

The role of iron regulatory proteins in mammalian iron homeostasis and disease

Tracey A Rouault

Iron regulatory proteins 1 and 2 (IRP1 and IRP2) are mammalian proteins that register cytosolic iron concentrations and post-transcriptionally regulate expression of iron metabolism genes to optimize cellular iron availability. In iron-deficient cells, IRPs bind to iron-responsive elements (IREs) found in the mRNAs of ferritin, the transferrin receptor and other iron metabolism transcripts, thereby enhancing iron uptake and decreasing iron sequestration. IRP1 registers cytosolic iron status mainly through an iron-sulfur switch mechanism, alternating between an active cytosolic aconitase form with an iron-sulfur cluster ligated to its active site and an apoprotein form that binds IREs. Although IRP2 is homologous to IRP1, IRP2 activity is regulated primarily by iron-dependent degradation through the ubiquitin-proteasomal system in iron-replete cells. Targeted deletions of IRP1 and IRP2 in animals have demonstrated that IRP2 is the chief physiologic iron sensor. The physiological role of the IRP-IRE system is illustrated by (i) hereditary hyperferritinemia cataract syndrome, a human disease in which ferritin L-chain IRE mutations interfere with IRP binding and appropriate translational repression, and (ii) a syndrome of progressive neurodegenerative disease and anemia that develops in adult mice lacking IRP2. The early death of mouse embryos that lack both IRP1 and IRP2 suggests a central role for IRP-mediated regulation in cellular viability.

Iron is indispensable for the function of many prosthetic groups, including heme and iron-sulfur clusters, and animals have accordingly developed sophisticated systems to maintain iron homeostasis. Cellular iron uptake, distribution and export must be tightly regulated, as insufficient iron concentrations impair the function of numerous iron proteins, whereas excess free iron can oxidize and damage the protein, nucleic acid and lipid contents of cells. In humans, iron deficiency is the most common cause of anemia in the world, and it interferes significantly with normal cognitive development in children. Conversely, the iron overload observed in common diseases such as hemochromatosis and thalassemia causes liver and heart failure. Thus, organisms and individual cells must regulate iron metabolism to ensure that sufficient iron is provided to supply heme, the iron-sulfur prosthetic groups of

mitochondrial respiratory-chain complexes, and cellular iron enzymes such as ribonucleotide reductase¹.

In mammals, dietary iron uptake across the duodenal mucosa is regulated through the expression of the intramembrane metal transporters ferroportin and divalent metal transporter 1 (DMT1) in response to signals from the liver^{1,2}. After iron has traversed the intestinal mucosa, it enters the bloodstream and binds tightly to transferrin³. Most cells can effectively control iron uptake by regulating the amount of transferrin receptor (TfR1)⁴ that is expressed on their plasma membranes. In addition, cells regulate expression of the cytosolic iron-sequestration protein ferritin⁵ as well as numerous other proteins to optimize availability of cytosolic iron.

In mammalian cells, the IRPs (which are derived from a duplicated gene pair) register metal availability mainly through direct interactions with iron in the cytosol^{6,7}. In iron-depleted cells, IRPs bind to IREs, RNA elements within mRNAs that encode ferritin, transferrin receptor and many other transcripts, and this IRP binding represses translation (**Fig. 1a**) or prolongs mRNA half-life (**Fig. 1b**) depending on where the IRP binds on the mRNA^{7,8}. The IRP-IRE regulatory system enables cells to rapidly adjust concentrations of available cytosolic iron and thereby optimize the functioning of numerous iron-dependent cellular components.

IRPs bind to IREs in iron metabolism transcripts

IREs are highly conserved IRP binding sites that are found in the 5' untranslated region (UTR) of transcripts that encode the H and L ferritin subunits (H, highly expressed in heart; L, highly expressed in liver), in the 3' UTR of TfR1 and in several other iron metabolism genes⁸. IRP1 and IRP2 are ubiquitously expressed mammalian members of the aconitase gene family⁹ that have adapted to sense cytosolic iron concentrations and accordingly modify gene expression. Aconitases are enzymes found in virtually all organisms that convert citrate to isocitrate through the intermediate *cis*-aconitate in the citric acid cycle, the central cycle of intermediary metabolism. In aconitases, a cubane iron-sulfur cluster is ligated by three cysteines within the enzymatic active site cleft, and the fourth iron of the cubane cluster binds substrate¹⁰. IRP1 and IRP2 are highly related to the aconitase family, but only IRP1 has retained its ability to function as an aconitase, whereas IRP2 apparently lost its aconitase activity sometime during evolution^{6,7}.

In cells that are depleted of iron, each IRP responds by binding to IREs (**Fig. 1**). By binding to a single IRE located in the 5' UTR of an mRNA, the IRP prevents translation of the mRNA (**Fig. 1a**), whereas by binding to IREs in the 3' UTR, the IRP protects the TfR transcript from endonucleolytic cleavage and degradation (**Fig. 1b**). In cells that are iron replete, IRPs do not bind IREs, and ferritin and other transcripts

Tracey A. Rouault is in the Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, Building 18T, Room 101, National Institutes of Health, Bethesda, Maryland 20892, USA.
e-mail: trou@helix.nih.gov

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that have an IRE in the 5' UTR are freely translated. Conversely, the TfR transcript undergoes cleavage by an uncharacterized endonuclease at a specific site flanked by IREs in the 3' UTR, and the cleavage products are subsequently degraded.

Acquisition of IRE binding ability by IRP1 may have occurred through natural selection: accumulation of IRP1 lacking an iron-sulfur cluster in iron-deficient cells could have allowed the apoprotein to bind to RNA stem-loop structures of appropriate size and shape that appeared in the highly mutable UTRs of some mRNAs. When expression from transcripts encoding iron metabolism proteins was favorably affected, IRP binding could have resulted in selective retention of the IRE within the transcript. Selection of IREs may be an ongoing evolutionary process, given that UTR sequences are not constrained by having to encode protein.

IRE stem loops have conserved sequence features, including a six-member loop in which the sequence of the first five residues is usually CAGUG, and upper and lower stems that are separated from one another by an unpaired cytosine on the 5' side (Fig. 2)^{11,12}. Fully functional IREs are likely to have longer lower stems than those depicted here, perhaps to allow more extensive interactions of the RNA with IRE-binding proteins (that is, IRPs) or to stabilize the conformation of the IRE stem loop^{13,14}. Base pairing between loop residues 1 and 5 of the IRE stabilizes the loop structure¹¹, and *in vitro* binding studies have shown that other residues may functionally substitute for the C1-G5 base pair of the loop if base pairing is maintained^{15,16}.

