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Mitochondrial [4Fe-4S] Proteins**

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Baffoni, Lucia Banci**

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ISCA1 orchestrates ISCA2 and NFU1 in the maturation of human mitochondrial [4Fe-4S] proteins

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ABSTRACT

The late-acting steps of the pathway responsible for the maturation of mitochondrial [4Fe-4S] proteins are still elusive. Three proteins ISCA1, ISCA2 and NFU1 were shown to be implicated in the assembly of [4Fe-4S] clusters and their transfer into mitochondrial apo proteins. We present here a NMR-based study showing a detailed molecular model of the succession of events performed in a coordinated manner by ISCA1, ISCA2 and NFU1 to make [4Fe-4S] clusters available to mitochondrial apo proteins. We show that ISCA1 is the key player of the [4Fe-4S] protein maturation process because of its ability to interact with both NFU1 and ISCA2. On the contrary, the two latter proteins do not interact each other. ISCA1 works as the promoter of the interaction between ISCA2 and NFU1 being indeed able to determine the formation of a transient ISCA1-ISCA2-NFU1 ternary complex. We also show that ISCA1, through its specific recognition with the C-domain of NFU1 that contains the cluster binding CXXC motif, mediates a [4Fe-4S] cluster transfer from the site where the cluster is assembled on the ISCA1-ISCA2 complex to a cluster binding site formed by ISCA1 and NFU1 in the ternary ISCA1-ISCA2-NFU1 complex. Such mechanism guarantees that the [4Fe-4S] cluster can be safely moved from the ISCA1-ISCA2 complex, where it is assembled, to NFU1, thus making the [4Fe-4S] cluster available for mitochondrial apo proteins specifically requiring NFU1 for their maturation.

Keywords: iron-sulfur protein, iron sulfur cluster assembly machinery, mitochondria, NMR, protein-protein interaction.

Abbreviations

Iron-sulfur: Fe-S; Heteronuclear single quantum coherence: HSQC; Size exclusion chromatography equipped with multiangle light scattering: SEC-MALS; Luria-Bertani: LB; Isopropyl- β -D-1-thiogalactopyranoside: IPTG; Bovine serum albumin: BSA; 1,4-dithiothreitol: DTT.

INTRODUCTION

In eukaryotes, mitochondria are the primary site for the biosynthesis of Fe-S clusters. Within the mitochondrial matrix, the biosynthesis of Fe-S clusters and their insertion into apo proteins is assisted by several proteins that are highly conserved from bacteria to mammals.^{1, 2} Among these proteins, three of them, ISCA1, ISCA2 and NFU1, were shown to be implicated in the assembly of [4Fe-4S] clusters and their insertion into mitochondrial apo proteins.³⁻¹⁰ ISCA1 and ISCA2 are two highly homologous members of the A-type evolutionarily conserved protein family, which is characterized by three highly conserved cysteine residues organized in a C-X_n-C-G-C sequence motif (n is usually 63-65, but is increased by a 21-residue insert in some eukaryotic proteins).¹¹ This motif has been shown to be able to bind both [2Fe-2S] and [4Fe-4S] cluster types.¹²⁻¹⁸ In humans, a ISCA1-ISCA2 complex was identified *in vivo*¹⁹ and in its heterodimeric state was shown to act *in vitro* as a platform to assemble a [4Fe-4S]²⁺ cluster.^{14, 20} The process, here named ISCA-dependent pathway, requires: i) GLRX5, the [2Fe-2S] metallochaperone of the mitochondrial matrix that donates two [2Fe-2S]²⁺ clusters to the ISCA1-ISCA2 complex; ii) ferredoxin FDX2, which supplies the two electrons required for the reductive coupling of two [2Fe-2S]²⁺ clusters to form a [4Fe-4S]²⁺ cluster; iii) IBA57, whose molecular role in the process is still not defined (**Fig. 1A**).^{14, 20-23} Concerning the role of IBA57 in the latter process, it was found that ISCA2 and IBA57 form a [2Fe-2S] ISCA2-IBA57 complex, suggesting that IBA57 might work with ISCA2 in the [4Fe-4S] cluster assembly process.²⁴⁻²⁶ This stringent IBA57-ISCA2 functional association was also documented *in vivo* by the finding that ISCA2, but not ISCA1, interacts with IBA57.¹⁹ In marked contrast to the just mentioned [4Fe-4S] cluster assembly process, *in vivo* data provided evidence that only human ISCA1 is essential for mitochondrial [4Fe-4S] proteins in skeletal muscle or primary neuronal cells under defined

physiological conditions, while ISCA2 is dispensable.¹⁹ These data, however, contrast with what was found in HeLa cells in which similar phenotypes were observed for ISCA1, ISCA2 and IBA57 knockdowns, supporting that the three proteins function together in the biosynthesis of mitochondrial [4Fe-4S] clusters,⁴ as well as they contrast with the finding that pathogenic mutations in either ISCA1, ISCA2, or IBA57 genes are associated with similar disease phenotypes that include neurodegenerative defects, suggesting that each encoded protein is crucial in neurons.²⁷⁻²⁹ Thus, the latter mentioned *in vivo* data support that the ISCA-dependent pathway described above is operative in mitochondria.

The other player of the ISCA1-ISCA2 pathway is NFU1, a universally present protein composed by folded, globular N- and C-domains connected by a flexible linker.³⁰⁻³² Human NFU1 contains a conserved CXXC-motif located in the C-domain, shown to be involved in [4Fe-4S] cluster binding.^{10, 31, 33} NFU1 was proposed to act downstream the ISCA1-ISCA2 complex.³⁴ This model was based on studies in *Saccharomyces cerevisiae*³⁵ and suggested that NFU1 receives the [4Fe-4S] cluster assembled on the ISCA complex and then transfers it to selected apo proteins (**Fig. 1A**). Supporting this model, it was found that yeast Nfu1 specifically interacts *in vivo* with the yeast homologues of both human ISCA1 and ISCA2.³⁵ On the other hand, human ISCA1 and ISCA2 were found to have distinct interacting partners. Indeed, while a specific interaction between ISCA1 and NFU1 was observed *in vivo* by immunoprecipitation experiments, NFU1 did not co-immunoprecipitate with ISCA2.¹⁹ In addition, it has been recently proposed that the ISCA-NFU1-dependent pathway might be not unique, since other alternative [4Fe-4S] cluster assembling pathways have been described. By employing genetic and biochemical techniques, it has been recently shown that the assembly of a [4Fe-4S] cluster on NFU1 requires the coordinate action of three proteins, which are ISCU2, the scaffold protein able to *de novo* assemble a [2Fe-

2S]²⁺ cluster,^{36, 37} ISCA1 and FDX2.³⁸ It was postulated a model (here named ISCA1-dependent pathway) where the [2Fe-2S]²⁺ cluster-bound forms of ISCA1 and ISCU2 interact with dimeric NFU1 to donate their clusters, which then reductively couple into a [4Fe-4S]²⁺ cluster upon the provision of two electrons by FDX2 (**Fig. 1B**). This mechanism is supported by the interactions observed *in vivo* between NFU1 and ISCU2, between NFU1 and ISCA1, between NFU1 and FDX2 and between two NFU1 molecules,³⁸ as well as by *in vitro* NMR and SAXS studies that showed that NFU1 and ISCU2 form a complex in which the cluster binding region of ISCU2 interacts with the C-terminal domain of NFU1.³⁹ Then, in the ISCA1-dependent pathway, a multi-protein complex composed by ISCA1, ISCU2, FDX2 and dimeric [4Fe-4S]-NFU1 was proposed to interact with mitochondrial apo proteins and to transfer to them the assembled [4Fe-4S]²⁺ cluster (**Fig. 1B**).³⁸ This proposed [4Fe-4S] cluster assembly mechanism bypasses the ISCA1-ISCA2 complex. Another alternative mechanism of [4Fe-4S] cluster assembly, which is possibly activated under oxidative cellular conditions only was proposed based on a NMR study.³⁵ It was shown indeed that the [2Fe-2S] GLRX5-BOLA3 complex^{40, 41} is able to promote the assembly of a [4Fe-4S]²⁺ cluster on dimeric NFU1 with no requirement of the ISCA1-ISCA2 complex.³³

In summary, the data available up to now in the literature do not still depict a molecular view of how ISCA1, ISCA2 and NFU1 proteins cooperate in the maturation of mitochondrial [4Fe-4S] proteins. In order to shed light on this matter, we report here a NMR-based study investigating the ISCA1, ISCA2 and NFU1 patterns of interactions and cluster transfer processes. Our data define a detailed molecular model of the succession of events performed in a coordinated manner by ISCA1, ISCA2 and NFU1 to make available [4Fe-4S] clusters for mitochondrial apo proteins.

This model provides a general view of the late-acting steps of the maturation of mitochondrial [4Fe-4S] proteins.

RESULTS

Interaction network among ISCA1, ISCA2 and NFU1 in their apo forms

Protein-protein interaction studies were characterized through NMR spectroscopy and analytical size exclusion chromatography, by titrating two or three proteins, which are ISCA1, ISCA2 and NFU1, in their apo forms with different mixing sequences. The used protein constructs were: i) the mitochondrial isoform of human NFU1 having, in the mature state, a molecular mass of ~22 kDa, as obtained upon removal of the N-terminal mitochondrial targeting sequence of 58 residues;¹⁰ ii) human ISCA2 without the predicted N-terminal mitochondrial targeting sequence of 43 residues and thus having a molecular mass of ~12 kDa;¹³ iii) human ISCA1 in its full-length form (molecular mass of ~14 kDa) as its mitochondrial presequence is not removed upon its mitochondrial import.²⁷

First, we investigated protein-protein interactions occurring between couples of proteins. When apo ¹⁵N ISCA2 was stepwise titrated with apo ¹⁵N NFU1 up to a 1:1 protein ratio (calculated considering monomeric protein concentrations), no chemical shift changes were observed along the ¹H-¹⁵N HSQC NMR experiments and the ¹H-¹⁵N HSQC map of the final 1:1 mixture is the sum of the ¹H-¹⁵N HSQC maps of the two isolated proteins (**Fig. 2A**), indicating that the two proteins do not interact each other. Analytical size exclusion chromatography data confirmed this result as no peaks, with apparent molar mass higher than those of the two isolated ISCA2 and NFU1 proteins (running, respectively, as a homodimer and a monomer with a low percentage of homodimer^{14, 33}) were observed in the final 1:1 mixture (**Fig. 2B**).

When apo ¹⁵N ISCA2 was stepwise titrated with apo ISCA1 up to a 1:1 protein ratio (calculated considering monomeric protein concentrations), chemical shift changes were observed in the ¹H-

^{15}N HSQC maps of apo ^{15}N ISCA2 in a slow exchange regime on the NMR time scale, which means that signals of apo ISCA2 decreased in intensity and those of a new species, assigned to the apo ISCA1-ISCA2 complex, appeared and increased in intensity along the titration (**Fig. 2C**). Once the 1:1 ISCA1-ISCA2 ratio was reached, the signals of apo ISCA2 completely disappeared and the signals of the apo ISCA1-ISCA2 complex reached their maximal intensity, indicating that the complex is fully formed at the 1:1 stoichiometric ratio. Analytical size exclusion chromatography of apo ISCA1 showed that the protein is essentially monomeric with a small fraction of dimer (**Fig. 2D**) and that of the final 1:1 mixture showed: i) a single peak with an apparent molar mass of ~24 kDa, which is close to the molecular weight calculated for a dimeric apo ISCA1-ISCA2 complex (26 kDa) and which contains both ISCA1 and ISCA2 proteins, as detected by SDS-PAGE (**Fig. S1**), ii) that the monomeric state of apo ISCA1 is no longer present in the mixture (**Fig. 2D**). Considering the concentration at which the complex is fully formed in the NMR and SEC-MALS experiments, the dissociation constant for the apo ISCA1-ISCA2 complex could be estimated to be lower than 50 μM . Overall, the NMR and analytical size exclusion chromatography data indicated the formation of a heterodimeric apo ISCA1-ISCA2 complex. The meaningful ($1\sigma \Delta\delta_{\text{avg}}(\text{HN}) > 0.05 \text{ ppm}$) ^1H and ^{15}N chemical shift changes between apo ISCA2 alone and in the 1:1 protein mixture (**Fig. S2**) are localized in a well-defined region of the protein, which matches with the subunit-subunit interface in dimeric apo ISCA2.¹⁴ The same results were previously found using a shorter ISCA1 construct without its predicted mitochondrial presequence of 1-23 amino acids,¹⁴ thus indicating that the latter stretch does not affect complex formation and does not take part as active contributor to complex formation.

