In this study, Camponeschi et al use NMR to characterize mitoNEET, a mitochondrial Fe_2S_2 protein. By using 1D NOE experiments, ^{13}C direct-detected experiments, and the optimization of NMR experiments for paramagnetic systems, the authors show significantly reduction of the "blind" sphere of the protein around the paramagnetic cluster, thus allowing the detection of residues possibly involved in the biological function of mitoNEET. The study has significant implications in the fields of paramagnetic NMR and FeS proteins. Some revisions are recommended.

We thank the reviewer for the comments and questions which allow us to better explain our work. We'll try to answer all the questions raised by the reviewer. Specifically:

1. I have some general questions about the mitoNEET protein I hope the authors can help answer.

a) If mitoNEET can repair Fe-S proteins by donating its own Fe_2S_2 cluster, how does it reacquire the Fe_2S_2 cluster? Can the authors comment on the source of its Fe_2S_2 cluster?

The source of mitoNEET cluster is still unknown. Ferecatu and coworkers (see Ferecatu et al., JBC, 2014, 289, 41, 28070-28086) demonstrated that the origin of iron and sulfur moieties required for mitoNEET maturation is mitochondrial, and that several components of the mitochondrial iron sulfur cluster (ISC) assembly and export machineries, such as ISCU, FXN, NFS1, HSC20, and ABCb7 are essential for the assembly of a [Fe_2S_2] cluster on mitoNEET, whereas early and late acting components of the cytosolic iron sulfur cluster assembly (CIA) machinery are not. However, the mechanism of mitoNEET cluster maturation was not clarified, and, to the best of our knowledge, a specific protein able to repair mitoNEET cluster has not been identified yet.

b) The redox states of mitoNEET are crucial for its function and stability. How are the redox states of mitoNEET regulated in cells?

Although *in vivo* studies addressing how the redox states of mitoNEET are regulated in the cell are still missing, several *in vitro* studies showed that mitoNEET clusters can be reduced by many cellular reductants. Indeed, mitoNEET redox state can be regulated *in vitro* by biological thiols such as reduced glutathione (GSH), L-cysteine, and *N*-acetyl-L-cysteine (Landry AP, Ding H, *J Biol Chem* 2014, 289, 4307–4315), human glutathione reductase (Landry AP et al *Free Radic Biol Med*. 2015, 81, 119–127), reduced flavin nucleotides (Landry AP et al. *Free Radic Biol Med.* 2017, 102, 240–247; Tasnim H et al. *Free Radic Biol Med* 2020, 156, 11–19) and human anamorsin (Camponeschi F et al *JACS*, 2017, 139, 9479–9482), while NAD(P)H and NADH are not able to reduce mitoNEET clusters (Landry AP, Ding H *J Biol Chem* 2014, 289, 4307–4315). A comment on this aspect will be added to the manuscript.

2. Some experimental details are needed.

a) For M9 media growth, how much $(^{15}NH_4)_2SO_4$ and ^{13}C-glucose were supplemented?

M9 media were supplemented with 1 g of $(^{15}NH_4)_2SO_4$ and 3 g ^{13}C-glucose per liter. These details will be added in the Materials and Method section.

b) What kind of anaerobic environment was used?

The protein was purified and handled inside an inert gas glove box, working with $O_2 < 1$ ppm. This is now specified in the Materials and Methods section.

c) Does the phosphate buffer contain any NaCl?

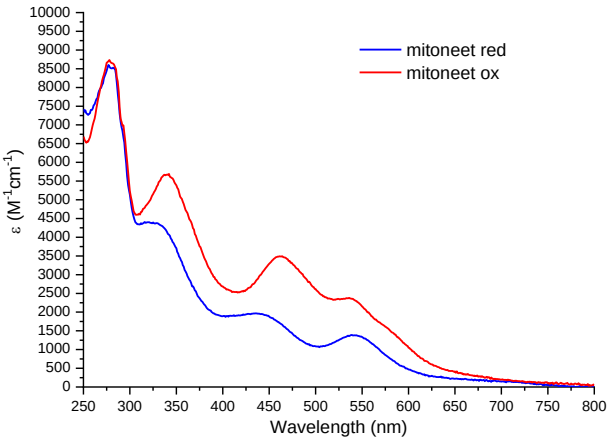
No, it doesn't

d) I assume there were additional steps to remove the extra $K_4Fe(CN)_6$ or sodium dithionite?

