

Antagonistic and autoinhibitory control of Fe-S cluster synthesis by FDX2 and FXN

Corresponding Author: Dr Benoit D'Autréaux

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.
This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

In this work Want et al have examined Fe-S cluster synthesis and in particular the mutually antagonistic roles of frataxin and ferredoxin. This is an important topic given the role of FeS clusters in multiple metabolic pathways. This approach is original, and the originality is added to by the diversity of approaches in this work. In several cases multiple approaches get complementary results.

The statistics are reasonably performed and well explained.

The references are well executed.

Overall, this is well executed, well integrated important work on a biochemical process of interest to all biological scientists.

My one comment on the models is that the Drosophila model provides an in vivo evaluation of interaction with frataxin and biochemical relevance, but it is not correct to say a model that lives less than 10 days has "hall marks of the disease". This discussion should be trimmed back. While this is interesting work, its applicability to treatment of the complex disease Friedreich ataxia in humans is still relatively distant.

Minor point_ FA should probably be abbreviated FRDA in formal writing since FA is usually reserved for Fanconi anemia

Referee #3

(Remarks to the Author)

Frataxin overexpression has been attempted as a therapy to treat patients harboring mutations within the gene encoding frataxin, which is required for iron-sulfur cluster synthesis and important cellular activities including oxidative phosphorylation. However, frataxin overexpression is quite toxic. Want and colleagues sought to determine the direct effect of frataxin (FXN) and FDX2 on the generation of iron sulfur cluster (2Fe-2S) biogenesis within mitochondria. Via an in vitro approach the authors established that in the absence of FXN the generation of 2Fe-2S was impaired, which was further impaired when the FXN concentration was increased.

In yeast, FXN competes with FDX2 for the same binding site on NIA/ISCU2 complex suggesting that excess FXN may interfere with FDX2 activity. Through elegant biochemistry, Want and colleagues demonstrate that the optimal rate of ISC generation occurred in the presence of equal quantities of FXN and FDX2. They also found that unbalanced levels of FXN/FDX2 impaired ISC production. Excess FDX2 also inhibits Fe-S synthesis even in the absence of FXN. But, in the presence of FXN, FDX2 can displace FXN from the NIAU complex indicating the two proteins compete for the same binding site with similar affinities.

Importantly, the authors found similar affinity constants for FXN and FDX2 binding to the NFS-1ISCU2 complex consistent with mutual exclusive binding. Lastly, the authors test their in vitro model indicating that decreased levels of FDX2 relative to FXN could increase Fe-S biogenesis in flies using a mutant line that harbored 42 GAA repeats in the gene encoding frataxin. Lifespan was increased pointing to the underlying cause of human disease.

In conclusion, this is a rigorous study supporting an elegant mechanism by which Frataxin mutations, or Frataxin overexpression perturb Fe-S cluster biogenesis and likely cause disease. The biochemistry is strong and supported by the in vivo experiments. The findings presented by Want and colleagues strongly support the findings by Meisel and colleagues, and vice versa. I support publication of this manuscript. The findings point towards therapeutic targets to treat FAA.

I am in favor of publication.

Referee #4

(Remarks to the Author)

In this work, the authors analyze the competitive binding of frataxin (FXN) and ferredoxin 2 (FDX2) to a joint site in the cysteine desulphurase NFS1. They biochemically dissect the competition of the two proteins for NFS1, and in large parts confirm, but also extend earlier work from others (refs 8-10). In the present work, they used, eg isothermal calorimetry and flow-induced dispersion analysis to measure binding constants and the competitive binding of FXN and FDX2, and they quantitate persulfide transfer and production to dissect the competitive action of the two proteins in the assembly process. Figure 1 nicely confirms previous findings made with yeast and human proteins (Refs 9, 10). They add that excess FDX2 inhibits a FXN free FeS synthesis reaction in vitro. It is not addressed whether FXN would also affect the background reaction observed with no added FDX2 (this analysis would possibly need longer reaction times, and could be analyzed for sulfur transfer as well, see below). In Fig. 2 the FIDA and ITC techniques were used to quantitatively examine the competitive character of FXN and FDX2 at NFS1. Similar binding constants for the two proteins were found. It is stated that "FDX2 has displaced FXN from the (NIAU)2 complex" and vice versa. Is this really an active process or the result of dynamic (reversible) on and off binding to the overlapping NFS1 binding site known from cryo EM studies? I wonder why the unphysiological Zn-ISCU2 was included in this experiment (NFS1 alone should suffice)? Of importance, it was not made clear what form of FDX2, red or ox, has been used in Fig. 2? Previous studies from the Markley and Lill labs have shown that there are significant differences for the two redox states, and mainly the reduced state may be relevant here. In Fig. 3, several assays show that FDX2 interferes with persulfide generation and transfer to ISCU2. Likely, this is explained by the known fact that FXN supports sulfur transfer (see the not cited Ref doi.org/10.1038/s41467-024-47310-8), and, if FXN is present in excess, FDX2 cannot readily bind to the FXN-blocked NFS1, reduce the ISCU persulfide, and finally assist FeS cluster formation. In fig. 4, the residues important for the (competitive) binding of FDX2 to NFS1 are analyzed. The authors find, in accordance to Refs. 9, 10, that some residues of helix F are important for FDX2 binding to NFS1 (ionic interaction). For two of the mutated FDX residues, the binding constants are weakened ca. 10 fold, but the FeS generation is hardly affected. How is this explained? For two other residues, the weakened affinity results in low FeS generation, which is suppressed by more FDX2, a nice finding showing that more FDX2 helps to compensate for reduced affinity. The authors also analyzed the FDX2 C-terminus which according to recent cryo EM structures binds to NFS1. The affinities are hardly changed by the mutations, and all these point mutations work fine (or even better) in FeS generation. If I understand the results from Ref 10 well, their findings differ marginally (even though the individual residues mutated in each study differ). In my view, the role of the C-terminus does not become clear yet from both studies and another one suggesting a role for FDX2 vs. FDX1 specificity (10.1038/s41589-022-01159-4). As a follow up of these biochemical data, the authors shift to genetics in flies (I suggest to add the word *Drosophila* to the sentence "...we aimed to determine whether lowering Fdx1 levels could be protective in vivo."), where the fly homolog of FDX2, termed Fdx1, is found increased by 62% in mRNA levels in a FXN knockdown animal (unfortunately no validation by immunostaining). I was surprised by this result because according to the results here and in Refs 9,10, I would have expected a lower Fdx1 level to avoid inhibition of FeS synthesis. Interestingly, exactly this expectation is suggested by the depletion of Fdx1 in frataxin-deficient animals where an increased life span was observed, fitting to the view that optimal ratios of frataxin and Fdx1 are needed. Why the frataxin knockdown animals seem to regulate Fdx1 gene expression in the opposite direction, is not further explained. In the Conclusion, the increased life span of double depleted animals is suggested to be "a therapeutic axis for FA". I wonder if, in Friedreich ataxia patients, one might consider reducing a critical component of FeS synthesis. Overall, the study presents a nice confirmation and extension of earlier studies on this subject, suggesting that there is a competitive behavior of Frataxin and FDX2 for binding to NFS1, and close to stoichiometric amounts are needed for optimal FeS synthesis rates.

Further comments:

1. Introduction: "...it is unclear whether FXN operates directly or indirectly on Fe-S cluster biosynthesis". Do the authors mean "overexpressed FXN"?
2. Introduction: Correct typo: "Complimenting work on..." to "Complementing work on..."
3. Results: Ref. 11 does not contain any data on FDX2. The citation in the sentence "...compete with FDX2 for the same binding site on the complex between NIA and ISCU2 (denoted (NIAU)2), and cryo-EM structures10,11" should be corrected here and in other places.
4. Results: The authors state "FXN and FDX2 operate at the same strength on [2Fe-2S] cluster synthesis". I do not see how "same strength" can be concluded at this! stage. The authors do not use high enough FDX2 concentrations to make this conclusion here (only 2 fold FDX2 vs. 50 fold for FXN).
5. Another reference should be cited for this sentence: "FXN accelerates persulfide transfer from NFS1 to iron loaded ISCU2 (Fe-ISCU2) (Fig. 3b).12,14" because this paper provides structural data on sulfur transfer (10.1038/s41467-024-47310-8).
6. Were the experiments in Fig. 2 and 3 performed under anaerobic conditions?

Reviewer comments:

Referee #1

(Remarks to the Author)

they have answered my questions

Referee #3

(Remarks to the Author)

The authors have addressed my concerns. I support publication.

Referee #4

(Remarks to the Author)

Figure 1 nicely confirms previous findings made with yeast and human proteins (Refs 9, 10). They add that excess FDX2 inhibits a FXN free FeS synthesis reaction in vitro. It is not addressed whether FXN would also affect the background reaction observed with no added FDX2 (this analysis would possibly need longer reaction times, and could be analyzed for sulfur transfer as well, see below).

