Functions of mitochondrial ISCU and cytosolic ISCU in mammalian iron-sulfur cluster biogenesis and iron homeostasis

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Summary

Iron-sulfur (Fe-S) clusters are required for the functions of mitochondrial aconitase, mammalian iron regulatory protein 1, and many other proteins in multiple subcellular compartments. Recent studies in *Saccharomyces cerevisiae* indicated that Fe-S cluster biogenesis also has an important role in mitochondrial iron homeostasis. Here we report the functional analysis of the mitochondrial and cytosolic isoforms of the human Fe-S cluster scaffold protein, ISCU. Suppression of human ISCU by RNAi not only inactivated mitochondrial and cytosolic aconitases in a compartment-specific manner but also inappropriately activated the iron regulatory proteins and disrupted intracellular iron homeostasis. Furthermore, endogenous ISCU levels were suppressed by iron deprivation. These results provide evidence for a coordinated response to iron deficiency that includes activation of iron uptake, redistribution of intracellular iron, and decreased utilization of iron in Fe-S proteins.

Introduction

Iron is an essential nutrient for all eukaryotes, serving as a cofactor for cellular processes including respiration, oxygen transport, intermediary metabolism, gene regulation, and DNA replication and repair. However, excess iron is cytotoxic, and misregulation of iron homeostasis can contribute to a number of hematologic, metabolic, and neurodegenerative diseases (Hentze et al., 2004). New insights into cellular iron metabolism have been provided by the recognition that certain diseases are associated with mitochondrial iron misregulation, although the genes involved in mitochondrial iron metabolism are not yet well characterized, and the relationship of mitochondrial iron trafficking to overall cellular iron homeostasis is still unclear (Napier et al., 2005). The human neurodegenerative disease, Friedreich ataxia, caused by loss of the mitochondrial protein frataxin, is characterized by mitochondrial iron overload, and studies in yeast and mouse models suggest that mitochondrial iron overload is a consequence of impaired Fe-S cluster assembly (Lill and Muhlenhoff, 2005; Pandolfo, 2003).

In mammalian cells, iron homeostasis is largely achieved by the coordinate regulation of transferrin-dependent iron uptake and iron sequestration by ferritin. The expression levels of transferrin receptor (TfR) and ferritin are controlled posttranscriptionally by the cytosolic RNA binding proteins iron regulatory protein 1 and 2 (IRP1 and IRP2), which bind to iron regulatory elements (IRE) in the transcripts of target genes (Pantopoulos, 2004). Although IRP1 and IRP2 are highly homologous, they sense cytosolic iron levels by different mechanisms. IRP1 registers cytosolic iron and oxidative stress through its labile Fe-S cluster. In tissue culture cells, when iron is abundant, IRP1 contains a [4Fe-4S] cluster, exhibits aconitase activity, and binds IREs with low affinity. When iron is scarce, IRP1 loses its Fe-S cluster and aconitase activity and binds IREs with high affinity. In contrast, IRP2 is regulated through protein degradation in the presence of iron and oxygen. Thus, oxidative stress or disrupted biogenesis of Fe-S clusters can activate IRE binding activities and lead to inappropriate repression of ferritin synthesis, increased TfR expression, and cellular iron toxicity. Studies of knockout mice suggested that IRP2 is the main functional iron sensor in vivo (Meyron-Holtz et al., 2004), whereas IRP1 may play an important role in some other physiologic settings (Cairo et al., 2002).

Much of what is known about Fe-S cluster biogenesis comes from the extensive genetic and biochemical studies of gene products of the bacterial *nif, isc*, and *suf* operons and their homologs in *S. cerevisiae* (Johnson et al., 2005; Lill and Muhlenhoff, 2005). Sulfur transfer from cysteine desulfurase (NifS, IscS, and SufS) (Smith et al., 2001; Urbina et al., 2001) and iron acquisition lead to assembly of [2Fe-2S] and [4Fe-4S] clusters on the scaffold proteins (NifU, IscU, Nfu, and IscA) for delivery to target proteins (Agar et al., 2000; Krebs et al., 2001; Nishio and Nakai, 2000; Ollagnier-de-Choudens et al., 2001). Redox proteins (ferredoxin and glutaredoxin 5) and chaperone proteins (HscA and HscB) may facilitate Fe acquisition and cluster assembly or transfer (Dutkiewicz et al., 2004; Lange et al., 2000; Silberg et al., 2004; Wingert et al., 2005; Wu et al., 2005). In yeast, the cysteine desulfurase Nfs1 is present in the mitochondria (Kispal et al., 1999; Nakai et al., 2001) and the nucleus (Nakai et al., 2001), but many other Fe-S cluster assembly proteins have thus far been detected only in the mitochondria (Garland et al., 1999; Gerber et al., 2004; Schilke et al., 1999), leading to the proposal that Fe-S cluster assembly occurs exclusively in the mitochondria, and that preformed Fe-S clusters are exported from the mitochondria into the cytosol, where delivery to cytosolic targets is enhanced by cytosolic Cfd1, Nar1, and Nbp35 (Lill and Muhlenhoff, 2005; Roy et al., 2003). The hypothesis that de novo Fe-S cluster synthesis takes place exclusively in the mitochondria led to the proposal that the essential metabolic functions of Fe-S proteins might have provided selection pressure for the permanent establishment of mitochondrial endosymbionts in early eukaryotic cells (Tovar et al., 2003). However, isoforms of several Fe-S cluster assembly proteins have been identified in the mitochondria, cytosol, and nucleus in

mammalian cells (Land and Rouault, 1998; Rouault and Tong, 2005), and in the chloroplast in plants (Leon et al., 2003; Yabe et al., 2004; Ye et al., 2005), suggesting that Fe-S cluster biogenesis may have evolved in multiple subcellular compartments of multicellular eukaryotes.

To investigate the functions of compartment-specific Fe-S cluster assembly, and more specifically the role of Fe-S cluster assembly in iron homeostasis, we suppressed the human Fe-S cluster scaffold protein ISCU by RNAi and examined the effect on Fe-S proteins and iron metabolism in mammalian cells. We have previously shown that alternative splicing gives rise to mitochondrial and cytosolic isoforms of ISCU (m-ISCU and c-ISCU) in mammalian cells (Tong and Rouault, 2000). In the present study, m-ISCU and c-ISCU were selectively suppressed with isoform-specific siRNAs, whereas a siRNA containing a sequence shared by the two different transcripts of ISCU suppressed both isoforms. The results confirmed alternative splicing of human ISCU, demonstrated that both m- and c-ISCU are functional, and showed that they are important not only in maintaining Fe-S protein functions in their respective cellular compartments but also in regulating the activity of the iron regulatory proteins, IRP1 and IRP2, in mammalian cells.

Results

Two assays for monitoring Fe-S cluster assembly in the cytosol and in the mitochondria of HeLa cells

Two nondenaturing gel assays, an aconitase activity assay and an IRE binding assay, were used to monitor the assembly and disassembly of Fe-S clusters in vivo. Previous studies have shown that an intact [4Fe-4S] cluster is required for aconitase activity (Kennedy et al., 1992). As shown in Figure 1A, the in-gel aconitase activity assay allowed clear separation of human m- and c-aconitases, providing a convenient method to simultaneously evaluate the status of Fe-S proteins in the two different subcellular compartments. Since complete disassembly of the Fe-S cluster in c-aconitase/IRP1 converts the protein to its RNA binding form, the Fe-S cluster status in the cytosol can also be assessed by monitoring the IRE binding activity of IRP1. In previous gel mobility shift studies, human IRP1 and IRP2 comigrated and were not distinguishable. Here, we modified the procedure to improve resolution, and the assignments of the two different bands in the RNA mobility shift gels were confirmed by antibody supershift assay (Figure 1B).