The structure of the IRE seems to function as a 'molecular ruler' that preserves a specific distance and spatial orientation between IRE residues that may directly and specifically contact IRE-binding proteins. Possible candidates for such specific contacts include the G3 of the loop, which has an unusual *syn* conformation in the NMR structure, and the bulge cytosine that separates the upper and lower stems¹¹, though substitution of other residues in the bulge position is most likely compatible with high-affinity binding¹⁷, and it is possible that IRP1 and IRP2 have individual specific targets that vary from the consensus sequence^{15,16,18}. Ferritin IREs have a more complex bulge and a lower stem, and they are often represented with a three-residue bulge^{19,20}, although data from selection studies¹⁶ and analysis of human mutations^{14,21} strongly imply that the middle residue of the proposed bulge forms a base pair with a residue on the opposite side of the stem (Fig. 3).

After initial characterization of the IRE stem-loop structure^{22,23}, IREs have been found in the 5' UTRs of the erythrocytic form of aminolevulinic acid synthase (eALAS, which catalyzes the first step in heme synthesis)^{24,25}, in mammalian mitochondrial aconitase²⁶⁻²⁸, in the succinate dehydrogenase b subunit of *Drosophila melanogaster*²⁹ and in the iron

exporter ferroportin³⁰. IREs have also been found in the 3' UTR of one isoform of the ferrous iron transporter, DMT1 (ref. 31), and in mammalian glycolate oxidase³² (also known as hydroxyacid oxidase 2), but it is not yet clear whether these IREs contribute to regulation of expression of DMT1 (ref. 33) or glycolate oxidase³⁴.

IRE mutations cause hyperferritinemia and cataract syndrome

Members of families with mutations in the IRE of the ferritin L chain have high serum concentrations of ferritin and are prone to development of early-onset cataracts. Since the initial description of the hereditary

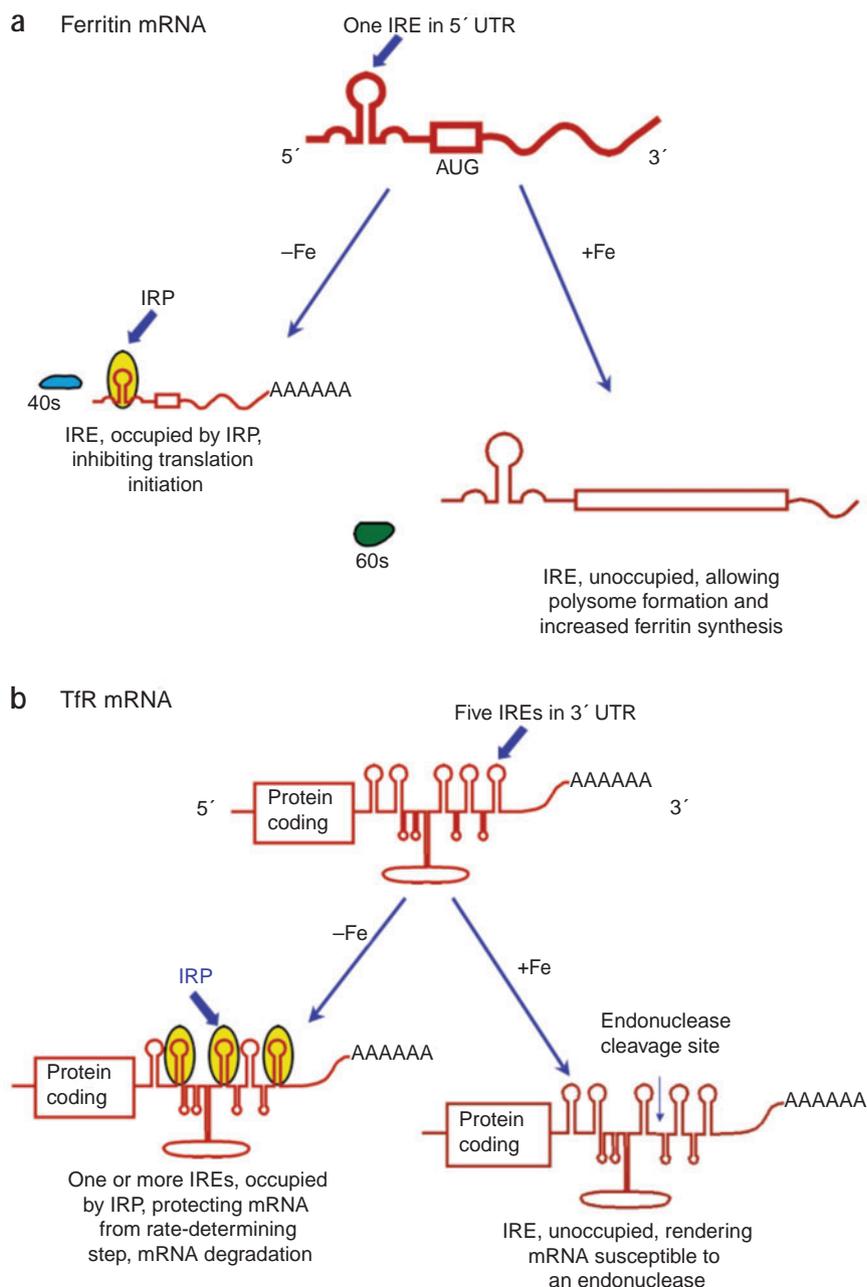


Figure 1 Ferritin translation and TfR mRNA degradation are regulated by IRP binding. (a) In iron-depleted cells, IRP binding to the IRE in the 5' UTR interferes with translational initiation. (b) Binding of IRPs to IREs in the TfR 3' UTR protects the transcript from endonucleolytic cleavage and degradation. The arrow marks the site at which an unknown endonuclease cleaves the TfR transcript in iron-replete cells when it is unprotected by IRP binding⁶.

hyperferritinemia cataract syndrome (HHCS)³⁵, numerous familial mutations have been described^{21,36}, and the degree of hyperferritinemia and cataract severity has been correlated with the degree to which the mutation impairs IRP binding¹⁴. Because patients with HHCS have high concentrations of ferritin in their blood, candidates for HHCS are easily identified by blood tests, and many mutations of the ferritin L-chain IRE have been described, some of which are depicted in **Figure 3**. Mutations of the ferritin L-chain IRE confer a dominant phenotype, because IRP binding to transcripts from the mutant allele is partially or completely attenuated, and ferritin L-chain expression increases even though translation of the other ferritin L-chain allele is repressed normally. Thus, HHCS illustrates the importance of ferritin translational repression in normal physiology.

