

Supporting Information

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SI Materials and Methods

Mice. IRP2^{-/-} mice were generated, propagated by breeding, and genotyped as described in ref. 1. Mice used in this study have a 129S4/SvJae × C57BL/6 mixed background (specific proportions of each strain are not known). Siblings were used to minimize phenotypic variation because of differences in genetic background in diet experiments. Mice of different genotypes and on different diets were age and sex matched. All protocols were approved by the National Institute of Child Health and Human Development Animal Care and Use Committee and met National Institutes of Health guidelines for the humane care of animals.

Chemicals. The synthetic [4Fe-4S] cluster (Bu₄N)₂[Fe₄S₄(SEt)₄] (2) was a generous gift from Richard Holm and Tom Scott (Harvard University, Cambridge, MA).

Diet Description. The mice were weaned at 3–4 weeks of age. Immediately after weaning, mice were maintained on either a Tempol or control diet. In the Tempol diet, powdered Tempol was uniformly mixed with bacon-flavored mouse chow (Bio-Serv) by a cold press technique (described at <http://www.bio-serv.com/newcatalog/mdfeed/rodent/meddiets.html>) at a concentration of 10 mg/g of food. Bacon-flavored chow without Tempol was used as a control diet. In the first batch, three mice each of age- and gender-matched wild-type, IRP2^{-/-}, and IRP1^{+/-} IRP2^{-/-} siblings were put in a control (without Tempol) group and three each in a Tempol group for 14 mo. Subsequently, four more mice for each category were studied for 8 mo. Because Tempol supplementation was found to prevent deterioration in the hang-time tests of IRP2^{-/-} but not IRP1^{+/-} IRP2^{-/-} mice, in the next batch we used six each of wild-type and IRP2^{-/-} mice on a control or Tempol diet for 9 months. In the last batch, we studied 11 each of wild-type and IRP2^{-/-} mice on a control or Tempol diet for 8 months. Two sets of these 24 had to be discontinued because of the death of one or more mice in these sets. The results for each time point during a diet trial were collated and graphed and statistics performed with the Student's *t* test. These results were combined according to age in Figs. 1 and 6C.

Assay of Tissue Tempol Concentrations. Female C3H/Hen mice at 9 wks of age were placed on a Tempol diet (10 mg of Tempol/g of food). After 2 weeks on this diet, blood and tissue were collected and wet weight was determined for total Tempol concentration as described in ref. 3. Briefly, blood samples were diluted and tissue samples were homogenized with a 4-fold volume of PBS. Ferricyanide (200 μ l of 10 mmol/liter solution) was added to each sample solution (800 μ l) to obtain the final concentration of 2 mmol/liter. The ferricyanide quantitatively converts the hydroxylamine produced as a result of *in vivo* reduction back to the oxidized form. The signal intensities of the 100- μ l samples were measured by using a Varian E-9 X-band electron paramagnetic resonance (EPR) spectrometer. The EPR spectrometer operating conditions were: modulation frequency, 100 kHz; microwave power, 1 mW; modulation amplitude, 1 G. Because there is linearity up to 2 mmol/liter between the concentration and EPR signal magnitude of Tempol, EPR signal magnitudes of homogenate mixtures were converted to the concentration by using previously obtained standard curves (100 μ mol/liter–2 mmol/liter). Tempol concentrations were determined for five

mice in heart, adipose, brain, kidney, and blood tissues, and the mean values and standard deviations were calculated.

Hang Test. In the hang test, mice were allowed to grip a wire mesh square that was then inverted. The length of time that a mouse could hang on to an inverted wire mesh square before falling (up to a maximum of 60 sec) was measured and recorded as described in ref. 4.