When used alone, the aconitase activity and IRE binding assays can provide useful information on the steady-state distributions of apo- and [4Fe-4S]-aconitases but not the dynamics of cluster assembly and disassembly. To study the function of Fe-S cluster assembly proteins, an oxidative challenge can be used to perturb the steady-state level of [4Fe-4S]-aconitases, and analysis of recovery can reveal the kinetics of Fe-S cluster repair/regeneration. Previous studies have shown that c-aconitase/IRP1 was converted to the IRE binding form when intact murine B6 fibroblast cells were treated with H_2O_2 (Pantopoulos and Hentze, 1995), and m-aconitase was inactivated when isolated rat cardiac mitochondria were treated with H_2O_2 (Bulteau et al., 2003). Other studies have shown that oxidative damage can increase the proteolytic susceptibility of IRP1 (Starzynski et al., 2005) or can convert IRP1 to an inactive form with a [3Fe-4S] cluster and no aconitase or IRE binding activities (Brazzolotto et al., 1999). In HeLa cells, treatment with H_2O_2 led to dose-dependent inactivation of c-aconitase (Figure 1C, lanes 1, 5, 9, 13). IRE binding assays and Western blot analyses indicated that the marked decrease in c-aconitase activity correlated with a marked increase in the IRE binding activity of IRP1 and only a slight decrease in protein levels, indictating that H_2O_2 treatment promoted disassembly of the Fe-S cluster in c-aconitase to generate apo-IRP1. Interestingly, m-aconitase activities were much less affected by the H_2O_2 treatment (Figure 1C), suggesting that m-aconitase is less accessible than c-aconitase to the effect of H_2O_2 in intact HeLa cells. More importantly, within 4 hr after removal of H_2O_2, c-aconitase and IRE binding activities of IRP1 had returned to baseline levels (Figure 1C, lanes 2, 6, 10, 14), indicating robust repair/regeneration of the [4Fe-4S] cluster in vivo.

To further study the dynamics of cluster disassembly and reassembly in vivo, we exposed cells to the iron chelator, desferroxamine (Dfo). Treatment with Dfo decreased both m- and c-aconitase activities and activated the IRE binding activities of both IRP1 and IRP2 (Figure 1D, lane 6). Subsequent withdrawal of Dfo led to reactivation of both aconitases and deactivation of both IRPs, consistent with reassembly of the Fe-S clusters, and iron-dependent degradation of IRP2. Taken together, these results indicate that Fe-S proteins are acutely sensitive to the iron and redox status of the cells, and that the assembly and disassembly processes of Fe-S clusters are highly dynamic.

Global silencing of human ISCU inactivates m- and c-aconitases and activates the IRE binding activities of IRP1 and IRP2

Previous studies have shown that m- and c-ISCU in mammalian cells are generated through alternative splicing of a common pre-mRNA (Tong and Rouault, 2000). Consistent with previous studies with human liver poly(A)$^+$ RNA, RT-PCR of human heart RNA using a primer for exon IA and a primer in the downstream common region generated the predicted product from *m-ISCU*, whereas RT-PCR using a primer for exon IB generated a product expected from *c-ISCU* (Figure 2A). Southern hybridization confirmed that *c-ISCU* contained exon IB, whereas *m-ISCU* contained exon IA but not exon IB. Consistent with previous studies in human RD4 cells, mature m-ISCU in HeLa cells is a 14 kDa protein, whereas c-ISCU is a 15 kDa protein that is much less abundant than m-ISCU (Figure 2A, Western blot).

To assess the function of human ISCU proteins in Fe-S cluster biogenesis, HeLa cells were transfected with an ISCU-univ siRNA duplex that is targeted to a sequence common to both ISCU transcripts (Figure 2A). Global silencing of human ISCU led to pronounced loss of both m- and c-aconitase activities (Figure 2C). Loss of m- and c-aconitase activity occurred with little change in protein levels (Figures 2B and 2C), but loss of c-aconitase activity was accompanied by an increase in the IRE binding activity of IRP1 (Figure 2C). Thus, assays of two distinct Fe-S proteins (m- and c-aconitases are distinct proteins with <30% homology) demonstrated that Fe-S cluster assembly/repair is impaired in mammalian cells that lack ISCU. Interestingly, silencing of ISCU also resulted in marked activation of the IRE binding activity of IRP2 (Figure 2C and Figure 2D, lane 6). Since IRP2 is not known to depend on an Fe-S cluster for its regulation, these results suggested that iron-dependent degradation of IRP2 was decreased (Hanson et al., 2003; Pantopoulos, 2004). Indeed, Western blot analysis confirmed that

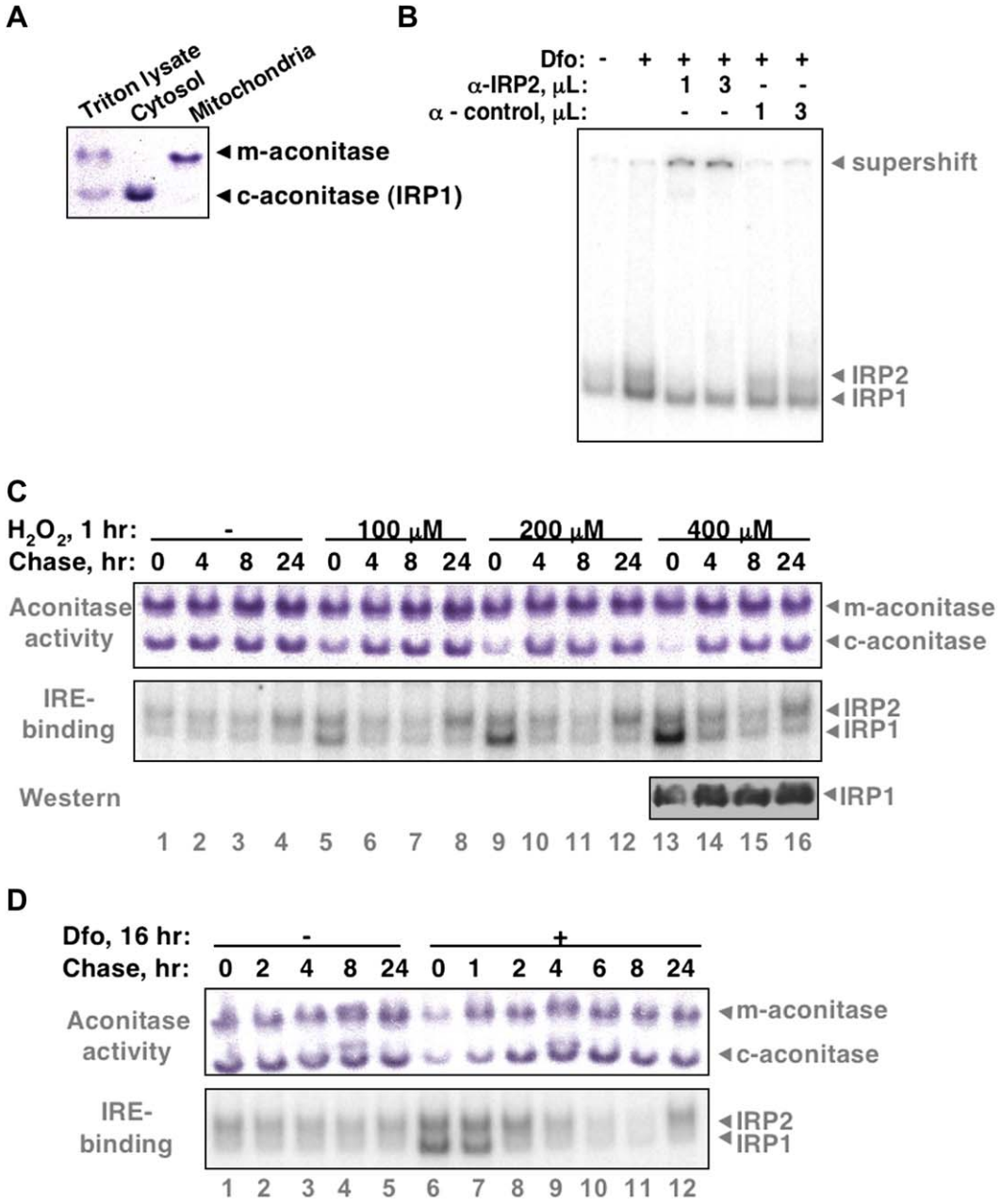

Figure 1. Monitoring Fe-S protein activities in the cytosol and in the mitochondria

