Mechanism of frataxin “bypass” in human iron-sulfur cluster biosynthesis with implications for Friedreich’s ataxia

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Running title: Frataxin-independent activation of Fe-S cluster biosynthesis

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Keywords: mitochondrial disease, iron-sulfur protein, enzyme kinetics, analytical ultracentrifugation, circular dichroism, fluorescent anisotropy, cysteine labeling, neurodegeneration, ISCU2 M140I, Fe-S assembly

ABSTRACT

In humans, mitochondrial iron-sulfur (Fe-S) cluster biosynthesis is an essential biochemical process mediated by the assembly complex consisting of cysteine desulfurase (NFS1), LYR protein (ISD11), acyl-carrier protein (ACP), and the iron-sulfur cluster assembly scaffold protein (ISCU2). The protein frataxin (FXN) is an allosteric activator that binds the assembly complex and stimulates the cysteine desulfurase and Fe-S cluster assembly activities. FXN depletion causes loss of activity of Fe-S-dependent enzymes and the development of the neurodegenerative disease Friedreich’s ataxia. Recently, a mutation that suppressed the loss of the \textit{FXN} homolog in \textit{Saccharomyces cerevisiae} was identified that encodes an amino acid substitution equivalent to the human variant ISCU2 M140I. Here, we developed Fe-S cluster synthesis and transfer functional assays and determined that the human ISCU2 M140I variant can substitute for FXN in accelerating the rate of Fe-S cluster formation on the monothiol glutaredoxin (GRX5) acceptor protein. Incorporation of both FXN and the M140I substitution had an additive effect, suggesting an acceleration of distinct steps in Fe-S cluster biogenesis. In contrast to the canonical role of FXN in stimulating the formation of [2Fe-2S]\textsuperscript{2+/1+} and [4Fe-4S]\textsuperscript{2+/1+} species, and commonly function in substrate activation and in electron transfer. Fe-S clusters are synthesized and distributed to apo target proteins by conserved biosynthetic pathways. In humans, an assembly complex located in the mitochondrial matrix is responsible for synthesizing Fe-S clusters (3,4).
The structural core of this assembly complex consists of cysteine desulfurase (NFS1), eukaryotic-specific LYR protein (ISD11), and acyl carrier protein (ACP) subunits and is referred to as the SDA (or SDA_{ec} if it includes E. coli ACP; Table 1) complex (5,6). The ISD11 and ACP subunits are required for function, stabilize NFS1, and favor different quaternary interactions for NFS1 than its prokaryotic homologs (6-10). NFS1 uses a pyridoxal 5'-phosphate (PLP) to convert L-cysteine to L-alanine and generate a persulfide intermediate on a cysteine residue of a mobile S-transfer loop (Fig. 1, step 1). Sulfur is then transferred from NFS1 to a cysteine residue on the scaffold protein ISCU2 (Fig. 1, step 2). ISCU2 combines the sulfane sulfur from persulfide intermediates with Fe^{2+} and electrons to produce [2Fe-2S] clusters (11-13) (Fig. 1, step 3) in a poorly understood process. To complete catalytic turnover, intact [2Fe-2S] cluster intermediates on ISCU2 are transferred to a cluster carrier protein, such as the monothiol glutaredoxin GRX5 (Fig. 1, step 4), as a part of the cluster distribution network.

An additional essential component of the eukaryotic Fe-S assembly system is the protein frataxin (FXN). The loss of FXN function leads to iron accumulation in mitochondria, increased oxidative stress and loss of activity of Fe-S enzymes, and is associated with the fatal neurodegenerative disease Friedreich’s ataxia (14-17). FXN binds to the SDA_{ec}U (SDA_{ec} with ISCU2; Table 1) complex and accelerates Fe-S cluster biosynthesis on the scaffold protein (18-22). Initially, FXN was proposed to be an iron chaperone for Fe-S cluster biosynthesis (23-26). More recent evidence supports a role for FXN as an allosteric activator that facilitates sulfur transfer chemistry, which is associated with persulfide intermediates required for Fe-S cluster synthesis (12,20,27,28). The mechanism and molecular details of FXN binding and activation are still poorly understood.

Interestingly, a suppressor mutation affecting Saccharomyces cerevisiae Isu1 (homolog of human ISCU2; Isu1^{M140I}) also known as Isu1^{Sup} was identified that rescues growth defects caused by the deletion of S. cerevisiae Yfh1 (homolog of human FXN) (29,30). Initial radiolabeling studies suggest that Isu1^{Sup} increases the accumulation of persulfide species on Nfs1 similar to the addition of FXN (12,27). In contrast, two subsequent studies using standard reconstituted assays of partial reactions failed to identify a mechanism by which the human ISCU2^{M140I} (equivalent to Isu1^{Sup}) variant could functionally replace FXN in Fe-S cluster biosynthesis (31,32).

In this manuscript, new functional assays were developed, which include both Fe-S cluster synthesis and transfer reactions, to test the effect of the human ISCU2^{M140I} substitution on activity. We found that human ISCU2^{M140I}, like S. cerevisiae Isu1^{Sup}, can accelerate Fe-S biosynthesis in the absence of FXN in complete reconstituted reactions. We also found that unlike FXN, which stimulates the cysteine desulfurase and [2Fe-2S] cluster biosynthetic reactions of the SDA_{ec}U complex, the substitution of ISCU2 with ISCU2^{M140I} primarily affects the transfer rate of intact [2Fe-2S] clusters from ISCU2 to GRX5. Overall, our results provide new mechanistic insights and indicate that FXN and ISCU2^{M140I} function at distinct stages of the Fe-S cluster biosynthetic pathway.

