

Nrf2 controls iron homeostasis in haemochromatosis and thalassaemia via Bmp6 and hepcidin

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Abstract

Iron is critical for life but toxic in excess because of iron-catalysed formation of pro-oxidants that cause tissue damage in a range of disorders. The Nrf2 transcription factor orchestrates cell-intrinsic protective antioxidant responses, and the peptide hormone hepcidin maintains systemic iron homeostasis, but is pathophysiologically decreased in haemochromatosis and beta-thalassaemia. Here, we show that Nrf2 is activated by iron-induced, mitochondria-derived pro-oxidants and drives Bmp6 expression in liver sinusoid endothelial cells, which in turn increases hepcidin synthesis by neighbouring hepatocytes. In Nrf2 knockout mice, the Bmp6-hepcidin response to oral and parenteral iron is impaired and iron accumulation and hepatic damage are increased. Pharmacological activation of Nrf2 stimulates the Bmp6-hepcidin axis, improving iron homeostasis in haemochromatosis and counteracting the inhibition of Bmp6 by erythroferrone in beta-thalassaemia. We propose that Nrf2 links cellular sensing of excess toxic iron to control of systemic iron homeostasis and antioxidant responses, and may be a therapeutic target for iron-associated disorders.

Iron facilitates the oxygen-carrying capacity of blood and serves as a critical component for many cellular processes including DNA replication and energy transduction¹. Defects in iron balance contribute to the pathogenesis of cancer, diabetes, cardiovascular disease, metabolic syndrome, as well as haemochromatosis, thalassaemia and anaemia. Oral and parenteral iron treatments are amongst the most commonly administered medical interventions worldwide. The amount and distribution of iron is regulated systemically by the liver-expressed hormone hepcidin (encoded by *Hamp1*)². Hepcidin induces the degradation of the iron exporter ferroportin³, limiting dietary iron absorption by enterocytes and iron recycling from senescent erythrocytes by macrophages. Appropriate regulation of hepcidin synthesis in response to fluctuating iron levels is critical to maintain iron homeostasis. Hepcidin deficiency causes pathological iron overload in the common genetic disorders hereditary haemochromatosis⁴ and beta-thalassaemia⁵. Hepcidin regulation in response to iron is controlled by BMP/SMAD signalling and by *Bmp6* expression⁶. *Bmp6*-deficient mice have very low hepcidin levels and accrue severe iron accumulation^{7,8}. Liver sinusoidal endothelial cells (LSECs) are the predominant source of hepatic *Bmp6*⁹. The intracellular mechanism by which iron is sensed and converted into a signal to regulate hepatic *Bmp6* expression is not known.

Excess iron, resulting from iron overload disorders, or because of oral or parenteral iron treatments, induces oxidative stress^{10,11}. By responding to toxic insults and controlling the expression of detoxification and antioxidant enzymes, the transcription factor Nrf2 maintains cellular health in the face of intracellular and environmental stresses¹², and so is a key player in carcinogenesis. At basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1 (Kelch-like ECH-associated protein 1), an adaptor subunit of Cullin3 E3 ubiquitin ligase, and targeted for ubiquitination and degradation. Oxidative stress-inducing stimuli destabilize the Nrf2-Keap1-Cullin3 complex and block Nrf2 degradation. This allows *de novo* synthesized Nrf2 to translocate into the nucleus, bind antioxidant response elements (AREs), and control the expression of genes encoding proteins with antioxidant and cytoprotective properties, such as NAD(P)H dehydrogenase quinone 1 (*Nqo1*) and Glutamate-Cysteine Ligase Catalytic Subunit (*Gclc*)^{12,13}. The Bach1 (Btb and Cnc Homology 1) transcriptional repressor negatively controls the expression of ARE-regulated genes, including heme oxygenase 1 (*Hmox1*)¹⁴.

Here we show that induction of *Bmp6* expression is regulated by the Nrf2-Keap1-Bach1 system in response to iron-induced mitochondrial reactive lipid species and heme. Nrf2 deficiency impairs control of hepcidin in the context of iron supplementation and associates with iron toxicity, while Nrf2 activation stimulates the *Bmp6*-hepcidin axis and enhances antioxidant defences. Therefore Nrf2 has systemic in addition to cell-intrinsic detoxifying properties that have relevance to a range of disorders in which iron accumulation is pathogenic.

Results

Iron activates Nrf2 and Bmp6 is regulated by Nrf2

Hepatic *Bmp6* is upregulated by iron in mice⁶ and in hereditary hemochromatosis^{15,16}. In separate studies, elevated hepatic iron content in mice fed high-iron diets was associated with increased Nrf2 activity^{17,18}. We tested whether concurrent Nrf2 activation and *Bmp6* upregulation was generalizable across several models of iron overload. We investigated how altering iron *in vivo* affected hepatic expression of *Bmp6*, downstream Bmp/Smad-target genes (*Hamp1*, *Id1*, *Smad7*) and Nrf2-target genes (*Nqo1*, *Hmox1*, *Gclc*). Mice fed high iron diet (1% carbonyl iron) for 3 weeks had increased liver iron content as well as raised *Bmp6*, *Hamp1*, *Id1* and *Smad7* gene expression, coupled with upregulation of *Nqo1*, *Hmox1* and *Gclc* consistent with Nrf2 activation (Fig. 1a). Intraperitoneal administration of 4mg FeDx increased liver iron content 6 hours later, with concomitant upregulation of hepatic *Bmp6*, *Nqo1*, *Hmox1*, *Gclc*, *Hamp1* and *Id1* mRNA expression (Supplementary Fig. 1a). The concurrent induction of these six mRNAs was dose-dependent over 24 hours and was observed in male and female mice (Supplementary Fig. 1b, c). To cause iron accumulation by genetic intervention, we deleted hepcidin in a previously described inducible *Hamp1*-knockout (*iHamp1*-KO) model¹⁹. Tamoxifen-treated *iHamp1*-KO mice were hepcidin-deficient, accumulated liver iron and had increased *Bmp6*, *Id1* and *Smad7* expression, concomitant with upregulation of *Nqo1*, *Hmox1* and *Gclc* expression (Supplementary Fig. 1d). Concurrent upregulation of *Bmp6* together with *Nqo1*, *Hmox1* and *Gclc* was also observed in LSECs purified *ex vivo* following *in vivo* iron loading in 129S6/SvEvTac mice fed 1% carbonyl iron (high) versus 37ppm iron (low) diets for 4 weeks (Fig. 1b). *In vitro*, ferric ammonium citrate (FAC) increased *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* expression in primary murine LSECs (Fig. 1c), C2C12 cells and MEF cells and upregulated *BMP6* in the human LSEC-derived cell-line TMNK-1 (Supplementary Fig. 1e). In L929 cells FeDx upregulated *Bmp6*, concurrently led to nuclear accumulation of Nrf2 and increased expression of *Nqo1*, *Hmox1* and *Gclc* (Fig. 1d).

Since concurrent upregulation of Nrf2 target genes and *Bmp6* was observed across several *in vitro* and *in vivo* models of iron loading, we hypothesized that *Bmp6* could be regulated by the Nrf2/Keap1/Bach1 system. Publicly available ChIP-seq data showed Nrf2 binds in intron 1 of the murine *Bmp6* locus in C2C12 cells treated with an agonist of Nrf2 (CDDO-Im²⁰⁻²²) and this binding site overlaps with an Nrf2 ChIP peak in *Keap1*^{-/-} MEF cells (Fig. 1e). The ChIP-seq peak overlaps with an H3K4-monomethylation (H3K4me1) enhancer mark and RNA polymerase II binding region in MEF cells, is highly conserved in mammals, and contains an ARE consensus sequence TGCTGAGTCA²³ (Fig. 1e). Nrf2 ChIP-seq peaks were also observed at well-characterised and similarly conserved AREs with the same TGCTGAGTCA consensus sequence in the *Hmox1* and *Nqo1* promoters²⁴ in both Nrf2 ChIP-seq datasets analysed (Supplementary Fig. 2a,b). To test whether baseline *Bmp6* expression was sensitive to changes in *Nrf2*, *Keap1* and *Bach1*, we knocked down expression of these three genes in C2C12, MEF and L929 cells. In C2C12 cells (Fig. 1f-h),

knockdown of Nrf2 led to a decrease of *Bmp6* expression, whereas knockdown of Keap1 (which targets Nrf2 for degradation) or Bach1 (which generally suppresses Nrf2 target genes) led to an increase in *Bmp6* expression; similar effects were observed on expression of Nrf2 target genes *Nqo1* and *Gclc*, while *Hmox1* expression was not altered by *Nrf2* or *Keap1* knockdown but strongly upregulated by *Bach1* knockdown as expected²⁴. Similar effects of *Nrf2*, *Keap1* and *Bach1* knockdown on expression of *Bmp6* and other Nrf2 target genes were observed in L929 and MEF cells (Supplementary Fig. 1f-k). The suppressive activity of Bach1 can be counteracted by heme¹⁴, which induces Bach1 degradation; we found that hemin (heme chloride) induced loss of whole cell and nuclear Bach1 protein, caused nuclear accumulation of Nrf2, and increased expression of *Bmp6* and other Nrf2 target genes in L929 cells (Supplementary Fig. 3a). In livers of mice injected with hemin, *Bmp6*, *Nqo1*, *Gclc* and *Hmox1* expression was also increased (Supplementary Fig. 3b). In summary, the *Bmp6* locus is bound by Nrf2 at a conserved ARE site, experimental manipulation of endogenous Nrf2 activity by siRNA alters expression of *Bmp6* and other Nrf2-regulated genes, and iron treatments that activate Nrf2 increase expression of *Bmp6* alongside canonical Nrf2 target genes.

Iron-induced mitochondria-derived pro-oxidants activate Nrf2

We further investigated how iron activates Nrf2 and induces *Bmp6* expression. Inflammation caused by LPS can activate Nrf2 in macrophages via induction of *Irg1* expression, leading to production of itaconate, which alkylates Keap1²⁵. However, *Irg1* mRNA was not detected after 40 cycles of quantitative RT-PCR in L929 or C2C12 cells at baseline or after iron treatment, and hepatic *Irg1* was not altered in mice injected with FeDx (in which mRNA levels of inflammatory genes *Fga1* and *Saa1* were also unaltered) or subjected to a high-iron diet for one week (in which *Fga1* was upregulated but *Saa1* was not altered) (Supplementary Fig. 4a, b). Therefore *Irg1*/itaconate is highly unlikely to be involved in regulation of *Bmp6* by iron and Nrf2. Instead we hypothesized that Nrf2 activation by iron could occur via the generation of ROS. By quantifying the lipid peroxidation product malondialdehyde (MDA) in liver lysates, we found that C57BL/6 mice fed a week of high iron diet experienced more hepatic lipid peroxidation than mice on a control iron diet (Fig. 2a). The mitochondrial electron transport chain is a major source of cellular ROS, and excess iron may promote mitochondrial ROS, oxidative damage and lipid peroxidation^{26,27}. To assess how the generation of ROS influences *Bmp6* induction by iron, we used the free radical scavenger mitoTEMPO²⁸, which concentrates in the mitochondria matrix and acts to quench ROS and oxidative damage (Fig. 2b). In L929 cells, mitoTEMPO prevented upregulation of *Bmp6* by FeDx, blunted *Nqo1* upregulation but did not alter upregulation of *Hmox1* (Fig. 2c). FeDx treatment increased the ratio of Nrf2:lamin A in the nucleus of L929 cells by >70%, but in the presence of mitoTEMPO, the ratio of Nrf2/lamin A was unchanged by FeDx, indicating a role for mitochondria-derived ROS production and/or oxidative damage for Nrf2 activation in response to iron (Supplementary Fig 4c). In mice, oral gavage with 2mg/kg iron did not measurably alter hepatic iron content by 6 hours but

acutely raised serum iron (Fig. 2d) as well as hepatic *Nqo1*, *Hmox1* and *Bmp6* expression (Fig. 2e). mitoTEMPO completely blocked the induction of *Bmp6* and *Hmox1* by iron gavage, although upregulation of *Nqo1* was maintained (Fig. 2e). We then tested the effects of generating mitochondrial ROS (specifically superoxide) in the absence of added iron by using the mitochondria-targeted redox cyclizer MitoParaquat (MitoPQ)²⁹; increased superoxide potentiates lipid peroxidation within mitochondria that leads to release of electrophilic reactive lipid species³⁰ (RLS) (Fig 2f). *Bmp6*, *Nqo1* and *Hmox1* were induced in cells treated with MitoPQ (Fig. 2g), but not by a control compound that similarly targets mitochondria but does not generate superoxide³¹ (Supplementary Fig. 4d,e). Therefore selective generation of ROS within mitochondria and the associated oxidative damage that can be induced by iron are necessary and sufficient to drive expression of Nrf2 target genes, including *Bmp6*. It is the pro-oxidant activity of iron, rather than iron *per se*, that activates Nrf2 and induces *Bmp6*.

Nrf2 regulates stimulation of the Bmp6-hepcidin axis by iron

Next we asked whether Nrf2 was required for upregulation of *Bmp6* by iron. Knockdown of *Nrf2* completely blocked the upregulation of *Bmp6* and *Nqo1* by FeDx in L929 (Fig. 3a), and in C2C12 cells the upregulation of *Bmp6* and *Nqo1* by FAC was blunted by knockdown of Nrf2 (Fig. 3b). *In vivo*, 24 hours after intraperitoneal injection of FeDx in *Nrf2*^{-/-}, *Nrf2*^{+/-} and *Nrf2*^{+/+} littermate mice, induction of hepatic *Bmp6* and *Nqo1* expression by FeDx was blunted in *Nrf2*^{-/-} mice, with an intermediate response in heterozygotes, and importantly, *Hamp1* induction by FeDx was also significantly blunted in *Nrf2* deficient mice (Supplementary Fig. 5). This decrease in hepcidin levels led us to test the effect of Nrf2 deficiency in the context of more chronic iron accumulation (increased liver iron was a previously unexplained finding in *Nrf2*^{-/-} mice³²). After one week on a high iron (1% carbonyl diet), *Nrf2*^{-/-} mice had decreased hepatic expression of *Nqo1*, *Gclc*, *Bmp6* and *Hamp1* compared to wild-type controls, and although liver iron accumulation was not different between the two groups as a whole, some *Nrf2*^{-/-} mice had accumulated more liver iron (Fig 3c). To test this further, *Nrf2*^{-/-} and *Nrf2*^{+/+} littermate mice were fed control (200ppm iron) or high iron (2% carbonyl iron) diet for 2 weeks. Again, *Nrf2*^{-/-} mice failed to upregulate hepatic *Bmp6* and had blunted *Nqo1* induction in response to hepatic iron accumulation, and furthermore the upregulation of Bmp/Smad-target genes *Id1* and *Smad7* was also abrogated (Fig. 3d). The upregulation of hepcidin by iron in *Nrf2*^{-/-} mice was blunted by about 25%, showing that Nrf2-controlled induction of *Bmp6* makes a non-redundant contribution to control of hepcidin in response to chronic iron overload (Fig 3e). Furthermore, in this experiment, *Nrf2*^{-/-} mice had ~ 27% higher levels of hepatic iron than wild-type controls (Fig 3f), and after 4 weeks of 1% carbonyl iron diet, *Nrf2*^{-/-} mice accumulated 30% more iron in the liver than wildtype animals (Fig. 3g). These findings could not be accounted for by differences in relative liver weight as we previously showed no difference in weight of livers between iron loaded wildtype and *Nrf2*^{-/-} animals¹⁸. Ferroportin is an Nrf2-regulated gene³³ and we considered

whether altered ferroportin expression could contribute to the iron loading phenotype. However, Nrf2 genotype did not affect hepatic ferroportin mRNA levels at baseline or 24 hours after intraperitoneal injection of FeDx; Nrf2 genotype also did not influence splenic ferroportin mRNA at baseline or after FeDx treatment, or affect splenic iron accumulation after one and four weeks of high iron diets (Supplementary Fig 6a-c). Hepatic *Slc39a14* (encoding Zip14) can also influence liver iron accumulation³⁴, but hepatic *Slc39a14* mRNA was not different between wild-type and *Nrf2*^{-/-} mice either at baseline or after 2 weeks of high iron diet, and so is not obviously Nrf2-regulated (Supplementary Fig 6d). Therefore the iron loading phenotypes we observe in *Nrf2*^{-/-} mice are unlikely to be due to altered transcriptional regulation of ferroportin or Zip14.

Nrf2 controls iron-mediated damage to hepatocytes and LSECs

Because Nrf2, as well as controlling the Bmp6-hepcidin axis, also regulates protective antioxidant effects, we analysed how Nrf2 deficiency would influence liver pathology. We observed a greater increase in lipid peroxidation (MDA) in *Nrf2*^{-/-} mice after 4 weeks of dietary iron loading; hepatic expression of liver fibrosis markers collagen type 1 (*Col1a1*) and transgelin (*Tagln*) were also higher in iron-loaded *Nrf2*^{-/-} mice (Fig. 4a). This suggested that as well as being disposed to over-accumulate iron, *Nrf2*^{-/-} mice are also more susceptible to iron-induced liver oxidative damage and subsequent tissue injury. Consistent with this idea, when *Nrf2*^{-/-} and wildtype mice were given a single intraperitoneal injection of FeDx, increased hepatic necroinflammatory foci developed over 30 days in *Nrf2*^{-/-} mice (Fig. 4b,c). Previously we described ultrastructural hepatocyte pathology in iron-exposed *Nrf2*^{-/-} mice¹⁸. Because LSECs are the key source of Bmp6⁹, and because Nrf2 is required for induction of Bmp6 by iron, we further examined ultrastructural features of LSECs in iron-loaded wild-type and *Nrf2*^{-/-} mice. In liver sinusoids of wild-type animals fed 2% carbonyl iron diet for two weeks, cristae of LSEC mitochondria were well-formed, as were hepatocyte mitochondria, and hepatocyte microvilli in the space of Disse were evident (Fig 4d). In contrast, in iron-loaded *Nrf2*^{-/-} mice, mitochondrial injuries were apparent in both LSECs and hepatocytes, including swelling and loss of cristae or matrix, microvilli were decreased, and some deposition of collagen fibres was observed (Fig 4e,f). In iron loaded *Nrf2*^{-/-} mice also treated daily with mitoTEMPO, a rescue of the phenotype occurred, with better preserved LSEC and hepatocyte mitochondria, and microvilli of the hepatocytes extending into the space of Disse (Fig 4g,h). Together these data show that Nrf2 is required for sensing and protecting against iron-induced mitochondrial ROS production and oxidative damage, and that the absence of Nrf2 in the context of iron loading leads to both defective hepcidin regulation and tissue damage.

Nrf2 controls serum iron levels in *Hfe* deficient mice

To build on these observations, we investigated the role of Nrf2 in hereditary hemochromatosis (HH). In humans the commonest form of HH is caused by mutations in *HFE*, but while *Hfe*^{-/-} mice model

the iron overload of the human disease, they do not recapitulate several other aspects of pathology, including liver fibrosis³⁵. *Hfe/Nrf2* double knockout (*Hfe/Nrf2*^{-/-}) mice on standard diet showed signs of increased liver damage and fibrosis over time, indicating that *Nrf2* deficiency modifies disease progression³⁶. We measured hepatic *Bmp6* expression at six-month intervals over two years in wildtype, *Nrf2*^{-/-}, *Hfe*^{-/-} and *Hfe/Nrf2*^{-/-} mice and analysed the data by 2-way ANOVA, stratifying on age of mice, to look at the effects of genotype on iron and hepatic gene expression. *Bmp6* was higher in *Hfe*^{-/-} mice compared with wildtypes, likely as a result of iron accumulation¹⁵; however *Nrf2* deficiency caused a decrease in *Bmp6* expression even in *Hfe*^{-/-} mice (Supplementary Fig. 7a). *Nrf2* deficiency (against a wild-type or *Hfe*^{-/-} background) also decreased *Nqo1* expression (Supplementary Fig. 7b). *Hamp1* levels were decreased in *Nrf2*^{-/-}, *Hfe*^{-/-} and *Hfe/Nrf2*^{-/-} mice, and liver iron was increased in *Hfe*^{-/-} and *Hfe/Nrf2*^{-/-} mice (Supplementary Fig. 7c,d). We noted a tendency for the lowest *Hamp1* mRNA and highest liver iron accumulation to occur in the oldest *Hfe/Nrf2*^{-/-} animals, and this prompted us to investigate iron loading in other organs affected in HH. We observed significant iron accumulation and stainable iron deposits in the heart and pancreas of 24-month old *Hfe/Nrf2*^{-/-} mice (Supplementary Fig. 7e). Together with our previous findings³⁶ these data indicate that in the context of murine HH, loss of *Nrf2* leads to increased parenchymal iron deposition and increased iron-associated tissue damage.