The structure of IRP1 and formation of the IRP-IRE complex

In mammalian cells, IRP1 and IRP2 bind to IREs and modify expression of ferritin, TFR and other transcripts in iron-depleted cells. Investigators originally purified and cloned IRP1 by using its ability to bind IREs as an assay^{37–40}, and they were able to clone IRP2 simultaneously with IRP1 because of sequence homology³⁷, although IRP2's role as a second IRE-binding protein was not recognized for several years^{41,42}. IRP1 and IRP2 are derived from duplicated mammalian genes, and the human forms are 56% identical to one another. IRP1 is a functional aconitase that interconverts citrate and isocitrate in the cytosol^{43,44}. This reaction is also catalyzed in the mitochondrial matrix by mitochondrial aconitase, a key enzyme of the citric acid cycle that is encoded by a separate gene⁴⁵. IRP1 contains a [4Fe-4S] cluster that is ligated to the active site by binding of Cys437, Cys503 and Cys506 to three iron atoms of the cluster, whereas the fourth iron binds solvent and substrate^{10,44}.

The recent solution of the crystal structure of IRP1 (ref. 46) reveals that the structure of IRP1 is very similar to that of mitochondrial aconitase⁴⁷, as was predicted on the basis of sequence conservation between IRP1 and mitochondrial aconitase¹⁰. When IRP1 loses its iron-sulfur cluster, it acquires the ability to bind to IREs with high affinity, and when the cysteines that ligate the iron-sulfur cluster are mutagenized, IRP1 becomes a constitutive IRE-binding protein^{48,49} that stabilizes Tfr mRNA and represses ferritin synthesis in cells^{50,51}. The iron-sulfur cluster of IRP1 is readily destabilized by oxidants^{52,53}, including nitric oxide⁵⁴ and hydrogen peroxide⁵⁵, and cluster degradation produces apoprotein that binds IREs and lacks iron and sulfur (**Fig. 4a**)^{44,56}.

Numerous cross-linking, mutagenesis and chemical modification studies have indicated that the IRE binding site of IRP1 overlaps with the aconitase active site^{48,49,57}. Given the known dimensions of several RNA stem-loop structures, it was hypothesized that IRE binding could occur within the active site cleft of IRP1 if the fourth domain of the protein, which is connected to domains one through three by a flexible hinge linker, were to swing open. Further mutagenesis⁵⁸ and footprinting of the IRE-IRP interactions⁵⁹ indicated that residues near the entrance to the active site cleft on both sides are important in IRE binding. **Figure 4b** shows the structure of IRP1 with an intact iron-sulfur cluster⁴⁶ as well as another view of the protein in which domain 4 has been displaced to accommodate docking of the IRE (created

according to the dimensions and shape determined in its NMR structure solution)¹¹. The crystal structure of the holoprotein shows a closed conformation that does not allow substrates to access the active site, unless dynamic motion of the structure allows small interdomain shifts within the holoprotein, as was previously predicted¹⁰. Notably, researchers have identified a partially connected network of hydrophilic cavities in the crystal structure of IRP1 at the domain interfacial region, and Dupuy *et al.* have proposed that these channel-like structural features may be involved in guiding the movement of substrates and products to and from the active site, perhaps aided by a small rotation of domain 4. The IRE has a shape much like that of the portion of domain 4 that faces domains 1–3, thereby enabling the IRE to potentially substitute for domain 4 and interact with the portions of domains 1–3 that form the active site cleft. In addition, electrostatic modeling of domain 4 has revealed numerous positive charges on the domain 4 surface that normally faces the active site cleft, and these positively charged residues may bind to the negatively charged phosphate backbone of the IRE in the IRP-IRE complex. Thus, one side of the IRE appears to mimic the size and conformational features of domain 4, allowing it to substitute for domain 4 by forming favorable contacts with domains 1–3, while on the opposite side of the IRE, charge interactions may allow the IRE to bind to the displaced fourth domain. Crystallization of the IRP-IRE complex⁶⁰ and solution of the crystal structure should allow a detailed characterization of the RNA binding site of IRP1.

Both IRP1 and IRP2 bind IREs with high affinity in iron-depleted cells, as demonstrated in many different cell lines by gel-shift assays that use radiolabeled IRE to reveal binding activity. Efforts to elucidate the mechanisms by which IRPs register cytosolic iron concentrations have been ongoing since their initial characterization.

IRP1 has an iron-sulfur switch that determines its activity

Researchers recognized a possible mechanism for regulation of the IRE-binding activity of IRP1 when it became apparent that IRP1 alternates between two major forms: the cytosolic aconitase form, which contains a [4Fe-4S] cluster bound to the enzymatic active site and which does not

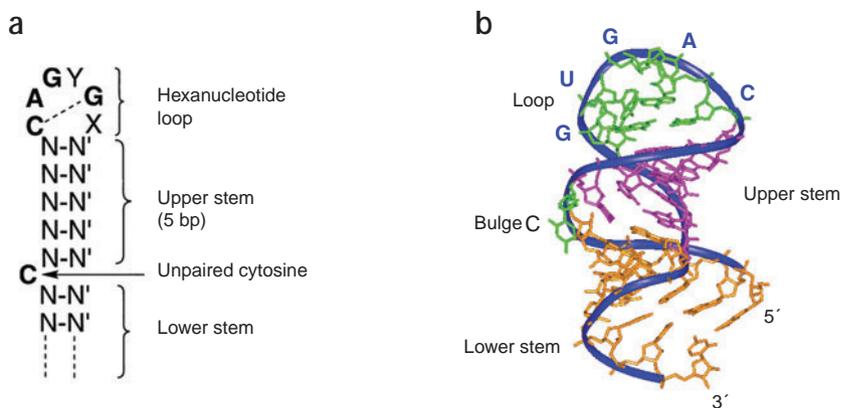


Figure 2 IRE secondary structure. (a) A schematic of a consensus IRE is shown. The IRE contains a six-residue loop, usually with the sequence CAGYGX, where Y represents U or C and X represents any residue except G. The upper and lower stems are composed of base pairs (bp) of variable sequence (N-N') that are separated by an unpaired C. (b) In the NMR solution structure of a consensus IRE, a bp forms between C1 and G5, and A2 stacks on G5 in the conserved loop sequence CAGUGX (modified from ref. 11, courtesy of K. Address, with permission). The helical upper and lower stems have an A-form conformation, and both the bulge C and the unpaired G residue at position 3 in the loop are disordered in solution. The 5-bp upper stem most likely functions as a molecular ruler that orients and correctly distances the bulge C from residues in the loop, allowing flexible residues to participate in sequence-specific interactions between the IRE and IRPs.