**Results**

**ISCU2 and the ISCU2^{M140I} variant have similar biochemical properties**

We purified native ISCU2 and the ISCU2^{M140I} variant and probed their secondary structure, oligomeric state, and ability to form Fe-S assembly complexes. ISCU2 and the ISCU2^{M140I} variant exhibited highly similar far UV circular dichroism (CD) spectra with negative ellipticity features at 208 and 222 nm and a positive feature at 193 nm, which are characteristic of \alpha-helices (Fig. S1). Analytical ultracentrifugation sedimentation velocity studies reveal that both ISCU2 and the ISCU2^{M140I} variant exist primarily as monomeric species in solution (Fig. 2). Next, the SDA_{ec}U and SDA_{ec}U^{M140I} complexes were generated by combining recombinantly expressed human NFS1-ISD11 complex that co-purified with Escherichia coli ACP (SDA_{ec}) (6,33) with ISCU2 or the ISCU2^{M140I} variant. To test for the ability of the Fe-S assembly complexes to bind FXN, the FXN^{S202C} variant was purified, labeled with the Texas Red C2 fluorophore, and titrated with either the SDA_{ec}U or SDA_{ec}U^{M140I} complex (Fig. 3). Changes in fluorescence anisotropy were fit to a binding equation and revealed that the FXN affinity to the complex is largely unaffected by the incorporation of the M140I substitution. Thus, the M140I...
substitution does not significantly affect the secondary structure and the oligomeric state of ISCU2, or its ability to form the activated Fe-S cluster assembly complex with SDA

Both FXN and the ISCU2^{M140I} substitution accelerate the formation of [2Fe-2S] clusters on GRX5

We then tested if Fe-S cluster assembly complexes in which human ISCU2 is substituted with the ISCU2^{M140I} variant can bypass the function of FXN in Fe-S cluster biosynthesis. A visible CD assay was developed to monitor an entire Fe-S cluster assembly and transfer reaction (Fig. 1, steps 1-4). This region of the CD spectrum is sensitive to the Fe-S cluster chromophore and can be used to distinguish cofactor association with different proteins. This assay, unlike previously reported assays that only monitor intermediate formation on NFS1 or ISCU2, better represents the in vivo biosynthetic pathway by building Fe-S clusters in situ on the scaffold protein ISCU2 and then transferring these clusters to GRX5 to complete a catalytic cycle. This assay takes advantage of the positive ellipticity from [2Fe-2S]-GRX5 and low ellipticity from [2Fe-2S]-ISCU2 at 450 nm (Fig. S2) to detect product formation. Reactions were initiated with the addition of L-cysteine and glutathione, which is required for binding the Fe-S cluster to GRX5. The SDA_{ec}U, SDA_{ec}U^{M140I}, and SDA_{ec}UF (SDA_{ec}U plus FXN; Table 1) complexes exhibited final CD spectra that include contributions from both [2Fe-2S]-ISCU2 and [2Fe-2S]-GRX5 (Fig. S3), consistent with the excess amount of substrates in the reaction and repopulating [2Fe-2S]-ISCU2 after cluster transfer to GRX5. Similar overall rates of [2Fe-2S]-GRX5 formation were observed for the SDA_{ec}UF and SDA_{ec}U^{M140I} complexes (Fig. 4 and Table 2), which were about 3 times greater than for the SDA_{ec}U complex. The results from this in vitro Fe-S cluster biosynthetic assay are consistent with both the stimulation of activity by FXN and by ISCU2^{M140I} in the absence of FXN.

**FXN and ISCU2^{M140I} operate at different steps in Fe-S cluster biosynthesis**

We also tested the effect of adding FXN to the SDA_{ec}U^{M140I} complex in complete cluster assembly and transfer reactions (Fig. 1, steps 1-4). The SDA_{ec}U^{M140I}F complex had a [2Fe-2S]-GRX5 formation rate that was greater than the SDA_{ec}U complex (nearly 7-fold greater) and was about twice the rate of either the SDA_{ec}UF or SDA_{ec}U^{M140I} complex (Fig. 4 and Table 2). This additive result in the Fe-S assembly assay hints that the stimulatory effects of FXN and ISCU2^{M140I} occur at different steps in Fe-S cluster biosynthesis. As FXN was previously shown to increase the rates of partial reactions, including the cysteine desulfurase activity of SDA_{ec}U and the assembly of [2Fe-2S] cluster intermediates on ISCU2 (6,20,22), we tested whether ISCU2^{M140I} also affected those steps of Fe-S cluster biosynthesis.

The M140I substitution had minor effects on the cysteine desulfurase and Fe-S cluster synthesis (Fig. 1, steps 1-3) activities of the SDA_{ec}U complex. SDA_{ec}U was previously shown to exhibit a low \( k_{cat} \) (0.60 min\(^{-1}\)) for the cysteine desulfurase reaction (6), which was not affected by the addition of ISCU2 but was stimulated ten-fold by the inclusion of both ISCU2 and FXN (Table 2) (6,20). The SDA_{ec}U^{M140I} complex exhibited kinetic parameters similar to those of the SDA_{ec}U complex, with a \( k_{cat} \) 10-fold less than that of the SDA_{ec}UF complex (Table 2 and Fig. 5). Addition of FXN to the SDA_{ec}U^{M140I} complex stimulated cysteine turnover in a manner reminiscent of the wild type system. Thus, the M140I variant behaves almost identically to ISCU2 in the cysteine desulfurase assay.

Next, the ISCU2^{M140I} variant was tested for its ability to mimic FXN in increasing the rate of [2Fe-2S] cluster formation on ISCU2. CD spectroscopy was used to monitor [2Fe-2S] cluster formation on ISCU2 and ISCU2^{M140I} by following changes in ellipticity at 330 nm, similar to a previously reported assay (22). Here, we show the addition of FXN to the SDA_{ec}U complex accelerates Fe-S cluster formation on ISCU2 (2.8 fold greater) under a comparable set of conditions (Fig. 6A; Fig. S4; Table 2). The SDA_{ec}U^{M140I} complex exhibited 1.5 times greater cluster synthesis activity than the native SDA_{ec}U complex, but only about half the activity of SDA_{ec}UF. Addition of FXN to the SDA_{ec}U^{M140I} complex further enhanced its activity (1.5 fold greater), consistent with FXN stimulation of the native system. Overall, the additive effect in complete synthesis and transfer assays coupled to the inability of ISCU2^{M140I} to fully replace FXN in stimulating cysteine desulfurase and Fe-S assembly reactions are consistent with FXN and ISCU2^{M140I}
affecting different steps of Fe-S cluster biosynthesis.