Tissue and Lysate Preparation. Animals were killed, and tissues were frozen in liquid N₂ immediately after harvesting and stored at –80°C under argon. Experiments were performed on tissues that were pulverized in liquid N₂-cooled mortars in an anaerobic chamber and then lysed in lysis buffer that was deaerated by cyclic freeze-thaw and air-removal with argon. Nuclei and debris were removed by centrifugation. Preparations of lysates for assays of IRP1 activity, Western blot analysis, carbonyl assay, and protein analysis were performed anaerobically.

Cells. Embryonic fibroblasts of 13-d-old embryos were isolated from wild-type, IRP1^{-/-}, and IRP2^{-/-} mice as described in ref. 1. The *myc*-tagged HEK293 Tet-on cell line, in which IRP2 expression was inducible, was prepared and cultured as described in ref. 5. Erythroblasts were harvested from bone marrow and purified as described in ref. 6.

RNA Mobility Shift Assays. Gel retardation assays were performed as described in ref. 7. Tissue lysates were prepared in an anaerobic chamber as described above in oxygen-depleted lysis buffer containing 10 mM Hepes (pH 7.2), 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 0.2% Nonidet P-40, 5 mM DTT, 1 mM the protease inhibitor AEBSF, 10 μ g/ml Leupeptin and Complete EDTA-free protease inhibitor mixture (Roche Applied Science). Lysate (*x* μ l) containing 10 μ g of total protein was added to (12.5 – *x*) μ l of bandshift buffer containing 25 mM Tris·HCl (pH 7.5) and 40 mM KCl. The samples were incubated for 5 min at room temperature (RT) with 12.5 μ l of a reaction mixture containing 20% glycerol, 0.2 units/ μ l Super RNAsin (Ambion), 0.6 μ g/ μ l yeast t-RNA, 5 mM DTT, and 20 nM ³²P-labeled IRE from human ferritin H chain gene in 25 mM Tris·HCl (pH 7.5) and 40 mM KCl. A measure of 20 μ l of this reaction mixture was loaded into a 10% acrylamide/TBE gel, which was run at 200 V for 2.15 h, and then the gel was fixed, dried, and exposed for autoradiography. Quantification was performed with the ImageQuant for Macintosh (GE Healthcare).

Western Blot Analysis and Antibodies. Protein analysis with the anaerobic lysates was carried out as described in ref. 1. Equal amounts of protein (20–40 μ g/lane) were separated on 13% SDS-PAGE gels and transferred to nitrocellulose membranes. The membrane was blocked with 5% nonfat milk and 0.1% Triton X-100 in PBS and probed at RT in the same blocking buffer. IRP1 antibody was prepared against purified hIRP1 and used at a 1:5,000 dilution. L-ferritin antiserum was raised in rabbit against L-ferritin protein purified from mouse livers. A mouse monoclonal TfR antibody (Zymed) was used at a 1:2,000 dilution. Monoclonal anti- α -tubulin and anti- β -actin antibodies (Sigma) were each used at a 1:5,000 dilution. Western blots were treated with secondary peroxidase-conjugated goat anti-rabbit IgG or sheep anti-mouse IgG antibodies (GE Healthcare) at 1:5,000 and 1:2,000 dilutions, respectively. Western blots were

developed by using ECL (Pierce). Quantification was performed with the National Institutes of Health Image program.

Aconitase Assay. Aconitase activity gels for human lysates were performed as described in ref. 8, and aconitase activity gels for mouse lysates were performed with the following modifications. The gel was composed of a separating gel containing 6% acrylamide, 132 mM Tris base, 66 mM borate, 3.6 mM citrate, and a stacking gel containing 4% acrylamide, 66 mM Tris base, 33 mM borate, and 3.6 mM citrate. The running buffer contained 25 mM Tris (pH 8.3), 96 mM glycine, and 3.6 mM citrate. Electrophoresis was carried out at 170 V at 4°C for 3.5 h.

