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Peer Review File

Manuscript Title: Structure of hepcidin-bound ferroportin reveals iron homeostatic mechanisms

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

N/A

Author Rebuttals to Initial Comments:

N/A

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The paper reports the structure of ferroportin, the only known cellular iron exporter which also functions as the receptor for the iron-regulatory hormone hepcidin. Importantly, the study determined not only the structure of the apo form of ferroportin but also the form bound to a metal substrate and the ligand hepcidin. This is a unique achievement that finally confirms how ferroportin binds metals, the way it interacts with its hormone ligand, and surprisingly, how the two processes are connected.

The study is important for two reasons. Ferroportin is a member of the Major Facilitator Superfamily (MFS), one of the largest classes of transporters found in most forms of life. MFS transporters are involved in many physiological processes and their dysfunction has been associated with many human diseases, thus they are of increasing interest as potential drug targets. Despite their importance, very few crystal structures of MFS members have been resolved, and almost none of them are based on human molecules. Thus, the current study will inform the entire field of transporter study.

Furthermore, structure of ferroportin and of its interaction with hepcidin is of specific interest for the field of iron disorders. Because the hepcidin-ferroportin interaction is the critical event for the regulation of iron homeostasis and it underlies the pathogenesis of iron disorders, having the structure of this complex is very important for the therapeutic targeting of these molecules, an area of significant activity at the moment.

Major comments:

1) Previously reported Ca++ interaction with FPN was not addressed in this manuscript, and should be incorporated when discussing both structural and functional aspects. How does proposed binding of Ca++ and its effect on the ferroportin transport function fits with the current findings? If authors disagree about the role of Ca++ in ferroportin function, they should indicate why.

2) Line 157: D39 coordinates Co++, it should be mentioned that this residue was reported to coordinate Ca++

3) If it is possible to do, it should be shown how the addition of calcium affects iron or cobalt transport in the calcein assay (calcein supposedly does not interact with calcium at physiological pH)

4) How does the addition of Ca++ in the RhoG assay affect hepcidin binding to FPN? What would be the structural explanation for how Ca++ binding affects accessibility of FPN central cavity for hepcidin

binding?

5) To address the specificity of the calcein assay, it would be important to test a metal that is not transported by FPN (Cu or Mn).

6) Figure 5. the authors need to further clarify their hypothesis for why the D325, C326 and H507 mutants cannot bind hepcidin but are still able to transport iron. The explanation is not sufficiently clear in its current form.

7) Figure 2d,e: if possible, it would be helpful to indicate where the intracellular gating residues are on the level of the whole molecule, for example using the view from Figure 2a and coloring the specific residues.

8) It should be described where RhoG is attached on hepcidin and how it fits in the Hepc-FPN interaction based on the new structure.

9) Comment on the possibility of ferroportin multimerization and whether the current study can inform us on the mechanism of ferroportin multimerization.

Minor:

1) Figure 2d, e. Consider using color other than red to highlight the residues that are mutated in FPN disease. The figure is just a tad too confusing this way, given that there are shades of red used for multiple things. Or at least use red font for those residues.

2) Figure 3 implies two metal-binding sites in FPN (N and C terminal site), but Figure 1 only shows one of them

3) Figure 1 is missing the label "b"

4) Supplementary figure 2, explain error bars. No statistics provided.

Referee #2 (Remarks to the Author):

The authors present cryo_EM structures of human ferroportin in the apo-state and in the Co2+- and hepcidin bound state. Although the structure by itself is not remarkable (MFS, very similar to previously determined structures of a bacterial homologue), the mode of interaction with hepcidin is new. The authors provide a novel hypothesis on how transport may be regulated by hepcidin, which may be useful future therapeutics.