Apo ISCA1 forms a heterocomplex also with apo NFU1. Indeed, when apo ^{15}N NFU1 was stepwise titrated with apo ISCA1 up to a 1:1 protein ratio (calculated considering monomeric

protein concentrations), chemical shift changes were observed on the ^1H - ^{15}N HSQC maps of apo NFU1 in an intermediate exchange regime on the NMR time scale (**Fig. 3A**). Indeed, almost all resonances of the C-domain of NFU1 shift and concomitantly broaden beyond detection upon additions of apo ISCA1 (**Fig. 3A** and **Fig. S3A**), due to the complex formation. This analysis therefore cannot allow to exactly quantify the amount of complex formed at the final point of the titration, even though the detection of chemical shift variations and broadening upon apo ISCA1 additions indicates the formation of the complex with the concomitant reduction of free monomeric apo NFU1. On the other hand, in the analytical size exclusion chromatography of the final 1:1 apo NFU1-apo ISCA1 mixture, a single peak, which contains both proteins (as detected by SDS-PAGE, **Fig. S1**), is present with an elution volume smaller than those of the two isolated proteins, while the peaks of the isolated monomeric apo ISCA1 and dimeric apo NFU1 are absent (**Fig. 3B**). Thus, the single peak containing both NFU1 and ISCA1 identifies exclusively the heterodimeric complex, which is fully formed at the 1:1 apo NFU1-apo ISCA1 ratio. SEC-MALS indicated that this single peak has a molar mass of 29.2 ± 0.5 kDa (**Fig. S3B**). This value is intermediate between those of the isolated proteins (apo ISCA1: 14 kDa, apo NFU1: 22 kDa) and that expected for a heterodimeric apo NFU1-ISCA1 complex (36 kDa). Considering the complex concentration in the NMR and SEC-MALS experiments where the complex formation was fully reached, a dissociation constant for the apo NFU1-ISCA1 complex lower than 50 μM can be estimated. Overall, both NMR and SEC-MALS data showed that a heterodimeric ISCA1-NFU1 complex is fully formed via a specific recognition between ISCA1 and the C-domain of NFU1. However, also a few resonances of the N-domain of NFU1 were affected by apo ISCA1 additions (residues 54-58, **Fig. S3A**). These residues are located at the interacting interface between the N- and C-domains of apo NFU1 when it is in its closed conformation.³³ This closed

conformation is in equilibrium with an open conformation where the N- and C-domains freely move in solution.^{31, 33} Considering these structural properties and the effect of ISCA1 addition on the NH signals of residues 54-58, we can propose that the ISCA1-NFU1 complex formation induces structural rearrangements at the interaction interface between the N- and C-domains of NFU1, possibly affecting the open-closed conformational equilibrium. This ISCA1-driven effect on the open-closed conformational equilibrium of NFU1 might also explain the lower than expected molar mass of the ISCA1-NFU1 complex as estimated by SEC-MALS data analysis. By mapping the meaningful chemical shift changes (both broadening beyond detection effects and chemical shift changes with $1\sigma \Delta_{\text{avg}}(\text{HN}) > 0.03$ ppm) on the closed conformation of apo NFU1, we see that the two helices of the C-domain are largely affected by the protein-protein interaction, while the β -sheet of the C-domain is essentially unaffected, as well as that only the first short helix of the N-domain in close contact with the C-domain is also affected (**Fig. 3C**). The two helices of the C-domain encase the cluster binding CXXC motif of NFU1, indicating that ISCA1 in the complex is positioned close to the cluster binding region, although the CXXC motif is not largely involved in protein-protein recognition.

The apo complexes, ISCA1-¹⁵N NFU1 and ISCA1-¹⁵N ISCA2, were then titrated with apo ISCA2 and apo ¹⁵N NFU1, respectively, and the resulting mixtures were again analyzed by NMR. In the first titration, the comparison of the ¹H-¹⁵N HSQC map of the final mixture, having a 1:1 ratio between apo ISCA1-¹⁵N NFU1 complex and apo ISCA2, with the maps of apo ¹⁵N NFU1 alone and in the complex with ISCA1, indicated that NFU1 remains complexed with ISCA1, thus ISCA2 not being able to extract ISCA1 from the ISCA1-NFU1 complex to form an isolated ISCA1-ISCA2 complex. Indeed, the signals of NFU1 complexed with ISCA1 were not affected by the addition of apo ISCA2 and the signals of isolated apo NFU1 were not detected,

thus indicating that free apo NFU1 was not released in solution, as it would have expected in the case of the formation of an isolated ISCA1-ISCA2 complex (**Fig. 4A**). In the second titration, when apo ISCA1-¹⁵N ISCA2 complex is titrated with apo ¹⁵N NFU1 up to a 1:1 ratio, ISCA2 remains complexed with ISCA1, thus NFU1 not being able to extract ISCA1 from the ISCA1-ISCA2 complex to form an isolated ISCA1-NFU1 complex. Indeed, the signals of ISCA2 complexed with ISCA1 were not affected by the addition of apo NFU1 and the signals of isolated apo ISCA2 were not detected (**Fig. 4B**), thus indicating that free apo ISCA2 was not released in solution, as it would have been expected in the case of the formation of an isolated ISCA1-NFU1 complex. However, the backbone NH signals of ¹⁵N NFU1 changed upon its addition to the ISCA1-ISCA2 complex and, in the final apo ISCA1-apo ¹⁵N ISCA2-apo ¹⁵N NFU1 1:1:1 mixture, they overlap with those of the apo ISCA1-¹⁵N NFU1 complex, while they differ from those of apo ¹⁵N NFU1 alone (**Fig. 4C**). Overall, from these data we can conclude that: i) apo NFU1 interacts with the apo ISCA1-ISCA2 complex via the C-domain of NFU1 similarly to what happens in the apo NFU1-ISCA1 complex; ii) apo ISCA1 is the protein mediating the interaction between apo NFU1 and apo ISCA2, being the latter two not interacting each other, in agreement with what observed when the two proteins are mixed (as described above). Thus, these data indicated that ISCA1, interacting with both ISCA2 and NFU1, mediates the formation of a ternary complex.

To further corroborate this model, apo ISCA1 was stepwise added to a 1:1 mixture containing the two not-interacting apo ¹⁵N ISCA2 and apo ¹⁵N NFU1 proteins. The ¹H-¹⁵N HSQC maps showed the occurrence of the interaction of apo ISCA1 with both apo ISCA2 and apo NFU1. In the final mixture, the NH signals of apo NFU1 overlap with those of apo NFU1 complexed with apo ISCA1, and not with those of isolated apo NFU1 (**Fig. S4A**), and the NH signals of apo

ISCA2 overlap with those of apo ISCA2 complexed with apo ISCA1, and not with those of isolated apo ISCA2 (**Fig. S4B**). The observed chemical shift changes are concurrent on both proteins along the titration and the chemical shift changes on both ^{15}N ISCA2/ ^{15}N NFU1 apo proteins are complete once one equivalent of apo ISCA1 was added to the 1:1 ISCA2/NFU1 apo mixture. Consistently, additions up to two equivalents of apo ISCA1 to the mixture did not significantly affect the ^1H - ^{15}N HSQC map. These results conclusively indicated that apo ISCA1 interacts with both apo ISCA2 and apo NFU1, by promoting the formation of a ternary complex, involving the two not interacting proteins apo ISCA2 and apo NFU1. The latter titration also allowed to estimate a dissociation constant for the apo ternary complex lower than 500 μM on the basis of the complex concentration reached once the complex was fully formed. On the other hand, analytical size exclusion chromatography of the final mixture did not show the presence of a peak with an elution volume smaller than those of the two isolated ISCA1-ISCA2 and ISCA1-NFU1 apo complexes, as it would be expected for the presence of a ternary complex. This result can be interpreted considering that the ternary complex is readily released at the lower protein concentrations used in the analytical size exclusion chromatography with respect to the higher protein concentrations used in the NMR experiments, suggesting its transient nature.

Protein-protein interaction studies between [4Fe-4S] ISCA1-ISCA2 and apo NFU1

Anaerobic purification of ISCA1 and ISCA2 led to a mixture of apo and $[2\text{Fe-2S}]^{2+}$ cluster-bound forms (see Materials and Methods for details). While chemical reconstitution of anaerobically purified ISCA2 led to the formation of a $[4\text{Fe-4S}]^{2+}$ cluster-bound dimeric form,¹⁴ chemical reconstitution of anaerobically purified ISCA1 does not lead to the formation of a $[4\text{Fe-4S}]$ cluster-bound form. Indeed, UV-visible spectrum of chemically reconstituted ISCA1

maintains, with a ~20% increase, the bands of the anaerobically purified ISCA1 at 325, 420 nm and a shoulder at 460 nm (**Fig. S5A**), typical of Cys-bound $[2\text{Fe-2S}]^{2+}$ clusters.⁴²⁻⁴⁴ The only difference is the presence of a band at ~650 nm in the chemically reconstituted protein (**Fig. S5A**), which is indicative of the presence of some Fe^{3+} -containing aggregates, that were not fully removed by the PD-10 desalting column performed at the end of chemical reconstitution protocol.⁴² The CD-visible spectrum of chemically reconstituted ISCA1 showed both positive and negative bands with well-defined peaks accompanied by shoulders and inflections, which are typical of $[2\text{Fe-2S}]^{2+}$ protein-bound clusters (**Fig. S5B**).^{42, 45} The CD-visible spectrum of chemically reconstituted ISCA1 showed the same bands detected in anaerobically purified $[2\text{Fe-2S}]^{2+}$ ISCA1 (**Fig. S5B**) and is also very similar to CD-visible spectrum of the $[2\text{Fe-2S}]^{2+}$ form of *Azotobacter vinelandii* NifHscA.¹² No formation of a $[4\text{Fe-4S}]$ cluster-bound ISCA1 species was confirmed by the 1D ^1H paramagnetic NMR spectrum of chemically reconstituted ISCA1, which has indeed the same signals of anaerobically purified $[2\text{Fe-2S}]^{2+}$ ISCA1, which are typical of $[2\text{Fe-2S}]^{2+}$ protein-bound clusters (**Fig. S5D** and **S5E**). On the other hand, chemical reconstitution of the 1:1 ISCA1-ISCA2 heterodimer (see Materials and Methods for details) led to the formation of a $[4\text{Fe-4S}]$ cluster-bound, heterodimeric species ($[4\text{Fe-4S}]$ ISCA1-ISCA2, hereafter), as shown by paramagnetic ^1H 1D NMR and UV-visible/CD-visible spectra. The UV-visible spectrum of the chemically reconstituted ISCA1-ISCA2 complex differs from that of anaerobically purified ISCA1, as it shows only a broad band at 420 nm characteristic of Cys-bound $[4\text{Fe-4S}]$ clusters (**Fig. S5A**).^{42, 46} The CD-visible spectrum of the chemically reconstituted ISCA1-ISCA2 complex exhibits negligible CD intensity compared to the signals of anaerobically purified ISCA1 (**Fig. S5B**), consistent with $[4\text{Fe-4S}]$ cluster formation in the ISCA1-ISCA2 complex.⁴² In agreement with the latter conclusion, the CD-visible spectrum of

the chemically reconstituted ISCA1-ISCA2 complex is also very similar to that of the $[4\text{Fe-4S}]^{2+}$ form of *Azotobacter vinelandii* IscA and differs from that of the $[2\text{Fe-2S}]^{2+}$ form of *A. vinelandii* IscA.¹² The 1D ^1H paramagnetic NMR spectrum of $[4\text{Fe-4S}]$ ISCA1-ISCA2 (**Fig. 5A**) has down-field shifted signals originating from βCH_2 of the Cys ligands and is similar to that of homodimeric $[4\text{Fe-4S}]$ ISCA2 (previously reported in ¹⁴), while it differs from that of $[2\text{Fe-2S}]^{2+}$ cluster-bound species of both ISCA1 and ISCA2 (compare **Fig. 5A** with 1D ^1H paramagnetic NMR spectra of $[2\text{Fe-2S}]^{2+}$ ISCA1 and $[2\text{Fe-2S}]^{2+}$ ISCA2 reported in **Fig. S5D** and in Brancaccio *et al.*,¹⁴ respectively). In conclusion, all the spectroscopic data indicated that ISCA1 binds a $[4\text{Fe-4S}]$ cluster only when it is in the ISCA1-ISCA2 heterodimeric complex.