Answer: We thank the referee for their positive comments. In the absence of FDX2, the mechanism of Fe-S cluster formation is very different, and is not considered as physiologically relevant. This has been described in Parent et al doi: 10.1038/ncomms6686, Gervason et al. doi:10.1038/s41467-019-11470-9 and reviewed in Gervason et al. doi.org/10.3390/inorganics8100055, Monfort et al. doi:10.3389/fnins.2022.838335. When FDX2 is lacking, L-cysteine acts as a reductant but preferentially reduces the persulfide of NFS1 (the reaction with ISCU2's persulfide is much slower), which leads to the release of hydrogen sulfide, then hydrogen sulfide combines with iron to generate Fe-S clusters, both [2Fe-2S] and [4Fe-4S]. Frataxin accelerates the reduction of NFS1's persulfide by a still unclear mechanism, which consequently accelerates Fe-S clusters formation. We refer to this process as the "thiol-based reaction" in previous works as opposed to the reaction with FDX2 referred to as the "FDX2-based reaction". Please see the reviewer-only figure comparing reactions with and without FDX2. This data shows that FXN accelerates the global rate of Fe-S clusters production of the thiol-based reaction; consistent with its stimulatory effect on the reduction of NFS1's persulfide by L-cysteine, but the overall kinetic of the reaction is much lower than the FDX2-based reaction. Its efficiency is also much lower with only about 5% of sulfide produced forming Fe-S clusters, to be compared with 95% in the FDX2-based reaction (see Gervason et al. doi:10.1038/s41467-019-11470-9). This is expected to be even worse in vivo, as the sulfide ions produced by the reduction of NFS1's persulfide will freely diffuse outside of the complex and react with other cellular components. Indeed, a *fdx2* mutant is lethal in vivo in yeast, confirming that the thiol-based reaction is not operative in vivo to support Fe-S cluster synthesis. Thereby, we consider the thiol-based reaction as not physiologically relevant. Since we are studying the physiological process, we prefer not to discuss the thiol-based reaction in the present manuscript.

R4-rev: Resolved. I agree that reactions lacking one of the ISC proteins may indeed be quite artificial and of minor (no?) relevance for the in vivo situation. So, these data should not be taken too serious.

In Fig. 2 the FIDA and ITC techniques were used to quantitatively examine the competitive character of FXN and FDX2 at NFS1. Similar binding constants for the two proteins were found. It is stated that "FDX2 has displaced FXN from the (NIAU)2 complex" and vice versa. Is this really an active process or the result of dynamic (reversible) on and off binding to the overlapping NFS1 binding site known from cryo EM studies?

Answer: We thank the referee for raising this interesting question. An active process implies that FXN and FDX2 would interact directly with each other and bind simultaneously to the (NIAU)2 complex, at least transiently. We have performed and included an additional ITC experiment showing that FDX2 and FXN do not directly interact (new Extended Figure 5.c). Moreover, our FIDA data indicate that FXN and FDX2 do not bind simultaneously to the (NIAU)2 complex. As a result of this, we have updated our interpretation to state that this competition is a result of statistical occupancies of FXN and FDX2 to the same binding site relying on their respective KD and concentrations, as opposed to active displacement.

R4-rev: I accept that there is no detectable interaction between FDX2 and FXN. My question, however, was a kinetic (dynamic) rather than thermodynamic one. In other words, are the off rates of FDX2 and FXN high enough to explain the rates of synthesis? This is an important point for the model.

I wonder why the unphysiological Zn-ISCU2 was included in this experiment (NFS1 alone should suffice)?

Answer: There are two reasons for including Zn-ISCU2. First, FXN does not interact with the (NIA)2 complex in the absence of ISCU2 (Figure 2f), NFS1 alone would thus not suffice, the presence of ISCU2 is needed to measure competitive binding. Second, the presence of a metal (iron or zinc) is needed to stabilize the ordered state of ISCU2. Under the aerobic conditions of the FIDA and ITC experiments, the Fe2+ ion in ISCU2 is readily oxidized and dissociates from the protein. We thus used Zn2+, which is redox stable, instead of Fe2+. The Zn2+ ion adopts the same coordination and geometry as Fe2+ in ISCU2 (Srouf et al. JACS (2022) doi: 10.1021/jacs.2c06338) and enables persulfide transfer as Fe2+ does (Gervason et al. doi:10.1038/s41467-019-11470-9). Zn2+ is thus a good structural surrogate of Fe2+. We further note that Zn-loaded ISCU2 interacts with the (NIA)2 complex with a higher binding affinity than apo-ISCU2, thus confirming that the presence of a metal is important (please see new Extended Figure 5.a-b and Extended Table 2).

R4-rev: OK, but the physiological ISCU-Fe would definitely be more convincing.

Of importance, it was not made clear what form of FDX2, red or ox, has been used in Fig. 2? Previous studies from the Markley and Lill labs have shown that there are significant differences for the two redox states, and mainly the reduced state may be relevant here.

Answer: We thank the referee for asking this important question. In light of the referee's suggestion, we have implemented FIDA with the reduced form of FDX2. All the samples were prepared under anaerobic conditions in septum-sealed vials with dithionite to reduce FDX2 and keep it reduced all the way throughout the experiment. We checked by UV-visible that FDX2 remained reduced for the duration of FIDA experiments. The new results have been added to Extended Figure 3 and Extended Table 1, and discussed in the main text. We measured KD values of 125 ± 13 nM and 144 ± 20 nM for oxidized and reduced FDX2 with the (NIAU-Zn)₂ complex, respectively, thus without any significant change within the margin errors. Equivalent KD values for reduced/oxidized FDX2 and FXN is consistent with the results of our Fe-S cluster synthesis assays pointing to competitive binding at similar strength between the two proteins. In agreement with our data, the group of R. Dutkiewicz found similar binding curves for the oxidized and chemically reduced yeast Yah1 with both the NIA and NIAU complexes, under aerobic and anaerobic conditions, respectively, by Biolayer Interferometry (BLI) (Uzarska et al doi: 10.1016/j.jbc.2022.101570).

In contrast, we note that the work from Markley's group comparing the binding affinity of oxidized and reduced FDX2 interacting with the (NIA)₂ complex was unsuccessful by ITC (doi:10.1021/acs.biochem.6b00447). Lill's group reported KD values of reduced Yah1 ferredoxin from *Chaetomium thermophilum* with the (NIA)₂ complex by microscale thermophoresis (MST) about 10-fold higher than for the oxidized form (Webert et al doi: 10.1038/ncomms6013). However, this higher value questions the mechanism of Fe-S cluster assembly, since the oxidized form would compete with the reduced one and thus strongly hamper persulfide reduction and Fe-S clusters formation. Furthermore, this value is not consistent with the competitive binding at similar strength we report here.

We note that a key difference between our studies is the labelling. While Lill's group labelled Nfs1-Icd11, we labelled FXN. Our FIDA and ITC data yielded KD for FXN of similar magnitude, indicating that FXN labelling for FIDA experiments on lysine residues does not interfere with binding to the NIAU complex. In contrast, we measured a much higher KD by FIDA than ITC for FDX2, indicating that FDX2 labelling perturbs the interaction with the NIAU complex. We thus used unlabelled FDX2 and FIDA competition assays where only FXN is labelled and FDX2 is a competitor of FXN, to measure the affinity of oxidized and reduced FDX2. Our ITC data confirmed that this approach is valid since similar KD values were obtained for oxidized FDX2 by FIDA competition assays and ITC. We can thus safely conclude that the labelling is not interfering in our analysis of oxidized and reduced FDX2 using this approach. Unfortunately, there was no control provided with another technique in Lill's study to ensure that the labelling did not interfere in their MST experiments. We thus prefer restraining from discussing their results in the present manuscript. Instead, we now refer to the work from Uzarska et al. They performed analysis using a tagged version of Yah1 which was confirmed to support activity in vivo alongside presenting no modification to the protein structure by CD.

R4-rev: Resolved.

In Fig. 3, several assays show that FDX2 interferes with persulfide generation and transfer to ISCU2. Likely, this is explained by the known fact that FXN supports sulfur transfer (see the not cited Ref doi.org/10.1038/s41467-024-47310-8), and, if FXN is present in excess, FDX2 cannot readily bind to the FXN-blocked NFS1, reduce the ISCU persulfide, and finally assist FeS cluster formation.

Answer: We agree with the referee, and this is our conclusion. However, acceleration of sulfur transfer by frataxin was not discovered in the non-cited reference doi: 10.1038/s41467-024-47310-8, but initially by Barondeau's group (Bridwell-Rabb et al. Biochemistry (2014), doi:10.1021/bi500532e) and our group (Parent et al Nat. Commun. (2015), doi: 10.1038/ncomms6686, Gervason et al. Nat. Commun. (2019), doi:10.1038/s41467-019-11470-9). We have added the citation of the initial paper from Barondeau's group and also the paper from Murphy's and Lill's groups as it provides the structural-basis of this process.

R4-rev: Resolved.

In fig. 4, the residues important for the (competitive) binding of FDX2 to NFS1 are analyzed. The authors find, in accordance to Refs. 9, 10, that some residues of helix F are important for FDX2 binding to NFS1 (ionic interaction). For two of the mutated FDX residues, the binding constants are weakened ca. 10 fold, but the FeS generation is hardly affected. How is this explained?

Answer: We thank the referee for raising this discussion. Indeed, two helix F mutants, E134A and D137A, are less affected than D138A and D141A in the presence of 10eq excess FXN, although all these mutants have close-to or lower-than 10-fold affinities for the (NIAU)₂ complex when compared to WT FDX2. It must be noted that E134A is more robust than D138A and D141A to the competition with FXN, due to higher affinity for the (NIAU)₂ complex (Fig. 4c-d), which could explain that it is less affected. This is less clear for D137A, which has similar affinity as D138A. However, both E134A and D137A mutants are notably faster than WT FDX2 when in the complete absence of FXN (Fig. 4h), indicating lower repressive effects on the mobile loop of NFS1. This lower direct repressive effect than the D138A and D141A mutants should participate to higher efficiency in Fe-S synthesis, thereby leading to the special observation of lower effect on [2Fe-2S] cluster synthesis than D138A and D141A when in the presence of excess FXN, despite decrease in their overall binding affinity.