I have a few issues that must be addressed:

- Nanodiscs. The NW11 and NW9 nanodiscs are used, but is is entirely unclear form the manuscript in which experiments each of the 2 is used. Since these nanodics can have large diameters, it would also be helpful if the can comment on the size of the particles used for structure determination: how much layers of lipids can surround the protein in the nanodiscs?
- The schematic presented in fig 5d seems inconsistent with the presented data. In the structure presented here, there are two sites occupied with Co2+. It is misleading to show only a single site in the righthand panel of fig 5d
- Related: There is another inconsistency in the proposed mechanism. The proposed regulatory site appears to have higher affinity for the transporter than the site near D39 (at least, that is what I understand from the MD simulations). If that is the case, Hepcidin would be able to bind in the absence of much transport. This needs to be explained/amended.

- Although Fab45D8 does not bind competitively with Hepcidin, it may affect the conformational ensemble. Does Fab45D8 affect transport activity? This can be tested easily using the assays presented.

Referee #3 (Remarks to the Author):

The study by Billesbolle et al. presents a high-quality structure of human ferroportin in an apo-state and a second structure bound to Co(2+) and the inhibiting endogenous hormone hepcidin. The two structures captured similar outward-open conformations of the transporter, but the degree of opening is a little larger in the hepcidin-bound structure given that the peptide hormone binds to the outward-facing cavity between the two halves of the transporter a little wider. The authors present a somewhat modest amount of biochemical data and some molecular dynamics simulations.

The structural studies are very nicely done and reach high resolution, which was accomplished with the use of a conformational Fab fragment binding a 3D epitope on the external side of ferroportin. The fold of the protein is not novel, as the protein looks very similar to the previously determined structures of the bacterial ferroportin homolog from the Nureki group (Taniguchi et al, 2015, a study describing both inward- and outward-open structures). Also, several results have been foreshadowed by the previous study, including the correct predictions of Fe(2+)-binding residues. I nevertheless find that the value of the having a high-quality structure of human ferroportin, in particular bound to divalent transition metal ions and hepcidin, brings in sufficient novelty to warrant publication in a general journal.
However, I do feel there are some biochemical experiments missing to strengthen the mechanistic conclusions, and there are some other issues to take care of:

1. Given that this is a structural/mechanistic study, the discussion and experimental probing of the transport mechanism is modest or even meagre. Is ferroportin a passive facilitatator or is there a driving force? Is liposome-reconstituted human FPN responsive to electrochemical gradients or at least sodium or proton gradients or membrane potentials? How does the study increase our insight into the transport mechanism?
2. Connected to the point above, Supplementary Fig. 1 details the (limited) transport studies the authors have conducted. They consist of a coupled fluorescence assay testing for passive diffusion of Fe(2+) or Co(2+) through FPN (downhill). In addition to the comments in point 1 above, what's missing here is how this system reacts to the conformational antibody fragment used for structural studies, as well as to hepcidin. For hepcidin, it would be critical to see inhibition if the hormone is enclosed in the liposome lumen (but not when added to the outside). For the Fab45D8, there might in fact be inhibition of transport if the conversion from outward-open to inward-open is prevented or slowed. Finally, there is no quantitation of the observed transport rate, nor is there proper quantitation of the reconstitution yield and transporter orientation. This type of data would greatly enhance the study. Instead, there is no need for schematics of columns (Suppl. Fig. 1a), I believe any reader interested in this paper will understand the sequential nature of a purification scheme.
Minor:
3. Suppl. Fig. 5a: I think ResMap is not the best program for visualizing the local resolution distribution. The reported local resolutions appear to be massively inflated, indicative of improper parameterization by ResMap. I doubt that there are large regions of the structure approaching 1.75Å resolution, as suggested by the coloring. I recommend that another package be used for local resolution estimation, such as the blocres function from Bsoft, or the windowed local FSC estimators within RELION or Cryosparc (anything but ResMap).
4. From the method section it is not clear if authors performed any kind of Bayesian polishing or CTF refinement, or if all frames from movies were taken into the processing. I am guessing that those jobs were performed (i.e. in Relion or CryoSPARC) but they are not described.
5. Probably it should be "imported into CryoSPARC" instead of "imported into RELION" in lines 590-591.

