A Glutaredoxin·BolA Complex Serves as an Iron-Sulfur Cluster Chaperone for the Cytosolic Cluster Assembly Machinery*◆

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Cells contain hundreds of proteins that require iron cofactors for activity. Iron cofactors are synthesized in the cell, but the pathways involved in distributing heme, iron-sulfur clusters, and ferrous/ferric ions to apoproteins remain incompletely defined. In particular, cytosolic monothiol glutaredoxins and BolA-like proteins have been identified as [2Fe-2S]-coordinating complexes in vitro and iron-regulatory proteins in fungi, but it is not clear how these proteins function in mammalian systems or how this complex might affect Fe-S proteins or the cytosolic Fe-S assembly machinery. To explore these questions, we use quantitative immunoprecipitation and live cell proximity-dependent biotinylation to monitor interactions between Glrx3, BolA2, and components of the cytosolic iron-sulfur cluster assembly system. We characterize cytosolic Glrx3·BolA2 as a [2Fe-2S] chaperone complex in human cells. Unlike complexes formed by fungal orthologs, human Glrx3-BolA2 interaction required the coordination of Fe-S clusters, whereas Glrx3 homodimer formation did not. Cellular Glrx3·BolA2 complexes increased 6–8-fold in response to increasing iron, forming a rapidly expandable pool of Fe-S clusters. Fe-S coordination by Glrx3·BolA2 did not depend on Ciapin1 or Ciao1, proteins that bind Glrx3 and are involved in cytosolic Fe-S cluster assembly and distribution. Instead, Glrx3 and BolA2 bound and facilitated Fe-S incorporation into Ciapin1, a [2Fe-2S] protein functioning early in the cytosolic Fe-S assembly pathway. Thus, Glrx3·BolA is a [2Fe-2S] chaperone complex capable of transferring [2Fe-2S] clusters to apoproteins in human cells.

Although hundreds of cellular proteins require iron-containing cofactors for activity (1), the machinery responsible for distributing these cofactors remains relatively obscure. Separate systems must exist for both the mitochondrial and the cytosolic/nuclear compartments and systems must selectively distribute ferrous iron ions, iron-sulfur (Fe-S) centers, and heme.

Recent studies indicate that the poly(rC)-binding proteins are involved in the distribution of ferrous iron to ferritin, the principal iron storage protein, and to mono- and dinuclear iron enzymes in the cytosol of mammalian cells (2–4). Studies in both bakers' yeast and vertebrates indicate that many proteins are involved in the assembly and distribution of Fe-S clusters in the cytosol (5–7). These proteins are structurally and functionally conserved across many species. One protein class, the monothiol glutaredoxins, has been functionally implicated in the trafficking of both ionic iron and Fe-S clusters (8–12).

Monothiol glutaredoxins are members of the thioredoxin (Trx)[3]-fold family of proteins. Most members of the Trx family utilize a dithiol active site to catalyze the oxido-reduction of thiol-disulfide residues. In contrast, monothiol glutaredoxins contain a Cys-Gly-Phe-Ser active site that lacks catalytic activity and instead coordinates a [2Fe-2S] cluster via the active site cysteine and the sulfhydryl residue of a molecule of glutathione, which is non-covalently bound adjacent to the glutaredoxin active site (10, 13–15). In vitro analysis of this Fe-S-containing species indicates that two glutathione-bound glutaredoxin proteins can coordinate a single [2Fe-2S] cluster as a bridging complex. In eukaryotes, distinct monothiol glutaredoxins are expressed in the mitochondria and cytosol. Genetic evidence suggests that mitochondrial glutaredoxins are involved in the transfer of newly assembled Fe-S clusters to recipient apoproteins (8, 9, 16, 17). Cytosolic monothiol glutaredoxins differ from their mitochondrial paralogs in that they contain an amino-terminal Trx-like domain followed by one or more glutaredoxin domains. Studies in fungi suggest these proteins are involved in iron homeostasis.

The yeast *Saccharomyces cerevisiae* expresses two cytosolic monothiol glutaredoxins, Grx3 and Grx4, which are functionally redundant. Genetic ablation of these or mutation of their active site cysteine results in a failure to activate enzymes requiring iron in the form of heme, Fe-S clusters, and di-iron centers, suggesting a critical role in the distribution of iron in both cytosol and mitochondria (11). Zebrafish embryos injected with morpholinos against the cytosolic zfGrx3 displayed profound hemoglobinization defects, but only small changes in the activity of heme and Fe-S enzymes, suggesting that the roles of Grx3 in yeast and fish differ (18). In mammalian cells, a single monothiol glutaredoxin, Glrx3 (also called PICOT, TXNL-2, HUSSY22, and Grx3) localizes to the cytosol.

[3] The abbreviations used are: Trx, thioredoxin; Dfo, desferrioxamine B; IP, immunoprecipitation; CIA, cytosolic iron sulfur assembly; EV, empty vector; WCE, whole cell extract.

Glrx3 in vertebrates differs structurally from the yeast proteins in that it contains, in addition to the amino-terminal Trx domain, two tandem carboxyl-terminal Grx domains, both of which can coordinate a [2Fe-2S] cluster *in vitro* (14). Depletion of Glrx3 in mammalian cells was associated with moderate deficiencies of cytosolic Fe-S cluster enzymes and evidence of altered iron homeostasis, whereas mitochondrial heme and Fe-S enzymes remained largely unaffected (18). Other studies from human cells suggest that Glrx3 may have a role in regulating growth, activation, or signaling, although mechanisms to account for these effects have not been characterized (19–21).

In yeast, the role of Glrx3 in the sensing or distribution of iron appears linked to its bound Fe-S cluster, but whether Glrx3 directly or indirectly mediates iron enzyme activation has not been determined in yeast or mammals. In many species, monothiol glutaredoxins are found in oligomeric complexes. Both yeast and mammalian Glrx3 can form Fe-S cluster-bridged homodimers *in vitro* (10, 14), and cluster coordination is required for yeast Glrx3 homodimerization *in vivo* (11, 22).

Monothiol glutaredoxins from many species form complexes with BolA-like proteins. BolA was initially described as a bacterial morphogen and was subsequently found to be highly conserved in prokaryotes and eukaryotes (23). Grx3 and BolA proteins are closely linked in prokaryotic genomes (24), and high throughput studies found physical interactions in bakers' yeast (25). The BolA proteins remained functionally uncharacterized, however, until genetic studies in bakers' yeast indicated that Fra2, the cytosolic BolA ortholog, functioned as a regulator of the iron-sensing transcription factor, Aft1, and formed a complex with Grx3/4 (26).

Fungi and mammals express three non-redundant BolA paralogs, with BolA2-like proteins localized to the cytosol/nucleus and BolA3-like proteins localized to the mitochondria. BolA1 proteins are largely uncharacterized. *In vitro*, both yeast and human BolA2 form complexes with Glrx3 that contain one or two bridging [2Fe-2S] clusters that are coordinated by a conserved histidine residue in BolA2 (10, 13, 14, 26). Moreover, *in vitro* studies indicate that the Glrx3 homodimers with [2Fe-2S] clusters can spontaneously undergo rearrangement in the presence of BolA2 to form Glrx3·BolA2 heterocomplexes with bridging [2Fe-2S] clusters. Although yeast Glrx3 and BolA2 form complexes with a 1:1 stoichiometry, human Glrx3 (which contains two tandem Grx domains) forms a heterotrimer containing two BolA2 proteins with two bridging [2Fe-2S] clusters. Recently, separate *in vitro* studies have shown that [2Fe-2S] clusters coordinated by Glrx3 homodimers or Glrx3·BolA2 hetero-oligomers can all be transferred to Ciapin1, an Fe-S protein involved in early steps of cytosolic Fe-S cluster assembly (27, 28). However, it is not clear which, if any, of these transfer reactions can occur in a cellular context.

The roles of Glrx3·BolA2 complexes have been extensively studied in yeast species, where BolA2 is called Fra2. In bakers' yeast, Glrx3·[2Fe-2S]·BolA2 complexes have a critical regulatory role in the iron-dependent inactivation of the transcription factor Aft1 (13, 26, 29). *In vitro*, Glrx3·[2Fe-2S]·BolA can bind and transfer a [2Fe-2S] cluster to Aft2, a paralog of Aft1 (30). *In vivo*, this transfer of the [2Fe-2S] cluster to Aft1 facilitates its dissociation from promoter DNA and subsequent export from

the nucleus (22, 26, 31, 32). Strains deleted for either Glrx3 or BolA2 fail to inactivate Aft1 under conditions of iron excess and fail to repress transcription of genes controlled by Aft1. In the fission yeast *Schizosaccharomyces pombe*, Glrx3 and BolA2 are also required for the regulation of the iron-dependent transcriptional repressors Fep1 and Php4. Glrx3·BolA2 complexes bind to both repressors independently and affect their DNA binding activity under different iron conditions; however, transfer of [2Fe-2S] clusters to either repressor is not proposed to account for the regulation (33–36). Rather, in the case of Fep1, the transfer of iron ions alone is implicated. Aft1 does not have a mammalian ortholog, and the iron-dependent transcriptional repression systems of Fep1 and Php4 do not operate in mammalian cells. Therefore, these regulatory events offer no clues as to the functions of Glrx3 and/or BolA2 in animal cells, and no studies examining the function of BolA2 in mammalian cells have been reported. Nevertheless, BolA2 and Glrx3 appear primed to respond to changes in cellular iron availability.