**The ISCU2<sup>M140I</sup> substitution accelerates cluster transfer to GRX5**

Given that ISCU2 acts as a scaffold for [2Fe-2S] cluster synthesis and functions as an intermediate to transfer these clusters to target proteins, we evaluated the ability of FXN and the ISCU2<sup>M140I</sup> substitution to affect the transfer of the [2Fe-2S] cluster intermediate from ISCU2 to GRX5 (Fig. 1, step 4). The SDA<sub>ec</sub>U and SDA<sub>ec</sub>U<sup>M140I</sup> complexes were incubated with L-cysteine and ferrous iron in the absence of GRX5 and the development of the characteristic [2Fe-2S]-ISCU2 CD spectrum was observed (Fig. S5). The reaction was allowed to progress for 160 min to ensure completion of the [2Fe-2S]-ISCU2 formation reaction. After 160 min, apo-GRX5 (with or without FXN) was added under anaerobic conditions (see arrow in Fig. S6). The cluster transfer reaction from ISCU2 to GRX5 was then followed by monitoring the increase in ellipticity at 450 nm (Fig. 6B and Fig. S6). The rates of cluster transfer for SDA<sub>ec</sub>U and SDA<sub>ec</sub>UF were the same within error (Table 2), establishing that FXN does not have a role in cluster transfer under these conditions. In contrast, Fe-S assembly complexes containing the ISCU2<sup>M140I</sup> variant transferred cluster at almost twice the rate of complexes with wild-type ISCU2.

One possible explanation for the enhanced transfer rate is the M140I substitution on ISCU2 could destabilize the [2Fe-2S] cluster to facilitate cluster exchange with GRX5. We, therefore, tested this possibility in three ways. First, we determined whether the incorporation of the M140I substitution affected the cluster equilibrium between ISCU2 and GRX5. Here, the ability of [2Fe-2S]-GRX5 to transfer its cluster to either excess apo-ISCU2 or apo-ISCU2<sup>M140I</sup> was monitored by changes in the CD spectra (Fig. S7). No significant loss of [2Fe-2S]-GRX5 signal was observed for either reaction after 1 h (in the presence or absence of glutathione), supporting an essentially irreversible [2Fe-2S] cluster transfer reaction from ISCU2 to GRX5.

Second, we used a SYPRO orange thermal stability assay to determine whether the M140I substitution affects the stability of ISCU2 in the presence or absence of a [2Fe-2S] cluster (Fig. S8). The assay takes advantage of an increase in fluorescence that occurs upon protein denaturation and dye binding to hydrophobic residues. ISCU2 and ISCU2<sup>M140I</sup> had similar melting temperatures with and without [2Fe-2S] cluster bound, suggesting that the M140I substitution does not greatly affect the stability of the apo or cluster bound state.

Third, we examined the susceptibility of [2Fe-2S]-ISCU2 and [2Fe-2S]-ISCU2<sup>M140I</sup> to loss of their clusters in the presence of DTT. DTT promotes the loss and transfer of [2Fe-2S] clusters from ISCU2 (11,22). Here, Fe-S clusters were enzymatically generated on ISCU2 (or ISCU2<sup>M140I</sup>), and the holo-proteins were combined with DTT (Fig. S9). Both samples lost a majority of their cluster-dependent CD signal within 30 min with a slightly greater rate of cluster extrusion for ISCU2 than the ISCU2<sup>M140I</sup> sample. These results indicate the greater [2Fe-2S] cluster transfer rate for ISCU2<sup>M140I</sup> samples is not simply due to decreased cluster stability on ISCU2. Overall, these studies indicate that the ISCU2<sup>M140I</sup> substitution, but not FXN, affects the Fe-S cluster transfer reaction from ISCU2 to GRX5.

**Discussion**

Friedreich’s ataxia is an incurable neurodegenerative disease associated with the loss of FXN function. The physiological role of FXN is still debated and two primary proposals for its function in Fe-S cluster biosynthesis have emerged: a chaperone that delivers iron and an allosteric activator that stimulates sulfur-based chemistry. Recently, an ISU1 mutation that suppressed the growth defects of *Saccharomyces cerevisiae* lacking the *YFHI* homolog of FXN was identified (29,30). This result was puzzling as it was not clear how an amino acid substitution affecting Isu1 might mimic FXN and function as an iron chaperone or stimulate sulfur-based chemistry. Moreover, it was also unclear if the analogous ISCU2 variant bypasses FXN function in the human Fe-S cluster biosynthetic system. Therefore, we decided to investigate whether the ISCU2<sup>M140I</sup> substitution stimulates human Fe-S cluster biosynthetic activity, which might provide new mechanistic insight into FXN function and opportunities for developing FRDA therapeutics.

Our results along with two recent studies (31,32) indicate that native ISCU2 and the ISCU2<sup>M140I</sup> variant have similar biochemical and
stability properties. First, we found that the M140I substitution does not affect the protein’s secondary structure (Fig. S1), consistent with a recent finding by Yue and coworkers (31). In contrast, the Markley group reported different secondary structures for native ISCU2 and the ISCU2M140I variant (32); similar differences have recently been correlated with apo and Zn-bound forms of ISCU2 (31). Second, we do not observe a significant difference between the stability of the as-isolated forms of ISCU2 and ISCU2M140I (Fig. S8), consistent with a previous study (31). Third, our results indicate that both ISCU2 and the M140I substitution exist as monomers in solution (Fig. 2).

Finally, we find that the M140I substitution also does not dramatically affect the ability of ISCU2 to form the SDAeUF complex (Fig. 3). We, therefore, concluded that the M140I substitution does not significantly alter the biochemical properties of ISCU2 and examined its effect on activity.