Author Rebuttals to First Revision:

Note: Author responses in black

We thank the referees for their careful consideration of our manuscript. Each of the three referees commented on key strengths of the work and cite the importance of this work to the broader understanding of iron homeostasis.

The referees' main concerns centered around interrogation of transport mechanism by ferroportin. Key considerations included a potential role for calcium, other ions, or pH in iron transport (Referees #1 & 3), effect of Fab45D8 (Referees #2 & 3), and whether hepcidin acutely influences iron transport (Referee #3). We have now performed a number of new experiments using ferroportin reconstituted into liposomes to address these concerns. Importantly, however, we believe a deep interrogation aimed at answering the atomic basis of iron transport remains outside the scope of this manuscript, which initially set out to describe how hepcidin binds to ferroportin, and how this binding influences iron homeostasis. An important caveat to our *in vitro* transport assays is that we cannot precisely control the orientation of ferroportin in liposomes.

As such, the observed changes in calcein fluorescence may reflect both forward and reverse transport directions. We have tried to control for this issue by altering the orientation of various perturbations, but believe this caveat precludes a definitive dissection of the transport mechanism.

We provide a point-by-point response to the referee comments below. Changes to the manuscript are highlighted as such: Revised or new text.

Referees' comments:

Referee #1 (Fe metabolism):

The paper reports the structure of ferroportin, the only known cellular iron exporter which also functions as the receptor for the iron-regulatory hormone hepcidin. Importantly, the study determined not only the structure of the apo form of ferroportin but also the form bound to a metal substrate and the ligand hepcidin. This is a unique achievement that finally confirms how ferroportin binds metals, the way it interacts with its hormone ligand, and surprisingly, how the two processes are connected.

The study is important for two reasons. Ferroportin is a member of the Major Facilitator Superfamily (MFS), one of the largest classes of transporters found in most forms of life. MFS transporters are involved in many physiological processes and their dysfunction has been associated with many human diseases, thus they are of increasing interest as potential drug targets. Despite their importance, very few crystal structures of MFS members have been resolved, and almost none of them are based on human molecules. Thus, the current study will inform the entire field of transporter study.

Furthermore, structure of ferroportin and of its interaction with hepcidin is of specific interest for the field of iron disorders. Because the hepcidin-ferroportin interaction is the critical event for the regulation of iron homeostasis and it underlies the pathogenesis of iron disorders, having the structure of this complex is very important for the therapeutic targeting of these molecules, an area of significant activity at the moment.

We thank the referee for highlighting the overall importance of this work, and its relation to the broader field of iron homeostasis.

Major comments:

1) Previously reported Ca++ interaction with FPN was not addressed in this manuscript, and should be incorporated when discussing both structural and functional aspects. How does proposed binding of Ca++ and its effect on the ferroportin transport function fits with the current findings? If authors disagree about the role of Ca++ in ferroportin function, they should indicate why.
2) Line 157: D39 coordinates Co++, it should be mentioned that this residue was reported to coordinate Ca++

We believe that the referee is referring to a recent manuscript (Deshpande CN et al *Nature Communications* 2018) that identified a calcium ion binding site within the N domain of the bacterial homolog bbFPN using a combination of structural, biochemical, and mutagenesis

studies. Mutagenesis studies on D39 and other residues in human FPN showed decreased

transport, which was ascribed to a calcium-dependent effect based on homology to bbFPN. We did not observe a density consistent with Ca^{2+} in an analogous position of human ferroportin, both for apo- and Co^{2+}/hepcidin-bound cryo-EM maps. In both cases, we did not explicitly incubate the sample with a physiological concentration of calcium prior to data collection; therefore, our ability to discuss the role of Ca^{2+} from our structural work remains rather limited. As the referee indicates here, one of the key residues (D39) previously proposed to coordinate Ca^{2+} instead directly binds Co^{2+} in our Co^{2+}/hepcidin-bound structure. We have now added some discussion on the previous study in the revised manuscript (lines 167-170) to highlight that the loss of transport observed previously likely directly resulted from perturbation of one of the key metal ion binding residues. We also test the effect of Ca^{2+} on reconstituted metal transport by FPN (outlined below).