Analytical size exclusion chromatography of the $[4\text{Fe-4S}]$ ISCA1-ISCA2 complex showed the presence of a single peak containing both ISCA1 and ISCA2 proteins, as detected by SDS-PAGE (**Fig. S1**), and eluting at a volume very similar to that of the dimeric ISCA2 with no presence of monomeric ISCA1, consistent with the complete formation of a dimeric hetero-complex (**Fig. 5B**). The ^1H - ^{15}N HSQC spectra of the 1:1 ISCA1- ^{15}N ISCA2 mixture before and after chemical reconstitution showed that the chemical shifts of most of the backbone NH signals did not vary, consistent with ISCA2 being still complexed with ISCA1. However, the residues of ISCA2 surrounding the cluster binding cysteines (Cys 79, Cys 144 and Cys 146), such as Gly 76, Gly 77, Gly 78, Gly 81, Gly 143 and Gly 147, broaden beyond detection upon chemical reconstitution (in **Fig. 5C** compare Gly residues in red with those in blue), due to the binding of the paramagnetic $[4\text{Fe-4S}]$ cluster. This indicates that the C-X₆₄-C-G-C conserved sequence motif of ISCA2 is involved in $[4\text{Fe-4S}]$ cluster binding in the ISCA1-ISCA2 complex.

When the $[4\text{Fe-4S}]$ ISCA1- ^{15}N ISCA2 complex was stepwise titrated with apo ^{15}N NFU1 up to a 1:1 ratio, the backbone NH signals of ISCA2 did not show chemical shift changes, with the

exception of the residues close to the Cys cluster ligands, whose backbone NH signals, at the end of the titration, became detectable again in the ^1H - ^{15}N HSQC map at the chemical shift values of the apo ISCA1-ISCA2 complex (in **Fig. 5C** compare Gly residues in green with those in blue and red). On the contrary, many spectral changes (mostly line broadening effects) were observed for the backbone NH signals of the C-domain of NFU1, and the affected residues are located on the same interacting region mapped in the ISCA1-NFU1 and ISCA1-ISCA2-NFU1 apo complexes. Overall, these NMR data indicate that: i) NFU1 interacts with the [4Fe-4S] ISCA1-ISCA2 complex via its C-domain similarly to what found in the apo ternary complex, thus with ISCA1 in the complex with ISCA2 mediating the interaction with NFU1 and promoting the formation of a ternary complex; ii) the cluster is no longer bound to ISCA2, possibly being shared by ISCA1 and NFU1 in the ternary ISCA1-ISCA2-NFU1 complex.

To further support this model, [4Fe-4S] ISCA1- ^{15}N ISCA2 complex was titrated with a ^{15}N labelled construct of apo NFU1 comprising only the C-domain, up to the 1:1 ratio. The spectral changes observed in the ^1H - ^{15}N HSQC NMR map of the final mixture were the same as those observed in the titration with full-length NFU1, both on ISCA2 (no spectral changes observed as ISCA2 still remains complexed with ISCA1, **Fig. S6**) and on NFU1 (the observed changes map on the same region of C-domain of NFU1 interacting with ISCA1, as identified in the apo ISCA1-apo NFU1 1:1 mixture, compare inset of **Fig. S6** with **Fig. 3C**). These data corroborate the proposed model that ISCA1 promotes the formation of a ternary complex between NFU1, via its the C-domain, and [4Fe-4S] ISCA1-ISCA2.

The still remaining question to be addressed is which kind of cluster is present in the ISCA1-ISCA2-NFU1 ternary complex and its redox state as well as which among the three proteins coordinate it. The paramagnetic 1D ^1H NMR spectrum of the 1:1 mixture of [4Fe-4S] ISCA1-

ISCA2 and apo NFU1 showed the presence of two hyperfine-shifted signals at 19.3 and 12.9 ppm and two close signals at around 10.5 ppm (**Fig. 6A**), all having an anti-Curie (i.e. the chemical shift of hyperfine-shifted signals increases as the temperature is increased) temperature dependence (**Fig. S7**). The chemical shift values of these signals, their anti-Curie temperature dependence and their linewidths are typical of βCH_2 signals of Cys residues bound to a $[\text{4Fe-4S}]^{2+}$ cluster with an $S=0$ electronic ground state, with the paramagnetism arising from excited states of the electron spin ladder, whose population is increased with temperature.^{47, 48} In particular, the anti-Curie temperature dependence of the paramagnetic signals allows to establish that the cluster bound to the complex is in its oxidized state, because an anti-Curie temperature dependence is typically observed for the cysteine hyperfine-shifted resonances in oxidized $[\text{4Fe-4S}]^{2+}$ ferredoxins.⁴⁸⁻⁵¹ The paramagnetic 1D ^1H NMR spectrum of the 1:1 $[\text{4Fe-4S}]$ ISCA1-ISCA2/apo NFU1 mixture is completely different with respect to that of the $[\text{4Fe-4S}]$ ISCA1-ISCA2 complex while it is closer, although not superimposable, to that of dimeric $[\text{4Fe-4S}]^{2+}$ NFU1 (compare **Fig. 6A** with **Figs 6B** and **5A**). These comparisons indicated that the $[\text{4Fe-4S}]^{2+}$ cluster coordination in the ternary ISCA1-ISCA2-NFU1 complex differs from both of those observed in the ISCA1-ISCA2 complex and in dimeric NFU1, although it is more similar to the latter case. The ^1H - ^{15}N HSQC NMR data described above (**Fig. 5C**), showing that ISCA2 is not involved in cluster binding in the ternary complex, suggested that the $[\text{4Fe-4S}]^{2+}$ cluster in the ternary complex is bridged between NFU1 and ISCA1. In order to validate this model, the heterodimeric ISCA1-NFU1 complex, obtained by mixing anaerobically purified ISCA1 and apo ^{15}N NFU1, was chemically reconstituted. Its paramagnetic 1D ^1H NMR spectrum (**Fig. 6C**) showed the presence of the same anti-Curie, hyperfine-shifted signals observed when the $[\text{4Fe-4S}]^{2+}$ cluster is bound to the ternary ISCA1-ISCA2-NFU1 complex. This data provides strong

hints to the model that the $[4\text{Fe-4S}]^{2+}$ cluster is shared by ISCA1 and NFU1 in the ternary complex. In addition, the latter data showed that the ISCA1-NFU1 complex can bind the $[4\text{Fe-4S}]^{2+}$ cluster, independently of the presence of ISCA2. To provide conclusive proofs that the $[4\text{Fe-4S}]^{2+}$ cluster is shared by ISCA1 and NFU1 in the ternary complex, 1D ^1H paramagnetic NMR data were acquired on $[4\text{Fe-4S}]^{2+} \text{ }^{15}\text{N}$ NFU1 titrated with apo ISCA1 (**Fig. 6D**). Once the 1:1 $[4\text{Fe-4S}]^{2+}$ NFU1-apo ISCA1 ratio was reached, the 1D ^1H paramagnetic NMR spectrum displayed the same hyperfine-shifted signals observed when the $[4\text{Fe-4S}]^{2+}$ cluster is bound to the ternary ISCA1-ISCA2-NFU1 complex (compare **Fig. 6A** with **Fig. 6D**), conclusively proving that the $[4\text{Fe-4S}]^{2+}$ cluster is shared by ISCA1 and NFU1 in the ternary ISCA1-ISCA2-NFU1 complex.

DISCUSSION

The late-acting steps of the maturation of mitochondrial [4Fe-4S] proteins, consisting of the assembly and insertion of [4Fe-4S] clusters into mitochondrial apo proteins, are not yet fully understood. The available genetic, biochemical and proteomic data do not yet provide a definitive picture on how the proteins that are essential for the assembly and insertion of the [4Fe-4S] clusters into the final proteins operate. Depending on the type of human cells or of the eukaryotic organisms, the genetic data pointed out at different mechanisms where either ISCA1 only,¹⁹ or both ISCA1 and ISCA2,^{3, 4, 52} are essential for the mitochondrial [4Fe-4S] protein biogenesis *in vivo* (**Fig. 1**). Recently, an *in vitro* biochemical study mimicking physiological conditions strongly supported the model that both human ISCA1 and ISCA2, with the assistance of IBA57, are required to assemble a [4Fe-4S] cluster.²¹ A significant discrepancy was also observed in the proteomic data. Indeed, human NFU1 was found to interact with ISCA1, but not with ISCA2,¹⁹ while yeast NFU1 interacts with both yeast ISCA1 and ISCA2.³⁵

The here presented NMR-based study provides a molecular model for the succession of events orchestrated by ISCA1, ISCA2 and NFU1 to make [4Fe-4S] clusters available to mitochondrial apo proteins. We have shown that ISCA1 is the key player, being able to interact either individually with ISCA2 or NFU1 or with both proteins, promoting the formation of a transient ISCA1-ISCA2-NFU1 ternary complex. ISCA1 works as the promoter of the interaction between ISCA2 and NFU1 that otherwise do not interact each other. The interaction between ISCA1 and NFU1 involves the C-domain of NFU1, while the N-domain of NFU1 does not take part to the protein-protein recognition. Specifically, the two helices of the C-domain of NFU1 encasing the cluster binding CXXC motif are involved in the recognition with ISCA1. We also found that the [4Fe-4S] cluster bound form of the ISCA1-ISCA2 complex, which is the physiological

mitochondrial [4Fe-4S] cluster assembler,^{14, 20, 21} interacts with apo NFU1. Upon this interaction, the cluster is not transferred from the ISCA1-ISCA2 complex to NFU1 to form dimeric [4Fe-4S]²⁺ NFU1. On the contrary, a ternary ISCA1-ISCA2-NFU1 complex, the same formed by the interaction among the three apo proteins, is formed. In the ternary complex, the cluster is transferred from a bridged ISCA1-ISCA2 coordination to a bridged ISCA1-NFU1 coordination, thus leaving ISCA2 no more involved in cluster binding. The same cluster coordination mode is also present in a ISCA1-NFU1 complex isolated in different ways (**Figs 6C and 6D**), suggesting that ISCA2 might not be required in the following step of cluster release to the mitochondrial [4Fe-4S] proteins. On the basis of these overall findings, the pathway of the maturation of mitochondrial [4Fe-4S] proteins (**Fig. 7**) can thus be outlined as it follows: i) the [4Fe-4S]²⁺ cluster is assembled on the ISCA1-ISCA2 heterodimer by reductively coupling two [2Fe-2S]²⁺ clusters donated by GLRX5, being the two required electrons provided by FDX2;^{14, 20, 21} ii) [4Fe-4S]²⁺ ISCA1-ISCA2 complex can transfer the cluster to proteins that do not require NFU1 for their maturation, such as aconitase;^{6, 7, 21} iii) ISCA1, through its specific recognition with the C-domain of NFU1, mediates the [4Fe-4S]²⁺ cluster transfer from the ISCA1-ISCA2 cluster binding site to the ISCA1-NFU1 cluster binding site upon the formation of a transient, ternary ISCA1-ISCA2-NFU1 complex; iv) the latter complex, coordinating the [4Fe-4S]²⁺ cluster via ISCA1 and NFU1, specifically directs the [4Fe-4S]²⁺ cluster to mitochondrial proteins that require NFU1 for their maturation, that are the respiratory complexes I and II and the radical SAM protein lipoyl synthase;^{6, 7, 53-55} v) upon ISCA2 release from the ternary complex (consistent with the transient nature of the ternary complex which might then be a transient intermediate in the pathway), the [4Fe-4S]²⁺ NFU1-ISCA1 adduct can also be able to transfer the [4Fe-4S]²⁺ cluster to mitochondrial proteins. Along this pathway (**Fig. 7**), the cluster can be

safely moved from where it is assembled on the ISCA1-ISCA2 complex towards the final proteins in a selective manner, with no risk of being released free in the mitochondrial matrix where it might damage the cellular environment. This pathway is in agreement with the *in vivo* proteomics data showing that ISCA2 interacts with ISCA1, but not with NFU1, and that ISCA1 interacts with both ISCA2 and NFU1 proteins.¹⁹ In conclusion, our results support the physiological relevance of the ISCA1-ISCA2-NFU1 pathway shown in **Figure 1A**. We have indeed defined for the first time the molecular function of the *in vivo*-detected ISCA1-ISCA2-NFU1 interactions: the ISCA1-ISCA2 interaction produces a complex, which, in its [4Fe-4S] bound form, is able to interact with apo NFU1 to form a ternary complex where the cluster is shared by ISCA1 and NFU1 to be ready for being inserted into mitochondrial proteins requiring NFU1 for their maturation.