A) In-gel activity assay of m-aconitase and c-aconitase (IRP1) in HeLa cell triton lysate and subcellular fractions.

B) IRE binding and antibody supershift assays showed the separation of human IRP1- and IRP2-IRE complexes.

C) Aconitase activity and IRE binding assays can be used to monitor the disassembly and reassembly of Fe-S cluster in cells subjected to oxidative stress. Cells were treated for 1 hr with H_2O_2, then washed and incubated in DMEM-complete medium for up to 24 hr (chase).

D) Aconitase activity and IRE binding assays revealed the disassembly and reassembly of Fe-S cluster in cells treated with iron chelator Desferal (Dfo).

IRP2 protein levels increased notably with global silencing of ISCU (Figure 2C), suggesting that there is a cytosolic iron deficit in these cells. Interestingly, these results also suggest that the loss of cytosolic aconitase activity and activation of IRP1 resulted from a primary defect in Fe-S cluster assembly compounded by a secondary defect in cytosolic iron availability. To assess the specificity of these effects, we also transfected HeLa cells with m-aconitase siRNA, which has no sequence homology to ISCU. Silencing of m-aconitase decreased m-aconitase but not c-aconitase activity (Figure 2C). More importantly, m-aconitase siRNA had no effect on the IRE binding activities of IRP1 and IRP2. Taken together, these results provided the first indication that global silencing of ISCU disrupts not only Fe-S cluster assembly but also intracellular iron homeostasis in mammalian cells.

In addition to lowering the steady-state level of Fe-S clusters, global silencing of human ISCU also disrupted the reassembly of Fe-S clusters in cells recovering from oxidative stress or iron depletion. Like untransfected cells (Figure 1C), treatment of mock-transfected cells with H_2O_2 lowered c-aconitase activity (see

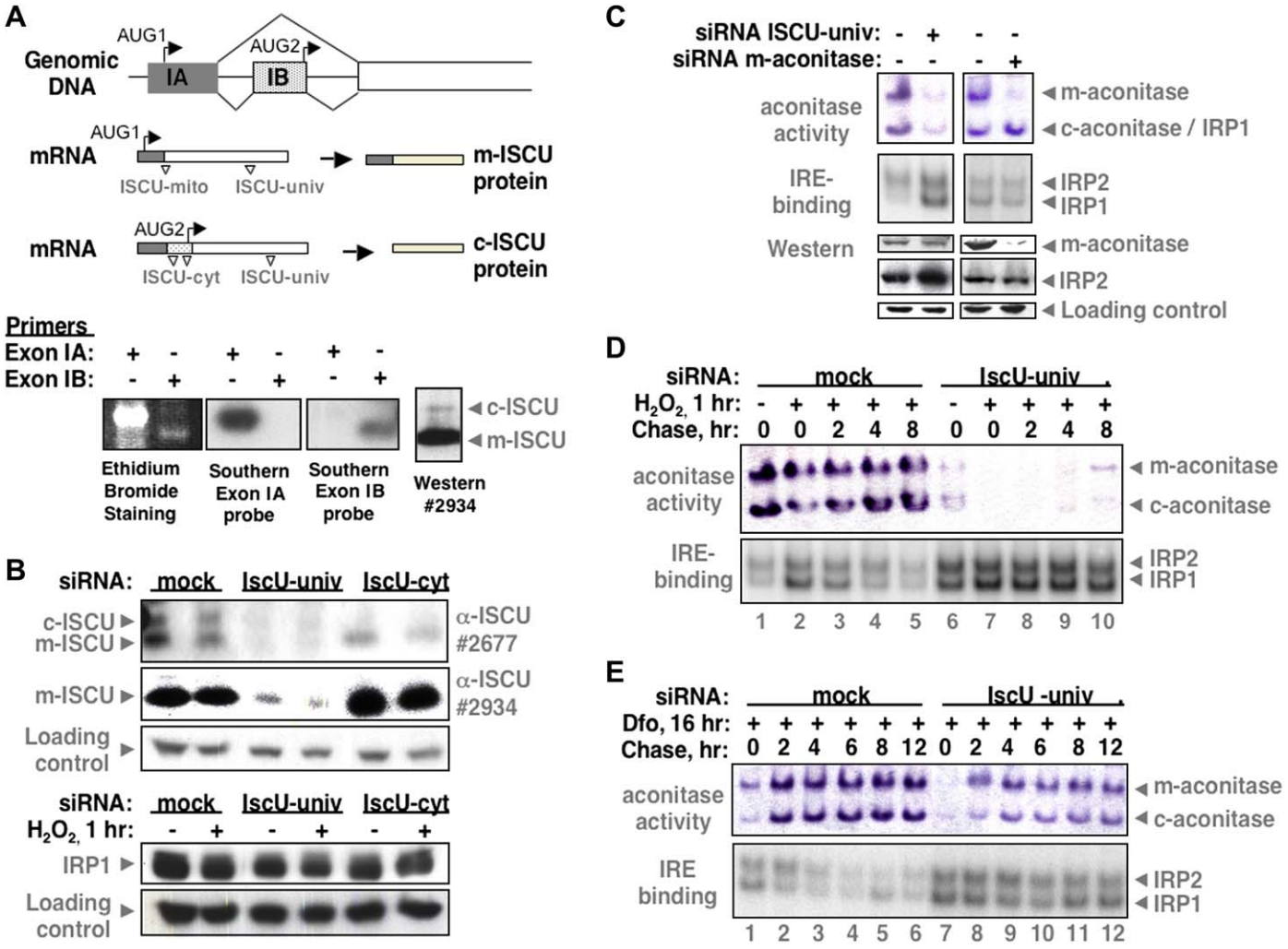

Figure 2. Global silencing of ISCU impaired Fe-S cluster assembly in both m- and c-aconitases and decreased degradation of IRP2

A) Alternative splicing generates m- and c-ISCU with different N-terminal sequences. Mitochondrial target sequences are shown in gray boxes, and inverted triangles denote siRNA targets. Nested PCRs of a 5′RACE product from human heart were carried out using primers specific to exon IA or exon IB, and Southern blots were carried out using exon IA or exon IB probe. Western blot with α-ISCU (#2934) indicated the presence of m- and c-ISCU in HeLa cells.

B) Western blots of cells transfected with ISCU-univ or control m-aconitase siRNA. Loading control: Western blots of m-ISCS.

C) Aconitase activity, IRE binding assays, and Western blots of cells transfected with ISCU-univ or control m-aconitase siRNA.