These findings led us to hypothesize that pharmacological activation of *Nrf2*, achieved using the small molecule CDDO-Im that activates *Nrf2* via destabilising the *Nrf2*/Keap1 interaction²¹, would improve the phenotype of *Hfe*^{-/-} mice. First, we established that after 6 hours, CDDO-Im increased *Bmp6* and *Hmox1* expression in primary murine LSECs and the human LSEC line TMNK-1 (Fig 5a), and in wild-type but not *Nrf2*^{-/-} murine livers (Fig. 5b). LSECs are also the source of *Bmp2*, which contributes to hepatic hepcidin regulation³⁷, however CDDO-Im did not influence LSEC *Bmp2* expression and had only very small effects on hepatic *Bmp2* expression *in vivo* (Fig 5a,b). To test the effects of *Nrf2* activation in the context of hemochromatosis, we next gave *Hfe*^{-/-} mice 30μmol/kg CDDO-Im for 6 hours. Compared to vehicle-treated *Hfe*^{-/-} mice, CDDO-Im increased hepatic expression of *Nqo1*, *Bmp6* (but not *Bmp2*), *Hamp1*, and decreased serum iron (Fig 5c). Hepcidin is unlikely to be directly regulated by CDDO-Im, because in mice expressing a constitutively active form of *Nrf2* in hepatocytes, in which hepatic expression of canonical *Nrf2* target genes are strongly upregulated³⁸, liver *Hamp1* expression is unchanged (Kohler UA and Werner S, personal communication). Nevertheless a requirement for *Hamp1* for CDDO-Im mediated decrease of serum iron was confirmed by treatment of *Hamp1*^{-/-} mice with CDDO-Im for 6 hours; CDDO-Im increased *Bmp6* (but not *Bmp2*), *Nqo1*, *Gclc* and *Hmox1* mRNA, but serum iron was not decreased (Supplementary Fig. 8a,b). As a further control, we tested *Bmp6*^{fl/fl}; *Tek-Cre*⁺ mice that lack *Bmp6* in LSECs, which are iron-loaded due to insufficient hepcidin expression⁹. CDDO-Im treatment of these mice increased hepatic expression of the canonical *Nrf2* target gene *Nqo1*, but did not increase hepatic

Bmp6, or *Hamp1*, or decrease serum iron levels (Supplementary Fig. 8c,d). Thus, in iron-loaded mice, Nrf2 activation decreases serum iron in a manner that requires LSEC *Bmp6* and hepcidin.

Activation of Nrf2 inhibits iron loading in haemochromatosis

To test the effects of chronic Nrf2 activation on organ iron accumulation, we gave 8-week old female *Hfe*^{-/-} mice 10 doses of 30µmol/kg CDDO-Im over 3 weeks. At the end of treatment, mice given CDDO-Im had increased Nrf2 protein in livers and upregulated hepatic *Bmp6* (with *Bmp2* unchanged), *Nqo1* and *Hmox1* expression, showing ongoing Nrf2 activation (Fig. 5d). *Hfe*^{-/-} mice given CDDO-Im accumulated three-fold less hepatic iron than vehicle-treated *Hfe*^{-/-} mice over the three-week treatment (Fig. 5e). Indeed, CDDO-Im exposed mice did not appear to accumulate liver iron over the three week treatment period as their liver iron levels at 11 weeks of age were not significantly different from 8 week old *Hfe*^{-/-} mice ('NS' in Fig 5e). CDDO-Im also decreased serum iron relative to vehicle-treated controls, and splenic iron was marginally although non-significantly reduced (Fig 5e). Together, these results indicate a decrease in systemic iron loading. The reduction in liver iron content was evident by Perls' staining, which showed less iron in CDDO-Im-treated mice (Fig. 5f). Despite the lower hepatic and serum iron, which normally decrease hepcidin expression, hepcidin mRNA and serum hepcidin protein were similar in CDDO-Im treated mice at the end of the treatment period, so that higher serum hepcidin to serum iron ratios were apparent in mice given CDDO-Im (Fig. 5g). We also noted decreased liver lipid peroxidation, a marker of oxidative damage, in CDDO-Im treated *Hfe*^{-/-} mice (Fig 5h). Consistent with the data on acute (6hour) Nrf2 activation on iron, a role for hepcidin in the decreased iron accumulation due to CDDO-Im was supported by experiments with *Hamp1*^{-/-} mice treated with CDDO-Im over 3 weeks. Unlike *Hfe*^{-/-} mice and despite increased hepatic expression of *Bmp6* and other Nrf2 target genes, CDDO-Im treated *Hamp1*^{-/-} mice did not have decreased hepatic iron accumulation or serum iron compared to vehicle-treated mice (Supplementary Fig. 8e,f). Thus the inhibition of systemic iron accumulation by CDDO-Im in *Hfe*^{-/-} mice is mediated by upregulation of the *Bmp6*-hepcidin axis, increasing the hepcidin:iron ratio over time and alleviating iron overload and hepatic oxidative damage.

Nrf2 counteracts suppression of hepcidin in thalassaemia

Toxic iron accumulation also occurs in non-transfusion-dependent thalassaemia, caused at least in part by chronic EPO-induced erythroblast expression of erythroferrone, which suppresses hepcidin synthesis³⁹. Erythroferrone inhibits the induction of hepcidin by blocking *Bmp6*⁴⁰, so we hypothesised that Nrf2 activation could counteract this pathophysiological mechanism by shifting the balance between *Bmp6* and erythroferrone, stimulating the *Bmp6*-hepcidin axis. CDDO-Im was administered to th3/+ mice (a model of beta-thalassaemia intermedia⁴¹) and six hours later we noted increased hepatic *Bmp6* and other Nrf2 target genes *Nqo1*, *Gclc* and *Hmox1* and increased *Hamp1* in CDDO-Im treated th3/+ mice (Fig 6a). Splenic *Fam132b* mRNA (encoding erythroferrone), *Slc40a1* mRNA and

kidney *Epo* mRNA were not altered by CDDO-Im (Fig 6b). Serum iron was markedly decreased in CDDO-Im treated th3/+ mice, while liver and spleen iron levels were not increased (Fig 6c). CDDO-Im-mediated Nrf2 activation augmented expression of *Bmp6* relative to its inhibitor erythroferrone, so increasing hepcidin and decreasing iron levels. Overall, the Nrf2-Bmp6-erythroferrone axis responds to excess iron, oxidative damage, hypoxia and anaemia, and regulates iron homeostasis and iron toxicity (Fig 6d).

Discussion

Maintaining iron levels at sufficient but non-toxic concentrations is critical for health. In mammalian cells, elevated iron alters the conformation and stability of Iron Response Proteins (IRPs), regulating post-transcriptional expression of Iron Response Element (IRE)-containing genes and controlling cellular iron homeostasis⁴². However, the IRE-IRP system does not directly modify *Bmp6* expression⁴³, and the IRE-IRP cell-intrinsic iron homeostatic mechanisms and hepcidin-controlled systemic iron equilibrium are not directly linked⁴⁴. We show instead that Nrf2 regulates *Bmp6* expression and is required for the induction of hepatic *Bmp6* in response to mitochondrial ROS and oxidative damage. Upon mitochondrial lipid peroxidation, electrophilic lipids are released from mitochondria and can react with cysteine residues on Keap1 to activate Nrf2³⁰. Hence iron-induced mitochondrial oxidative damage likely activates Nrf2 (and increases *Bmp6* expression) by causing mitochondrial lipid peroxidation and the release of electrophilic reactive lipid species. In this model, ‘toxic iron’ rather than iron itself is the key entity influencing *Bmp6*-driven hepcidin synthesis. This mechanism may allow a distinction to be drawn between levels of iron accumulation that are not harmful and can be tolerated, and the development of potentially pathogenic iron overload. This sensing apparatus sits alongside hepatocytes that directly detect transferrin saturation via HFE and TFR2 to regulate hepcidin¹. Thus both transferrin saturation sensing by hepatocytes and iron-driven LSEC *Bmp6* synthesis (controlled by Nrf2) protect against iron accumulation.

However, *Nrf2*, unlike lack of *Hfe*, *Tfr2* or *Bmp6*, also regulates antioxidant defences. A single injection of iron dextran caused hepatic necroinflammation in *Nrf2*^{-/-} mice but not wildtype controls, showing that iron is more cytotoxic in the absence of Nrf2. Iron-loaded *Nrf2*^{-/-} mice demonstrated elevated liver lipid peroxidation and fibrotic markers; injured LSEC and hepatocyte mitochondria and disrupted sinusoid architecture were also apparent and likely caused by iron-induced mitochondrial oxidative damage. Oral and parenteral iron formulations, frequently administered worldwide for a range of conditions, cause varying degrees of transient labile non-transferrin bound iron that catalyses free-radical generation^{10,45,46}. In our experiments, Nrf2 defends against oxidative damage and controls iron homeostasis in response to oral and parenteral iron.

Supraphysiological doses of recombinant BMP6 increase hepcidin and reduce serum iron in *Hfe*^{-/-} mice⁴⁷, and increasing hepcidin in beta-thalassaemia is also therapeutic⁴⁸. We activated Nrf2 with CDDO-Im in *Hfe*^{-/-} mice and th3/+ mice and observed robust upregulation of *Bmp6* and hepcidin

expression and decreased serum iron accumulation. Genes involved in the antioxidant response were also activated, and in *Hfe*^{-/-} mice hepatic lipid peroxidation was decreased. Pharmacological Nrf2 activation in the context of iron overload boosts the capability to detoxify iron-induced ROS and stimulates the Bmp6-hepcidin axis. We demonstrate that Nrf2 links cellular health to systemic iron homeostasis, and constrains the cause and effects of iron-induced oxidative damage. This concept is relevant to a range of disease processes and treatments. Our findings suggest therapeutic approaches for iron overload disorders, and extend the possible indications for Nrf2-activating drugs^{49,50}.

Author Contributions

Designed research: PJJ, TLD, CBW, DGS, PP, AEA, SRP, HD

Collected data: PJJ, TLD, AEA, JA, HM, DGS, AH, SW, SRP, JRH, AGS, ALB, ASG, AM, ES, CYW, JLB

Analyzed and interpreted data: PJJ, HD, TLD, DGS, PP, PK, AEA, SRP, ES, RCH, MPM, CYW, JLB, GP

Wrote the manuscript: PJJ, HD

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Competing financial interests

The authors declare no competing financial interests.

Figure legends

Figure 1 The transcription factor Nrf2 regulates *Bmp6* gene expression. **(a)** 3-week old C57BL/6 male mice were fed 3 weeks of control (200ppm iron) or high iron diet (1% CI). Liver non-heme iron content and hepatic gene expression of *Bmp6*, well-characterised Nrf2 target genes (*Nqo1*, *Hmox1* and *Gclc*) and Bmp-Smad target genes (*Hamp1*, *Id1* and *Smad7*) were quantified (n=5 mice). **(b)** Liver sinusoidal endothelial cells (LSECs) were purified from mice fed 4 weeks control (37ppm iron) or high iron diet containing 1% carbonyl iron (CI). Gene expression was quantified by qRT-PCR (n=4 mice). **(c)** Primary LSECs were cultured with 200ug/mL ferric ammonium citrate (FAC) for 6 hours and expression of *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* was quantified (n=3 biologically independent samples). **(d)** L929 cells were treated with iron-dextran (FeDx) for 6 hours. Nrf2 protein was detected by Western blot in nuclear, cytoplasmic and whole cell extracts, with lamin A as nuclear marker and GAPDH as cytoplasmic marker. Gene expression of *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* was quantified by qRT-PCR (n=4 biologically independent samples). **(e)** 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 sequence in intron1 of *Bmp6* is highlighted in yellow. **(f-h)** siRNA-mediated knockdown of **(f)** Nrf2, **(g)** Keap1 and **(h)** Bach1 was performed in MEF cells. Knockdown efficiency was validated by Western blot and qRT-PCR. Gene expression of *Bmp6*, *Nqo1*, *Hmox1* and *Gclc* was quantified by qRT-PCR (n=3 biologically independent samples). Data represented with centre values as mean and error bars as SEM. Two-tailed t-tests performed between iron-treated and control groups, and between NTC and Nrf2/Keap1/Bach1 siRNA groups.

Figure 2 Iron induces oxidative stress to activate Nrf2 and upregulate *Bmp6* expression. **(a)** 7-week old C57BL/6 male mice were fed control (200ppm iron) or high iron diet (1% CI) for a week. Hepatic malondialdehyde (MDA) was quantified by the Thiobarbituric acid reactive substances (TBARS) assay (n=4 mice). **(b)** MitoTEMPO decreases mitochondrial oxidative damage. **(c)** L929 cells were pretreated with 500µM mitoTEMPO for 1 hour, followed by 1mg/ml FeDx for 6 hours. Gene expression of *Bmp6*, *Nqo1* and *Hmox1* was quantified by qRT-PCR (n=5 biologically independent samples). **(d,e)** 4-week old C57BL/6 male mice were fed 2ppm iron diet for 3 weeks, gavaged with 2mg/kg iron (FeSO₄), injected with 0.25mg mitoTEMPO 1 hour after iron gavage, and sacrificed 5 hours after mitoTEMPO injection (n=10 (water-treated groups), n=8 (FeSO₄-treated groups) mice). **(d)** Liver non-heme iron and serum iron and **(e)** hepatic gene expression was quantified. **(f)** Mito-Paraquat (mitoPQ) localises to mitochondria, selectively induces superoxide and leads to selective mitochondrial oxidative damage release of electrophilic reactive lipid species (RLS). **(g)** C2C12 cells

were exposed to 100uM mitoPQ for 6 hours and then gene expression was quantified (n=3 biologically independent samples). Data represented with centre values as mean and error bars as SEM. Statistics: Two-tailed t-tests.

Figure 3 Induction of *Bmp6* and hepcidin by iron is blunted in the absence of Nrf2; *Nrf2*-knockout mice load with more hepatic iron. **(a)** Nrf2 was knocked-down for 24 hours, followed by 1mg/ml FeDx treatment for 6 hours in L929 cells. Gene expression of *Nfe2l2*, *Bmp6* and *Nqo1* was quantified by qRT-PCR (n=5 biologically independent experiments). **(b)** Nrf2 was knocked-down for 24 hours, followed by 200µg/ml FAC treatment for 6 hours in C2C12 cells. Gene expression of *Nfe2l2*, *Bmp6* and *Nqo1* was quantified by qRT-PCR (n=5 biologically independent experiments). **(c)** wild-type and *Nrf2*^{-/-} mice were fed 1% carbonyl iron diet for one week, and then hepatic gene expression of *Nqo1*, *Gclc*, *Bmp6* and *Hamp1* was quantified, and liver non-heme iron levels were measured (n=7 (WT) and n=8 (*Nrf2*^{-/-}) mice). **(d-f)** 12-week old male *Nrf2*-knockout and wildtype littermate mice were fed 200ppm iron or 2% CI diet for 2 weeks. **(d)** Hepatic gene expression of *Nqo1*, *Bmp6*, *Hamp1*, *Id1* and *Smad7* was quantified by qRT-PCR (n=4 (WT 200ppm, *Nrf2*^{-/-} 200ppm), n=6 (WT 2%CI) and n=8 (*Nrf2*^{-/-} 2%CI) mice). **(e)** Fold change in hepatic *Bmp6* and *Hamp1* was calculated in mice fed 2% CI diet relative to mice fed 200ppm iron diet within each genotype. **(f)** Liver non-heme iron content was quantified. **(g)** 4-week old male *Nrf2*^{-/-} and wildtype littermate mice were fed 1% CI diet for 4 weeks (n=8 (WT) and n=5 (*Nrf2*^{-/-}) mice) and non-heme liver iron was quantified. Data represented with centre values as mean and error bars as SEM. Statistics: **(a)** two-tailed paired t-test and **(b-g)** two-tailed t-test

Figure 4 Liver and LSEC mitochondrial pathology in iron loaded *Nrf2*^{-/-} mice. **(a)** 7-week and 4-week old male *Nrf2*-knockout and wildtype littermate mice were fed 1% CI diet for 1 week (n=7 (WT), n=8 (*Nrf2*^{-/-}) mice) or 4 weeks (n=8 (WT), n=5 (*Nrf2*^{-/-}) mice), respectively, and culled at 8-week old. Malondialdehyde (MDA) and gene expression of *Colla1* and *Tagln* was quantified. **(b)** *Nrf2*^{-/-} and WT mice were given a single intraperitoneal injection of 4mg FeDx or Dx. Liver necroinflammatory foci were quantified 30 days post-injection (n=5 (WT) and n=4 (*Nrf2*^{-/-}) mice); **(c)** representative liver sections stained with HE (first, third images) or Perls Prussian blue (second, fourth images), necroinflammatory foci in *Nrf2*^{-/-} mouse livers at 30 days post-FeDx injection containing hepatocyte apoptotic bodies and iron pigment in histiocytes are shown (arrows). **(d)** Transmission electron microscopy (TEM) of a liver sinusoid of a WT mouse fed iron-rich diet (2% CI) for 2 weeks (representative of n=3 mice) depicting intact mitochondria in an LSEC (blue arrow) and adjacent hepatocyte (yellow arrow), and hepatocyte microvilli (orange *). **(e,f)** TEM of hepatic sinusoidal areas of *Nrf2*^{-/-} mice on the same diet (representative of n=3 mice), showing deposition of extracellular matrix proteins in Disse's spaces (white arrows), with continuous basement membranes evident on the basal side of LSECs. Note the presence of mitochondrial injuries in both LSECs (blue

arrows) and hepatocytes (yellow arrows), including mitochondrial swelling and loss of cristae. **(g,h)** In *Nrf2*^{-/-} mice fed iron-rich diet and treated daily with mitoTEMPO (10 mg/kg intraperitoneally for 2 weeks, from the day before the start of dietary iron supplementation) (representative of n=3 mice), microvilli of the hepatocytes extend into the space of Disse (*) and sinusoids are lined by fenestrated, incomplete endothelium. The mitochondria of LSECs and hepatocytes (blue and yellow arrows, respectively) are better preserved with evident matrix and cristae. Images from d-h are representative of 3 mice. Stain, uranyl acetate and lead citrate; scale bar, 0.5 μ m. LSEC, liver sinusoidal endothelial cell; RBC, red blood cell. Data in **a, b** represented with centre values as mean and error bars as SEM. Statistics: two-tailed t-test.