Incorporating the M140I substitution and generating the SDAeU M140I complex does not replace FXN in stimulating the cysteine desulfurase and Fe-S cluster assembly reactions (Fig. 1, steps 1-3). The SDAeU M140I complex has nearly identical cysteine desulfurase kinetic parameters and ability to be stimulated by FXN as the SDAeU complex (Table 2), consistent with recent activity measurements performed at a single substrate concentration (31,32). The lack of stimulation for the SDAeU M140I complex compared to SDAeU is surprising for two reasons. First, if this substitution mimicked FXN, we would expect a stimulation of activity for the SDAeU M140I complex. Second, previous single-turnover experiments showed that Isu1 increases the accumulation of persulfide label on Nfs1 in the S. cerevisiae system (30). It is unclear if these different results and conclusions are due to the experimental systems (human vs. yeast), nature of the assays (multi-turnover vs. single turnover), or some other factor. Further, we found that complexes that contain the M140I substitution exhibit a modest (1.5 fold) increase in the rate of [2Fe-2S] cluster synthesis but were unable to fully reproduce the stimulation by FXN (2.8 fold). This result is again consistent with previous assays that showed Fe-S assembly complexes containing the M140I substitution are unable to replace the activation role of FXN in Fe-S cluster synthesis (32); quantitative comparisons between our CD-based results and these assays are challenging as their absorbance peak has contributions from [2Fe-2S] cluster synthesis on ISCU2, [2Fe-2S] cluster oxidation of FDX2, and potential formation of Fe-S mineral-like species (22). We find the M140I variant functions similar to ISCU2 and can be activated by FXN (Table 2), consistent with previous DTT-containing but not FDX2-containing assays (32). Overall, all three studies on human Fe-S assembly systems indicate that the M140I substitution on ISCU2 is unable to replace the role of FXN and stimulate cysteine desulfurase and Fe-S assembly reactions.

Here, we provide evidence that the M140I substitution unexpectedly affects the transfer of intact Fe-S clusters (Figs. 4 and 6; Table 2). A new assay was developed that monitors the synthesis of [2Fe-2S] clusters on ISCU2 (Fig. 1, steps 1-3) and their subsequent cluster transfer to GRX5 (Fig. 1, step 4). Assembly complexes in which native ISCU2 was substituted with the M140I variant or supplemented with FXN accelerate the rate of [2Fe-2S] cluster formation on GRX5 by about a factor of three (Fig. 4; Table 2). Interestingly, when both the M140I substitution and FXN are included the rate of cluster formation on GRX5 is further stimulated (6.6 fold; Table 2), suggesting that these effects occur at different steps of the pathway. In a separate experiment, we show that the M140I substitution, but not FXN, accelerates cluster transfer from ISCU2 to GRX5 (Fig. 6). Our working model is that the rate-limiting step in this biosynthetic system is a transfer of intact [2Fe-2S] clusters from ISCU2 to GRX5, which appears to be an essentially irreversible reaction (Fig. S7). The rate of this reaction is directly proportional to the amount of [2Fe-2S] cluster on ISCU2 and the rate constant for cluster transfer to GRX5. Our data support a role for FXN in increasing the concentration of [2Fe-2S] cluster on ISCU2 and the rate constant for cluster transfer to GRX5. We show that the M140I substitution, but not FXN, accelerates cluster transfer from ISCU2 to GRX5 (Fig. 1, steps 1-3) and their subsequent cluster transfer to GRX5 (Fig. 1, step 4). Assembly complexes in which native ISCU2 was substituted with the M140I variant or supplemented with FXN accelerate the rate of [2Fe-2S] cluster formation on GRX5 by about a factor of three (Fig. 4; Table 2). Interestingly, when both the M140I substitution and FXN are included the rate of cluster formation on GRX5 is further stimulated (6.6 fold; Table 2), suggesting that these effects occur at different steps of the pathway. In a separate experiment, we show that the M140I substitution, but not FXN, accelerates cluster transfer from ISCU2 to GRX5 (Fig. 6). Our working model is that the rate-limiting step in this biosynthetic system is a transfer of intact [2Fe-2S] clusters from ISCU2 to GRX5, which appears to be an essentially irreversible reaction (Fig. S7). The rate of this reaction is directly proportional to the amount of [2Fe-2S] cluster on ISCU2 and the rate constant for cluster transfer to GRX5. Our data support a role for FXN in increasing the concentration of [2Fe-2S] cluster on ISCU2 and the M140I substitution in enhancing the rate constant for cluster transfer. This model explains why the effects of FXN and the ISCU2 M140I substitution are additive in this system and provides a possible mechanism for how Isu1 may replace FXN function.

We then aimed to better understand how the M140I substitution might facilitate cluster transfer. First, we examined if the [2Fe-2S] cluster bound to the M140I variant was inherently less stable, which might facilitate cluster transfer. However, a thermal stability assay revealed similar melting temperatures for the chemically reconstituted [2Fe-
2S] cluster bound forms of both proteins (Fig. S8). Second, we examined if the [2Fe-2S] cluster binding equilibrium between ISCU2 and GRX5 was altered by the M140I substitution. There was very little, if any, back transfer of Fe-S clusters from [2Fe-2S]-GRX5 to either apo ISCU2 or the apo M140I variant (Fig. S7). Third, we tested the susceptibility of the [2Fe-2S] clusters to extrusion from the protein through the addition of DTT. A decrease in [2Fe-2S] cluster CD signal was observed in both cases with the rate of signal loss slightly higher for ISCU2 than for the M140I variant (Fig. S9). Together, these results do not support a model in which decreased stability of the [2Fe-2S] cluster bound to the M140I variant promotes increased cluster transfer kinetics. Interestingly, residues other than isoleucine that have a higher hydropathy index (34) than methionine (valine and leucine) but not those with a lower index (threonine, proline, histidine, and glycine) are also able to replace FXN function (29). We hypothesize that during cluster transfer, the [2Fe-2S]-containing ISCU2 undergoes a conformational change, likely involving a hydrophobic residue at position 140, to an activated form that is critical for interactions with apo-GRX5 and lowering the activation energy for cluster transfer.

