THE
EMBO
JOURNAL

Structures of the heart specific SERCA2a Ca^{2+}-ATPase

Aljona Sitsel, Joren De Raeymaecker, Nikolaj Düring Drachmann, Rita Derua, Susanne Smaardijk, Jacob Lauwring Andersen, Ilse Vandecaetsbeek, Jialin Chen, Marc De Maeyer, Etienne Waelkens, Claus Olesen, Peter Vangheluwe, Poul Nissen

Review timeline:

Submission date:	10th Jun 2018
Editorial Decision:	18th Jul 2018
Revision received:	13th Nov 2018
Editorial Decision:	5th Dec 2018
Revision received:	29th Dec 2018
Accepted:	10th Jan 2019

Editor: Anne Nielsen / Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18th Jul 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript but they also raise a number of points that you will have to address before they can support publication here. In particular, while the referees all acknowledge that value in presenting the first crystal structure of SERCA2a, they would like to see additional experimental data to explain the different behaviour of SERCA1 and SERCA2. This is mainly focused on the MDS analysis where both refs #1 and #2 ask for experimental validation of (at least some) of the computational findings. In addition, ref #3 is concerned about the conclusiveness of the MD simulations and would like to see additional clarification. The referees would also like to see the PTM data more integrated with the rest of the study.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS:

Referee #1:

General Summary and Opinion:

The paper by Nissen and co-workers describes and compares crystal structures of SERCA2a to previously obtained SERCA1a structures that have been determined in most states of the transport

cycle. SERCA2a is a heart specific Ca2+-ATPase and is reported to bind Ca2+ with an higher affinity than SERC1a and has a lower turnover. The mechanistic basis for this difference is unclear and in this paper the comparison between the SERC2a and SERCA1a structures are used to dissect this.

Despite some amino acid sequence differences, however, the structures are highly similar. Rather than differences for the coordination of Ca2+ per se, the authors speculate if inherent dynamics differences could explain the kinetic differences between the two pumps. They conclude using MD simulations that their are indeed different hydrogen bonding networks being formed as well as differences in post-translational modifications as analysed by MS, which could explain the differences in the kinetic behaviour. Furthermore, it was shown before that a replacement of L7/8 in SERCA1a by the corresponding loop of SERCA2a alters the kinetic properties (Clausen et al, 2012). However, after making these swap mutants both proteins had a lowered Vmax, which although indicating these loops have been fine-tuned to the relative isoforms, does not shed any light into their differences in kinetic behaviour.

Overall, whilst I think this is a solid paper the conclusions reached in the abstract are not supported by experimental data, i.e., "We show that the isoform specific motifs of SERCA2a allow a distinct regulation by post-translational modifications and affect the dynamic behavior, which may explain specific properties and regulation". Whilst the authors have attempted to explain the kinetic differences, as yet they have not actually mechanistically explained this. From my point of view, the SERCA2a structures themselves are major achievements and the fact that these types of questions are being examined (as a result of the highly similar structures) is important step in the right direction. As such, I think it is unreasonable to expect that mechanistic differences can be explained in one paper. That said, I think its important that the paper does not "over-claim" mechanistic insights that are not supported by data that at this stage is mostly observational.

Major points:

1. The kinetic differences between SERCA1a and SERCA2a are reported to be due to differences in the affinity for Ca2+. However, as far as I am aware, the affinity differences between SECA1a and SERCA2a have not been measured directly. It would strengthen this paper if isothermal titration calorimetry (ITC) or an equivalent method was used to show that the binding affinities (Kd) were similar. This would be consistent with the high similar structures and put the current focus on examining the differences in dynamics and allosteric regulation on a firmer footing.

2. MD simulations were carried out to show the two pumps can form different hydrogen bond interactions. This result was used to imply they had different dynamic behaviour. Given that one has to assume the protonation state of the charged residues in the MD simulations how much confidence can be placed on this analysis? Is there any experimental data that can support the networks observed in the MD simulations as alternating dynamic behaviour? Furthermore, would it not be more straightforward to compare the average rmsd differences across the chains after a much longer time period, i.e., 1 us? That is, rather than assessing what networks are being formed can one see differences in their dynamic behaviour, i.e., breathing. The caveat should also be made clear that these MD simulations are being carried out in POPC membranes.

3. The list of identified PTM are not really integrated into this paper. Judging from Supplementary Fig. 3 it seems all the lysine residues shown to be new acetylation sites are conserved between SERCA1a and SERC2a. As such, it is unclear to me how differences in PTM could cause altered kinetic behaviour?

Minor points:

1. Please define in the methods the criterion or using the chosen relsolution cut-offs. Typically CC1/2 or CC* is used, but these are not listed in Table 1.

2. Structural imposition differences were calculated using Align in PyMol. Please add details as to the number of Caplha atoms that this superimposition is based on.