Figure 5 Small molecule Nrf2 agonist CDDO-Im alleviates iron accumulation in *Hfe*-knockout hemochromatosis mice. **(a)** Primary mouse LSECs and human TMNK-1 cells were treated with 100nM CDDO-Im for 6 hours. Gene expression of *Bmp6*, *Nqo1* and *Hmox1* (and human equivalents) and *Bmp2*, was quantified by qRT-PCR (n=3 biologically independent samples). **(b)** 6-week old male and female WT and *Nrf2*^{-/-} mice were gavaged with 30 μ mol/kg CDDO-Im and culled 6 hours post-treatment (n=5 (per group in WT male vehicle or CDDO-Im) and n=3 (*Nrf2*^{-/-} male vehicle or CDDO-Im, WT female vehicle or CDDO-Im, *Nrf2*^{-/-} female vehicle or CDDO-Im) mice). Hepatic gene expression of *Bmp6*, *Bmp2*, *Nqo1* and *Hmox1* was quantified by qRT-PCR. **(c)** *Hfe*-knockout mice were given a single dose of vehicle or 30 μ mol/kg CDDO-Im by oral gavage (n=5 mice per group) and 6 hours later hepatic expression of *Nqo1*, *Bmp6*, *Bmp2* and *Hamp1* was determined and serum iron was quantified. **(d-h)** 8-week old female *Hfe*-knockout mice were given vehicle or 30 μ mol/kg CDDO-Im by oral gavage for 10 doses over 3 weeks (n=9 mice per group). **(d)** Western blot on liver lysates to detect Nrf2 protein and qRT-PCR to quantify liver gene expression of *Bmp6*, *Bmp2*, *Nqo1*, *Hmox1* and *Hamp1* were performed. **(e)** Liver non-heme iron was quantified and calculated as a percentage of untreated 8-week old female *Hfe*-knockout mice. Serum and spleen iron were also quantified. **(f)** Liver sections from vehicle-treated (left) and CDDO-Im-treated (right) were stained with Perls Prussian blue. **(g)** Serum hepcidin was quantified by ELISA and calculated as a ratio against serum iron. **(h)** Hepatic malondialdehyde (MDA) was quantified by TBARS assay. Statistics for **(a,c,d,e,g,h)**: two-tailed t-test. Statistics for **(b)**: Asterisks (*): two-tailed t-test between vehicle and CDDO-Im groups within same genotype. Hash (#): 2-way ANOVA between wildtype and *Nrf2*-knockout mice given CDDO-Im, stratified by sex. Statistics for left-most graph in **(e)**: Hash: two-tailed t-test between vehicle or CDDO-Im treated mice and untreated 8-week old mice; asterisks (*): two-tailed t-test between vehicle and CDDO-Im group. Data represented with centre values as mean and error bars as SEM.

Figure 6 CDDO-Im alters the Bmp6-erythroferrone axis and decreases serum iron in thalassaemia mice. **(a)** 12-16 week-old male th3/+ mice treated with 100nM CDDO-Im for 6 hours had increased

hepatic expression of *Bmp6*, *Nqo1*, *Gclc*, *Hmox1* and *Hamp1*. **(b)** CDDO-Im did not significantly alter splenic expression of *Fam132b* (encoding erythroferrone) or *Slc40a1* (encoding ferroportin) or kidney expression of *Epo*. **(c)** CDDO-Im significantly decreased serum iron but not liver or splenic non-heme iron concentration. (n=6 mice per group for a-c) **(d)** Proposed scheme, depicting activation of Nrf2 in LSECs and hepatocytes by iron-induced mitochondrial ROS and subsequent released RLS, or by CDDO-Im, leading to Keap1 destabilisation, enhanced *Bmp6* (counteracting anaemia-induced erythroferrone) and raised hepcidin, and increased cytoprotection. Two-tailed t-tests were performed between mice treated with vehicle and CDDO-Im. Data represented with centre values as mean and error bars as SEM.

Methods

Mice

Unless otherwise stated, animal procedures were performed under the authority of UK Home office project and personal licenses in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the University of Oxford ethical review committee. Mice were housed in individually ventilated cages and fed *ad-libitum* with standard diet containing 188 ppm iron (SDS Dietex Services, diet 801161) C57BL/6 mice were purchased from Envigo. *Nrf2*^{-/-} mice (RIKEN) were generated previously⁵¹. *iHamp1*-KO mice were generated previously¹⁹. *Hfe*^{-/-} mice were generated previously⁵², backcrossed onto C57BL/6 background for 10 generations and were a kind gift from Helene Coppin (Toulouse, France). *Hamp1*^{-/-} mice on a C57BL/6 background⁵³ were a kind gift from Sophie Vaulont (Institut Cochin, France); *Bmp6*^{fl/fl}; *Tek-Cre*⁺ mice were generated previously⁹.

Nrf2^{+/+}, *Nrf2*^{+/-} and *Nrf2*^{-/-} littermates treated with FeDx or carbonyl iron dietary loading, *Hfe*^{-/-}, *Bmp6*^{fl/fl}; *Tek-Cre*⁺ and *Hamp1*^{-/-} mice treated with CDDO-Im for 6 hours or 3 weeks, and *Hfe/Nrf2*^{-/-} mice were bred and received humane care according to the EU Directive 2010/63/EU and the experimental procedures were approved by the *Instituto de Biologia Molecular e Celular* Animal Ethics Committee. LSECs isolation studies were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital.

C57BL/6 mice heterozygous for the β -globin gene deletion (*th3/+*)⁴¹ were maintained and bred in the animal facility at the Lady Davis Institute for Medical Research. The *th3/+* mice were used as a model of thalassemia intermedia⁴¹ and were kindly donated by Stefano Rivella.

Mouse treatments

Dietary iron loading: Mice were fed 200ppm iron control diet (Harlan, TD.07801), 1% carbonyl iron diet (Harlan, TD.09077) or 2% carbonyl iron diet (Harlan, 2018S) *ad-libitum*; diets were matched for other constituents. For LSEC isolation studies, wildtype 129S6/SvEvTac male mice were fed control 37ppm iron or 1% carbonyl iron diets (Research Diets) for 4 weeks. **Iron-dextran (FeDx):** Mice were given intraperitoneal (i.p.) injections of FeDx (Sigma, D8517) or dextran Dx control (Sigma, D9260) for 6 or 24 hours. ***iHamp1*-knockout:** *iHamp1*-Ctrl and *iHamp1*KO mice were given 1mg tamoxifen (Sigma, T5648) in 90% Corn Oil / 10% Ethanol i.p. for 4 consecutive days. **Ferrous sulphate:** Mice were fed 2ppm iron diet *ad-libitum* for 3 weeks, followed by oral gavage with 2mg/kg elemental iron (as FeSO₄) (Sigma, F7002) and culled 6 hours later. **Hemin:** Mice were administered two i.p. doses of 50 μ mol/kg hemin (Sigma, 512800) or saline 12-hours apart and culled 12 hours after the last dose. **mitoTEMPO:** 0.25mg mitoTEMPO (Sigma, SML0737) in saline was administered i.p. 1 hour after gavage with 2mg/kg elemental iron (as FeSO₄) and culled 6 hours later. **CDDO-Imidazole (CDDO-Im):** Mice were given 30 μ mol/kg CDDO-Im (Tocris Bioscience, 4737) dissolved in DMSO and

reconstituted in PBS / 5% Tween80 / 5% PEG300 by oral gavage. Mice were culled in increasing CO₂. Tissues were harvested for non-heme iron measurement, gene expression, Nrf2 protein, and histology analysis. Serum was collected for iron and hepcidin measurements.

Cell cultures

L929 fibroblast, C2C12 myoblast and MEF mouse embryonic fibroblast cells were maintained in Dulbecco's modified Eagle's medium (*DMEM*) supplemented with 10% fetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin. CD1 mouse primary liver sinusoidal endothelial cells (Cell Biologics) were maintained in complete mouse endothelial cell medium with growth factor supplement following manufacturer's instructions. The LSEC cell line (TMNK-1⁵⁴) was kindly provided by Dr Naoya Kobayashi at the Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan.

***In vitro* treatments**

siRNA-mediated knockdown was performed with Lipofectamine RNAiMAX reagent with 100nM Dharmacon siGENOME SMARTpool siRNA targeting mouse Nrf2 (M-040766-01-0005), Keap1 (M-041104-01-0005) or Bach1 (M-042956-01-0005) in antibiotic-free media for 24 hours (Nrf2) or 48 hours (Keap1 and Bach1). L929 cells were treated with 0.1mg/ml or 1mg/ml FeDx with or without 500μM mitoTEMPO, 2μM or 10μM hemin. C2C12, MEF and mouse primary LSECs were treated with 200μg/ml ferric ammonium citrate, FAC (Sigma). LSECs were treated with 100nM CDDO-Im (Tocris Bioscience). Cells were harvested for Western blot and/or qRT-PCR analysis. MitoPQ was synthesised as described²⁹ and used at 100uM; the mitochondria targeted MitoPQ control compound was also used at 100uM and has been described³¹, and its full synthesis will be published separately. LSEC cell line (TMNK-1) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine (Sigma Aldrich) and non essential amino acids NEAA (Invitrogen); cells were passaged twice a week using trypsin EDTA (Sigma). LSECs were plated at 0.1x10⁶/well in 100 uL of culture Medium in 96 well flat bottom plate +/- CDDO-Im or +/- FAC as indicated.

LSEC isolation

Livers were dissected after cardiac perfusion with 25 ml sterile PBS. Single cell suspension was made by incubating scissor cut 200mg liver 10minutes at 37°C in 1ml cocktail containing 450U/ml collagenase I, 125U/ml collagenase XI, 60U/ml Dnase I, 60U/ml Hyaluronidase and 20mM HEPES (Sigma-Aldrich). After cell suspension was filtered through 40 μm cell strainer, non-parenchymal cells were pelleted down at 750 x g for 7minutes at 4°C and resuspended with PBS containing 1% FBS and 0.5% BSA. The following monoclonal antibodies were used for cell sorting: anti-Ly-6C

(clone AL-21, BD Biosciences), anti-CD45.2 (clone 104, BD Biosciences), anti-CD3e (clone 145-2C11, ebioscience), anti-CD90.2 (clone 53-2.1, BD Biosciences), anti-CD19 (clone 6D5, Biolegend), anti-MHC-II (clone AF6-120.1, BD Biosciences), anti-F4/80 (clone BM8, Biolegend), anti-NK1.1 (clone PK136, BD Biosciences), anti-Ly-6G (clone 1A8, BD Biosciences), anti-CD11b (clone M1/70, BD Biosciences), anti-CD146 (clone ME-9F1, BioLegend), anti-CD31 (clone 390, BioLegend), anti-CD326 (clone G8.8, BioLegend), anti-CD64 (clone X54-5/7.1, BioLegend). Cells were first gated using FSC/SSC characteristics, and doublets were sequentially excluded by comparing FSC- and SSC-height and -area signals. Specifically, endothelial cells were identified as CD45-, CD31+, Lin-, MHCII-, CD64-, CD146+. Lineage was defined as: CD3e, CD19, CD90.2, CD326, Ly6G, NK1.1. Cells were sorted on a FACS Aria II (BD Biosciences). See Supplementary Figure 9 for FACS gating strategy, and Supplementary Table 1 for details of antibodies used.

Western blot

Cells were pelleted for nuclear and cytoplasmic fractionation using the NEPER protein extraction kit (Thermo Scientific, 78833). Whole cell extracts were harvested using the RIPA lysis buffer (ThermoFisher, 89900) with protease and phosphatase inhibitor cocktail. Liver tissues were agitated with glass beads (Sigma, G8772) and lysed in RIPA buffer (ThermoFisher, 89900). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred onto 0.2µm nitrocellulose membrane, and probed with antibodies against Nrf2 (Cell Signaling Technology, D1Z9C), Bach1 (R&D systems, AF5777), lamin A (Abcam, ab8980), and GAPDH (Proteintech, 60004). See Supplementary Figure 10 for all uncropped gels, and Supplementary Table 1 for details of antibodies used.

Gene expression analysis by quantitative real time PCR (qRT-PCR)

RNA was extracted from liver explants and cells with RNeasy plus kit (Qiagen, 74136) and reverse transcribed to cDNA (Life Technologies, 4387406) for quantitative PCR on the Applied Biosystems 6500 Fast Real-Time PCR system machine using TaqMan assays listed in Supplementary Table 2 according to the manufacturers' protocols, except for *Hfe/Nrf2*^{-/-} and LSEC isolation experiments. In *Hfe/Nrf2*^{-/-} experiment, RNA was extracted using TriZol, DNase-treated, and reverse transcribed to cDNA (ThermoScript RT-PCR System). Relative gene expression levels were quantified using iQ5 Real-Time PCR Detection System. All reactions were performed with iQ SYBR Green Supermix, with primers listed in Supplementary Table 3. The quantity of each transcript was estimated against the respective standard curve and normalized against the quantity of the endogenous control gene Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). In LSECs experiments, total RNA was isolated using PicoPure RNA Isolation Kit (Thermo Scientific). cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). PCR reactions were carried out by using the

PowerUp SYBR Green Master Mix (Applied Biosystems) on the QuantStudio3 Real-Time PCR system (Applied Biosystems), with primers listed in Supplementary Table 4.

Tissue non-heme iron measurement

Tissue samples were dried for 4–6 h at 100 °C before weighing. Dried tissue was digested with 10% trichloroacetic acid/30% hydrochloric acid for 20 h at 65 °C. Non-heme iron content was measured colorimetrically (OD 535 nm) against a standard curve generated from ferric ammonium citrate (F5879, Sigma) serial dilutions following reaction with chromogen reagent containing 0.1% (w/v) batho-phenoldisulphonic acid (BPS, 146617, Sigma)/0.8% thioglycolic acid (88652, Sigma).

Tissue histology

Liver and spleen samples from *Hfe*^{-/-} mice given CDDO-Im, liver tissue from *Nrf2*^{-/-} mice injected with FeDx, and liver, heart and pancreas tissues from *Hfe/Nrf2*^{-/-} mice were fixed in neutral formalin 10% and embedded in paraffin. 3µm-thick sections were stained with Perls' Prussian blue reaction for ferric iron using standard procedures at Ipatimup Diagnostics (Ipatimup, Porto, Portugal).

Transmission electron microscopy

Liver tissue samples were immersed immediately on isolation into 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight and then postfixed in 2.0% osmium tetroxide for 2 h, dehydrated through a series of ethanol solutions, and embedded in Epon. Ultrathin sections (50 nm) stained with uranyl acetate and lead citrate were visualized with a JEM 1400 electron microscope (Jeol, Tokyo, Japan) operated at 120 kV. Electron micrographs were captured with an Orius CCD (Gatan, Warrendale, PA, USA).

Liver malondialdehyde (MDA)

Liver tissues were agitated with glass beads (Sigma, G8772) and lysed in RIPA buffer (ThermoFisher, 89900). Lysates were used for quantification of MDA using the thiobarbaturic acid reactive substance (TBARS) kit according to the manufacturer's protocols (Cayman Chemical, 700870). Briefly, liver lysates were allowed to react with thiobarbaturic acid at 95°C for 1 hour, and absorbance of the solution was measured at 530nm.

Serum measurements

Blood was taken by cardiac puncture immediately after euthanising mice. Serum was prepared by centrifugation of clotted blood at 8000 x g for 5minutes in BD Microtainer SST tubes (Beckton Dickinson). Serum hepcidin was quantified by competitive ELISA using the Hepcidin-Murine Compete Kit (Intrinsic Lifesciences) according to the manufacturer's protocol. For mice given FeSO₄ gavage, serum iron was quantified using the MULTIGENT Iron Kit on the Abbott Architect c16000

automated analyzer (Abbott Laboratories) at John Radcliffe Hospital, UK. For *Hfe*^{-/-} and *Hamp1*^{-/-} mice given CDDO-Im, serum irons were quantified in a Cobas C8000 analyzer (Roche Diagnostics, Mannheim, Germany) at *Centro Hospitalar do Porto*.

ChIP-sequencing

Previously published Nrf2 ChIP-seq datasets generated from differentiated C2C12 myotube cells treated with 100nM CDDO-Im for 3 hours²² and from *Keap1*-knockout MEF cells⁵⁵ were mapped onto the mouse mm9 genome build on UCSC genome browser. 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 also mapped onto the mm9 mouse genome.

Statistics

Standard randomization procedures were used for cell-line groups and mice of the same age and sex (experimental and control mice were chosen at random from among littermates). The investigators were not blinded to allocation during experiments and assessments. The required number of mice for each experiment was determined from availability of bred animals, power calculations, prior experience of performing experiments with the same strains of mice and data from pilot experiments. Statistical analyses were performed using Prism version 6 (GraphPad Software). Details of specific statistical tests are given in figure legends.

Reporting summary

Further information on experimental design is available in the Reporting Summary.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References:

- 1 Muckenthaler, M. U., Rivella, S., Hentze, M. W. & Galy, B. A Red Carpet for Iron Metabolism. *Cell* **168**, 344-361, doi:10.1016/j.cell.2016.12.034 (2017).
- 2 Ganz, T. Systemic iron homeostasis. *Physiological reviews* **93**, 1721-1741, doi:10.1152/physrev.00008.2013 (2013).
- 3 Nemeth, E. *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306**, 2090-2093 (2004).

- 4 Pietrangelo, A. Genetics, Genetic Testing, and Management of Hemochromatosis: 15 Years since Hfe. *Gastroenterology* **149**, 1240-1251e1244, doi:10.1053/j.gastro.2015.06.045 (2015).
- 5 Gupta, R., Musallam, K. M., Taher, A. T. & Rivella, S. Ineffective Erythropoiesis: Anemia and Iron Overload. *Hematology/oncology clinics of North America* **32**, 213-221, doi:10.1016/j.hoc.2017.11.009 (2018).
- 6 Kautz, L. *et al.* Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. *Blood* **112**, 1503-1509 (2008).
- 7 Andriopoulos, B., Jr. *et al.* BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet* **41**, 482-487 (2009).
- 8 Meynard, D. *et al.* Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet* **41**, 478-481 (2009).
- 9 Canali, S. *et al.* Endothelial cells produce bone morphogenetic protein 6 required for iron homeostasis in mice. *Blood* **129**, 405-414, doi:10.1182/blood-2016-06-721571 (2017).
- 10 Koskenkorva-Frank, T. S., Weiss, G., Koppenol, W. H. & Burckhardt, S. The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: Insights into the potential of various iron therapies to induce oxidative and nitrosative stress. *Free Radic Biol Med* **65C**, 1174-1194, doi:10.1016/j.freeradbiomed.2013.09.001 (2013).
- 11 Hershko, C. Pathogenesis and management of iron toxicity in thalassemia. *Annals of the New York Academy of Sciences* **1202**, 1-9, doi:10.1111/j.1749-6632.2010.05544.x (2010).
- 12 Suzuki, T. & Yamamoto, M. in *Free Radical Biology and Medicine* Vol. 88 93-100 (2015).
- 13 Kobayashi, A. *et al.* Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Molecular and cellular biology* **26**, 221-229, doi:10.1128/MCB.26.1.221-229.2006 (2006).
- 14 Igarashi, K. & Watanabe-Matsui, M. Wearing red for signaling: the heme-bach axis in heme metabolism, oxidative stress response and iron immunology. *Tohoku J. Exp. Med.*, 229-253, doi:10.1620/tjem.232.229.Correspondence (2013).
- 15 Kautz, L. *et al.* BMP/Smad signaling is not enhanced in Hfe-deficient mice despite increased Bmp6 expression. *Blood* **114**, 2515-2520 (2009).
- 16 Ryan, J. D., Ryan, E., Fabre, A., Lawless, M. W. & Crowe, J. Defective bone morphogenic protein signaling underlies hepcidin deficiency in HFE hereditary hemochromatosis. *Hepatology* **52**, 1266-1273, doi:10.1002/hep.23814 (2010).
- 17 Moon, M. S. *et al.* Elevated hepatic iron activates NF-E2-related factor 2-regulated pathway in a dietary iron overload mouse model. *Toxicological sciences : an official journal of the Society of Toxicology* **129**, 74-85, doi:10.1093/toxsci/kfs193 (2012).
- 18 Silva-Gomes, S. *et al.* Transcription factor NRF2 protects mice against dietary iron-induced liver injury by preventing hepatocytic cell death. *Journal of Hepatology* **60**, 354-361, doi:10.1016/j.jhep.2013.09.004 (2014).
- 19 Armitage, A. E. *et al.* Induced disruption of the iron-regulatory hormone hepcidin inhibits acute inflammatory hypoferremia *Journal of Innate Immunity* (2016).
- 20 Liby, K. *et al.* The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer research* **65**, 4789-4798, doi:10.1158/0008-5472.CAN-04-4539 (2005).
- 21 Cleasby, A. *et al.* Structure of the BTB domain of Keap1 and its interaction with the triterpenoid antagonist CDDO. *PloS one* **9**, e98896, doi:10.1371/journal.pone.0098896 (2014).
- 22 Uruno, A. *et al.* Nrf2-Mediated Regulation of Skeletal Muscle Glycogen Metabolism. *Molecular and Cellular Biology* **36**, 1655-1672, doi:10.1128/MCB.01095-15 (2016).
- 23 Yamamoto, T. *et al.* Predictive base substitution rules that determine the binding and transcriptional specificity of Maf recognition elements. *Genes to Cells* **11**, 575-591, doi:10.1111/j.1365-2443.2006.00965.x (2006).