D) Cells transfected with ISCU-univ siRNA were treated for 1 hr with 200 μM H_2O_2, and the recovery of Fe-S clusters was monitored by aconitase and IRE binding assays.

E) Cells transfected with ISCU-univ siRNA were treated for 16 hr with 50 μM Dfo, and the recovery of Fe-S clusters was monitored by aconitase and IRE binding assays.

lane 1 and 2 of Figure 2D), and inactivation of aconitase activities was readily reversed after removal of H_2O_2 (Figure 2D, lanes 3–5). Interestingly, m-aconitase activity was also slightly reduced (~20%) (Figure 2D, lane 2), suggesting that some cytotoxicity caused by siRNA transfection might have increased the sensitivity of m-aconitase to the H_2O_2 treatment. In contrast to mock-transfected cells, recoveries of both m and c-aconitase activities were severely impaired in cells transfected with ISCU-univ siRNA, and IRPs remained activated many hours after H_2O_2 withdrawal (Figure 2D, lanes 7–10). The slight increase in aconitase activity at the 8 hr timepoint (Figure 2D, lane 10) is consistent with the presence of a small amount of residual ISCU that remained in the cells after knockdown (Figure 2B, lanes 3 and 4). Western blot analyses of IRP1 showed that the marked decreases in c-aconitase activity in cells depleted of ISCU were not caused by decreases in IRP1 protein levels (Figure 2B). Recoveries of both aconitases after Dfo treatment were also markedly impaired in cells transfected with ISCU-univ siRNA (Figure 2E, lanes 8–12). Furthermore, IRP2 remained activated many hours after Dfo withdrawal, indicating that ISCU-deficient cells fail to respond normally to increases in iron availability.

Isoform-specific silencing of cytosolic ISCU impairs the recovery of Fe-S clusters in the cytosol

If c-ISCU is nonfunctional and Fe-S clusters are made solely in the mitochondria, as suggested by the studies in *S. cerevisiae* (Gerber et al., 2004), then c-aconitase activities and IRE binding activities of IRP1 would be predicted to be unaffected when c-ISCU expression is selectively silenced. To investigate the function of c-ISCU in mammalian cells, HeLa cells were transfected with ISCU-cyt siRNAs that are targeted to an exon that is present exclusively in the c-ISCU mRNA (exon IB in Figure 2A). Western blot analysis indicated that the ISCU-cyt siRNA reduced the expression of c-ISCU but not m-ISCU (Figure 2B), providing

additional evidence for alternative splicing of human ISCU. Silencing of the c-ISCU in HeLa cells under normal growth conditions resulted in only slight changes in the steady-state levels of c-aconitase activities (see lanes 1 and 6 of Figure 3A). Since the exon uniquely present in c-ISCU is only 90 bp, the search for effective siRNA sequences for c-ISCU was highly constrained. We reasoned that the presence of residual c-ISCU due to incomplete silencing may be sufficient to maintain the steady-state level of c-aconitase activity in HeLa cells grown in normal conditions. To further evaluate the function of c-ISCU, we then asked if HeLa cells depleted of c-ISCU could appropriately respond to an H_2O_2 challenge. After 1 hr of H_2O_2 treatment, c-aconitase activity in mock-transfected cells was reduced to 48% (Figure 3A, lane 2) compared to control (lane 1), whereas in cells depleted of c-ISCU, c-aconitase activity was reduced to 6% (lane 7). These results suggested that during the H_2O_2 treatment, repair/regeneration was ongoing in control cells, whereas in cells depleted of c-ISCU, repair/regeneration was impaired. Likewise, quantitative analysis at 2 and 4 hr after removal of H_2O_2 indicated that c-aconitase activity in mock-transfected cells had recovered ~60% and 90% (Figure 3A, lanes 3 and 4), but only 20% and 40% in cells depleted of c-ISCU (lanes 8 and 9). Consistent with the aconitase assay, IRE binding activity of IRP1 was higher in cells depleted of c-ISCU (Figure 3A, lanes 7–10) compared to mock-transfected cells (lanes 2–5).

Defects in cytosolic Fe-S cluster assembly in cells depleted of c-ISCU were further confirmed with a Dfo challenge experiment. Greater than 90% of c-aconitase activity was recovered in mock-transfected cells at 2 and 4 hr after removal of Dfo (Figure 3B, lanes 3 and 4), whereas only 20% and 70% of c-aconitase activity was recovered in cells depleted of c-ISCU at the corresponding time points (lanes 8 and 9). In comparison, recoveries of m-aconitase activity were similar in the presence or absence of ISCU-cyt siRNA (Figure 3B, see lanes 3 and 8). These results indicated that, despite the abundance of m-ISCU in these cells (Figure 2B), depletion of c-ISCU impairs Fe-S cluster assembly in the cytosol. Thus, c-ISCU plays an active role in facilitating Fe-S cluster assembly in the cytosol of mammalian cells, and m-ISCU is not sufficient to compensate for the loss of cytosolic ISCU.

Silencing of mitochondrial ISCU induces changes in aconitase and IRP activities in a dose-dependent manner

Having established that c-aconitase was affected by silencing of c-ISCU, we then asked if silencing of m-ISCU, by using a siRNA targeted to an exon-exon boundary that is present exclusively in the transcript of the mitochondrial isoform (ISCU-mito), would have a selective effect on m-aconitase activity. Western blot analysis indicated that ISCU-mito siRNA reduced the expression of m-ISCU, but not c-ISCU (Figure 4A), and aconitase gel and IRE binding assays indicated only slight changes in the steady-state levels of Fe-S clusters in m- and c-aconitases in unchallenged HeLa cells (Figure 4A). We then examined Fe-S cluster repair/regeneration in cells depleted of m-ISCU after a Dfo challenge. Indeed, a modest (~2-fold) suppression of m-ISCU led to impaired recovery of m-aconitase activities (Figure 4B, lanes 4–6). Quantitative analysis of multiple Dfo challenge experiments indicated that, at 4 and 8 hr after removal of Dfo, m-aconitase activity had recovered only ~50%, whereas c-aconitase activity had recovered 80%–100% compared to mock-transfected cells (Figure 4B, bar graph). These experiments suggested that m-aconitase is more sensitive than c-aconitase to the depletion of m-ISCU, in keeping with the presence of a functional c-ISCU in the cytosol.

Interestingly, RNAi transfections that lowered m-ISCU levels more than 2-fold exhibited impaired recovery of both m- and c-aconitases (Figure 4C). More importantly, IRE binding activities of both IRP1 and IRP2 remained elevated for many hours after Dfo withdrawal (Figure 4C). Since IRP2 activation is a marker for cytosolic iron depletion, these results suggested that high levels of m-ISCU silencing disrupted intracellular iron homeostasis. These findings are consistent with studies in yeast which showed that disruption of Fe-S cluster biogenesis can result in mitochondrial iron overload and cytosolic iron depletion (Foury and Talibi, 2001; Knight et al., 1998; Li et al., 1999). Since iron is essential for Fe-S cluster assembly, cytosolic iron depletion together with impaired mitochondrial Fe-S cluster biogenesis can account for elevated IRE binding activities of both IRP1 and IRP2 and decreased m- and c-aconitase activities.

Misregulation of iron homeostasis in cells depleted of ISCU

Previous studies have shown that IRP functions are important in mammalian iron homeostasis (Pantopoulos, 2004). Therefore, we evaluated whether iron homeostasis was disrupted in cells depleted of ISCU. Perls' DAB staining indicated that cells depleted of ISCU show punctate ferric iron staining (Figure 5B) that is not found in control cells (Figure 5A). Furthermore, when cultured in iron-supplemented medium, iron accumulation was substantially increased in cells transfected with ISCU-univ (Figure 5E) compared to mock-transfected cells (Figure 5D), suggesting that cells depleted of ISCU were unable to register and respond to iron overload as a result of inappropriate activation of IRP1 and IRP2. As shown in Figure 4B, extensive disruption of mitochondrial Fe-S cluster biogenesis also inappropriately activates IRPs, and Perls' DAB staining confirmed that iron homeostasis was also disrupted in cells transfected with ISCU-mito siRNA (Figures 5C and 5F).