R4-rev: This answer means that we do not understand the role of the residues and the importance of the binding strength for the synthesis rate.

For two other residues, the weakened affinity results in low FeS generation, which is suppressed by more FDX2, a nice finding showing that more FDX2 helps to compensate for reduced affinity.

Answer: We thank the referee for this positive comment.

The authors also analyzed the FDX2 C-terminus which according to recent cryo EM structures binds to NFS1. The affinities are hardly changed by the mutations, and all these point mutations work fine (or even better) in FeS generation. If I understand the results from Ref 10 well, their findings differ marginally (even though the individual residues mutated in each study differ). In my view, the role of the C-terminus does not become clear yet from both studies and another one suggesting a role for FDX2 vs. FDX1 specificity (10.1038/s41589-022-01159-4).

Answer: The study from ref 10 reports the effects of truncations of the FDX2 C-terminus as opposed to point mutations of key interacting residues in our case. The authors find that some of these FDX2 truncations ($\Delta C1$, $\Delta C5$, $\Delta C10$) improve the rate of Fe-S cluster synthesis under 1:1 FXN/FDX2 ratios and slightly less with 2eq. excess FDX2, while the large $\Delta C12$ mutation is similar to WT in both cases.

Our study highlights the key interacting residues of the C-terminus of FDX2 (Y177A, D179A, H181A), which are included in the aforementioned $\Delta C5$ (H181A), $\Delta C10$ (Y177A, D179A, H181A), and $\Delta C12$ (Y177A, D179A, H181A) truncations. The substitutions of these residues are indeed marginally better than WT at equal FDX2/FXN concentrations, significantly better in excess-FDX2 concentrations, and, critically, faster under conditions lacking FXN (i.e. recapitulating conditions of FRDA). It is not immediately clear why the complete removal of the last 12 amino acids from the C-terminus do not show enhancement of Fe-S cluster synthesis in ref10, but it is likely that such large truncation might have side effects, notably on the structure of the nearby Fe-S cluster.

Our two studies thus seem consistent with each other. We have added a sentence on page 10 to specifically cite this work. Importantly, our data show that these mutations do not significantly affect binding affinities (ITC and FIDA data) but specifically impact the repression on persulfide transfer between NFS1 and ISCU2 (ARBS data) and Fe-S cluster synthesis. We can thus conclude that the interaction between the C-terminus of FDX2 and the mobile loop of NFS1 mediates a direct repression on persulfide generation and transfer, possibly by decreasing the flexibility required for persulfide generation and transfer.

We agree with the referee that complete truncation of the FDX2 C-terminus ($\Delta C12$) was shown to be critical for yeast growth (as reported in ref 10.1038/s41589-022-01159-4). However, in light of the in vitro data showing that the $\Delta C12$ is still active in [2Fe-2S] cluster synthesis, this suggests the involvement of FDX2 C-terminus in other cellular processes beyond [2Fe-2S] cluster synthesis, likely in the assembly of [4Fe-4S] clusters by the ISCA scaffold (ref 10.1073/pnas.2003982117), which is a key function for cellular Fe-S cluster biogenesis. The C-terminus of FDX2 might thus play different roles depending on its interacting partner.

R4-rev: The authors agree that the published studies from others and their findings are similar. However, upon re-reading the Steinhilper paper, I saw that these authors also analyzed a point mutant (residue 175, not studied here) that behaves similarly to the C12del rather than the wild type (and wild type is NOT similar to C12del as noted above), indicating that residue 175 is a decisive one, but overall the role of the C-terminus remains unclear.

As a follow up of these biochemical data, the authors shift to genetics in flies (I suggest to add the word *Drosophila* to the sentence "...we aimed to determine whether lowering Fdx1 levels could be protective in vivo."), where the fly homolog of FDX2, termed Fdx1, is found increased by 62% in mRNA levels in a FXN knockdown animal (unfortunately no validation by immunostaining).

Answer: We thank the referee for their suggestion. The word *drosophila* has been added to the sentence. Unfortunately, there is no antibody available against the *drosophila* Fdx1 protein. However, we tested an antibody directed against the human protein (sc-515121 from Santa Cruz Biotechnology), but it failed to recognise the *Drosophila* protein.

R4-rev: Resolved.

I was surprised by this result because according to the results here and in Refs 9,10, I would have expected a lower Fdx1 level to avoid inhibition of FeS synthesis. Interestingly, exactly this expectation is suggested by the depletion of Fdx1 in frataxin-deficient animals where an increased life span was observed, fitting to the view that optimal ratios of frataxin and Fdx1 are needed. Why the frataxin knockdown animals seem to regulate Fdx1 gene expression in the opposite direction, is not further explained.

Answer: We interpret the cellular increase in Fdx1 to arise as a normal physiological response to increase components of the ISC system in attempt to enhance Fe-S biogenesis during cellular conditions of low Fe-S biogenesis.

R4-rev: Well possible, but if excess Fdx1 would inhibit the synthesis rate, as suggested here, this would decrease FeS synthesis even further. The in vivo result therefore is more consistent with the idea that at least in flies, the proposed mechanism is less relevant.

In the Conclusion, the increased life span of double depleted animals is suggested to be "a therapeutic axis for FA". I wonder if, in Friedreich ataxia patients, one might consider reducing a critical component of FeS synthesis.

Answer: We agree that it may be challenging to target a critical component, although we note that other promising treatments within the field are facing a similar difficulty with this disease, especially gene therapy for which overdosage of FXN is toxic. We agree that we are yet distant from a treatment for FRDA, and we have thus softened our conclusions on the application of treatment to Friedreich's ataxia throughout the article.

R4-rev: I agree that we are far from using these results for therapy. Other regimes such as mimicking FXN's structural role in stimulating S transfer are more likely to help FRDA patients.

Overall, the study presents a nice confirmation and extension of earlier studies on this subject, suggesting that there is a competitive behavior of Frataxin and FDX2 for binding to NFS1, and close to stoichiometric amounts are needed for optimal

FeS synthesis rates.

Answer: We thank the referee for their positive comments.

Further comments:

1. Introduction: "...it is unclear whether FXN operates directly or indirectly on Fe-S cluster biosynthesis". Do the authors mean "overexpressed FXN"?

Answer: Indeed, overexpressed FXN was intended. This has now been updated in the text.

R4-rev: Resolved.

2. Introduction: Correct typo: "Complimenting work on..." to "Complementing work on..."

Answer: This has been corrected.

R4-rev: Resolved.

3. Results: Ref. 11 does not contain any data on FDX2. The citation in the sentence "...compete with FDX2 for the same binding site on the complex between NIA and ISCU2 (denoted (NIAU)2), and cryo-EM structures^{10,11}" should be corrected here and in other places.

Answer: The references were misplaced, and we have thus corrected this. Ref 10 is needed here as it reports the first cryo-EM structure of FXN-bound (NIAU)2 complex. Combined with the cryo-EM structure of Ref 11, these data show that FXN and FDX2 bind to the same site.

R4-rev: Resolved.

4. Results: The authors state "FXN and FDX2 operate at the same strength on [2Fe-2S] cluster synthesis". I do not see how "same strength" can be concluded at this stage. The authors do not use high enough FDX2 concentrations to make this conclusion here (only 2 fold FDX2 vs. 50 fold for FXN).

Answer: To complete our titration, we performed titrations at higher FDX2 ratios (5 and 10 equivalents, new Figure 1.c-d). The results confirmed that the effects of FDX2 and FXN are of similar magnitude, with an optimal ratio close to 1:1, consistent with nearly equal affinity constants for the (NIAU)2 complex as determined by FIDA and ITC.

R4-rev: The data are difficult to interpret for me. If I get it right, the same behavior is seen with 10 eq FDX2 and 50 eq FXN, i.e. a considerable difference for the two proteins.

5. Another reference should be cited for this sentence: "FXN accelerates persulfide transfer from NFS1 to iron loaded ISCU2 (Fe-ISCU2) (Fig. 3b).^{12,14}" because this paper provides structural data on sulfur transfer (10.1038/s41467-024-47310-8).

Answer: This reference has been added.

R4-rev: Resolved.

6. Were the experiments in Fig. 2 and 3 performed under anaerobic conditions?

Answer: Experiments in Figure 3 (as in Figure 1) were performed under anaerobiosis, this is now specified in the text. Measurements in Figure 2 were performed under aerobiosis as already specified in the text, except for reduced FDX2 which was performed under anaerobic conditions (see New Extended Figure 3 and Extended Table 1).

R4-rev: Does this mean that the binding studies in Fig. 2 use oxidized FDX2, i.e. the entity that should leave the NFS complex?

R4-rev: Overall, the study has been improved, and in most parts agrees well with what we know from earlier studies on this subject.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We thank the referees for their insightful feedback, comments, and suggestions. We have revised the text and included several new experiments to support the suggestions of the reviewers. Attached is our response to comments from the referees.