Overall, we demonstrate that the human ISCU2M140I variant can overcome the loss of FXN function but, surprisingly, not by accelerating the same chemical step in Fe-S cluster biosynthesis. Our results indicate the ISCU2M140I variant accelerates the cluster transfer reaction from ISCU2 to GRX5 rather than mimicking the FXN role in stimulating Fe-S cluster assembly. These results provide new insight into Fe-S cluster biosynthesis and suggest new potential strategies to overcome the loss of FXN function and treat FRDA.

Experimental Procedures

Protein Preparations

Plasmids containing human NFS1 (Δ1-55) and ISD11 (pZM4) were generously provided by S. Leimkühler (35). The NFS1 and ISD11 plasmids were transformed into E. coli strain BL21(DE3) cells and copurified with the bacterial ACP (ACPec) as the SDAec complex (6). Human ISCU2 (Δ1-35) and FXN (Δ1-55) were separately expressed and purified as previously described (20). The spontaneous conversion of Δ1-55 FXN to the truncated form (Δ1-80) was confirmed by SDS-PAGE (20). The QuikChange protocol (Agilent) was used to introduce the M140I point mutation into the ISCU2-pET11a plasmid (20), the C381A point mutation into the NFS1-pET15b plasmid (35), and the S202C point mutation into the FXN-pET11a plasmid (20). The MEGAWHOP (36) method was used to substitute human GRX5 for FDX in the pHis-GFP-TEV-FDX plasmid (37) and produce pHis-GFP-TEV-GRX5. Sequences were confirmed by the Gene Technologies Lab (Texas A&M University).

The pHis-GFP-TEV-GRX5 plasmid was transformed into Rosetta (DE3) cells (VWR). The cells were grown in LB media (VWR) at 37 °C until the ODb00 reached 0.5 and then β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The temperature was decreased to 16 °C and the cells were grown for an additional ~16 h. The harvested cell pellets were resuspended in Buffer A (50 mM Tris and 250 mM NaCl at pH 7.5) with 5 mM imidazole and lysed by sonication (Branson sonifier 450). The soluble fraction was loaded on a Ni-NTA column (5 mL; GE Life Sciences) and the his-GFP-TEV-GRX5 fusion was eluted using Buffer A and a linear gradient between 5 and 500 mM imidazole. The green fractions were combined and dialyzed into Buffer B (50 mM Tris, pH 7.5). The sample was then incubated overnight at room temperature with TEV protease (1:50 molar ratio of protease to fusion protein). The cleaved material was then loaded onto an anion exchange column (27 mL; 16 mm × 13.5 cm, POROS HQ 50) and eluted with Buffer B and a linear gradient from 0 to 1 M NaCl. The GRX5 fractions were concentrated and loaded onto a 26/60 Sephadex 100 column (GE Life Sciences) equilibrated with 50 mM HEPES and 150 mM NaCl at pH 7.5. Monomeric fractions with > 96% purity were concentrated, frozen in liquid nitrogen and stored at −80 °C.

Apo protein concentrations were estimated using the following extinction coefficients: SDAec using 10.9 M−1cm−1 at 420 nm, ISCU2 using 8490 M−1cm−1 at 280 nm, FXN using 26030 M−1cm−1 at 280 nm, and GRX5 using 17780 M−1cm−1 at 280 nm (20,38). Protein variants were assumed to have the same extinction coefficient as the native proteins. The concentration of [2Fe-2S]-GRX5 was determined with a sulfide-detection assay (39). 10 and 20 µL of as purified [2Fe-2S]-GRX5 was
Frataxin-independent activation of Fe-S cluster biosynthesis
diluted to 800 µL with buffer C and incubated at 37 °C for 20 min. 100 µL of 20 mM N,N'-diphenyl-p-phenylenediamine (DPD) (in 7.2 M HCl) and 30 mM FeCl₃ (in 1.2 M HCl) were added and the solutions were incubated at 37 °C for 20 min. The samples were centrifuged at 10000g for 5 min and the absorbance at 670 nm was converted to the concentration of sulfide using a standard curve. The concentration of sulfide was plotted against the absorbance at 670 nm and used to calculate the [2Fe-2S]-GRX5 amounts of sample, fit to a straight line through zero, and used to calculate the [2Fe-2S]-GRX5 concentration.

Oligomeric state of ISCU2 and ISCU2M140I

Purified ISCU2 (300 µL of 538 µM) and ISCU2M140I (400 µL of 434 µM) were passed through an analytical size exclusion chromatography column (Superdex 200 10/300 GL, GE Healthcare Life Sciences) that was equilibrated in Buffer C (50 mM HEPES, 250 mM NaCl, pH 7.5). Each protein eluted as a single peak that was collected and concentrated. ISCU2 and ISCU2M140I stock solutions were diluted with Buffer C to produce 29.5 (A₂₈₀ = 0.25) and 88.3 (A₂₈₀ = 0.75) µM samples. The 500 µL samples were examined with sedimentation velocity analytical ultracentrifugation (AUC) experiments at 50000 rpm at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies (CAUMA) under UT Health Science Center, San Antonio. The molar mass was estimated from the measured sedimentation and diffusion coefficients for each sample, using a partial specific volume based on the protein sequence of the analyte. The calculation was performed using the formula: MW = R.T.s/[D-(1-\(\bar{\nu}\)-\(\rho\))], where MW is the molar mass, R is the gas constant, T is the absolute temperature, s is the sedimentation coefficient corrected to 20 °C in water, D is the diffusion coefficient corrected to 20 °C in water, \(\bar{\nu}\) is the partial specific volume, and \(\rho\) is the solvent density. CRC reference values were used for viscosity and density. The \(\bar{\nu}\) values of 0.746 mL/g for ISCU2M140I and 0.744 mL/g for ISCU2 were calculated by UltraScan (https://alamo.uthscsa.edu/uslims3/uslims3_CAUMA/index.php) (40).