- 24 Reichard, J. F., Motz, G. T. & Puga, A. Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. *Nucleic Acids Research* **35**, 7074-7086, doi:10.1093/nar/gkm638 (2007).
- 25 Mills, E. L. *et al.* Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* **556**, 113-117, doi:10.1038/nature25986 (2018).
- 26 Urrutia, P. J., Mena, N. P. & Nunez, M. T. The interplay between iron accumulation, mitochondrial dysfunction, and inflammation during the execution step of neurodegenerative disorders. *Frontiers in pharmacology* **5**, 38, doi:10.3389/fphar.2014.00038 (2014).
- 27 Huang, H. *et al.* Iron-induced generation of mitochondrial ROS depends on AMPK activity. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* **30**, 623-628, doi:10.1007/s10534-017-0023-0 (2017).
- 28 Liang, H. L. *et al.* Partial attenuation of cytotoxicity and apoptosis by SOD1 in ischemic renal epithelial cells. *Apoptosis : an international journal on programmed cell death* **14**, 1176-1189, doi:10.1007/s10495-009-0393-z (2009).
- 29 Robb, E. L. *et al.* Selective superoxide generation within mitochondria by the targeted redox cycler MitoParaquat. *Free Radic Biol Med* **89**, 883-894, doi:10.1016/j.freeradbiomed.2015.08.021 (2015).
- 30 Higdon, A., Diers, A. R., Oh, J. Y., Landar, A. & Darley-Usmar, V. M. Cell signalling by reactive lipid species: new concepts and molecular mechanisms. *The Biochemical journal* **442**, 453-464, doi:10.1042/BJ20111752 (2012).
- 31 Fazakerley, D. J. *et al.* Mitochondrial oxidative stress causes insulin resistance without disrupting oxidative phosphorylation. *The Journal of biological chemistry*, doi:10.1074/jbc.RA117.001254 (2018).
- 32 Yanagawa, T. *et al.* Nrf2 deficiency causes tooth decolourization due to iron transport disorder in enamel organ. *Genes to cells : devoted to molecular & cellular mechanisms* **9**, 641-651, doi:10.1111/j.1356-9597.2004.00753.x (2004).
- 33 Marro, S. *et al.* Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. *Haematologica* **95**, 1261-1268, doi:10.3324/haematol.2009.020123 (2010).
- 34 Jenkitkasemwong, S. *et al.* SLC39A14 Is Required for the Development of Hepatocellular Iron Overload in Murine Models of Hereditary Hemochromatosis. *Cell metabolism* **22**, 138-150, doi:10.1016/j.cmet.2015.05.002 (2015).
- 35 Lebeau, A. *et al.* Long-term sequelae of HFE deletion in C57BL/6 x 129/O1a mice, an animal model for hereditary haemochromatosis. *European journal of clinical investigation* **32**, 603-612 (2002).
- 36 Duarte, T. L. *et al.* Genetic disruption of NRF2 promotes the development of necroinflammation and liver fibrosis in a mouse model of HFE-hereditary hemochromatosis. *Redox biology* **11**, 157-169, doi:10.1016/j.redox.2016.11.013 (2017).
- 37 Koch, P. S. *et al.* Angiocrine Bmp2 signaling in murine liver controls normal iron homeostasis. *Blood* **129**, 415-419, doi:10.1182/blood-2016-07-729822 (2017).
- 38 Kohler, U. A. *et al.* Activated Nrf2 impairs liver regeneration in mice by activation of genes involved in cell-cycle control and apoptosis. *Hepatology* **60**, 670-678, doi:10.1002/hep.26964 (2014).
- 39 Kautz, L. *et al.* Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet* **46**, 678-684, doi:10.1038/ng.2996 (2014).
- 40 Arezes, J. *et al.* Erythroferrone inhibits the induction of hepcidin by BMP6. *Blood* **132**, 1473-1477, doi:10.1182/blood-2018-06-857995 (2018).
- 41 Yang, B. *et al.* A mouse model for beta 0-thalassemia. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 11608-11612 (1995).

- 42 Wilkinson, N. & Pantopoulos, K. The IRP/IRE system in vivo: insights from mouse models. *Frontiers in pharmacology* **5**, 176, doi:10.3389/fphar.2014.00176 (2014).
- 43 Sanchez, M. *et al.* Iron regulatory protein-1 and -2: transcriptome-wide definition of binding mRNAs and shaping of the cellular proteome by iron regulatory proteins. *Blood* **118**, e168-179, doi:10.1182/blood-2011-04-343541 (2011).
- 44 Hentze, M. W., Muckenthaler, M. U., Galy, B. & Camaschella, C. Two to tango: regulation of Mammalian iron metabolism. *Cell* **142**, 24-38 (2010).
- 45 Bhandari, S., Pereira, D. I. A., Chappell, H. F. & Drakesmith, H. Intravenous Irons: From Basic Science to Clinical Practice. *Pharmaceuticals* **11**, doi:10.3390/ph11030082 (2018).
- 46 Brittenham, G. M. *et al.* Circulating non-transferrin-bound iron after oral administration of supplemental and fortification doses of iron to healthy women: a randomized study. *The American journal of clinical nutrition* **100**, 813-820, doi:10.3945/ajcn.113.081505 (2014).
- 47 Corradini, E. *et al.* BMP6 treatment compensates for the molecular defect and ameliorates hemochromatosis in Hfe knockout mice. *Gastroenterology* **139**, 1721-1729, doi:10.1053/j.gastro.2010.07.044 (2010).
- 48 Casu, C. *et al.* Minihepcidin peptides as disease modifiers in mice affected by beta-thalassemia and polycythemia vera. *Blood* **128**, 265-276, doi:10.1182/blood-2015-10-676742 (2016).
- 49 Bollong, M. J. *et al.* A metabolite-derived protein modification integrates glycolysis with KEAP1-NRF2 signalling. *Nature* **562**, 600-604, doi:10.1038/s41586-018-0622-0 (2018).
- 50 Dodson, M. *et al.* Modulating NRF2 in Disease: Timing Is Everything. *Annual review of pharmacology and toxicology*, doi:10.1146/annurev-pharmtox-010818-021856 (2018).
- 51 Itoh, K. *et al.* An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications* **236**, 313-322, doi:10.1006/bbrc.1997.6943 (1997).
- 52 Bahram, S. *et al.* Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13312-13317 (1999).
- 53 Lesbordes-Brion, J. C. *et al.* Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. *Blood* **108**, 1402-1405, doi:10.1182/blood-2006-02-003376 (2006).
- 54 Matsumura, T. *et al.* Establishment of an immortalized human-liver endothelial cell line with SV40T and hTERT. *Transplantation* **77**, 1357-1365 (2004).
- 55 Malhotra, D. *et al.* Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Research* **38**, 5718-5734, doi:10.1093/nar/gkq212 (2010).

