAC (200ug/mL) or FeDx (1mg/mL) for 6h and *BMP6*, *NQO1*, *HMOX1* and *GCLC* mRNA levels were quantified (n=3 biologically independent experiments). **(f-h)** In L929 cells, siRNA-mediated knockdown of **(f)** Nrf2, **(g)** Keap1 and **(h)** Bach1 was performed (n=5 biologically independent experiments). **(i-k)** In MEF cells, siRNA-mediated knockdown of **(i)** Nrf2, **(j)** Keap1 and **(k)** Bach1 was performed. Knockdown efficiency was validated by Western blot and qRT-PCR. Gene expression of *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* was quantified (n=3 biologically independent experiments)). Data represented with centre values as mean and error bars as SEM except for *Hamp1* expression in **(e)** where data is represented as geometric mean ± SD and statistics performed on $\log_{10}$-transformed data for normalisation. Two-tailed t-tests performed between iron-loaded and control groups, and between NTC and Nrf2/Keap1/Bach1 siRNA groups.

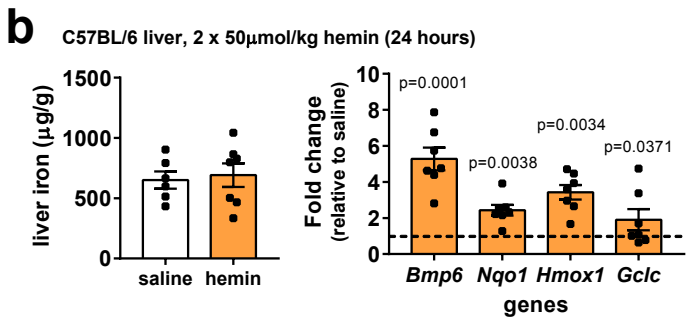
Supplementary Figure 2



Supplementary Figure 2 Nrf2 binds to conserved ARE in proximity to Nqo1 and Hmox1. Nrf2 ChIP-seq datasets from C2C12 cells treated with CDDO-Im for 3 hours and Keap1-knockout MEF cells, ChIP-seq tracks of H3K4-monomethylation (H3K4me1) depicting enhancer regions, H3K4-trimethylation (H3K4me3) depicting promoter regions, and RNA polymerase II (RNA pol II) on MEF cells, as well as mammalian conservation based on multiple alignments of 30 vertebrate species were mapped onto the mm9 mouse genome build on UCSC genome browser. The consensus ARE sequences (a) in the enhancer regions of Hmox1 and (b) in the promoter of Nqo1 are highlighted in yellow.