Here, we use a combination of genetic and biochemical approaches to characterize Glrx3·BolA2 complexes isolated from cultured human cells. We show that BolA2 formed dynamic complexes with Glrx3 that required bridging Fe-S clusters; complex formation was dependent on the availability of iron, inorganic sulfur, and amino acid residues required for glutathione and [2Fe-2S] coordination in Glrx3. Both Glrx3 and BolA2 interacted with Ciapin1. This interaction resulted in the acquisition of an iron-containing cofactor by Ciapin1, which was required for its stability in living cells. Collectively, our data demonstrate that the Glrx3·BolA2 complex represents a highly elastic reservoir of cytosolic [2Fe-2S] clusters that can be transferred to enzymes via direct protein-protein interactions, consistent with the role of [2Fe-2S] cluster chaperone.

Results

Characterization of Glrx3·BolA2 as an Fe-S Cluster-binding Complex in Vivo—To determine whether BolA2 and Glrx3 form a complex in mammalian cells, we constructed HEK293 stable cell lines expressing tetracycline-inducible BolA2-FLAG or Glrx3-FLAG and performed co-immunoprecipitations. Western blotting using antibodies against BolA2 and Glrx3 indicated that the induced proteins were both expressed at levels roughly similar to those of the respective endogenous proteins (Fig. 1*A*, *left panel*), with BolA2-FLAG 2–4-fold higher than endogenous BolA and Glrx3-FLAG 0.5–1.5-fold of endogenous Glrx3. Endogenous BolA2 was detected as a single species with an apparent molecular mass of 10 kDa, rather than the 17-kDa size predicted by annotated coding sequences utilizing an upstream in-frame AUG codon. Accordingly, the BolA2-FLAG construct contains the conserved coding sequence from the second in-frame start codon, which is immediately preceded by a favorable translation initiation (Kozak) sequence. Immunoprecipitation using anti-FLAG resin revealed that Glrx3 was readily detectable in immune complexes from cells expressing BolA2-FLAG, but not from cells containing an empty vector, confirming the specificity of the interaction (Fig. 1*A*, *right panel*). Similarly, BolA2 was abundant in immunoprecipitates from cells expressing Glrx3-FLAG but undetectable in cells containing an empty vector. Thus, these results confirm
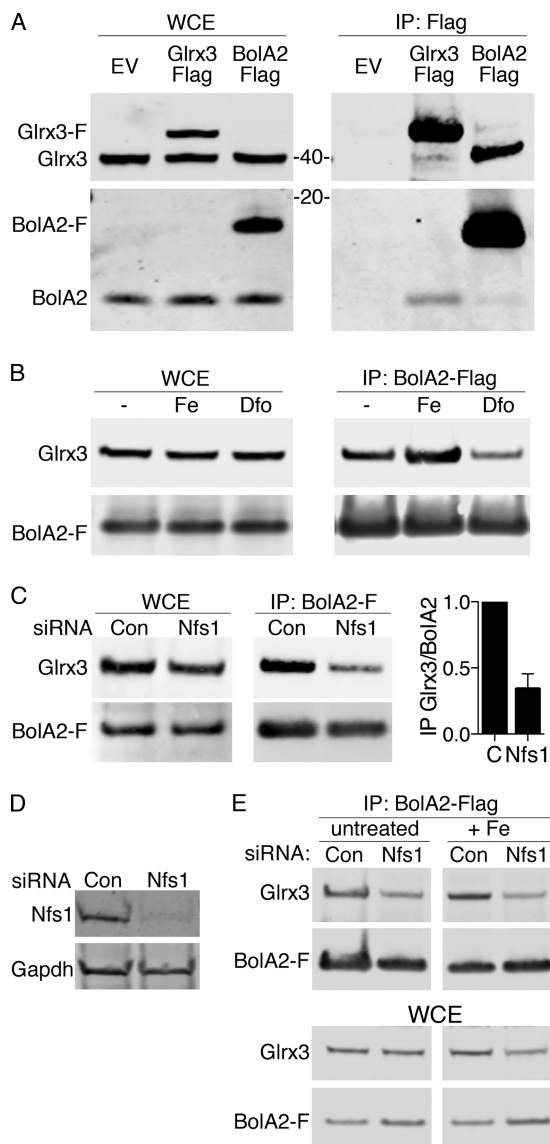
FIGURE 1. **Glrx3·BolA2 forms an Fe-S-dependent complex in human cells.** *A,* physical interaction between Glrx3 and BolA2 in cells. HEK293 cells containing an empty vector (*EV*) or inducible Glrx3-FLAG (*Glrx3-F*) or BolA2-FLAG (*BolA2-F*) were treated overnight with doxycycline. Cells were harvested and lysed, and anti-FLAG immunoprecipitation (*IP*) was performed. Whole cell extracts (*WCE*) and immune complexes (*IP: FLAG*) were analyzed by immunoblotting using antibodies against Glrx3 and BolA2. *B,* iron-dependent formation of the Glrx3·BolA2 complex. BolA2-FLAG cells were treated with doxycycline and 50 μM Dfo or 100 μM FeCl$_3$ for 16 h. WCE and anti-FLAG immune complexes were analyzed by immunoblot. *C,* requirement of Nfs1 for Glrx3·BolA2 complex formation. BolA2-FLAG cells were treated with siRNAs against Nfs1 or a non-targeting control (*Con*), induced with doxycycline, and analyzed by IP and immunoblotting. Ratio of co-precipitated Glrx3 to IP BolA2-FLAG, normalized to control, is shown at *right.* $n = 3$, *error bars* represent S.E. *D,* depletion of Nfs1 using siRNA. Immunoblotting analysis of whole cell extracts from BolA2-FLAG cells treated with control siRNA or siRNA against Nfs1 for 4 days. *E,* failure of iron to restore Glrx3·BolA2 complex formation in Nfs1-depleted cells. BolA2-FLAG cells were depleted of Nfs1 as in *A,* then treated with or without 25 μM FeCl$_3$ overnight. Immunoblotting analysis of whole cell extracts and anti-FLAG immune complexes is shown.

the presence of a Glrx3·BolA2 complex in mammalian cells. Notably, endogenous Glrx3 was not reproducibly detected in Glrx3-FLAG immunoprecipitates, as might be expected for a homodimer. Homodimer formation was similarly not detected in Glrx3 carrying an amino-terminal FLAG tag (data not shown).

Although the Glrx3·BolA2 complex has been shown to bind bridging 2Fe-2S clusters *in vitro* (10), cluster ligation was not required for the formation of the complex *in vitro* or in yeast cells (26). To investigate whether Glrx3·BolA2 bound Fe-S clusters in human cells, we performed co-immunoprecipitations of the Glrx3·BolA2 complex in cells depleted of or supplemented with iron (Fig. 1*B*). Cells stably expressing BolA2-FLAG were untreated or treated with the iron chelator desferrioxamine B (Dfo) or ferric iron salts for ~16 h, which produced no significant changes in the amount of Glrx3 or BolA2-FLAG in cell extracts. In contrast to cells grown in untreated media, cells treated with iron displayed a robust increase in the amount of Glrx3 precipitating with BolA2-FLAG, whereas cells treated with Dfo displayed a clear loss of Glrx3 co-precipitating with BolA2-FLAG. This result suggested a critical role for iron in stabilizing the physical interaction between Glrx3 and BolA2.

We next determined whether inorganic sulfur was required for this interaction. Nfs1 encodes the sole cysteine desulfurase in mammalian cells, and cells lacking this enzyme are unable to produce sulfur for both mitochondrial and cytosolic Fe-S cluster synthesis (37). Using small interfering RNA (siRNA), we depleted Nfs1 in BolA2-FLAG cells to 20% of the level in control siRNA-treated cells. BolA2-FLAG immunoprecipitates from cells depleted of Nfs1 contained only 30% of the Glrx3 detected in cells treated with control siRNA (Fig. 1, *C* and *D*). Furthermore, treatment of Nfs1-depleted cells with iron did not restore Glrx3·BolA2 complex formation (Fig. 1*E*), suggesting that both iron and sulfur were critical for complex formation.