$K_4Fe(CN)_6$/sodium dithionite were removed after oxidation/reduction of the cluster using a PD10 desalting column. This detail has been added to the Material and Methods section. Thanks for the comment.

2) What's the Fe_2S_2: protein ratio 'as purified'? It would be helpful to include UV data to show the load of Fe_2S_2 on the protein in both redox states.

Non-heme iron and acid-labile sulfide quantification data (not reported in the manuscript) obtained for anaerobically purified mitoNEET following a previously published procedure (Banci L. et al. *Chem. Biol.* 2011, *18*, 794–804), gave 2.0 ± 0.1 Fe/mitoNEET and 1.9 ± 0.2 S^{2-}/mitoNEET (mol/mol of monomeric protein; error is the standard deviation of 4 measurements), meaning that we purified mitoNEET with ~ one $[Fe_2S_2]$ cluster per monomer. UV-visible data are reported here for the reviewer and will be later included in the revised manuscript, as suggested by the reviewer. ε values are based on monomeric protein concentration (determined with Bradford assay).



3) The authors purified the protein in an anaerobic environment, I assume it's because the Fe_2S_2 is susceptible to oxidative damage. Would addition of 10mM $K_4Fe(CN)_6$ to the protein solution damage the Fe_2S_2 cluster?

In order to avoid oxidation of the $[Fe_2S_2]^+$ clusters of mitoNEET or oxidative damage of the $[Fe_2S_2]^{2+}$ clusters of mitoNEET upon exposure to O_2, we worked in an anaerobic environment using an inert gas glove box. This ensured long term stability of mitoNEET $[Fe_2S_2]^{+/2+}$ clusters. Indeed, during the NMR experiments we didn't observe changes in the cluster-bound protons signals in the paramagnetic NMR experiments or changes in the HN amide backbone signals in the diamagnetic and paramagnetic ^1H-^{15}N experiments. Indeed, when the $[Fe_2S_2]$ cluster of mitoNEET is disassembled the protein undergoes a folded-unfolded conformational change and the HSQC spectrum of the protein changes significantly (Ferecatu et al., JBC, 2014, 289, 41, 28070-28086; Golinelli-Cohen et al. *J Biol Chem.* 2016, 291, 7583–7593). Such changes were not observed in the HSQC spectra of reduced or oxidized mitoNEET over a period of roughly 12 h, suggesting that the cluster is stably bound to the protein for all the NMR experimental time.

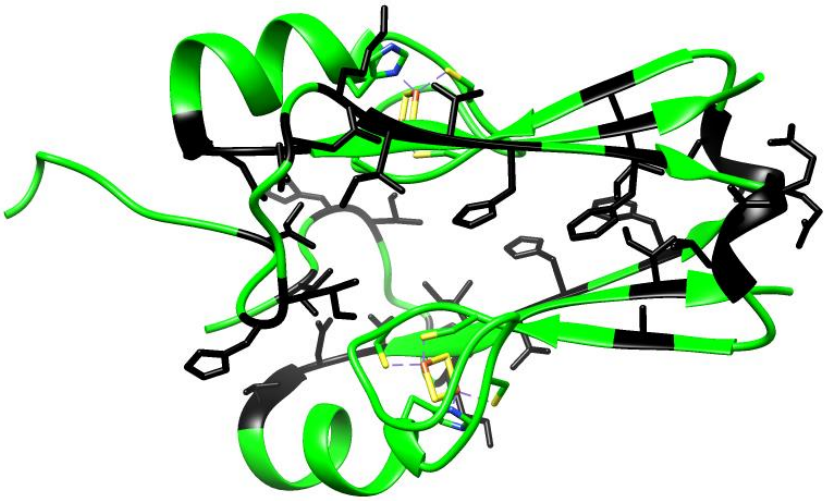
The same behavior was observed when 10 mM $K_4Fe(CN)_6$ was added to the protein solution and the removed by PD10. Indeed, it can be stated that damaging of the $[Fe_2S_2]$ cluster was not observed upon addition of $K_4Fe(CN)_6$.

4) Is the purified mitoNEET protein a homodimer as shown in Fig. 1A?

Yes, the protein was purified as a homodimer, as suggested by size exclusion chromatography data and by ^{15}N relaxation measurements. Indeed, the latter account for a τ_R value of 11.6 ± 0.8 ns, which is consistent with a dimeric state of the protein, whose molecular weight is ~18 kDa. The data will be added in appendix X in the revised version of the manuscript.