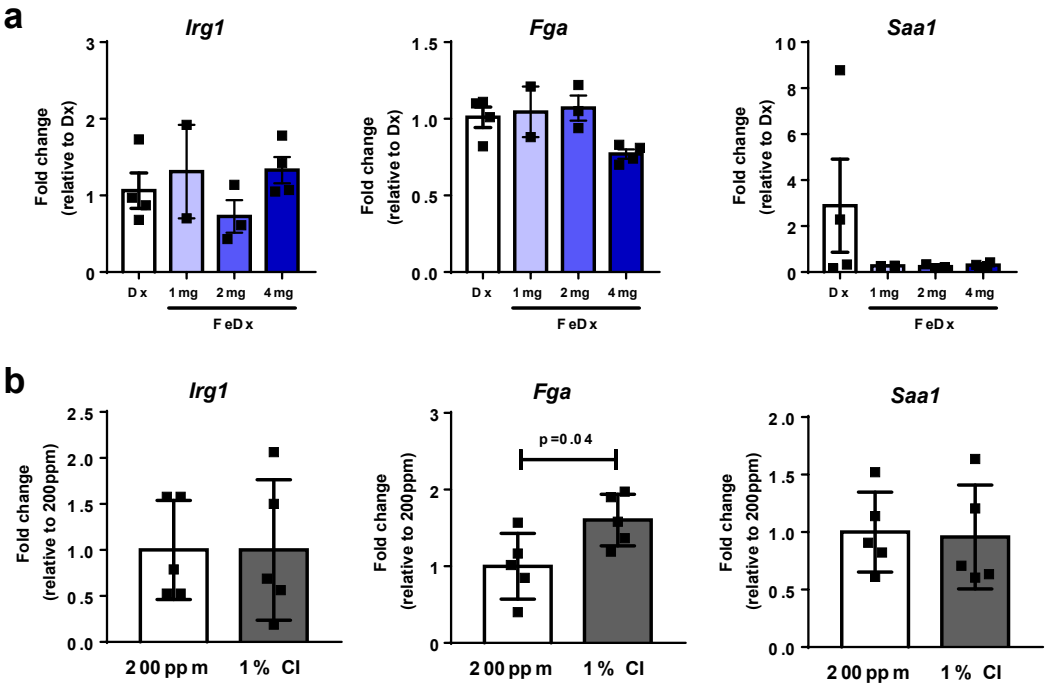
Supplementary Figure 3

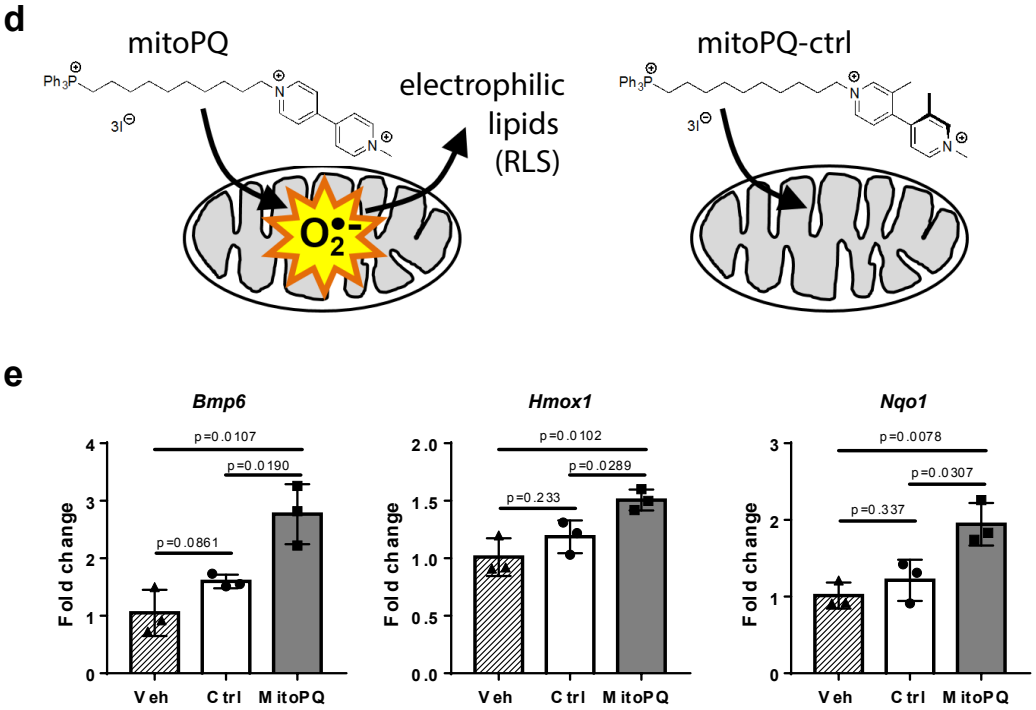


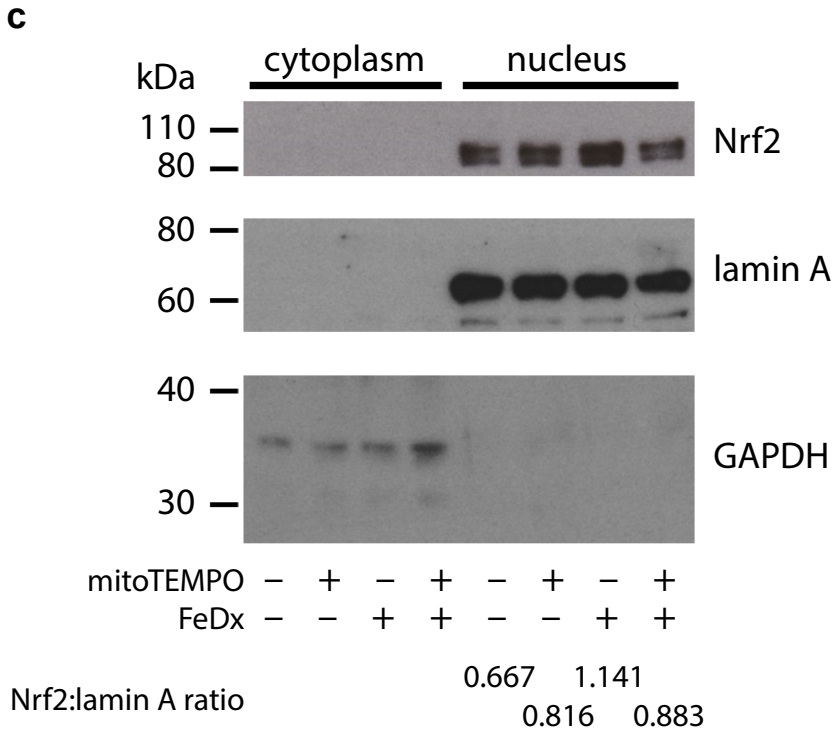


Supplementary Figure 3 Hemin-induced Bach1 degradation and Nrf2 stabilisation upregulated Bmp6 expression. (a) L929 cells were treated with hemin for 6 hours (n=4 biologically independent experiments). Immunoblot of Bach1 and Nrf2 protein in nuclear and whole cell extracts, and quantification of Bmp6, Nqo1, Hmox1 and Gclc expression were performed. (b) 6-week old C57BL/6 male mice were injected with two doses of 50μmol/kg hemin or saline intraperitoneally (n=6 (saline) and n=7 (hemin) mice). Liver non-heme iron and hepatic gene expression of Bmp6, Nqo1, Hmox1 and Gclc were quantified 12 hours after last injection. Data represented with centre values as mean and error bars as SEM. Two-tailed t-tests performed between hemin and control groups.

Supplementary Figure 4

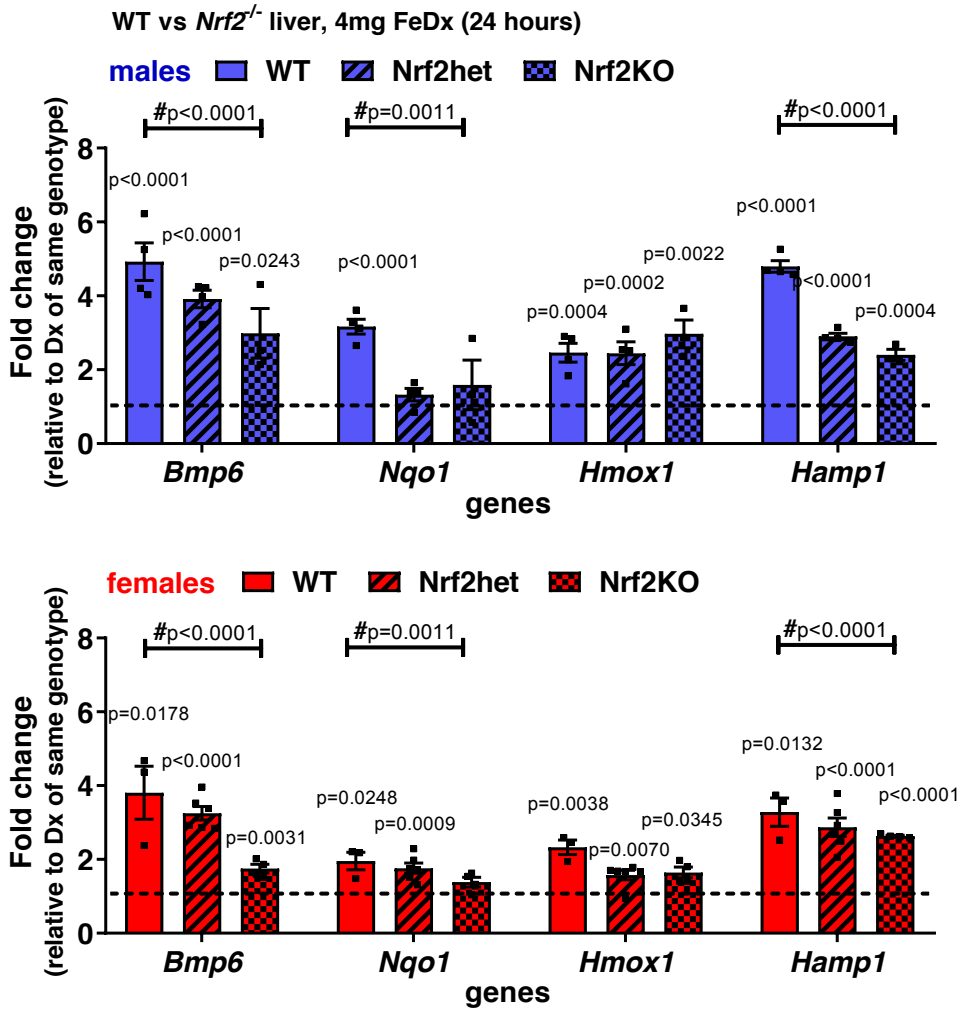






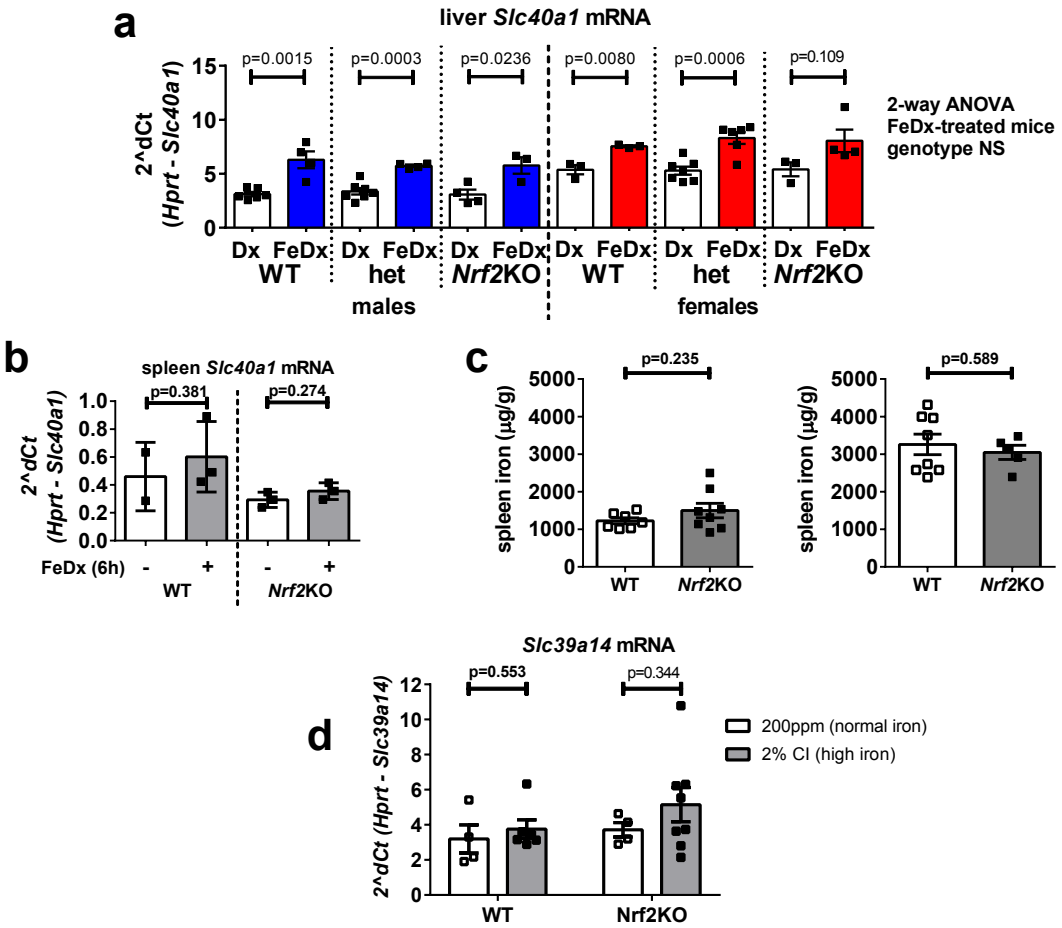
Supplementary Figure 4 Roles of Irg1 and superoxide in iron-mediated activation of Nrf2. (a, b) Mice were either injected intraperitoneally with increasing doses of FeDx for 24 hours (Dx (n=4), 1mg FeDx (n=2), 2mg FeDx (n=3) or 4mg FeDx (n=4) mice) or placed on a 1% carbonyl iron diet or control diet (200ppm Fe) for one week (n=5 mice per group), and then hepatic Irg1, Fga and Saa1 gene expression was measured. Expression of Fg1 and Saa1 are generally strongly induced by inflammation (two-tailed t-test). (c) L929 cells were pretreated with 500uM mitoTEMPO or vehicle for one hour then exposed to 1mg/mL FeDx or vehicle for then 6 hours, then cytoplasmic and nuclear Nrf2, lamin A and GAPDH protein levels were assessed by western blot and band density quantified by Image J analysis. Nuclear Nrf2 to lamin A ratios are given. (d) mitoPQ localises to mitochondria and induces superoxide generation and RLS, whereas the related control molecule mitoPQ-ctrl localises similarly but does not induce superoxide. (e) Gene expression of Bmp6, Hmox1 and Nqo1 was quantified in C2C12 cells exposed to 100uM mitoPQ, 100uM mitoPQ-ctrl, or vehicle (EtOH) for 6 hours (n=3 biologically independent samples, two-tailed t-test). Data represented with centre values as mean and error bars as SEM.