Referee #1 (Remarks to the Author):

In this work Want et al have examined Fe-S cluster synthesis and in particular the mutually antagonistic roles of frataxin and ferredoxin. This is an important topic given the role of FeS clusters in multiple metabolic pathways. This approach is original, and the originality is added to by the diversity of approaches in this work. In several cases multiple approaches get complementary results.

The statistics are reasonably performed and well explained.

The references are well executed.

Overall, this is well executed, well integrated important work on a biochemical process of interest to all biological scientists.

Answer: We thank the referee for their very positive comments.

My one comment on the models is that the *Drosophila* model provides an *in vivo* evaluation of interaction with frataxin and biochemical relevance, but it is not correct to say a model that lives less than 10 days has “hall marks of the disease”. This discussion should be trimmed back. While this is interesting work, its applicability to treatment of the complex disease Friedreich ataxia in humans is still relatively distant.

Answer: The Iron-sulfur cluster machinery is highly conserved and *drosophila* models have proven to be a valuable approach for uncovering mechanisms of cellular and physiological dysfunctions induced by frataxin deficiency (as reviewed in Monnier et al; Int J Mol Sci DOI: 10.3390/ijms19071989). Here, we used *Drosophila* as an *in vivo* proof-of-concept that ferredoxin reduction could be protective. We agree that the term “hallmarks of the disease” might be not be appropriate and we have thus modified it. Similarly, we have toned down the application for the treatment to Friedreich’s ataxia throughout the article. We have changed the title and modified the last sentences of the abstract, the introduction and the conclusion.

Minor point_ FA should probably be abbreviated FRDA in formal writing since FA is usually reserved for Fanconi anemia

Answer: This has been updated throughout the paper.

Referee #3 (Remarks to the Author):

Frataxin overexpression has been attempted as a therapy to treat patients harboring mutations within the gene encoding frataxin, which is required for iron-sulfur cluster synthesis and important cellular activities including oxidative phosphorylation. However, frataxin overexpression is quite toxic. Want and colleagues sought to determine the direct effect of frataxin (FXN) and FDX2 on the generation of iron sulfur cluster (2Fe-2S) biogenesis within mitochondria. Via an in vitro approach the authors established that in the absence of FXN the generation of 2Fe-2S was impaired, which was further impaired when the FXN concentration was increased.

In yeast, FXN competes with FDX2 for the same binding site on NIA/ISCU2 complex suggesting that excess FXN may interfere with FDX2 activity. Through elegant biochemistry, Want and colleagues demonstrate that the optimal rate of ISC generation occurred in the presence of equal quantities of FXN and FDX2. They also found that unbalanced levels of FXN/FDX2 impaired ISC production. Excess FDX2 also inhibits Fe-S synthesis even in the absence of FXN. But, in the presence of FXN, FDX2 can displace FXN from the NIAU complex indicating the two proteins compete for the same binding site with similar affinities.

Importantly, the authors found similar affinity constants for FXN and FDX2 binding to the NFS-1ISCU2 complex consistent with mutual exclusive binding. Lastly, the authors test their in vitro model indicating that decreased levels of FDX2 relative to FXN could increase Fe-S biogenesis in flies using a mutant line that harbored 42 GAA repeats in the gene encoding frataxin. Lifespan was increased pointing to the underlying cause of human disease.

In conclusion, this is a rigorous study supporting an elegant mechanism by which Frataxin mutations, or Frataxin overexpression perturb Fe-S cluster biogenesis and likely cause disease. The biochemistry is strong and supported by the in vivo experiments. The findings presented by Want and colleagues strongly support the findings by Meisel and colleagues, and vice versa. I support publication of this manuscript. The findings point towards therapeutic targets to treat FAA.

I am in favor of publication.

Answer: We thank the referee for their very positive comments.

Referee #4 (Remarks to the Author):

In this work, the authors analyze the competitive binding of frataxin (FXN) and ferredoxin 2 (FDX2) to a joint site in the cysteine desulphurase NFS1. They biochemically dissect the competition of the two proteins for NFS1, and in large parts confirm, but also extend earlier work from others (refs 8-10). In the present work, they used, eg isothermal calorimetry and flow-induced dispersion analysis to measure binding constants and the competitive binding of FXN and FDX2, and they quantitate persulfide transfer and production to dissect the competitive action of the two proteins in the assembly process.

Figure 1 nicely confirms previous findings made with yeast and human proteins (Refs 9, 10). They add that excess FDX2 inhibits a FXN free FeS synthesis reaction in vitro. It is not addressed whether FXN would also affect the background reaction observed with no added FDX2 (this analysis would possibly need longer reaction times, and could be analyzed for sulfur transfer as well, see below).

Answer: We thank the referee for their positive comments. In the absence of FDX2, the mechanism of Fe-S cluster formation is very different, and is not considered as physiologically relevant. This has been described in Parent et al doi: 10.1038/ncomms6686, Gervason et al. doi:10.1038/s41467-019-11470-9 and reviewed in Gervason et al. doi.org/10.3390/inorganics8100055, Monfort et al. doi:10.3389/fnins.2022.838335. When FDX2 is lacking, L-cysteine acts as a reductant but preferentially reduces the persulfide of NFS1 (the reaction with ISCU2's persulfide is much slower), which leads to the release of hydrogen sulfide, then hydrogen sulfide combines with iron to generate Fe-S clusters, both [2Fe-2S] and [4Fe-4S]. Frataxin accelerates the reduction of NFS1's persulfide by a still unclear mechanism, which consequently accelerates Fe-S clusters formation. We refer to this process as the "thiol-based reaction" in previous works as opposed to the reaction with FDX2 referred to as the "FDX2-based reaction". Please see the **reviewer-only figure** comparing reactions with and without FDX2. This data shows that FXN accelerates the global rate of Fe-S clusters production of the thiol-based reaction; consistent with its stimulatory effect on the reduction of NFS1's persulfide by L-cysteine, but the overall kinetic of the reaction is much lower than the FDX2-based reaction. Its efficiency is also much lower with only about 5% of sulfide produced forming Fe-S clusters, to be compared with 95% in the FDX2-based reaction (see Gervason et al. doi:10.1038/s41467-019-11470-9). This is expected to be even worse in vivo, as the sulfide ions produced by the reduction of NFS1's persulfide will freely diffuse outside of the complex and react with other cellular components. Indeed, a *fdx2* mutant is lethal in vivo in yeast, confirming that the thiol-based reaction is not operative in vivo to support Fe-S cluster synthesis. Thereby, we consider the thiol-based reaction as **not physiologically relevant**. Since we are studying the physiological process, we prefer not to discuss the thiol-based reaction in the present manuscript.

In Fig. 2 the FIDA and ITC techniques were used to quantitatively examine the competitive character of FXN and FDX2 at NFS1. Similar binding constants for the two proteins were found. It is stated that "FDX2 has displaced FXN from the (NIAU)₂ complex" and vice versa. Is this really an active process or the result of dynamic (reversible) on and off binding to the overlapping NFS1 binding site known from cryo EM studies?

Answer: We thank the referee for raising this interesting question. An active process implies that FXN and FDX2 would interact directly with each other and bind simultaneously to the (NIAU)₂ complex, at least transiently. We have performed and included an additional ITC experiment showing that FDX2 and FXN do not directly interact (**new Extended Figure 5.c**). Moreover, our FIDA data indicate that FXN and FDX2 do not bind simultaneously to the (NIAU)₂ complex. As a result of this, we have

updated our interpretation to state that this competition is a result of statistical occupancies of FXN and FDX2 to the same binding site relying on their respective K_D and concentrations, as opposed to active displacement.

I wonder why the unphysiological Zn-ISCU2 was included in this experiment (NFS1 alone should suffice)?

Answer: There are two reasons for including Zn-ISCU2. First, FXN does not interact with the (NIA)₂ complex in the absence of ISCU2 (Figure 2f), NFS1 alone would thus not suffice, the presence of ISCU2 is needed to measure competitive binding. Second, the presence of a metal (iron or zinc) is needed to stabilize the ordered state of ISCU2. Under the aerobic conditions of the FIDA and ITC experiments, the Fe²⁺ ion in ISCU2 is readily oxidized and dissociates from the protein. We thus used Zn²⁺, which is redox stable, instead of Fe²⁺. The Zn²⁺ ion adopts the same coordination and geometry as Fe²⁺ in ISCU2 (Srouf et al. JACS (2022) doi: 10.1021/jacs.2c06338) and enables persulfide transfer as Fe²⁺ does (Gervason et al. doi:10.1038/s41467-019-11470-9). Zn²⁺ is thus a good structural surrogate of Fe²⁺. We further note that Zn-loaded ISCU2 interacts with the (NIA)₂ complex with a higher binding affinity than apo-ISCU2, thus confirming that the presence of a metal is important (please see **new Extended Figure 5.a-b and Extended Table 2**).

Of importance, it was not made clear what form of FDX2, red or ox, has been used in Fig. 2? Previous studies from the Markley and Lill labs have shown that there are significant differences for the two redox states, and mainly the reduced state may be relevant here.