bind IREs, and the apoprotein form, which lacks an iron-sulfur cluster and binds IREs. It is now well accepted that the 'iron-sulfur cluster switch' is an important determinant of whether IRP1 has cytosolic aconitase or IRE-binding activity^{1,6,7,53,61}. Synthesis of the iron-sulfur cluster requires iron, sulfur and dedicated biosynthetic proteins, whereas cluster turnover results mainly from cluster oxidation and spontaneous degradation. The strength and capacity of the forces that enhance new synthesis relative to that of the forces that favor cluster degradation determine the way in which IRP1 functions (Fig. 4a). Thus, to understand IRP1 function, it is important to understand the factors involved in assembly and turnover of iron-sulfur clusters in mammalian cells.

Mammalian iron-sulfur cluster assembly

Assembly of iron-sulfur clusters is a complex process involving many enzymes and scaffold proteins^{62,63}, and mammalian iron-sulfur clusters are synthesized by homologs of bacterial and yeast proteins⁶⁴ (Fig. 5). Through alternative splicing and alternative initiation, mammalian cells generate cytosolic isoforms of iron-sulfur cluster assembly proteins that directly facilitate assembly of the iron-sulfur cluster of IRP1 in the cytosol^{65,66}. Mammalian iron-sulfur assembly proteins known to be present in both mitochondria and cytosol include the cysteine desulfurase that provides sulfur in the correct oxidation state⁶⁷, the primary scaffold on which nascent clusters are assembled (iron sulfur cluster U assembly protein)⁶⁸ and the scaffold protein NFU (a protein required for maturation of FeS clusters of nitrogenase in nitrogen-fixing bacteria)⁶⁹. Other proteins involved in iron-sulfur cluster assembly include (i) frataxin, which may serve as an iron donor, (ii) reducing proteins such as ferredoxin and glutaredoxin and (iii) chaperone and co-chaperone proteins, which facilitate protein folding reactions when nascent clusters are transferred from scaffold to recipient proteins (Fig. 5). Clearly, iron-sulfur cluster assembly requires sufficient sources of sulfur and iron, and deficiency of iron-sulfur cluster assembly proteins, cysteine or iron can potentially inhibit iron-sulfur cluster assembly. Expression of the iron-sulfur cluster assembly scaffold protein ISCU decreases in iron-deficient cells⁶⁵, thereby offering the possibility that the iron-sulfur switch of IRP1 may be regulated not only through iron availability, but also through regulation of the expression of iron-sulfur cluster assembly proteins.

There are several recent examples in which impairment of the iron-sulfur cluster assembly machinery activates the IRE binding activity of IRP1 in a physiologically relevant setting. In zebrafish, mutations in glutaredoxin 5 (an enzyme important in iron-sulfur cluster biogenesis)^{70,71} cause heme deficiency, profound anemia and early death. Rescue experiments indicate that the first step of heme biosynthesis is repressed by binding of IRP1 to the eALAS transcript of zebrafish, which, as in mammals, contains an IRE in its 5' UTR. The zebrafish studies establish an important genetic linkage between heme biosynthesis and the iron-sulfur cluster biogenesis of IRP1 (ref. 72), and they constitute an important independent confirmation of the iron-sulfur switch model of IRP1 regulation⁷³. In mammalian cells, knockdown of ISCU markedly reduces mitochondrial aconitase activity and shifts IRP1 from the aconitase form to the IRE-binding form. Moreover, specific knockdown of the cytosolic ISCU isoform shows that the iron-sulfur switch of IRP1 can be activated by interfering directly with the cytosolic iron-sulfur cluster assembly machinery⁶⁵. However, it is not yet clear how many iron-sulfur assembly proteins are present in mammalian cytosol.

Iron-sulfur cluster disassembly

The state of the iron-sulfur switch of IRP1 depends not only on synthesis of the iron-sulfur cluster but also on the rate of cluster turnover. The iron-sulfur cluster of IRP1 can be oxidized and destabilized by

oxygen, nitric oxide and peroxynitrite⁷⁴, and it can be indirectly disassembled by hydrogen peroxide-mediated activation of a signaling pathway⁷⁵. In macrophages in which IRP1 could be exposed to endogenously generated reactive oxygen species and nitric oxide, there is little apparent cytosolic aconitase activity⁷⁶. Notably, silencing of cytosolic superoxide dismutase in *Drosophila melanogaster* results in activation of cytosolic aconitase but not mitochondrial aconitase, whereas silencing of mitochondrial superoxide dismutase (SOD) decreases mitochondrial but not cytosolic aconitase activity⁷⁷, implying that superoxide can be an important cause of intracellular iron-sulfur cluster disassembly. Similarly, in mice with cytosolic SOD deficiency, cytosolic aconitase activity decreases as IRE binding activity concomitantly increases⁷⁸. In yeast with cytosolic SOD deficiency, iron-sulfur enzymes in both the cytosol and the mitochondria have decreased activity⁷⁹. Thus, results in fly, yeast and mouse model systems implicate superoxide as a potentially potent cause for disassembly of the iron-sulfur clusters of both mitochondrial and cytosolic aconitases.