To confirm that Glrx3·BolA complex formation was dependent on a bound [2Fe-2S] cluster, we constructed Glrx3 mutants containing conservative amino acid substitutions at the active site cysteine and the glutathione-binding residues in each of the two Grx domains (Fig. 2*A*). Because the amino-terminal Trx domain has been variably implicated in Glrx3 protein-protein interactions (29, 38), we also constructed a Glrx3 mutant lacking the Trx domain. These mutants were constructed in Glrx3 carrying an amino-terminal BirA-FLAG-fusion tag, which permitted affinity purification through both the FLAG epitope and live-cell biotinylation (39). All BirA-FLAG-Glrx3 protein products were present at similar levels in whole cell extracts from cells expressing the respective mutants (Fig. 2*B*). Moreover, these fusions were expressed at levels nearly identical to that of endogenous Glrx3. Similar to Glrx3-FLAG, endogenous BolA2 was readily detectable in anti-FLAG immunoprecipitates from cells expressing wild type BirA-FLAG Glrx3, but not in immunoprecipitates from cells expressing a BirA-FLAG negative control (Fig. 2*C*). In mutants lacking GrxA or GrxB Fe-S cluster binding residues, levels of co-precipitating BolA2 were reduced to 30% of wild type. BolA2 was undetectable in immunoprecipitates when both GrxA and GrxB sites were mutated, indicating that Fe-S cluster binding is necessary for the formation of the Glrx3·BolA2 complex. These data also suggested that both Grx domains might simultaneously bind BolA2 via an Fe-S cluster in cells. Conversely, a Glrx3 mutant harboring a deletion of the Trx domain showed increased co-precipitation of BolA2, although this difference was not statistically significant. Nonetheless, this result indicated that the
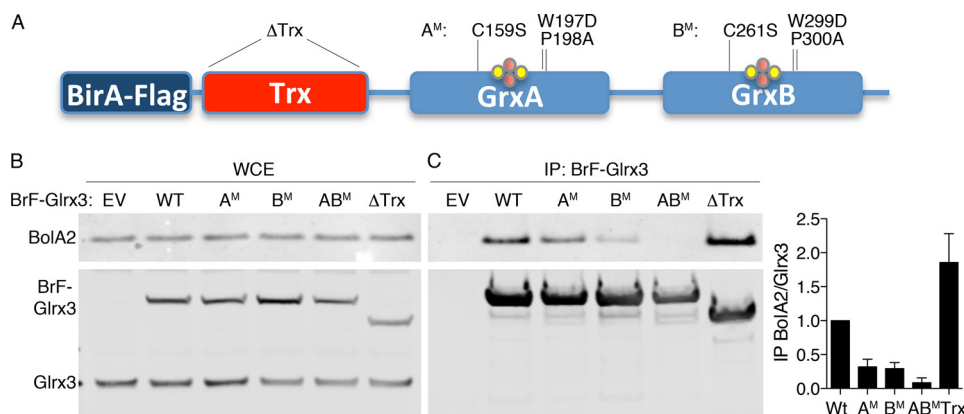
FIGURE 2. **Fe-S cluster binding residues of Glrx3 are required for stabilizing the Glrx3·BolA2 interaction.** *A,* schematic of BirA*-FLAG-Glrx3. Deletion and amino acid substitutions of BrF-Glrx3 mutants are shown. *B,* impaired interaction between BolA2 and Glrx3 Fe-S-binding mutants. Anti-FLAG immune complexes were isolated from cells expressing EV or the indicated allele of BrF-Glrx3. WCE (*B*) and immune complexes (*C*) were analyzed by immunoblot. Ratio of co-precipitated BolA2 to BrF-Glrx3 in IPs, normalized to WT, is shown at *right. n* = 3, *error bars* represent S.E.

Trx domain is not required for Glrx3·BolA2 Fe-S cluster acquisition in cells.

Glrx3·[2Fe-2S]·BolA2 Complex Represents an Expandable Pool of Cytosolic Fe-S Clusters—Our results in Figs. 1 and 2 indicated that the Fe-S cluster-bridged Glrx3·BolA2 complex responds to iron availability and may represent an exchangeable pool of cytosolic Fe-S clusters. To characterize the iron responsiveness of the Glrx3·BolA2 complex in detail, we measured the interaction of endogenous Glrx3 with BolA2-FLAG in cells depleted of or supplemented with different amounts of iron. Because the interaction of Glrx3 and BolA2 is dependent on bound Fe-S clusters when the proteins are expressed at endogenous levels, the amount of Glrx3 bound to BolA is proportional to the amount of bound Fe-S clusters. We detected a 6-fold increase in the amount of Glrx3 co-precipitated with BolA2 when cells supplemented with iron were compared with those depleted of iron (Fig. 3, *A* and *B*), with complex formation increasing over the whole range of iron conditions.

We next determined how rapidly the Glrx3 apoprotein could be converted to the Glrx3·[2Fe-2S]·BolA2 complex. BolA2-FLAG cells were depleted of iron overnight and then resupplied with iron, and complex formation was measured. An increase in Glrx3·BolA2 complexes was detected within 1 h and reached its maximum level, a 6-fold increase, by 4 h (Fig. 3, *C* and *D*). Western blotting analysis of immunodepleted cell extracts (Fig. 3*C, Post-IP Supernatant*) demonstrated that cellular Glrx3 was largely depleted by immunoprecipitation of BolA2-FLAG, with only 25% of the initially present Glrx3 remaining at 5–6 h. These data indicated that a majority of the Glrx3 pool in an iron-replete cell is BolA2-bound. The iron depletion-repletion treatment did not perturb protein expression levels of endogenous Glrx3 or BolA-FLAG, confirming that changes in these complexes were a result of differences in Fe-S cluster coordination. A similar experiment performed using immunoprecipitation of FLAG-Glrx3 and measurement of co-precipitating BolA2 showed a similar response to iron over a similar time scale (Fig. 3*E*). Together, these findings indicate that the Glrx3·[2Fe-2S]·BolA2 complex responded to a wide range of cellular iron conditions, constituted a major Glrx3-containing complex within the cell, and represented a rapidly expandable pool of cytosolic Fe-S clusters.
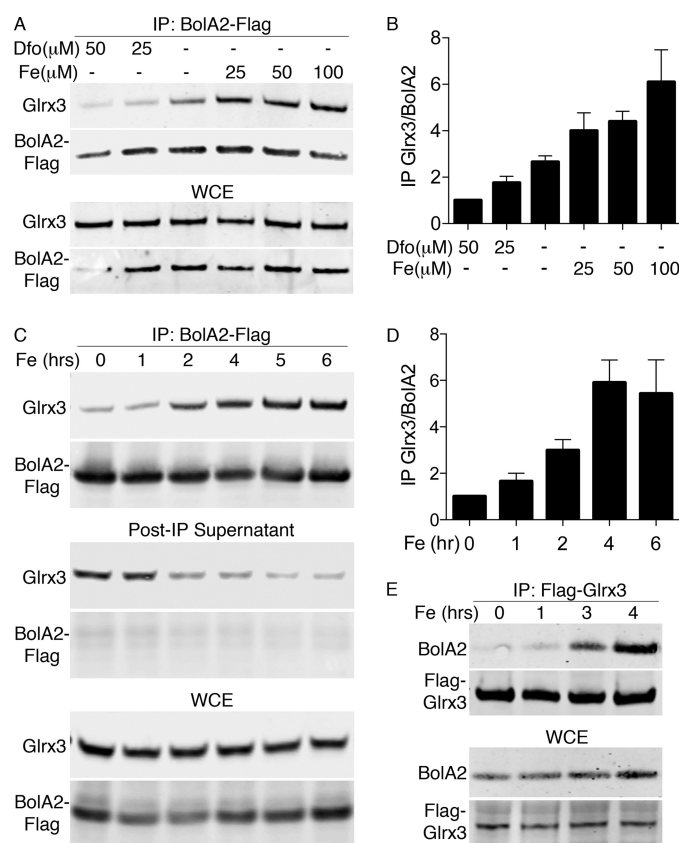

FIGURE 3. **Glrx3·BolA2 complex coordinates a rapidly expandable pool of Fe-S clusters.** *A,* iron-dependent increases in Glrx3·BolA2 complexes. BolA2-FLAG cells were induced and treated with the indicated concentration of Dfo or FeCl$_3$ for 16 h. WCE and anti-FLAG immune complexes were prepared and analyzed by immunoblotting using antibodies against Glrx3 and FLAG. *B,* quantitation of IPs in *A*. Ratio of co-precipitated Glrx3 to BolA2-FLAG in IP was normalized to 50 μM Dfo. *C,* rapid reformation of Glrx3·BolA2 complexes from apo-BolA2 after cellular iron repletion. Induced BolA2-FLAG cells were treated overnight with 50 μM Dfo to promote formation of apo-BolA2-FLAG. Dfo medium was replaced with 50 μM FeCl$_3$ medium for the indicated times. Whole cell extracts, anti-FLAG IPs, and post-IP supernatants were prepared and analyzed by immunoblotting against Glrx3 and FLAG. Quantitative comparisons of *Post-IP Supernatant* and *WCE* were made from samples loaded in parallel on same immunoblot. *D,* quantitation of IPs in *C*. *E,* rapid reformation of Glrx3·BolA2 complexes from apo-FLAG-Glrx3. FLAG-Glrx3 cells were grown and analyzed as in *C*, above, using antibodies against BolA2 and FLAG. In all experiments *n* = 3, *error bars* represent S.E.

Glrx3 Forms Homodimers Independent of Bound Fe-S Clusters—Previous work in yeast and *in vitro* demonstrated that, in addition to the Glrx3·BolA2 heterodimer, yeast Glrx3 also forms a [2Fe-2S] cluster-bridged homodimer that positively responds to iron availability *in vivo* (10, 11, 22). We tested for the presence of a Glrx3-Glrx3 homodimer in mammalian cells using co-immunoprecipitations in a HEK293 cell line stably expressing inducible Glrx3-FLAG or FLAG-Glrx3. Although BolA2 was readily detectable in anti-FLAG immunoprecipitates from both cell lines (Figs. 1*A* and 3*E*), we failed to reproducibly detect endogenous Glrx3 in these fractions, even when the precipitations were performed anaerobically, used large amounts of whole cell extract, and included reduced glutathione (data not shown). Because the Fe-S ligand bridging the Glrx3 homodimer was labile *in vitro* (13), we turned to a cell-based assay designed to detect transient, labile Glrx3 interactions in living cells.