Figure 1

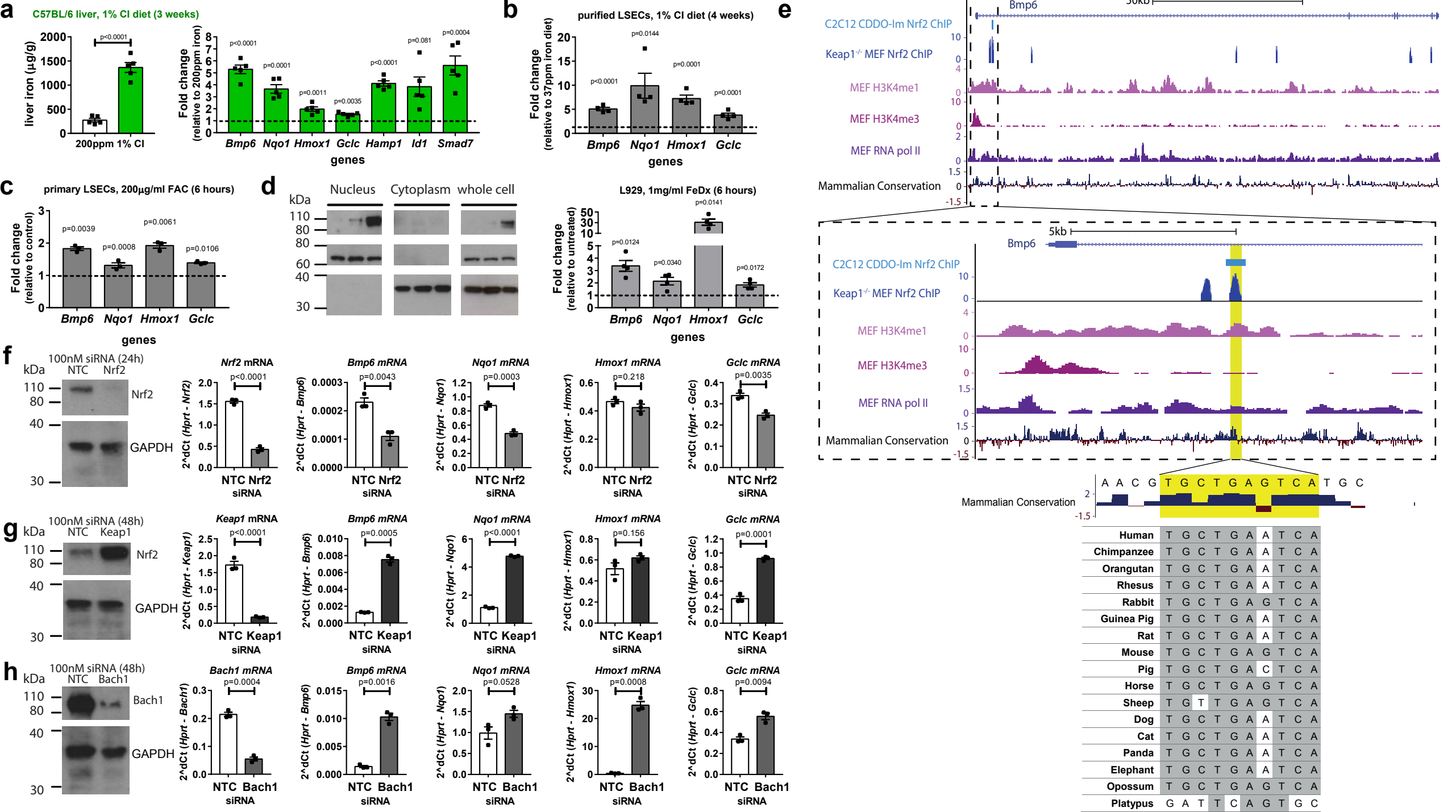


Figure 2

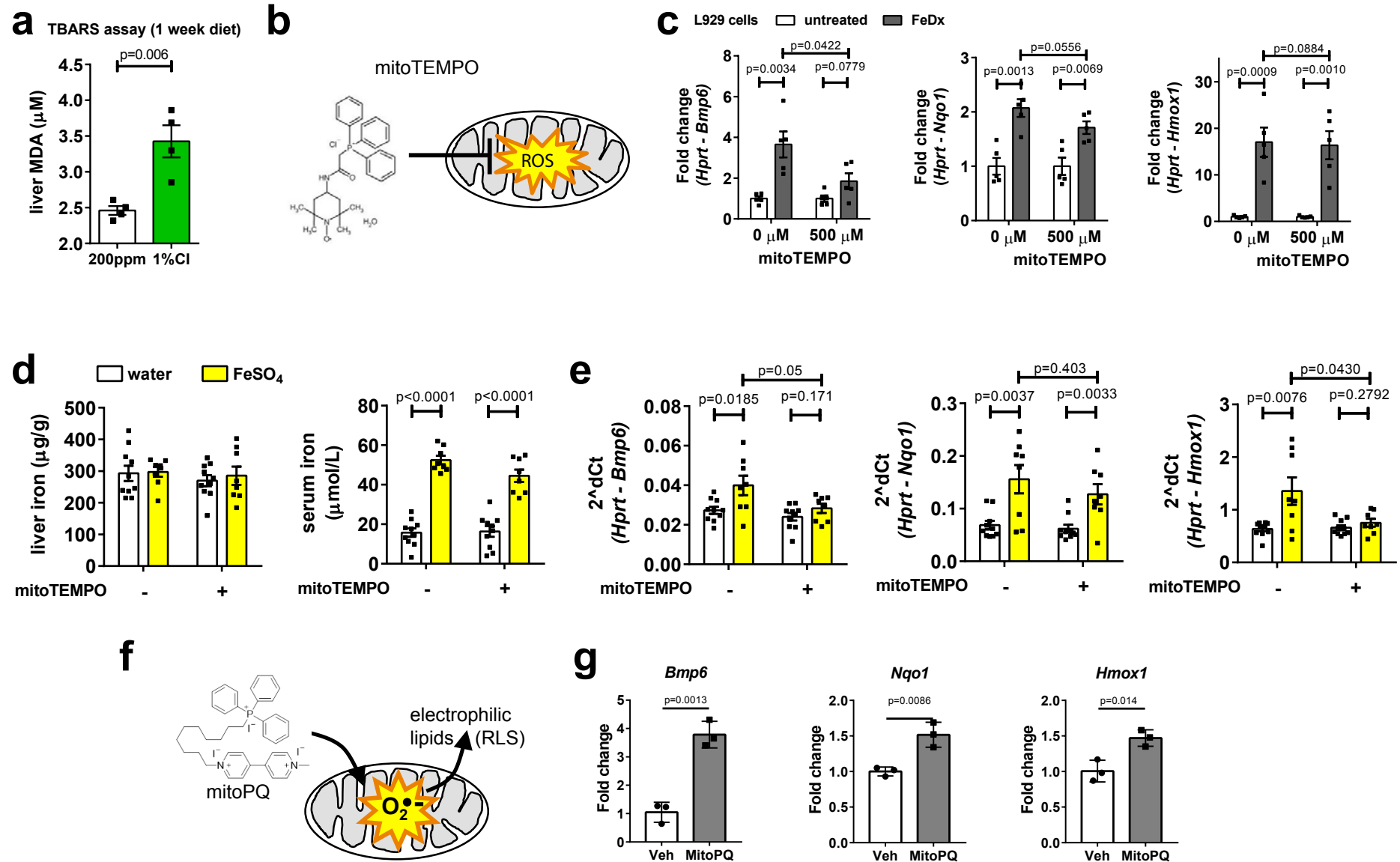


Figure 3

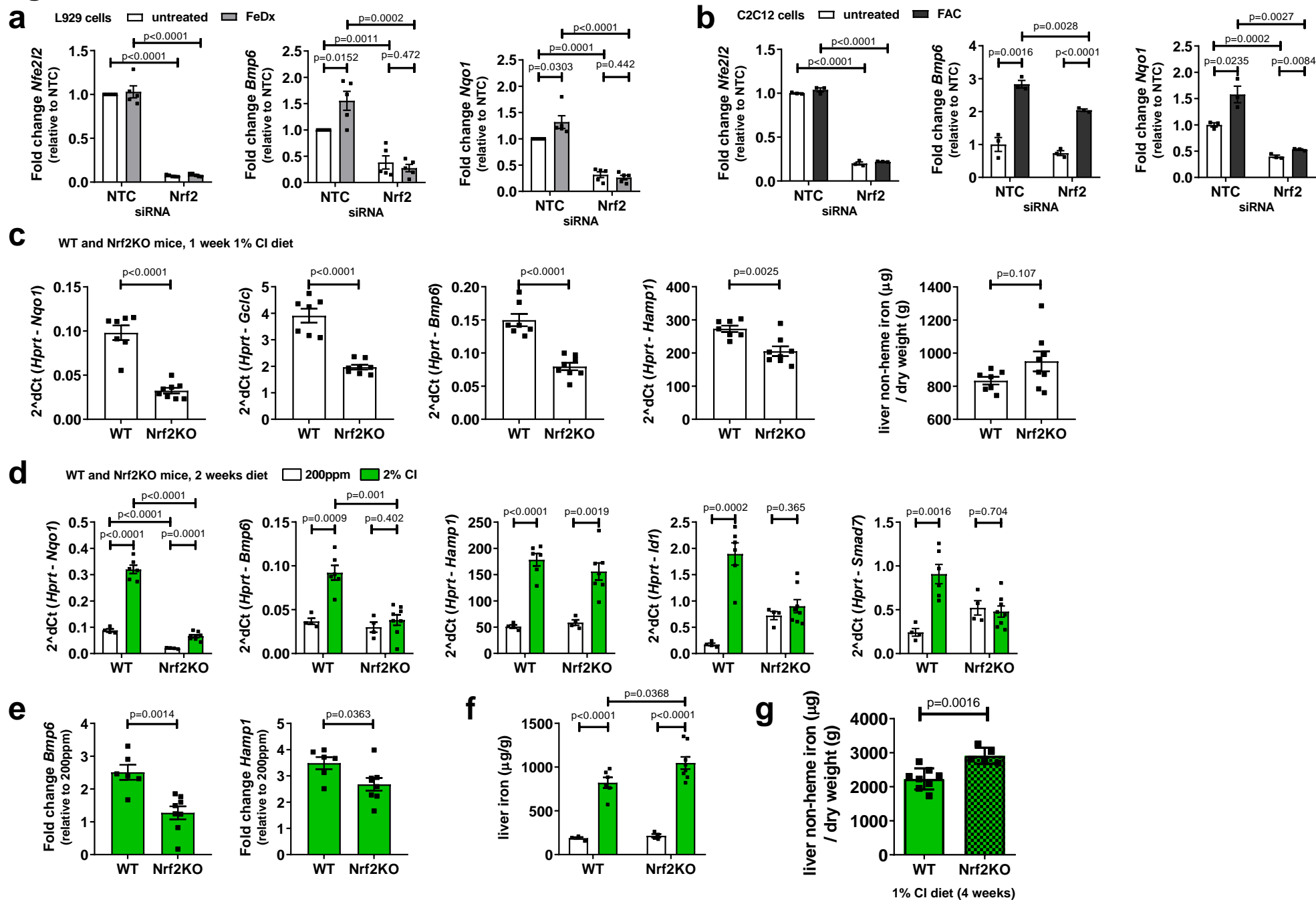


Figure 4

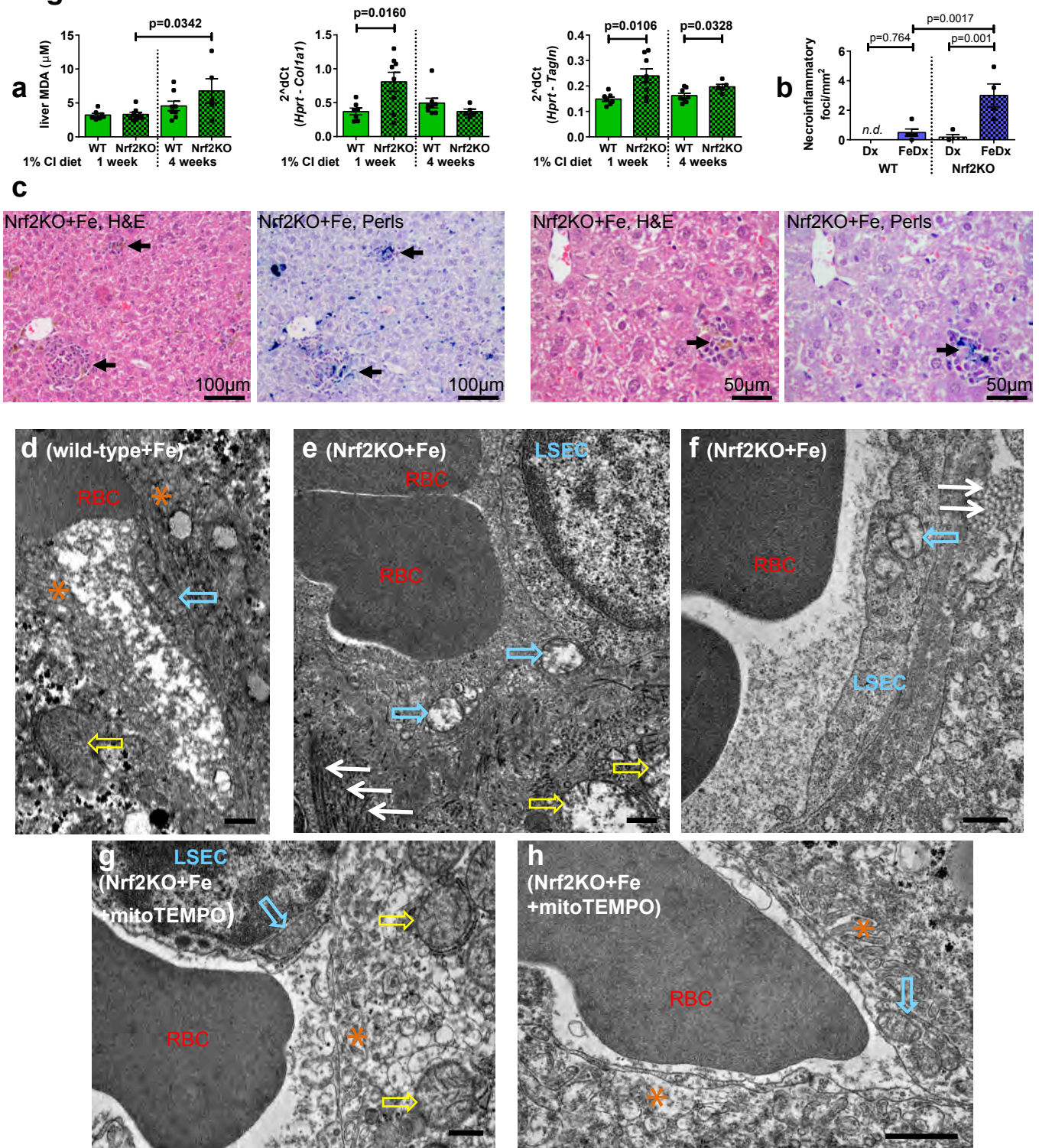


Figure 5

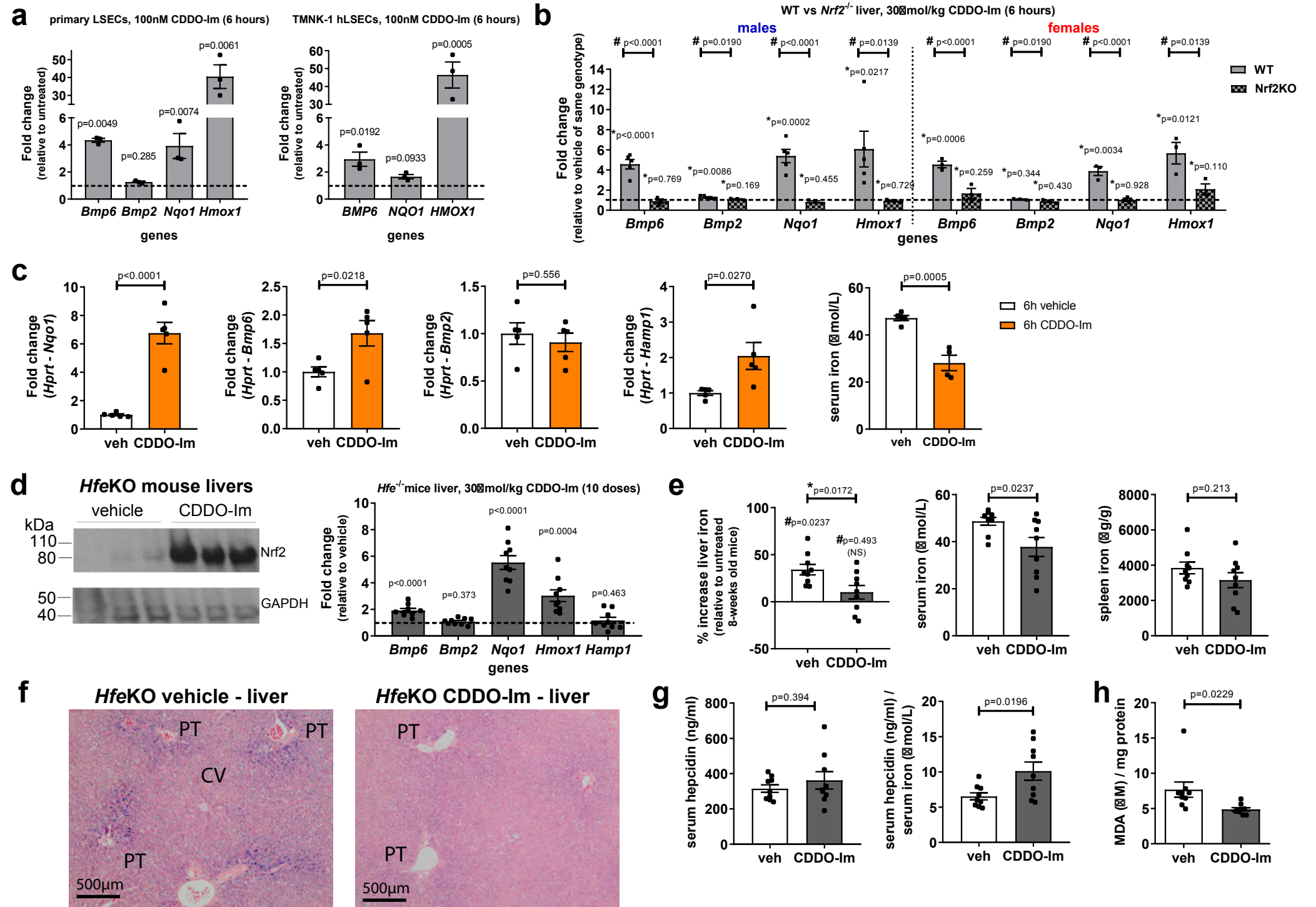
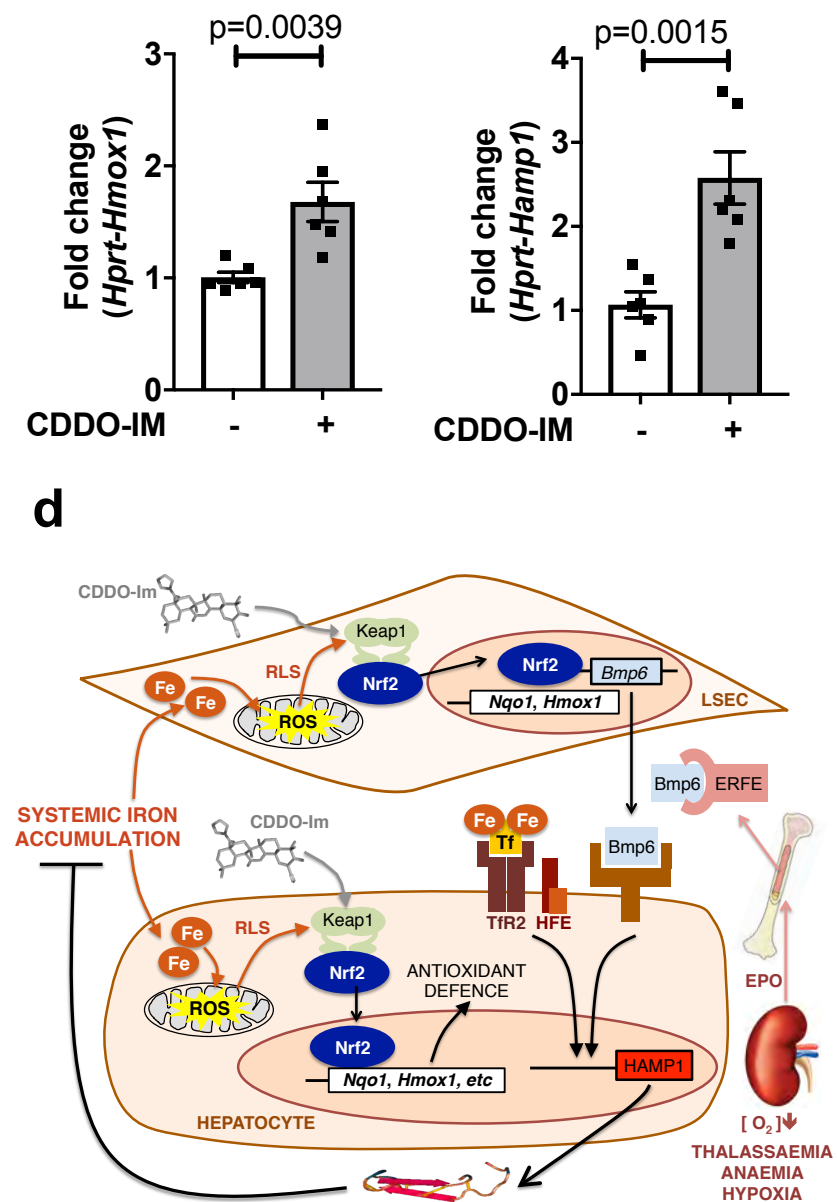
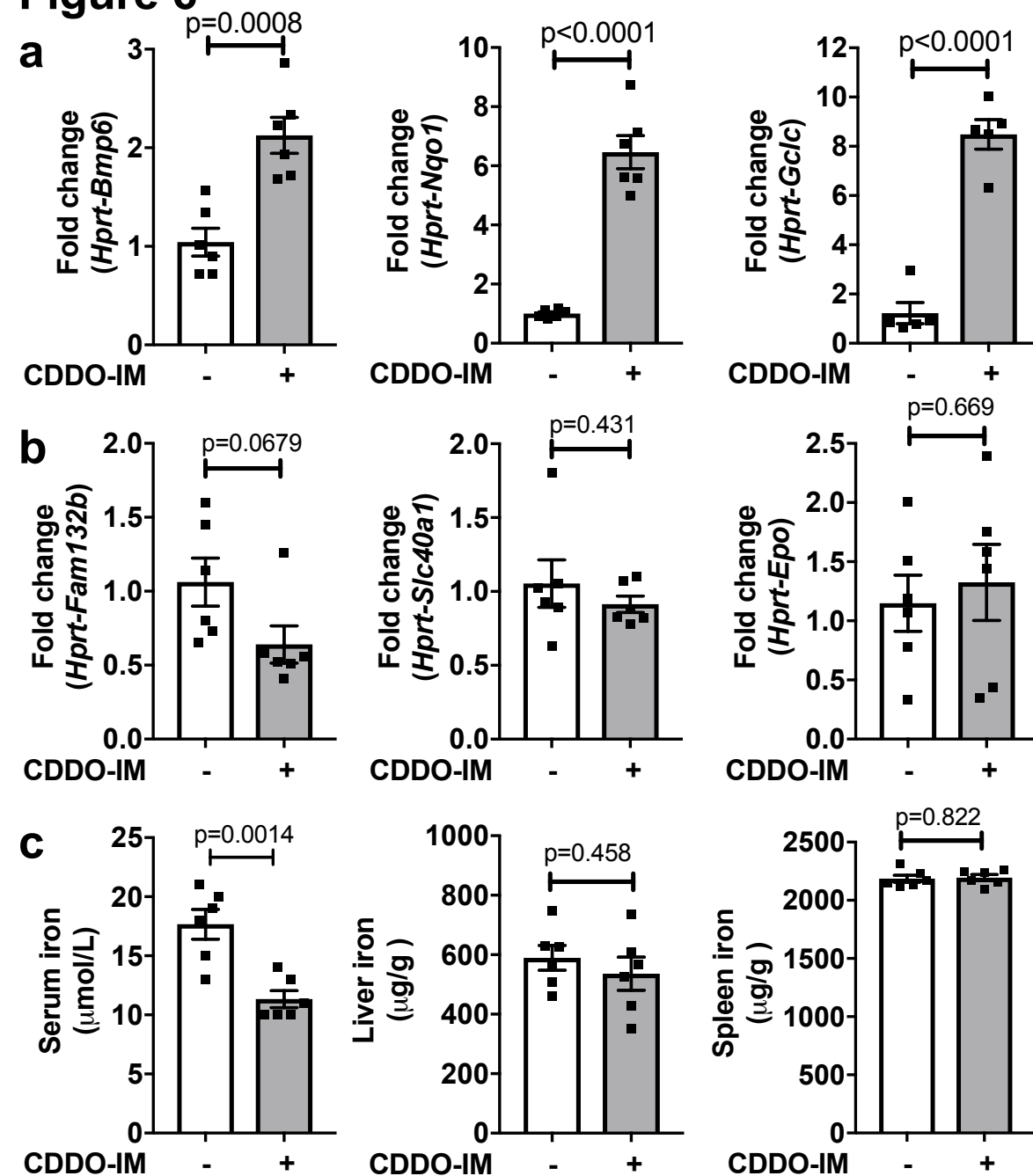
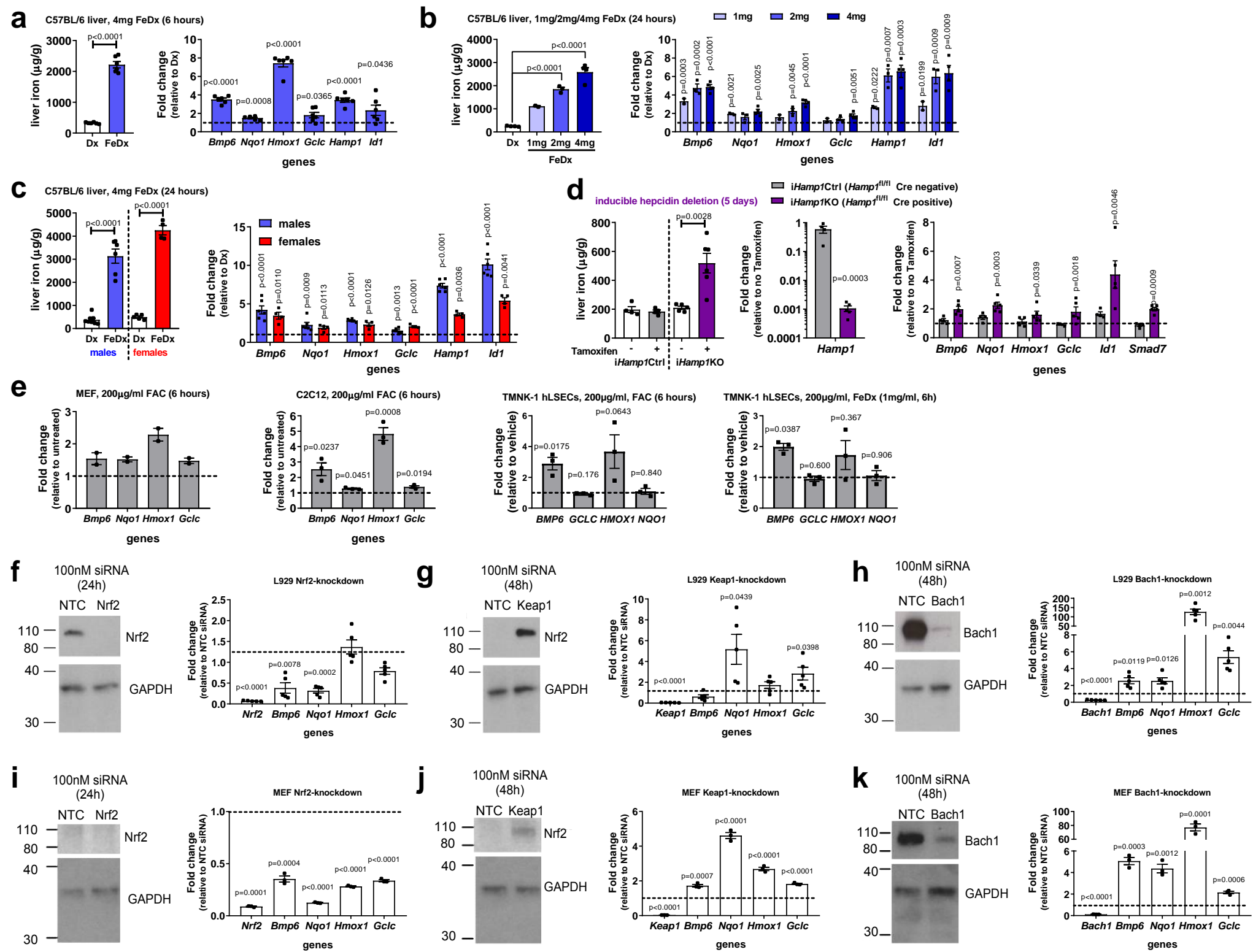


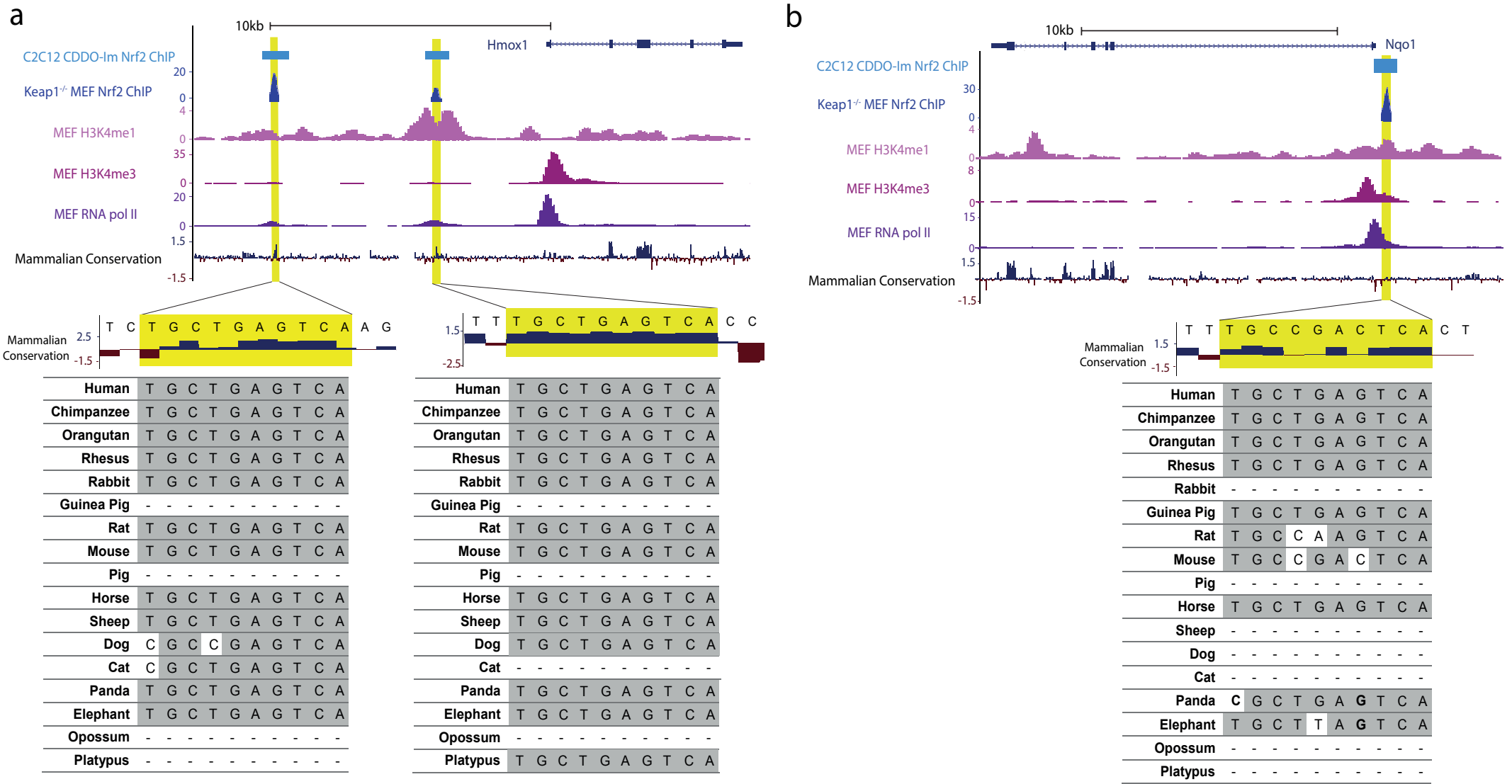
Figure 6

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 *iHamp1*-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 \pm SD and statistics performed on log₁₀-transformed data for normalisation. Two-tailed t-tests performed between iron-loaded and control groups, and between NTC and Nrf2/Keap1/Bach1 siRNA groups.