MATERIALS AND METHODS

Protein expression and purification

pDONR221 plasmid containing full-length ISCA1 gene (UniProt: Q9BUE6) was purchased by Genscript. Gateway cloning technology (Invitrogen) was then applied to clone full-length ISCA1 gene into pDEST-HisMBP plasmid to express N-terminal 6His-tag-MBP-ISCA1 protein. The latter plasmid was transformed in *Escherichia coli* BL21-Gold(DE3) (Agilent) competent cells. Cells were cultivated at 37°C in LB media adding ampicillin (100 µg/mL), 4 mL of Solution Q (40 mM HCl, 50 mg/l FeCl₂ 4H₂O, 184 mg/l CaCl₂ 2H₂O, 64 mg/l H₃BO₃, 18 mg/l CoCl₂ 6H₂O, 4 mg/l CuCl₂ 2H₂O, 340 mg/l ZnCl₂, 355 mg/l Na₂MoO₄ 2H₂O, 40 mg/l MnCl₂ 4H₂O) and 250 µM of FeCl₃ per liter of sterile LB until OD₆₀₀ reached 0.8-1. Protein expression was induced with 0.2 mM IPTG at 18°C for 16 hours. The cells were harvested by centrifugation at 5000 rpm for 20 min (JA-10, Beckman Coulter) and then resuspended in binding buffer (50 mM Tris-HCl, 500 mM NaCl, 15 mM Imidazole, pH 8.0). The cells were lysed by sonication at 4 °C. The N-terminal 6His-tag-MBP-ISCA1 protein was purified from the lysate using a HisTrap HP column (GE Healthcare) and then the 6His-MBP tag was cleaved by tobacco etch virus protease treatment over-night at room temperature in binding buffer. HisTrap column followed by amylose resin column were performed to separate the digested ISCA1 from MBP-6His tag and from undigested 6His-tag-MBP-ISCA1 protein. The final yield of ISCA1 was ~15 mg/L of LB culture. Apo ISCA1 was obtained performing all the purification steps under aerobic conditions, while a mixture of apo and [2Fe-2S]²⁺ ISCA1 was purified under anaerobic conditions (anaerobically purified ISCA1). The anaerobically purified ISCA1 protein was characterized by UV- and CD-visible absorption and 1D ¹H paramagnetic NMR spectroscopies, analytical size exclusion chromatography (**Fig. S5**), and iron and acid-labile sulfide content. Protein quantification was carried out with the Bradford

protein assay, using BSA as a standard. Non-heme iron and acid-labile sulfide content was determined as described previously.⁵⁶ In the UV-visible absorption spectrum of anaerobically purified ISCA1, the extinction coefficients at 325 and 420 nm (ranging from 4200 to 5700 and from 2100 to 3900 M⁻¹ cm⁻¹ depending on protein preparation, respectively) are far from the lower end of the range of values that are considered typical for [2Fe-2S] clusters (11000 and 8000 M⁻¹ cm⁻¹, respectively⁵⁷), indicating a partial [2Fe-2S] cluster occupancy in the homodimer. Iron and acid-labile sulfide analyses of anaerobically purified ISCA1 samples indicate ~0.3 [2Fe-2S]²⁺ cluster per homodimer (Fe, 0.6 ± 0.1; acid-labile S, 0.5 ± 0.1 per mol of homodimer; Fe and S measurements are the averages of three independent samples).

The production of human ISCA2, and of full-length and C-domain NFU1 in their apo and Fe-S cluster-bound forms were obtained as previously described in literature.^{13, 33} As previously reported,¹⁴ the purification under anaerobic conditions of ISCA2 resulted in a dimer, which is composed by a mixture of apo and [2Fe-2S]²⁺ cluster-bound dimeric species with a [2Fe-2S]²⁺ cluster occupancy of 0.1-0.2 cluster per homodimer.

Production of Fe-S cluster-bound species

A 1:1 mixture of ISCA1 and ISCA2 was produced by stepwise titrating anaerobically purified ¹⁵N ISCA2 with anaerobically purified ISCA1 and monitoring changes in the ¹H-¹⁵N HSQC NMR maps. Chemical shift changes occur in a slow exchange regime on the NMR time scale, which were completed once the 1:1 protein ratio was reached, indicating that an ISCA1-ISCA2 hetero-complex is fully formed at this stoichiometric ratio. The ISCA1-ISCA2 complex was then chemically reconstituted to obtain the [4Fe-4S] cluster-bound hetero-complex. Chemical

reconstitution was anaerobically performed in 50 mM Tris-HCl, 100 mM NaCl and 5 mM DTT buffer at pH 8.0 adding eight equivalents of FeCl₃ and Na₂S to a protein solution of ~40-80 μM. The reaction was incubated for 16 h at room temperature. Anaerobic conditions were obtained performing the chemical reconstitution in glove-box (MBraun Labstar 130) with less than 2 ppm of oxygen and by using all buffers degassed. The excess of FeCl₃ and Na₂S as well as Fe-S aggregates/precipitates were removed by PD-10 desalting column following a widely used methodology applied in Fe-S protein reconstitution protocols.⁴² Anaerobically purified ISCA1 and a 1:1 mixture of anaerobically purified ISCA1 and apo NFU1 were chemically reconstituted following the same procedure. This led to dimeric [2Fe-2S]²⁺ and dimeric [4Fe-4S]²⁺ cluster-bound species, respectively. Iron and acid-labile sulfide analyses indicated an Fe:S²⁻ ratio close to 1:1 for both chemically reconstituted ISCA1-ISCA2 and ISCA1-NFU1 complexes. Moreover, the analytical data on three independent preparations of both latter complexes indicated a 3.5 ± 0.1 Fe and 3.7 ± 0.1 S²⁻ content per heterodimer, consistent with the presence of ~0.9 [4Fe-4S] cluster per heterodimer. Dimeric [4Fe-4S]²⁺ NFU1 with 90% of cluster loading was produced following the protocol previously reported.³³

Analytical size exclusion chromatography and SEC-MALS

For analytical size exclusion chromatography analysis, purified samples were loaded on a SuperdexTM 200 Increase 10/300 GL column attached to an AKTA pure chromatography unit with a continuous flow rate of 0.65 mL/min. The column was calibrated with gel filtration marker calibration kit, 6500–66000 Da (Sigma-Aldrich), to obtain the apparent molecular masses of the detected species. The column was equilibrated with degassed phosphate buffer 50 mM, 150 mM NaCl, 5 mM DTT and pH 7.0. SEC-MALS data were acquired by attaching a SuperdexTM 200

Increase 10/300 GL column to a DAWN HELEOS system with a continuous flow rate of 0.6 mL/min using a filtered buffer (50 mM, 150 mM NaCl, 5 mM DTT and pH 7.0). Each experiment was successfully repeated at least three times.

UV- and CD-visible spectroscopy

UV- and CD-visible spectra were performed to characterize the cluster bound to anaerobically purified and chemically reconstituted ISCA1 as well as chemically reconstituted ISCA1-ISCA2 heterodimeric complex. The experiments were performed under anaerobic conditions, preparing the samples in glove-box with less than 5 ppm of oxygen with degassed buffers and using a gas-tight cuvette. UV- and CD-visible spectra were performed at room temperature (25 °C) in 50 mM phosphate buffer, 150 mM NaCl, 5 mM DTT and pH 7.0 on a Cary 50 Eclipse spectrophotometer and JASCO J-810 circular dichroism spectropolarimeter, respectively. Each experiment was successfully repeated three times.

NMR spectroscopy

^1H - ^{15}N HSQC spectra were acquired at 298 K in 50 mM phosphate buffer, 150 mM NaCl, 5 mM DTT pH 7.0, 10% (v/v) D_2O . All NMR spectra were recorded on Bruker AVANCE 700, 900 and 950 MHz, processed using the standard Bruker software (Topspin) and analyzed with CARA program⁵⁸. To monitor the possible interaction among ISCA1, ISCA2 and NFU1 in their apo and Fe-S cluster-bound states, ^{15}N labeled protein(s) were stepwise titrated in anaerobic conditions with increasing amounts of unlabeled or ^{15}N -labelled protein(s). Chemical shifts of the backbone NHs observed in the ^1H ^{15}N HSQC spectra along the additions of the unlabeled or ^{15}N -labelled

protein(s) were compared with chemical shifts of ^{15}N -labelled protein(s) in the initial state(s). The observed chemical shift changes were reported as backbone weighted average chemical shift differences $\Delta\delta_{\text{avg}}(\text{HN})$, i.e. $((\Delta\text{H})^2 + (\Delta\text{N}/5)^2/2)^{1/2}$, where ΔH and ΔN are chemical shift differences for backbone amide ^1H and ^{15}N nuclei, respectively. Chemical shift assignment of full-length NFU1 and of the C-domain of NFU1 were available in the Biological Magnetic Resonance Bank (under accession codes BMRB: 26801 [full-length NFU1] and BMRB: 19068 [NFU1 C-domain]).³¹ Chemical shift assignment of apo ISCA2 was also already available.¹⁴ Each titration was successfully repeated three times.

1D ^1H paramagnetic NMR experiments were acquired at 400 MHz with a ^1H optimized 5 mm probe at temperatures ranging from 283 K and 298 K, with protein samples in 50 mM phosphate buffer, 150 mM NaCl, 5 mM DTT pH 7.0, 99% (v/v) D_2O . The protein concentration was 0.5-0.8 mM. Water signal was suppressed via fast repetition experiments and water selective irradiation.⁵⁹

⁶⁰ Experiments were typically performed using an overall recycle delay of 90 ms. Squared cosine and exponential multiplications were applied prior to Fourier transformation.⁶¹ Manual baseline correction was performed, using polynomial functions. Each experiment was successfully repeated three times.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Dafne Suraci: Investigation, Methodology, Validation, Visualization. **Giovanni Saudino:** Investigation, Methodology, Validation, Visualization. **Veronica Nasta:** Investigation, Validation. **Simone Ciofi-Baffoni:** Conceptualization, Supervision, Writing - Original Draft, Writing - Review & Editing. **Lucia Banci:** Conceptualization, Writing - Review & Editing, Project administration, Funding acquisition.

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Figure 1. Proposed models of [4Fe-4S] cluster assembly and transfer to mitochondrial targets. (A) The ISCA1-dependent pathway requires ISCA1 and ISCA2 to assemble a [4Fe-4S]²⁺ on the ISCA1-ISCA2 complex that received two [2Fe-2S]²⁺ clusters from GLRX5 and two electrons (2e⁻) from ferredoxin FDX2.^{14, 21} IBA57 is required to activate this process, but, since its molecular role is still undefined, it was not shown in the figure. Then, the [4Fe-4S]²⁺ ISCA1-ISCA2 complex was proposed to transfer the cluster to specific protein targets with the assistance of NFU1.^{7, 35} (B) In the ISCA1-dependent pathway, two [2Fe-2S] clusters, each ligated by ISCA1 and ISCU2, are transferred to dimeric NFU1 and assembled into a [4Fe-4S]²⁺ cluster by a reductive coupling mediated by FDX2, which provide two electrons (2e⁻).³⁸ Then, a complex formed by dimeric NFU1, monomeric ISCA1 and ISCU2 has been proposed to transfer the assembled [4Fe-4S]²⁺ cluster to specific protein targets.

