Iron-regulatory protein 1 (IRP1) belongs to a family of RNA-binding proteins that modulate metazoan iron metabolism. Multiple mechanisms are employed to control the action of IRP1 in dictating changes in the uptake and metabolic fate of iron. Inactivation of IRP1 RNA binding by iron primarily involves insertion of a [4Fe-4S] cluster by the cytosolic iron–sulfur cluster assembly (CIA) system, converting it into cytosolic aconitase (c-acon), but can also involve iron-mediated degradation of IRP1 by the E3 ligase FBXL5 that also targets IRP2. How CIA and FBXL5 collaborate to maintain cellular iron homeostasis through IRP1 and other pathways is poorly understood. Because impaired Fe-S cluster biogenesis associates with human disease, we determined the importance of FBXL5 for regulating IRP1 when CIA is impaired. Suppression of FBXL5 expression coupled with induction of an IRP1 mutant (IRP1<sup>3C<sup>&gt;</sup>3S</sup>) that cannot insert the Fe-S cluster, or along with knockdown of the CIA factors NUBP2 or FAM96A, reduced cell viability. Iron supplementation reversed this growth defect and was associated with FBXL5-dependent polyubiquitination of IRP1. Phosphorylation of IRP1 at Ser-138 increased when CIA was inhibited and was required for iron rescue. Impaired CIA activity, as noted by reduced c-acon activity, was associated with enhanced FBXL5 expression and a concomitant reduction in IRP1 and IRP2 protein level and RNA-binding activity. Conversely, expression of either IRP induced FBXL5 protein level, demonstrating a negative feedback loop limiting excessive accumulation of iron-response element RNA-binding activity, whose disruption reduces cell growth. We conclude that a regulatory circuit involving FBXL5 and CIA acts through both IRPs to control iron metabolism and promote optimal cell growth.

Iron-regulatory proteins (IRPs)<sup>2</sup> are central regulators of cellular iron metabolism in vertebrates (1, 2). IRP1 and IRP2 are iron-regulated RNA-binding proteins that control the fate of mRNAs encoding critical modulators of iron metabolism (1, 2). IRP1 RNA binding is primarily controlled through insertion or loss of the Fe-S cluster, the so-called Fe-S switch mechanism. Insertion of a [4Fe-4S] cluster into the IRP1 apoprotein converts it to the cytosolic isoform of aconitase (c-acon) and alters iron metabolism by eliminating IRP1 RNA-binding activity. In some cell types, c-acon is present in vast excess compared with the RNA-binding form (3, 4). The fact that only a small portion of c-acon is recruited to IRP1 even in severe iron deficiency (3) suggests that unregulated conversion of this large pool of c-acon into the RNA-binding form would have negative consequences unless a compensatory mechanism limits IRP1 activation. Interestingly, IRP1 can be degraded when the Fe-S switch is inhibited, but the mechanistic links underlying this have not been fully explored (5–11). These studies suggest that the level at which IRP1 RNA-binding activity is increased in response to inhibition of Fe-S cluster biogenesis depends on the extent to which the apoprotein is degraded. The fact that the E3 ligase, FBXL5, which controls degradation of IRP2, also targets IRP1 suggests a role for this proteolytic mechanism in managing IRP1 accumulation in response to CIA inhibition (12, 13). Furthermore, the unanticipated finding that CIA inhibition alters IRP2 protein level suggests the existence of a regulatory circuit between Fe-S cluster biogenesis activity, FBXL5 action, and the steady-state level of each IRP (10). Taken together, CIA and FBXL5 appear to collaborate with IRP in sensing cellular iron status and needs to ensure that iron levels are optimal for cell viability.

Two dedicated systems exist for Fe-S cluster biogenesis (14). The mitochondrial iron–sulfur cluster (ISC) assembly system not only assembles Fe-S clusters for mitochondrial proteins, but its activity is required for the cytosolic iron–sulfur cluster assembly (CIA) system (15). The CIA system is essential for maturation of Fe-S proteins in the cytosol and nucleus (14, 16). Disruption of either the ISC or CIA system substantially impairs cell function with potentially lethal consequences (17, 18). In humans, highly debilitating diseases, including some anemias, ataxia, and encephalopathies, are linked to impaired Fe-S cluster biogenesis (17, 18). Whereas many recent studies have identified the major components of Fe-S biogenesis systems and the impact of inborn metabolic errors in their genes on cell function, much remains to be defined concerning the interrelationships between this key
cellular process and the control of the metabolism of its central substrate, iron.

Recent studies have shed much light on the pathway of cytosolic Fe-S cluster biogenesis (14, 16). The heterotetramer composed of two copies of NUBP1 (also known as NBP35) and NUBP2 (also known as CFD1) serves as a scaffold for formation of transient Fe-S clusters that are transferred to IOP1, a key factor linking early and late steps of cytosolic Fe-S biogenesis. Subsequently, the CIA pathway divides into two branches with a complex containing FAM96B (also known as CIA2B) targeting general Fe-S cytosolic or nuclear apoproteins, whereas for the second branch, a complex including FAM96A (also known as CIA2A) targets IRP1 for cluster insertion. Knockdown of FAM96A causes a loss of IRP1 RNA-binding activity and protein level and an unexpected similar response of IRP2, whereas knockdown of FAM96B unexpectedly increased IRP2 without altering IRP1 (10). The mechanism(s) responsible for these unanticipated changes in IRP protein level and RNA-binding activity are not understood but further suggest a functional link between CIA and FBXL5.

In this study, the response of cells to CIA system inhibition was investigated, including asking what role FBXL5 has in limiting potentially deleterious consequences of overaccumulation of IRP1. Knockdown of FBXL5 itself failed to affect cell viability, but cell growth was strongly inhibited when this was combined with expression of an IRP1 mutant with constitutive RNA binding or if CIA activity was impaired by suppression of either NUBP2 or FAM96A. In response to CIA inhibition, IRP1 and IRP2 protein level and total IRP RNA-binding activity was reduced. Ser-138 phosphorylation of IRP1 increased when CIA was inhibited, and this phosphorylation event was required for complete iron rescue of cell growth. Interestingly, CIA inhibition or induction of either IRP1 or IRP2 was associated with increased FBXL5 protein level, demonstrating the existence of a feedback loop limiting the overaccumulation of either IRP. We conclude that an interactive regulatory network between the CIA system, both IRPs, and FBXL5 is critical for the appropriate management of the uptake and metabolic fate of iron in cell proliferation.