Supplementary Figure 5



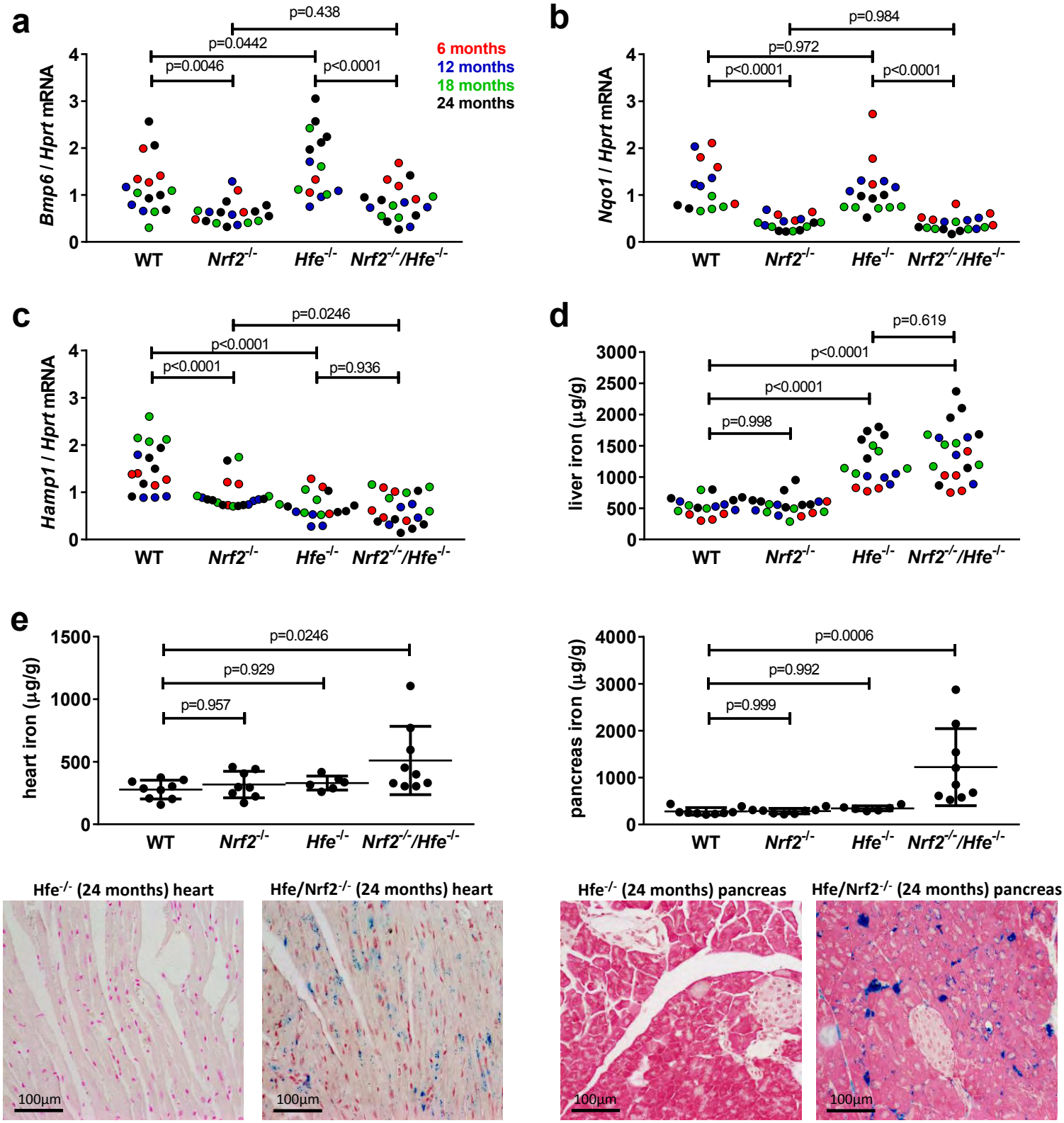
Supplementary Figure 5 Iron-induced *Bmp6* and hepcidin response is blunted in Nrf2-deficient mice. Wildtype (WT), heterozygous (Nrf2het) and homozygous (Nrf2KO) Nrf2-knockout mice were injected with 4mg FeDx or Dx (n=3 (male KO FeDx, female WT Dx, female WT FeDx, female KO Dx), n=4 (male WT FeDx, male het FeDx, male KO Dx, female KO FeDx), n=6 (male WT Dx, female het FeDx) and n=7 (male het Dx, female het Dx) mice). Liver non-heme iron and hepatic gene expression of *Bmp6*, *Nqo1*, *Hmox1* and *Hamp1* were assayed after 24 hours in males (blue) and females (red). Statistics: two-tailed t-test between Dx and FeDx groups within same genotype, with p-values labelled above each FeDx group; hash (#): 2-way ANOVA across WT, Nrf2het and Nrf2KO mice given FeDx, stratified by sex. Data represented with centre values as mean and error bars as SEM.