Non-Essential suggestions:

Naturally, the lipid bilayer is likely to shape the energetic landscape and ultimately the kinetic barriers (dynamics) separating the different states. Is it possible to functionally reconstitute SERCA2a and SERC1a into liposomes made from POPC and measure real pumping (not just ATP hydrolysis) and, if so, does one see differences in their kinetic behaviour ?

Referee #2:

Summary: This manuscript describes the first crystal structure of the cardiac isoform of the SERCA calcium pump, SERCA2a, in both the calcium bound and calcium free conformations. This is a very important contribution. The skeletal muscle isoform has been solved in dozens of enzymatic states, but not the cardiac isoform, so the study represents a welcome advance for the field.
Major concerns: None
Minor concerns:
1) A primary reason to determine the structure was to compare the structure/function relationships of the two isoforms. While the overall transport mechanisms are apparently conserved, there are important functional differences. In particular, the skeletal muscle isoform has lower affinity and faster turnover compared to the cardiac enzyme. These differences must be physiologically critical, as the relative expression of the two isoforms is highly tissue-specific. It is therefore somewhat disappointing that the investigators were not able to deduce a structural basis for the different functional properties. The challenge for such an analysis is that there are only modest differences in the structures, none of which suggest a mechanistic rationale for different affinity or transport kinetics. The authors hypothesize that the functional differences may be due to different relative regulation by post-translational modifications. They demonstrate by mass spectrometry that the two isoforms have significantly different PTM profiles, though they do not show that these different PTMs account for the functional differences. Moreover, PTMs are not observed as electron densities in the X-ray structure, so it may be that the modifications are not relevant for this study.
2) The authors offer the compelling suggestion that the isoform functional differences are due to different structural dynamics, rather than differences in the architecture that would be revealed in the single crystal structure. To test this, the investigators performed 50 ns MD simulations of the SERCA2a and SERCA1a structures in PC membranes and looked for differences in salt bridges and H-bond interactions. Interestingly, they found differences in the internal bond networks, and the unique bonds were largely attributable to isoform-specific residues. They infer that the different interactions would result in different structural dynamics for the two isoforms, which could affect transport function. Is it possible to demonstrate that the different bonding networks do indeed alter aspects of protein motion? In the MD simulations, did the investigators observe differences in RMSD of key regions? Did they see a different range of motion or different rates of motion between the isoforms? Was there a difference in the dynamic motion of calcium-coordinating residues in the membrane region that could explain the different stability (affinity) of the calcium bound states?
Overall this was a rigorous study that provides a long sought-after structure. While there are still unanswered questions, it is likely that the present structure will allow those puzzles to be solved in the future by the authors and by others in the field.

Referee #3:

The authors reported, for the first time, crystal structures of SERCA2a in H+-occluded [H2-3]E2-AlF4 and [Ca2]E1-ATP conformations. Technically, this study is of importance, as it for the first time shows that native SERCA2a can yield high quality crystals. Unfortunately, the structures are not new, with regard to the overall structure and domain arrangements, as compared to the previously determined structures of SERCA1a. The authors have made great efforts to discuss details and specific aspects of SERCA2a, which in part succeeded in highlighting interesting differences between the subtypes. However, some figures and tables are not well represented, and discussion on the different kinetics of these subtypes are not clear. Overall, this manuscript is appropriate for publishing, if the authors address following points.

Major comments
1. Authors describe "These SERCA2a specific regions may possibly play a role in the dimerization of SERCA2a in cardiomyocytes (Blackwell et al, 2016)". This is somewhat unlikely, because the

observed interaction in the crystal packing allows more than dimer, such as trimer, tetramer or higher oligomers. Any mutation or functional analysis for these region? Given that there are so many controversial studies on oligomeric state of P-type ATPases, I would not support this discussion, at least without any experimental evidence.

2. The authors performed MD simulation, using the [H+]E2Pi conformation as the starting model, to discuss different dynamics between the two subtypes. However this is not appropriate in this case, because dynamics, especially turnover rate, should be highly dependent on the interaction rearrangement during the transport cycle. Why was E2Pi selected for the MD simulation? In addition, the conclusion here - differences in the luminal loop interaction between the subtypes could affect dynamics - is very obscure. It seems that the SERCA2a subtypes have fewer luminal interactions as compared to SERCA1a. Is this difference possibly explaining the different conformational transition?