Answer: We thank the referee for asking this important question. In light of the referee's suggestion, we have implemented FIDA with the reduced form of FDX2. All the samples were prepared under anaerobic conditions in septum-sealed vials with dithionite to reduce FDX2 and keep it reduced all the way throughout the experiment. We checked by UV-visible that FDX2 remained reduced for the duration of FIDA experiments. **The new results have been added to Extended Figure 3 and Extended Table 1, and discussed in the main text.** We measured K_D values of 125 ± 13 nM and 144 ± 20 nM for oxidized and reduced FDX2 with the (NIAU-Zn)₂ complex, respectively, thus without any significant change within the margin errors. Equivalent K_D values for reduced/oxidized FDX2 and FXN is consistent with the results of our Fe-S cluster synthesis assays pointing to competitive binding at similar strength between the two proteins. In agreement with our data, the group of R. Dutkiewicz found similar binding curves for the oxidized and chemically reduced yeast Yah1 with both the NIA and NIAU complexes, under aerobic and anaerobic conditions, respectively, by Biolayer Interferometry (BLI) (Uzarska et al doi: 10.1016/j.jbc.2022.101570).

In contrast, we note that the work from Markley's group comparing the binding affinity of oxidized and reduced FDX2 interacting with the (NIA)₂ complex was unsuccessful by ITC (doi:10.1021/acs.biochem.6b00447). Lill's group reported K_D values of reduced Yah1 ferredoxin from *Chaetomium thermophilum* with the (NIA)₂ complex by microscale thermophoresis (MST) about 10-fold higher than for the oxidized form (Webert et al doi: 10.1038/ncomms6013). However, this higher value questions the mechanism of Fe-S cluster assembly, since the oxidized form would compete with the reduced one and thus strongly hamper persulfide reduction and Fe-S clusters formation. Furthermore, this value is not consistent with the competitive binding at similar strength we report here.

We note that a key difference between our studies is the labelling. While Lill's group labelled Nfs1-Isd11, we labelled FXN. Our FIDA and ITC data yielded K_D for FXN of similar magnitude, indicating that FXN labelling for FIDA experiments on lysine residues does not interfere with binding to the NIAU complex. In contrast, we measured a much higher K_D by FIDA than ITC for FDX2, indicating that FDX2 labelling perturbs the interaction with the NIAU complex. We thus used unlabelled FDX2 and FIDA competition assays where only FXN is labelled and FDX2 is a competitor of FXN, to measure the affinity of oxidized and reduced FDX2. Our ITC data confirmed that this approach is valid since similar K_D values were obtained for oxidized FDX2 by FIDA competition assays and ITC. We can thus safely conclude that the labelling is not interfering in our analysis of oxidized and reduced FDX2 using this approach. Unfortunately, there was no control provided with another technique in Lill's study to ensure that the labelling did not interfere in their MST experiments. We thus prefer restraining from discussing their results in the present manuscript. Instead, we now refer to the work from Uzarska *et al.* They performed analysis using a tagged version of Yah1 which was confirmed to support activity in vivo alongside presenting no modification to the protein structure by CD.

In Fig. 3, several assays show that FDX2 interferes with persulfide generation and transfer to ISCU2. Likely, this is explained by the known fact that FXN supports sulfur transfer (see the not cited Ref doi.org/10.1038/s41467-024-47310-8), and, if FXN is present in excess, FDX2 cannot readily bind to the FXN-blocked NFS1, reduce the ISCU persulfide, and finally assist FeS cluster formation.

Answer: We agree with the referee, and this is our conclusion. However, acceleration of sulfur transfer by frataxin was not discovered in the non-cited reference [doi: 10.1038/s41467-024-47310-8](https://doi.org/10.1038/s41467-024-47310-8), but initially by Barondeau's group (Bridwell-Rabb *et al.* Biochemistry (2014), [doi:10.1021/bi500532e](https://doi.org/10.1021/bi500532e)) and our group (Parent *et al.* Nat. Commun. (2015), [doi: 10.1038/ncomms6686](https://doi.org/10.1038/ncomms6686), Gervason *et al.* Nat. Commun. (2019), [doi:10.1038/s41467-019-11470-9](https://doi.org/10.1038/s41467-019-11470-9)). We have added the citation of the initial paper from Barondeau's group and also the paper from Murphy's and Lill's groups as it provides the structural-basis of this process.

In fig. 4, the residues important for the (competitive) binding of FDX2 to NFS1 are analyzed. The authors find, in accordance to Refs. 9, 10, that some residues of helix F are important for FDX2 binding to NFS1 (ionic interaction). For two of the mutated FDX residues, the binding constants are weakened ca. 10 fold, but the FeS generation is hardly affected. How is this explained?

Answer: We thank the referee for raising this discussion. Indeed, two helix F mutants, E134A and D137A, are less affected than D138A and D141A in the presence of 10eq excess FXN, although all these mutants have close-to or lower-than 10-fold affinities for the (NIAU)₂ complex when compared to WT FDX2. It must be noted that E134A is more robust than D138A and D141A to the competition with FXN, due to higher affinity for the (NIAU)₂ complex (**Fig. 4c-d**), which could explain that it is less affected. This is less clear for D137A, which has similar affinity as D138A. However, both E134A and D137A mutants are notably faster than WT FDX2 when in the complete absence of FXN (**Fig. 4h**), indicating lower repressive effects on the mobile loop of NFS1. This lower direct repressive effect than the D138A and D141A mutants should participate to higher efficiency in Fe-S synthesis, thereby leading to the special observation of lower effect on [2Fe-2S] cluster synthesis than D138A and D141A when in the presence of excess FXN, despite decrease in their overall binding affinity.

For two other residues, the weakened affinity results in low FeS generation, which is suppressed by more FDX2, a nice finding showing that more FDX2 helps to compensate for reduced affinity.

Answer: We thank the referee for this positive comment.

The authors also analyzed the FDX2 C-terminus which according to recent cryo EM structures binds to NFS1. The affinities are hardly changed by the mutations, and all these point mutations work fine (or even better) in FeS generation. If I understand the results from Ref 10 well, their findings differ marginally (even though the individual residues mutated in each study differ). In my view, the role of the C-terminus does not become clear yet from both studies and another one suggesting a role for FDX2 vs. FDX1 specificity (10.1038/s41589-022-01159-4).

Answer: The study from ref 10 reports the effects of truncations of the FDX2 C-terminus as opposed to point mutations of key interacting residues in our case. The authors find that some of these FDX2 truncations ($\Delta C1$, $\Delta C5$, $\Delta C10$) improve the rate of Fe-S cluster synthesis under 1:1 FXN/FDX2 ratios and slightly less with 2eq. excess FDX2, while the large $\Delta C12$ mutation is similar to WT in both cases.

Our study highlights the key interacting residues of the C-terminus of FDX2 (Y177A, D179A, H181A), which are included in the aforementioned $\Delta C5$ (H181A), $\Delta C10$ (Y177A, D179A, H181A), and $\Delta C12$ (Y177A, D179A, H181A) truncations. The substitutions of these residues are indeed marginally better than WT at equal FDX2/FXN concentrations, significantly better in excess-FDX2 concentrations, and, critically, faster under conditions lacking FXN (i.e. recapitulating conditions of FRDA). It is not immediately clear why the complete removal of the last 12 amino acids from the C-terminus do not show enhancement of Fe-S cluster synthesis in ref10, but it is likely that such large truncation might have side effects, notably on the structure of the nearby Fe-S cluster.

Our two studies thus seem consistent with each other. **We have added a sentence on page 10 to specifically cite this work.** Importantly, our data show that these mutations do not significantly affect binding affinities (ITC and FIDA data) but specifically impact the repression on persulfide transfer between NFS1 and ISCU2 (ARBS data) and Fe-S cluster synthesis. We can thus conclude that the interaction between the C-terminus of FDX2 and the mobile loop of NFS1 mediates a direct repression on persulfide generation and transfer, possibly by decreasing the flexibility required for persulfide generation and transfer.

We agree with the referee that complete truncation of the FDX2 C-terminus ($\Delta C12$) was shown to be critical for yeast growth (as reported in ref 10.1038/s41589-022-01159-4). However, in light of the *in vitro* data showing that the $\Delta C12$ is still active in [2Fe-2S] cluster synthesis, this suggests the involvement of FDX2 C-terminus in other cellular processes beyond [2Fe-2S] cluster synthesis, likely in the assembly of [4Fe-4S] clusters by the ISCA scaffold (ref 10.1073/pnas.2003982117), which is a key function for cellular Fe-S cluster biogenesis. The C-terminus of FDX2 might thus play different roles depending on its interacting partner.

As a follow up of these biochemical data, the authors shift to genetics in flies (I suggest to add the word *Drosophila* to the sentence "...we aimed to determine whether lowering Fdx1 levels could be protective *in vivo*."), where the fly homolog of FDX2, termed Fdx1, is found increased by 62% in mRNA levels in a FXN knockdown animal (unfortunately no validation by immunostaining).

Answer: We thank the referee for their suggestion. The word *drosophila* has been added to the sentence. Unfortunately, there is no antibody available against the *drosophila* Fdx1 protein. However,

we tested an antibody directed against the human protein (sc-515121 from Santa Cruz Biotechnology), but it failed to recognise the Drosophila protein.

I was surprised by this result because according to the results here and in Refs 9,10, I would have expected a lower Fdx1 level to avoid inhibition of FeS synthesis. Interestingly, exactly this expectation is suggested by the depletion of Fdx1 in frataxin-deficient animals where an increased life span was observed, fitting to the view that optimal ratios of frataxin and Fdx1 are needed. Why the frataxin knockdown animals seem to regulate Fdx1 gene expression in the opposite direction, is not further explained.

Answer: We interpret the cellular increase in Fdx1 to arise as a normal physiological response to increase components of the ISC system in attempt to enhance Fe-S biogenesis during cellular conditions of low Fe-S biogenesis.