Fluorescence anisotropy measurements

As native FXN contains no cysteine residues, the FXN520C variant was constructed for maleimide labeling. The FXN520C variant (50 µL of 1420 µM) in Buffer D (20 mM NaH₂PO₄, 150 mM NaCl, pH 7.2) was reacted with 10 µL of a 9.84 mM solution of Texas Red™ C2 maleimide (TRM; ThermoFisher) in 100% DMSO for 2 h. The excess fluorophore was removed using a 1 mL anion column (HiTrap Q HP, GE Healthcare Life Science). The concentration of FXN520C in the labeled aliquot was determined using a Bradford assay (using purified FXN520C as standard). The concentration of the TRM fluorophore in the labeled protein was determined using \(\varepsilon_{595} = 112000\) M⁻¹cm⁻¹. The final concentration of TRM labeled FXN520C was 18.5 µM with a labeling efficiency of 55%.

The fluorophore-labeled FXN520C (0.1 µM) was combined with 100 µM L-cysteine, 200 µM Fe²⁺, and either 30 µM ISCU2 or ISCU2M140I. The samples were then incubated with different concentrations of the SDAec complex for 15 min. The fluorescence was measured at room temperature with an excitation wavelength of 560 nm and perpendicular excitation polarizer and an emission wavelength of 605 nm with either a perpendicular or parallel emission polarizer (TECAN infinite F200 PRO). The Kd was determined by fitting the data to the equation below. The \(k_A\) and \(k_B\) values are the measured anisotropies for the SDAecU (or SDAecU(M140I)) and SDAecUF (or SDAecU(M140F)) complexes (at saturation), respectively. The total concentration of SDAecU and FXN are represented by [A] and [FXN], respectively.

\[
\text{Anisotropy} = k_A + \frac{(k_B - k_A)}{[A]} \cdot \frac{b - \sqrt{b^2 - 4 \cdot [A] \cdot [FXN]}}{2}
\]

where \(b = [A] + [FXN] + K_d\)

Complete Fe-S cluster synthesis and transfer reactions

The complete reaction assays included 0.5 µM SDAec, 20 µM ISCU2 (or ISCU2M140I), 20 µM (or 0 µM) FXN, 40 µM GRX5, and 400 µM

\[\text{Complete reaction} \quad \begin{align*}
\text{SDAec} + \text{ISCU2} + \text{FXN} + \text{GRX5} & \rightarrow \text{Complete Fe-S cluster synthesis} \\
& \rightarrow \text{Transfer reactions}
\end{align*}\]
Fe(NH$_4$)$_2$(SO$_4$)$_2$ and Buffer E (50 mM HEPES, 150 mM KCl and 10 mM MgCl$_2$ at pH 7.5). The reactions were initiated by the addition of 100 μM L-cysteine and 10 mM GSH at room temperature. Cluster formation on GRX5 was then measured for SDA$_{ec}$U, SDA$_{ec}$UF, SDA$_{ec}$UM$_{140I}$ and SDA$_{ec}$UM$_{140I}$Fb by monitoring the ellipticity change at 450 nm using a Chirascan circular dichroism (CD) spectrometer (Applied Photophysics). Cuvettes (1 cm path length) were sealed with a rubber septum and electrical tape in a glove box. The kinetic data were fit to an exponential rise equation ([y = y$_0$ + A0(1 - exp(-kt)))] where the k is the apparent rate of cluster formation on GRX5) using Kaleidograph (Synergy Software, Reading, PA).

Cysteine desulfurase activity measurements
Cysteine desulfurase activities were measured for each complex using a slightly modified methylene blue assay (20, 39). Protein complexes were generated with final concentrations of 0.5 μM SDA$_{ec}$, 1.5 μM ISCU2 (or ISCU2$^{M_{140I}}$), and 1.5 μM FXN (when included). The complexes were combined with 4 mM D,L-DTT and incubated for 15 min anaerobically on a heating block at 37 °C. Different concentrations of L-cysteine were added, incubated for 6 min, and quenched with 20 mM DPD (in 7.2 M HCl) and 30 mM FeCl$_3$ (in 1.2 M HCl). The samples were centrifuged after 20 min and the absorbance was measured at 670 nm. The amount of sulfide produced was determined for each data point using a standard curve. Rates ([S$^2^-$]/([NFS1]*min)) were plotted against the amount of L-cysteine added and fit to the Michaelis-Menten equation using Kaleidograph.

Fe-S cluster assembly reactions on ISCU2
Fe-S assembly reactions on ISCU2 contained 10 μM SDA$_{ec}$, 30 μM ISCU2 (or ISCU2$^{M_{140I}}$), 30 μM FXN (when added), 400 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and Buffer E. The reactions were initiated with 1 mM L-cysteine at room temperature. The formation of [2Fe-2S] clusters was monitored by the change in ellipticity at 330 nm (22). The initial increase in ellipticity was plotted with time and fit to a linear equation ($R^2$ values ≥ 0.97) using Kaleidograph. A 1.50 μM/mdeg (see Fig. S2) factor was used to convert ellipticity at 330 nm to [2Fe-2S]-ISCU2 concentration.

[2Fe-2S] cluster transfer reactions from enzymatic reconstituted ISCU2 to GRX5
Clusters were enzymatically generated on ISCU2 (or ISCU2$^{M_{140I}}$) using 0.5 μM SDA$_{ec}$, 20 μM ISCU2 (or ISCU2$^{M_{140I}}$), and 400 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and Buffer E. The reactions were initiated with 100 μM L-cysteine and 10 mM GSH at room temperature. After 160 min, the enzymatic formation of [2Fe-2S] clusters appeared to be complete based on the lack of changes in ellipticity at 450 nm. A solution containing 40 μM GRX5 (with or without 20 μM FXN) was then injected with an air-tight syringe into the sealed cuvette to initiate the transfer reactions. The change in ellipticity was plotted with time and fit to a linear equation with Kaleidograph ($R^2$ values ≥ 0.97). A 3.77 μM/mdeg (see Fig. S2) factor was used to convert ellipticity at 450 nm to [2Fe-2S]-GRX5 concentration.