3) If it is possible to do, it should be shown how the addition of calcium affects iron or cobalt transport in the calcein assay (calcein supposedly does not interact with calcium at physiological pH)

We have now obtained data from the calcein-transport assay for ferroportin reconstituted into liposomes in the presence of either external or internal $CaCl_2$. We observe very little effect of external $CaCl_2$. For $CoCl_2$ transport, we observed a decrease in both $_{max}$ and K_M when $CaCl_2$ was included inside liposomes. By contrast, $FeCl_2$ transport was potentiated by internal $CaCl_2$. The latter result is concordant with Deshpande *et al,* who reported that extracellular CaCl2 potentiates metal efflux. We are unable to account for the discrepancy in transport inhibition/potentiation between CoCl2 and FeCl2. A key caveat to the reductionist calcein assay is the use of synthetic lipids without other cellular membrane components. This new data is now provided in Supplementary Fig. 7, and the main text has been revised to include this discussion (lines 167-178).

4) How does the addition of Ca++ in the RhoG assay affect hepcidin binding to FPN? What would be the structural explanation for how Ca++ binding affects accessibility of FPN central cavity for hepcidin binding?

We have now obtained new data to query the effect of Ca^{2+} on RhoG-hepcidin binding. Unlike Fe^{2+} or Co^{2+}, $CaCl_2$ led to a small increase in RhoG-hepcidin affinity, and this effect titrated at millimolar concentrations of $CaCl_2$. This modest potentiation suggests that, unlike Co^{2+}, calcium is unlikely to directly coordinate an interaction between ferroportin and hepcidin. We have now included this data in Supplementary Fig. 7 and also discuss this in the main text of the manuscript (lines 292-294).

5) To address the specificity of the calcein assay, it would be important to test a metal that is not transported by FPN (Cu or Mn).

We have done several additional experiments to ascertain whether the calcein assay specifically measures metal transport by ferroportin. Empty liposomes show minimal calcein quenching with the addition of high concentrations of $CoCl_2$, while FPN-reconstituted liposomes show a saturable decrease in fluorescence. This experiment indicates that the liposomes are not leaky to divalent cations. We have assessed whether other divalent transition metals (Mn^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) yield responses in the calcein assay. We see responses for each, but Cu^{2+} itself yields an artifact and significantly quenches calcein fluorescence in liposomes without ferroportin. While Ni^{2+} and Zn^{2+} have previously been characterized to be ferroportin substrate, experiments in oocytes indicate that Mn^{2+} is not a ferroportin efflux substrate. Due to the lack of response seen for empty liposomes, we believe that Mn^{2+} quenching may indeed reflect ferroportin transport that is observed in the synthetic lipid mixture for this *in vitro* assay. This control data is now included in Supplementary Fig. 2. Our confidence that this assay truly measures metal transport by ferroportin is further increased by a direct inhibitory of hepcidin on transport activity, as shown in the revised Fig. 4b,c.

6) Figure 5. the authors need to further clarify their hypothesis for why the D325, C326 and H507 mutants cannot bind hepcidin but are still able to transport iron. The explanation is not sufficiently clear in its current form.

We thank the referee for this suggestion. We have now rewritten this section (lines 181-187) for clarity and hope the central message is now clearer.

7) Figure 2d,e: if possible, it would be helpful to indicate where the intracellular gating residues are on the level of the whole molecule, for example using the view from Figure 2a and coloring the specific residues.

This is a great suggestion to improve this figure. We have now provided a revised version of Fig. 2 to provide landmarks for the reader to better assess Fig. 2d,e.

8) It should be described where RhoG is attached on hepcidin and how it fits in the Hepc-FPN interaction based on the new structure.

We have now included a description of RhoG attachment to FPN in the Methods section (line 549) and have highlighted the attachment site in Fig. 4e.

9) Comment on the possibility of ferroportin multimerization and whether the current study can inform us on the mechanism of ferroportin multimerization.

