*Putative ferritin G-subunit*

Immunoprecipitates of ferritin from sera of WT, IRP2−/− and Fe-dextran overloaded mice were separated on SDS-PAGE and proteins were stained with Coomassie or subjected to Western blot. A prominent band was detected with an approximate molecular weight of 25kD (p25) (Fig. S1). The expected 21 and 17 kD bands for serum ferritin were detected by Western blot of the same samples, as protein concentration was not high enough for detection by Coomassie staining in non-iron overloaded mouse-sera. Analysis of the 25 kD band by mass spectrometry showed that this band contained no ferritin and was identified as Apolipoprotein A1 (ApoA1). This implied that Apo A1 is associated with ferritin and may represent the earlier described G-subunit (1). In the sera of Fe-dextran overloaded mice, various amounts of Apo A1 were detected. We hypothesize that less ApoA1 is associated with serum ferritin in the highly iron overloaded mice because serum ferritin levels in these mice are extremely high, while the ApoA1 levels remain unchanged and therefore many ferritin molecules under these conditions may not be associated with ApoA1.

*Serum ferritin as iron transport protein*

To compare the iron content of serum ferritin with the iron transport capacity of transferrin, the following calculation was made: a normal (high-end) serum-ferritin concentration of 300ng/ml with an average iron content of 700 iron atoms/molecule (Fig. 3a) can provide $2 \times 10^{14}$ iron atoms/ml blood, while iron levels expected from transferrin would provide $2 \times 10^{16}$ iron atoms/ml blood (taking 5mg/ml transferrin and the lower end of transferrin saturation of 30% into account (2)). Very little is known about the distribution, affinity and prevalence of ferritin receptors, and therefore this calculation remains just a theoretical number for consideration. As stated earlier, we hypothesize that secreted ferritin may play a role in local iron transport in specific tissues.

*Mice*

In addition to wild type control and iron overloaded mice from the C57bl/6 strain we also used our IRP1−/− and IRP2−/− mice and Hfe−/− mice that were a generous gift of Joanne Levy (deceased) and Nancy Andrews (Pediatrics and Pharmacology & Cancer Biology, Duke University, Durham, North Carolina, USA). These mice were used for analysis of the tissue and serum evaluation of the S-/L-subunit ratio and for the quantification of serum ferritin by ELISA before and after ConA binding. Results of these experiments were identical to wt, in that we observed high representation of the S-subunit in sera and no ConA binding of mouse serum ferritin.

Mice used for splenectomy and sham operated mice contained loxP sites on both IRP genes. As these mice did not express CRE recombinase, IRP genes and ferritin were expressed in those mice normally, as in mice not genetically modified at all.

*Mass spectrometry (MS)*

Following gel filtration the aliquots of the fractionated serum ferritin were separated on reducing SDS-PAGE and stained with Coomassie Brilliant blue R-250. Relevant bands were cut out, digested by trypsin, analyzed by LC-MS/MS on LTQ-Orbitrap (Thermo) and identified by Pep-Miner and Sequest software against the human, mouse, rat, bovine and rabbit part of the non-redundant database.
Transmission Electron Microscopy
TEM analysis of liver ferritin and serum was performed in a Jeol 2000 FXII microscope operated at 200 kV. Samples were prepared by placing a drop of the liquid on a carbon-coated copper grid and leaving them to dry in a vacuum chamber for 4h. Kidney cortical sections and bone marrow derived macrophages were prepared for ultrastructural analysis by fixation with 2.5% glutaraldehyde and reduced osmium, followed by dehydration and embedding in Epon resin. Microtome sections were mounted on copper grids, stained with lead acetate, and analyzed with a Tecnai T12 microscope.