**Results**

**A synthetic growth defect in human embryonic kidney (HEK) 293 cells is induced by uncoupling IRP1 from the Fe-S switch along with FBXL5 knockdown**

Two approaches were used to determine the regulatory interrelationships between the Fe-S cluster biogenesis and FBXL5 mechanisms in the control of cellular iron metabolism by IRP1. First, two CIA factors, one acting early (NUBP2) and one acting late (FAM96A) in the CIA pathway, were knocked down with or without combined knockdown of FBXL5. NUBP2 is one of two scaffold proteins functioning as the initial site of formation of cytosolic Fe-S clusters before they are ultimately transferred to apo-Fe-S proteins functioning in the cytosol or nucleus (14, 16). FAM96A is downstream of NUBP2, inactivates apo-IRP1 by promoting cluster insertion, and has a stabilizing effect on IRP2 (10). Second, we determined the impact of FBXL5 knockdown in cells expressing either wild-type IRP1 or an Fe-S cluster mutant of IRP1 (IRP1^{3C->3S}) that is constitutionally present in the RNA-binding form.

Using previously validated siRNA protocols (10), we determined the response of HEK 293 cells to the individual or combined knockdown of a CIA factor and FBXL5. Individual knockdown of NUBP2, FAM96A, and FBXL5 failed to affect cell number (Fig. 1A, lanes 2–4). In contrast, the combined knockdown of NUBP2 along with FBXL5 significantly impaired cell growth (Fig. 1A, lane 5). The same response was observed when FBXL5 and the CIA specificity factor for IRP1, FAM96A, were simultaneously knocked down (Fig. 1A, lane 6). A potential explanation for the reduction in cell growth in response to combined knockdown of CIA factors and FBXL5 could be iron toxicity caused by an excessive accumulation of IRE RNA-binding activity, presumably resulting in increased iron uptake (TFR) and reduced storage (ferritin). However, in contrast to what was expected, iron treatment blocked the decrease in cell viability in response to impaired CIA and FBXL5 function and was associated with an increase in FBXL5 protein level (Fig. 1B; see Fig. 7C for FBXL5 data). We conclude that FBXL5 protects cells from the negative consequences of reduced CIA activity.

Given the link between CIA activity and the regulation of IRP1 RNA-binding activity, we sought to determine the extent to which the protective effect of FBXL5 was linked to IRP1 dysregulation. Cells capable of expressing Myc-tagged IRP1^{3C->3S} or, as a control, Myc-tagged IRP1^{WT} were treated without or with tetracycline (tet) and transfected with either non-targeting (NT) or FBXL5-targeting siRNAs. In the absence of tet, when IRP1^{3C->3S} is not expressed, FBXL5 siRNA did not affect cell viability (Fig. 1C, lane 2). Similarly, induction of IRP1^{3C->3S} in the presence of an NT siRNA failed to impact cell growth but was associated with a strong increase in FBXL5 protein level (Fig. 1C, lane 3). However, when FBXL5 expression was knocked down in the presence of IRP1^{3C->3S}, cell viability was significantly reduced (Fig. 1C, lane 4). In contrast, simultaneous treatment with FBXL5 siRNA along with tet induction of IRP1^{WT} was without effect on cell viability (Fig. 1D). Interestingly, when the level of FBXL5 expression was normalized to that of Myc-tagged IRP1^{WT} or IRP1^{3C->3S}, the E3 ligase accumulated to a 3-fold higher level in the latter case (Fig. 1E). As was observed with combined knockdown of CIA and FBXL5, iron supplementation prevented the reduction in viability of HEK cells expressing IRP1^{3C->3S} in the presence of FBXL5 siRNA (Fig. 1F, lane 6) perhaps because iron stabilizes FBXL5 protein (12, 13) and increases its expression relative to cells treated with FBXL5 siRNA alone (Fig. 1F, compare lanes 5 and 6). Iron chelation with desferal failed to rescue the cells (results not shown). We conclude that when the Fe-S switch mechanism is not functional, as occurs when CIA is inhibited, the action of FBXL5 is critical in limiting the negative impact of excess IRP1 RNA-binding activity on cell function.

The impact of FBXL5 knockdown on the level of both IRP1^{3C->3S} and endogenous IRP2 was determined (Fig. 1, F and G). When compared with HEK cells treated with the NT siRNA, FBXL5 siRNA treatment significantly increased the level of IRP1^{3C->3S} protein (Fig. 1F, compare lanes 4 and 5). The endog-
The enous level of IRP2 protein was also increased when FBXL5 was knocked down (Fig. 1G, compare lanes 2 and 1). The impact of IRP1<sup>3C</sup> on IRP2 protein level was also examined. Whereas induction of IRP1<sup>3C</sup> failed to statistically increase IRP2 protein level (p = 0.083), such that there was no longer a significant impact of concurrent knockdown of FBXL5 on IRP2 protein level (compare lane 4 with lane 3 and lane 2 with lane 1 in Fig. 1G). This is consistent with the previous studies showing that the RNA-binding forms of IRP1 and IRP2 are both subject to FBXL5 regulation (12, 13). Taken together, these results suggest that in the face of reduced CIA activity, dysregulation of both IRPs may occur unless sufficient FBXL5 is present to prevent their overaccumulation.

Impact of CIA knockdown on IRP1 and IRP2 protein level

Because apoprotein mutants of IRP1 that cannot assemble an Fe-S cluster exhibit iron-dependent regulation of protein stability (6, 19), and knockdown of some CIA factors, such as FAM96A (10), reduces IRP1 protein level, the role of FBXL5 in controlling IRP1 protein level in response to knockdown of CIA factors was investigated. Knockdown of NUBP2 reduced IRP1 protein level by 20%, but this difference did not reach statistical significance at this time point (p = 0.086) (Fig. 2A, lanes 1 and 2). Western blots were performed on cell lysates using the 9E10 anti-Myc monoclonal antibody to detect Myc-tagged IRP1WT or IRP1<sup>3C</sup> or using antibodies directed against FBXL5, IRP2, or tubulin. Representative blots from n = 3 experiments are shown. Antibodies against Fam96a are not available.
However, extended treatment from the standard 9-day to 12-day treatment with NUBP2 siRNA decreased IRP1 protein level without affecting the level of another Fe-S protein (GPAT) or tubulin (Fig. 2B, lanes 1 and 2). IRP2 responded similarly to IRP1. Extending treatment to 15 days resulted in loss of IRP1, IRP2, and GPAT but not tubulin (Fig. 2B, lanes 3 and 4). Knockdown of FAM96A reduced IRP1 protein level more effectively than was the case with NUBP2, with a nearly 50% reduction compared with cells treated with the NT siRNA, after the standard 9-day transfection protocol (Fig. 2A). This effect of FAM96A knockdown on IRP1 protein level was blocked by simultaneous transfection with FBXL5 siRNAs (Fig. 2A, compare lanes 6 and 3 with lane 1) (Fig. 7C contains FBXL5 protein expression data for this experiment).

