Supplementary Data

Materials and methods

Reagents

Liver perfusion medium, liver digest medium, Williams-E with Glutamax-I medium, fetal bovine serum, penicilin/streptomycin, horseradish peroxidase-conjugated sheep anti-rabbit IgG, anti-rabbit IgG AlexaFluor 568, TriZol, Turbo DNA-free kit and ThermoScript RT-PCR System were obtained from Life Technologies (Carlsblad, CA, USA). ChemiDoc XRS System, Image Lab Software, iQ SYBR Green Supermix and the iQ5 Real-Time PCR Detection System were purchased from Bio-Rad (Hercules, CA, USA). Staurosporine was obtained from LC Laboratories (Woburn, MA, USA). All other chemicals and reagents were purchased from Sigma–Aldrich (Munich, Germany) unless otherwise stated.

Primary hepatocyte cultures

Fourteen to eighteen week-old mice were sacrificed by CO_2 inhalation and the inferior vena cava was cannulated with a nineteen gauge needle attached to a tube connected to a peristaltic pump. The portal vein was immediately cut to allow fluid to drain. Liver Perfusion Medium was perfused at 7 ml/min for 7 min at 37ºC. Liver Digest Medium was then perfused for an additional 10 min. After digestion, the gall bladder was removed and the liver was excised and gently disrupted in a Petri dish with 10ml of Williams-E with Glutamax-I medium supplemented with 4% fetal bovine serum and 1% penicilin/streptomicin (Williams' complete medium). The cell suspension was filtered through a 100 µm nylon strainer (BD Falcon) to a 50 ml tube containing 20 ml of Williams' complete medium and centrifuged at 50*g* for 5 min without brake at 20ºC. The cell pellet was re-suspended in 20 ml of Williams'

complete medium and carefully layered on top of an equal volume of 0.672 g/ml Percoll. The Percoll solution was prepared by diluting Percoll to 1.12 g/ml with 10X PBS and further diluting it with Williams' complete medium to 0.672 g/ml. The mixtures were centrifuged at 750*g* for 25 min without brake at 20ºC. The pellet containing the hepatocytes was washed twice in 20 ml of Williams' complete medium and re-suspended in 4ml of the same medium. After counting the number of viable cells (cells that were able to exclude trypan blue) in a haemocytometer, hepatocytes were seeded at 3×10^4 cells/cm^2 on culture plates that were pre-coated with 0.2 % gelatin for 30 min. Cells were allowed to adhere for 2 h, after which the medium was replaced by fresh medium containing either ferric ammonium citrate (FAC), ammonium citrate (AC) or staurosporine. All cell preparations were used within 24 h of isolation. It is noteworthy that the iron content in our batch of FAC was 18%, which means that the concentration of FAC that we used in most experiments (1.71 µg/ml) corresponded to approximately 0.3 µg/ml of iron.

MTT cell viability assay

After 24 h of treatment, hepatocytes were washed with PBS and the medium was replaced by 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Following incubation at 37 °C for 1.5 h, the purple formazan crystals formed in the mitochondria of viable cells were dissolved in dimethyl sulfoxide and the absorbance read at 540 nm. Experiments were performed on five different occasions.

Determination of hepatocyte necrosis and apoptosis

After 24 h of various treatments, cells were incubated with 2.5 µg/ml propidium iodide (PI) for 15 min. Cells were then fixed in 4% paraformaldehyde and the nuclei

were counterstained with 4'6'-diamidino-2-phenylindole (DAPI). Cells were visualized in a wide-field fluorescence microscope (Zeiss AxioSkop, Carl Zeiss, Jena, Germany). Quantification of total and PI-positive cells was performed by counting cells in 5 different fields at 200× magnification. Experiments were performed on three different occasions.