3. The authors describe "The results suggest that isoform specific residues alter intramolecular salt bridge and hydrogen bond interactions that affect the protein dynamics (Supplementary Table 3)". While salt bridge interactions during the simulation are presented in Supplemental Table 3, hydrogen bonding interactions are not presented, which makes it difficult to interpret Fig. 5, as it mainly shows hydrogen-bonding interactions.
Also, the Authors describe "In both isoforms, Gln965 (Gln966 in SERCA1a) of L9/10 is involved in hydrogen bond interactions with isoform specific residues of L7/8 (Glu895 and Asp861 in SERCA1a and Tyr894 and Gly860 in SERCA2a) with different interaction times (72% and 26%, respectively)", but readers cannot judge how much this difference could affect dynamics. Overall, Supplemental Table 3 is less informative, and it should be revised as to provide better information for the readers (Ex. It should include hydrogen bonding interactions as well).

Minor comments
1. Crystal packing of the [Ca2]E1-AMPPCP form should be shown, as the authors describe "In the [Ca2]E1-AMPPCP crystals, the TM regions of neighboring proteins interact in an antiparallel packing (i.e. not reflecting physiological contacts)". Only from this description, readers cannot understand how molecules are packed in the crystals.

2. Labeling for A, N and P-domains should be included in Fig.2 and also in Supplemental Figure 1 or its legend, to help better understanding for the non-specialists of P-type ATPase.

3. I do not understand the sentence, "We cannot exclude that specific interactions at the N-domain are Other SERCA2a regulators have been described (Vandecaetsbeek et al, 2009, 2011; Nelson et al, 2016; Anderson et al, 2015), but their interaction site are less well characterized", which is probably a typo.

4. In the abstract, authors describe "we present the first crystal structures of SERCA2a that were determined in the CPA-stabilized and H+-occluded [H2-3]E2-AlF4 - (3.3 Å) form, arranged as parallel dimers, and the Ca2+-occluded [Ca2]E1-ATP (4.0 Å) form", but they solved the structure in complex with AMPPCP, not with ATP. This should be described correctly, although its complex structure would be referred as the ATP-bound form.

5. Two different color codes of dotted lines (cyan and red) in Fig. 5 are not explained in the corresponding legend. What do these different lines mean?

6. Add appropriate citation for the description, "In addition, only in SERCA2 isoforms L7/8 serves as an acceptor site for the luminal extension of the SERCA2b Cterminus, indicating that L7/8 is important for the isoform specific properties".

1st Revision - authors' response 13th Nov 2018

Editor comments

As you will see from the reports, all referees express interest in the findings reported in your manuscript but they also raise a number of points that you will have to address before they can support publication here. In particular, while the referees all acknowledge that value in presenting the first crystal structure of SERCA2a, they would like to see additional experimental data to explain the different behaviour of SERCA1 and SERCA2.

- This is mainly focused on the MDS analysis where both refs #1 and #2 ask for experimental validation of (at least some) of the computational findings.

  We now included experimental observations that are in line with the MD predictions. We illustrated the functional relevance of several isoform specific residues that are predicted to form intramolecular interactions in the MDs. This provides strong support of our conclusions.

  First, we highlighted that three isoform specific residues that participate in intramolecular salt bridges or hydrogen bounds are functionally relevant. Indeed, two of these residues are sites of known Darier disease mutations (loss of function), while one residue, K956, leads to an impaired ATPase and Ca^{2+} transport activity when mutated.

  Second, we switched luminal loop L7/8 between SERCA1a and SERCA2a, which led to a loss of function in both isoforms. This is in line with the MD predictions that L7/8 forms salt bridges and hydrogen bonds with the neighbouring luminal loops L3/4 and L9/10 in an isoform specific manner. This adds credit to our claim that isoform specific intramolecular interactions are functionally important.

  Finally, we compared the dynamics of the two SERCA isoforms by assessing the root mean square deviations (RMSD) of atomic positions in various protein domains during the MD simulation. We noticed that some, but not all domains are more dynamic in SERCA1a as compared to SERCA2a, which may at least partially explain the faster conformational transitions in SERCA1a than SERCA2a, which were reported before.

- In addition, ref #3 is concerned about the conclusiveness of the MD simulations and would like to see additional clarification.

  We've now performed additional MD simulations (n=3), which allowed us to assess reproducibility and we included a detailed analysis of the RMSD of atomic positions in various protein domains. Moreover, we explained the purpose of the MD analysis and the implications more clearly and specified the limitations of the MD analysis in the discussion.

  The 50 ns MD simulations were not designed to simulate major conformational transitions in the catalytic cycle (which requires a completely different MD approach that is beyond reach of regular atomistic MD). Instead, our MDs were designed to look at intramolecular dynamics within one conformational state. This focus allowed us to compare the intramolecular interactions between the SERCA1a and SERCA2a E2 states, which are clearly isoform specific.

  The MD analysis predicts that isoform specific residues influence the network of intramolecular interactions, which we from a mechanistic point of view find very important. Moreover, we show that some of the residues in these networks have a direct functional impact. We therefore argue in the discussion that the isoform specific intramolecular interactions modify the rate of larger domain movements, which may be responsible for the observed kinetic differences between SERCA1a and SERCA2a. Also the analysis of RMSD values of atomic positions supports this view, since several cytosolic protein domains of SERCA1a appear more dynamic than in SERCA2a.