BirA* is a promiscuous biotin ligase from *E. coli* that biotinylates surface-exposed lysine residues on proteins in close proximity to the enzyme (40). BirA* fused to a cellular protein has been used as a tool to detect transient and stable protein-protein interactions in living cells (39). We incubated cells expressing the BirA-FLAG-Glrx3 (BrF-Glrx3) fusion protein in medium supplemented with biotin, we then used streptavidin affinity capture and immunoblot analyses to detect proteins interacting with and biotinylated by BrF-Glrx3. Endogenous Glrx3 was readily detectable in streptavidin-captured fractions from biotin-treated cells expressing BrF-Glrx3 but was absent in cells expressing a BirA-FLAG control (Fig. 4*A*), indicating that BrF-Glrx3 specifically interacted with endogenous Glrx3 in living cells and could form a homodimeric complex. Antibiotin immunoblotting analysis demonstrated that the cell lines exhibited roughly similar levels of biotin ligase activity and that biotinylated proteins were quantitatively recovered by streptavidin affinity purification (Fig. 4*A*). We next examined the effects of iron depletion or supplementation on the BrF-Glrx3-Glrx3 interaction. Again, endogenous Glrx3 was readily detected in cells treated with Dfo (Fig. 4*B*). Surprisingly, much less endogenous Glrx3 (20% of Dfo-treated levels) was observed in captured fractions from iron-replete cells, suggesting that iron did not promote Glrx3 homodimerization, as it did for Glrx3·BolA2. We questioned whether long incubations with biotin might disproportionally allow detection of proteins interacting transiently rather than stably with BrF-Glrx3. However, shorter biotin incubations, which more closely capture interactions at a single point in time, did not change the inhibitory effects of iron on the relative detection of Glrx3 homodimers (Fig. 4*C*). Even with only 2–4 h of labeling, iron-replete cells exhibited only 10% of the interaction between BrF-Glrx3 and endogenous Glrx3 when compared with iron-depleted cells. Overall levels of protein biotinylation were not reduced (instead slightly increased) in iron-treated cells (Fig. 4*D*), ruling out a decrease in biotin ligase activity as a potential explanation. None of these data suggested that coordination of an Fe-S cluster could stabilize the formation of Glrx3 homodimers in cells.

Because a large percentage of Glrx3 in an iron-treated cell is in a complex with BolA2, the pool of uncoordinated Glrx3
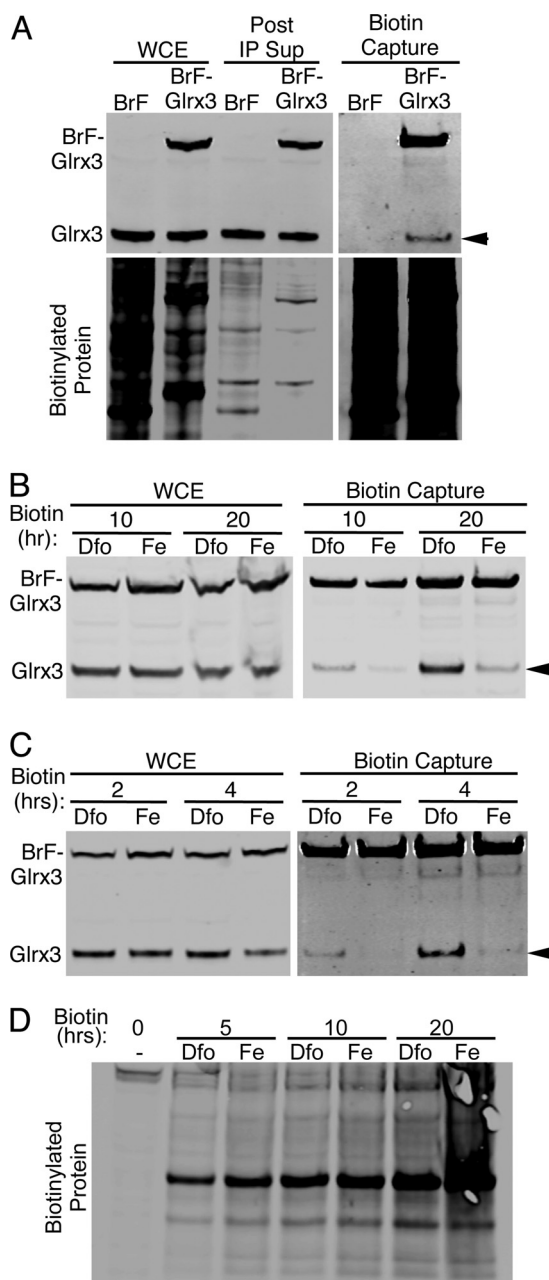

FIGURE 4. **Glrx3 homodimers are detectable in live cells and more abundant in iron-limited cells.** *A,* detection of Glrx3 homodimers in human cells using BrF-Glrx3. Lysates from biotin-supplemented cells stably expressing inducible BirA*-FLAG (*BrF*) or BirA*-FLAG-Glrx3 (*BrF-Glrx3*) were subjected to biotin capture using streptavidin-agarose. Captured biotinylated proteins were eluted and probed for endogenous Glrx3 using immunoblotting analysis (*arrowhead*). Total biotinylated protein was detected using IR dye-conjugated streptavidin. *B* and *C,* increased Glrx3 homodimer formation in iron-deficient cells. Cells expressing BrF-Glrx3 were made iron-deficient or -replete by overnight treatment with 25 μM Dfo or 100 μM FeCl$_3$, respectively. Following biotin supplementation for the indicated times, cells were harvested and lysed, and the resulting whole cell extracts were subjected to biotin capture and immunoblot analyses as described in *A*. *D,* similar levels of biotinylated proteins in WCE from iron- and Dfo-treated cells. Induced BrF-Glrx3 cells were treated as in *B* and *C* for the indicated times. Immunoblotting analysis of total biotinylated protein is shown.

available for homodimerization may be small. We therefore depleted BolA2 with siRNA in BrF-Glrx3 cells and again evaluated Glrx3 homodimerization (Fig. 5*A*). Despite similar levels of endogenous Glrx3 in cell extracts (Fig. 5*A, left panel*) and
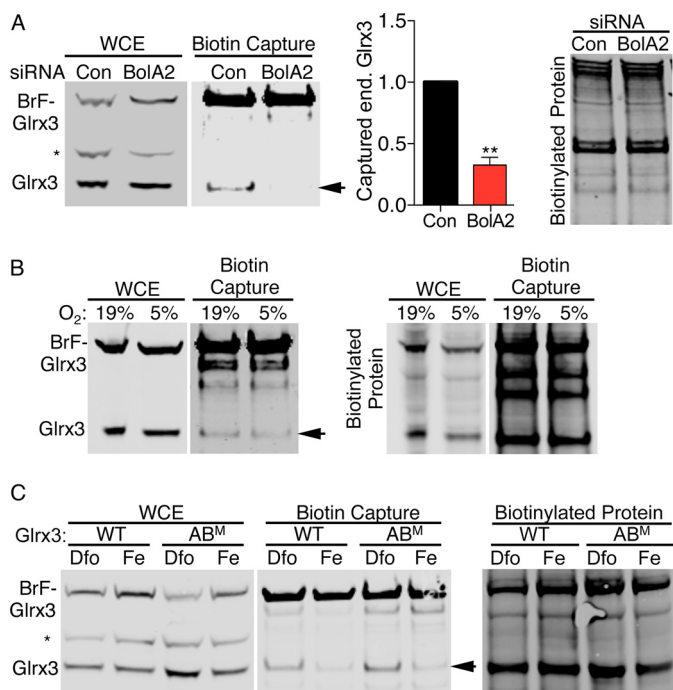
FIGURE 5. **Glrx3 homodimers are not stabilized by Fe-S clusters.** *A,* BolA2 deficiency does not increase Glrx3 homodimerization. Cells expressing BrF-Glrx3 were treated with siRNA against BolA2 and analyzed for Glrx3 homodimer formation by capture of biotinylated Glrx3 (*arrow*). Quantitation of captured endogenous Glrx3 is shown in *middle*. n = 4; *error bars* represent S.E. Immunoblotting analysis of total biotinylated proteins is shown at *right*. *Con,* control. n = 4; *error bars* represent S.E.; **, $p < 0.002$. *B,* lack of effect of hypoxia on Glrx3 homodimer formation. BrF-Glrx3 cells supplemented with biotin were grown under 19 or 5% O_2 for 20 h. Cells lysates were prepared, and biotinylated proteins were captured using streptavidin-agarose resin. Captured proteins were eluted and probed for Glrx3 and total biotin using immunoblotting analysis. *C,* lack of effect Glrx3 Fe-S cluster coordination on homodimerization. Biotinylated endogenous Glrx3 was captured from Dfo- and iron-treated cells stably expressing wild type (*WT*) or Fe-S binding mutant (AB^M) of BrF-Glrx3.
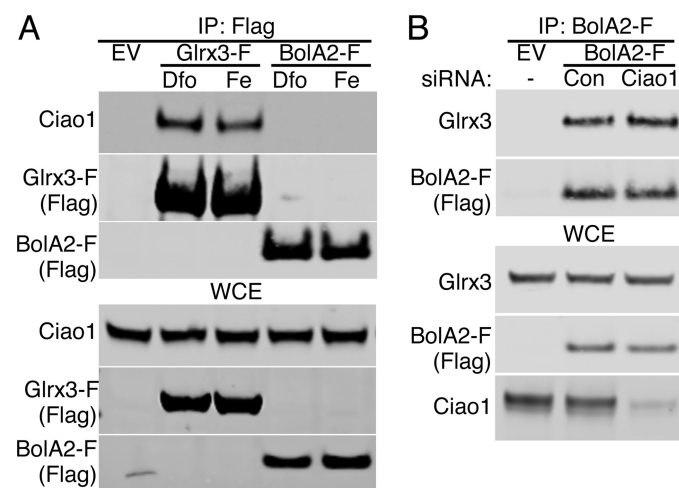

FIGURE 6. **Ciao1 binds Glrx3 but is not required for Glrx3·BolA2 cluster acquisition.** *A,* binding of endogenous Ciao1 with Glrx3 but not BolA2. EV, Glrx3-FLAG (*Glrx3-F*), or BolA2-FLAG (BolA2-F) cells were treated with 50 μM Dfo or 100 μM $FeCl_3$. WCE were prepared and analyzed by anti-FLAG IP and immunoblotting analysis using antibodies against Ciao1 and FLAG. *B,* no effect of Ciao1 depletion on Glrx3·BolA2 interaction. Cells expressing BolA2-FLAG were treated with control (*Con*) or Ciao1 siRNA. WCE and anti-FLAG immune complexes were analyzed by immunoblot.