Because previous work has shown that IRP2 protein level (10) is reduced when FAM96A is knocked down, we asked whether a similar response occurred with NUBP2 knockdown and what role FBXL5 had in each scenario. IRP2 protein level declined by 40% upon knockdown of NUBP2 and by 80% when FAM96A was knocked down (Fig. 2C, lanes 2 and 3 versus lane 1). The reduction in IRP2 protein expression in response to knockdown of NUBP2 or FAM96A was completely blocked by

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**Figure 2. Impact of CIA knockdown on IRP1, IRP2, and GPAT protein level and cytosolic aconitase activity.** A–C, Western blots for endogenous IRP1, IRP2, NubP2, GPAT, or tubulin were performed on cell lysates as described under “Experimental procedures.” A, wild-type HEK 293 cells were transfected with NT, NubP2, or Fam96a targeting siRNA and then with or without FBXL5 siRNA as described in the legend to Fig. 1. B, the siRNA transfections were extended such that the wild-type HEK 293 cells were transfected on day 9 with harvest on day 12 (lanes 1 and 2) or transfected on days 9 and 12 with harvest on day 15 (lanes 3 and 4). Representative blots from n = 3 experiments are shown. C, wild-type HEK 293 cells were treated as described for A. D, cells were transfected with NubP2 or Fam96a siRNA, and cytosolic and mitochondrial fractions were obtained after digitonin permeabilization as described under “Experimental procedures” (11). The mean aconitase activities for the cytosolic and mitochondrial fractions were 5.38 and 1.31 milliunits/mg, respectively. Results were determined using a Student’s two-tailed t test and are expressed as mean ± S.E. (error bars) for n = 3–6 separate experiments.
co-transfection with FBXL5 siRNA (Fig. 2C, lanes 5 and 6) (see Fig. 6C for FBXL5 expression level). Taken together, it is apparent that the response of IRP1 and IRP2 to knockdown of NUBP2 or FAM96A occurred earlier than was the case for the cytosolic Fe-S protein GPAT (Fig. 2B). These results indicate that targeted degradation of both IRPs by FBXL5 represents an adaptive response necessary for maintenance of cell viability when cytosolic Fe-S biogenesis is impairead.

Knockdown of NUBP2 or FAM96A decreases the cytosolic aconitase activity of IRP1

To further understand how impaired CIA assembly affects IRP1 function, the impact of NUBP2 and FAM96A knockdown on c-acon activity was assessed. Knockdown of NUBP2 or FAM96A decreased c-acon activity by 52 and 60%, respectively (Fig. 2D). The activity of mitochondrial aconitase was not significantly affected. Interestingly, the observation that NUBP2 knockdown decreased c-acon activity indicates that it is a bona fide CIA component in mammalian cells. This result demonstrates that like FAM96A (10), NUBP2 has an important role in human CIA activity, including regulating the function of IRP1.

The impact of CIA factor knockdown on IRE RNA-binding activity

The impact of CIA factor knockdown on IRP protein level suggests that RNA-binding activity is also altered. IRP RNA-binding activity was determined by electrophoretic mobility shift assay (EMSA). Because IRP1 and IRP2 co-migrate in human cells, the sum of IRP1 plus IRP2 RNA-binding activity was determined first. The RNA-binding activity obtained in as-isolated cell lysates (Spontaneous) and the total cellular RNA binding (Total) was determined after activation of the latent pool of IRP1 with 2-mercaptoethanol (Fig. 3A). An antibody specific to IRP1 was used to quantify IRP1-specific binding by gel supershift (Fig. 3B). IRP1 RNA-binding activity, measured by supershift with an antibody specific to IRP1, showed a substantial reduction in RNA-binding activity in response to NUBP2 siRNA or FAM96A siRNA (Fig. 3B). FBXL5 siRNA did not reverse the
reduction in IRP1 RNA-binding activity induced by knockdown of NUBP2 or FAM96A. 2-ME-induced IRP1 RNA-binding activity showed a similar response. The lack of impact of FBXL5 knockdown in reversing the decline in RNA-binding activity of IRP1 when CIA was inhibited contrasted with the partial rescue of IRP1 protein level noted in Fig. 2A, suggesting accumulation of a form of IRP1 that neither binds RNA or is an aconitase. Previous work in HEK cells or when IRP1 was expressed in yeast observed that IRP1 can accumulate in a form that neither binds RNA nor exhibits aconitase activity (20, 21).

In contrast, the non-supershifted band, which represents IRP2 RNA-binding activity, did exhibit an FBXL5-dependent response. IRP2 RNA binding was reduced upon knockdown of NUBP2 or FAM96A, but when FBXL5 was simultaneously knocked down, RNA binding increased significantly (Fig. 3B, bottom histogram, compare lanes 5 and 6 with lanes 2 and 3, respectively).

It should be noted that, upon knockdown of CIA components in HEK cells, the fraction of IRP1 present in the RNA-binding form, measured as spontaneous over 2-ME-inducible RNA binding, was not increased in our study. This contrasts with earlier studies in HeLa cells where knockdown of Fe-S biogenesis activity increased the fraction of IRP1 in the RNA-binding form (5, 9–11) but is similar to the impact of longer-term reduction in Fe-S biogenesis in liver, which included induction of FBXL5 expression (8). Importantly, our previous studies (22) in HeLa cells found a level of IRP1 RNA-binding activity that was about 10-fold higher than what we observed in the current study with HEK cells, suggesting a different set point for regulation of IRPs in HeLa cells. To address this, we determined the relative abundance of FBXL5, IRP1, and IRP2 at the protein level in these cell lines. HEK cells were found to express substantially higher levels of FBXL5 as compared with HeLa cells in the absence or presence of added iron (Fig. 4). Conversely, HEK cells expressed a lower level of both IRP1 and IRP2 protein compared with HeLa cells. Taken together, these observations suggest that cell type–specific differences in FBXL5 expression influence the steady-state level of RNA binding by both IRPs as well as their response to changes in Fe-S biogenesis activity.