Comet assay

Oxidatively modified DNA in mouse hepatocytes was measured using the alkaline comet assay in conjunction with the hOGG1 repair glycosylase as described [6]. DNA damage was expressed as the median of percentage of DNA in the comet tails. The level of hOGG1 sensitive sites was obtained as the difference in score between gels that had been incubated with enzyme or buffer. Experiments were performed on two different occasions, each using duplicate cultures.

Animals and experimental design

C57BL/6J mice and Nrf2-/- mice on a C57BL/6J background [1] purchased from Riken (Japan) were housed and bred at the 'Instituto de Biologia Molecular e Celular' animal facility. Male mice (16 week-old) were fed standard rodent chow (Global rodent diet, Harlan Laboratories, Barcelona, Spain; n = 8 each group) or iron-rich diet (Global rodent diet supplemented with 2.0 % carbonyl iron; n = 6 each group) for 2 weeks. In a subsequent experiment, Nrf2-/- animals were maintained on the iron-rich diet for the same time period and injected daily with either (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-xoethyl)-triphenylphosphonium chloride monohydrate (mito-TEMPOL, Santa Cruz Biotechnology, Heidelberg, Germany) (10 mg/kg intraperitoneally, n = 6) or saline (control, n = 5), starting from the day before

the start of dietary supplementation. At the end of the dietary treatment, mice were anaesthetized with isoflurane (B. Braun, Portugal), blood was collected by retro-orbital bleeding, and the animals were sacrificed for organ collection. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent RNA and protein analysis.

Since the saline injections in *Nrf2*$^{-/-}$ animals on iron-rich diet had no effect on any of the parameters assessed when comparing with *Nrf2*$^{-/-}$ animals on the same diet, samples from both groups were pooled for statistical analysis.

Experiments were carried out in compliance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). The animal experimental protocol was approved by the competent national authority Direcção Geral de Veterinária (DGV) and by the Institute's ethical committee.

Serum biochemistry

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was measured according to the International Federation of Clinical Chemistry reference method [2]. Serum iron and total iron binding capacity were quantified by the guanidine-ferrozine and the ferrozine methods, respectively. All measurements were performed in a Cobas C8000 analyzer (Roche Diagnostics, Mannheim, Germany) at Centro Hospitalar do Porto.

Liver iron quantification

Non-heam iron content in liver samples was measured by the bathophenantroline method as described [3].

Liver histology, histochemistry and immunofluorescence

Liver samples were fixed with 4% paraformaldehyde in phosphate buffer and embedded in paraffin. Five-micrometer sections were stained with haematoxylin-eosin and Perls' prussian blue using standard procedures. The grading of liver necrosis was performed according to Ishak *et al.* [4]. By this method grade 0 represents absence of confluent necrosis, 1 represents focal confluent necrosis, 2 represents zone 3 necrosis in some areas, 3 represents zone 3 necrosis in most areas, 4 represents zone 3 necrosis + occasional portal-central bridging, 5 represents zone 3 necrosis + multiple portal-central bridging, and 6 represents panacinar or multiacinar necrosis. Histological sections stained by Perls' method were graded for stainable iron content of parenchymal cells according to Nash *et al.* [5]. By this method grade 0 represents absence of stainable liver cell iron, and grades 1-3 stand for increasing degrees of parenchymal siderosis ranging from a predominant periportal iron deposition (grade 1) to the deposition of iron in midzone (grade 2) or centrilobular hepatocytes (grade 3). When the intensity of the siderosis was regarded as intermediate between two grades (usually reflecting uneven distribution) the higher value was accepted. The presence of reticuloendothelial siderosis was recorded but did not influence the grading of the specimen. The histological examinations were performed by a pathologist (J.L.) with no previous knowledge of the sample identification.