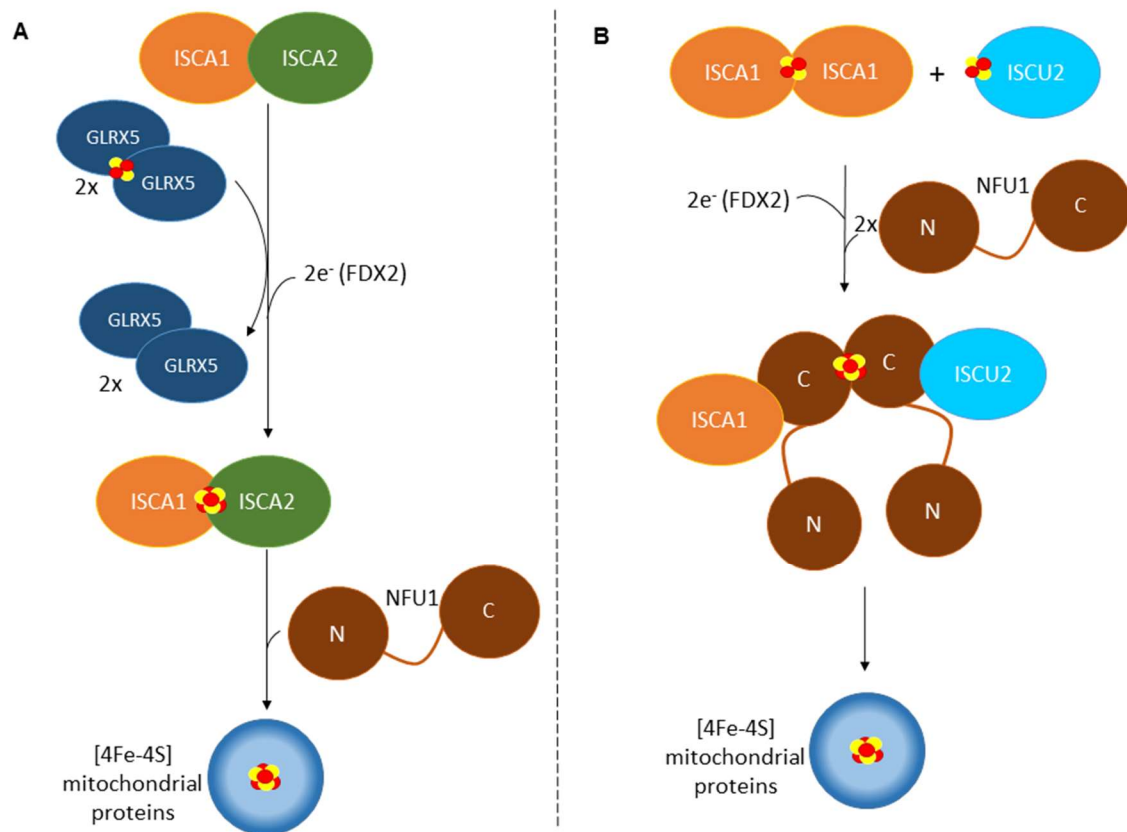


Figure 2. Apo ISCA2 interacts with apo ISCA1 and not with apo NFU1. A schematic cartoon depicting the NMR titration related to each panel is reported. (A) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 apo ^{15}N ISCA2-apo ^{15}N NFU1 mixture (red) with the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N ISCA2 (cyan) and on isolated apo ^{15}N NFU1 (black). (B) Analytical size exclusion chromatography of apo ISCA2 (cyan), apo NFU1 (black) and a 1:1 apo ISCA2-apo NFU1 mixture (red). (C) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 apo ^{15}N ISCA2-apo ISCA1 mixture (red) with the ^1H - ^{15}N HSQC spectrum acquired on apo ^{15}N ISCA2 (black). In the inset, overlays of three different regions of ^1H - ^{15}N HSQC spectra, acquired on isolated apo ^{15}N ISCA2 (black) and on apo ^{15}N ISCA2-apo ISCA1 mixtures at 1:1 (red) and 1:0.5 (green) ratios, are shown. (D) Analytical size exclusion chromatography of apo ISCA2 (black), apo ISCA1 (violet) and a 1:1 apo ISCA2-apo ISCA1 mixture (red).

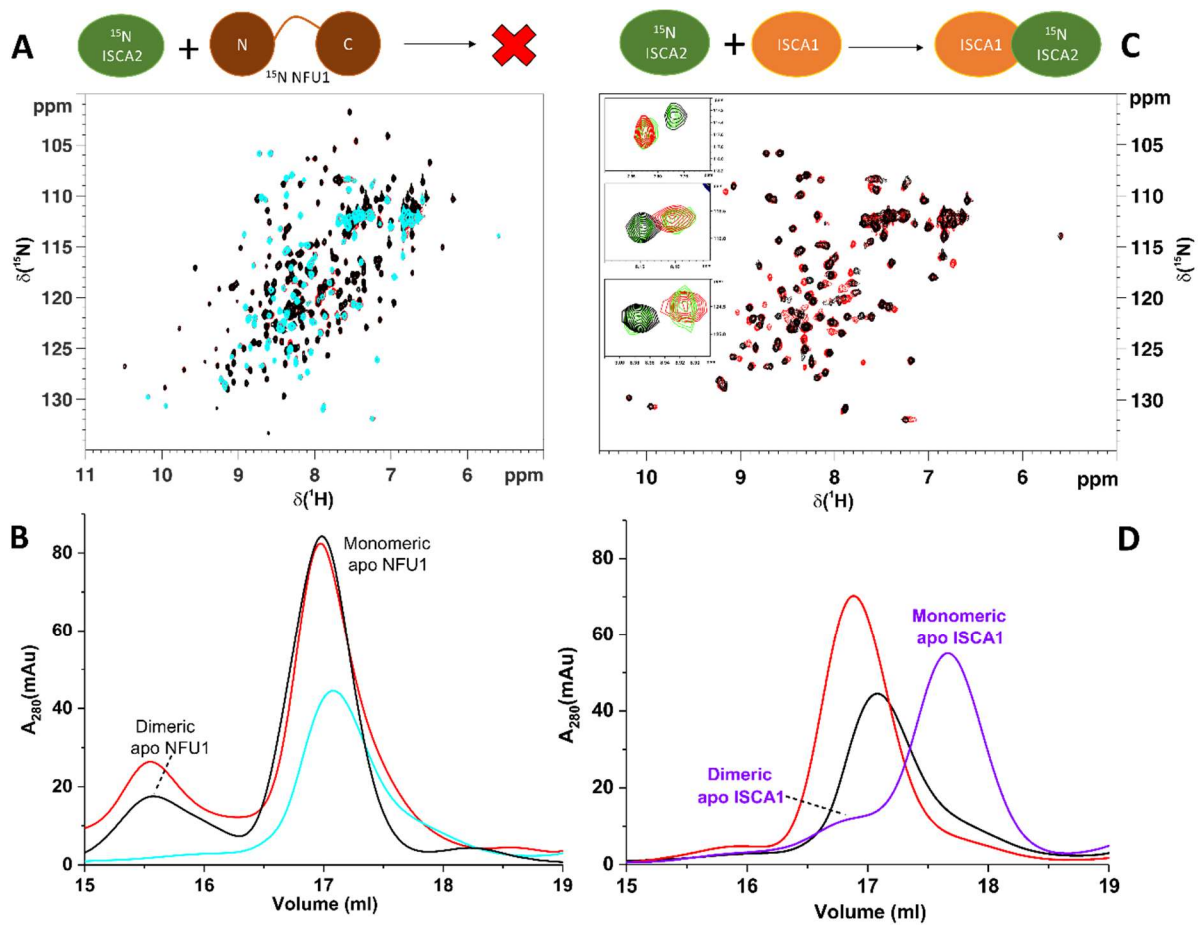


Figure 3. Apo ISCA1 interacts with the C-domain of apo NFU1. (A) A schematic cartoon depicting the NMR titration related to this panel is reported. Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 apo ^{15}N NFU1-apo ISCA1 mixture (red) with the ^1H - ^{15}N HSQC spectrum acquired on apo ^{15}N NFU1 (black). In the inset, overlay of a region of the ^1H - ^{15}N HSQC spectra of mixtures obtained by adding apo ISCA1 at increasing concentration to apo ^{15}N NFU1. (B) Analytical size exclusion chromatography of apo ISCA1 (violet), apo NFU1 (black) and a 1:1 apo NFU1-apo ISCA1 mixture (red). (C) Meaningful chemical shift changes for the backbone NHs of apo ^{15}N NFU1 upon the addition of 1 equivalent of apo ISCA1 are mapped on a structural model of monomeric apo NFU1 in a closed conformation.³³ In orange are the sidechains of the solvent exposed (>50%) residues showing chemical shift changes (line broadening beyond detection effects and/or meaningful chemical shift changes). The protein stretches in blue represent residues showing chemical shift changes but with a solvent exposure below 50%. The sidechains of the proline residues are shown in gray.

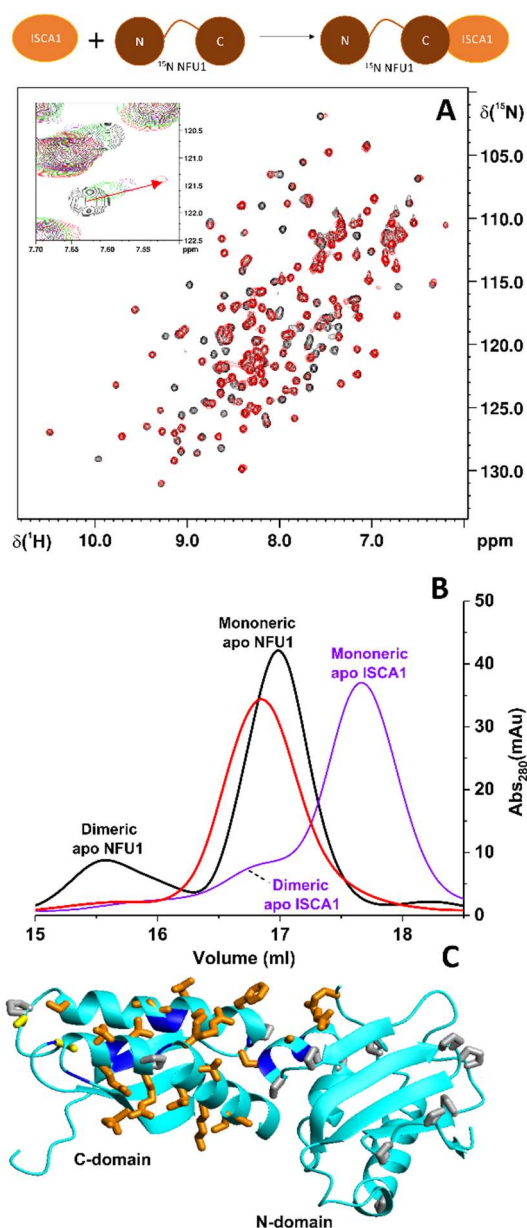


Figure 4. Apo ISCA1-ISCA2 heterodimeric complex interacts with the C-domain of apo NFU1. A schematic cartoon depicting the NMR titration related to each panel is reported. (A) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a mixture containing apo ^{15}N NFU1-ISCA1 complex and apo ISCA2 at 1:1 ratio (green) with the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N NFU1 (black) and on a 1:1 apo ^{15}N NFU1-apo ISCA1 complex (red). (B) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a mixture containing apo ^{15}N ISCA2-ISCA1 complex and apo ^{15}N NFU1 at 1:1 ratio (black) with the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 apo ^{15}N ISCA2-apo ISCA1 complex (red). (C) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a mixture containing apo ^{15}N ISCA2-ISCA1 complex and apo ^{15}N NFU1 at 1:1 ratio (black) with the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N NFU1 (red) and on a 1:1 apo ^{15}N NFU1-apo ISCA1 complex (green).