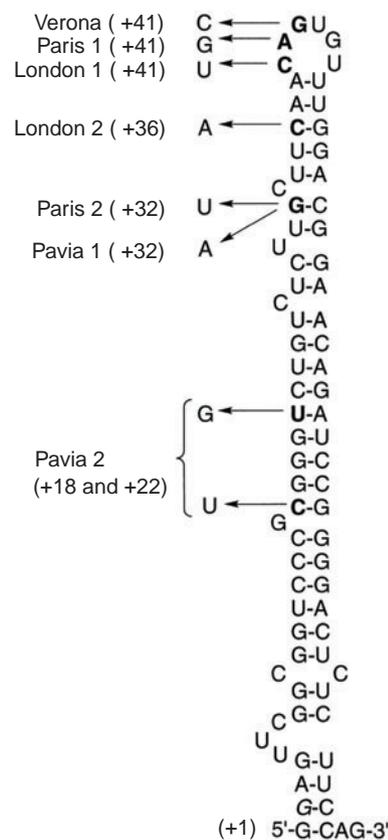


Figure 3 Mutations of the ferritin L-chain IRE cause HHCS. Multiple mutations in the ferritin L-chain transcript interfere with IRP binding, resulting in derepression of ferritin L-chain translation, high serum ferritin concentrations and cataract formation. The ferritin L-chain transcriptional start site (+1) is shown, and the first 77 nucleotides are depicted in an extended stem-loop structure with residues numbered in sequence¹⁴. Multiple mutations are labeled to show the nucleotide change, along with the name of the city in which patients affected by the mutation were first identified. Mutations affect the loop and the upper and lower stems, including a G32U substitution in the Paris 2 mutation and a G32A substitution in the Pavia 1 mutation (shown), which is also the site of a recently described G32C mutation²¹ that affects the potential base pair formation of G32 within a region of the ferritin IRE that is often represented as an unpaired residue within a three-nucleotide 'bulge'¹⁴.

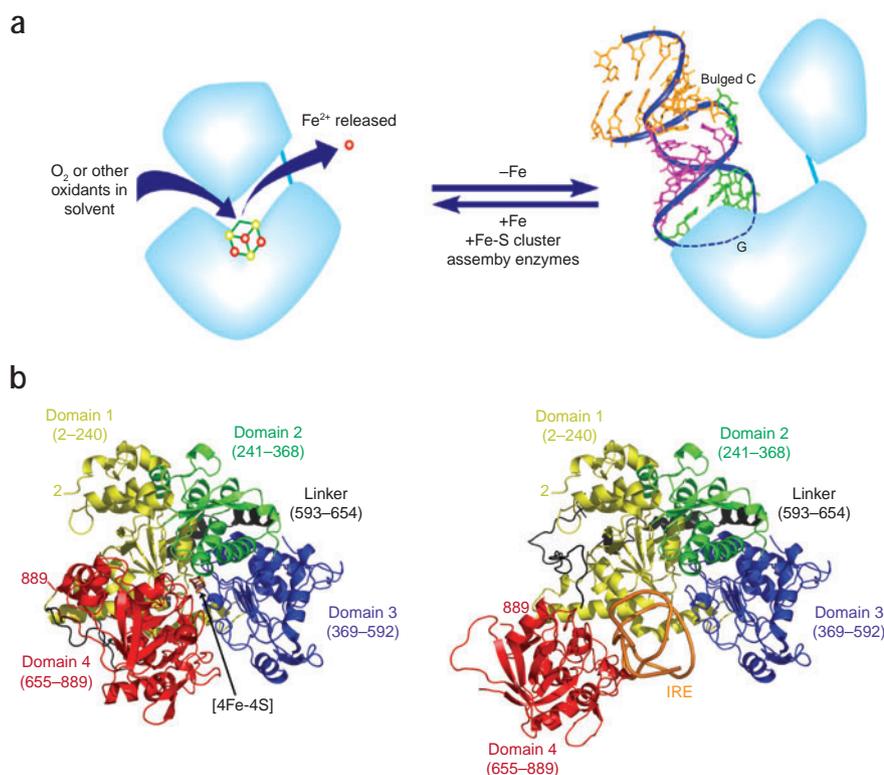


Figure 4 The iron-sulfur switch of IRP1. (a) A cartoon showing the iron-sulfur switch of IRP1. IRP1 is a bifunctional protein that can exist as a functional cytosolic aconitase, interconverting citrate and isocitrate, or as an apoprotein that binds IREs. Studies indicate that the active site cleft responsible for aconitase activity overlaps extensively with the region that binds IREs. Thus, IRP1 is bifunctional, and the ratio of the holoprotein and apoprotein forms most likely determines the way IRP1 functions in cells. Instability of the iron-sulfur prosthetic group may be a key determinant of the ratio between holoprotein and apoprotein. Numerous enzymes and proteins facilitate iron-sulfur cluster assembly and formation of active cytosolic aconitase. (b) Left, the IRP1 structure with its cluster; right, IRP1 modeled with the IRE bound. Structures are depicted according to the recently solved holoprotein structure⁴⁶; illustrations created by J. Dupuy. Domains are labeled: domains 1–3 are connected to domain 4 by a flexible hinge linker (black) that is depicted as seen in the crystal structure (left) or in a conformation that could accommodate the IRE, based on structural modeling (right) in which the position of domain 4 is moved to accommodate the IRE within an enlarged cleft.

Some IRP1s may lack function as either a cytosolic aconitase or an IRE binding protein⁸⁰, but the physiologic relevance and size of this functionless pool of IRP1 is not yet clear. Nitration of IRP1 in activated macrophages leads to inactivation of the aconitase and IRE binding activities of IRP1 without causing degradation⁸¹. In addition, iron-dependent degradation of IRP1 occurs in some cells and can be enhanced by phosphorylation at Ser138 (ref. 78), thereby providing a potential link between growth signaling pathways and IRP1 activity. Phosphorylation of IRP1 at Ser711 can also modulate aconitase activity *in vivo*^{82,83}. Thus, various factors that do not directly affect iron-sulfur cluster assembly and disassembly may modify operation of the iron-sulfur switch.

The role of IRP1 in mammalian physiology

Because IRP1 is abundant in animal tissues and regulates its IRE binding activity in tissue culture and in rats subjected to iron depletion⁸⁴, investigators have assumed that IRP1 is important in intracellular iron regulation. However, genetic ablation studies have revealed that the homologous protein, IRP2, is important in the regulation of iron metabolism in mice^{85,86}, whereas ablation of IRP1 has little effect on regulation of tissue iron homeostasis, except in kidney and brown fat, in which IRP1 expression is very high⁸⁷. Unlike in cells grown in room air, most IRP1 in animal tissues is in the aconitase form, and IRP1 does not convert to the IRE-binding form in cells given a low-iron diet that is sufficient to activate IRP2 (ref. 87). Even when IRP2 is absent, IRP1 binding activity does not increase, whereas when IRP1 is absent, IRP2 binding activity and concentration increases in a compensatory fashion^{87,88}.