- The referees would also like to see the PTM data more integrated with the rest of the study.

  We have expanded and improved the PTM section. The purpose of the PTM section is to highlight residues in SERCA1a and SERCA2a that are sites of post-translational control. We observed that many reported PTMs take place on isoform specific residues, which leads to an isoform specific PTM fingerprint. Furthermore, by determining the PTMs in our purified SERCA1a and SERCA2a samples, we obtained experimental evidence that confirms that some PTMs are isoform specific. Thus, SERCA1a and SERCA2a clearly

present different post-translational modifications, which points to isoform-specific regulatory control.

Referee #1:

General Summary and Opinion

The paper by Nissen and co-workers describes and compares crystal structures of SERCA2a to previously obtained SERCA1a structures that have been determined in most states of the transport cycle. SERCA2a is a heart specific Ca^{2+}-ATPase and is reported to bind Ca^{2+} with a higher affinity than SERCA1a and has a lower turnover. The mechanistic basis for this difference is unclear and in this paper the comparison between the SERCA2a and SERCA1a structures are used to dissect this.

Despite some amino acid sequence differences, however, the structures are highly similar. Rather than differences for the coordination of Ca^{2+} per se, the authors speculate if inherent dynamics differences could explain the kinetic differences between the two pumps. They conclude using MD simulations that they are indeed different hydrogen bonding networks being formed as well as differences in post-translational modifications as analysed by MS, which could explain the differences in the kinetic behaviour. Furthermore, it was shown before that a replacement of L7/8 in SERCA1a by the corresponding loop of SERCA2a alters the kinetic properties (Clausen et al, 2012). However, after making these swap mutants both proteins had a lowered Vmax, which although indicating these loops have been fine-tuned to the relative isoforms, does not shed any light into their differences in kinetic behaviour.

Overall, whilst I think this is a solid paper, the conclusions reached in the abstract are not supported by experimental data, i.e., "We show that the isoform specific motifs of SERCA2a allow a distinct regulation by post-translational modifications and affect the dynamic behaviour, which may explain specific properties and regulation". Whilst the authors have attempted to explain the kinetic differences, as yet they have not actually mechanistically explained this.

From my point of view, the SERCA2a structures themselves are major achievements and the fact that these types of questions are being examined (as a result of the highly similar structures) is important step in the right direction. As such, I think it is unreasonable to expect that mechanistic differences can be explained in one paper. That said, I think it's important that the paper does not "over-claim" mechanistic insights that are not supported by data that at this stage is mostly observational.

We thank Reviewer 1 for the positive evaluation and critical remarks. We now tempered our conclusions in the abstract and manuscript to not over-claim mechanistic insights, and we separated experimental evidence from justifiable claims and hypotheses.

Major points:

1. The kinetic differences between SERCA1a and SERCA2a are reported to be due to differences in the affinity for Ca^{2+}. However, as far as I am aware, the affinity differences between SERCA1a and SERCA2a have not been measured directly. It would strengthen this paper if isothermal titration calorimetry (ITC) or an equivalent method was used to show that the binding affinities (Kd) were similar. This would be consistent with the high similar structures and put the current focus on examining the differences in dynamics and allosteric regulation on a firmer footing.

Reviewer 1 requests experimental proof that the kinetics of SERCA1a and SERCA2a are different, which would add credit to a focus on the changes in intramolecular dynamics as a mechanistic explanation for the isoform specific properties. Differences in the kinetic behaviour of SERCA1a and SERCA2a were already reported before (Clausen et al, 2012; Dode et al, 2003, 2002). However, the kinetic differences between SERCA1a and SERCA2a are not due to a change in the intrinsic Ca^{2+} affinity. The rate of Ca^{2+} dissociation in E1 (Ca^{2+} off rate) is remarkably similar for both isoforms; and the Ca^{2+} on rate during the E1 to E1Ca2 transition is most likely comparable. Indeed, SERCA2a displays a two-fold lower rate of the E2 to E1PCa2 transition as compared to SERCA1a, which is mainly explained by a slower E2 to E1 conversion. The comparable intrinsic Ca^{2+} affinity

of SERCA1a and SERCA2a is in good agreement with the similar E1-Ca2 structures (Clausen et al, 2012; Dode et al, 2003, 2002).