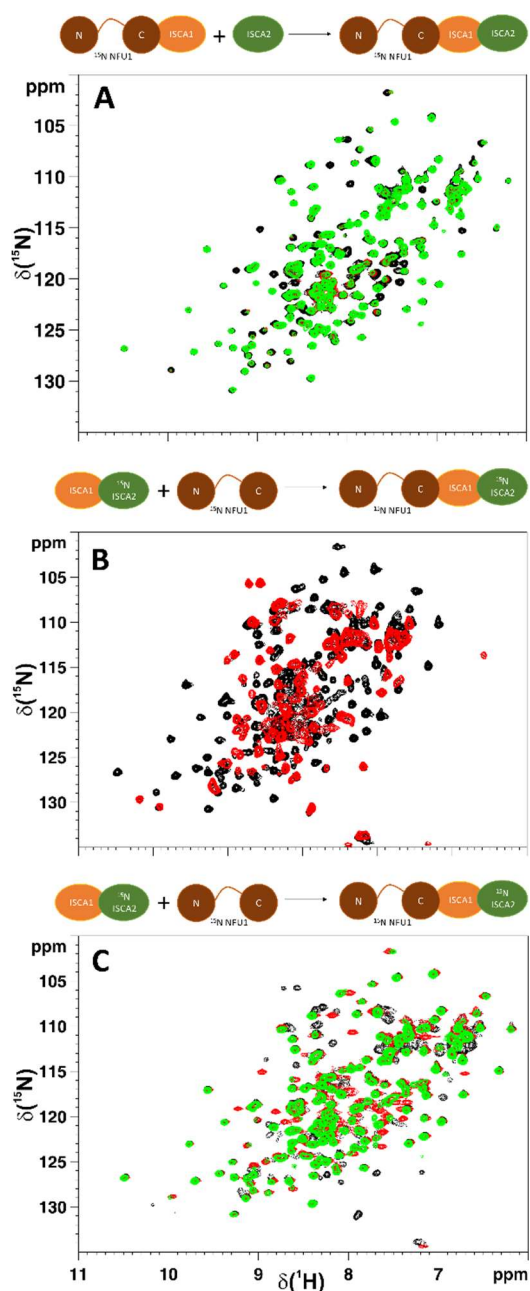


Figure 5. The ISCA1-ISCA2 heterodimer binds a [4Fe-4S] cluster. (A) 1D ^1H paramagnetic NMR spectrum at 283 K of [4Fe-4S] ISCA1-ISCA2, obtained by chemically reconstituting a 1:1 mixture of anaerobically purified ISCA1 and ISCA2. **(B)** Analytical size exclusion chromatography of [4Fe-4S] ISCA1-ISCA2 (red), apo ISCA2 (black) and apo ISCA1 (violet). **(C)** A schematic cartoon depicting the NMR titration related to this panel is reported. Overlay of the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N ISCA2 (black), on apo ^{15}N ISCA2-ISCA1 complex (red), on [4Fe-4S] ^{15}N ISCA2-ISCA1 complex (blue) and on a mixture containing [4Fe-4S] ^{15}N ISCA2-ISCA1 complex and apo ^{15}N NFU1 at 1:1 ratio (green). The backbone NH signals of ISCA2 residues (Gly 46, Gly 48, Ile 63, Gly 76, Gly 77, Gly 78, Gly 81 and Gly 143) are shown, and those of the N-domain, the linker and the C-domain of NFU1 are indicated as N-, L- and C-NFU1, respectively.

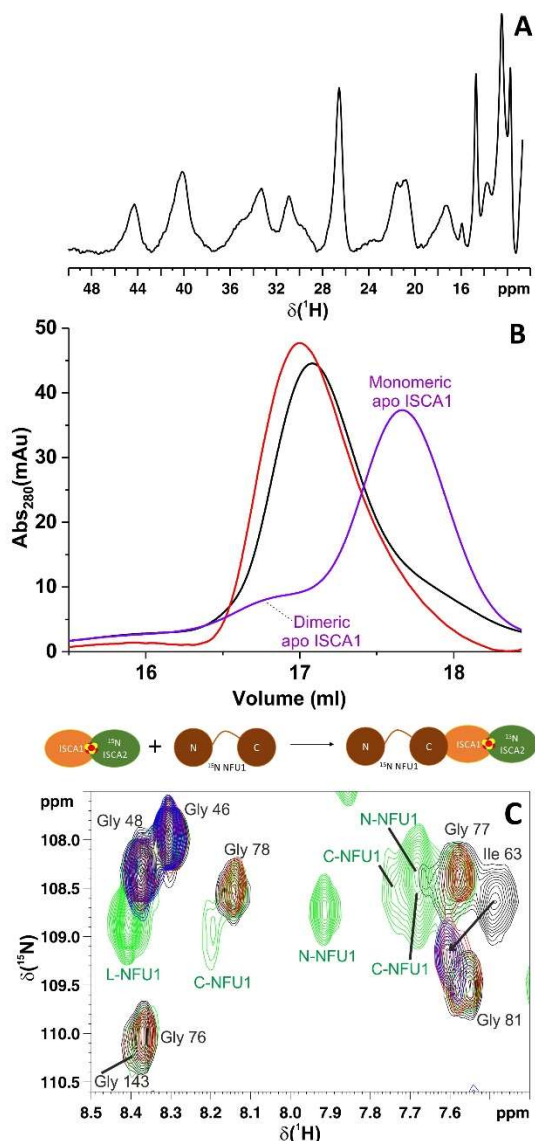


Figure 6. A $[4\text{Fe-4S}]^{2+}$ cluster is bound to the ternary ISCA1-ISCA2-NFU1 complex. 1D ^1H paramagnetic NMR spectra acquired on (A) a mixture containing $[4\text{Fe-4S}]$ ISCA1-ISCA2 complex and apo NFU1 at 1:1 ratio, (B) dimeric $[4\text{Fe-4S}]^{2+}$ NFU1, (C) a chemically reconstituted mixture of anaerobically purified ISCA1 and apo NFU1 at 1:1 ratio, (D) a 1:1 dimeric $[4\text{Fe-4S}]^{2+}$ NFU1-apo ISCA1 mixture. The arrows in panel C indicate signals with the same chemical shift values of $[4\text{Fe-4S}]^{2+}$ NFU1 (compare them with signals of panel B), indicating that upon the chemical reconstitution, a small fraction of dimeric $[4\text{Fe-4S}]^{2+}$ NFU1 is formed.

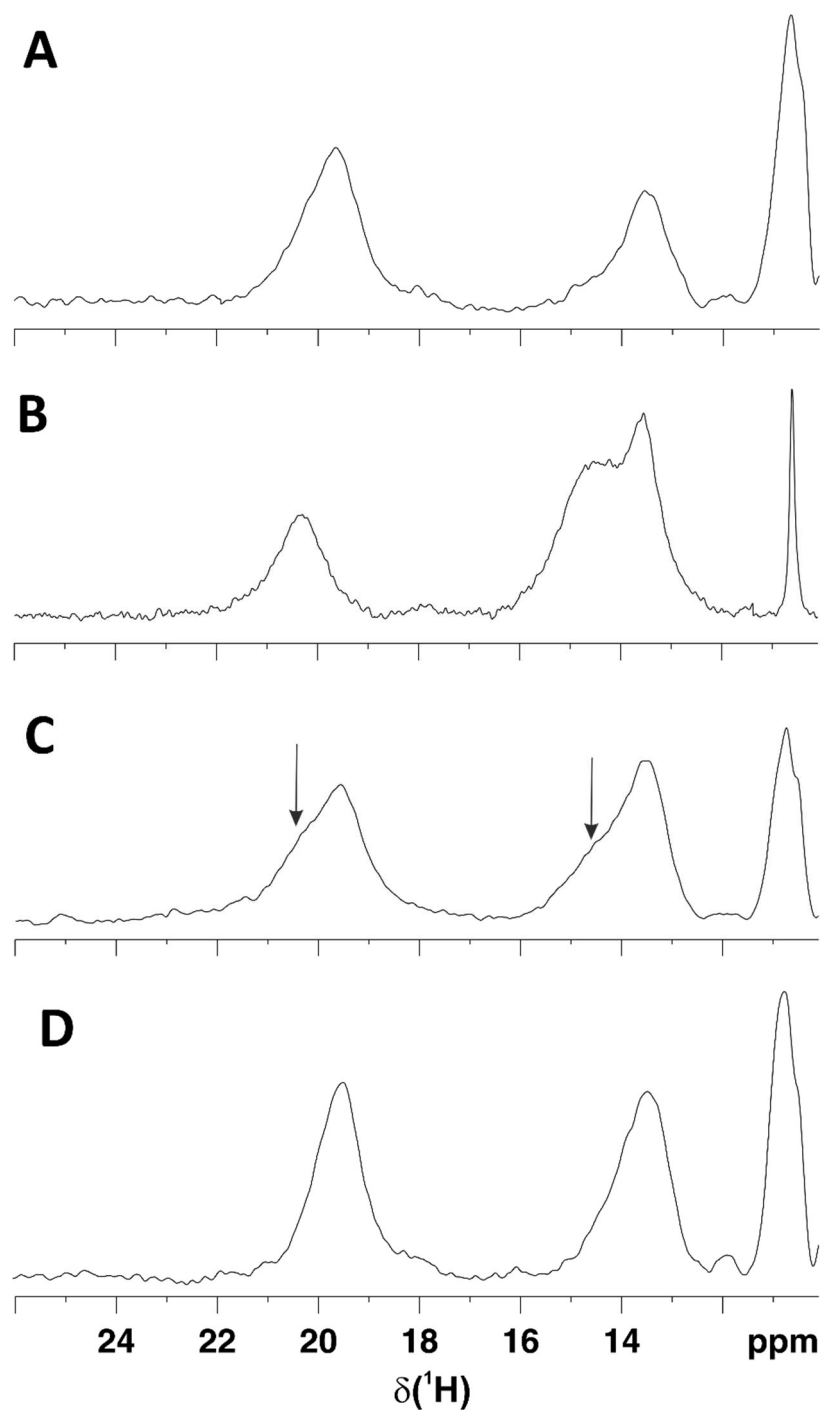
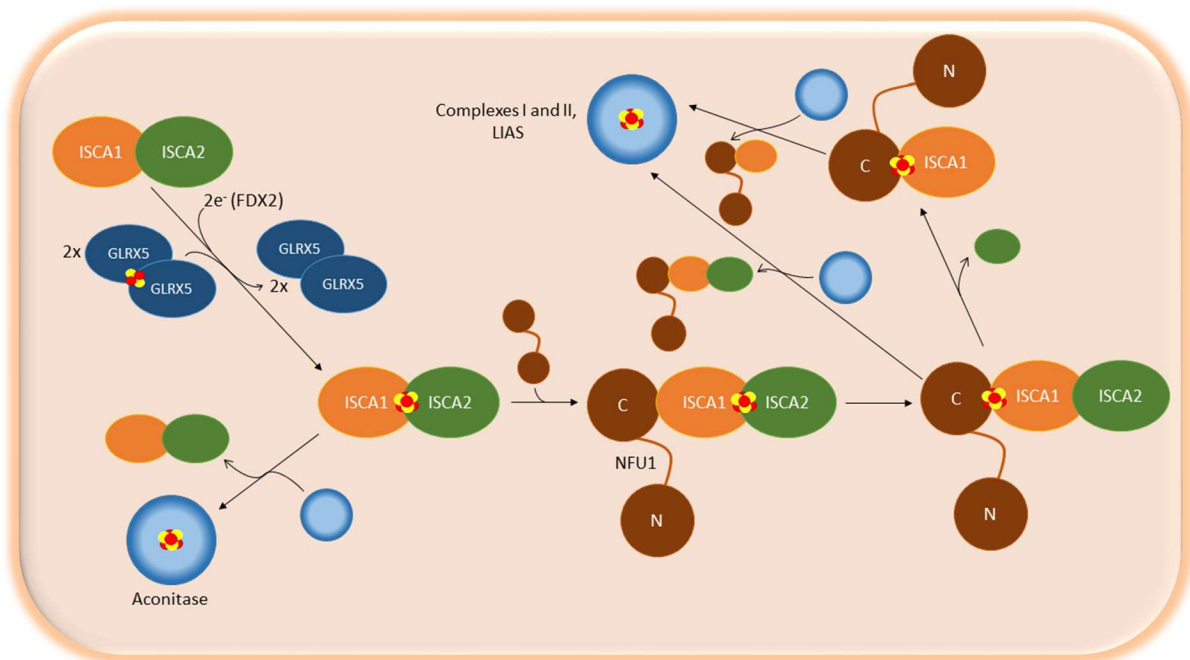


Figure 7. Iron-sulfur cluster assembly pathways for the maturation of mitochondrial [4Fe-4S] proteins. The ISCA-dependent pathway requires ISCA1 and ISCA2 to mature [4Fe-4S] mitochondrial proteins: 1) the ISCA1-ISCA2 complex receives two [2Fe-2S]²⁺ clusters from GLRX5¹⁴ and two electrons (2e⁻) from ferredoxin FDX2²¹ to assemble a [4Fe-4S]²⁺ cluster by means of IBA57, whose molecular role is still undefined and for this reason is not shown in the figure; 2) the [4Fe-4S]²⁺ ISCA1-ISCA2 complex can transfer the cluster to aconitase maturing it or can specifically interact with the C-domain of apo NFU1 forming a transient ternary [4Fe-4S]²⁺ NFU1-ISCA1-ISCA2 complex; 3) the [4Fe-4S]²⁺ cluster is moved within the ternary complex from a bridged ISCA1-ISCA2 coordination to a bridged ISCA1-NFU1 coordination; 4) the [4Fe-4S]²⁺ ISCA1-ISCA2-NFU1 complex might transfer the cluster to mature respiratory complexes I and II and lipoyl synthase (LIAS), with the help of other accessory proteins when required (not shown in the Figure); 5) upon ISCA2 release by the transient ternary complex, a [4Fe-4S]²⁺ NFU1-ISCA1 adduct is formed and it might another species directing the [4Fe-4S]²⁺ cluster to mitochondrial proteins.