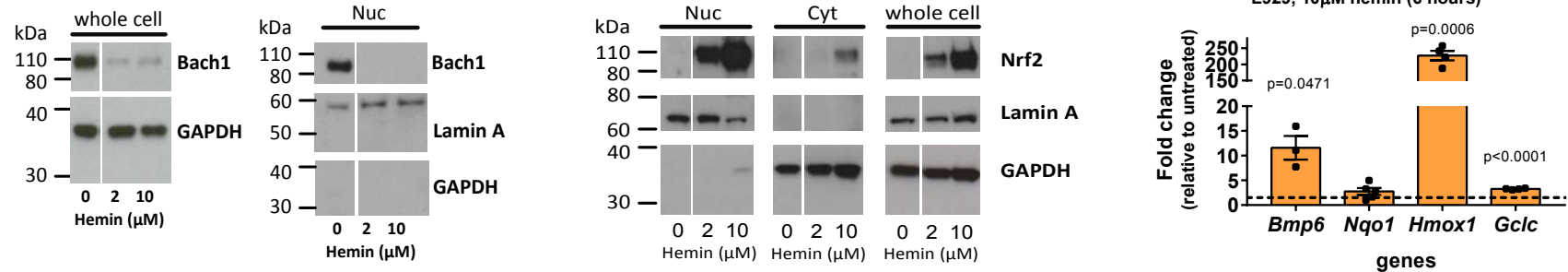
Supplementary Figure 2



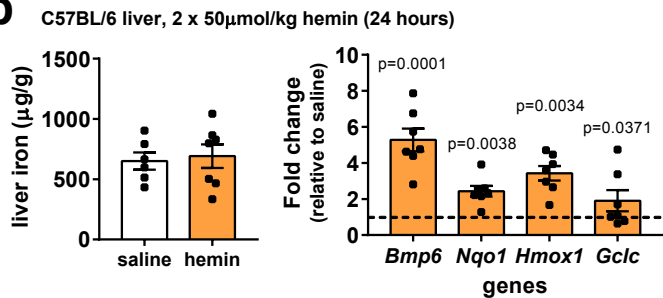
Supplementary Figure 2 Nrf2 binds to conserved ARE in proximity to Nqo1 and Hmox1. Nrf2 ChIP-seq datasets from C2C12 cells treated with CDDO-Im for 3 hours and Keap1-knockout MEF cells, ChIP-seq tracks of H3K4-monomethylation (H3K4me1) depicting enhancer regions, H3K4-trimethylation (H3K4me3) depicting 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

a

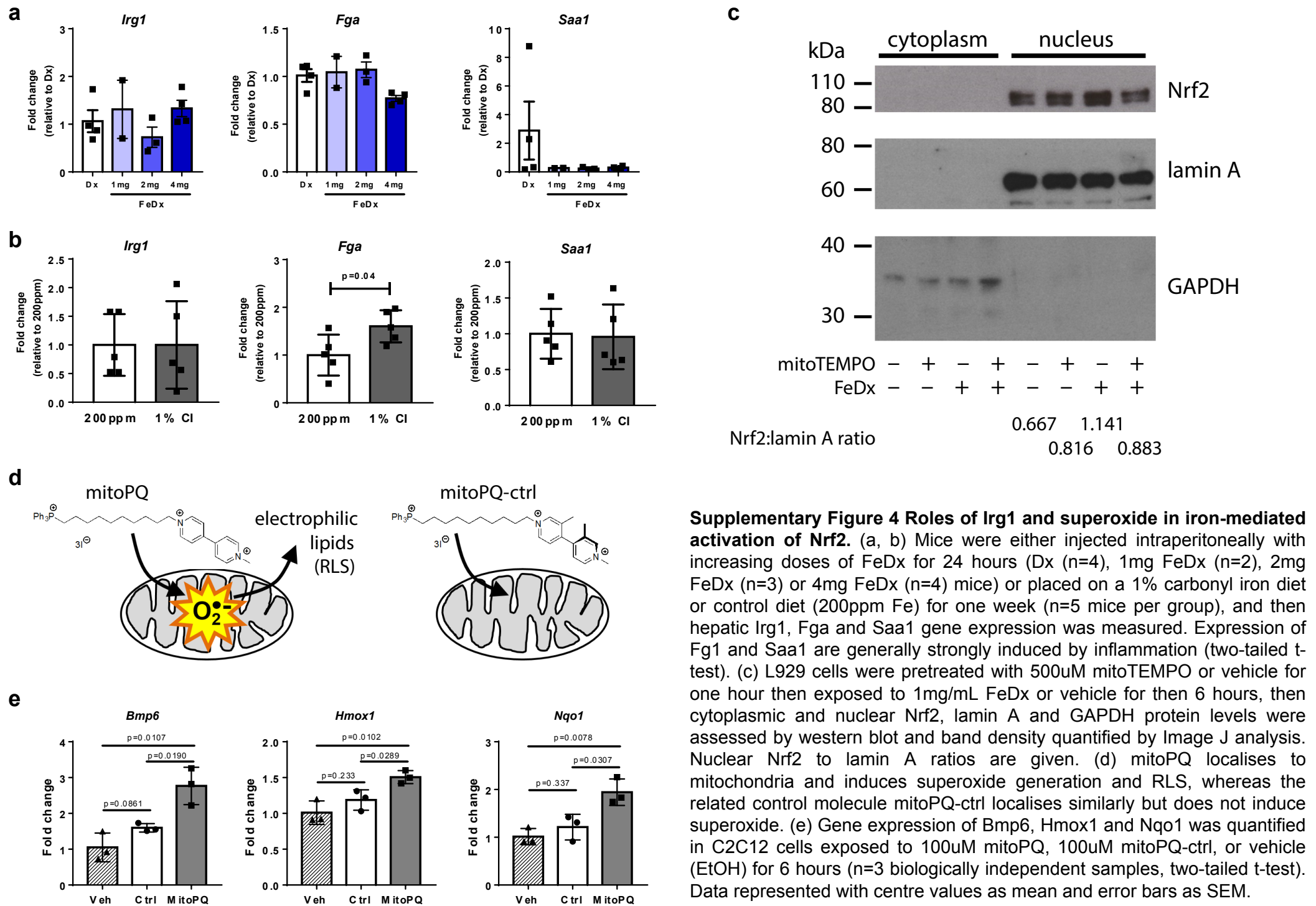


b

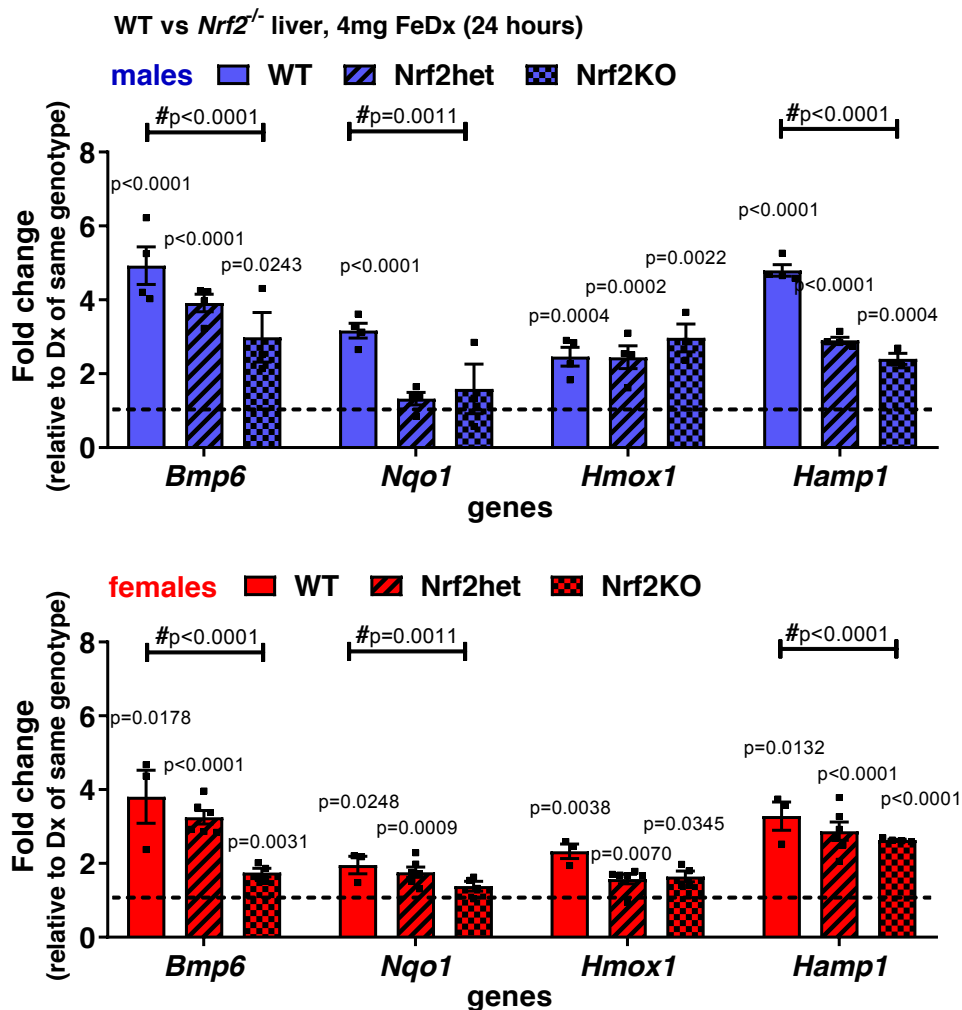


Supplementary Figure 3 Hemin-induced Bach1 degradation and Nrf2 stabilisation upregulated Bmp6 expression. (a) L929 cells were treated with hemin for 6 hours (n=4 biologically independent experiments). Immunoblot of Bach1 and Nrf2 protein in nuclear and whole cell extracts, and quantification of Bmp6, Nqo1, Hmox1 and Gclc expression were performed. (b) 6-week old C57BL/6 male mice were injected with two doses of 50 μ mol/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

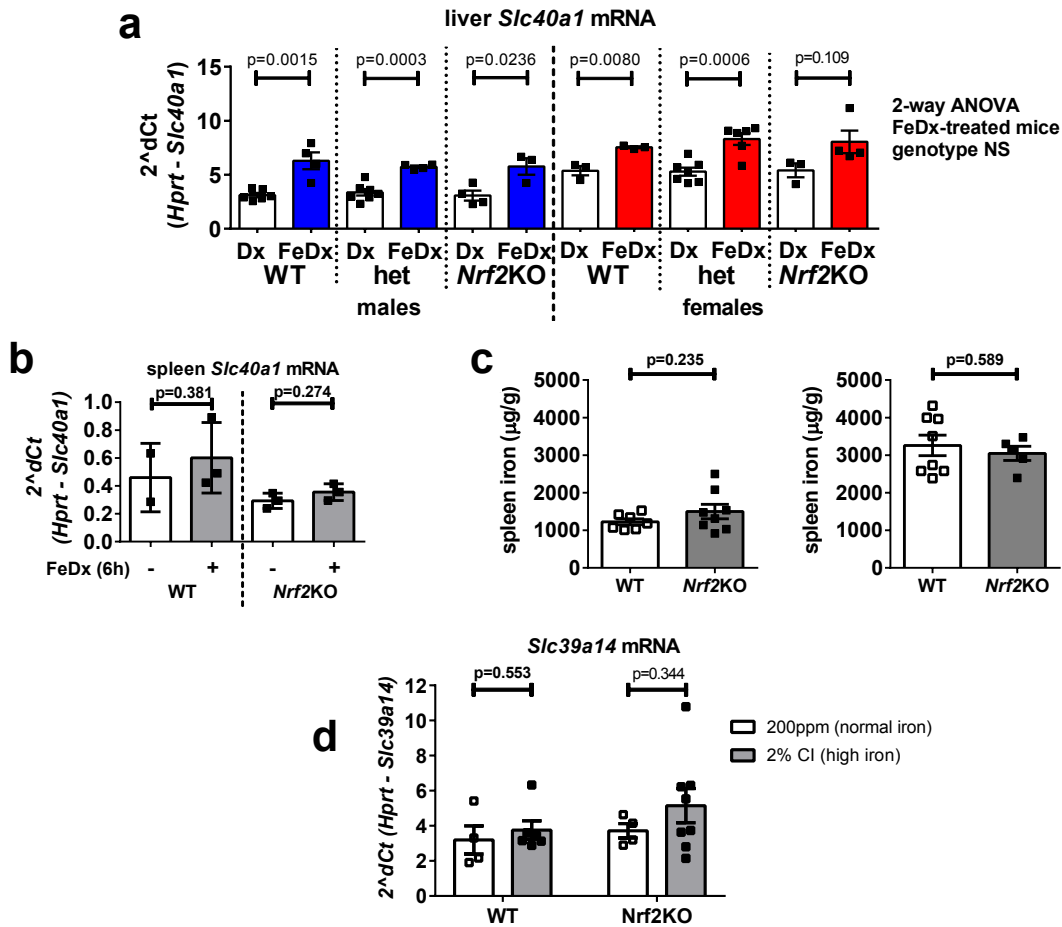


Supplementary Figure 5



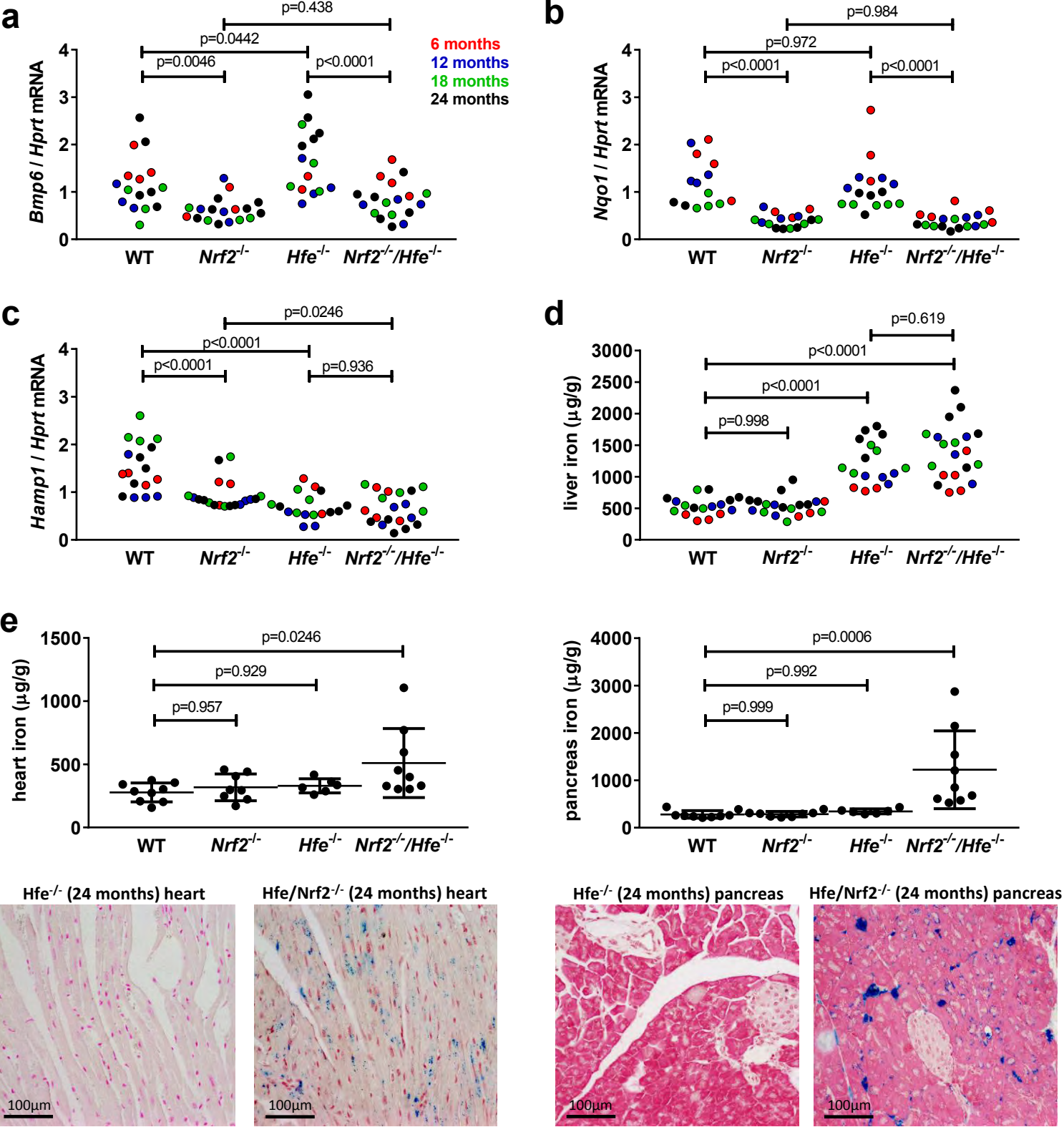
Supplementary Figure 5 Iron-induced *Bmp6* and hepcidin response is blunted in *Nrf2*-deficient mice. Wildtype (WT), heterozygous (*Nrf2*het) and homozygous (*Nrf2*KO) *Nrf2*-knockout mice were injected with 4mg FeDx or Dx (n=3 (male KO FeDx, female WT Dx, female WT FeDx, female KO Dx), n=4 (male WT 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, *Nrf2*het and *Nrf2*KO mice given FeDx, stratified by sex. Data represented with centre values as mean and error bars as SEM.