Thus, it seems that the primary physiologic role of IRP1 may be to function as a cytosolic aconitase. By interconverting citrate and isocitrate in the cytosol, cytosolic aconitase may allow cells to balance the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated by cytosolic isocitrate dehydrogenase with the amount of acetyl coenzyme A generated from cytosolic citrate by the citrate lyase reaction. Fatty acid synthesis requires (i) the acetyl coenzyme A building blocks

derived from citrate and (ii) NADPH, which is generated by cytosolic isocitrate dehydrogenase when it catalyzes conversion of isocitrate to 2-oxoglutarate. Recent studies indicate that cytosolic isocitrate dehydrogenase is one of the main sources of NADPH in mammalian cells⁸⁹. In energy-rich cells, large NADH to NAD⁺ ratios repress mitochondrial isocitrate dehydrogenase activity⁹⁰, leading to accumulation of citrate and isocitrate in the mitochondrial matrix and driving export of these citric acid intermediates to the cytosol through the tricarboxylic acid transporter, where they can serve as fatty acid precursors⁹¹. Notably, IRP1 is most highly expressed in brown fat, liver, kidney and testicular epididymal cells, potentially important sites for energy storage and fatty acid synthesis.

The difference between the potential of IRP1 to regulate iron metabolism and its actual role in animals is most likely attributable to the role of oxygen in destabilizing iron-sulfur clusters. In tissue culture, cells are usually exposed to the high atmospheric oxygen concentrations of room air, whereas in animals, oxygen concentrations are much lower, in the range of 3–6%⁸⁸. IRP1 provides most of the IRE binding activity detected in cells grown in room air, whereas IRP1 converts to the cytosolic aconitase form at the low oxygen tensions that prevail in mammalian tissues and is less important than IRP2 in regulation of iron metabolism (Fig. 6). Thus, turnover of the labile iron-sulfur cluster is likely to be markedly lower in animal tissues than in tissue culture cells, and cluster turnover in animal tissues may depend more on the reactive oxygen species and nitric oxide produced by stimuli such as infection and inflammation⁵³.

Role of IRP2 in regulation of iron metabolism

IRP2 is a ubiquitously expressed regulatory protein that has a central role in mammalian iron metabolism^{85,86}. Mice that lack IRP2 develop anemia due to insufficient erythroid expression of TfR, which combines with overexpression of ferritin to deplete cells of iron that is needed for heme synthesis. *IRP2*^{-/-} mice do not appropriately repress translation of eALAS, which has an IRE at its 5' end; consequently, they produce 200-fold more

of the porphyrin heme precursor protoporphyrin IX than do wild-type mice²⁵. In adulthood, *IRP2*^{-/-} mice develop progressive neurodegenerative disease associated with degeneration of axons followed by death of neuronal cell bodies in cells that overexpress ferritin and synthesize too little TfR^{85,92,93}. Anemia without progressive neurodegeneration has been reported in one *IRP2*^{-/-} mouse model⁸⁶, but neurodegeneration is most likely the primary consequence of IRP loss, given that both neurodegeneration and anemia are exacerbated in *IRP1*^{+/-} *IRP2*^{-/-} mice compared with *IRP2*^{-/-} mice (Fig. 7a–f)⁹², a result indicating that IRP1 and IRP2 function in the same pathway and that there is a gene dosage effect.

Although IRP1 and IRP2 are equally able to regulate ferritin translation *in vitro*⁹⁴ and are both ubiquitously expressed, IRP2 dominates regulation of iron homeostasis⁸⁷. The reason for this domination is likely to be intimately related to IRP2's unique mechanism for sensing and regulating iron concentrations. In iron-replete cells, IRP2 is efficiently degraded in an iron-dependent manner, and its concentration is not regulated by an iron-sulfur switch^{17,41,42}. In addition, cells activated by expression of the *c-myc* oncogene increase expression of IRP2 mRNA, indicating that regulation of IRP2 expression may be affected by the growth status of cells⁹⁵. Thus, regulation of transcription and protein turnover most likely affect IRP2 expression.

Mechanism of iron-dependent degradation of IRP2

The mechanism by which IRP2 undergoes iron-dependent degradation is incompletely understood. Relative to IRP1, IRP2 contains an extra cysteine-rich exon. Previously referred to as the iron-dependent degradation domain, this exon has been thought to be responsible for characteristic iron-dependent degradation owing to its ability to facilitate iron-dependent oxidation⁹⁶, ubiquitination and proteasomal degradation^{97,98}. However, the initial steps in iron-dependent degradation of IRP2 are not well characterized, and the role of the iron-dependent degradation domain has been questioned^{99,100}. Other molecules or factors that may signal the iron-replete state and facilitate degradation of IRP2 include heme^{100–103}, 2-oxoglutarate-dependent oxygenases^{99,104} and phosphorylation status¹⁰⁵, and the contribution of various pathways to IRP2 degradation may depend in part on the cells involved¹⁰³. It is possible that IRP2 has evolved to 'sense' cytosolic iron concentrations through multiple pathways: direct binding of iron or heme, as well as activation of cytosolic oxygenases, may combine to robustly target IRP2 for ubiquitination and proteasomal degradation in iron-replete cells. It