Instead, the higher Ca^{2+} affinity and lower turnover rate of SERCA2a are a direct consequence of an altered kinetic behaviour (Clausen et al, 2012; Dode et al, 2003, 2002). Compared to SERCA1a, SERCA2a presents slower conversion rates of E2 to E1, and E2P to E2 (Clausen et al, 2012; Dode et al, 2003, 2002). Thus, the different rates of conformational transitions are responsible for the altered biochemical properties, which puts the relevance of the molecular dynamics on a firmer footing. We now referred to these published kinetic differences at several points in the manuscript.

Since the different biochemical properties between both isoforms are not explained by a major change in the intrinsic Ca^{2+} affinity, we believe that additional experiments that compare the true Ca^{2+}-affinity of both isoforms may not add novel information. Instead, we now described the previously reported kinetic differences between SERCA1a and SERCA2a in the paper. This justifies our focus on the molecular dynamics of both isoforms as a mechanistic explanation for their distinct biochemical properties.

2. MD simulations were carried out to show the two pumps can form different hydrogen bond interactions. This result was used to imply they had different dynamic behaviour.

a) Given that one has to assume the protonation state of the charged residues in the MD simulations how much confidence can be placed on this analysis? The caveat should also be made clear that these MD simulations are being carried out in POPC membranes.

While SERCA is not very specific to particular lipids, we agree with Reviewer 1 that the presented network of intramolecular interactions may be affected by several parameters, such as the protonation state of the charged residues, the local ion concentrations or the membrane composition. However, the 50 ns MD simulations were designed to compare, with the same set of MD parameters, the intramolecular network of interactions between of SERCA1a and SERCA2a. Our studies represent a genuine justification of distinct, isoform-specific properties.

Testing multiple possible MD variables would fall beyond the scope of the current study. Even with altered parameters, our simulations would still be an approximation of the real molecular dynamics. Thus, instead of adding further MD runs with different parameters, we decided to discuss more clearly the scope of our MD study in the manuscript:

"The presented list of intramolecular interactions was obtained with a fixed number of MD parameters for SERCA2a and SERCA1a. Albeit a limitation of the study, this facilitates a direct comparison of the interaction network of SERCA2a and SERCA1a in the same conditions, which revealed isoform specific differences in the dynamic behavior of a subset of regions. However, the type and duration of intramolecular salt bridges and hydrogen bonds most likely depends on the local environment, which may differ for SERCA1a and SERCA2a in a cellular context where also interacting proteins, ions, PTMs and lipids are present."

b) Is there any experimental data that can support the networks observed in the MD simulations as alternating dynamic behaviour?

Reviewer 1 requested experimental data demonstrating that the observed networks in the MDs are affecting the dynamic behaviour. We therefore included experimental observations showing that isoform-specific interactions are functionally relevant, which reflects their impact on the kinetic properties and dynamic behaviour in the catalytic cycle.

1) Two isoform specific residues are sites of known Darier disease point mutations (loss of function mutations Leu32Pro/Phe and Thr982Met), while one other residue, K956, leads to an impaired ATPase and Ca^{2+} transport activity when mutated (Xu et al, 2008).

2) We focused on luminal loop L7/8, which is one of the regions with highest sequence variation between SERCA1a and SERCA2a. We switched luminal loop L7/8 between SERCA1a and SERCA2a, which led to a loss of function in both isoforms. This is in line with the MD predictions that L7/8 forms salt bridges and hydrogen bonds with the neighbouring luminal loops L3/4 and L9/10 in an isoform-specific manner. Our experiments on the L7/8 chimera therefore lends credits to our claim that isoform-specific intramolecular interactions are functionally important. The mild functional loss of the L7/8 chimeras are most likely caused by impaired intramolecular interactions that affect the overall dynamics and activity of the protein. Moreover, L3/4 is identical in SERCA1a

and SERCA2a, but the RMSD analysis suggests that its dynamic behaviour is isoform specific. This can only be explained by differences in the interaction with L7/8.

We've included these experimental observations in the results.

c) Furthermore, would it not be more straightforward to compare the average rmsd differences across the chains after a much longer time period, i.e., 1 us? That is, rather than assessing what networks are being formed can one see differences in their dynamic behaviour, i.e., breathing.

We thank Reviewer 1 for the suggestion to compare the RMSD values of both isoforms. We analyzed the root mean square deviations (RMSD) of atomic positions of various protein domains during the MD simulations. This allowed us to compare the dynamics of protein domains between the two isoforms. The RMSD values are similar for SERCA1a and SERCA2a for the N-domain and membrane region M6. However, the statistically different RMSD values for the A- and P-domains indicate that these domains are more flexible in SERCA1a than in SERCA2a. The higher flexibility may facilitate larger domain movements, possibly contributing to the faster conformational transitions in SERCA1a than in SERCA2a, which were reported before. This information is now included in the results section and in a new Figure 5.