similar levels of total biotinylation (*right panel*), affinity captured fractions from cells lacking BolA2 contained 66% less Glrx3 than control siRNA-treated cells (*center panels*). These data indicated that, in the absence of BolA2, Glrx3 homodimer formation was impaired rather than facilitated and that BolA2 was not preventing cluster coordination between Glrx3 homodimers. We also tested the effects of oxygen on the formation of Glrx3 homodimers, and we found that BrF-Glrx3 cells grown under hypoxic (5% O_2) conditions, which would be expected to stabilize an O_2-sensitive cluster, exhibited the same degree of homodimer formation as cells grown under normoxic conditions (Fig. 5B). Finally, when we compared Glrx3 homodimerization using the wild type BrF-Glrx3 and the Fe-S cluster-binding mutant, AB^M, we observed near-identical amounts of biotinylated endogenous Glrx3 bound to both wild type and mutant, in both iron-depleted and iron-supplemented cells (Fig. 5C). Taken together, these data suggested that, in human cells, Glrx3 homodimers were a transient, labile species that did not require Fe-S clusters for formation; furthermore, Glrx3 homodimers with bridging [2Fe-2S] clusters did not accumulate to a detectable degree.

Glrx3·BolA2 Complex Formation Is Independent of Ciao1— Previous work demonstrates that Glrx3 interacts with components of the cytosolic Fe-S cluster assembly (CIA) machinery in

cells (41). We therefore questioned whether any of these interactions were involved in the Fe-S cluster-dependent formation of Glrx3·BolA2 complexes. Ciao1, a late-acting CIA-targeting factor, was previously identified in proteomic screens as a protein interacting with Glrx3, and we confirmed this interaction using co-immunoprecipitations. The presence of endogenous Ciao1 in Glrx3-FLAG immunoprecipitates was readily detectable and remained largely unaffected by iron depletion or supplementation (Fig. 6A). In contrast, Ciao1 was nearly undetectable in BolA2-FLAG immunoprecipitates, suggesting the two proteins do not form a stable complex and that the Ciao1·Glrx3 complex does not include stably associated BolA2. However, Glrx3-Ciao1 interactions could possibly be important for the acquisition of [2Fe-2S] clusters by Glrx3·BolA2. To address this, we depleted Ciao1 in BolA2-FLAG cells using siRNA and measured the amount of Glrx3 interacting with BolA2 (Fig. 6B). Despite >70% depletion of Ciao1 protein, Glrx3·BolA2 complex formation was not impaired. Thus, although Ciao1 is clearly an interacting protein partner of Glrx3, it is not required for Fe-S cluster incorporation into Glrx3-BolA2.

Glrx3 and BolA2 Form a Complex with Ciapin1 and Ndor1— Cytokine-induced apoptosis inhibitor 1 (Ciapin1, Cpn) was also identified in previous studies as a protein that interacts with Glrx3 (42). Ciapin1 is an early acting factor in the maturation of newly synthesized [4Fe-4S] clusters in the CIA pathway (43, 44). Ciapin1 itself contains two bound Fe-S clusters that are thought to function in the transfer of reducing equivalents from the flavoprotein Ndor1 to downstream components of the CIA (45, 46). We confirmed the presence of the Glrx3·Ciapin1 complex in cells by detecting endogenous Ciapin1 in Glrx3-FLAG immunoprecipitates (Fig. 7A). To determine whether this interaction occurs in the context of a Glrx3·BolA2 complex or independently of BolA2, we also performed co-immunoprecipitations using BolA2-FLAG cells. Endogenous Ciapin1 was also detected in immunoprecipitates from BolA2-FLAG cells,
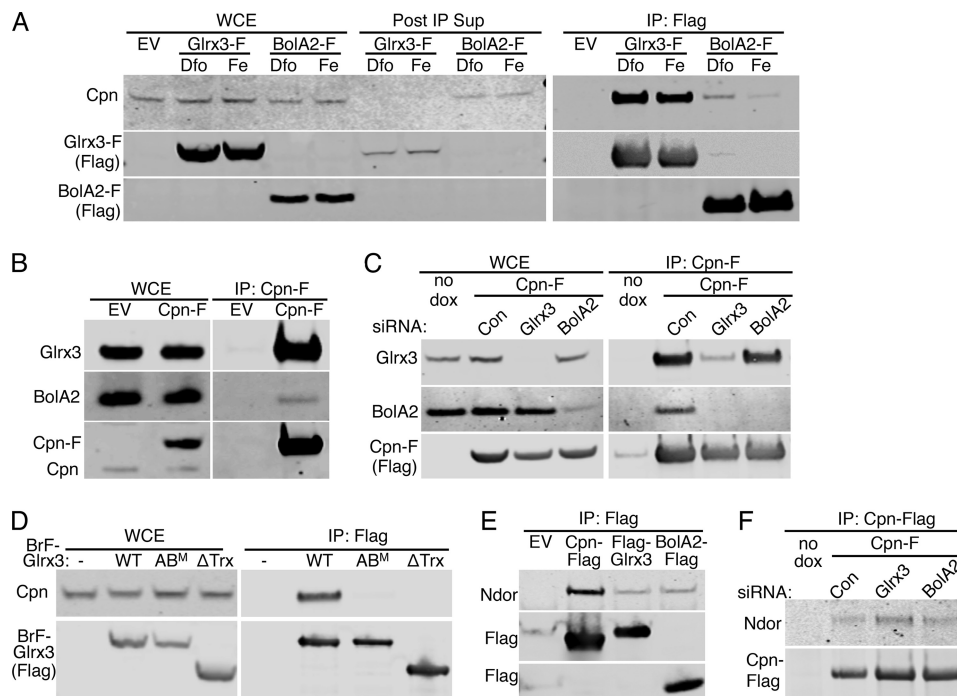
FIGURE 7. **Ciapin1 primarily interacts with Glrx3 via its Fe-S clusters and Trx domain.** *A,* co-precipitation of endogenous Ciapin1 with Glrx3 or BolA2. Glrx3-FLAG (*Glrx3-F*) and BolA2-FLAG (*BolA2-F*) cells were treated with 50 μM Dfo or 100 μM FeCl$_3$, followed by IP. Immunoblotting analysis of Ciapin1 (*Cpn*) from WCE, post-IP supernatant (*post-IP Sup*), and anti-FLAG immune complexes was performed. *B,* interaction of endogenous Glrx3 and BolA2 with Ciapin1-FLAG. Cells containing an EV or expressing inducible Ciapin1-FLAG (*Cpn-F*) were analyzed by anti-FLAG IP and immunoblotting using antibodies against Glrx3, BolA2, and Cpn. *C,* requirement of Glrx3 for interaction of BolA2 with Ciapin1. Cells expressing Ciapin1-FLAG were treated with control, Glrx3, or BolA2 siRNA. Whole cell extracts were subjected to IP and immunoblotting analysis as in *C. Dox,* doxycycline. *D,* requirement of Fe-S cluster and Trx domain of Glrx3 in interaction with Ciapin1. Lysates from cells expressing BrF alone or BrF-Glrx3 wild type (*WT*), thioredoxin deletion mutant (ΔTrx), or Fe-S cluster mutant (*AB^M*) alleles were subjected to anti-FLAG IP. Whole cell extracts and anti-FLAG immune complexes were analyzed by immunoblotting using antibodies against FLAG and Ciapin1. *E,* co-precipitation of Ndor1 with Ciapin1, Glrx3, and BolA2. Immunoblotting analysis for Ndor1 from anti-FLAGs IPs of Ciapin1-FLAG, Glrxe-FLAG, and BolA2-FLAG cells. *F,* co-precipitation of Ndor1 with Ciapin1 in cells lacking Glrx3 or BolA2. Cells expressing Ciapin1-FLAG were treated as in *C,* above, and IPs subjected to immunoblotting analysis for Ndor1.

but at a much lower level than in Glrx3-FLAG cells. This low level of interaction was specific, however, as it was not detected in control cells that did not express a FLAG-tagged protein. A greater stability of the Ciapin·Glrx3 complex than the Ciapin·BolA2 complex was also suggested by the observation that endogenous Ciapin1 was depleted in cell extracts after immunoprecipitation of Glrx3 (Fig. 7A, *Post-IP Sup.*), but not after precipitation of BolA2.

To further study the interactions of Ciapin1 with Glrx3 and BolA2, we developed a HEK293 cell line stably expressing Ciapin1-FLAG. Both endogenous Glrx3 and BolA2 were detected in Ciapin1-FLAG immunoprecipitates from these cells and were absent from cells containing an empty vector, although, again, the amount of co-precipitating BolA2 was low (Fig. 7B). We questioned whether Glrx3 or BolA2 could bind to Ciapin1 independent of the other partner. Therefore, we treated Ciapin-FLAG cells with control siRNA or siRNA directed against Glrx3 or BolA2, and we measured the amount of BolA2 or Glrx3, respectively, bound to Ciapin1-FLAG (Fig. 7C). BolA2 was undetectable in Ciapin1-FLAG immunoprecipitates from cells lacking Glrx3, whereas Glrx3 remained bound to Ciapin1 in cells partially depleted of BolA2. Thus, our results support a model in which Ciapin1 formed a stable complex with Glrx3, although BolA2 only transiently associated with Ciapin1. This transient association was absolutely dependent on the presence of Glrx3.