Supplementary Materials

ISCA1 orchestrates ISCA2 and NFU1 in the maturation of mitochondrial [4Fe-4S] proteins

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Figure S1. SDS-PAGE of SEC peaks to identify the constituents of the protein complexes. 1. apo ISCA1; 2. apo NFU1; 3. diluted apo NFU1; 4. apo ISCA2; 5. peak of analytical size exclusion chromatography performed on [4Fe-4S] ISCA1-ISCA2 (red peak in Figure 5B); 6. peak of analytical size exclusion chromatography performed on a 1:1 apo NFU1-apo ISCA1 mixture (red peak in Figure 3B); 7. peak of analytical size exclusion chromatography performed on 1:1 apo ISCA2-apo ISCA1 mixture (red peak in Figure 2D).

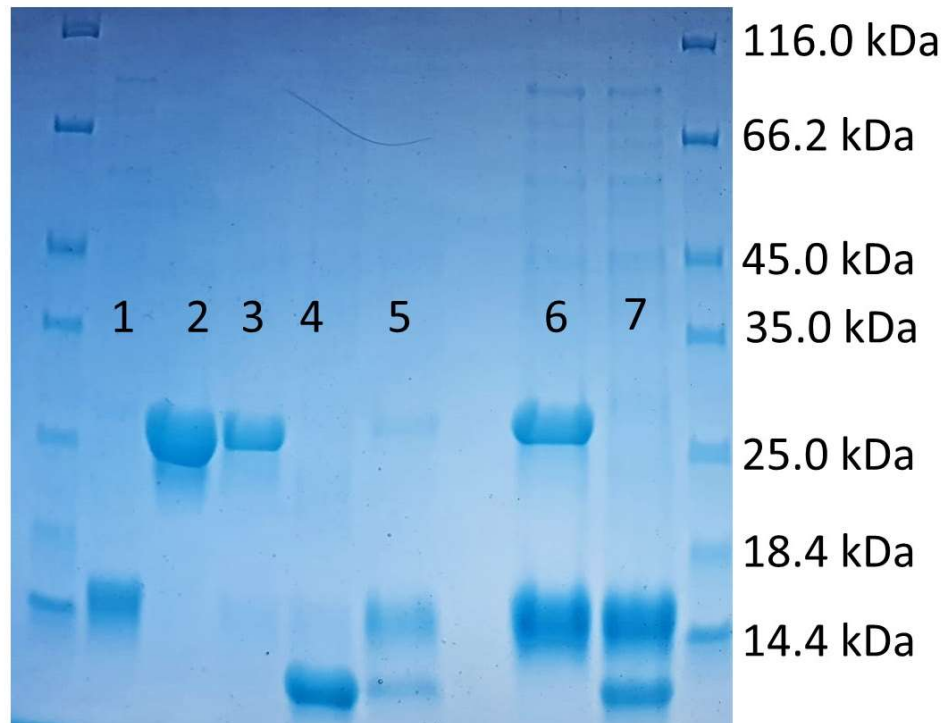


Figure S2. Apo ISCA1 and apo ISCA2 form a heterodimeric complex. Weighted-average chemical shift differences $\Delta\delta_{\text{avg}}$ (that is, $[(\delta_{\text{HN}})^2 + (\delta_{\text{N}}/5)^2]/2)^{1/2}$, where δ_{HN} and δ_{N} are chemical shift differences for ^1HN and ^{15}N , respectively) between apo ^{15}N -labelled ISCA2 alone and in a 1:1 mixture with unlabeled apo ISCA1. The cyan bars represent proline residues. The blue bars represent residues whose NHs are not detected or too broad to be analyzed. The dotted horizontal line indicates the threshold to consider chemical shift changes as meaningful ($1\sigma \Delta\delta_{\text{avg}} > 0.04$ ppm). In the inset, meaningful chemical shift changes for the backbone NHs of apo ^{15}N -labeled ISCA2 upon the addition of 1 equivalent of unlabeled apo ISCA1 are mapped on the structural model of monomeric ISCA2. In red are the sidechains of the residues showing meaningful chemical shift changes. The protein stretches in blue and cyan represent residues whose NHs are not detected or too broad to be analyzed and proline residues, respectively.

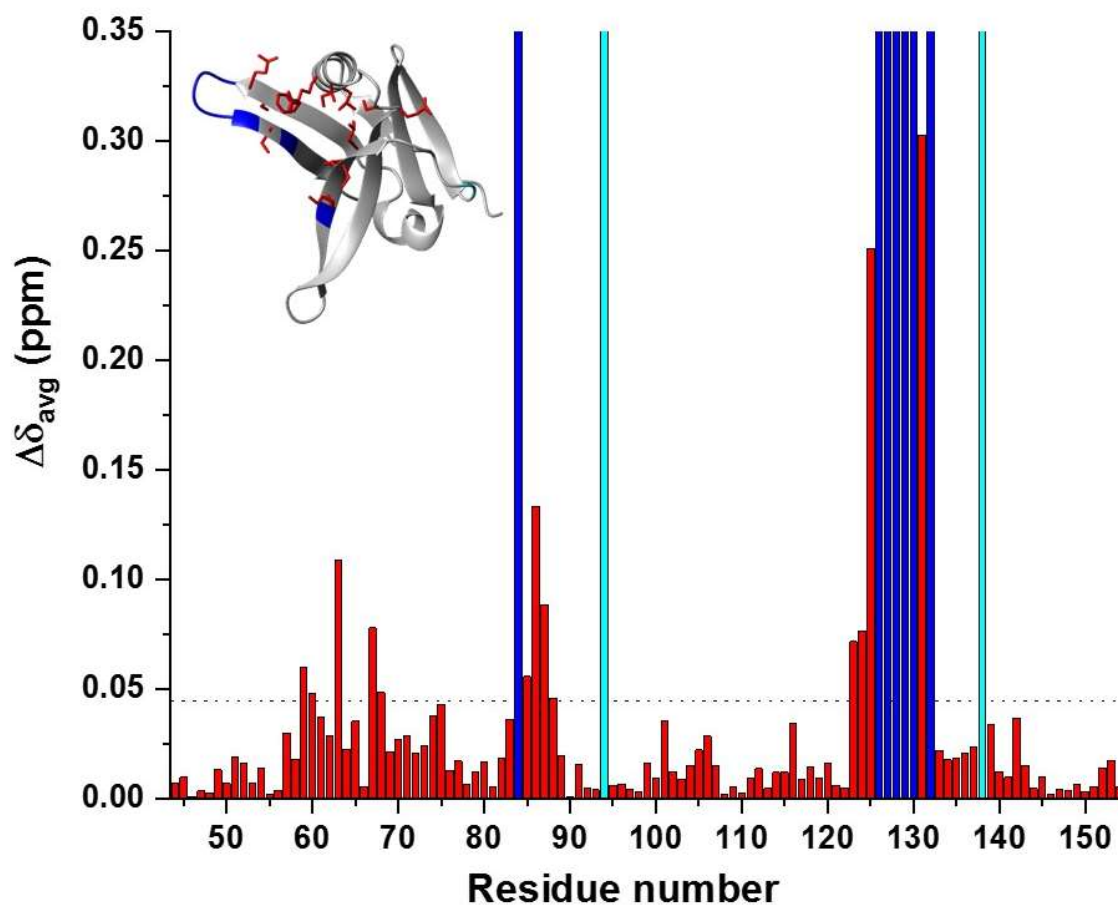


Figure S3. Apo ISCA1 interacts with the C-domain of apo NFU1 forming a heterodimeric complex. (A) Weighted-average chemical shift differences $\Delta\delta_{\text{avg}}$ (that is, $[(\delta_{\text{HN}})^2 + (\delta_{\text{N}}/5)^2]/2)^{1/2}$, where δ_{HN} and δ_{N} are chemical shift differences for ^1HN and ^{15}N , respectively) between apo ^{15}N NFU1 alone and in a 1:1 mixture with apo ISCA1. The cyan bars represent proline residues. The yellow bars represent residues whose NHs broaden beyond detection or are very broad upon additions of apo ISCA1. The dotted horizontal line indicates the threshold to consider chemical shift changes as meaningful ($1\sigma \Delta\delta_{\text{avg}} > 0.03$ ppm). (B) SEC-MALS of a 1:1 apo NFU1-apo ISCA1 mixture.

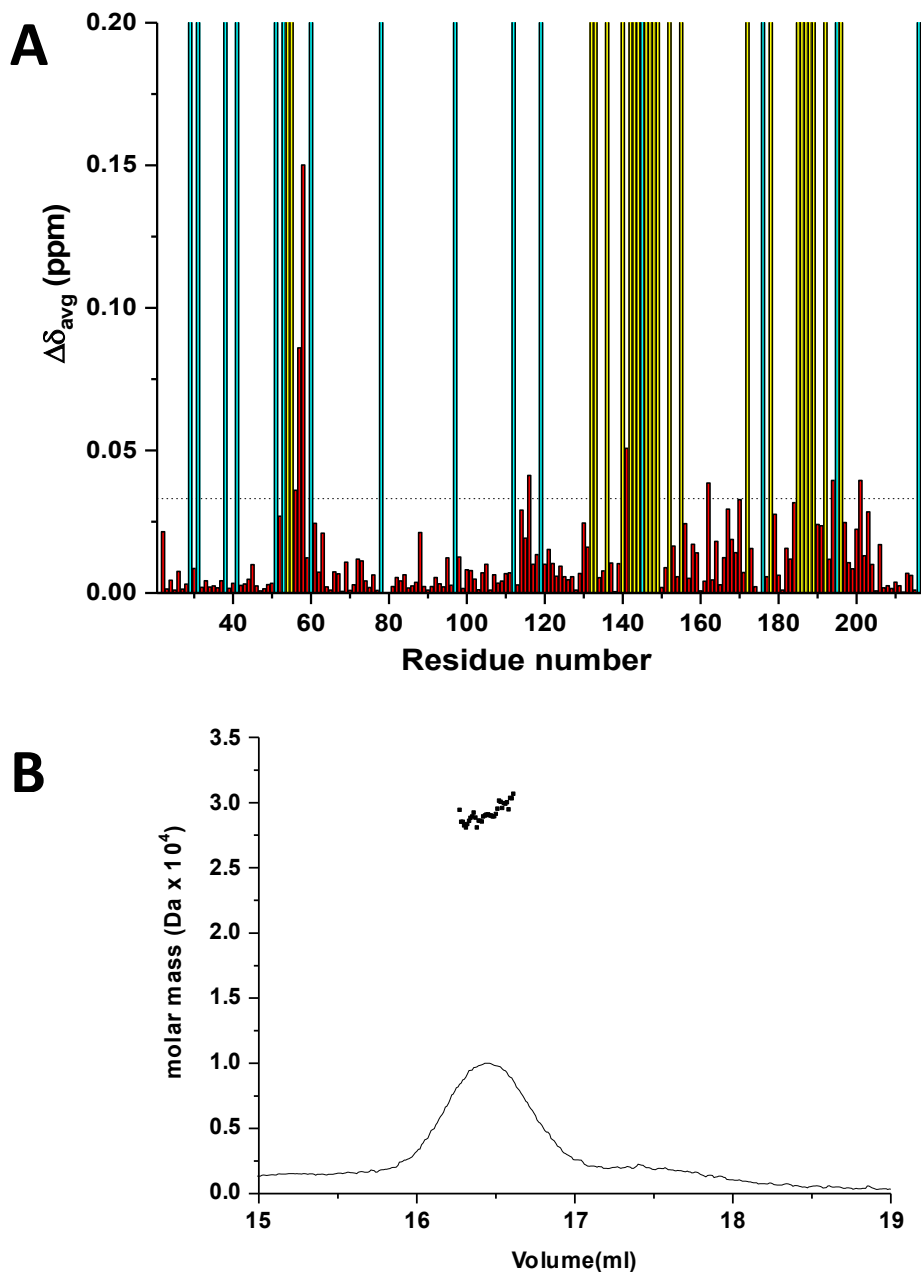


Figure S4. Apo ISCA1 promotes the formation of a ternary complex between apo ISCA2 and apo NFU1. A schematic cartoon depicting the NMR titration related to each panel is reported. **(A)** Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a 1:1:1 ^{15}N apo ISCA2-apo ^{15}N NFU1-apo ISCA1 mixture (black) with the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N NFU1 (red) and on a 1:1 apo ^{15}N NFU1 and apo ISCA1 mixture (green). **(B)** Overlay of the ^1H - ^{15}N HSQC spectrum acquired on a 1:1:1 apo ^{15}N ISCA2-apo ^{15}N NFU1-apo ISCA1 mixture (black) with the ^1H - ^{15}N HSQC spectra acquired on isolated apo ^{15}N ISCA2 (cyan) and on a 1:1 apo ^{15}N ISCA2-apo ISCA1 mixture (red). In the inset, an overlay of a region of the here overlapped ^1H - ^{15}N HSQC spectra is shown. The arrow indicates a backbone NH signal of ISCA2 that monitors the formation of ISCA2 complexed with ISCA1 at the 1:1:1 mixture. The ^1H - ^{15}N HSQC spectrum of the 1:1 apo ^{15}N NFU1-apo ISCA1 mixture is shown in green to identify in the inset the NH signals of NFU1 complexed with ISCA1.