In the Conclusion, the increased life span of double depleted animals is suggested to be “a therapeutic axis for FA”. I wonder if, in Friedreich ataxia patients, one might consider reducing a critical component of FeS synthesis.

Answer: We agree that it may be challenging to target a critical component, although we note that other promising treatments within the field are facing a similar difficulty with this disease, especially gene therapy for which overdosage of FXN is toxic. We agree that we are yet distant from a treatment for FRDA, and we have thus softened our conclusions on the application of treatment to Friedreich's ataxia throughout the article.

Overall, the study presents a nice confirmation and extension of earlier studies on this subject, suggesting that there is a competitive behavior of Frataxin and FDX2 for binding to NFS1, and close to stoichiometric amounts are needed for optimal FeS synthesis rates.

Answer: We thank the referee for their positive comments.

Further comments:

1. Introduction: “...it is unclear whether FXN operates directly or indirectly on Fe-S cluster biosynthesis”. Do the authors mean “overexpressed FXN”?

Answer: Indeed, *overexpressed FXN* was intended. This has now been updated in the text.

2. Introduction: Correct typo: “Complimenting work on...” to “Complementing work on...”.

Answer: This has been corrected.

3. Results: Ref. 11 does not contain any data on FDX2. The citation in the sentence “...compete with FDX2 for the same binding site on the complex between NIA and ISCU2 (denoted (NIAU)₂), and cryo-EM structures^{10,11}” should be corrected here and in other places.

Answer: The references were misplaced, and we have thus corrected this. Ref 10 is needed here as it reports the first cryo-EM structure of FXN-bound (NIAU)₂ complex. Combined with the cryo-EM structure of Ref 11, these data show that FXN and FDX2 bind to the same site.

4. Results: The authors state “FXN and FDX2 operate at the same strength on [2Fe-2S] cluster synthesis”. I do not see how “same strength” can be concluded at this! stage. The authors do not use high enough FDX2 concentrations to make this conclusion here (only 2 fold FDX2 vs. 50 fold for FXN).

Answer: To complete our titration, we performed titrations at higher FDX2 ratios (5 and 10 equivalents, **new Figure 1.c-d**). The results confirmed that the effects of FDX2 and FXN are of similar magnitude, with an optimal ratio close to 1:1, consistent with nearly equal affinity constants for the (NIAU)₂ complex as determined by FIDA and ITC.

5. Another reference should be cited for this sentence: “FXN accelerates persulfide transfer from NFS1 to iron loaded ISCU2 (Fe-ISCU2) (Fig. 3b).12,14” because this paper provides structural data on sulfur transfer (10.1038/s41467-024-47310-8).

Answer: This reference has been added.

6. Were the experiments in Fig. 2 and 3 performed under anaerobic conditions?

Answer: Experiments in Figure 3 (as in Figure 1) were performed under anaerobiosis, this is now specified in the text. Measurements in Figure 2 were performed under aerobiosis as already specified in the text, except for reduced FDX2 which was performed under anaerobic conditions (see **New Extended Figure 3 and Extended Table 1**).

Referee #4 (Remarks to the Author):

Figure 1 nicely confirms previous findings made with yeast and human proteins (Refs 9, 10). They add that excess FDX2 inhibits a FXN free FeS synthesis reaction in vitro. It is not addressed whether FXN would also affect the background reaction observed with no added FDX2 (this analysis would possibly need longer reaction times, and could be analyzed for sulfur transfer as well, see below).

Answer: We thank the referee for their positive comments. In the absence of FDX2, the mechanism of Fe-S cluster formation is very different, and is not considered as physiologically relevant. This has been described in Parent et al doi: 10.1038/ncomms6686, Gervason et al. doi:10.1038/s41467-019-11470-9 and reviewed in Gervason et al. doi.org/10.3390/inorganics8100055, Monfort et al. doi:10.3389/fnins.2022.838335. When FDX2 is lacking, L-cysteine acts as a reductant but preferentially reduces the persulfide of NFS1 (the reaction with ISCU2's persulfide is much slower), which leads to the release of hydrogen sulfide, then hydrogen sulfide combines with iron to generate Fe-S clusters, both [2Fe-2S] and [4Fe-4S]. Frataxin accelerates the reduction of NFS1's persulfide by a still unclear mechanism, which consequently accelerates Fe-S clusters formation. We refer to this process as the "thiol-based reaction" in previous works as opposed to the reaction with FDX2 referred to as the "FDX2-based reaction". Please see the reviewer-only figure comparing reactions with and without FDX2. This data shows that FXN accelerates the global rate of Fe-S clusters production of the thiol-based reaction; consistent with its stimulatory effect on the reduction of NFS1's persulfide by L-cysteine, but the overall kinetic of the reaction is much lower than the FDX2-based reaction. Its efficiency is also much lower with only about 5% of sulfide produced forming Fe-S clusters, to be compared with 95% in the FDX2-based reaction (see Gervason et al. doi:10.1038/s41467-019-11470-9). This is expected to be even worse in vivo, as the sulfide ions produced by the reduction of NFS1's persulfide will freely diffuse outside of the complex and react with other cellular components. Indeed, a *fdx2* mutant is lethal in vivo in yeast, confirming that the thiol-based reaction is not operative in vivo to support Fe-S cluster synthesis. Thereby, we consider the thiol-based reaction as not physiologically relevant. Since we are studying the physiological process, we prefer not to discuss the thiol-based reaction in the present manuscript.

R4-rev: Resolved. I agree that reactions lacking one of the ISC proteins may indeed be quite artificial and of minor (no?) relevance for the in vivo situation. So, these data should not be taken too serious.

In Fig. 2 the FIDA and ITC techniques were used to quantitatively examine the competitive character of FXN and FDX2 at NFS1. Similar binding constants for the two proteins were found. It is stated that "FDX2 has displaced FXN from the (NIAU)2 complex" and vice versa. Is this really an active process or the result of dynamic (reversible) on and off binding to the overlapping NFS1 binding site known from cryo EM studies?

Answer: We thank the referee for raising this interesting question. An active process implies that FXN and FDX2 would interact directly with each other and bind simultaneously to the (NIAU)2 complex, at least transiently. We have performed and included an additional ITC experiment showing that FDX2 and FXN do not directly interact (new Extended Figure 5.c). Moreover, our FIDA data indicate that FXN and FDX2 do not bind simultaneously to the (NIAU)2 complex. As a result of this, we have updated our interpretation to state that this competition is a result of statistical occupancies of FXN and FDX2 to the same binding site relying on their respective KD and concentrations, as opposed to active displacement.

R4-rev: I accept that there is no detectable interaction between FDX2 and FXN. My question, however, was a kinetic (dynamic) rather than thermodynamic one. In other words, are the off rates of FDX2 and FXN high enough to explain the rates of synthesis? This is an important point for the model.

Rev-Answer: BLI analysis of the association and dissociation processes of the yeast homologues of FXN and FDX2 to the NIAU complex (Uzarska et al doi: 10.1016/j.jbc.2022.101570) showed that these interactions are dynamic and in the same range, with on and off rates of the range of 10^3 - 10^4 $M^{-1}.s^{-1}$ and 10^{-1} to 10^{-3} s^{-1} , respectively, thus sufficiently high to explain the rate of Fe-S cluster synthesis.

I wonder why the unphysiological Zn-ISCU2 was included in this experiment (NFS1 alone should suffice)?

Answer: There are two reasons for including Zn-ISCU2. First, FXN does not interact with the (NIA)₂ complex in the absence of ISCU2 (Figure 2f), NFS1 alone would thus not suffice, the presence of ISCU2 is needed to measure competitive binding. Second, the presence of a metal (iron or zinc) is needed to stabilize the ordered state of ISCU2. Under the aerobic conditions of the FIDA and ITC experiments, the Fe²⁺ ion in ISCU2 is readily oxidized and dissociates from the protein. We thus used Zn²⁺, which is redox stable, instead of Fe²⁺. The Zn²⁺ ion adopts the same coordination and geometry as Fe²⁺ in ISCU2 (Srour et al. JACS (2022) doi: 10.1021/jacs.2c06338) and enables persulfide transfer as Fe²⁺ does (Gervason et al. doi:10.1038/s41467-019-11470-9). Zn²⁺ is thus a good structural surrogate of Fe²⁺. We further note that Zn-loaded ISCU2 interacts with the (NIA)₂ complex with a higher binding affinity than apo-ISCU2, thus confirming that the presence of a metal is important (please see new Extended Figure 5.a-b and Extended Table 2).

R4-rev: OK, but the physiological ISCU-Fe would definitely be more convincing.

Rev-Answer: As an addendum to our previous answer, it is also important to highlight that Fe²⁺ has a much lower affinity for ISCU2 than Zn²⁺ which would lead to a partially metalated protein during interaction studies and therefore may introduce significant bias. As already mentioned, structural, spectroscopic and functional studies comparing Fe²⁺ and Zn²⁺ loaded ISCU2 all converge to the conclusion that Zn²⁺ is a reliable substitute for Fe²⁺, at least for persulfide transfer, we therefore remain confident that the binding parameters using Zn-ISCU2 cross-verified by FIDA and ITC represent a physiologically-relevant system.

Of importance, it was not made clear what form of FDX2, red or ox, has been used in Fig. 2? Previous studies from the Markley and Lill labs have shown that there are significant differences for the two redox states, and mainly the reduced state may be relevant here.