[2Fe-2S] cluster transfer reactions from holo-GRX5 to apo-ISCU2/ISCU2$^{M_{140I}}$
Reactions (200 μL) were initiated in an anaerobic cuvette by mixing a final concentration of 20 μM [2Fe-2S]-GRX5 with 50 μM ISCU2 (or ISCU2$^{M_{140I}}$) in Buffer C in the presence and absence of 10 mM GSH. The cuvette was sealed with a rubber septum in an anaerobic glovebox (O$_2$ < 0.5 ppm). The cluster transfer reaction was tracked at room temperature by monitoring the spectrum from 300 nm to 600 nm with time.

Chemical reconstitution of Proteins
[2Fe-2S] clusters were chemically generated on ISCU2 (or ISCU2$^{M_{140I}}$) by reacting 30 μM (final concentrations) of apo protein in Buffer C with 600 μM ferric ammonium citrate, 600 μM Na$_2$S and 10 mM D,L-DTT for 1 h at room temperature. The DTT and the excess iron and sulfide were then removed with a desalting column (5 mL, GE Healthcare) equilibrated in Buffer C. The iron concentration was quantitated with the ferrozine assay (extinction coefficient of 28,000 M$^{-1}$cm$^{-1}$ at 562 nm) (41) and sulfide was quantitated using a methylene blue assay with an additional pre-treatment of the protein with NaOH and zinc acetate to release bound sulfide (42). The protein concentration was determined using the Bradford assay. Chemically reconstituted ISCU2 had 2.1 ±
0.3 iron and 2.0 ± 0.6 sulfide atoms per protein, whereas ISCU2M140I had 1.9 ± 0.5 iron and 2.2 ± 0.1 sulfide atoms per protein.

**SYPRO-Orange assay for thermal stability analysis of proteins**

The samples were prepared anaerobically in a glovebox by mixing 5 µL of a 1 mM SYPRO orange (Thermo Fisher) stock solution in water with 5 µM (final concentration) of ISCU2 or ISCU2M140I (with or without chemically reconstituted [2Fe-2S] cluster) in a 20 µL (final volume) reaction in buffer C (50 mM HEPES, 250 mM NaCl, pH 7.5). The samples were first equilibrated at 25 °C for 5 minutes and then the temperature was gradually increased to 100 °C in 75 cycles (1°C increase per cycle with 1 min waiting period) using a qPCR instrument (Stratagene Mx3005P, Agilent Technologies) and the change in fluorescence (λex – 491 nm, λem – 610 nm) was monitored. The raw data were analyzed and fluorescence vs. temperature plots were generated using MxPro – Mx3005P software. Melting temperatures were calculated from the peak value of the first derivative plot. All the species had melting temperature (Tm) of 50 ± 2 °C.

**[2Fe-2S] cluster extrusion assay**

[2Fe-2S] clusters were assembled on 30 µM ISCU2 (or ISCU2M140I) in the presence of 10 µM SDAex, 100 µM L-cysteine, 400 µM Fe(NH4)2(SO4)2, and 10 mM GSH in Buffer C. 4 mM D,L-DTT was added to the samples to initiate the cluster extrusion reaction, which was followed by the loss of ellipticity at 330 nm. The extrusion rates were determined by fits to a linear equation.

**Acknowledgments:** We thank Professor Tadhg Begley for the generous use of his CD spectrometer. We also thank Seth Cory and Chris Putnam for helpful discussions. This work was supported in part by NIH grant R01GM096100, NSF grant CHE 1508269 and the Robert A. Welch grant A-1647. The LIMS cluster at the Bioinformatics Core Facility at the University of Texas Health Science Center at San Antonio and multiple High-Performance Computing clusters supported by NSF XSEDE Grant #MCB070038 (to Borries Demeler).

**Conflict of Interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions:** All authors designed the project, interpreted experimental results, and wrote the manuscript. D.D, S.P. and J.B.R. conducted experiments.

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**Abbreviations:** CD, circular dichroism; DPD, *N,N’*-diphenyl-*p*-phenylenediamine; DTT, dithiothreitol; FRDA, Friedreich’s Ataxia; GSH, glutathione; SDA<sub>ec</sub>, human NFS1-ISD11 in complex with E. coli ACP; SDA<sub>ec</sub>U, human NFS1-ISD11-ISCU2 in complex with E. coli ACP; SDA<sub>ec</sub>UF, human NFS1-ISD11-ISCU2-FXN in complex with E. coli ACP.
Table 1. Descriptions of the protein complexes.

| Complexes      | Description                                      |
|----------------|--------------------------------------------------|
| SDA<sub>ec</sub> | NFS1 + ISD11 + ACP<sub>ec</sub>                 |
| SDA<sub>ecU</sub> | NFS1 + ISD11 + ACP<sub>ec</sub> + ISCU2         |
| SDA<sub>ecU<sup>M140I</sup></sub> | NFS1 + ISD11 + ACP<sub>ec</sub> + ISCU2<sup>M140I</sup> |
| SDA<sub>ecUF</sub> | NFS1 + ISD11 + ACP<sub>ec</sub> + ISCU2 + FXN  |
| SDA<sub>ecU<sup>M140F</sup></sub> | NFS1 + ISD11 + ACP<sub>ec</sub> + ISCU2<sup>M140F</sup> + FXN |

**Note:** ec denotes *E. coli* protein that copurifies with human proteins. ISCU2<sup>M140I</sup> denotes M140I variant of ISCU2.
Table 2. Kinetic data for Fe-S assembly complexes.