**Impact of knockdown of CIA factors on TfR1 and ferritin protein level**

To relate the impact of knockdown of CIA factors and FBXL5 on IRP action, the expression of TfR1 and ferritin heavy chain protein was determined. Like what was shown when FAM96A was knocked down (10), knockdown of NUBP2 resulted in a paradoxical IRP-independent increase in TfR and decline in H-ferritin protein level (Fig. 5, A and B, lanes 2 and 3), although IRP1 and IRP2 protein level (see Fig. 2) and total as well as IRP1 RNA-binding activity (Fig. 3) were decreased upon treatment with CIA targeting siRNAs. To extend this finding, the impact of simultaneous knockdown of FBXL5 was examined. Interestingly, TfR1 protein level was further increased in response to simultaneous knockdown of FBXL5 (Fig. 5A, lanes 5 and 6) when total IRE-binding activity (Fig. 3A) and IRP2 protein level increased (Fig. 2C). These findings suggest that cells act to limit IRP action in response to CIA inhibition.

**FBXL5 targets IRP1 for proteasomal degradation**

Because previous studies demonstrated that IRP1\(^{3C>3S}\) is stabilized by depletion of FBXL5 (12), we reasoned that this E3 ligase was required for control of IRP1 protein stability when CIA is impaired or in response to iron overload. Cells capable of expressing Myc-tagged IRP1\(^{WT}\) or IRP1\(^{3C>3S}\) were transiently transfected with a HA-tagged ubiquitin construct (23). The ubiquitination state of IRP1 was assessed by immunoprecipitation of Myc-tagged IRP1 followed by immunoblotting for the HA epitope. IRP1\(^{WT}\) exhibited a significant level of mono- or perhaps oligoubiquitinated species, the abundance of which was not detectably affected by iron status or the proteasome inhibitor MG115 (Fig. 6A, lanes 3–4). In contrast, a substantial amount of polyubiquitinated IRP1\(^{3C>3S}\) was detected, the abundance of which was iron-regulated (Fig. 6A, lanes 9–12). The polyubiquitinated species of IRP1\(^{3C>3S}\) increased upon treatment of cells with MG115 and iron, whereas desferal eliminated it. Polyubiquitination of IRP1\(^{3C>3S}\) was substantially inhibited by FBXL5 knockdown (results not shown); others

**Figure 4. Expression level of IRPs and FBXL5 in HEK 293 and HeLa Cells.**

The protein level of FBXL5, IRP1, IRP2, and tubulin was determined in HEK cells or HeLa cells grown in the absence or presence of 8 \mu M FAC for 4 h. For all panels, representative results for \(n = 3–4\) experiments are shown in the Western blots. Results are expressed as mean ± S.E. (error bars).
IRP1 and FBXL5 synergism and iron metabolism in cell growth

Figure 5. Impact of CIA knockdown on TfR1 and ferritin protein expression. HEK 293 cells were transfected with various siRNAs as described in the legend to Fig. 1. A, TfR1 protein expression was determined in cell lysates by immunoblotting with an antibody against TfR1. B, ferritin heavy chain protein expression was determined in the same lysates by immunoblotting with antibody against H-ferritin. α-Tubulin was used as a control. Representative blots from n = 3 experiments are shown. A two-tailed Student’s t test was used to determine statistical significance of three independent experiments.

have noted that FBXL5 overexpression increases the polyubiquitination of IRP1<sup>3C≡3S</sup> (13). We conclude that the apoprotein form of IRP1 is the preferred substrate for iron-dependent FBXL5-mediated polyubiquitination.

IRP1 is polyubiquitinated when CIA is impaired

The failure to detect polyubiquitination of transfected IRP1<sup>WT</sup> in HEK cells may relate to the fact that in some cell types, <5% of IRP1 is present in the apoprotein (RNA-binding) form (3, 4, 21). To address this, HEK cells were treated with NUBP2 siRNA to decrease formation of c-acon from IRP1 and co-transfected with the HA-ubiquitin expression plasmid. NUBP2 knockdown led to increased accumulation of polyubiquitinated IRP1<sup>WT</sup> (Fig. 6B, lane 2). Inhibition of the proteasome with MG115 substantially increased the level of polyubiquiti-
in the absence of added iron (Fig. 7A, lane 7). About 8-fold more FBXL5 protein was detected in cells expressing IRP1WT and IRP13C relative to IRP1WT in the absence of added iron (Fig. 7A, compare lanes 7 and 2). As was the case with IRP1WT, MG115 did not further increase FBXL5 protein level in cells simultaneously treated with iron (Fig. 7A, compare lane 9 with lane 8). Desferal blocked the induction of FBXL5 by either IRP1 allele.

To determine whether the induction of FBXL5 was specific to IRP1, a HEK cell line that expresses FLAG-tagged IRP2 was transfected with either NT siRNA or NuBP2 siRNA on days 0, 3, and 6. On day 6, all cells were transfected with the HA-ubiquitin expression construct, and cells capable of expressing IRP1WT were transfected with either NT siRNA or NuBP2 siRNA followed by FBXL5 siRNA using the 9-day protocol as in Fig. 1. On day 9, cells were treated with tet for 12 h prior to harvesting. During the last 4 h of tet induction, cells were treated with various combinations of 8 μM FAC and 2.5 μM MG115. Cells lysates were then immunoprecipitated and examined by Western blotting using the indicated antibodies (n = 3). C, HEK 293 cells were transfected with either NT siRNA or NuBP2 siRNA on days 0, 3, and 6. On day 6, all cells were transfected with the HA-ubiquitin expression construct, and cells in lane 3 were also transfected with FBXL5 siRNA. Cell extracts were then immunoprecipitated using an antibody that recognizes endogenous IRP1. The immunoprecipitates were then examined by Western blotting for HA-ubiquitin or IRP1WT (n = 3). D, wild-type HEK 293 cells were plated for 24 h and transfected with the HA-ubiquitin expression plasmid for 20 h. Cells could grow for another 24 h and were treated with 100 μM Df for 12 h followed by treatment with 2.5 μM MG115 and 8 μM FAC for 1, 2, and 12 h (n = 3). Cell extracts were immunoprecipitated and probed for endogenous IRP1 as described in C.