Supplementary figure 6



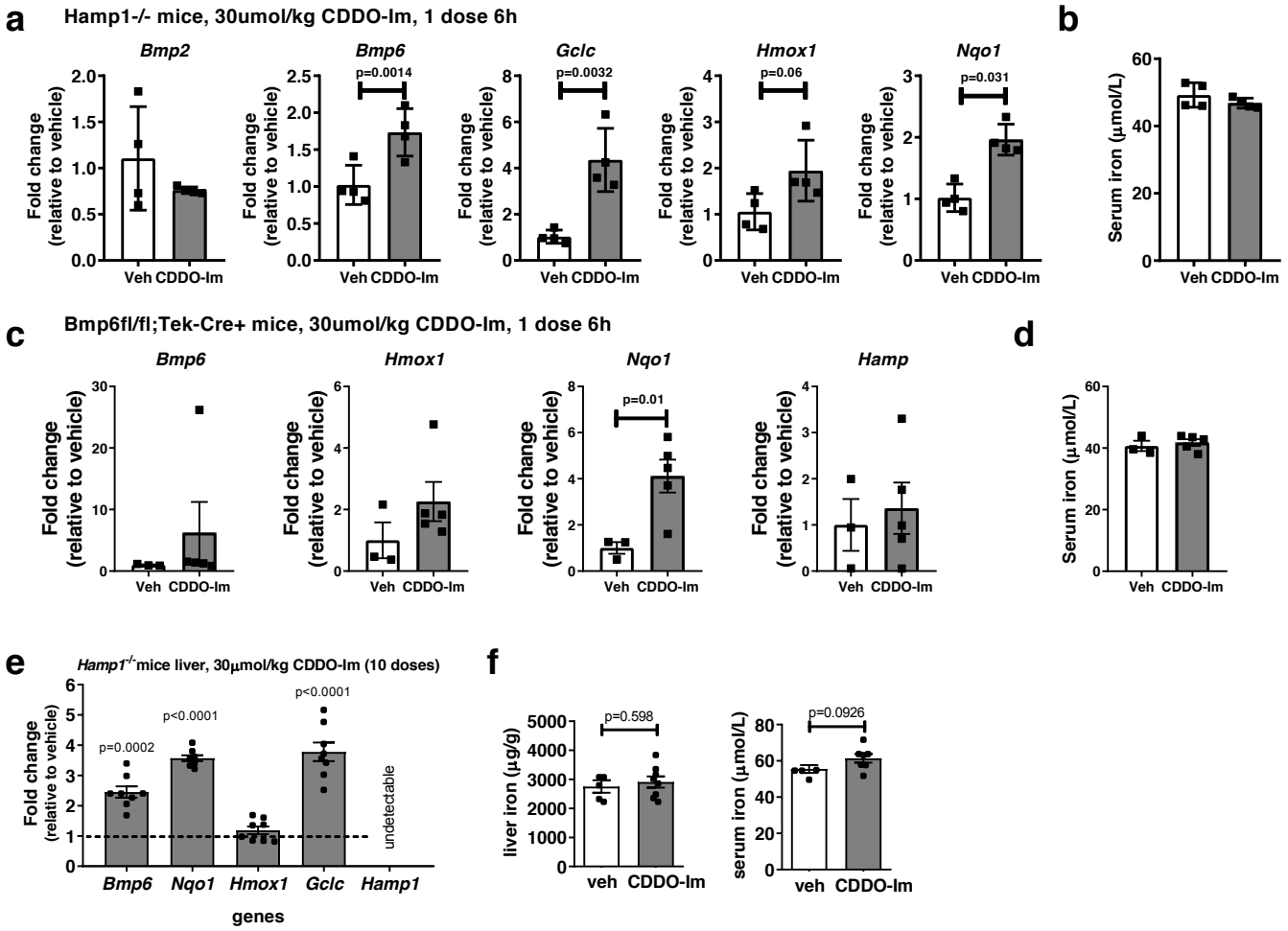
Supplementary Figure 6 Effects of Nrf2 deficiency on ferroportin and Zip14 mRNA expression. (**a**) Liver ferroportin (*Slc40a1*) expression in wildtype, heterozygous and homozygous *Nrf2* knockout mice 24 hours post FeDx injection (n=3 (male KO FeDx, female WT Dx, female WT FeDx, female KO Dx), n=4 (male WT FeDx, male het FeDx, male KO Dx, female KO FeDx), n=6 (male WT Dx, female het FeDx) and n=7 (male het Dx, female het Dx) mice). Two-tailed t-tests were performed between Dx and FeDx groups of same sex and genotype; 2-way ANOVA was performed for fold change induction in FeDx group relative to Dx control stratified on sex and genotype). (**b**) Spleen *Slc40a1* expression in wildtype and *Nrf2* knockout mice 6 hours post FeDx injection (n=2 (WT Dx) and n=3 (WT FeDx, KO Dx, KO FeDx) mice). Two-tailed t-test was performed. (**c**) Spleen iron in 7-week old mice fed 1% CI diet for 1 week (left, n=7 (WT) and n=8 (KO) mice) and 4-week old mice fed 1% CI diet for 4 weeks (right, n=8 (WT) and n=5 (KO) mice). Two-tailed t-tests were performed. **(d)** Hepatic *Slc39a14* (encoding Zip14) expression in wildtype and Nrf2 knockout mice fed 2 weeks of normal or high iron diet (n=4 (WT 200ppm, KO 200ppm), n=6 (WT 2%CI) and n=8 (KO 2%CI) mice. Data represented with centre values as mean and error bars as SEM. Statistics: two-tailed t-test.

Supplementary Figure 7



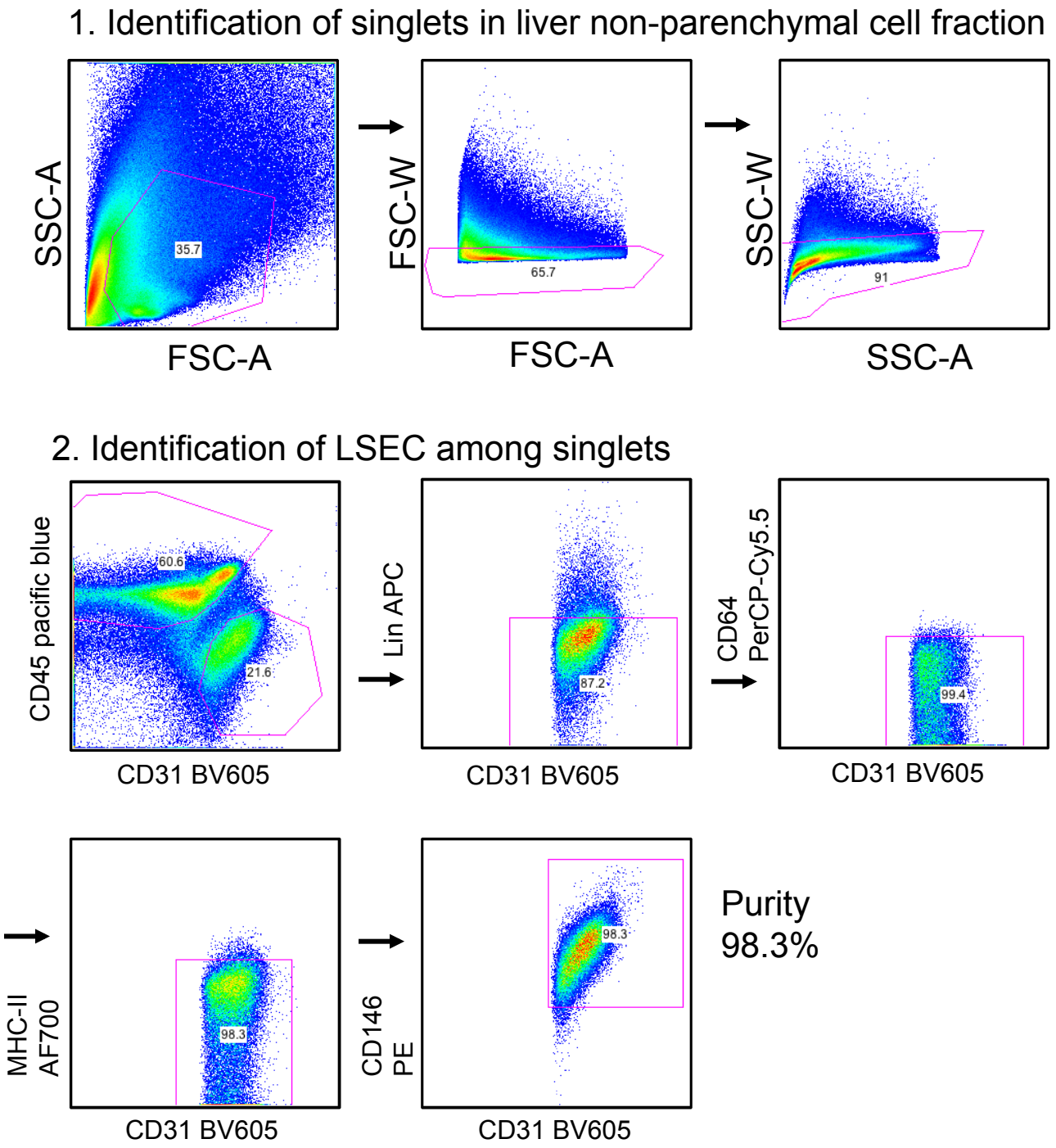
Supplementary Figure 7 Severe iron accumulation in Hfe/Nrf2 double knockout mice. Wildtype (WT) (n=17), Nrf2-/- (n=19), Hfe-/- (n=17) and Hfe/Nrf2-/- female mice (n=20) were fed a standard diet and culled at 6 (red), 12 (blue), 18 (green) or 24 (black) months of age. Hepatic gene expression of (a) Bmp6, (b) Nqo1 and (c) Hamp1 was quantified, and liver non-heme iron was assayed (d). (e) Non-heme iron was quantified in the heart and pancreas of 24 month-old mice (WT n=9, Nrf2-/- n=8, Hfe-/- n=6, Hfe/Nrf2-/- n=9 mice). The mean and SD are shown along with individual data points corresponding to different animals. Representative Perls Prussian blue staining of heart and pancreas sections of 24-month old Hfe-/- (n=6) and Hfe/Nrf2-/- mice (n=9). Statistics: 2-way ANOVA performed between each genotype, stratified by age.