Supplementary figure 6



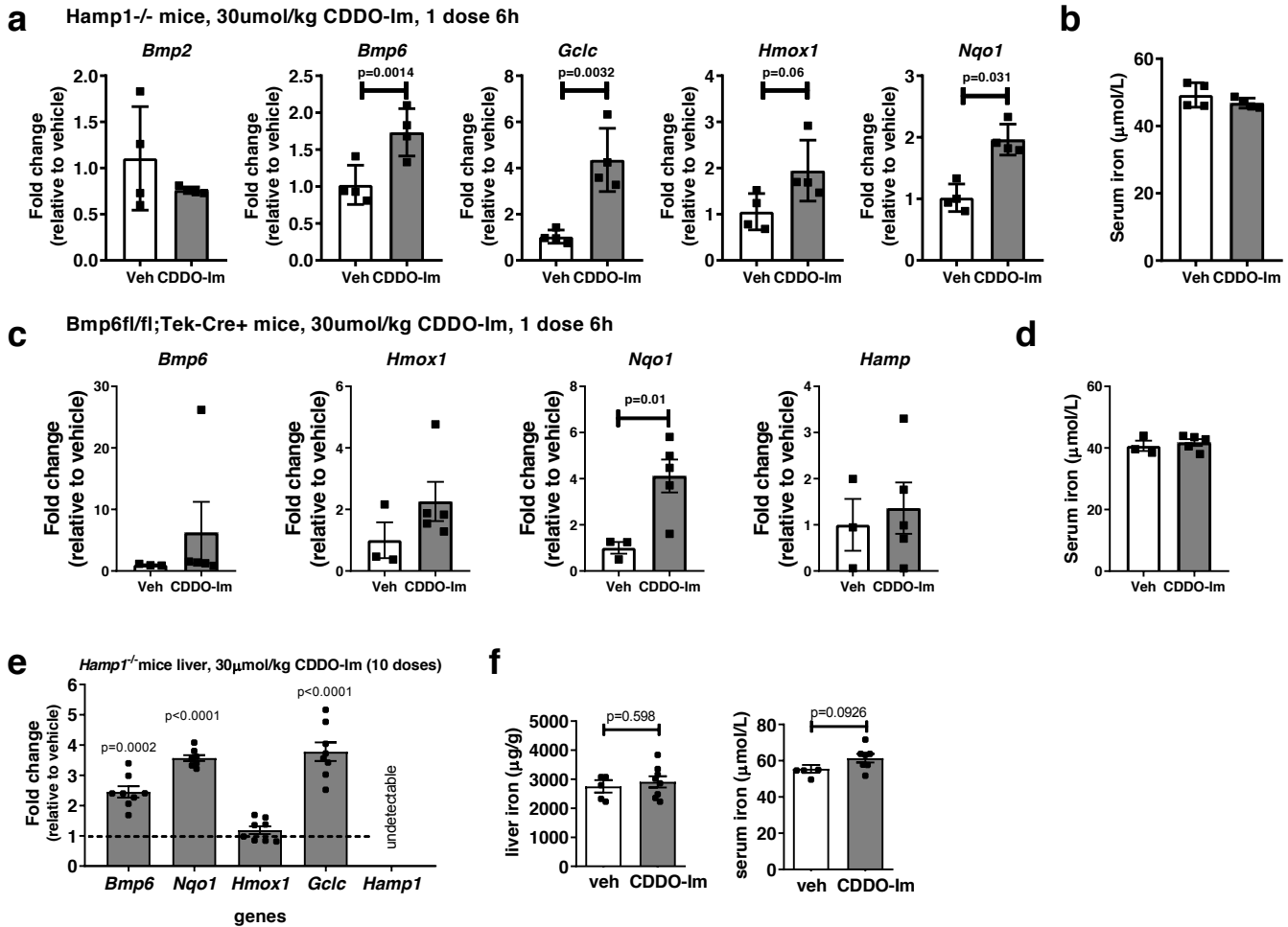
Supplementary Figure 6 Effects of Nrf2 deficiency on ferroportin and Zip14 mRNA expression. **(a)** Liver ferroportin (*Slc40a1*) expression in wildtype, heterozygous and homozygous *Nrf2* knockout mice 24 hours post FeDx injection (n=3 (male KO FeDx, female WT Dx, female WT FeDx, female KO Dx), n=4 (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.

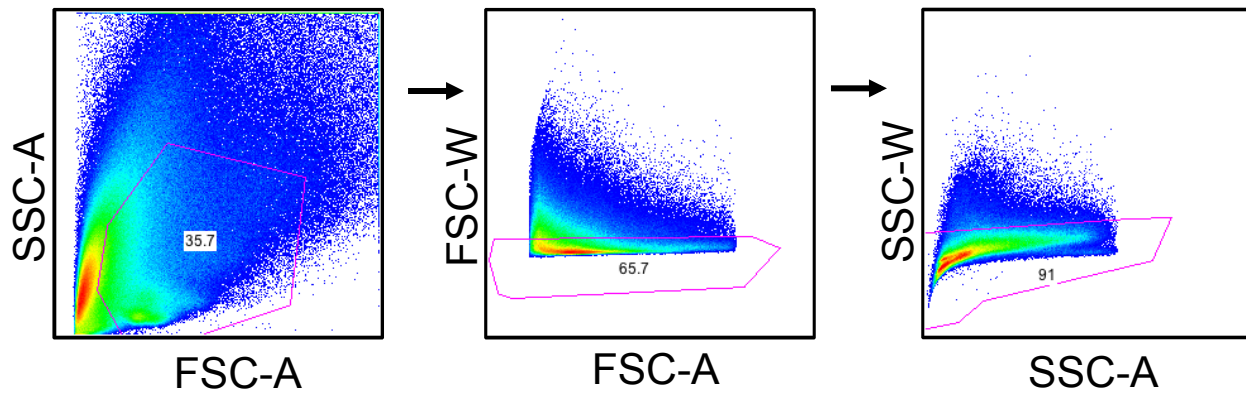
Supplementary Figure 8



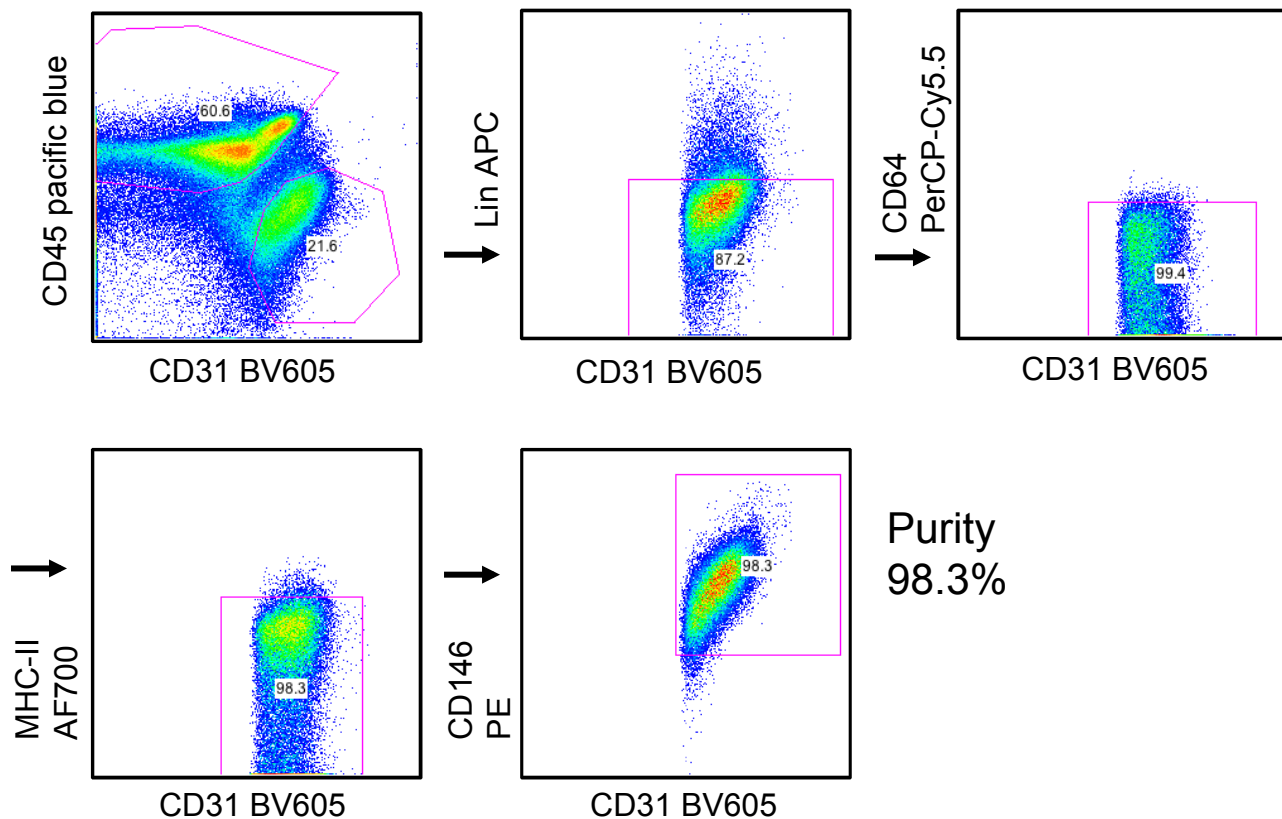
Supplementary Figure 8 Nrf2 agonist CDDO-Im in *Hamp1*-knockout mice and *Bmp6*^{fl/fl}; *Tek-Cre*⁺ mice. (a, b) Female *Hamp1*-knockout mice were given a single dose of vehicle or 30μmol/kg CDDO-Im by oral gavage and 6 hours later hepatic *Bmp2*, *Bmp6*, *Gclc*, *Hmox1* and *Nqo1* were measured (*Hamp1* was undetectable) and serum iron was quantified (n=4 mice per group). (c, d) *Bmp6*^{fl/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

1. Identification of singlets in liver non-parenchymal cell fraction



2. Identification of LSEC among singlets

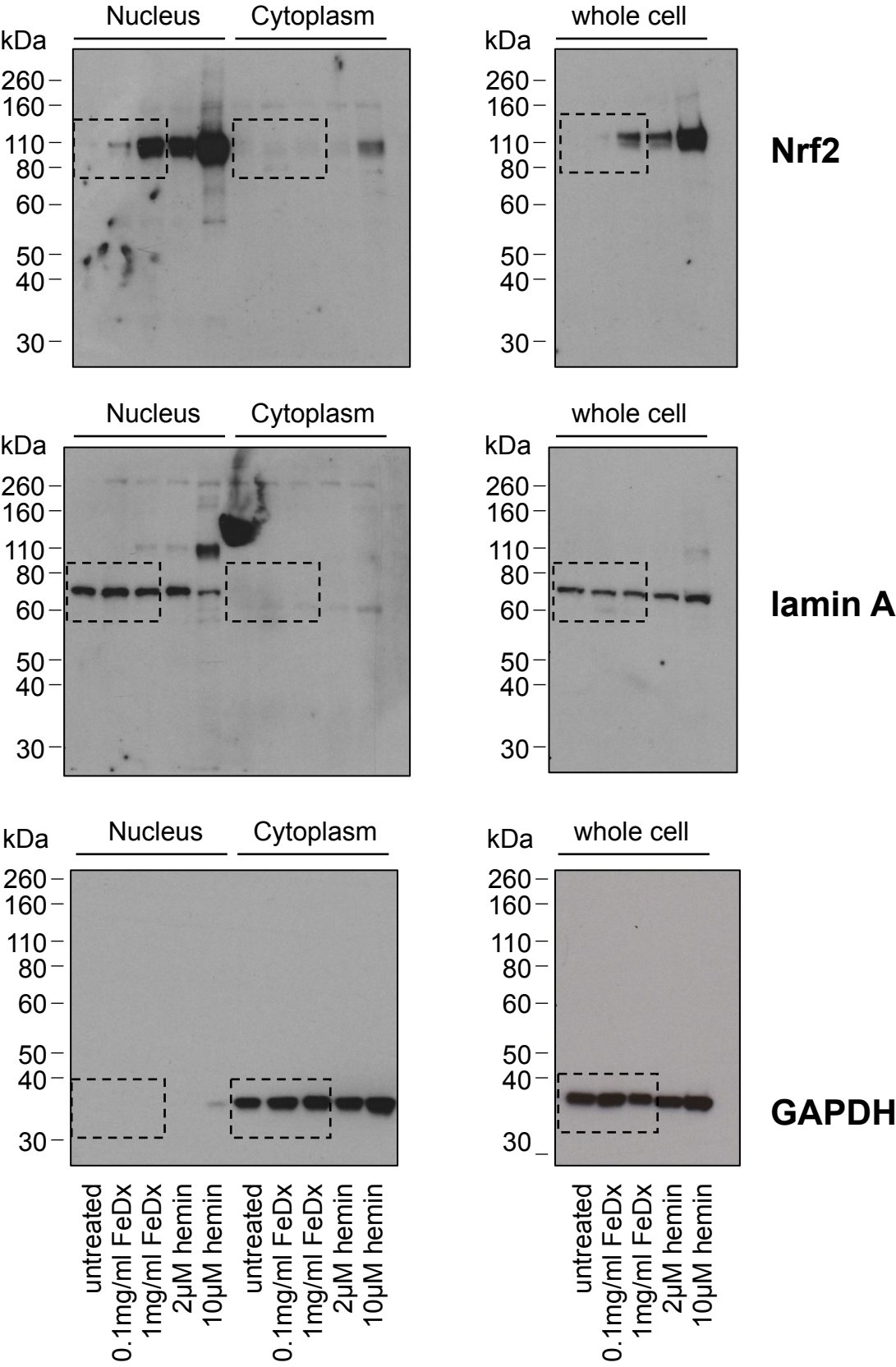


Supplementary Figure 9. Gating/sorting strategy for LSEC enrichment.

LSECs are defined as CD45- CD31+ Lin- CD64- MHC-II- CD146+

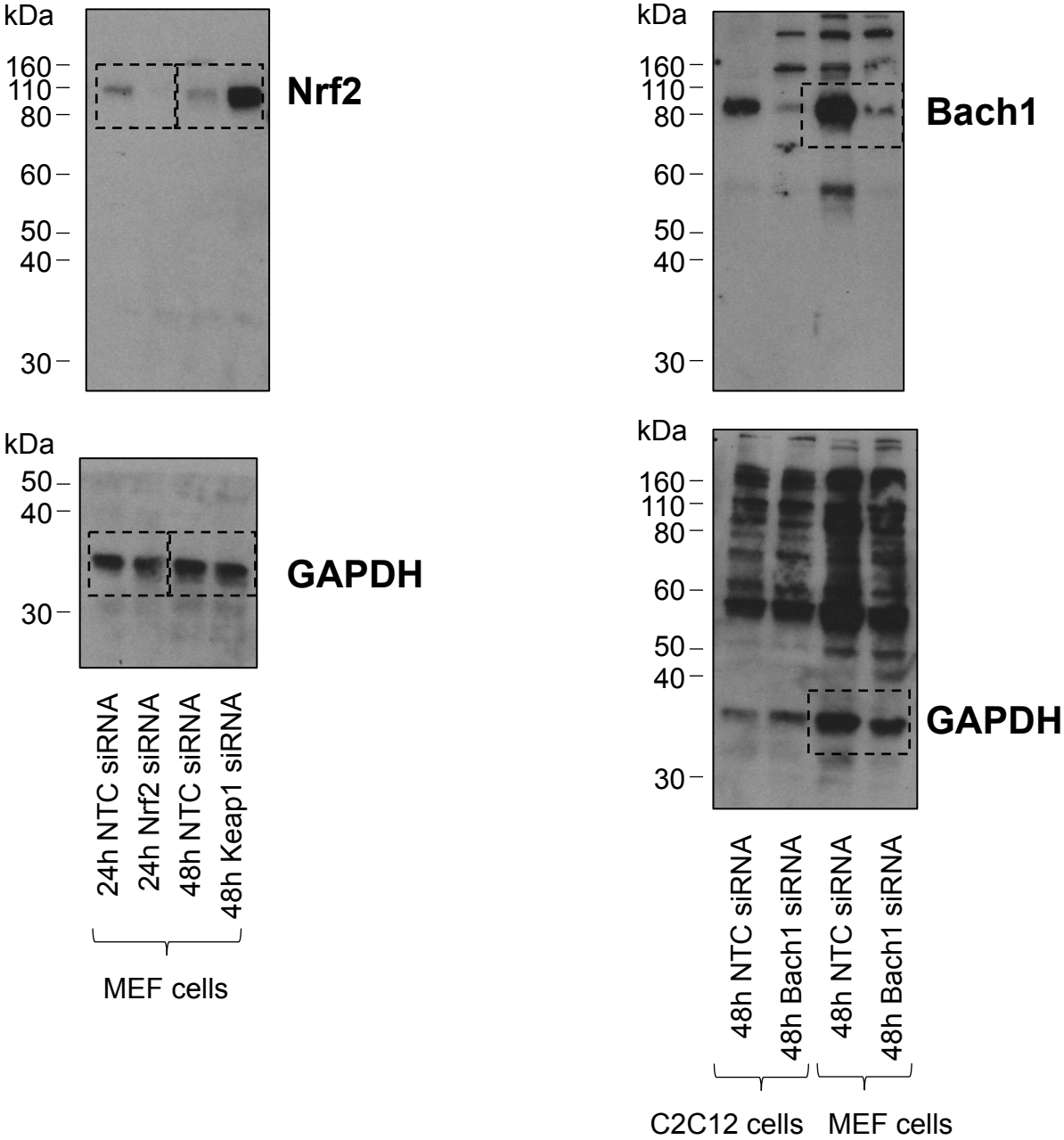
Lineage includes leukocyte markers (CD3, CD19, NK1.1, Ly6G, CD90.2) and an epithelial marker (CD326).