Reviewer 1 also suggested to run longer MDs to follow larger dynamic movements of the protein. The purpose of these simulations would be to connect differences in the dynamics with the reported kinetic differences. However, our MD analyses do not intend to follow transitions of states that may simulate kinetic differences, but are merely performed for validation and justification of our central proposal that isoform-specific residues support different interaction networks, and therefore also most likely different kinetics. We are currently not in a position to provide the funding and computing time needed to extend our MD simulation at the scale requested.

Instead, we now repeated each 50 ns MD simulation (n=3), which renders a total simulation time of 150 ns for each isoform. This allowed us to assess the reproducibility of the simulations. We've now updated the Tables to include all H-bond and salt bridge interactions for each individual MD analysis, and we've calculated the average +-/ SD interaction times, which were used in the new Figure 6 to represent the isoform specific interactions.

We included the data of the additional MD runs and of the RMSD values in the results. We also improved the description of our rationale and included the limitations of our MD analysis.

3. The list of identified PTM are not really integrated into this paper. Judging from Supplementary Fig. 3 it seems all the lysine residues shown to be new acetylation sites are conserved between SERCA1a and SERCA2a. As such, it is unclear to me how differences in PTM could cause altered kinetic behaviour?

We now integrated the PTM section in the flow of the manuscript. The analysis of the PTMs points out that several conserved isoform specific residues in SERCA1a and SERCA2a are PTM acceptor sites. Indeed, many reported PTMs in the database take place on isoform specific residues, which leads to an isoform specific PTM fingerprint. Furthermore, by determining the PTMs in our purified SERCA1a and SERCA2a samples, we also experimentally confirmed that several PTMs are isoform specific.

Reviewer 1 is correct that many of the newly identified acetylated amino acids are identical in both SERCA isoforms. However, other PTMs take place on residues that differ between SERCA1a and SERCA2a, and are therefore considered as isoform specific PTMs: K572 in SERCA1a, and K476, K533 and S663 in SERCA2a. Based on this, we conclude that SERCA1a and SERCA2a undergo distinct post-translational modifications.

We therefore conclude that a subset of isoform specific amino acids may be dedicated to provide isoform-specific regulatory control. However, we don't want to claim that the reported or experimentally observed PTMs mechanistically explain the functional differences between SERCA1a and SERCA2a. While a functional role of the observed PTMs may be expected, it is currently unclear how they affect the functional properties of SERCA1a or SERCA2a.

These distinctions and rationale are now better explained in the results and discussion.

Minor points:

4. Please define in the methods the criterion or using the chosen resolution cut-offs. Typically CC1/2 or CC* is used, but these are not listed in Table 1.

The CC1/2 for the [Ca2]E1-AMPPCP structure is 0.991 (0.449) and [H2-3]E2-AlF4- structure is 0.996 (0.598). These are added to the Table 1.

5. Structural imposition differences were calculated using Align in PyMol. Please add details as to the number of Caplha atoms that this superimposition is based on.

The structural imposition differences were calculated using Super in PyMol (not Align). Root mean square deviation (r.m.s.d.) for Cα atoms between the SERCA2a and the corresponding SERCA1a structure in the E1-Ca^{2+}-AMPPCP state [as determined in by the operation 'super' in PyMol] is 1.73 Å (918 atoms) and between the E2-Pi-CPA SERCA2a and SERCA1a isoform r.m.s.d. is 0.89 Å (895 atoms). This information is now provided in the main text, and we thank the Reviewer for pointing that out.

Non-Essential suggestions:

6. Naturally, the lipid bilayer is likely to shape the energetic landscape and ultimately the kinetic barriers (dynamics) separating the different states. Is it possible to functionally reconstitute SERCA2a and SERC1a into liposomes made from POPC and measure real pumping (not just ATP hydrolysis) and, if so, does one see differences in their kinetic behaviour?

As highlighted before in comment 2a, several parameters will influence the MD behaviour of SERCA1a and SERCA2a, including the membrane environment. Testing multiple parameters in the MD simulation falls beyond the scope of the current study. Instead, we described the limitations of our MD analysis in the discussion.

We recently put a lot of effort in optimizing a protocol for reconstituting purified SERCA2a, however, our attempts were so far unsuccessful. This is unfortunate and somewhat surprising, since we were able to reconstitute other purified Ca^{2+} pumps like SERCA1a (Gorski *et al*, 2012), SPCA1a (Chen *et al*, 2017) and SPCA2 in parallel, but like crystallization it cannot be predicted if reconstitution will work or not for a given membrane protein.

Referee #2:

Summary:

This manuscript describes the first crystal structure of the cardiac isoform of the SERCA calcium pump, SERCA2a, in both the calcium bound and calcium free conformations. This is a very important contribution. The skeletal muscle isoform has been solved in dozens of enzymatic states, but not the cardiac isoform, so the study represents a welcome advance for the field.

We thank Reviewer 2 for the overall positive feedback and to consider our work as an important contribution to the field.