For immunofluorescence staining, slides were deparaffinized with xylene and hydrated by a passage through a grade of alcohols. Fragmented DNA was detected through a TdT-mediated dUTP nick-end labeling (TUNEL) staining (Roche Diagnostics, Mannheim, Germany). For the immunohistochemical detection of CD45+ cells, samples were subjected to antigen retrieval with citrate buffer, endogenous peroxidases were blocked with 3% H_2O_2 and non-specific antigens were blocked with 10 % fetal bovine serum. Samples were incubated with goat anti-mouse CD45 antibody (R&D Systems, Minneapolis, MN, USA) followed by biotinylated anti-goat IgG (Millipore, Billerica, MA, USA) and horseradish peroxidase-streptavidin complex. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB). For the detection of cleaved caspase-3, samples were subjected to antigen retrieval with citrate buffer, blocked with 5% fetal bovine serum and 0.3% Triton X-100 in phosphate buffer and incubated with rabbit anti-cleaved caspase-3 (Cell Signalling, Danvers, MA, USA) followed by anti-rabbit IgG AlexaFluor 568. Cell nuclei were stained with DAPI. Samples were analysed in a widefield fluorescence microscope (Zeiss AxioSkop, Carl Zeiss, Jena, Germany). As a positive control, we used Jurkat cells treated with the topoisomerase II inhibitor drug etoposide (25 µM, 5 hrs) as supplied by the manufacturer (Cell Signaling). For the detection of 8-OHdG, samples were treated with RNase (100 µg/mL) and incubated with 6N HCl followed by 50 mM Tris base in order to denature DNA. 8-OHdG was detected by immunohistochemistry using a monoclonal antibody (clone N45.1, JaICA, Japan) and M.O.M. Immunodetection Kit (Vector, USA), according to the manufacturer's instructions. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB). The quantification of immunohistochemical data was performed blindly by an independent observer (C.M.S.) who was not involved in image acquisition and

coding. Quantification was performed by counting positive cells or nuclei in 5 different fields at 200× magnification.

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) of periportal areas stained with uranile acetate and lead citrate were visualized with a JEM 1400 electron microscope (Jeol, Tokyo, Japan) operated at 120 kV. Electron micrographs at a magnification of 6000–30,000 were captured with an Orius CCD (Gatan, Warrendale, PA, USA).

Real-time reverse-transcription (RT)-PCR

Total RNA was extracted using TriZol, followed by DNase treatment (Turbo DNA-free kit). First-strand cDNA was prepared using the ThermoScript RT-PCR System according to the manufacturer's instructions. Relative gene expression levels were quantified using an iQ5 Real-Time PCR Detection System. Primer sequences are listed in Supplementary Table 1. All reactions were performed in a total volume of 20 µl with iQ SYBR Green Supermix. The amplification protocol consisted of denaturation at 95°C for 4 min and 40 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 30 s. The quantity of each transcript was estimated against the respective standard curve and normalized against the quantity of the endogenous control gene actin, beta (Actb).

Protein analysis

Liver samples were homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing protease inhibitors. Liver extracts (30 µg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a 0.45 µm nitrocellulose membrane (GE Healthcare, Portugal). The membrane was incubated with rabbit anti-ferritin, rabbit anti-NQO or rabbit anti-β-actin (all from Abcam, Cambridge, UK), followed by a horseradish peroxidase-conjugated sheep anti-rabbit IgG. Immunoreactivity was visualized using a chemiluminescence substrate (Millipore, Billerica, MA, USA) and the signal was recorded on a ChemiDoc XRS System. Band densitometry was performed using Image Lab Software.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, California USA). Results are expressed as the mean and standard deviation. Differences between two groups were compared by t-test. Differences among multiple groups were compared by one-way or two-way analysis of variance with Tukey's multiple comparison test. Statistical significance was assumed at $p < 0.05$.

Supplementary Table 1. Primer sequences.