Interestingly, in untransfected cells, the expression of m-ISCU is regulated by iron. Iron depletion decreased m-ISCU protein biosynthesis (Figure 6A) and transcript levels (Figure 6B), suggesting that either the transcription of *ISCU* or the stability of *ISCU* mRNA was decreased. These results implied that iron deprivation can decrease ISCU expression and thus provide an additional mechanism to further activate IRPs in response to iron deficiency.

Discussion

Iron-sulfur clusters are cofactors in many important cellular processes, including electron transport, enzymatic catalysis, and regulation (Rouault and Tong, 2005). Thus, it is not surprising that the enzymes that catalyze de novo synthesis of Fe-S clusters are essential for viability in many different organisms (Li et al., 1999; Schilke et al., 1999; Seidler et al., 2001; Zheng et al., 1998). In addition, because of the sensitivity of Fe-S clusters toward species such as H_2O_2, NO, and O_2^-, Fe-S proteins are targets of oxidative stress (Bouton and Drapiers, 2003; Caltagirone et al., 2001; Gardner, 1997; Mueller et al., 2001; Pantopoulos and Hentze, 1995; Schriner et al., 2005). Thus, optimal maintenance of Fe-S protein functions would require both protection against oxidative damage as well as mechanisms for

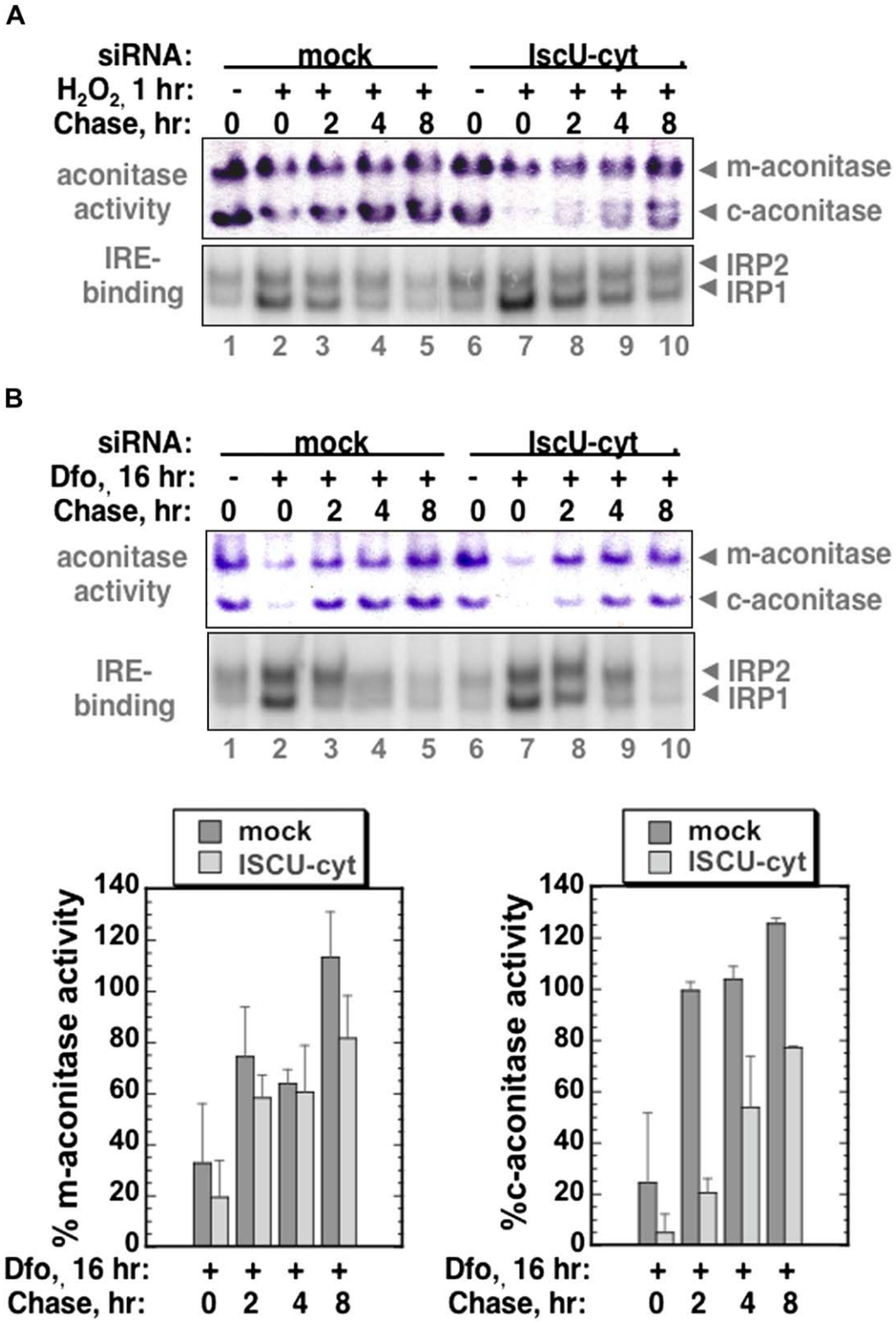

Figure 3. Isoform-specific silencing of c-ISCU impaired recovery of c-aconitase but not m-aconitase after a H_2O_2 or a Dfo challenge

A) Impaired recovery of c-aconitase activities in cells transfected with the ISCU-cyt siRNA after a H_2O_2 challenge. The results are representative of three or more H_2O_2 challenge experiments.

B) Impaired recovery of c-aconitase activities in cells transfected with the ISCU-cyt siRNA after a Dfo challenge. The results are representative of three or more Dfo challenge experiments. Aconitase activity in mock-transfected, untreated cells is set at 100% in the bar graphs. The error bars indicate a one standard deviation error.