Previous *in vitro* work indicates that both apo-Glrx3 and [2Fe-2S]-bound Glrx3 interact with Ciapin1 solely through the Trx domain (28). We evaluated the roles of bound Fe-S clusters and the Trx domain of Glrx3 in the interaction with Ciapin1 in cells using the BrF-Glrx3 mutants described in Fig. 1. Although wild type Glrx3 clearly formed a stable complex with Ciapin1, Glrx3-AB^M, which cannot coordinate [2Fe-2S] clusters and failed to bind BolA2, also failed to form a complex with Ciapin1 (Fig. 7D). Similarly, Ciapin1 was not detectable in immunoprecipitates of Glrx3-ΔTrx. These data suggested that in cells the Trx domain of Glrx3 was not sufficient to mediate stable complex formation with Ciapin1, and both the Trx domain and an Fe-S cluster bound to Glrx3 were required for the Glrx3 interaction with Ciapin1.

In yeast, Ciapin1 forms a complex with Ndor1. We tested whether Ndor1 was present in complexes with Ciapin1, Glrx3, or BolA2 in HEK293 cells. Endogenous Ndor1 was detected in IPs from Ciapin1-FLAG, Glrx3-FLAG, and BolA2-FLAG cells (Fig. 7E), confirming that the complex of Ndor1 and Ciapin1 exists in mammalian cells and that Glrx3 and BolA2 can interact with Ciapin1 in complex with Ndor1. To test whether Glrx3 or BolA2 was required for the formation of the Ciapin1·Ndor1 complex, we depleted Glrx3 or BolA2 in the Ciapin1-FLAG cell line and measured the amount of Ndor1 that co-precipitated with Ciapin1. Similar amounts of Ndor1 were present in Ciapin1-FLAG IPs under each condition (Fig. 7F), suggesting that
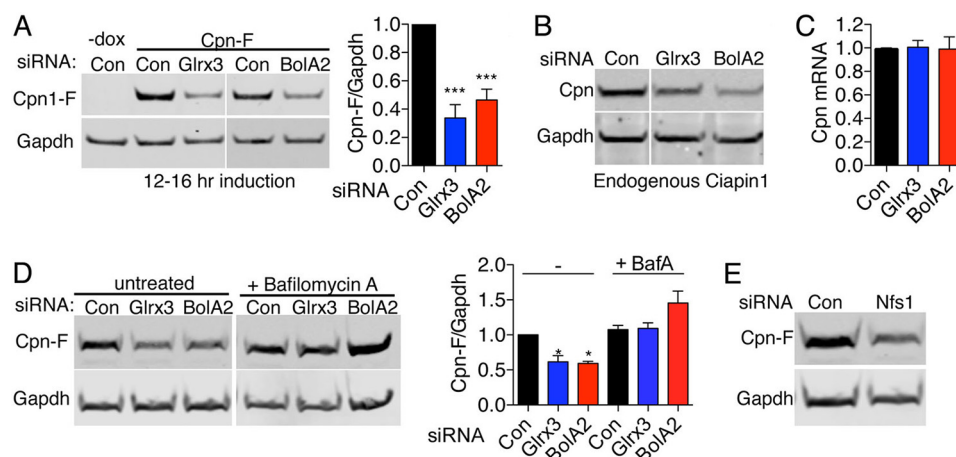
FIGURE 8. **Glrx3 and BolA2 are required for stabilization of Ciapin1 protein.** *A,* loss of Ciapin1 protein in cells lacking Glrx3 or BolA2. Immunoblotting analysis of cells expressing inducible Ciapin1-FLAG (*Cpn-F*) following two sequential treatments with siRNA against Glrx3 or BolA2. Quantitation at *right*. $n = 4$, ***, $p < 0.002$. *Con,* control; *dox,* doxycycline. *B,* decrease in endogenous Ciapin1 after depletion of Glrx3 or BolA2. HEK293T cells were treated with the indicated siRNA for 4 days. Cell lysates were then prepared and subjected to immunoblotting analysis using antibodies against Ciapin1 (*Cpn*) and Gapdh. *C,* no loss of Ciapin1 mRNA. RT-PCR analysis of Ciapin1 mRNA isolated from HEK293T cells treated with siRNA as described in *B*. Ciapin1 mRNA levels were normalized to Gapdh and expressed relative to control siRNA-treated cells. *D,* restoration of Ciapin1-FLAG levels by lysosomal inhibition. Ciapin1-FLAG cells received two treatments of control, Glrx3, or BolA2 siRNA. Cells were then induced with doxycycline in the presence or absence of 80 nM bafilomycin A for 12–16 h and then analyzed by immunoblotting. Quantitation at *right*. $n = 3$, *error bars* represent S.E., *, $p < 0.05$. *E,* decrease in Ciapin1-FLAG in Fe-S cluster-deficient cells. Ciapin1-FLAG (*Cpn-F*) cells were treated with control siRNA or siRNA against Nfs1 for 4 days. After Nfs1 depletion, Ciapin1-FLAG expression was induced with doxycycline. Whole cell extracts were analyzed by immunoblot using anti-FLAG and Gapdh antibodies.

Glrx3-BolA is not required to form the Ndor1·Ciapin1 complex.

BolA2·Glrx3 Complexes Transfer Fe-S Clusters to Ciapin1 in Cells—Ciapin1 can coordinate either a pair of [2Fe-2S] clusters (46) or a [2Fe-2S] cluster and a [4Fe-4S] cluster *in vitro* and in yeast (43, 47). *In vitro* work also demonstrates the transfer of [2Fe-2S] clusters from Glrx3 homodimers and Glrx3·BolA2 complexes to Ciapin1 (27, 28). These studies raise the possibility that [2Fe-2S] cluster exchange could occur between Ciapin1, Glrx3, and BolA2 in mammalian cells. Ciapin1 could theoretically donate its bound Fe-S clusters to Glrx3, which could rapidly form a complex with BolA2. However, our data indicate that the Glrx3-ΔTrx mutant, which fails to interact with Ciapin1, readily forms an Fe-S cluster-bridged complex with BolA2 (Fig. 2*C*); thus, cluster transfer from Ciapin1 to Glrx3·BolA2 in cells is unlikely. Instead, we reasoned that Glrx3·BolA2 could deliver its bound [2Fe-2S] clusters to Ciapin1.

Because Fe-S cluster incorporation is required for the stability of many proteins that bind the ligand, we measured the accumulation of Ciapin1-FLAG in cells lacking BolA2 or Glrx3 (Fig. 8*A*). Ciapin1 accumulated to only 35 and 45% of control levels in cells depleted of Glrx3 and BolA2, respectively. Similar decreases in endogenous Ciapin1 were measured in HEK293T cells depleted of Glrx3 or BolA2 (Fig. 8*B*). Neither of these observations could be attributed to differences in Ciapin1 mRNA, as quantitative PCR analysis indicated no change in Ciapin1 transcript levels (Fig. 8*C*) and translational regulation is unlikely, as the this regulation is typically mediated through 5′ or 3′ UTR sequences and the Ciapin-FLAG construct contains neither the 5′ nor 3′ UTRs of the native transcript. To confirm that Ciapin1 is more rapidly degraded in Glrx3- or BolA2-deficient cells, we inhibited protein turnover by treating cells with bortezomib (to inhibit the proteasome) or bafilomycin A (to inhibit the lysosome). Although bortezomib treatment had little effect on Ciapin1 levels, treatment of Ciapin1-FLAG

cells lacking Glrx3 or BolA2 with bafilomycin A restored Ciapin1 levels to that of control cells, indicating that Ciapin1 was destabilized in cells lacking Glrx3 or BolA2 (Fig. 8*D*). These data also suggested that Ciapin1 or a protein required for Ciapin1 turnover was degraded in the lysosome. We also observed that cells lacking Nfs1 exhibited enhanced turnover of Ciapin1 (Fig. 8*E*). These data suggested that the instability of Ciapin1 in Glrx3-, BolA2-, or Nfs1-depleted cells could be due to the absence of its [2Fe-2S] cofactors.