Enhanced FBXL5 expression in response to CIA inhibition

The reduction in IRP2 protein level when CIA factors NUBP2 and FAM96A (10) are knocked down (Fig. 2C) suggests that cytosolic iron is increased, which is in line with increased TfR1 expression (Fig. 5A) and binding of iron-loaded transferrin to cells with impaired CIA activity (10, 11). Consistent with this, a strong impact of CIA knockdown on FBXL5 protein level was observed (Fig. 7C), reflecting the impact of iron (Fig. 7A). NUBP2 knockdown resulted in a nearly 2.3-fold increase in FBXL5 protein accumulation relative to cells treated with NT siRNA (Fig. 7C, lane 2). FAM96A siRNA treatment resulted in a 3.3-fold increase (Fig. 7C, lane 3). The relative impact of knockdown of these two CIA factors mirrors their impact on IRP2 and IRP1 protein abundance, IRP RNA binding, and c-acon activity. FAM96A knockdown had the stronger effect, followed by NUBP2. Furthermore, a reciprocal impact of the knockdown of CIA factors on FBXL5 relative to IRP protein levels was observed (compare Fig. 2 (A and C) with Fig. 7C). FBXL5 protein level is increased, whereas that of both IRP1 and IRP2 is decreased.

Figure 6. FBXL5-dependent regulation of IRP1 in response to impaired CIA. A, HEK 293 cells that can express Myc-tagged IRP1WT or IRP13C were transfected with an HA-ubiquitin (HA-UB) expression construct for 20 h. Cells were then treated with tet to induce expression of Myc-IRP1WT or Myc-IRP13C for 36 h followed by a 4-h treatment with various combinations of 8 μM FAC, proteasome inhibitor MG115 (2.5 μM), or 100 μM Df. Cell lysates were subjected to immunoprecipitation (IP) with the 9E10 anti-Myc monoclonal antibody followed by immunoblotting (IB) for HA-ubiquitin or Myc-IRP1. B, HEK 293 cells capable of expressing IRP1WT were transfected with either NT siRNA or NuBP2 siRNA followed by FBXL5 siRNA using the 9-day protocol as in Fig. 1. On day 9, cells were treated with tet for 12 h prior to harvesting. During the last 4 h of tet induction, cells were treated with various combinations of 8 μM FAC and 2.5 μM MG115. Cells lysates were then immunoprecipitated and examined by Western blotting using the indicated antibodies (n = 3). C, HEK 293 cells were transfected with either NT siRNA or NuBP2 siRNA on days 0, 3, and 6. On day 6, all cells were transfected with the HA-ubiquitin expression construct, and cells in lane 3 were also transfected with FBXL5 siRNA. Cell extracts were then immunoprecipitated using an antibody that recognizes endogenous IRP1. The immunoprecipitates were then examined by Western blotting for HA-ubiquitin or IRP1WT (n = 3). D, wild-type HEK 293 cells were plated for 24 h and transfected with the HA-ubiquitin expression plasmid for 20 h. Cells could grow for another 24 h and were treated with 100 μM Df for 12 h followed by treatment with 2.5 μM MG115 and 8 μM FAC for 1, 2, and 12 h (n = 3). Cell extracts were immunoprecipitated and probed for endogenous IRP1 as described in C.

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IRP2 is decreased when CIA factor expression is impaired. These findings support the concept that FBXL5 is an integral component of the adaptive processes influencing the IRP-dependent control of cellular iron metabolism when Fe-S biogenesis is dysregulated.

Phosphorylation of IRP1 at Ser-138 is required to maintain cellular growth rate in response to CIA inhibition

Previous studies have suggested that Ser-138 phosphorylation of IRP1 targets the RNA-binding form and favors its accumulation due to destabilization of the Fe-S cluster in c-acon (6, 21, 25). Hence, we reasoned that induction of Ser-138 phosphorylation might represent an adaptive response to CIA inhibition, perhaps by promoting TfR accumulation and iron uptake. Knockdown of NUBP2 resulted in a 3-fold increase in Ser-138-phosphorylated IRP1 (Fig. 8A, lane 2 versus lane 1). The abundance of Ser-138-phosphorylated IRP1 was not affected by knockdown of FBXL5 as expected based on previous studies with Ser-138 phosphomutants (6). Interestingly, CIA inhibition resulted in a level of Ser-138 phosphorylation of IRP1 similar to that observed in response to the addition of the strong PKC agonist phorbol 12-myristate 13-acetate (Fig. 8A, lane 4).

To determine whether Ser-138 phosphorylation of IRP1 played a role in the coordination of iron metabolism and cell proliferation, the impact of the non-phosphorylatable mutant IRP1<sup>3C>3S</sup>/S<sup>138A</sup> on the response of HEK cells to FBXL5 knockdown was determined. As noted previously, coordinate induction of IRP1<sup>3C>3S</sup> and knockdown of FBXL5 impaired cell growth, and this response was reversed by iron (Fig. 8B). In contrast, in the presence of IRP1<sup>3C>3S</sup>/S<sup>138A</sup> and FBXL5 knockdown, cell viability was not rescued by iron (Fig. 8B, compare lanes 6 and 3). These findings support the concept that induction of Ser-138 phosphorylation of IRP1 is an adaptive response.

Figure 7. Stimulation of FBXL5 protein expression by induction of IRP1 or IRP2 or knockdown of FBXL5. A and B, HEK cells were treated with tet for 24 h to induce the expression of Myc-tagged IRP1<sup>WT</sup> or IRP1<sup>3C>3S</sup>. A and B, the 24-h treatment without or with tet was followed by a 4-h treatment with various combinations of 8 µM FAC, 2.5 µM MG115, and 100 µM Df. Cell lysates were probed for IRP2, FBXL5, and Myc-tagged-IRP1 (n = 4 experiments). B, HEK cells were treated with tet to induce the expression of IRP2<sup>FLAG</sup> for 24 h followed by a 4-h treatment with various combinations of 8 µM FAC, 2.5 µM MG115, and 100 µM Df. Cell lysates were then examined by immunoblotting using the indicated antibodies (n = 4). C, wild-type HEK 293 cells were transfected and harvested as in Fig. 1. Western blots using antibodies against FBXL5 and α-tubulin were conducted. For C, the samples used were from the same experiment as Fig. 5A, and the same α-tubulin blot is shown as a loading control for C and Fig. 5A. For all blots shown in this figure, representative blots from n = 3 experiments are shown, and tubulin was used as a loading control. Results are expressed as mean ± S.E. (error bars) for n = 3 experiments.
antibodies against FBXL5, tubulin, and the Myc epitope on IRP13C trypan blue exclusion. Cell lysates were subjected to Western blotting using Myc-tagged IRP13C of siRNA treatment, cells were treated with tet to induce the expression of n phosphospecific IRP1 antibodies (6) (targets synonymous IRP1 was immunoprecipitated from cell lysates using a pan-IRP1 anti-

Figure 8. Ser-138 phosphorylation of IRP1 in response to CIA inhibition. A, wild-type HEK 293 cells were transfected and harvested as in Fig. 1. Endogenous IRP1 was immunoprecipitated from cell lysates using a pan-IRP1 antibody and immunoblotted for Ser-138-phosphorylated IRP1 using Ser-138-phosphospecific IRP1 antibodies (6) (n = 3). B, wild-type HEK 293 cells were transfected with either NT or FBXL5 siRNA for 48 h. During the last 24 h of siRNA treatment, cells were treated with tet to induce the expression of Myc-tagged IRP13S35 or IRP13S35/S138A. Cell viability was determined by trypan blue exclusion. Cell lysates were subjected to Western blotting using antibodies against FBXL5, tubulin, and the Myc epitope on IRP13S35 or IRP13S35/S138A (n = 3). Error bars, S.E.

to maintain cell viability in the face of inhibition of Fe-S cluster biogenesis.