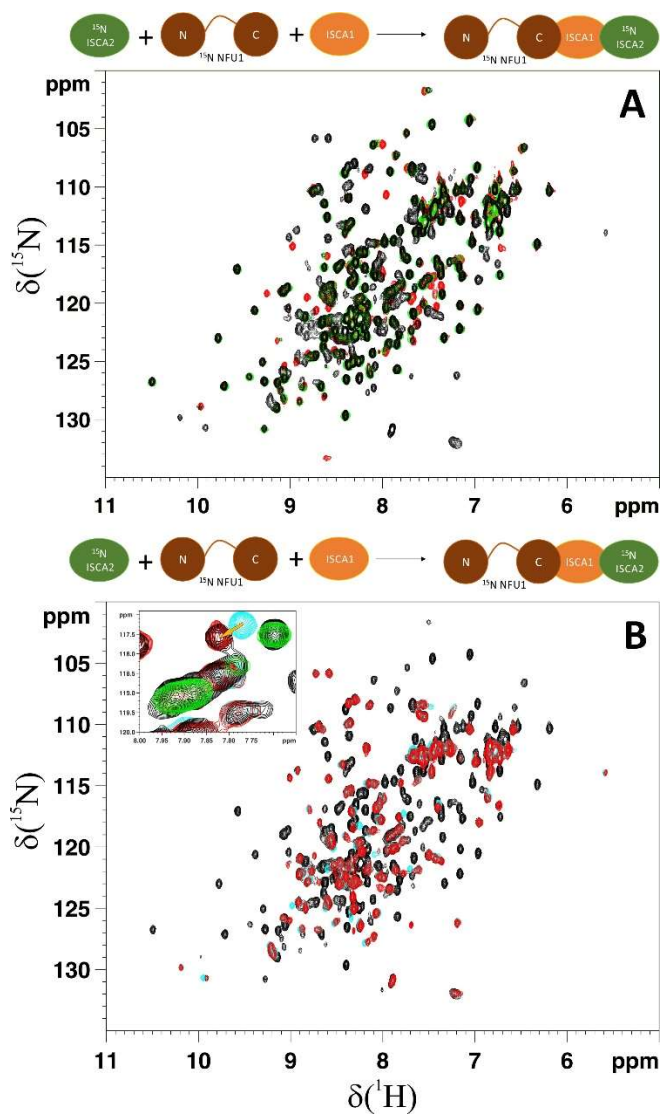


Figure S5. Dimeric ISCA1 binds $[2\text{Fe-2S}]^{2+}$ cluster. UV-visible (A) and CD-visible (B) absorption spectra of “as purified” (black), chemically reconstituted (red) ISCA1 and chemically reconstituted 1:1 ISCA1-ISCA2 mixture (green). (C) Analytical gel filtration of “as purified” ISCA1. Paramagnetic 1D ^1H NMR spectra of “as purified” (D) and chemically reconstituted (E) ISCA1 at 298 K. The signal-to-noise ratio of the NMR spectrum is worse in (E) than in (D) because the protein largely precipitates during the acquisition time of the NMR experiment in (E). All data were recorded in 50 mM phosphate buffer pH 7.0, 150 mM NaCl, 5 mM DTT.

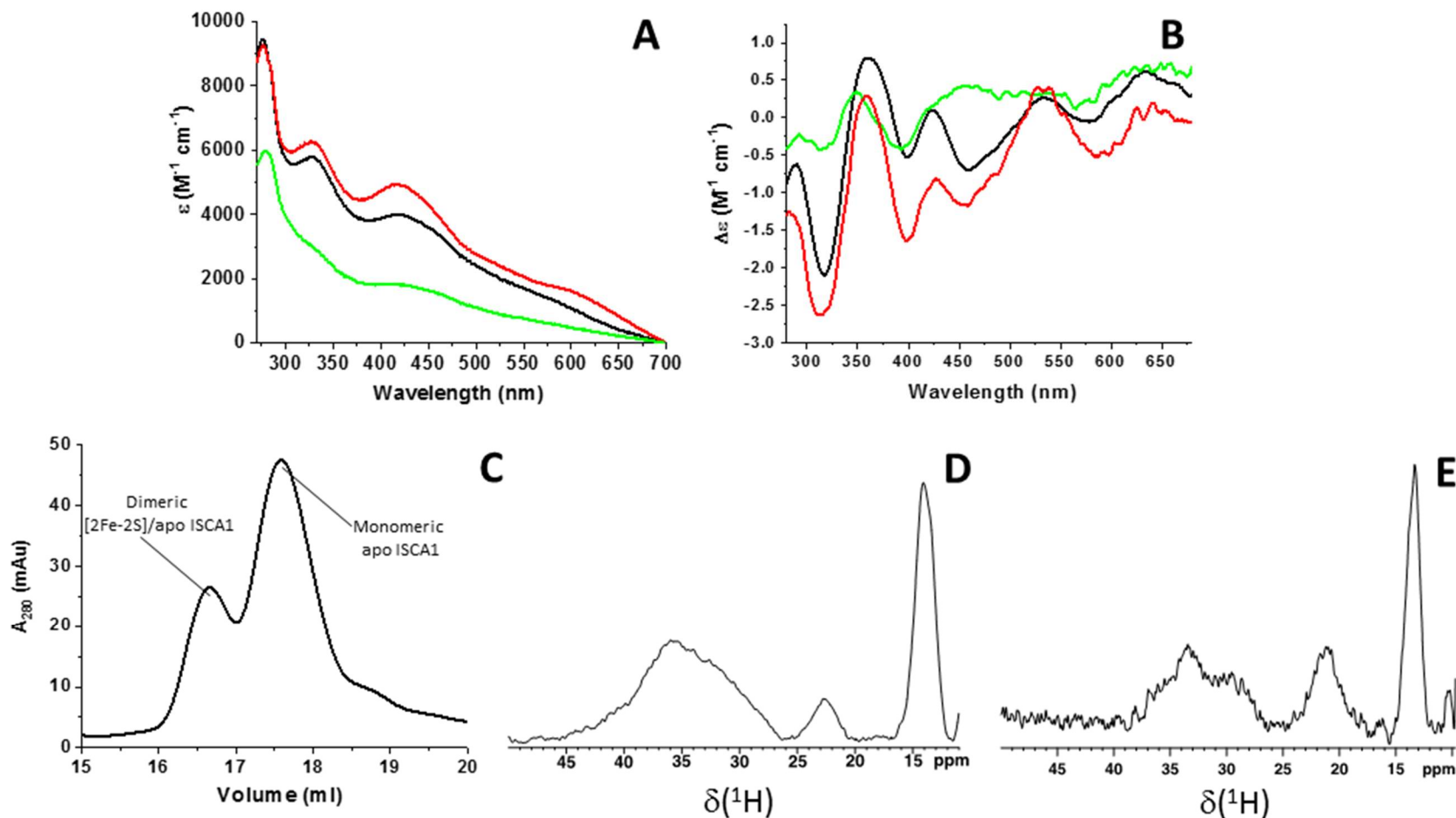


Figure S6. [4Fe-4S] ISCA1-ISCA2 interacts with apo NFU1 via its C-domain. A schematic cartoon depicting the NMR titration related to each panel is reported. (A) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on apo ^{15}N ISCA2-ISCA1 complex (red) with the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 mixture of [4Fe-4S] ^{15}N ISCA2-ISCA1 complex and C-domain of apo ^{15}N NFU1 (black). (B) Overlay of the ^1H - ^{15}N HSQC spectrum acquired on the C-domain of apo ^{15}N NFU1 (red) with the ^1H - ^{15}N HSQC spectrum acquired on a 1:1 mixture of [4Fe-4S] ^{15}N ISCA2-ISCA1 complex and C-domain of apo ^{15}N NFU1 (black). The residues of the C-domain of NFU1 whose backbone NH chemical shifts were affected (in terms of line broadening beyond detection effects and/or meaningful chemical shift changes) in the final mixture when compared to those of isolated apo NFU1 are indicated in the ^1H - ^{15}N HSQC spectrum and are shown in the inset on the structure of the C-domain of NFU1 (PDB ID 2M5O) as orange sidechains. The sidechains of proline residues are shown in gray.

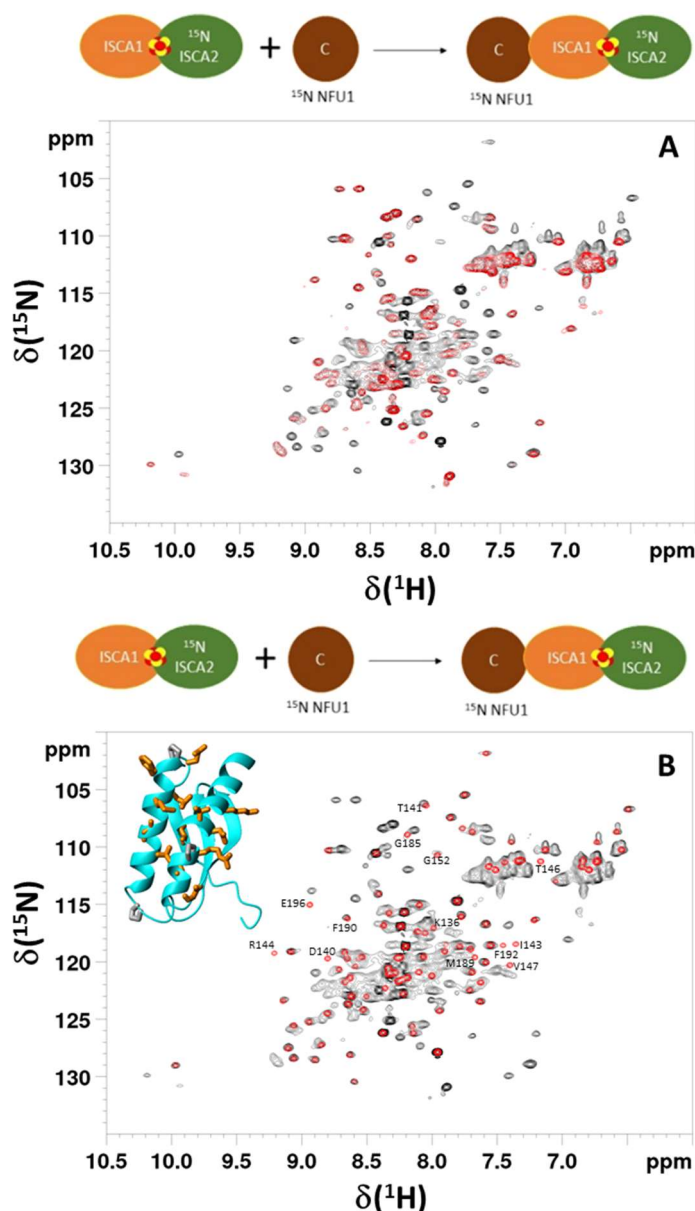


Figure S7. Temperature dependence of hyperfine-shifted signals of cluster ligands in the paramagnetic 1D ^1H NMR spectrum acquired on the 1:1 mixture of [4Fe-4S] ISCA1-ISCA2 and apo NFU1. The paramagnetic 1D ^1H NMR spectra were recorded on a Bruker NMR spectrometer operating at 400 MHz at 283 K (black) and 298 K (red) in degassed 50 mM phosphate, 150 mM NaCl buffer pH 7.0, and 5 mM DTT.