5) In Fig. 1A, can the authors highlight the residues that are affected by different redox states?

As suggested by the reviewer, we will modify figure 1 highlighting the residues affected by the different redox states. These residues belong to the inter-subunit region as pointed out also by the reviewer. Residues number involved in the redox switch are 45, 48, 49, 53, 55, 56, 57, 58, 60, 63, 64, 65, 69, 94, 95, 96, 97, 103. They are colored in black in the figure.



6) Fig 1B, how were the chemical shift differences between two redox states calculated?

The chemical shifts differences have been calculated using the following equation.

$\Delta_{HN} = ((\delta_H)^2 + (\delta_N/5)^2)^{1/2}$. In the revised version of the manuscript this will be included in the Materials and Method Section.

7) It's intriguing to me that the redox state change would mainly affect the regions involved in inter-subunit contacts. Do the authors have any hypothesis why?

We completely agree, it is very intriguing and interesting that the region affected by the redox state change is the inter-subunit one. Our hypothesis is that in order to perform its function mitoNEET has to switch between different conformational states, with the redox state change being one of the ways of regulating these transitions. Indeed, when mitoNEET passes from the "inactive", reduced state to the "active", oxidized state it might adopt a less tight conformation that facilitates the cluster transfer to IRP1 or to other apo recipient proteins, possibly driven by higher solvent accessibility of the cluster itself.

8) There is no mention of Fig. 1C in the text. The author might add some.

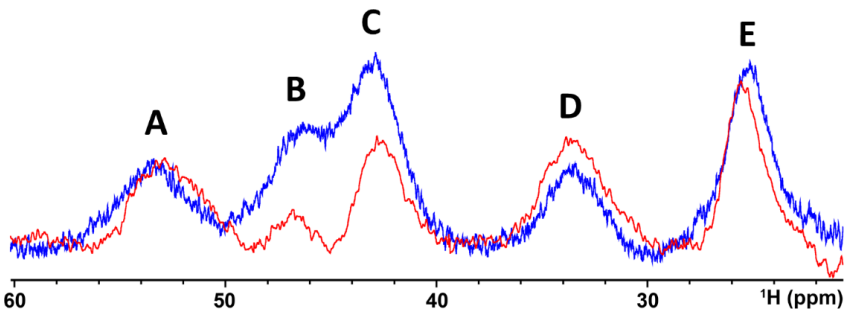
We will refer to Fig 1C in the manuscript according to the suggestion of the reviewer.

9) Can the authors provide some explanations why no hyperfine shifted signals were observed for the reduced $[Fe_2S_2]^+$-bound form of mitoNEET?

As reported in previous work (J Biol Inorg Chem. 2018; 23(4): 665–685), this a typical effect in mammalian $[Fe_2S_2]^+$, in particular in the case of the two irons ion pairs with delocalized valence.
This has been first described by J Markley and coworkers and interpreted as due to the fundamentally different patterns of electron delocalization observed, for reduced $[Fe_2S_2]^+$ centers in plant and vertebrate feredoxins (Skjeldal et al, Biochemistry. 1991; 30 (37), 9078-9083). When valence is delocalized, the iron ions have much slower electron spin relaxation rates than in the localized valence pairs, thus determining much broader lines often undetectable for ^1H signals and eventually detectable, as very broad signals, only by ^2H NMR measurements (Xia et al, Archives Biochem, Biophys, 2000, 373 (2), 328-334.)

10) The authors should provide the data showing the broadening of signal B collected in D_2O.

We report here for the reviewer the data showing the broadening of signal B in D_2O. The figure will be also added in the revised version of the manuscript.



11) The authors might want to highlight the additional residues assigned by ^{15}N-IR-HSQC-AP in the structure of mitoNEET.

Actually, IR-HSQC-AP and CON experiments pointed out a number of resonances, unobserved in the diamagnetic experiments, that belong to the residues in the proximity of the cluster. However, the sequence specific assignment of these resonances, requires a quantitative analysis of R_1 and R_2 H_N and H_C rates, a series of triple resonance experiments optimized to provide scalar connectivities, ^{13}C paramagnetic HSQC data and an “a-la-carte” analysis in order to identify the scalar and dipolar connectivities to confirm the assignment. This is beyond the aim of this work.

12) The labels in Fig.3 are too small to read, the authors might want to improve that.

We will provide a figure with improved and bigger labels. Thanks for the comment.