Discussion

Our findings describe key roles for a regulatory network involving FBXL5 and both IRP1 and IRP2 that responds to changes in CIA activity and is central to the regulation of iron metabolism during cell proliferation. This conclusion is based on the following observations (Fig. 9). First, a CIA-FBXL5 regulatory circuit was shown to be a critical factor controlling cellular iron metabolism. Induction of FBXL5 expression is a required component of the cellular adaptive response to CIA inhibition. Second, a negative feedback loop between IRP1 and FBXL5 was shown to be essential for maintenance of cellular iron metabolism. Furthermore, FBXL5 expression was enhanced in response to induction of either IRP1 or IRP2. Third, our findings support the concept that differences in CIA activity and FBXL5 expression are used to selectively control the action of IRP1 to meet unique iron needs of specific cell types under diverse physiological scenarios. Fourth, the CIA system and FBXL5 respond to perturbations of the same or interconnected cellular pool(s) of iron that appears to be necessary to properly coordinate the uptake and metabolic fate of iron. Fifth, the requirement for phosphorylation of IRP1 at Ser-138 to maintain optimal cell proliferation in response to CIA inhibition suggests the existence of novel signaling pathways that act to limit the dysregulation of iron metabolism. Taken together, our studies support the concept that the CIA-FBXL5-IRP axis is central for integrating a range of cellular regulatory pathways and coordinates changes in iron status with central aspects of cell physiology, including growth rate.

FBXL5 responds to CIA activity

We observed a strong induction of FBXL5 when either of the CIA components, NUBP2 or FAM96A, was impaired. The induction of FBXL5 presumably was a factor contributing to the decline in IRP1 and IRP2 protein expression and probably contributes to the “stabilizing effect” of FAM96A expression on IRP2 expression (10). Further support for this relationship came from the observation that the increased FBXL5 expression in response to induction of either IRP1 or IRP2 was iron-dependent. These data are consistent with a model where FBXL5 and CIA respond to the same cytosolic iron pool (8, 26). Paradoxically, although IRE binding is low and the cytosolic IRP1 is not required for cellular iron metabolism. Induction of FBXL5 expression is a required component of the cellular adaptive response to CIA inhibition. Second, a negative feedback loop between IRP1 and FBXL5, perhaps by limiting iron for formation of holo-FBXL5 or by generation of a signal indicative of increased demand for Fe-S biogenesis. In this condition, suppression of FBXL5 would enhance cellular iron uptake to meet the needs for increased Fe-S biogenesis. Once the CIA system is saturated with iron, activation of FBXL5 occurs, which then suppresses IRP1.

Figure 9. Model of regulatory processes controlling IRP1. IRP1 RNA-binding activity can be inactivated through insertion of an Fe-S cluster by the CIA pathway or by targeted polyubiquitination and degradation initiated by the ubiquitin E3 ligase FBXL5. Thus, IRP1 senses changes in cellular iron levels either through supply of iron for CIA followed by incorporation of an Fe-S cluster or by the iron-dependent activation of FBXL5, leading to degradation of the RNA-binding form of IRP1. FBXL5 protein stability is controlled by a constitutive and iron-induced mechanism (12, 13, 30). Both CIA and FBXL5 require iron, and as cells progress from an iron-deficient to an iron-replete state, inhibition of IRP1 prevents iron overload. Iron-dependent activation of these mechanisms leads to a suppression of IRP1, which reduces iron uptake. Presumably, the CIA system “acquires” iron more avidly than does FBXL5, but this remains to be determined. CIA acts to inhibit FBXL5, as evidenced by RNAi knockdown of CIA components, leading to increased expression of the ligase. The model predicts that as the CIA system increases in activity, it suppresses FBXL5, perhaps by limiting iron for formation of holo-FBXL5 or by generation of a signal indicative of increased demand for Fe-S biogenesis. In this condition, suppression of FBXL5 would enhance cellular iron uptake to meet the needs for increased Fe-S biogenesis. The limitations in iron homeostasis are critical for cell physiology, including cell growth rate.
ferritin is decreased and the iron import protein TfR1 is elevated, as noted here and elsewhere (10). Our findings support this paradoxical observation and further demonstrate the importance of FBXL5 in regulating the RNA-binding activity of IRP1 and IRP2 in response to impaired CIA, presumably because excessive induction of IRP would be deleterious. The significant increase in total spontaneous IRE-binding activity when FBXL5 and CIA factors are simultaneously suppressed, compared with cells with only CIA knockdown, supports this view. We favor the view that limiting the level of IRP1 and IRP2 RNA-binding activity by FBXL5 is crucial for the adaptive response to CIA inhibition, but it is important to note that FBXL5 has been reported to have other protein targets, and their dysregulation may contribute to the phenotypes we observed (27).

A negative feedback loop between IRPs and FBXL5 is essential for optimal cell growth

Insertion and removal of Fe-S has widely been accepted as the dominant mechanism for regulating IRP1 RNA-binding activity (28). However, like IRP2, which lacks the ability to insert a Fe-S, IRP1 can also be controlled by the iron-dependent E3 ubiquitin ligase FBXL5 (12, 13). We therefore sought to determine conditions under which FBXL5-mediated degradation of IRP1 is important for regulating cellular iron metabolism. Interestingly, a decrease in cell viability was observed when FBXL5 expression was suppressed in combination with either impaired CIA or cells expressing a mutant form of IRP1 that cannot insert an Fe-S cluster, IRP13C (29). The reduction in cell number in response to FBXL5 suppression was presumably due to iron-dependent dysregulation of IRP1 and IRP2 as total IRP RNA-binding activity was increased. Our findings suggest that FBXL5 is needed to limit IRP1 action when Fe-S biogenesis is impaired presumably to prevent unacceptably high levels of IRE RNA-binding activity. Notable examples in support of this concept include the shiraz zebrafish mutant, where overactivation of IRP1 causes lethality; the human equivalent of the shiraz zebrafish mutant developing sideroblastic-like microcytic anemia associated with cellular iron overload and decreased IRP1 expression; and Fbxl5 −/− mice that die in utero unless rescued by simultaneous ablation of IRP2 (29–31). Conversely, recent work in liver deficient in the mitochondrial Fe-S biogenesis factor frataxin demonstrated that IRP1 is essential for adaptive processes when Fe-S biogenesis is impaired (8). In that study, IRP1 RNA binding was initially activated but subsequently declined due to significant loss of IRP1 protein that was associated with induction of FBXL5 (8). Taken together, these studies indicate that a delicate balance exists between IRP1, IRP2, and FBXL5 essential for cell viability when Fe-S biogenesis is impaired. Dysregulation of this balance as produced by FBXL5 knockdown when CIA is impaired reduces cell growth and may be lethal in some circumstances.