To directly measure the amounts of Fe-S clusters associated with Ciapin1, we induced expression of Ciapin1-FLAG-labeled cells with ^{55}FeCl$_3$, and we measured the amount of ^{55}Fe co-precipitating with Ciapin1-FLAG (Fig. 9*A*). The amount of ^{55}Fe associated with Ciapin1 complexes was 8–10-fold greater than the ^{55}Fe precipitated on anti-FLAG resin in uninduced control cells, indicating that we could quantitatively measure the Fe-S clusters associated with Ciapin1. To test whether the Glrx3· BolA2 complex was required for delivery of iron to Ciapin1, we depleted Glrx3 or BolA2, then induced Ciapin1-FLAG expression, and labeled cells with ^{55}Fe (Fig. 9*B*). We used short periods (8 h) of induction and labeling to eliminate differences in the expression levels of Ciapin1-FLAG. Ciapin1·FLAG immune complexes from Glrx3- or BolA2-depleted cells contained less than half the level of iron found in complexes from control cells, even after adjusting for lower levels of ^{55}Fe labeling in the BolA2-deficient cells. To determine whether Fe-S clusters from Glrx3·BolA2 were actually transferred to Ciapin1, rather than simply remaining on Glrx3·BolA complexes that interact with Ciapin1, we subjected ^{55}Fe-labeled Ciapin1·FLAG immune complexes to high salt washes. These effectively removed Glrx3 from the Ciapin1·FLAG immune complexes but had no effects on the ^{55}Fe retained in the complex (Fig. 9*C*). These data indicated that cells lacking Glrx3·[2Fe-2S]·BolA2 complexes exhibited defects in the incorporation of Fe-S clusters into Ciapin1 and strongly suggested that Fe-S clusters coordinated by the
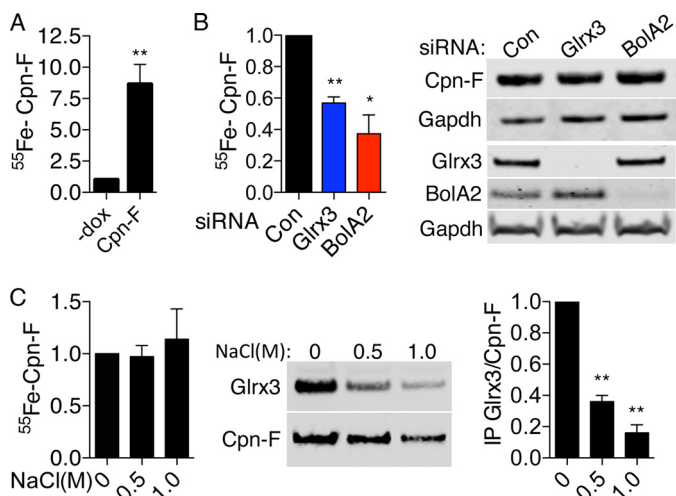
FIGURE 9. Glrx3 and BolA2 are required for iron incorporation into Ciapin1. *A,* isolation of ^{55}Fe-containing Ciapin1 complexes from cells. Ciapin1-FLAG cells were labeled with ^{55}FeCl$_3$ for 16 h. Whole cell extracts were analyzed by FLAG IP and scintillation counting. Retained ^{55}Fe is expressed relative to uninduced (−*dox*) cells. *Dox,* doxycycline. *B,* loss of ^{55}Fe in Ciapin1 complexes from cells lacking Glrx3 or BolA2. Ciapin1-FLAG cells treated with control (*Con*), Glrx3, or BolA2 siRNAs were given ^{55}FeCl$_3$ for 8 h. ^{55}Fe specifically bound to Cpn-F was measured as in *A* and normalized to total ^{55}Fe accumulation. Ciapin1-FLAG from whole cell extracts shown at *right.* $n = 3,$ *error bars* represent S.E., **, $p < 0.01.$ Immunoblot analyses of Ciapin1-FLAG, Glrx3, and BolA2 are shown at *right panel.* Immunoblots of Glrx3, BolA2, and Gapdh were made from same lysates shown in Fig. 8*D.* Gapdh blot also shown in Fig. 8*D. C,* transfer of Glrx3·BolA2 Fe-S clusters to Ciapin1. Ciapin1-FLAG cells treated with or without doxycycline were ^{55}Fe-labeled as described. Anti-FLAG IPs were performed in buffers and washes containing the indicated concentration of NaCl. Immune complexes were divided for ^{55}Fe quantitation by scintillation counting (*left*) and immunoblotting analysis of Glrx3 and Cpn1 with quantitation (*right*). $n = 3,$ *error bars* represent S.E., **, $p < 0.01.$

BolA2·Glrx3 complex could be directly transferred to Ciapin1 in cells.

Discussion

The function of cytosolic BolA proteins in animal cells is largely unknown. Here, we present cell-based studies that reveal functions of BolA2 in complex with the cytosolic monothiol glutaredoxin Glrx3. These studies indicate a role for BolA2·Glrx3 complexes in [2Fe-2S] cluster coordination and distribution. They also reveal that the characteristics of these proteins in animal cells differ significantly from those observed in yeast and *in vitro*, highlighting the importance of cell-based studies and revealing the functional adaptations manifest by orthologous proteins in different niches.

Several studies in yeast have underscored the critical role of cytosolic monothiol glutaredoxins and their BolA-binding partners in iron homeostasis (26, 33, 48), and a recent *in vitro* study of human Glrx3 and BolA2 characterized their capacity to bind and transfer [2Fe-2S] clusters (14, 27). Here, we show that Glrx3 and BolA2 form a complex in human cells and that complex formation is highly dependent on coordination of bridging Fe-S clusters. Although cytosolic Glrx·BolA complexes from fungi do not require the Fe-S ligand for formation or stability, multiple observations from this work suggest [2Fe-2S] cluster coordination is essential for this complex to form in cells. 1) Depleting cellular iron disrupts Glrx3·BolA2 formation, whereas iron supplementation augments its formation. 2) Limiting production of inorganic sulfur for Fe-S biogenesis reduces

Glrx3·BolA2 complex levels. 3) Mutating the Glrx3 residues required *in vitro* for Fe-S cluster binding prevents binding of BolA2.

Surprisingly, our studies indicated that Glrx3·[2Fe-2S]·BolA2 complexes can serve as a rapidly expandable pool of Fe-S clusters. Glrx3·BolA2 complexes exhibited a 6-fold increase in response to increased iron availability. Moreover, a majority of the cellular Glrx3 pool was bound to BolA2 in iron-replete cells, indicating that the Glrx3·[2Fe-2S]·BolA2 complex represents a major functional form of Glrx3 in the cell. The physiological roles of the [2Fe-2S] clusters elastically and transiently stored in Glrx3·[2Fe-2S]·BolA2 are not completely known, but we show at they can be transferred to Ciapin1 in cells. Glrx3·[2Fe-2S]·BolA2 complexes may be analogous to the labile iron pool and serve to make [2Fe-2S] clusters available to apoproteins or the cytosolic [4Fe-4S] distribution machinery, essentially functioning as [2Fe-2S] cluster chaperones. The wide dynamic range and kinetic lability of the Glrx3·[2Fe-2S]·BolA2 complexes are reminiscent of the iron-sensing, iron-regulatory protein 1 (IRP1), which responds to increases in cellular iron availability by rapidly coordinating a [4Fe-4S] cluster (49, 50). Cluster coordination allows IRP1 to shift from an RNA-binding protein to an enzyme; thus, IRP1 acts as an iron sensor and post-transcriptional regulator of gene expression. We show that Glrx3 and BolA2 are clearly primed to respond to changes in cellular iron status. Glrx3 or BolA2 may have functions independent of their binding partner, and complex formation may competitively inhibit those activities. Alternatively, Glrx3 or BolA may directly impact the systems that control iron uptake. Future studies will determine whether the iron responsiveness of human Glrx3 and BolA2 can serve a storage and/or regulatory function, as occurs in their fungal counterparts.

Data from our studies indicate that Glrx3-Glrx3 homodimers represent a rare species of Glrx3 in cells that is labile and transient but does not appear to require a stabilizing Fe-S cluster for dimerization. In contrast to Glrx3·[2Fe-2S]·BolA2, which was readily detectable using co-immunoprecipitation, we repeatedly failed to detect endogenous Glrx3 in Glrx3·FLAG immune complexes, even under conditions that are permissive for complex formation *in vitro*. We were, however, able to detect Glrx3 homodimer formation using cell-based and proximity-dependent biotinylation with BirA*-FLAG-Glrx3, which suggests that the interaction is transient and labile. This experimental approach indicated that Glrx3 homodimerization did not require the coordination of an Fe-S cluster. Quantitative measurements of homodimerization indicated that the species was predominantly detected in iron-deprived cells, rather than iron-replete cells, and that Fe-S cluster-binding mutants of Br-F-Glrx3 dimerized with endogenous Glrx3 as well as wild type Br-F-Glrx3. Although Fe-S cluster-bridged Glrx3 homodimers are oxygen-sensitive *in vitro* (10), we found no evidence that hypoxia could stabilize Glrx3 dimers in cells. Although we cannot completely rule out the transient formation of cluster-bridged Glrx3 homodimers, they do not represent a Glrx3 species that accumulates in cultured cells.

The discrepancies between these studies characterizing the interactions between Glrx3 and BolA2 and previous studies in yeast and *in vitro* likely have multiple origins. Chief among

these is the effect of protein concentration in driving protein-protein interactions. The high concentrations used in *in vitro* work and occasionally in cell-based overexpression studies can potentially result in non-native interactions that do not readily occur in cells expressing proteins at endogenous levels. Similarly, cellular levels of Glrx3 and BolA2 in bakers' and fission yeasts may be high enough to permit interactions that are not seen in mammalian cells. These differences could account for the previously reported formation of Glrx3 homodimers containing Fe-S clusters and Glrx3·BolA2 species without Fe-S clusters in these systems. Perhaps a more important consideration is that clearly Glrx3 and possibly BolA2 are involved in multiple competing protein-protein interactions that occur simultaneously in cells, and the relative affinities of these competing partners determine the associations and functions of Glrx3 and BolA2.

Our work confirms that Glrx3-[2Fe-2S]-BolA2 can directly bind Ciapin1 and facilitate Ciapin1 iron acquisition in cells, consistent with the transfer of a [2Fe-2S] cluster, but that Ciapin1 is not required for cluster acquisition by Glrx3·BolA2. Similar to other cytosolic Fe-S cluster proteins, Ciapin1 that did not acquire its cluster was more rapidly degraded than holo-Ciapin1. Surprisingly, apo-Ciapin1 degradation appeared to occur via an autophagosomal/lysosomal pathway. This observation suggests that Ciapin1 may be part of a large cytosolic complex that undergoes turnover via autophagy rather than through the proteasome. Previous *in vitro* studies of Ciapin1 and Glrx3 demonstrated the Trx domain of Glrx3 mediated a direct interaction with an amino-terminal region of Ciapin1 (28). Our findings support the requirement for the Trx domain but also indicate that a bound Fe-S cluster (and presumably BolA2) is required for the initial interaction of Glrx3 and Ciapin1. BolA2 appears not to remain stably associated with the Ciapin·Glrx3 complex. Although Glrx3 remained stably bound to Ciapin1, dissociation of Glrx3 with high ionic strength buffers did not affect the iron bound to Ciapin1, which demonstrated that the iron cofactors were transferred to Ciapin1. The importance of apo-Glrx3 remaining bound to Ciapin1 after cluster transfer is not clear, but it may promote the association of Ciapin1 with other components of the CIA machinery, such as Ciao1 or Iop1 (51).