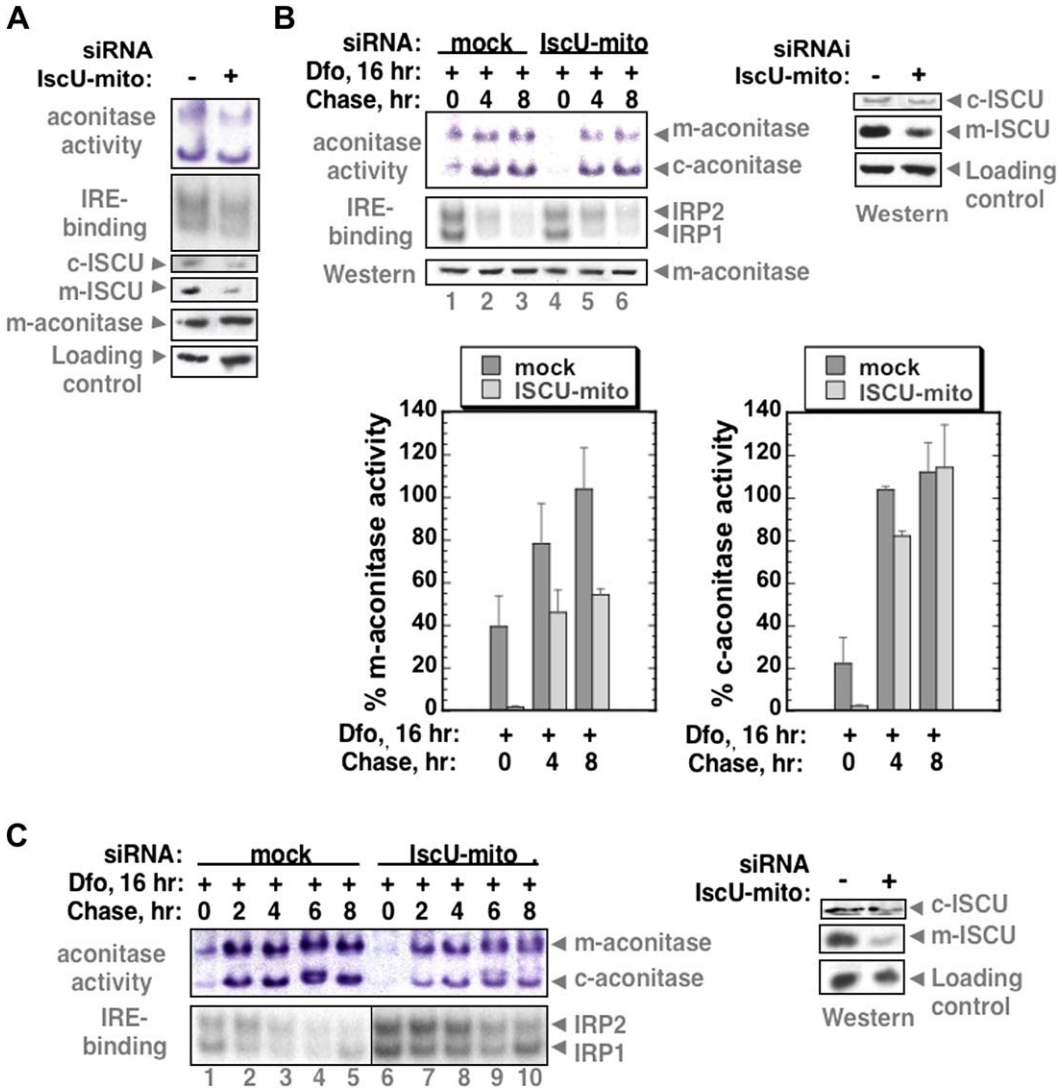

Figure 4. Silencing of m-ISCU changes aconitase and IRP activities in a dose-dependent manner

A) Aconitase activity, IRE binding assays, and Western blots of cells transfected with ISCU-mito siRNA. Loading control: Western blot of m-ISCS.

B) Moderate level of m-ISCU silencing (2-fold decrease as judged by Western blot analysis) impaired recovery of m-aconitase activities after Dfo treatment. Aconitase activity in mock-transfected, untreated cells is set at 100%. The error bars indicate a one standard deviation error.

C) Higher level of m-ISCU knockdown (4-fold decrease as judged by Western blot analysis) impaired recovery of both m- and c-aconitase activities and activated the IRE binding activities of both IRPs. The results are representative of three or more Dfo challenge experiments.

repair/regeneration. Studies in *S. cerevisiae* and *Drosophila* have shown that the mitochondrial and cytosolic superoxide dismutases provide protection to Fe-S proteins against damage by O_2^- generated under oxidative stress (Missirlis et al., 2003; Wallace et al., 2004), whereas the results described here indicate that m- and c-ISCU facilitate robust repair/regeneration of Fe-S clusters that are damaged by oxidative stress or iron deprivation.

An important finding of this study is that silencing of ISCU disrupted not only Fe-S cluster biogenesis but also iron sensing and regulation in human cells. In contrast to mock-transfected cells, IRP2 remained activated long after withdrawal of the iron chelator in cells that were depleted of both m- and c-ISCU (Figure 2E), suggesting that ISCU-deficient cells did not respond normally to changes in iron availability. Abnormal IRP activation may explain why these cells failed to register and defend against iron overload (Figure 5E). We have previously shown that compromised iron metabolism due to malfunction of IRPs can have severe physiological consequences. Genetically engineered mice that lack IRP2 ($IRP2^{-/-}$) showed ferritin accumulation and TfR deficiency in multiple tissues and developed adult-onset neurodegenerative disease (LaVaute et al., 2001), and genetic ablation of both IRPs is embryonic lethal (Smith et al., 2004). Furthermore, recent studies suggested that increased IRP1 activity can contribute to iron overload in the mitochondria of cells depleted of frataxin (Seznec et al., 2005). Taken together, these results further underscore the importance of IRPs and Fe-S cluster biogenesis in protecting cells from iron starvation and iron toxicity.

Whereas global silencing of human ISCU disrupted Fe-S cluster biogenesis in both m- and c-aconitases, the isoform-specific knockdowns provided evidence for functional specialization of

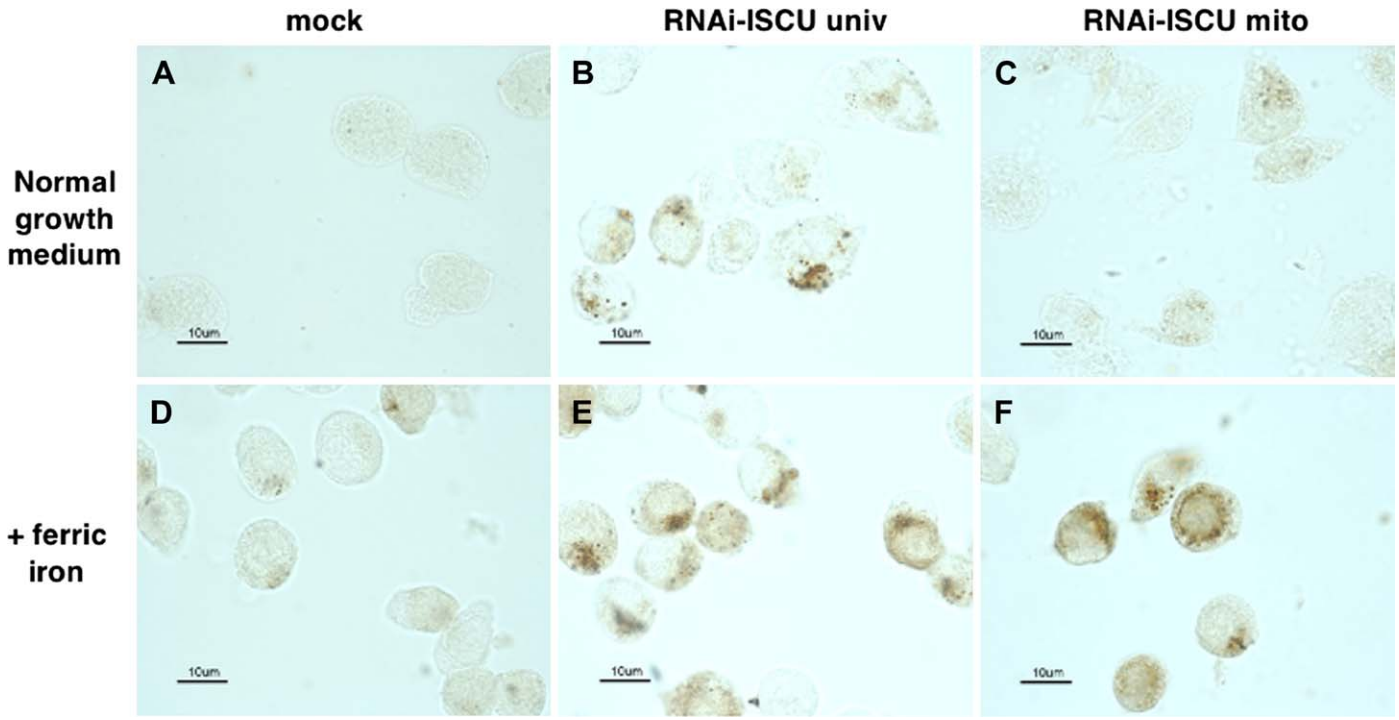

Figure 5. Misregulation of iron homeostasis in cells depleted of ISCU

Ferric iron staining of mock-transfected cells (**A** and **D**), cells transfected with ISCU-univ siRNA (**B** and **E**), and cells transfected with ISCU-mito siRNA (**C** and **F**) in normal or iron-supplemented medium.