Supplementary Figure 8



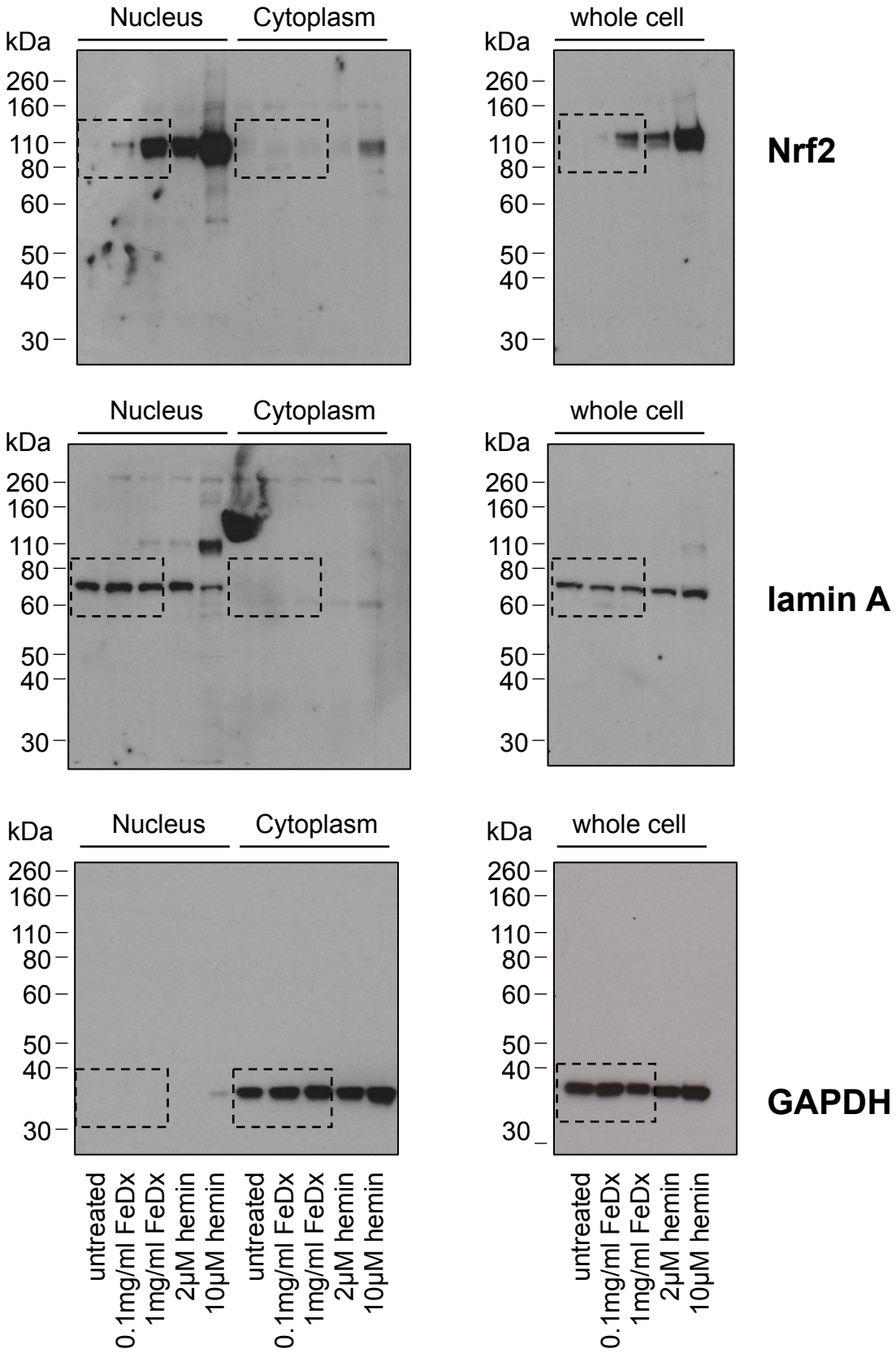
Supplementary Figure 8 Nrf2 agonist CDDO-Im in *Hamp1*-knockout mice and *Bmp6fl/fl;Tek-Cre+* mice. (**a, b**) Female *Hamp1*-knockout mice were given a single dose of vehicle or 30µmol/kg CDDO-Im by oral gavage and 6 hours later hepatic *Bmp2, Bmp6, Gclc, Hmox1* and *Nqo1* were measured (*Hamp1* was undetectable) and serum iron was quantified (n=4 mice per group). (**c, d**) *Bmp6fl/fl;Tek-Cre+* mice were given a single dose of vehicle or 30µmol/kg CDDO-Im by oral gavage and 6 hours later hepatic, *Bmp6, Hmox1, Nqo1* and *Hamp1* were measured and serum iron was quantified (n=3 (veh) and n=4 (CDDO-Im) mice per group). (**e, f**) Female *Hamp1*-knockout mice were given vehicle or 30µmol/kg CDDO-Im by oral gavage for 10 doses over 3 weeks (n=9 mice per group). Hepatic gene expression of *Bmp6*, *Nqo1*, *Hmox1*, *Gclc* and *Hamp1* was measured. Liver non-heme iron and serum iron were also quantified. Data represented with centre values as mean and error bars as SEM. Two-tailed t-test performed between mice treated with vehicle and CDDO-Im.

Supplementary Figure 9



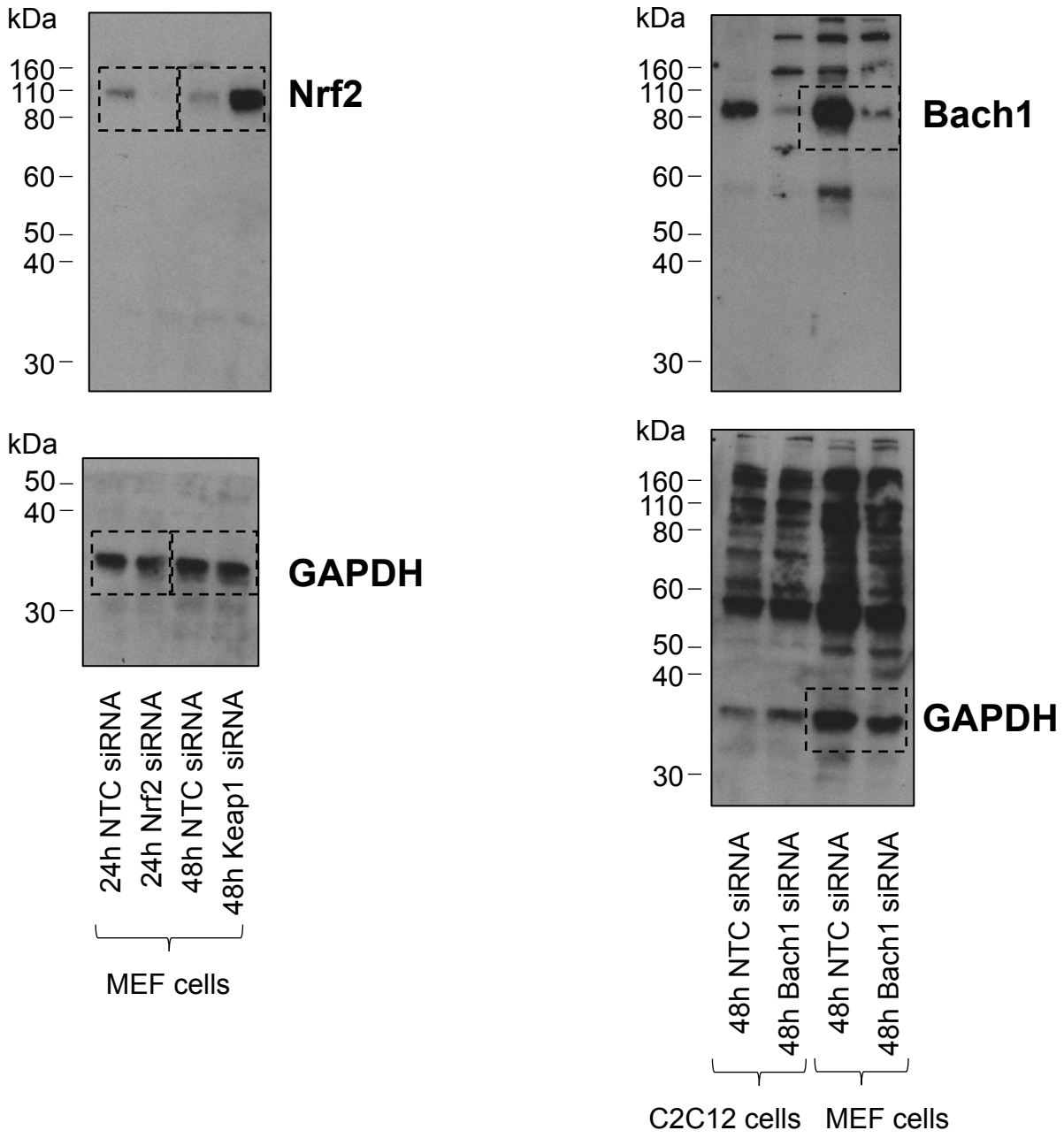
Supplementary Figure 9. Gating/sorting strategy for LSEC enrichment.
LSECs are defined as CD45- CD31+ Lin- CD64- MHC-II- CD146+
Lineage includes leukocyte markers (CD3, CD19, NK1.1, Ly6G, CD90.2) and a epithelial marker (CD326).