Immunoprecipitation (IP)
Sera were buffered with IP-lysis buffer to a final concentration of 10mM Tris-HCl pH7.5, 150mM NaCl, 1% Triton-X-100 and Protease-inhibitor-cocktail (Complete without EDTA, Roche) and incubated with Sepharose protein A beads (Amersham) for 1 hour to remove all proteins that bind non-specifically to the beads. Pre-cleared supernatant was incubated with 25µl slurry of Sepharose protein A beads bound to rabbit anti mouse liver-ferritin antibody (a kind gift of Dr. A.M. Konijn, Hebrew University of Jerusalem) for 2 hours. Beads were washed three times with 1% Triton buffer (20mM Tris-HCl pH8, 13.7mM NaCl, 1% Triton-X-100, 10% glycerol) and once with PBS. Immunoprecipitated samples were further analyzed by SDS-PAGE (14% gels) under reducing conditions either by protein staining or by Immunoblotting.

Immunoblot
Following fractionation on SDS-PAGE samples Immunoblot was performed as described (3). Primary antibody was polyclonal rabbit anti-mouse H-subunit ferritin antibody (produced from purified H-ferritin from a plasmid that was a kind gift from Dr. Paolo Santambrogio) or anti liver-ferritin antibody. Secondary Horseradish-peroxidase conjugated antibody (Amersham) and ECL (Pierce) were used according to manufacturer’s instruction.

Kidney and BMDM Immunofluorescence (IF)
Kidney IF was performed as described (4). BMDMs were fixed in 4% paraformaldehyde and incubated over night with 1:200 rat anti mouse LAMP1(abcam) and 1:400 rabbit anti mouse liver ferritin antibody. Alexa chicken anti rat 488 and goat anti rabbit 568 were used as secondary antibodies, diluted 1:1000 and incubated for 1 hour. Negative controls were done with secondary antibodies only and one primary antibody followed by both secondary antibodies. Image visualization on an LSM 510 META laser scanning confocal microscope from Zeiss with a Plan-Apochromat 63×/1.4 oil DIC lens and scan zoom 2.0, resulting in a final magnification of ×126. Pinhole aperture for green channel: 108µm and red channel 116µM. Acquisition software: LSM5 software program.

Iron assays: Ferrozine-assay
Ferritin was immunoprecipitated as above and eluted into 50µl SDS sample buffer with mercaptoethanol without bromophenol-blue at 95°C for 5 minutes. 11µl concentrated HCl (11.6 M) were added to 50µl eluate and incubated at 95°C for 20 minutes. After centrifugation for 10 min. at 14 000 rpm, 45µl of supernatant were added to 18µl ascorbate (75 mM) to reduce the iron. 18µl ferrozine (10 mM) was added and the reaction was stopped with 36µl saturated ammonium acetate (NH₄OAc). Absorbance of the samples was measured at 562 nm and compared to a standard curve.
**Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)**

Elemental analysis was performed after microwave acid digestion with a solution 4:1 (v/v) of HNO₃ (65%) and H₂O₂ (30%) of the same freeze-dried samples that were magnetically characterized. The elemental analysis was focused on the determination of iron, but the presence of other magnetogenic elements as Co, Ni, Mn and Cu, which might contribute to the magnetic susceptibility, was included in the analysis.

**Macrophage-related experiments**

Primary cultures of bone-marrow derived macrophages (BMDMs) were prepared, metabolically labeled and ferritin was immunoprecipitated as described (3). ⁵⁵Fe-transferrin was prepared as in (5) and splenectomy was performed as described in (6).

**Data analysis and densitometry**

Average and standard deviation was done with Excel and for densitometry films were scanned into .jpg format using an HP Officejet 6213, and images were analyzed and quantified in Adobe Photoshop following the method outlined at [http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html](http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html).

**REFERENCES**

Figure S1. A putative G-subunit is not ferritin
Ferritin was immunoprecipitated with an anti mouse liver ferritin antibody from serum of wild type, \( IRP2^{-/-} \) and chemically iron overloaded mice. Immunoprecipitate was separated by SDS-PAGE and stained with Coomassie or analyzed by Western blot with the same antibody. P25 was cut from the coomassie gel and analyzed by mass spectrometry.