m- and c-ISCU. Previously, the function of c-ISCU was questioned. Lill and coworkers reported that expression of the human m-ISCU, but not human c-ISCU, partially complemented the defects in yeast strains depleted of Isu1 and Isu2 and concluded that c-ISC is not functional (Gerber et al., 2004). However, the present study indicated that c-ISCU is active in mammalian cells. Despite the presence of abundant m-ISCU, silencing of c-ISCU impaired the recovery of the Fe-S cluster in c-aconitase (Figure 3). Conversely, moderate m-ISCU silencing had a stronger impact on the recovery of m-aconitase than c-aconitase (Figure 4B). These results indicated that both c- and m-ISCU are involved in Fe-S cluster biogenesis in mammalian cells. Since many key factors in mammalian iron metabolism, including IRP1, IRP2, and their regulatory targets, TfR, ferritin, and ferroportin, are absent in yeast, marked differences in iron metabolism pathways may result in different requirements for compartmentalized Fe-S cluster assembly in these divergent organisms. Furthermore, protein complexes of ISCU/ISCS/frataxin and ISCU/HSCA/HSCB have been reported in bacteria and yeast (Gerber et al., 2003; Ramazzotti et al., 2004; Tokumoto et al., 2002; Urbina et al., 2001), and coimmunoprecipitation experiments have demonstrated protein complexes containing cytosolic ISCS and ISCU in human cells (Tong and Rouault, 2000), suggesting that specific physical interactions among these proteins may be crucial for in vivo Fe-S cluster biogenesis. It is possible that expression of human c-ISCU alone in yeast cytosol was not sufficient to complement the yeast mutants lacking ISU1 or ISU2, due to lack of its natural interacting partners.

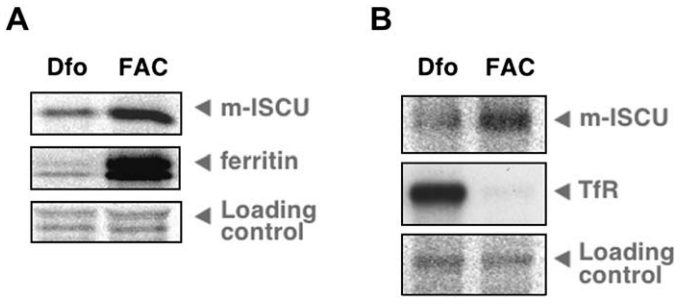

Figure 6. Iron-dependent regulation of ISCU

A) Immunoprecipitation of ISCU in metabolically labeled RD4 cells indicated that m-ISCU biosynthesis decreased in iron-depleted cells. Immunoprecipitations of ferritin and a loading control (nonspecific band) are shown for comparison.

B) Northern blot analysis showed decreased ISCU mRNA in iron-depleted cells. Northern blots of TfR and a loading control (one of the DMT1 isoforms) are shown for comparison.

Interestingly, extensive loss of m-ISCU not only impaired the recovery of Fe-S clusters in both m- and c-aconitases but also impaired deactivation of the IRE binding activities of both IRP1 and IRP2 (Figure 4C). Since Fe-S cluster biogenesis requires iron, sulfur (from cysteine), and Fe-S cluster assembly proteins, we interpreted the crosscompartment effect of extensive m-ISCU silencing to be a result of cytosolic iron depletion, as indicated by the persistent activation of IRP2 (Figure 4C). Previous studies have shown that IRP2 accumulates in iron-deficient cells and is targeted for degradation through the proteosome in cells replete with iron (Hanson et al., 2003; Pantopoulos, 2004). Various studies have suggested that iron might be involved in targeting IRP2 for degradation by directly binding to IRP2 or indirectly through a mechanism that involves heme or Fe(II)

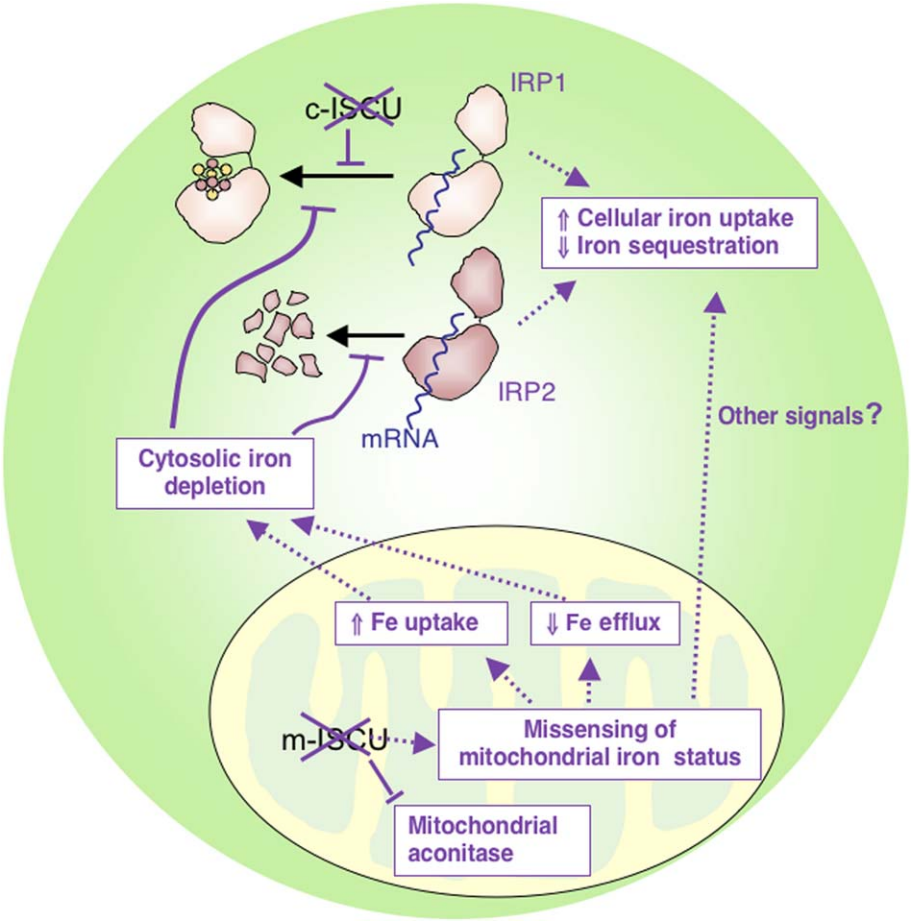

Figure 7. Proposed mechanism for disruption of mammalian iron homeostasis in cells with defective Fe-S cluster biogenesis

In analogy to the cytosolic iron sensor/regulator IRP1, a mitochondrial Fe-S protein might function as a biosensor for mitochondrial iron status. In cells depleted of m-ISCU, decreased mitochondrial Fe-S cluster biogenesis could be registered by the cell as mitochondrial iron deficiency, which could result in increased delivery of iron to mitochondria and decreased mitochondrial iron export. Cytosolic iron pools could become functionally depleted because of avid mitochondrial sequestration. As a result, IRP1 and IRP2 would be inappropriately activated, thus compromising cellular iron metabolism. In cells depleted of both m-ISCU and c-ISC, silencing of c-ISCU can also activate IRP1 directly and further exacerbate disruption of iron homeostasis.