Supplementary Figure 10 (page 1/8)



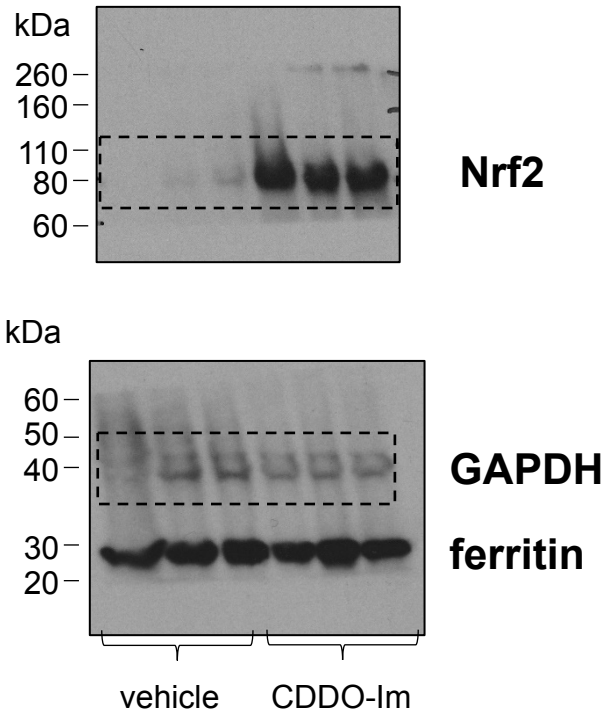
Complete blots corresponding to Figure 1d

Supplementary Figure 10 (page 2/8)



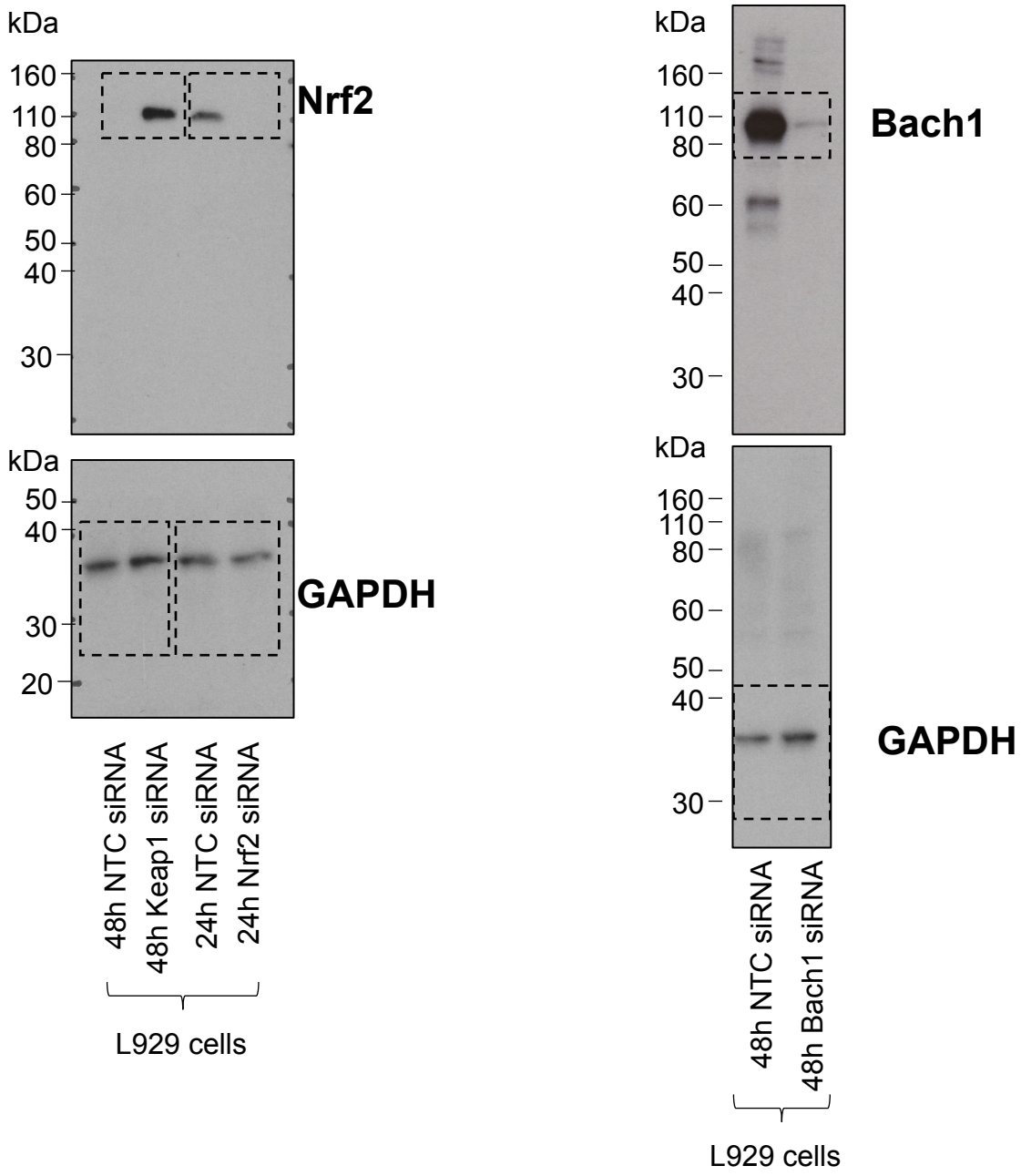
Complete blots corresponding to Figure 1f,g,h

Supplementary Figure 10 (page 3/8)



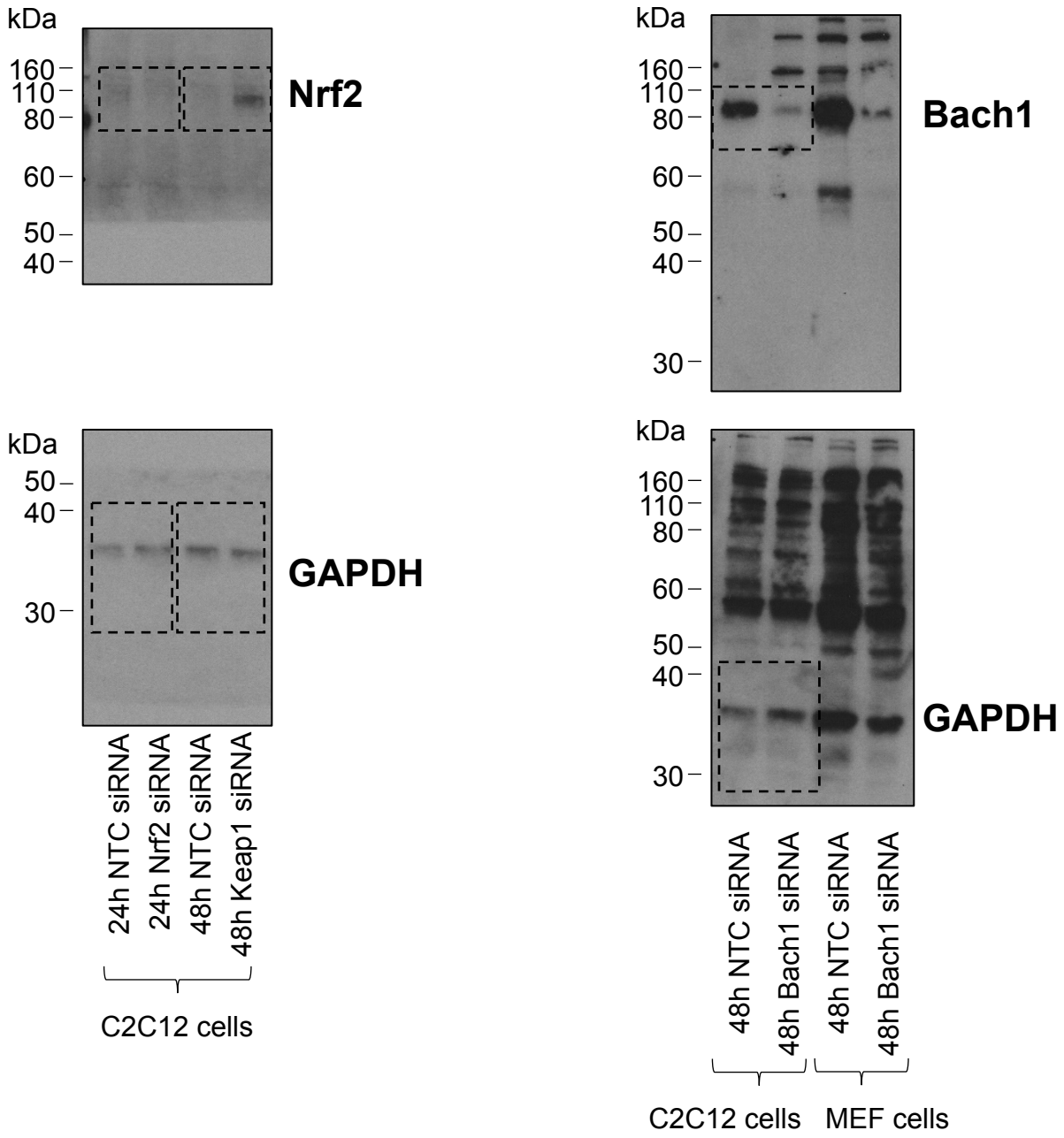
Complete blots corresponding to Figure 5d