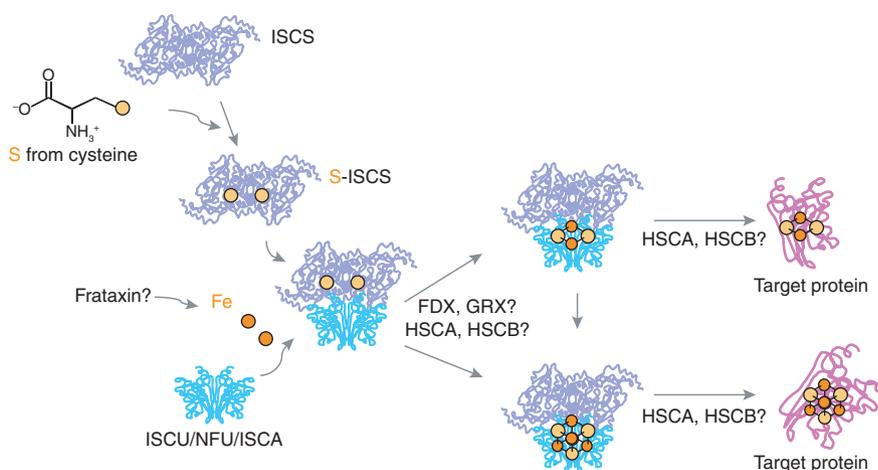


Figure 5 Proposed schematic of mammalian iron-sulfur cluster assembly. The cysteine desulfurase ISCS generates sulfur, which it donates to ISCU. ISCU binds iron, perhaps donated by frataxin, and serves as a scaffold on which nascent iron-sulfur clusters are assembled. ISCA and NFU may also function as scaffold proteins. Ferredoxin (FDX) and glutaredoxin 5 (GRX) provide reducing equivalents, and the chaperones HSCA and HSCB (heat shock cognate proteins A and B, respectively) are likely to enhance scaffold folding and transfer of clusters to recipient apoproteins such as IRP1 (refs. 63,64).

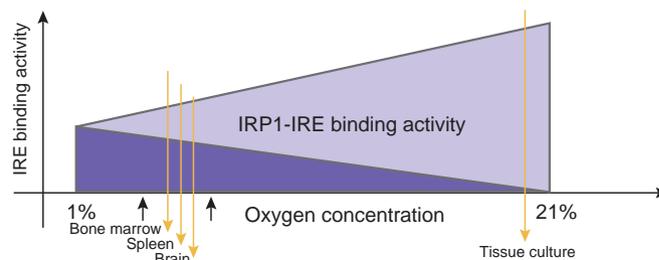
is also possible, but unlikely, that IRP2 transiently assembles an iron-sulfur cluster¹⁷.

A critical feature that allows IRP2 to predominate in the regulation of iron metabolism is IRP2's relative stability and IRE-binding activity at the low oxygen concentrations that prevail in mammalian tissues. IRP1, in contrast, is mainly active as an aconitase at these low oxygen concentrations (Fig. 6). Another important difference is that IRP2 can compensate for loss of IRP1 (ref. 88), perhaps because loss of IRP1 results in mild iron deficiency due to minor decreases in TfR expression and increases in ferritin, and decreased cytosolic iron concentrations partially stabilize IRP2. Though recent data on regulation of ISCU indicate that iron deficiency decreases cellular ISCU expression and may thereby reduce the amount of IRP1 that has an iron-sulfur cluster⁶⁵, regulation of iron-sulfur cluster assembly proteins has yet to be characterized in intact animals.

The physiologic importance of the IRE-IRP regulatory system

Developing embryos that lack both copies of IRP1 and IRP2 die at the blastocyst stage, before implantation¹⁰⁶. This embryonic lethality of the *IRP1*^{-/-} *IRP2*^{-/-} genotype underscores the fact that the IRP-IRE regulatory system is critical for regulation of iron metabolism and that IRP1 and IRP2 have redundant functions. Their redundancy is further underscored by the fact that the neurodegeneration and anemia of *IRP2*^{-/-} mice is greatly exacerbated in animals that also lack at least one copy of IRP1 (Fig. 7)^{25,92}. The gene dosage effect of IRP loss is illustrated by

Figure 6 IRP2 has greater IRE-binding activity than IRP1 at the characteristically low oxygen concentrations found in mammalian tissues. At these low oxygen concentrations IRP2 is highly active as an IRE-binding protein, whereas IRP1 acts mainly as a cytosolic aconitase and has low IRE binding activity. The schematic shows IRP binding activity over a range of oxygen concentrations, along with markings that indicate the reported mammalian tissue oxygen concentrations of bone marrow, spleen and brain (yellow arrows). The oxygen concentrations of many other tissues are between 3% and 6% (the area between the two vertical black arrows on the x axis), whereas most cells grown in traditional tissue culture flasks are exposed to oxygen concentrations closer to the 21% oxygen concentration of room air.