Major concerns:

None

Minor concerns:

7) A primary reason to determine the structure was to compare the structure/function relationships of the two isoforms. While the overall transport mechanisms are apparently conserved, there are

important functional differences. In particular, the skeletal muscle isoform has lower affinity and faster turnover compared to the cardiac enzyme. These differences must be physiologically critical, as the relative expression of the two isoforms is highly tissue-specific. It is therefore somewhat disappointing that the investigators were not able to deduce a structural basis for the different functional properties. The challenge for such an analysis is that there are only modest differences in the structures, none of which suggest a mechanistic rationale for different affinity or transport kinetics.

The authors hypothesize that the functional differences may be due to different relative regulation by post-translational modifications. They demonstrate by mass spectrometry that the two isoforms have significantly different PTM profiles, though they do not show that these different PTMs account for the functional differences. Moreover, PTMs are not observed as electron densities in the X-ray structure, so it may be that the modifications are not relevant for this study.

This comment overlaps with Comment 3 of Reviewer 1, which was already addressed above.

The goal of the PTM analysis was to investigate the roles of the highly conserved isoform specific residues in SERCA1a or SERCA2a. We agree that the reported differences in PTM fingerprints for SERCA1a and SERCA2a may not be sufficient to explain their functional differences. However, our PTM analysis shows that a significant subset of isoform specific residues serve as elements for isoform specific regulatory control. This points to an important role for some of the isoform specific residues, which is relevant to mention in the manuscript.

However, we explained that isoform specific residues not only provide regulatory control via PTMs, but also change the intramolecular network of salt bridge and hydrogen bond interactions, which most likely affect the dynamic behaviour and functional properties of the pump. The regulatory control by PTMs and intramolecular dynamics will together determine the isoform specific properties.

We modified the PTM section in the text to clarify the rationale behind our approach. We now also explained the implications of our findings.

8) The authors offer the compelling suggestion that the isoform functional differences are due to different structural dynamics, rather than differences in the architecture that would be revealed in the single crystal structure. To test this, the investigators performed 50 ns MD simulations of the SERCA2a and SERCA1a structures in PC membranes and looked for differences in salt bridges and H-bond interactions. Interestingly, they found differences in the internal bond networks, and the unique bonds were largely attributable to isoform-specific residues. They infer that the different interactions would result in different structural dynamics for the two isoforms, which could affect transport function.

a) In the MD simulations, did the investigators observe differences in RMSD of key regions?

We thank Reviewer 2 for this suggestion, which was also highlighted by Reviewer 1 (comment 2b). In short, we analyzed the RMSDs of various protein domains and noticed isoform specific differences in the dynamic behaviour of the A- and P-domains and luminal loops. The cytosolic regions are more dynamic in SERCA1a than in SERCA2a, which may facilitate larger domain movements. This may contribute to the kinetic differences such as the higher turnover rate of SERCA1a. This information and description is now included in the manuscript and in a new Figure 5.

b) Did they see a different range of motion or different rates of motion between the isoforms? Is it possible to demonstrate that the different bonding networks do indeed alter aspects of protein motion?

Compared to SERCA1a, SERCA2a displays a slower rate of E2P to E2 and E2 to E1 conversion (Clausen et al, 2012; Dode et al, 2003, 2002). As already highlighted earlier (Reviewer 1, comment 2b), our MD analysis was not designed to follow transitions of state that may simulate these kinetic differences. Such an MD analysis requires a very different and far more extensive approach and represents a dedicated project of the future. Instead, our current MDs offer insight in the intramolecular interaction network within one state that may be at the origin of larger scale dynamic differences. It seems that our simulation provides a rather static view of one particular state and does

not show major conformational transitions or larger protein motions. Our simulation time is too short to describe such aspects of protein motions.

We improved the description of our rationale and conclusions of the MDs. Also, the limitations of our MD analysis are now described in the discussion.

c) Was there a difference in the dynamic motion of calcium-coordinating residues in the membrane region that could explain the different stability (affinity) of the calcium bound states?

We simulated the non Ca^{2+} bound E2 conformation, so it is not possible to look at the Ca^{2+} coordination in this state. We selected the E2 form for the MDs, because of its higher resolution than the [Ca2]E1-AMPPCP state, which on the other hand we deem unfit for detailed analysis and simulations.

9) Overall this was a rigorous study that provides a long sought-after structure. While there are still unanswered questions, it is likely that the present structure will allow those puzzles to be solved in the future by the authors and by others in the field.

We appreciate this constructive feedback of Reviewer 2. To further address the impact of the isoform specific residues on the conformational transitions throughout the cycle, more advanced MD studies will be required in the future.