Supplementary Figure 10 (page 1/8)



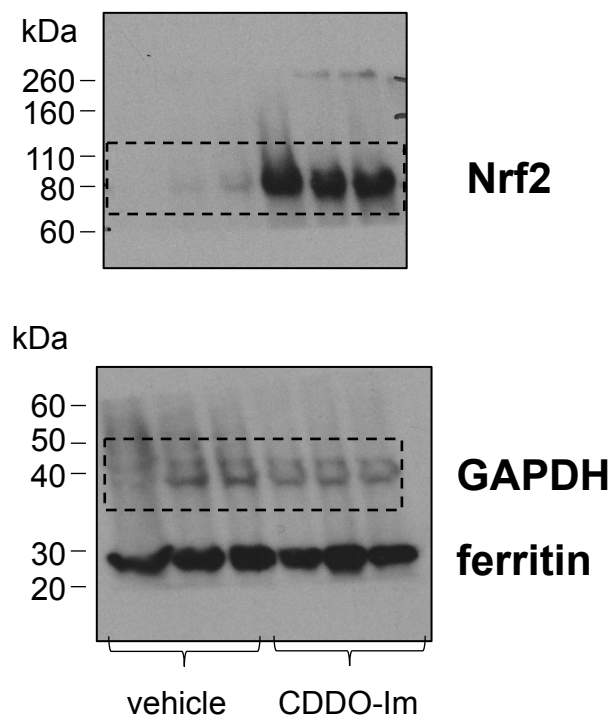
Complete blots corresponding to Figure 1d

Supplementary Figure 10 (page 2/8)



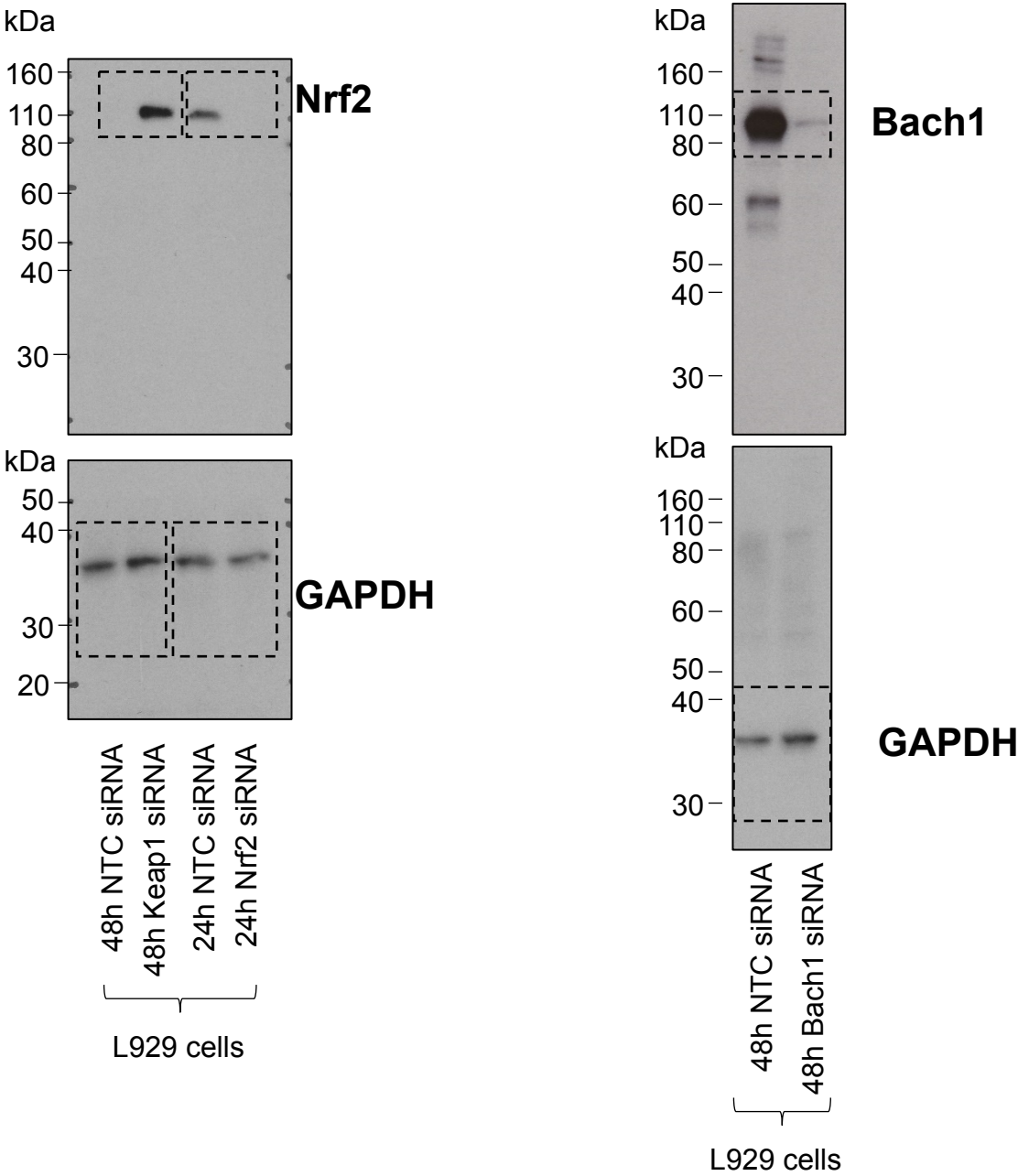
Complete blots corresponding to Figure 1f,g,h

Supplementary Figure 10 (page 3/8)



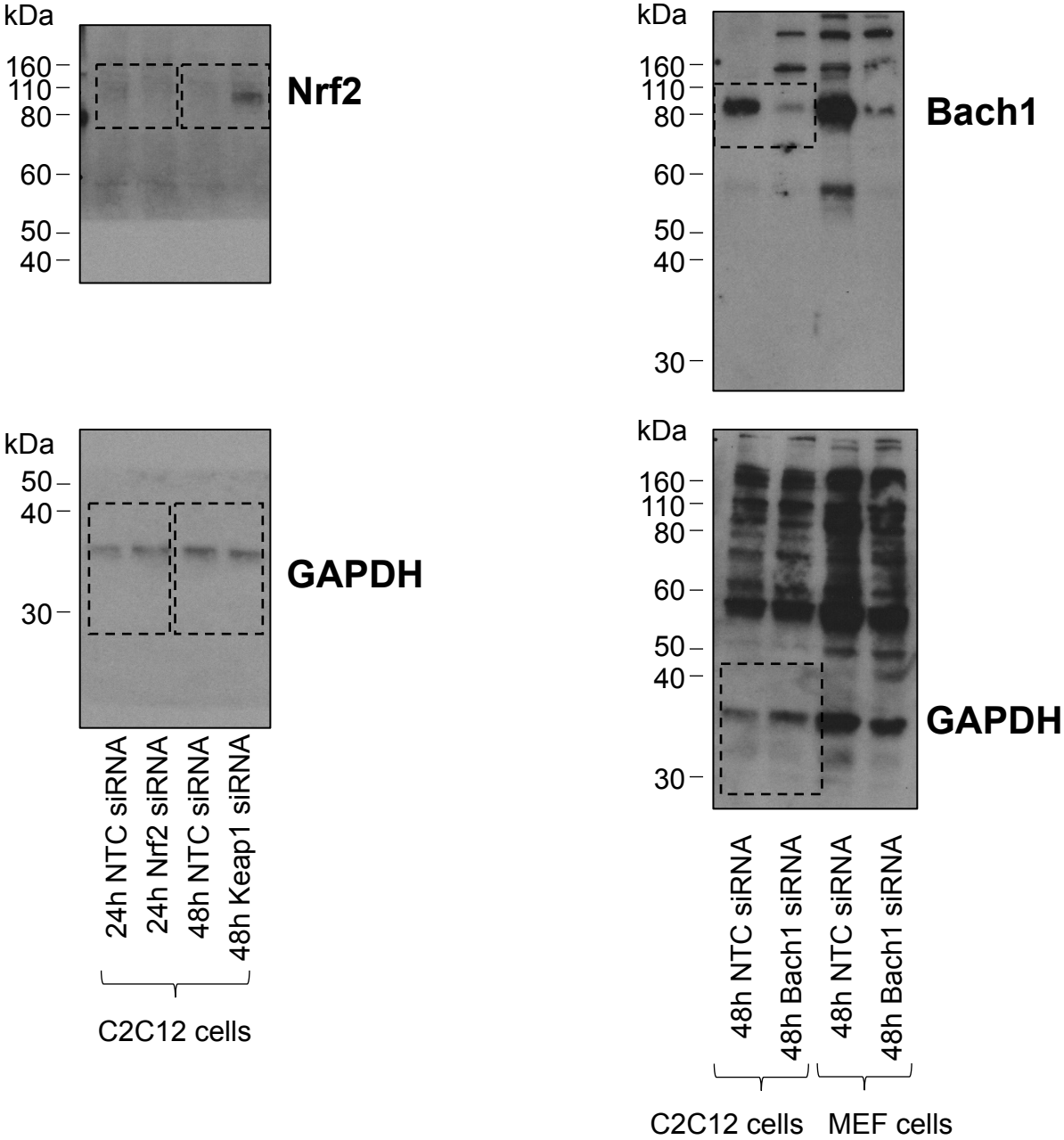
Complete blots corresponding to Figure 5d

Supplementary Figure 10 (page 4/8)



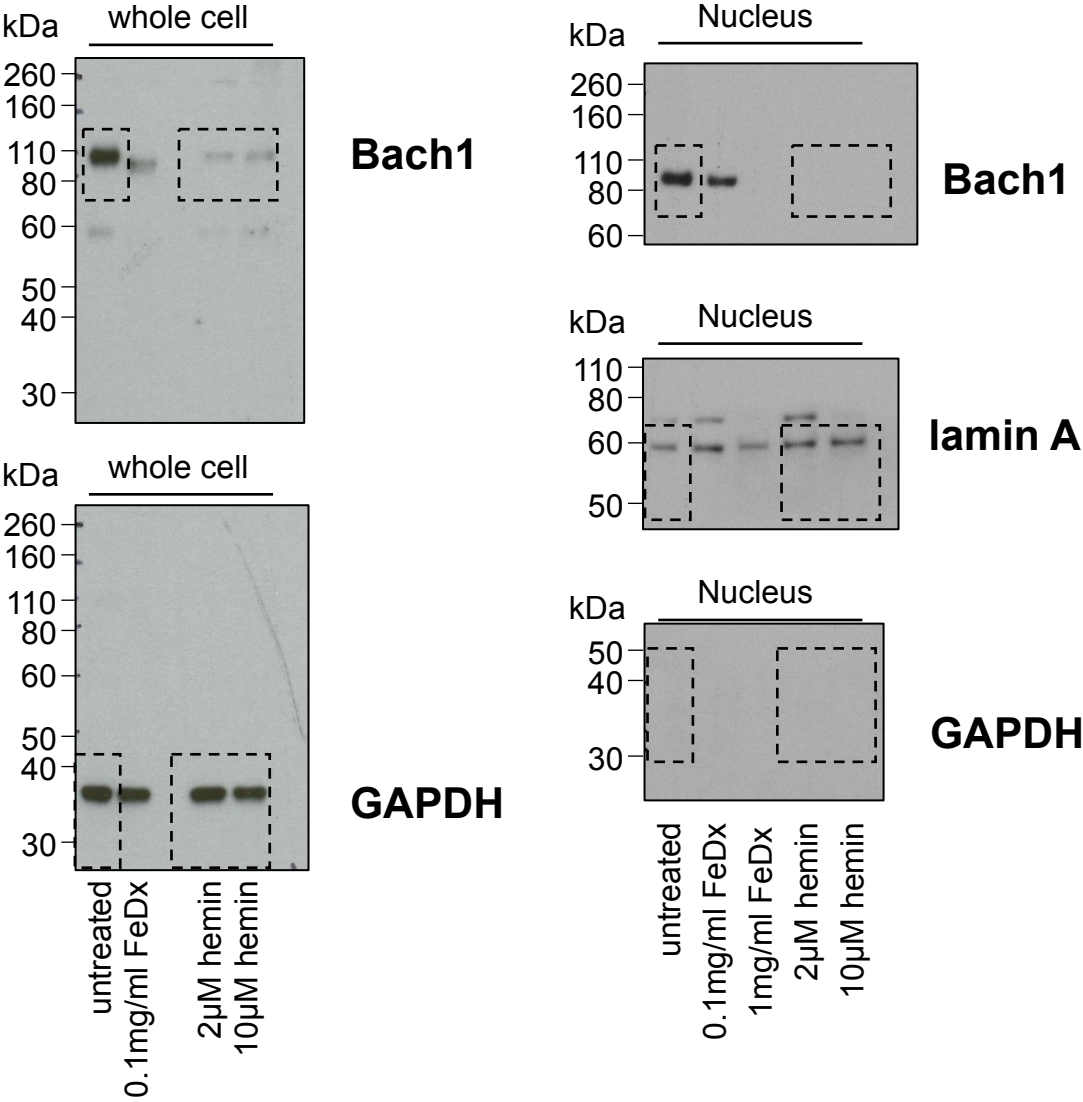
Complete blots corresponding to Supplementary Figure 1f,g,h

Supplementary Figure 10 (page 5/8)



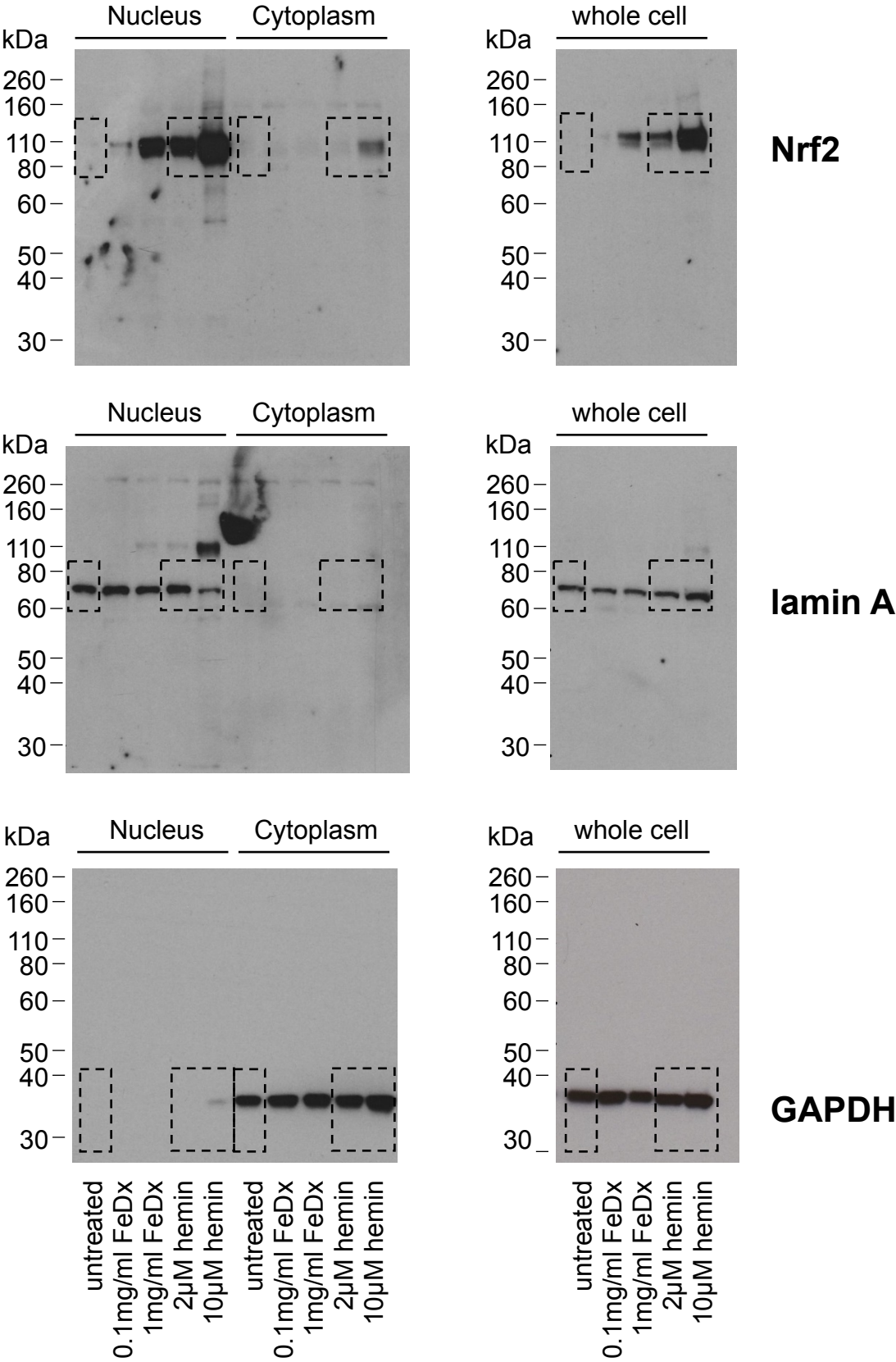
Complete blots corresponding to Supplementary Figure 1i,j,k

Supplementary Figure 10 (page 6/8)



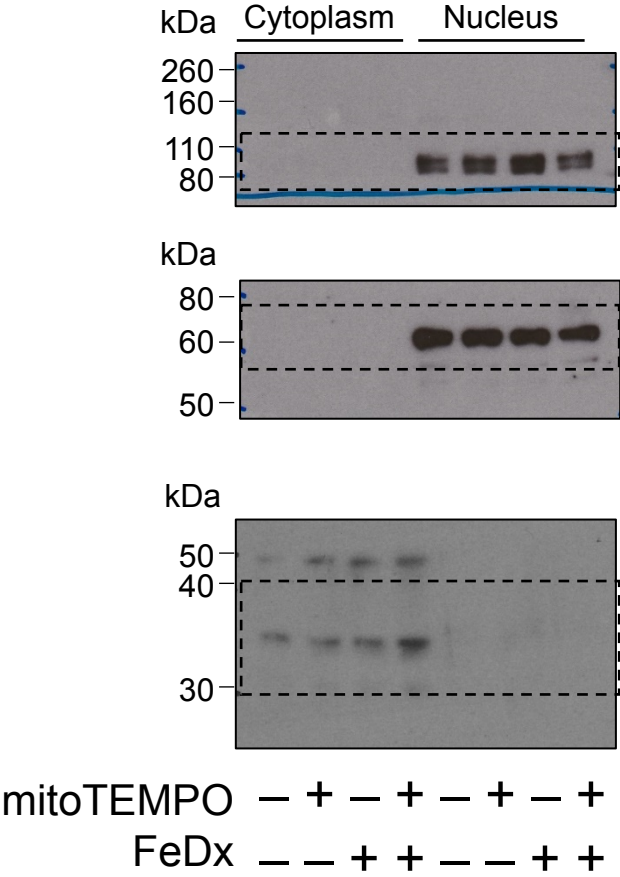
Complete blots corresponding to Supplementary Figure 3a

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Complete blots corresponding to Supplementary Figure 3a

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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	<i>Hprt1</i>	Mm01545399_m1
Bone morphogenetic protein 6	<i>Bmp6</i>	Mm01332882_m1
NAD(P)H dehydrogenase, quinone 1	<i>Nqo1</i>	Mm01253561_m1
Heme oxygenase 1	<i>Hmox1</i>	Mm00516005_m1
Glutamate-cysteine ligase catalytic subunit	<i>Gclc</i>	Mm00802655_m1
Hepcidin	<i>Hamp1</i>	Mm04231240_s1
Nuclear factor (erythroid-derived 2)-like 2	<i>Nfe2l2 (Nrf2)</i>	Mm00477784_m1
Kelch-like ECH associated protein 1	<i>Keap1</i>	Mm00497268_m1
BTB And CNC Homology 1	<i>Bach1</i>	Mm01344527_m1
Inhibitor of DNA-binding protein 1	<i>Id1</i>	Mm00775963_g1
Sons of mothers against decapentaplegic 7	<i>Smad7</i>	Mm00484742_m1
Bone morphogenetic protein 2	<i>Bmp2</i>	Mm01340178_m1
Collagen type I alpha I chain	<i>Colla1</i>	Mm00801666_g1
Transgelin	<i>Tagln</i>	Mm00441661_g1
Ferroportin	<i>Slc40a1</i>	Mm01254822_m1
Zip14	<i>Slc39a14</i>	Mm01317439_m1
Immunoresponsive gene 1	<i>Irg1</i>	Mm01224532_m1
Erythroferrone	<i>Fam132b</i>	Mm01224532_m1
Erythropoietin	<i>Epo</i>	Mm01202755_m1
Glyceraldehyde 3-phosphate dehydrogenase (human)	<i>GAPDH</i>	Hs99999905_m1
Heme oxygenase 1 (human)	<i>HMOX1</i>	Hs01110250_m1
Bone morphogenetic protein 6 (human)	<i>BMP6</i>	Hs01099594_m1
Glutamate-cysteine ligase catalytic subunit (human)	<i>GCLC</i>	Hs00155249_m1
NAD(P)H dehydrogenase, quinone 1 (human)	<i>NQO1</i>	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')
<i>Hprt1</i>	AGATGGGAGGCCATCACATTGT	ATGTCCCCCGTTGACTGATCAT
<i>Bmp6</i>	TCCCCACATCAACGACACCA	TCCCCACCACACAGTCCTTG
<i>Nqo1</i>	GTGCAGAAGCGAGCTGGAAATACTC	CGAATCTTGATGGAGGACTGGATGC
<i>Hamp1</i>	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA

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

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>Rpl19</i>	AGGCATATGGGCATAGGGAAGAG	TTGACCTTCAGGTACAGGTGTG
<i>Bmp6</i>	AGCACAGAGACTCTGACCTATTTT	CCACAGATTCTAGTTGCTGTGA
<i>Nqo1</i>	GCCCGCATGCAGATCCT	GGTCTCCTCCCAGACGGTTT
<i>Hmox1</i>	CAGCCCCACCAAGTTCAA	TCAGGTGTCATCTCCAGAGTGTC