Gene ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Actb[a]	GGCGGACTGTTACTGAGCTGCGTTT	AAAGCCATGCCAATGTTGTCTCTT
Gclc[a]	AGGTTGACGAGAACATGAAAGTGGC	CCGCCTTTGCAGATGTCTTTCCTGA
Gsta1[4]	GACTGTGAGCTGAGTGGAGAAGAA	CCGGCCATTGCAGCAA
Hamp [5]	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA
Hamp2 [5]	CCTATCTCCATCAACAGATG	AACAGATACCACAGGAGGGT
Nfe2l2/Nrf2[a]	CTAAAGCACAGCCAGCACATTCTCC	TTGGGATTCACGCATAGGAGCAC
Nqo1[a]	GTGCAGAAGCGAGCTGGAAATACTC	CGAATCTTGATGGAGGACTGGATGC

[a] designed with Primer 3 software [6]

References

[1] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313-322.

[2] Bergmeyer HU, Horder M. International federation of clinical chemistry. Scientific committee. Expert panel on enzymes. IFCC document stage 2, draft 1; 1979-11-19 with a view to an IFCC recommendation. IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase. J Clin Chem Clin Biochem 1980;18:521-534.

[3] Neves JV, Olsson IA, Porto G, Rodrigues PN. Hemochromatosis and pregnancy: iron stores in the Hfe-/- mouse are not reduced by multiple pregnancies. Am J Physiol Gastrointest Liver Physiol 2010;298:G525-529.

[4] Sugimoto H, Okada K, Shoda J, Warabi E, Ishige K, Ueda T, et al. Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice. Am J Physiol Gastrointest Liver Physiol 2010;298:G283-294.

[5] Ilyin G, Courselaud B, Troadec MB, Pigeon C, Alizadeh M, Leroyer P, et al. Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. FEBS Lett 2003;542:22-26.

[6] Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365-386.

Supplementary Figure legends

Supplementary Fig. 1. Typical morphology of wild-type and *Nrf2*$^{-/-}$ hepatocytes incubated with ferric ammonium citrate (FAC) or ammonium citrate (AC). Hepatocytes were isolated from the mouse liver as described in Materials and Methods and plated on a 12-well plate. Cells were allowed to adhere for 2 h, after which the medium was replaced by fresh medium containing either 22.9 µg/ml FAC or AC. The images were captured at the end of 24 h of incubation. FAC treatment caused morphological alterations that included rounding up of *Nrf2*$^{-/-}$ hepatocytes.

Supplementary Fig. 2. Oncotic necrosis of hepatocytes and severe necroinflammation in the liver of *Nrf2*$^{-/-}$ mice fed iron-rich diet. *Nrf2*$^{-/-}$ mice were fed iron-rich diet for 2 weeks. H&E stain of liver sections depicting representative histopathological features is shown. Oncotic necrosis of hepatocytes is illustrated by increased eosinophilia (A-D), and cell swelling with cytoplasmic clumping and lysis (A). Nuclei in these hepatocytes were either in advanced stage of karyolysis or completely absent (B-C). Confluent areas of necrosis resulted in loss of architecture and hemorrhage (B-C). Necrosis was associated with infiltrates of mononuclear cells into the adjacent parenchyma and destruction of individual hepatocytes along the edges of the portal tract (D). Although less frequent, apoptotic cells were found, as illustrated by the presence of a round, hypereosinophilic cytoplasm surrounded by a clear halo resulting from fragmentation and contraction of the dying hepatocyte (acidophilic bodies) (arrows).

Supplementary Fig. 3. Detection of DNA fragmentation and caspase 3 activation in the liver of wild-type and *Nrf2*$^{-/-}$ mice fed iron-rich diet. Wild-type or *Nrf2*$^{-/-}$ mice were fed iron-rich diet for 2 weeks. (A) Extensive TUNEL staining of contiguous hepatocytes is shown in the liver of two *Nrf2*$^{-/-}$ animals fed iron-rich diet. As depicted, only a few scattered cells display activation of caspase 3 (arrows). (B) As a positive control for the anti-cleaved caspase 3 antibody, we used Jurkat cells that were either left untreated (left) or treated with the topoisomerase II inhibitor drug etoposide at 25 μM for 5h (right).