Answer: We thank the referee for asking this important question. In light of the referee's suggestion, we have implemented FIDA with the reduced form of FDX2. All the samples were prepared under anaerobic conditions in septum-sealed vials with dithionite to reduce FDX2 and keep it reduced all the way throughout the experiment. We checked by UV-visible that FDX2 remained reduced for the duration of FIDA experiments. The new results have been added to Extended Figure 3 and Extended Table 1, and discussed in the main text. We measured K_D values of 125 ± 13 nM and 144 ± 20 nM for oxidized and reduced FDX2 with the (NIAU-Zn)₂ complex, respectively, thus without any significant change within the margin errors. Equivalent K_D values for reduced/oxidized FDX2 and FXN is consistent with the results of our Fe-S cluster synthesis assays pointing to competitive binding at similar strength between the two proteins. In agreement with our data, the group of R. Dutkiewicz found similar binding curves for the oxidized and chemically reduced yeast Yah1 with both the NIA

and NIAU complexes, under aerobic and anaerobic conditions, respectively, by Biolayer Interferometry (BLI) (Uzarska et al doi: 10.1016/j.jbc.2022.101570).

In contrast, we note that the work from Markley's group comparing the binding affinity of oxidized and reduced FDX2 interacting with the (NIA)2 complex was unsuccessful by ITC (doi:10.1021/acs.biochem.6b00447). Lill's group reported KD values of reduced Yah1 ferredoxin from *Chaetomium thermophilum* with the (NIA)2 complex by microscale thermophoresis (MST) about 10-fold higher than for the oxidized form (Webert et al doi: 10.1038/ncomms6013). However, this higher value questions the mechanism of Fe-S cluster assembly, since the oxidized form would compete with the reduced one and thus strongly hamper persulfide reduction and Fe-S clusters formation. Furthermore, this value is not consistent with the competitive binding at similar strength we report here.

We note that a key difference between our studies is the labelling. While Lill's group labelled Nfs1-Isd11, we labelled FXN. Our FIDA and ITC data yielded KD for FXN of similar magnitude, indicating that FXN labelling for FIDA experiments on lysine residues does not interfere with binding to the NIAU complex. In contrast, we measured a much higher KD by FIDA than ITC for FDX2, indicating that FDX2 labelling perturbs the interaction with the NIAU complex. We thus used unlabelled FDX2 and FIDA competition assays where only FXN is labelled and FDX2 is a competitor of FXN, to measure the affinity of oxidized and reduced FDX2. Our ITC data confirmed that this approach is valid since similar KD values were obtained for oxidized FDX2 by FIDA competition assays and ITC. We can thus safely conclude that the labelling is not interfering in our analysis of oxidized and reduced FDX2 using this approach. Unfortunately, there was no control provided with another technique in Lill's study to ensure that the labelling did not interfere in their MST experiments. We thus prefer restraining from discussing their results in the present manuscript. Instead, we now refer to the work from Uzarska et al. They performed analysis using a tagged version of Yah1 which was confirmed to support activity in vivo alongside presenting no modification to the protein structure by CD.

R4-rev: Resolved.

In Fig. 3, several assays show that FDX2 interferes with persulfide generation and transfer to ISCU2. Likely, this is explained by the known fact that FXN supports sulfur transfer (see the not cited Ref doi.org/10.1038/s41467-024-47310-8), and, if FXN is present in excess, FDX2 cannot readily bind to the FXN-blocked NFS1, reduce the ISCU persulfide, and finally assist FeS cluster formation.

Answer: We agree with the referee, and this is our conclusion. However, acceleration of sulfur transfer by frataxin was not discovered in the non-cited reference doi: 10.1038/s41467-024-47310-8, but initially by Barondeau's group (Bridwell-Rabb et al. *Biochemistry* (2014), doi:10.1021/bi500532e) and our group (Parent et al *Nat. Commun.* (2015), doi: 10.1038/ncomms6686, Gervason et al. *Nat. Commun.* (2019), doi:10.1038/s41467-019-11470-9). We have added the citation of the initial paper from Barondeau's group and also the paper from Murphy's and Lill's groups as it provides the structural-basis of this process.

R4-rev: Resolved.

In fig. 4, the residues important for the (competitive) binding of FDX2 to NFS1 are analyzed. The authors find, in accordance to Refs. 9, 10, that some residues of helix F are important for FDX2 binding to NFS1 (ionic interaction). For two of the mutated FDX residues, the binding constants are weakened ca. 10 fold, but the FeS generation is hardly affected. How is this explained?

Answer: We thank the referee for raising this discussion. Indeed, two helix F mutants, E134A and D137A, are less affected than D138A and D141A in the presence of 10eq excess FXN, although all

these mutants have close-to or lower-than 10-fold affinities for the (NIAU)₂ complex when compared to WT FDX2. It must be noted that E134A is more robust than D138A and D141A to the competition with FXN, due to higher affinity for the (NIAU)₂ complex (Fig. 4c-d), which could explain that it is less affected. This is less clear for D137A, which has similar affinity as D138A. However, both E134A and D137A mutants are notably faster than WT FDX2 when in the complete absence of FXN (Fig. 4h), indicating lower repressive effects on the mobile loop of NFS1. This lower direct repressive effect than the D138A and D141A mutants should participate to higher efficiency in Fe-S synthesis, thereby leading to the special observation of lower effect on [2Fe-2S] cluster synthesis than D138A and D141A when in the presence of excess FXN, despite decrease in their overall binding affinity.

R4-rev: This answer means that we do not understand the role of the residues and the importance of the binding strength for the synthesis rate.

Rev-Answer: We reiterate that residue-specific contributions to the rate of [2Fe-2S] synthetic are more complex than just the binding strength via helix F, since there are two phenomena occurring at the same time: tight binding to the (NIAU)₂ complex via helix F, and an interaction with the mobile loop of NFS1. It is not unlikely that the mutants in the helix F domain also affects the conformation of FDX2 on the NIAU complex (either the proximal or distal conformation) which would, in turn, modulate the repressive effect on the mobile loop. Future studies may aim to evolve this question by determining the structure of the (NIAU-FDX2)₂ complex when carrying each of the aforementioned FDX2 mutations, which is out of scope for the present manuscript. Rather, we observe and describe a significant correlation between several FDX2 mutations on binding affinity, adoption of the proximal/distal conformation, location on either Helix F or the C-terminal tail, and the corresponding Fe-S synthetic rate. This allows us to make strong conclusions on the mechanism of regulation by FDX2, which may be further complemented by future structural studies.

For two other residues, the weakened affinity results in low FeS generation, which is suppressed by more FDX2, a nice finding showing that more FDX2 helps to compensate for reduced affinity.

Answer: We thank the referee for this positive comment.

The authors also analyzed the FDX2 C-terminus which according to recent cryo EM structures binds to NFS1. The affinities are hardly changed by the mutations, and all these point mutations work fine (or even better) in FeS generation. If I understand the results from Ref 10 well, their findings differ marginally (even though the individual residues mutated in each study differ). In my view, the role of the C-terminus does not become clear yet from both studies and another one suggesting a role for FDX2 vs. FDX1 specificity (10.1038/s41589-022-01159-4).

Answer: The study from ref 10 reports the effects of truncations of the FDX2 C-terminus as opposed to point mutations of key interacting residues in our case. The authors find that some of these FDX2 truncations (Δ C1, Δ C5, Δ C10) improve the rate of Fe-S cluster synthesis under 1:1 FXN/FDX2 ratios and slightly less with 2eq. excess FDX2, while the large Δ C12 mutation is similar to WT in both cases. Our study highlights the key interacting residues of the C-terminus of FDX2 (Y177A, D179A, H181A), which are included in the aforementioned Δ C5 (H181A), Δ C10 (Y177A, D179A, H181A), and Δ C12 (Y177A, D179A, H181A) truncations. The substitutions of these residues are indeed marginally better than WT at equal FDX2/FXN concentrations, significantly better in excess-FDX2 concentrations, and, critically, faster under conditions lacking FXN (i.e. recapitulating conditions of FRDA). It is not immediately clear why the complete removal of the last 12 amino acids from the C-terminus do not show enhancement of Fe-S cluster synthesis in ref10, but it is likely that such large truncation might have side effects, notably on the structure of the nearby Fe-S cluster. Our two studies thus seem

consistent with each other. We have added a sentence on page 10 to specifically cite this work. Importantly, our data show that these mutations do not significantly affect binding affinities (ITC and FIDA data) but specifically impact the repression on persulfide transfer between NFS1 and ISCU2 (ARBS data) and Fe-S cluster synthesis. We can thus conclude that the interaction between the C-terminus of FDX2 and the mobile loop of NFS1 mediates a direct repression on persulfide generation and transfer, possibly by decreasing the flexibility required for persulfide generation and transfer. We agree with the referee that complete truncation of the FDX2 C-terminus (Δ C12) was shown to be critical for yeast growth (as reported in ref 10.1038/s41589-022-01159-4). However, in light of the in vitro data showing that the Δ C12 is still active in [2Fe-2S] cluster synthesis, this suggests the involvement of FDX2 C-terminus in other cellular processes beyond [2Fe-2S] cluster synthesis, likely in the assembly of [4Fe-4S] clusters by the ISCA scaffold (ref 10.1073/pnas.2003982117), which is a key function for cellular Fe-S cluster biogenesis. The C-terminus of FDX2 might thus play different roles depending on its interacting partner.