Supplementary Figure 10 (page 4/8)



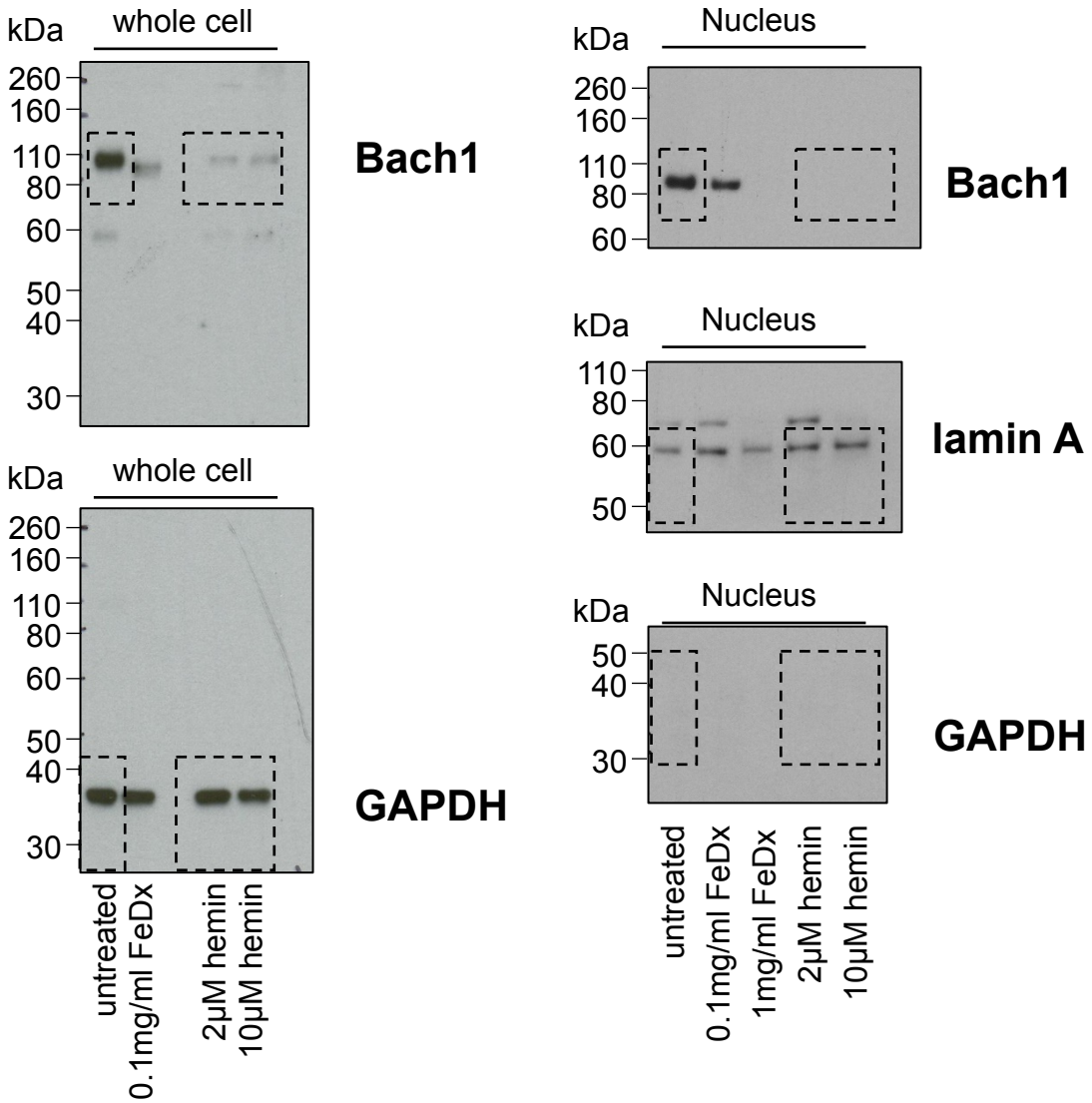
Complete blots corresponding to Supplementary Figure 1f,g,h

Supplementary Figure 10 (page 5/8)



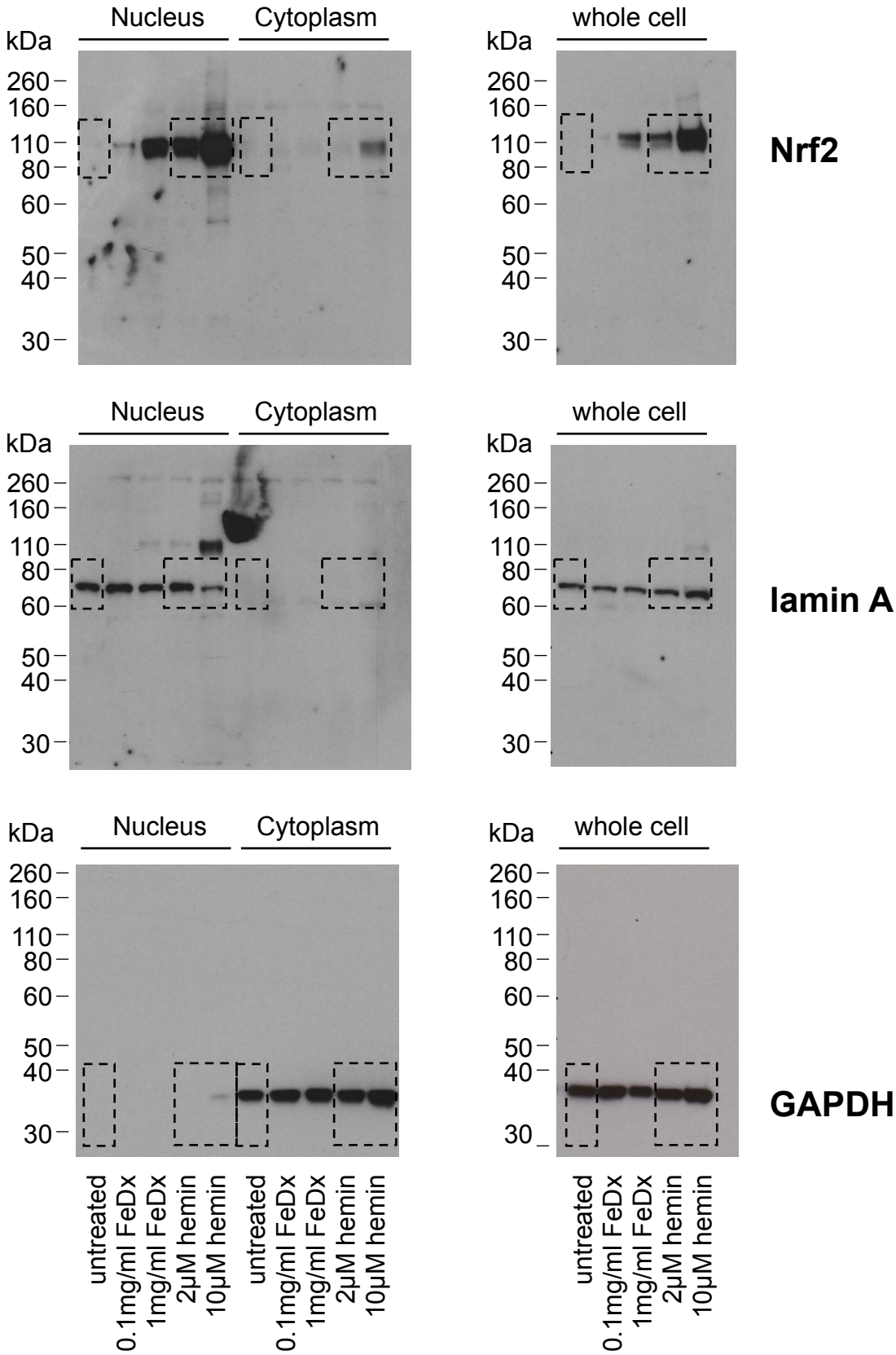
Complete blots corresponding to Supplementary Figure 1i,j,k