The referee brings up an important set of observations in the ferroportin field that suggest ferroportin may multimerize; a key result is that certain mutations cause a dominant negative effect on iron transport. Our structures of ferroportin are monomeric, likely due to the use of detergent to extract the transporter from membranes for purification. We would prefer to refrain from speculation on ferroportin multimerization as our experiments do not tackle this issue in any direct manner and the manuscript is incredibly constrained for textual space.

Minor:

1) Figure 2d, e. Consider using color other than red to highlight the residues that are mutated in FPN disease. The figure is just a tad too confusing this way, given that there are shades of red used for multiple things. Or at least use red font for those residues.

We thank the referee for this suggestion. We have now modified the figure for clarity.

2) Figure 3 implies two metal-binding sites in FPN (N and C terminal site), but Figure 1 only shows one of them

We have now revised Figure 1 to omit a specific binding site for ferroportin, opting instead to simply show iron efflux by FPN. We prefer to lead with the primary data in Fig. 3 before proposing a model for iron binding sites in FPN.

3) Figure 1 is missing the label "b"

We have now fixed this oversight.

4) Supplementary figure 2, explain error bars. No statistics provided.

We have now carefully gone through the manuscript to provide explanations for error bars and statistics.

Referee #2 (transporter structure/function):

The authors present cryo_EM structures of human ferroportin in the apo-state and in the Co2+- and hepcidin bound state. Although the structure by itself is not remarkable (MFS, very similar to previously determined structures of a bacterial homologue), the mode of interaction with hepcidin is new. The authors provide a novel hypothesis on how transport may be regulated by hepcidin, which may be useful future therapeutics.

We thank the referee for highlighting the importance of this work and the novelty of the proposed interaction between iron and hepcidin.

I have a few issues that must be addressed:

- Nanodiscs. The NW11 and NW9 nanodiscs are used, but is is entirely unclear form the manuscript in which experiments each of the 2 is used. Since these nanodics can have large diameters, it would also be helpful if the can comment on the size of the particles used for structure determination: how much layers of lipids can surround the protein in the nanodiscs?

This is a very helpful suggestion. We have now explicitly included this information in each panel in the figures and on relevant axes for plotted data. Our studies use the NW9 and NW11 nanodiscs from Gerhard Wagner's lab, with diameters of either 9 or 11 nm, respectively. Ferroportin is roughly ellipsoidal and based on simple modeling using lipids from snapshots of MD simulations and the cryoEM maps of FPN in NW9 nanodiscs, we estimate 2-3 lipid layers between FPN and the nanodisc belt protein in the major axis, and between 5-6 layers in the minor axis. We have performed hepcidin binding experiments on FPN in both NW9 and NW11 discs and found no difference in hepcidin affinity, suggesting dynamics relevant to binding are unperturbed in NW9 discs. The reason for choosing NW11 for the FP assay is the enhanced binding response due to a larger delta in hydrodynamic radius.

- The schematic presented in fig 5d seems inconsistent with the presented data. In the structure presented here, there are two sites occupied with Co2+. It is misleading to show only a single site in the righthand panel of fig 5d

- Related: There is another inconsistency in the proposed mechanism. The proposed regulatory site appears to have higher affinity for the transporter than the site near D39 (at least, that is what I understand from the MD simulations). If that is the case, Hepcidin would be able to bind in the absence of much transport. This needs to be explained/amended.

This is another helpful suggestion and we agree that the manuscript provides little experimental evidence for a singly occupied metal binding site, both in the presence or absence of hepcidin. We have now revised the cartoon in Fig. 5 to fix this, with the hepcidin-bound state occupied with two iron molecules. We believe this change also addresses an implicit inconsistency mentioned by the reviewer here regarding relevant affinities of iron binding to the N and C domain sites. We have no direct evidence that either site is higher affinity, so only presume their relative transport/regulatory roles based on human mutations and experimental mutagenesis. The manuscript has been carefully combed to remove any speculation regarding relative affinity of the metal at either site.