Spectrophotometric Study on the Effect of Tempol on a [4Fe–4S] Cluster. In an anaerobic chamber, solid $(\text{Bu}_4\text{N})_2[\text{Fe}_4\text{S}_4(\text{SEt})_4]$ (9) was dissolved in deaerated acetonitrile to make a $\approx 60 \mu\text{M}$ solution, which was then divided into two aliquots. A solution of Tempol made in anaerobic acetonitrile was added to the second fraction to make a final concentration of 9 mM Tempol. These solutions of [4Fe–4S] cluster without (first fraction) or with (second fraction) Tempol were then transferred into two quartz cuvettes, which were closed with rubber septum caps and Parafilm. These cuvettes were then taken out of the anaerobic chamber, and the spectral changes (250–800 nm) were monitored at RT (20°C) at 20 min intervals.

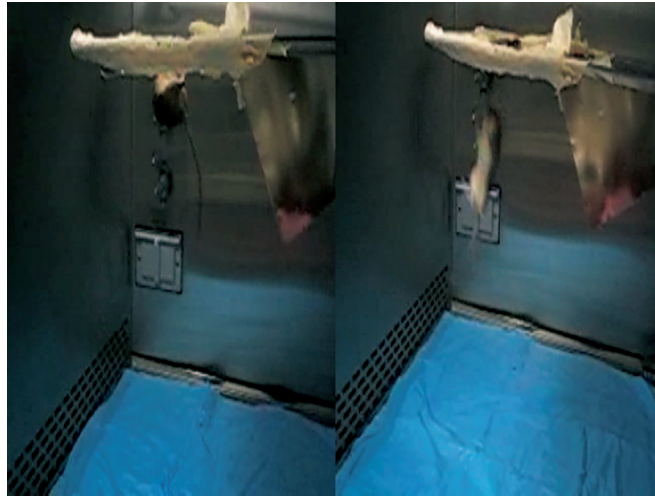
Immunohistochemistry. Ferritin immunohistochemistry was performed as described in ref. 10. Paraffin-embedded tissue sections

were boiled in a microwave oven for 15 min for antigen retrieval in 10 mM citrate buffer (pH 6.4) after dewaxing and rehydrating. The sections were blocked in blocking buffer [Tris-buffered saline (pH 7.4), 5% normal goat serum, 0.1% Tween-20] for 30 min and incubated with polyclonal rabbit anti-ferritin antisera for 2 h at RT, and then the protein-antibody complex was labeled with Cy3-donkey anti-rabbit antibody (Jackson ImmunoResearch), and nuclei were labeled with DAPI. The slides were observed and the pictures were recorded with a Nikon Eclipse E600 fluorescence microscope.

Perls' 3,3'-Diaminobenzidine (DAB) Iron Staining. After dewaxing and rehydrating, paraffin-embedded tissue sections were stained in prewarmed staining solution (5% potassium ferrocyanide, 15.0 ml; 5% hydrochloric acid, 15.0 ml; heated for 15 sec in a microwave oven) for 5 min. The sections were rinsed with distilled (10) and Tris-buffered saline (pH 7.4), then were stained with DAB staining solution [20 mg of DAB, 50 μl of 30% H_2O_2 , 20 ml of Tris-buffered saline (pH 7.4)] for 30 min at RT. After being rinsed with distilled water, the sections were mounted with Crystal Mount mounting solution (Sigma) and were observed, and pictures were taken with a Nikon Eclipse E600 fluorescence microscope.

Statistics. We tested differences between means of hang-time by a paired Student's *t* test. Results with $P < 0.05$ were considered statistically significant.

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Movie S1. The IRP2^{-/-} mouse shows compromise of neuromuscular function in comparison with the wild-type mouse. Wild-type (*Left*) and IRP2^{-/-} (*Right*) mice were hang-tested by allowing them to grip a wire mesh, which was then slowly inverted. The IRP2^{-/-} mouse lost its grip and fell after 11 seconds, whereas its wild-type counterpart was able to retain its grip for a full minute, a finding that was reproducible and for which statistics are shown in Figs. 1 and 6C.

[Movie S1 \(AVI\)](#)