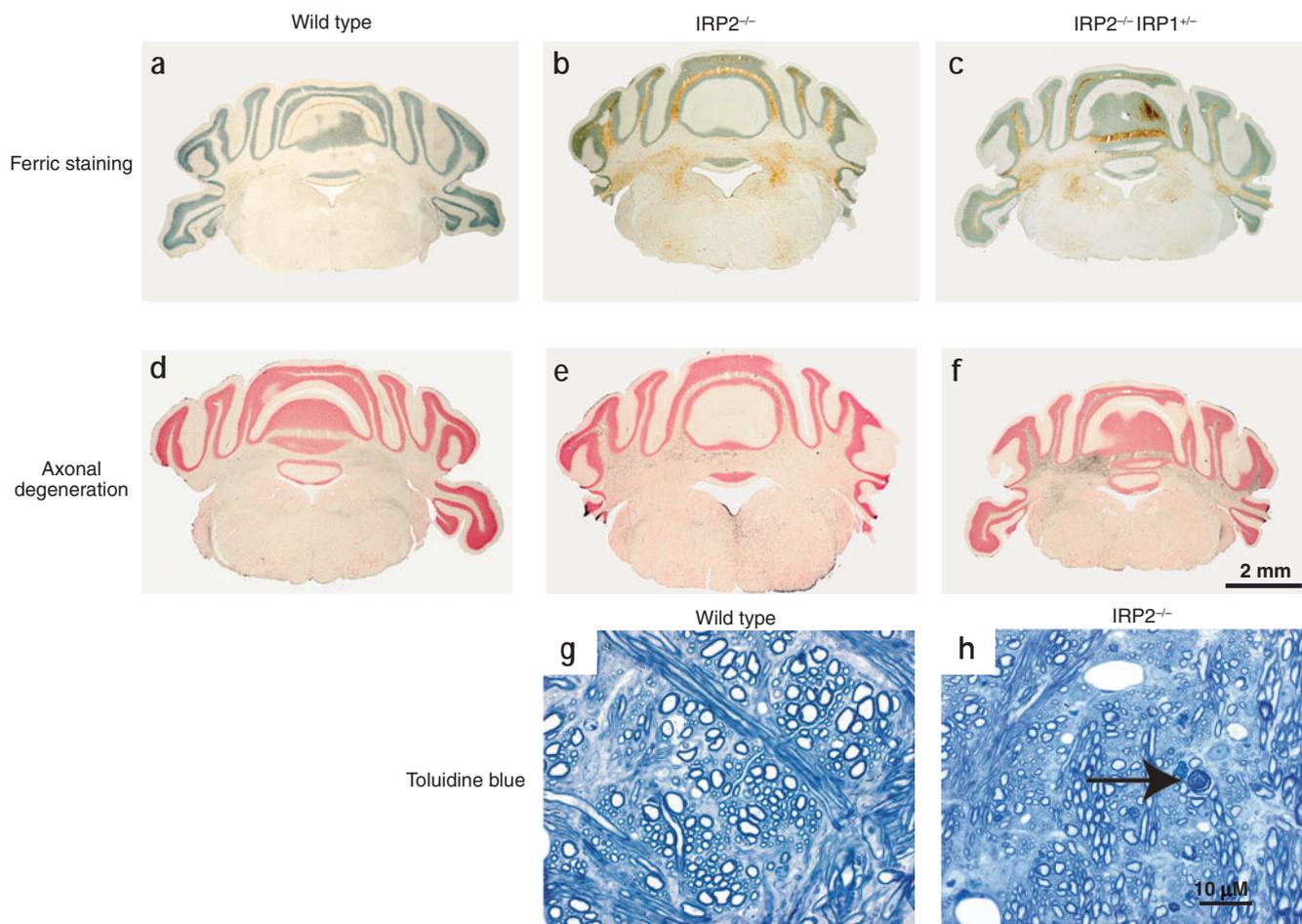


Figure 7 Neurodegeneration in adult $IRP2^{-/-}$ and $IRP1^{+/+} IRP2^{-/-}$ animals is characterized by ferric iron accumulations in distinctive white-matter areas of the brain and by axonal degeneration in the affected white-matter areas. (a–f) Coronal sections of whole mouse brains through the cerebellum show little ferric iron accumulation or axonal degeneration in wild-type mice (a,d). However, ferric iron accumulations (golden-brown stain in a–c) are present in $IRP2^{-/-}$ mice (b) and are markedly increased in $IRP2^{-/-} IRP1^{+/+}$ mice (c), and there is concomitant axonal degeneration in $IRP2^{-/-}$ mice (e), which is more marked in $IRP2^{-/-} IRP1^{+/+}$ mice (f), as indicated by deposition of black thread-like silver deposits within white matter in degenerating axons. A green counterstain for nuclei heavily stains regions that are rich in cell bodies (traditional gray matter) in a–c, whereas a red nuclear counterstain is used in d–f to allow visualization of distinctive landmarks near the back of the brain, particularly the cerebellar folia. (g,h) In brain tissue fixed in epoxy and stained with toluidine blue for improved morphologic visualization at high magnification, myelin-dense bodies indicative of axonal degeneration are commonly found (arrow) in $IRP2^{-/-}$ (h) but not in wild-type mice (g). In this stain, normal axons appear as blue rings with white centers; the thick blue rim represents the myelin sheath, whereas the white center represents the normal axon. Myelin-dense bodies are created when axons degenerate and the myelin sheath collapses to fill the space formerly occupied by an axon. Figures reproduced from ref. 92 with permission from Blackwell Publishing.

ferric iron accumulations, which are detectable in many axon-rich regions of the brain in 1-year-old $IRP2^{-/-}$ mice (Fig. 7c) and are pronounced in age-matched $IRP1^{+/+} IRP2^{-/-}$ mice (Fig. 7e) but are not detectable in age-matched wild-type mice (Fig. 7a). The ferric iron is most likely sequestered by ferritin and is relatively inaccessible, even though increased ferric iron staining is usually thought to signify increased iron availability^{85,92}. In cells, the combination of ferritin overexpression with decreased Tfr1 expression may result in a state of functional iron deficiency that adversely affects axons, which depend on iron-replete mitochondria to generate the ATP that supports axonal transport and function. Notably, axons in white matter areas of the brain that accumulate ferric iron show degeneration, as evidenced by a special staining procedure known as the amino cupric silver stain, in which black deposits of silver are observed in $IRP2^{-/-}$ and $IRP1^{+/+} IRP2^{-/-}$ brains (Fig. 7e,f)⁹². Intact axons of the

wild type prevent penetration of silver (Fig. 7b), whereas degenerating axons allow the silver to penetrate and bind to negatively charged neurofilaments, creating threadlike black deposits that follow the course of axons. Ultimately, complete loss of axonal integrity results in collapse of the myelin sheath around axonal remnants in mutant mice (Fig. 7h), but not in wild-type mice (Fig. 7g).

Notably, animals having some residual IRP activity ($IRP1^{+/+} IRP2^{-/-}$ animals) can survive to adulthood with apparent functional compromise of only the hematopoietic and neurologic systems, whereas other cells and tissues are spared. In neurons, swelling and inflammation initially affects axons, but ultimately neuronal cell bodies die, particularly in the substantia nigra, the area of the brain affected in Parkinson disease⁹². The cause of neuronal dysfunction and death is not yet clear, but based on an analogy to erythroid cells, it is possible that iron deficiency may adversely

affect neurons by compromising synthesis of the iron-sulfur complexes of the mitochondrial respiratory chain.

The fact that IRP2 loss causes adult-onset neurodegenerative disease with Parkinsonian features raises the possibility that IRP2 dysfunction will prove to be a cause of adult-onset human neurodegenerative disease. Affected individuals carrying IRP2 mutations are also likely to have microcytic anemia and elevated red cell protoporphyrin IX concentrations, although the movement disorder and anemia can be very subtle if only one IRP2 allele is inactivated⁸⁵.

Future directions

Animal studies indicate that the IRP-IRE regulatory system is important in physiology. Future studies are likely to bring more knowledge about mammalian iron-sulfur cluster assembly and the factors that operate the iron-sulfur cluster switch of IRP1. Much remains to be determined about the mechanisms by which IRP2 is targeted for iron-dependent degradation and those by which IRP transcription is affected by growth signals. The neurodegeneration of *IRP2*^{-/-} mice provides a useful model in which the causes of neuronal death can be dissected. If functional iron deficiency causes mitochondrial dysfunction, which in turn causes free radical stress and failure of energy production in axons, this central insight may provide understanding of the pathophysiology of many neurodegenerative diseases in which iron misregulation is suspected to have a role.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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