- Although Fab45D8 does not bind competitively with Hepcidin, it may affect the conformational ensemble. Does Fab45D8 affect transport activity? This can be tested easily using the assays presented.

We thank the referee for spurring us to do this control experiment. We have now performed transport assays in the presence of a saturating concentration of Fab45D8, which revealed a decrease in Co2+ transport $_{max}$ and K_M. This suggests that Fab45D8 does indeed influence the ferroportin ensemble, but does not stabilize any given state to a significant extent as this would more profoundly inhibit transport activity. This new data is now included in Supplementary Fig. 2 and is mentioned in the main text (lines 111-114).

Referee #3 (cryo-EM, transport systems):

The study by Billesbolle et al. presents a high-quality structure of human ferroportin in an apo-state and a second structure bound to Co(2+) and the inhibiting endogenous hormone hepcidin. The two structures captured similar outward-open conformations of the transporter, but the degree of opening is a little larger in the hepcidin-bound structure given that the peptide hormone binds to the outward-facing cavity between the two halves of the transporter a little wider. The authors present a somewhat modest amount of biochemical data and some molecular dynamics simulations.

The structural studies are very nicely done and reach high resolution, which was accomplished with the use of a conformational Fab fragment binding a 3D epitope on the external side of ferroportin. The fold of the protein is not novel, as the protein looks very similar to the previously determined structures of the bacterial ferroportin homolog from the Nureki group (Taniguchi et al, 2015, a study describing both inward- and outward-open structures). Also, several results have been foreshadowed by the previous study, including the correct predictions of Fe(2+)-binding residues. I nevertheless find that the value of the having a high-quality structure of human ferroportin, in particular bound to divalent transition metal ions and hepcidin, brings in sufficient novelty to warrant publication in a general journal.

We thank the referee for a balanced, enthusiastic perspective on this work in the context of recent developments of the structural biology of iron homeostasis.

However, I do feel there are some biochemical experiments missing to strengthen the mechanistic conclusions, and there are some other issues to take care of:

1. Given that this is a structural/mechanistic study, the discussion and experimental probing of the transport mechanism is modest or even meagre. Is ferroportin a passive facilitatator or is there a driving force? Is liposome-reconstituted human FPN responsive to electrochemical gradients or at least sodium or proton gradients or membrane potentials? How does the study increase our insight into the transport mechanism?

Our main focus for this manuscript was to elucidate how hepcidin interacts with ferroportin, and how this is coordinated by the metal itself. We had provided transport data mostly to provide evidence that our protein preparations were functionally active. The referee's point, however, is well taken and we have performed a number of new experiments using the calcein quenching assay to start to dissect mechanisms of transport by ferroportin. We observe that, like the bacterial homolog bbFPN, transport by human FPN is dependent on proton gradients, but not a membrane potential driven by an opposing potassium gradient. This new data is provided in Supplementary Fig. 7. Consistent with prior reports in oocytes,

we observe an increase in iron transport in the presence of $CaCl_2$. We believe that further careful dissection of transport mechanism deserves sufficient space in a future manuscript to discuss rigorously controlled experiments connecting ferroportin structure and function.

2. Connected to the point above, Supplementary Fig. 1 details the (limited) transport studies the authors have conducted. They consist of a coupled fluorescence assay testing for passive diffusion of Fe(2+) or Co(2+) through FPN (downhill). In addition to the comments in point 1 above, what's missing here is how this system reacts to the conformational antibody fragment used for structural studies, as well as to hepcidin. For hepcidin, it would be critical to see inhibition if the hormone is enclosed in the liposome lumen (but not when added to the outside). For the Fab45D8, there might in fact be inhibition of transport if the conversion from outward-open to inward-open is prevented or slowed.

We thank the referee for spurring us to do these experiments. We have now performed transport assays in the presence of hepcidin and Fab45D8.