FBXL5 regulates both IRP1 and IRP2 when CIA is impaired

Previous studies have demonstrated that when Fe-S biogenesis components, such as FAM96A, are impaired, IRP1 protein levels decrease significantly (9, 32–37). Our work further demonstrates knockdown of the CIA decrease IRP1 levels in an FBXL5-dependent manner. Surprisingly, although IRP2 does not possess a Fe-S cluster, we noted a decrease in IRP2 protein with CIA knockdown, a result that has previously been observed with the knockdown of FAM96A (10). We also observed that impairment of the CIA components NUBP2 and FAM96A had a greater effect on IRP2 protein stability than IRP1, presumably due in part to the pool of c-aconitase being resistant to FBXL5-mediated degradation, as observed in frataxin-deficient liver (8). In support of FBXL5 regulation of IRP1 during impaired Fe-S cluster biogenesis, we also observed FBXL5-dependent polyubiquitination of IRP1 with impaired NUBP2 function. This supports work completed by others demonstrating that overexpression of FBXL5 induces the polyubiquitination of IRP1 (12, 13). Further, support for both IRP utilizing the same protein degradation machinery came from the observation that overexpression of apo-IRP1, presented as IRP13C−3S, interferes with IRP2 degradation. Our findings further support a key interaction of IRP1 and FBXL5 in maintaining cell viability/growth.

Role of Ser-138 phosphorylation of IRP1 in the cellular response to CIA inhibition

Previous studies demonstrated that Ser-138 of IRP1 is a protein kinase C phosphorylation site (38). On the basis of studies with phosphomimetic mutants of Ser-138 (e.g. IRP1S138E), phosphorylation of IRP1 allows its conversion to c-acon (6, 7, 25), but the Fe-S cluster is much more unstable and, unlike IRP1S138E, undergoes spontaneous non-oxidative demetallation, allowing its preferential accumulation in the RNA-binding form in cells (6, 21). Ser-138 phosphomutants of IRP1 are more sensitive to iron-mediated protein degradation (6, 7). In the context of CIA inhibition, the strong increase in Ser-138 phosphorylation of IRP1 is predicted to promote the increased TfR and reduced ferritin expression noted in the current study and is consistent with the fact that iron supplementation reversed the inhibitory effect of CIA and FBXL5 knockdown in cell growth. Finally, it remains to be determined whether phosphorylation of IRP1 reflects activation of broad scale signaling pathways that respond to CIA activity perhaps through an Fe-S cluster–regulated kinase or phosphatase as is the case with the bacterial kinase NreB (39).

Summary and future directions

Our studies demonstrate that both Fe-S cluster insertion and protein degradation are critical components of a regulatory network controlling the level of IRP1 RNA-binding activity. Whereas previous studies have shown that IRP1 is subject to these modes of regulation (40, 41), the work described here identified novel interactions between the Fe-S biogenesis and protein degradation pathways that control IRP1 action. Several issues need to be addressed in future studies. First, over the continuum of iron levels that cells experience, from deficient to overloaded, is there a hierarchical role for the iron-dependent processes of the Fe-S switch versus protein degradation that control IRP1? Second, do tissue-specific differences in oxygenation selectively influence the control of IRP1 by FBXL5 versus the CIA system? FBXL5 requires oxygen to function. In contrast, CIA components with labile Fe-S clusters may be less...
active at the oxygen levels that are optimal for FBXL5. Thus, in contrast to iron, oxygen would appear to have opposing effects on the Fe-S switch versus protein degradation pathways that may contribute to tissue-specific control of IRP1 action. Third, how is IRP2 regulation integrated with that of IRP1 in relation to CIA and FBXL5 action? Considering the two branches of the CIA pathway, how does inhibition of the FAM 96B branch lead to increased IRP2 protein level without affecting IRP1 (10)? Does inhibition of the FAM 96B pathway, which delivers Fe-S clusters to nearly all cytosolic and nuclear apo-Fe-S proteins, increase FBXL5 expression, as is the case when the IRP-1 targeting FAM 96A branch is inhibited? Is the response of FBXL5 to inhibition of both arms of the CIA pathway, as shown here with knockdown of the upstream factor NuBP2, the same when FAM 96B is impaired? Fourth, what is the mechanism through which FBXL5 senses CIA activity? Is it merely changes in iron availability influencing assembly of the heme-thrin center in FBXL5, or is there some molecular species in the CIA pathway whose iron-dependent accumulation influences FBXL5 action? What is the protein kinase that phosphorylates IRP1 when CIA is inhibited? Future studies will address these questions.

**Experimental procedures**

**Mammalian cell culture and reagents**

HEK 293 cells were grown in DMEM with 10% FBS from Gibco. HeLa cells capable of expressing HFE were grown as described but in the absence of neomycin or antibiotics (22). HEK cells capable of tet-induced (1 μg/ml tet) expression of Myc-tagged IRP1<sup>3C</sup>–3S, IRP1<sup>3C</sup>–3S/S138A, or Myc-tagged IRP1<sup>WT</sup> were maintained as described (6). Cell viability was determined by trypan blue. Protein concentration was determined by a BCA assay (Thermo Fisher). Wild-type HEK cells were determined by trypan blue. Protein concentration was determined by measuring the conversion of isocitrate to α-ketoglutarate as described previously (46). Rabbit phosphospecific antibodies for Ser-138 of IRP1 were generated in collaboration with the laboratory of Rick Bruick (46). Rabbit phosphospecific antibodies for Ser-138 of IRP1 were generated in collaboration with the laboratory of Rick Bruick (46). Rabbit phosphospecific antibodies for Ser-138 of IRP1 were generated in collaboration with the laboratory of Rick Bruick (46). Rabbit phosphospecific antibodies for Ser-138 of IRP1 were generated in collaboration with the laboratory of Rick Bruick (46).