α-ketoglutarate-dependent oxygenases (Ishikawa et al., 2005; Pantopoulos, 2004). Studies in *S. cerevisiae*, and in the human disease Friedreich ataxia, have indicated that disruption of Fe-S cluster biogenesis leads to mitochondrial iron overload (Babcock et al., 1997; Garland et al., 1999; Kispal et al., 1999; Knight et al., 1998; Li et al., 1999; Pandolfo, 2003; Rotig et al., 1997; Schilke et al., 1999; Strain et al., 1998) and cytosolic iron depletion (Knight et al., 1998; Li et al., 1999), suggesting that an Fe-S protein may be involved in sensing mitochondrial iron status, and mis-sensing of mitochondrial iron status may result in increased delivery of iron to mitochondria and decreased mitochondrial iron export. In mammalian cells, disruption of mitochondrial Fe-S cluster biogenesis may lead to functional iron deficiency in the cytosol and thereby inactivate c-aconitase and activate IRP1 and IRP2. These events, when combined with the activation of IRP1 due to silencing of c-ISCU, can act synergistically to further disrupt iron homeostasis (Figure 7).

Taken together, these results suggest that Fe-S cluster biogenesis in the cytosol and in the mitochondria coordinately drive the reprogramming of iron metabolism in different subcellular compartments to regulate overall cellular iron trafficking in response to iron needs. Interestingly, recent studies in yeast have demonstrated that, in response to iron deprivation, the RNA binding protein Cth2 downregulates multiple Fe-dependent pathways, including many Fe-S proteins and two Fe-S cluster scaffold proteins, Nfu1 and Isa1, to limit the utilization of scarce available iron (Puig et al., 2005). The iron-dependent regulation of mammalian ISCU (Figure 6) is consistent with a model in which mammalian cells respond to iron deficiency by activating cytosolic iron uptake through activation of IRPs, increasing mitochondrial iron uptake to preserve vital mitochondrial functions that are Fe dependent, and decreasing utilization of limited cellular iron by decreasing ISCU levels and curtailing Fe-S cluster biogenesis. It is tempting to speculate that the importance of precise coupling of iron trafficking with the changing metabolic needs in the different compartments might have provided selection pressure for the development of multiple Fe-S cluster assembly machineries in multicellular eukaryotes. Future experiments will be directed toward understanding the evolution and coordination of Fe-S cluster biogenesis in the different intracellular compartments of eukaryotic cells.

Experimental procedures

Cell cultures, transfections, lysates, and subcellular fractions

HeLa S3 cells (ATCC) were cultivated in DMEM-complete medium (DMEM medium supplemented with 10% fetal calf serum, 100 U/ml PenStrep, and 2 mM L-glutamine). Treatments with H_2O_2 were performed in EMEM medium with 100 U/ml PenStrep, and 2 mM L-glutamine to avoid reactivity of H_2O_2 with iron present in serum (Pantopoulos and Hentze, 1995). Cells were treated for 1 hr with H_2O_2, then washed and incubated in DMEM-complete medium. At different time-points, cells were washed and harvested for analysis. Treatments with Dfo were performed by incubating cells for 16 hr in DMEM-complete medium with 50 μM Dfo, then washed and incubated in DMEM-complete medium for up to 24 hr. Triton lysates were prepared as previously described with addition of 2 mM citrate (Tong et al., 2003). Subcellular fractions were prepared as previously described (Tong et al., 2003).

RNA oligonucleotide duplexes were purchased from Dharmacon and Qiagen. The target sequences are: ISCU-univ: TCAAGGCCGCCCTGGCTGA; ISCU-cyt: GTATTGTAGAGGAGACACA and TTGACATGAGTGTAGACCT; ISCU-mito: TCACAAGAAGGTTGTTGATC; m-aconitase: CGGGAAGACAT TGCCAATCTA. HeLa cells at a confluency of 30%–50% were transfected with 100 nM siRNA duplex using Oligofectamine or Lipofectamine 2000 (Invitrogen) every 72 hr for 3–7 days (Elbashir et al., 2002).

Aconitase activities

Aconitase activity gels are composed of a separating gel containing 8% acrylamide, 132 mM Tris base, 132 mM borate, 3.6 mM citrate, and a stacking gel containing 4% acrylamide, 67 mM Tris base, 67 mM borate, 3.6 mM citrate. The running buffer contains 25 mM Tris pH 8.3, 192 mM glycine, and 3.6 mM citrate. Samples contain cell lysates, 25 mM Tris-Cl, pH 8.0, 10% glycerol, and 0.025% bromophenol blue. Electrophoresis was carried out at 180 V at 4ºC. Aconitase activities were assayed by incubating the gel in the dark at 37ºC in 100 mM Tris (pH 8.0), 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM $MgCl_2$, 1.2 mM MTT, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase, and quantitation was performed using NIH Image software.

RNA mobility shift assays

Gel retardation assays were performed as described (Meyron-Holtz et al., 2004), with the following modifications: a 8% acrylamide/TBE gel, pre-run at 200 V for >30 min, and run at 200 V for 3.5 hr at room temperature. In the antibody supershift assays, lysates from cells treated with 50 μM Dfo for 16 hr were incubated for 1 hr at 4ºC with 0, 1, or 3 ul of rabbit serum raised against human IRP2 or an unrelated protein (control) and analyzed on an 8% acrylamide/TBE gel run at 200 V for 4.5 hr at 4ºC.

RT-PCR, Western blot, Northern blot, and immunoprecipitation

RT-PCR analyses were performed using human heart poly(A)$^+$ RNA (Clontech) using procedures described previously (Tong and Rouault, 2000). Western analyses of ISCU were carried out using two different affinity-purified antibodies, #2934 and #2677, which are raised against different peptide sequences common to both c- and m-ISCU. In Western blots and immunoprecipitation experiments, antibody #2934 detects a strong band for m-ISCU and a much weaker band for c-ISCU, consistent with Northern and RT-PCR data. In contrast, comparisons between Western blot and immunoprecipitation studies of steady-state and newly synthesized proteins using antibody 2677 indicated that a posttranslational modification results in weaker detection of the mature m-ISCU in Western blots by #2677 (Tong and Rouault, 2000). Antibodies to human IRP2 were described previously (Meyron-Holtz et al., 2004). Antibody to m-aconitase was raised against peptide YDLLEKNINIVRKRLNR. IRP1 antibody was prepared against amino acid #2-16 of mouse IRP1 and can detect human IRP1. RD4 cells were grown for 16 hr in medium supplemented with 100 μM of Dfo or 100 mg/ml ferric ammonium citrate (FAC), and Northern analysis, metabolic labeling, and immunoprecipitation experiments were carried out as previously described (Tong and Rouault, 2000).

Iron staining

Transfected cells were treated with or without 100 mg/ml FAC for 16 hr. Cells were fixed and stained simultaneously in 4% formalin and Perls' solution (1% $K_4Fe(CN)_6$ and 1% HCl) for 30 min at room temperature. After rinsing in PBS, cells were incubated with 0.75 mg/ml diaminobenzidine (DAB), 0.07% H_2O_2 in 1 M Tris pH 7.5 for 30–60 min. The reaction was stopped by rinsing in PBS. Negative control slides were prepared with control cells through the DAB intensification without the Perls' solution preincubation. No positive staining was detected in the negative control sections.

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