Our calcein quenching assay reports transport of metals into the liposome lumen either due to ferroportin transport in the forward direction (cellular efflux) or reverse direction (cellular influx). Ferroportin reconstituted with hepcidin within liposomes shows a clear decrease in transport activity. Because hepcidin binds the extracellular side of ferroportin, this result suggests that a large fraction of the calcein quenching signal arises from metal transport in the forward direction (cellular efflux) into the liposome lumen. By contrast, there is essentially no change in Co^{2+} transport when hepcidin is added to the external solution, suggesting that the calcein assay is relatively insensitive to transport in the reverse direction (cellular influx). Reconstitution of a precomplexed ferroportin-hepcidin complex shows inhibited transport activity that can be rescued by beta-mercaptoethanol, which is predicted to reduce hepcidin disulfides and thereby inactivate the hormone. These experiments provide further evidence that hepcidin can directly inhibit iron transport, which was recently proposed by Elizabeta Nemeth and colleagues (Aschemyer S *et al Blood* 2018). We have now included this data in Fig. 4b,c.

We have also now performed transport assays in the presence of Fab45D8, which revealed a decrease in Co^{2+} transport $_{max}$ and K_M, suggesting that Fab45D8 does indeed influence the ferroportin ensemble. This new data is now included in Supplementary Fig. 2 and is mentioned in the main text (lines 111-114).

Finally, there is no quantitation of the observed transport rate, nor is there proper quantitation of the reconstitution yield and transporter orientation. This type of data would greatly enhance the study. Instead, there is no need for schematics of columns (Suppl. Fig. 1a), I believe any reader interested in this paper will understand the sequential nature of a purification scheme.

This point is well taken. We now provide $_{max}$ and K_M values for iron and cobalt transport. For cobalt, we have quantified these parameters in the presence of internal $CaCl_2$ and Fab45D8 (main text and Supplementary Table 4). We have been unable to accurately determine transporter orientation, despite significant attempts to cleave a C-terminal tag and assess relative orientation by SDS-PAGE. However, the inhibitory effect of internal hepcidin in the calcein transport assay indicates that a reasonably large fraction of ferroportin is oriented with the extracellular side facing into the liposome lumen. We have also determined reconstitution yields by the BCA protein assay. This is now reported in the methods section. A qualitative assessment of FPN and Fab incorporation by SDS-PAGE is provided in Supplementary Fig. 2.

Minor:

3. Suppl. Fig. 5a: I think ResMap is not the best program for visualizing the local resolution distribution. The reported local resolutions appear to be massively inflated, indicative of improper parameterization by ResMap. I doubt that there are large regions of the structure approaching 1.75A resolution, as suggested by the coloring. I recommend that another package be used for local resolution estimation, such as the blocres function from Bsoft, or the windowed local FSC estimators within RELION or Cryosparc (anything but ResMap).

Local resolution estimates calculated through cryoSPARC, similar algorithm to blocres, have replaced those performed in ResMap. The methods have been updated to reflect this change.

4. From the method section it is not clear if authors performed any kind of Bayesian polishing or CTF refinement, or if all frames from movies were taken into the processing. I am guessing that those jobs were performed (i.e. in Relion or CryoSPARC) but they are not described.

We did not perform Bayesian polishing or CTF refinement on the final particle stack for the apo state. Instead, dose weighted sums outputted from UCSF version of MotionCor2 were used for all further image processing. Local CTF refinement was run for the hepcidin-bound state particle stack and the methods have been updated to reflect this.

5. Probably it should be "imported into CryoSPARC" instead of "imported into RELION" in lines 590-591.

Reviewer Reports on the Second Revision:

Referee #1 (Remarks to the Author):

The authors appropriately addressed all the comments by performing additional experiments and

reframing discussion.

Referee #3 (Remarks to the Author):

The authors have provided additional functional data that address my main concerns in a satisfactory manner. Additional biochemical data might allow more detailed mechanistic conclusions to be drawn, but I accept the authors' argument that pursuing these studies will likely take a lot of time. I now support publication of the paper.
There is a typo in the legend to Suppl. Fig. 2: Panel "d" is mentioned twice; The second time it should read "e". Further down, "e" should be replaced with "f".

Author Rebuttals to Second Revision:

N/A