**Aconitase activity**

HEK 293 cells were harvested and fractionated using digitonin treatment as described previously (5, 42). Cytosolic and mitochondrial aconitase activity was measured as described (21). In brief, cells were permeabilized with digitonin and fractionated into cytosolic and mitochondrial fractions by spinning lysates at 15,000 × g for 10 min. Aconitase activity was determined by measuring the conversion of isocitrate to α-ketoglutarate at 240 nm.

**siRNA knockdown experiments**

All siRNA transfection mixtures were prepared under low light using Opti-MEM (Invitrogen), Lipofectamine 2000 (Invitrogen), and 30 nM siRNA. siRNA transfection mixtures were prepared in 0.5 ml of Opti-MEM as described previously and plated with 0.5 × 10<sup>6</sup> HEK 293 cells in 3.5 ml antibiotic-free media (DMEM + 10% FBS) for the indicated times (12). FBXL5 siRNA transfection mixture was added to Myc-tagged IRP1–expressing cell lines 48 h prior to harvesting. To increase the extent to which CIA component expression was reduced, cells were transfected on day 0 and retransfected on days 3 and 6 with CIA siRNA as described previously (11). FBXL5 siRNA was added with CIA siRNA on day 6. Fam96a Silencer<sup>®</sup> Select predesigned individual siRNA (catalog no. s38637) was from Ambion. NuBP2 individual siRNA (D-004705-02-0005), non-targeting siRNA (D-001210-03-05), and FBXL5 siRNA (D-012424-04-0010) were from Dharmaco.

**IRP1 ubiquitination state**

Cells were plated in DMEM + 10% FBS 24 h prior to transfection. Cells were transfected with a HA-ubiquitin expression plasmid for 20 h (23) before induction of IRP1 with 1 μg/ml tetracycline for 36 h in DMEM + 10% FBS. Cells were then treated with various combinations of FAC, desferal (DF), and proteasome inhibitor MG115 (Calbiochem) for 4 h. To assess the effect of FBXL5 on Myc-tagged IRP1 ubiquitination species, cells were cotransfected with the HA-ubiquitin expression plasmid and FBXL5 siRNA in 25% Opti-MEM and 75% DMEM + 10% FBS for 48 h. Myc-tagged IRP1 was induced during the last 24 h of incubation using tet as described (6).

To evaluate IRP ubiquitination state, cells were lysed as described (6) with the addition of 10 mM N-ethylmaleimide, 3 mM iodoacetamide, and 0.5 mM dithiothreitol to inhibit deubiquitination (44, 45). IRP1 was immunoprecipitated from 0.5 mg of cell lysate by incubation with 5 μg of anti-Myc monoclonal antibody (9E10) for 12 h at 4 °C with orbital shaking. During the last hour of incubation, 30 μl of 5% BSA-blocked protein L beads was added (Thermo Fisher). Samples were centrifuged at 1500 × g at 4 °C for 2 min. The protein L beads were washed with 1 ml of 0.1% Triton X-100, 50 mM Tris, pH 7.4, 300 mM NaCl, 5 mM EDTA. The beads were washed three more times in this manner followed by resuspension in 0.1% SDS, 0.1% sodium deoxycholate in PBS, pH 7.4. The final wash was in PBS alone. Protein was treated with Laemmli reducing sample buffer and heated at 70 °C for 12 min. The samples were then run in an 8% SDS-polyacrylamide gel.

**Antibodies and immunoblotting**

Myc-tagged IRP1 was determined using anti-Myc antibody (9E10). IRP1 monoclonal antibodies (clone 295B) were made against recombinant rabbit IRP1 by Neoclone (Madison, WI). Other antibodies were obtained as follows: α-tubulin (T5168) and anti-FLAG M2 (F3165) (Roche); human NUBP2 (H-79) antibody, ferritin heavy chain (H-53), and IRP2 antibody (7H6) (Santa Cruz Biotechnology); anti-HA (clone 3F10) (Roche Applied Science); and human TIR1 antibody (H68.4) (Zymed Laboratories Inc.). Mouse and human FBXL5 antibodies were generated in collaboration with the laboratory of Rick Bruick (46). Rabbit phosphospecific antibodies for Ser-138 of IRP1 have been described (6). Rabbit anti-GPAT antibody was generously provided by Oliver Stehling and Roland Lill (11). A plasmid encoding rat NUBP2 cDNA (MRN1768-98073773) (Thermo Scientific) was transfected into HEK 293 cells and used to confirm the molecular weight of human NUBP2 in our Western blot analyses (data not shown). Samples were electrophoresed through an 8 or 12% SDS-polyacrylamide gel and transferred to nitrocellulose at 60 mA for 12 h. After incubation with antibodies, proteins were detected using SuperSignal West Pico (Thermo Scientific). Blots were exposed to Blue Light.
Ultra X-ray film (GeneMate), and multiple exposures were used and quantified by densitometry.

To prevent excessive overcrowding of figures, the molecular weight markers are shown when a given target protein is analyzed for the first time (e.g. NuBP2 in Fig. 1A) as a representative example of where the markers migrate.

RNA-binding assays

IRP RNA-binding activity in HEK cell lysates was determined by EMSA using a 32P-labeled RNA of the first 65 nucleotides of the rat t1-ferritin 5’-UTR with specific radioactivity of 10,000–20,000 dpm/fmol (47). RNA binding was determined in the absence and presence of 2% 2-ME to 20 ng/ml in the presence of 2% 2-ME. After 90 min at 4 °C, [32P]RNA was added, and the 4 °C incubation continued for 10 min in the absence of 2-ME or 20 min in the presence of 2% 2-ME.

To separate human IRP1 from IRP2, gel supershift assays were performed. Cell lysate (20 µg of protein) was diluted into RNA-binding buffer (38) containing anti-IRP1 IgG (10 µg) without or with 2% 2-ME. After 90 min at 4 °C, [32P]RNA was added, and the 4 °C incubation continued for 10 min in the absence of 2-ME or 20 min in the presence of 2% 2-ME.

Statistical analyses

Means of data sets were compared by the two-tailed Student’s t test.

Author contributions—N. B. J., K. M. D., and C. P. N. performed the experiments. N. B. J., K. M. D., and R. S. E. interpreted the data. N. B. J. and R. S. E. designed the study. N. B. J. and R. S. E. wrote the paper with input from all authors.

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IRP1 and FBXL5 synergy and iron metabolism in cell growth

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