|                         | SDA_{ec} U | SDA_{ec} U^M140I | SDA_{ec} UF | SDA_{ec} U^M140I F | Relative Rate with respect to SDA_{ec} U |
|-------------------------|------------|-------------------|------------|--------------------|-----------------------------------------|
| **Complete reaction**   |            |                   |            |                    |                                         |
| Cluster synthesis &     | 4.1 ± 0.3  | 11.3 ± 0.2        | 14.4 ± 0.2 | 27.2 ± 0.9         | 2.8                                     |
| transfer to GRX5 (min^-1 X 10^-3) |            |                   |            |                    |                                         |
| **Cysteine desulfurase**|            |                   |            |                    |                                         |
| activity                |            |                   |            |                    |                                         |
| \( k_{cat} \) (min^{-1})| 0.82 ± 0.03^a | 0.90 ± 0.02      | 10.1 ± 0.2^a | 7.34 ± 0.34       | 1.1                                     |
| \( K_M \) (\mu M)      | 0.62 ± 0.11^a | 0.65 ± 0.09      | 11.6 ± 0.9^a | 8.0 ± 2.0         | 12.3                                    |
| **Cluster synthesis**   |            |                   |            |                    |                                         |
| Cluster formation on ISCU2 (\mu M/min) | 0.36 ± 0.02 | 0.56 ± 0.03      | 1.02 ± 0.03 | 0.83 ± 0.02       | 1.5                                     |
| **Cluster transfer**    |            |                   |            |                    |                                         |
| Cluster transfer ISCU2 to GRX5 (\mu M/min) | 0.26 ± 0.02 | 0.49 ± 0.04      | 0.26 ± 0.02 | ND                 | 1.9                                     |

^aData from reference 6; ND – Not determined.
Figure 1. Sulfur transfer and [2Fe-2S] synthesis reactions.
Figure 2. ISCU2 and ISCU2\textsuperscript{M140I} are primarily monomeric in solution. Analytical ultracentrifugation (AUC) experiments revealed that ISCU2 (88.3 μM red and 29.5 μM green) and ISCU2\textsuperscript{M140I} (88.3 μM blue and 29.5 μM black) were primarily monomeric species with little evidence of aggregate or higher order oligomer. Inset: ISCU2 and ISCU2\textsuperscript{M140I} samples were eluted from an analytical size exclusion chromatography as a single peak (elution volume = 17.2 mL) prior to the AUC analysis. The estimated molecular weights from the AUC experiment is 15 kDa, consistent with the expected mass of 14.35 kDa.
Figure 3. The M140I substitution does not significantly affect the ability of ISCU2 to form the Fe-S assembly complex. Fluorophore labeled FXN (0.1 μM) was combined with 100 μM L-cysteine, 200 μM Fe^{2+}, and either 30 μM ISCU2 (black) or ISCU2^{M140I} (red). Fluorescence anisotropy (excitation 560 nm, emission 605) was measured upon titration with the SDA_{ec} complex. Each measurement is an average from 3 experiments with a maximum standard deviation of 5 millianisotropy units. The dashed lines are the fit to a binding curve. The $K_d$ values for SDA_{ec}UF and SDA_{ec}^{M140I}F are 1.5 ± 0.1 and 2.8 ± 0.5 μM, respectively.
Figure 4. FXN and the ISCU2^{M140I} substitution accelerate different steps in Fe-S cluster biosynthesis. A) Kinetics of Fe-S cluster synthesis and transfer to GRX5 were monitored for different assembly complexes by the change in ellipticity at 450 nm. The average change in ellipticity (n = 3; maximum error of 1.8 in ellipticity) is plotted and fit with an exponential rise equation. The final CD spectra are shown in Fig. S3. Color scheme: SDA_{ec}U (red), SDA_{ec}UF (blue), SDA_{ec}U^{M140I} (black) and SDA_{ec}U^{M140I}F (green).
Figure 5. Fe-S assembly complexes containing native ISCU2 or ISCU2\textsuperscript{M140I} exhibit similar kinetic parameters for the cysteine desulfurase reaction. Cysteine desulfurase activities for complexes A) without (SDA\textsubscript{ec}U in red and SDA\textsubscript{ec}U\textsuperscript{M140I} in black) and B) with FXN (SDA\textsubscript{ec}UF in blue and SDA\textsubscript{ec}U\textsuperscript{M140F} in green) at different concentrations of L-cysteine. Lines through the data are the fits to the Michaelis-Menten equation. Experiments were performed in triplicate. The native SDA\textsubscript{ec}U and SDA\textsubscript{ec}UF data is reported in reference 6 and shown here for comparison.
Figure 6. FXN and the ISCU2<sup>M140I</sup> substitution accelerate different steps in Fe-S cluster biosynthesis. 
A) Fe-S cluster assembly reactions on ISCU2 were monitored for assembly complexes by the change in ellipticity at 330 nm. The average change in ellipticity (n = 3; maximum error of 1.8 in ellipticity) is plotted and fit with a linear equation. The complete time course of the reactions and final CD spectra are shown in Fig. S4. 
B) Kinetics of cluster transfer from pre-formed holo-ISCU2 to apo-GRX5 were monitoring by the change in ellipticity at 450 nm. Reactions were initiated (time = 0) by the anaerobic injection of GRX5 (with or without FXN). The average change in ellipticity (n = 3; maximum error of 0.8 in ellipticity) is plotted and fit with a linear equation. The CD spectra before addition of GRX5 and the complete time course of the reaction are shown in Figs. S5 and S6. Color scheme: SDA<sub>ec</sub>U (red), SDA<sub>ec</sub>UF (blue), SDA<sub>ec</sub>U<sup>M140I</sup> (black) and SDA<sub>ec</sub>U<sup>M140I-F</sup> (green).
Mechanism of frataxin "bypass" in human iron-sulfur cluster biosynthesis with implications for Friedreich's ataxia
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J. Biol. Chem. published online April 11, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.007716

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