Supplementary Figure 10 (page 6/8)



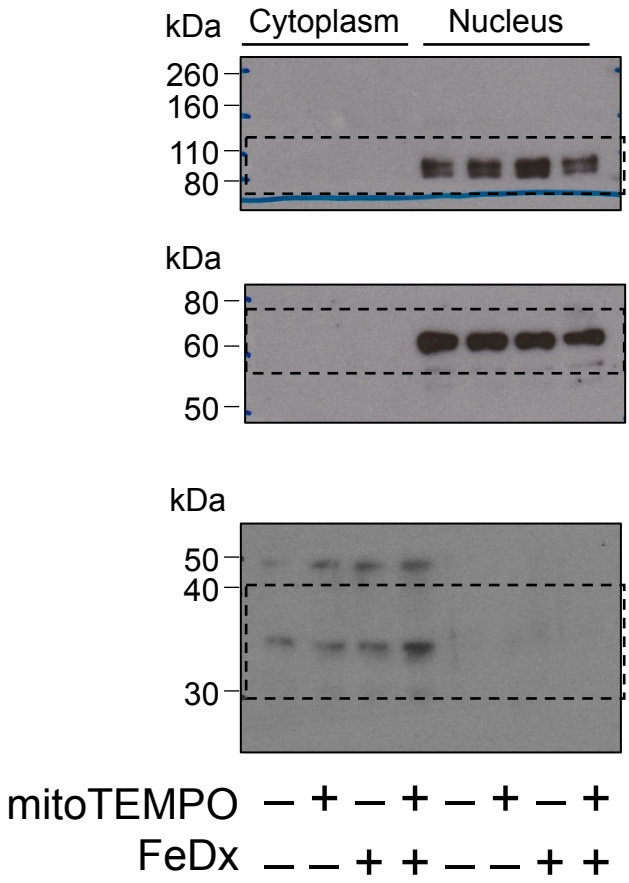
Complete blots corresponding to Supplementary Figure 3a

Supplementary Figure 10 (page 7/8)



Complete blots corresponding to Supplementary Figure 3a

Supplementary Figure 10 (page 8/8)



Complete blots corresponding to Supplementary Figure 4c

Supplementary Tables 1-4

Supplementary Table 1: List of antibodies used for LSEC isolation and for Western blot

Antibody	Manufacturer, catalog#, clone/lot	Dilution used
FITC Ly-6C	BioLegend #128006, clone HK1.4	1:600
PE CD146	BioLegend #134704, clone ME-9F1	1:600
PerCP-Cy5.5 CD64	BioLegend #139307, clone X54-5/7.1	1:600
PE-Cy7 F4/80	BioLegend #123114, clone BM8	1:600
Pacific Blue CD45.2	BioLegend #109819, clone 104	1:600
BV605 CD31	BioLegend #102427, clone 390	1:600
APC-Cy7 CD11b	BioLegend #101226, clone M1/70	1:600
AF700 MHC Class II	eBioscience #56-5321-80, clone M5/114.15.2	1:600
APC CD326	BioLegend #118214, clone G8.8	1:600
APC CD90.2	BioLegend #140311, clone 53-2.1	1:600
APC NK-1.1	BioLegend #108709, clone PK136	1:600
APC CD19	BioLegend #115512, clone 6D5	1:600
APC CD3ε	BioLegend #100312, clone 145-2C11	1:600
APC Ly-6G	BioLegend #127613, clone 1A8	1:600
Nrf2	Cell Signaling Technology, #D1Z9C	1:500
Bach1	R&D systems, #AF5777	1:400
Lamin A	Abcam, #ab8980	1:1000
Gapdh	Proteintech, # HRP-60004	1:20000
Anti-mouse IgG HRP-conjugated	Santa Cruz, #sc-2005, lot K0714	1:2000
Anti-goat IgG HRP-conjugated	Santa Cruz, #sc-2020, lot H2113	1:5000
Anti-rabbit IgG HRP-conjugated	R&D Systems, #HAF008, lot FIN1716111	1:2000

Supplementary Table 2: List of TaqMan Gene Expression assays (Applied Biosystems)

Protein	**Gene**	**Assay code**
Hypoxanthine-guanine phosphoribosyltransferase	*Hprt1*	Mm01545399_m1
Bone morphogenetic protein 6	*Bmp6*	Mm01332882_m1
NAD(P)H dehydrogenase, quinone 1	*Nqo1*	Mm01253561_m1
Heme oxygenase 1	*Hmox1*	Mm00516005_m1
Glutamate-cysteine ligase catalytic subunit	*Gclc*	Mm00802655_m1
Hepcidin	*Hamp1*	Mm04231240_s1
Nuclear factor (erythroid-derived 2)-like 2	*Nfe2l2 (Nrf2)*	Mm00477784_m1
Kelch-like ECH associated protein 1	*Keap1*	Mm00497268_m1
BTB And CNC Homology 1	*Bach1*	Mm01344527_m1
Inhibitor of DNA-binding protein 1	*Id1*	Mm00775963_g1
Sons of mothers against decapentaplegic 7	*Smad7*	Mm00484742_m1
Bone morphogenetic protein 2	*Bmp2*	Mm01340178_m1
Collagen type I alpha I chain	*Col1a1*	Mm00801666_g1
Transgelin	*Tagln*	Mm00441661_g1
Ferroportin	*Slc40a1*	Mm01254822_m1
Zip14	*Slc39a14*	Mm01317439_m1
Immunoresponsive gene 1	*Irg1*	Mm01224532_m1
Erythroferrone	*Fam132b*	Mm01224532_m1
Erythropoietin	*Epo*	Mm01202755_m1
Glyceraldehyde 3-phosphate dehydrogenase (human)	*GAPDH*	Hs99999905_m1
Heme oxygenase 1 (human)	*HMOX1*	Hs01110250_m1
Bone morphogenetic protein 6 (human)	*BMP6*	Hs01099594_m1
Glutamate-cysteine ligase catalytic subunit (human)	*GCLC*	Hs00155249_m1
NAD(P)H dehydrogenase, quinone 1 (human)	*NQO1*	Hs01045993_g1

Supplementary Table 3: List of primers for *Hfe*/*Nrf2*$^{-/-}$ liver qRT-PCR

Gene	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**
Hprt1	AGATGGGAGGCCATCACATTGT	ATGTCCCCCGTTGACTGATCAT
Bmp6	TCCCCACATCAACGACACCA	TCCCCACCACACAGTCCTTG
Nqo1	GTGCAGAAGCGAGCTGGAAATACTC	CGAATCTTGATGGAGGACTGGATGC
Hamp1	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA

Supplementary Table 4: List of primers for LSECs qRT-PCR

Gene	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**
Rpl19	AGGCATATGGGCATAGGGAAGAG	TTGACCTTCAGGTACAGGTGTG
Bmp6	AGCACAGAGACTCTGACCTATTTTT	CCACAGATTCTAGTTGCTGTGA
Nqo1	GCCCGCATGCAGATCCT	GGTCTCCTCCCAGACGGTTT
Hmox1	CAGCCCCACCAAGTTCAAA	TCAGGTGTCATCTCCAGAGTGTTC