Despite a clear loss of ^{55}Fe incorporation into Ciapin1-FLAG when cells were depleted of Glrx3 or BolA2, significant amounts of residual ^{55}Fe were retained in Ciapin1 immune complexes. Ciapin1 may be capable of acquiring Fe-S clusters from an alternative source, *e.g.* the mitoNEET/miner1 family of [2Fe-2S] proteins (52). This alternative source of Fe-S clusters could account for the relatively milder phenotypes associated with depletion of Glrx3 in mammalian cells *versus* yeast. Alternatively, iron that remained in Ciapin1 immune complexes could be coordinated by other iron-binding proteins associated with Ciapin1. As Ciapin1-Ndor1 is involved in the transfer of electrons to the CIA machinery, other Fe-S cluster components of the CIA may be present in Ciapin1 immune complexes. Our evidence that Glrx3·BolA2 transfers Fe-S clusters to Ciapin1 also suggests that some of the phenotypes associated with Glrx3 depletion in yeast or mammalian cells may be due to the failure of Ciapin1-Ndor to transfer electrons or possibly Fe-S clusters

to downstream components of the CIA or to other enzymes requiring reducing equivalents for activation, such as the tyrosyl radical in the small subunit of ribonucleotide reductase (11, 18, 43, 53, 54). Finally, Glrx3·[2Fe-2S]·BolA2 may function as an Fe-S chaperone, distributing its bound cofactors to other enzymes requiring [2Fe-2S] clusters, such as xanthine dehydrogenase or aldehyde oxidase. The precise mechanism by which Glrx3·[2Fe-2S]$_2$·BolA2$_2$ acquires its cluster in cells, as well as the physiological importance of dynamic transient 2Fe-2S cluster pool, are also important questions that await further investigation.

Experimental Procedures

Plasmids Used in This Study—Plasmids pcDNA5-FLAG-Glrx3 and pcDNA5-Glrx3-FLAG were generated by amplifying the wild type human Glrx3 open reading frame from pcDNA3–6×-myc-Glrx3 (gift of J. Wohlschleigel) and subcloning the resulting fragment into pcDNA5/FRT/TO vectors (Thermo Fisher Scientific) containing 2×FLAG at each terminus using Gibson Assembly (New England Biolabs). pcDNA5-BirA*-FLAG-Glrx3 was constructed by amplifying the BirA* ORF from pcDNA5-BirA*-FLAG (39) (gift of J. Wohlschleigel) and subcloning the resulting fragment into pcDNA5-FLAG-Glrx3 by Gibson Assembly (55). pcDNA5-BirA*-FLAG Glrx3 mutants were constructed by whole plasmid PCR-mediated site-directed mutagenesis. Plasmids pcDNA5-FLAG-BolA2 and pcDNA5-BolA2-FLAG were created by amplifying BolA2 from pETDuet1-hBolA2$^{\Delta1-66}$ (14) and subcloning the fragment into pcDNA5/FRT/TO vectors as described above. pcDNA5-Ciapin1-FLAG was created by amplifying the Ciapin1 ORF from human Ciapin1 cDNA (Open Biosystems, accession no. BC002568) and subcloning the resulting fragment into pcDNA/FRT/TO containing 2×FLAG at the carboxyl terminus. All plasmids were confirmed by sequencing.

Cell Culture and Stable Cell Lines—HEK293T cells were grown in DMEM supplemented with 10% FBS, 50 units/ml penicillin G, and streptomycin. Flp-In T-Rex 293 cell lines (Thermo Fisher Scientific) containing stably integrated pcDNA5/FRT/TO vectors were constructed according to the manufacturer's instructions and maintained in DMEM supplemented with 10% tetracycline-free FBS, 50 μg/ml hygromycin, and 15 μg/ml blasticidin. Where indicated, doxycycline was used at 1 μg/ml and bortezomib at 25 nM. Iron was used as ferric ammonium citrate.

Immunoblotting analysis—Whole cell extracts were prepared by lysing cells in IP buffer (100 mM Tris-HCl, pH 7.5, 40 mM KCl, 0.1% Nonidet P-40, 5% (v/v) glycerol, 5 mM GSH, 1× protease inhibitor mixture; Roche Applied Science). Membranes were incubated with the indicated primary antibodies, followed by detection using IR dye-conjugated secondary antibodies (1:10,000, Li-Cor). Antibodies used for Western blotting were rat anti-FLAG (1:2000, BioLegend), rabbit anti-Nfs1 (1:1000) (56), rabbit anti-Ciao1 (1:1000, Santa Cruz Biotechnology), mouse anti-Ndor1 (1:250, Abcam), and mouse anti-Ciapin1 (1:500, Abcam). Rabbit anti-Glrx3 (1:10,000) was a gift from R. Lill. Rabbit antisera against Glrx3 (Covance) was also raised using recombinant human Glrx3 corresponding to amino acid residues 130–335. Antibodies against human

TABLE 1
siRNA used in this study

Gene	Targeting sequence
Glrx3 (Stealth siRNA-Thermo Fisher)	5′–CCUACCUAUCCUCAGCUCUAUGUUU–3′
BolA2 (Stealth siRNA-Thermo Fisher)	5′–UGGUGUCGGCUAAGUUCGAGGGAAA–3′
Ciao1 (Silencer Select-Thermo Fisher)	5′–GGAAGAGGAUGACUGGGUA–3′
Nfs1 (Dharmacon ON-TARGETplus pool)	5′–GGAGCUGAUCCUCGUGAGA–3′
	5′–UCUCUAUAUGGAUGUGCAA–3′
	5′–UCCCUUACCUAAUCAACUA–3′
	5′–CUUAUGGCUGGGAGAGUGA–3′

recombinant BolA2 conjugated to keyhole limpet hemocyanin (1:2,000, Covance) were raised in chickens and purified from yolks. Purified recombinant Glrx3 and BolA2 were gifts of C. Outten (14). Anti-Gapdh (Calbiochem) was used to confirm equal loading. Biotinylated proteins were detected using IR-dye-conjugated streptavidin (Li-Cor). Detection and quantitative analysis were performed using Odyssey CLx (Li-Cor) software.

Co-immunoprecipitations—Cells expressing indicated FLAG epitope-tagged proteins were treated with doxycycline to induce expression of the respective gene. Cells were lysed in IP buffer, and clarified whole cell extracts were subjected to immunoprecipitation using anti-FLAG-agarose for 30 min at 4 °C. Following four washes with IP buffer, immune complexes were eluted in 2× sample buffer containing 10% β-mercaptoethanol and analyzed by immunoblotting.

Protein Depletion by siRNA—Cells were treated with 5–20 nM of the targeting siRNA (Table 1) according to the manufacturer's protocol. Unless otherwise stated, cells were treated with two sequential siRNA treatments spaced by 24 h and harvested 4 days after the initial transfection. Non-targeting scrambled sequence pools of siRNA were used as a control.

BioID of Glrx3—Flp-In T-Rex 293 cells containing inducible BirA*-FLAG-Glrx3 were treated overnight with doxycycline to induce expression of the protein. Cells were then supplemented with 1 mM biotin at the indicated times, and whole cell extracts were prepared using IP buffer. Biotinylated proteins were affinity captured by incubation with High Capacity streptavidin-agarose (Thermo Fisher Scientific) for 1 h at 4 °C with gentle tumbling. Following four washes with IP buffer, biotinylated proteins were eluted with 2× sample buffer containing 10% β-mercaptoethanol.

RNA Extraction and RT-PCR—Total RNA was isolated from cells using the RiboPure™ kit (Life Technologies, Inc.). Complementary DNA (cDNA) was prepared using High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real time PCR (AB7500, Applied Biosystems) was performed using Quantitect Primer Assays (Qiagen) for human Ciapin1 (catalog no. QT00002205) and Gapdh. Values were analyzed using the $\Delta\Delta C_T$ method.

55Fe Labeling of Ciapin1—Flp-In T-Rex 293 cells expressing Ciapin1-FLAG were treated with 1 μg/ml doxycycline and 2 μM ^{55}FeCl$_3$ (PerkinElmer Life Sciences) for the indicated time points. Cells were washed three times, including one wash with PBS + 10 mM EDTA, and whole cell extracts were prepared using IP buffer. Immunoprecipitation was then performed using anti-FLAG-agarose for 30 min at 4 °C with gentle tumbling. Immune complexes were washed four times with IP buffer, and the retained ^{55}Fe was quantitated by liquid scintil-

lation counting (LSC500, Beckman Coulter). IPs from cells not treated with doxycycline were used to determine nonspecific ^{55}Fe background. Co-precipitating ^{55}Fe was normalized to total cellular ^{55}Fe accumulation and Ciapin1-FLAG in whole cell extracts.

Statistical Analysis—Data were analyzed using Prism 5 (GraphPad). Results were reported as means ± S.E. Differences between the two groups were determined by unpaired Student's *t* test.

Author Contributions—A. G. F. conceived and coordinated the study, designed, performed, and analyzed experiments, prepared figures, and wrote the paper. D. J. P. and J. D. W. performed the experiments. C. C. P. conceived and coordinated the study, prepared the figures, and wrote the paper. All authors analyzed results and approved the final version of the manuscript.

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