R4-rev: The authors agree that the published studies from others and their findings are similar. However, upon re-reading the Steinhilper paper, I saw that these authors also analyzed a point mutant (residue 175, not studied here) that behaves similarly to the C12del rather than the wild type (and wild type is NOT similar to C12del as noted above), indicating that residue 175 is a decisive one, but overall the role of the C-terminus remains unclear.

Rev-Answer: The finding that an N175A mutant supports efficient [2Fe-2S] synthesis at high concentrations strengthens our discovery and conclusion that FDX2 directly modulates persulfide generation on NFS1 and subsequent persulfide transfer to ISCU2 via a mechanism exclusively involving its C-terminal tail. The finding that N175A is less optimal than WT FDX2 at low concentrations is not apparently clear, and was not discussed nor further explored in the corresponding paper by Steinhilper *et al.* However, a FXN-containing ISC system was used throughout all of their [2Fe-2S] assembly assays since the auto-inhibitory role of FDX2 had not yet been discovered nor characterized. Thereby, their conclusion on the importance of the N175A mutant is that of mediating the switch between the proximal and distal conformation, which is in line with the main discussion of their study. However, our ITC data shows that, for the range sampled in our study, C-terminal mutants do not alter proximal-distal binding thermodynamics, and our FIDA assays show that these mutants retain WT-like binding affinities. Critically, and to reiterate the important conclusion that we propose, we find that C-terminal mutants on FDX2 in a FXN-free ISC system is significantly more efficient than WT FDX2. This enhancement in [2Fe-2S] cluster synthesis can only be attributed to an increase in the rate of persulfide transfer, which is rate limiting when in the absence of FXN, and not due to altering the thermodynamics of binding causing any increase in the rate of persulfide reduction by FDX2.

As a follow up of these biochemical data, the authors shift to genetics in flies (I suggest to add the word *Drosophila* to the sentence "...we aimed to determine whether lowering Fdx1 levels could be protective in vivo."), where the fly homolog of FDX2, termed Fdx1, is found increased by 62% in mRNA levels in a FXN knockdown animal (unfortunately no validation by immunostaining).

Answer: We thank the referee for their suggestion. The word *drosophila* has been added to the sentence. Unfortunately, there is no antibody available against the *drosophila* Fdx1 protein. However, we tested an antibody directed against the human protein (sc-515121 from Santa Cruz Biotechnology), but it failed to recognise the *Drosophila* protein.

R4-rev: Resolved.

I was surprised by this result because according to the results here and in Refs 9,10, I would have expected a lower Fdx1 level to avoid inhibition of FeS synthesis. Interestingly, exactly this expectation is suggested by the depletion of Fdx1 in frataxin-deficient animals where an increased life span was observed, fitting to the view that optimal ratios of frataxin and Fdx1 are needed. Why the frataxin knockdown animals seem to regulate Fdx1 gene expression in the opposite direction, is not further explained.

Answer: We interpret the cellular increase in Fdx1 to arise as a normal physiological response to increase components of the ISC system in attempt to enhance Fe-S biogenesis during cellular conditions of low Fe-S biogenesis.

R4-rev: Well possible, but if excess Fdx1 would inhibit the synthesis rate, as suggested here, this would decrease FeS synthesis even further. The in vivo result therefore is more consistent with the idea that at least in flies, the proposed mechanism is less relevant.

Rev-Answer: The positive effect observed by knocking down Fdx1 in flies strongly supports the *in vitro* mechanism indicating that Fdx1 exerts a repressive effect on Fe-S cluster biosynthesis. We consider that the induced expression of Fdx1 under conditions of poor Fe-S cluster synthesis (due to a low level of frataxin) is an unintentional cellular maladaptive response in an attempt to upregulate components involved in Fe-S homeostasis. We reason that adaptive mechanisms able to counteract a sudden frataxin deficiency (i.e. by lowering Fdx1) have not been naturally selected. A more general response to low Fe-S synthesis which could be induced through environmental conditions such as variations in sources of iron and sulfur, and aiming to boost the machinery by increasing the

expression of ISC components is more likely to occur. **TEXTS REDACTED**

In the Conclusion, the increased life span of double depleted animals is suggested to be “a therapeutic axis for FA”. I wonder if, in Friedreich ataxia patients, one might consider reducing a critical component of FeS synthesis.

Answer: We agree that it may be challenging to target a critical component, although we note that other promising treatments within the field are facing a similar difficulty with this disease, especially gene therapy for which overdosage of FXN is toxic. We agree that we are yet distant from a treatment for FRDA, and we have thus softened our conclusions on the application of treatment to Friedreich's ataxia throughout the article.

R4-rev: I agree that we are far from using these results for therapy. Other regimes such as mimicking FXN's structural role in stimulating S transfer are more likely to help FRDA patients.

Rev-Answer: We agree that we are far from using these results for therapy, but not much further than the search for drugs mimicking FXN structural role in stimulating S transfer, unfortunately. Targeting FDX2 might be seen as an innovative complementary approach.

Overall, the study presents a nice confirmation and extension of earlier studies on this subject, suggesting that there is a competitive behavior of Frataxin and FDX2 for binding to NFS1, and close to stoichiometric amounts are needed for optimal FeS synthesis rates.

Answer: We thank the referee for their positive comments.

Further comments:

1. Introduction: "...it is unclear whether FXN operates directly or indirectly on Fe-S cluster biosynthesis". Do the authors mean "overexpressed FXN"?

Answer: Indeed, overexpressed FXN was intended. This has now been updated in the text.

R4-rev: Resolved.

2. Introduction: Correct typo: "Complimenting work on..." to "Complementing work on..."

Answer: This has been corrected.

R4-rev: Resolved.

3. Results: Ref. 11 does not contain any data on FDX2. The citation in the sentence "...compete with FDX2 for the same binding site on the complex between NIA and ISCU2 (denoted (NIAU)2), and cryo-EM structures^{10,11}" should be corrected here and in other places.

Answer: The references were misplaced, and we have thus corrected this. Ref 10 is needed here as it reports the first cryo-EM structure of FXN-bound (NIAU)2 complex. Combined with the cryo-EM structure of Ref 11, these data show that FXN and FDX2 bind to the same site.

R4-rev: Resolved.

4. Results: The authors state "FXN and FDX2 operate at the same strength on [2Fe-2S] cluster synthesis". I do not see how "same strength" can be concluded at this! stage. The authors do not use high enough FDX2 concentrations to make this conclusion here (only 2 fold FDX2 vs. 50 fold for FXN).

Answer: To complete our titration, we performed titrations at higher FDX2 ratios (5 and 10 equivalents, new Figure 1.c-d). The results confirmed that the effects of FDX2 and FXN are of similar magnitude, with an optimal ratio close to 1:1, consistent with nearly equal affinity constants for the (NIAU)2 complex as determined by FIDA and ITC.

R4-rev: The data are difficult to interpret for me. If I get it right, the same behavior is seen with 10 eq FDX2 and 50 eq FXN, i.e. a considerable difference for the two proteins.

Rev-Answer: We do not understand what the referee means by the same behavior is seen with 10 eq FDX2 and 50 eq FXN. It was not possible to test FDX2 at 50 eq, so we cannot answer whether the optimal rate is seen at a 1:1 ratio at these concentrations. Furthermore, at these very high protein concentrations there might be some side effects. Overall, the titrations show, within the margin errors, that the optimal rate is reached for FXN to FDX2 ratios very close to 1:1, which is in agreement with the very close K_D values of FXN and FDX2 for the NIAU complex. Altogether, these data support the idea that FXN and FDX2 operate at similar strength on the Fe-S cluster assembly process.

5. Another reference should be cited for this sentence: "FXN accelerates persulfide transfer from NFS1 to iron loaded ISCU2 (Fe-ISCU2) (Fig. 3b).^{12,14}" because this paper provides structural data on sulfur transfer (10.1038/s41467-024-47310-8).

Answer: This reference has been added.

R4-rev: Resolved.

6. Were the experiments in Fig. 2 and 3 performed under anaerobic conditions?

Answer: Experiments in Figure 3 (as in Figure 1) were performed under anaerobiosis, this is now specified in the text. Measurements in Figure 2 were performed under aerobiosis as already specified in the text, except for reduced FDX2 which was performed under anaerobic conditions (see New Extended Figure 3 and Extended Table 1).

R4-rev: Does this mean that the binding studies in Fig. 2 use oxidized FDX2, i.e. the entity that should leave the NFS complex?

Rev-Answer: The experiment reported in Fig. were performed with oxidized FDX2, except those on reduced FDX2. Our data clearly show that oxidized FDX2 bind to the NIAU complex with affinity in the same range. Therefore, there is apparently no mechanism such that the affinity of oxidized FDX2 would be lower than the reduced form to promote its dissociation once electron would have been transferred, as inferred by the referee.

R4-rev: Overall, the study has been improved, and in most parts agrees well with what we know from earlier studies on this subject.

Rev-Answer: We thank the referee for the positive comments. Our studies not only agree with other studies (thought submitted after we submitted our own manuscript), but also highlight a novel and complex antagonistic cross-regulation by FDX2 and FXN as well as a direct repressive effect of FDX2 (revealed in the absence of FXN). In addition, our studies in *Drosophila* indicate that this mechanism operates in vivo.