Referee #3:

The authors reported, for the first time, crystal structures of SERCA2a in H+-occluded [H2-3]E2-AlF4 and [Ca2]E1-ATP conformations. Technically, this study is of importance, as it for the first time shows that native SERCA2a can yield high quality crystals. Unfortunately, the structures are not new, with regard to the overall structure and domain arrangements, as compared to the previously determined structures of SERCA1a. The authors have made great efforts to discuss details and specific aspects of SERCA2a, which in part succeeded in highlighting interesting differences between the subtypes. However, some figures and tables are not well represented, and discussion on the different kinetics of these subtypes are not clear. Overall, this manuscript is appropriate for publishing, if the authors address following points.

We thank Reviewer 3 for this overall positive evaluation and for acknowledging the importance of our study.

Major comments

10. Authors describe "These SERCA2a specific regions may possibly play a role in the dimerization of SERCA2a in cardiomyocytes (Blackwell et al, 2016)". This is somewhat unlikely, because the observed interaction in the crystal packing allows more than dimer, such as trimer, tetramer or higher oligomers. Any mutation or functional analysis for these region? Given that there are so many controversial studies on oligomeric state of P-type ATPases, I would not support this discussion, at least without any experimental evidence.

Neighboring proteins in all reported SERCA1a crystal forms and also the current SERCA2a E1 crystal form interact in an antiparallel packing. Only the SERCA2a E2 crystal form is marked by an unusual parallel packing. We also find that only the dimer interaction fits a single membrane plane, while the higher order oligomers do not. We therefore maintain the note of this interaction being compelling, although we do acknowledge that it is too early to firmly conclude if these dimers also occur in a cell membrane or in cardiomyocytes; or whether the dimers are functionally different from the monomeric state.

We therefore rephrased this sentence: “These SERCA2a specific regions may play a role in the formation of SERCA2a dimers, which have also been reported in cardiomyocytes (Blackwell et al, 2016).”

11. a) Why was E2Pi selected for the MD simulation?

The E2Pi conformation was selected for the MD analysis because of its higher resolution than the [Ca2]E1-AMPPCP conformational state, which was only determined at 4 Å resolution and with problematic data quality for proper structural refinement. We included this rationale in the paper.

b) The authors performed MD simulations, using the [H+]E2Pi conformation as the starting model, to discuss different dynamics between the two subtypes. However this is not appropriate in this case, because dynamics, especially turnover rate, should be highly dependent on the interaction rearrangement during the transport cycle.

We addressed this remark earlier, which was also raised by the other Reviewers (Reviewer 1, Comment 2b; Reviewer 2, Comment 8).

c) In addition, the conclusion here – 'differences in the luminal loop interaction between the subtypes could affect dynamics' - is very obscure. It seems that the SERCA2a subtypes have fewer luminal interactions as compared to SERCA1a. Is this difference possibly explaining the different conformational transition?

This description was indeed too compact and therefore confusing. We now described the implications of our MD simulations and functional validation more clearly in the manuscript.

We focused on the isoform specific interactions involving two different luminal loops (new Figure 6), which may contribute to the functional differences between SERCA1a and SERCA2a. Indeed, the MDs predicted a distinct network of interactions in SERCA1a and SERCA2a between L7/8 and other luminal loops. Also, we show in a new Figure 5 that luminal loops are more dynamic in SERCA2a than in SERCA1a. Since L3/4 has the same sequence in both isoforms, the distinct dynamic behaviour should be a consequence of isoform specific interactions with neighbouring loops. The different dynamics of the luminal loops may be functionally relevant, since the L7/8 chimera present a loss-of-function phenotype. This is most likely a consequence of disturbed isoform specific contributions between L7/8 and other luminal loops. Presumably, these interactions affect the rates of conformational transitions and kinetic properties of the pump, leading to an impact on the functional properties.

12. The authors describe "The results suggest that isoform specific residues alter intramolecular salt bridge and hydrogen bond interactions that affect the protein dynamics (Supplementary Table 3)".

a) While salt bridge interactions during the simulation are presented in Supplemental Table 3, hydrogen bonding interactions are not presented, which makes it difficult to interpret Fig. 5, as it mainly shows hydrogen-bonding interactions.
Overall, Supplemental Table 3 is less informative, and it should be revised as to provide better information for the readers (Ex. It should include hydrogen bonding interactions as well).

We apologize for not including the Table with the H-bond interactions. This is a very long list, which is now included as a new Appendix Table S4.
We also included a new Figure (now Figure 6) that captures the isoform specific H-bonds and salt bridges between luminal loop L7/8 and other luminal loops L3/4 and L9/10. The isoform specific interactions between luminal loops explain well why exchanging L7/8 between SERCA1a and SERCA2a causes a functional loss.

b) Also, the Authors describe "In both isoforms, Gln965 (Gln966 in SERCA1a) of L9/10 is involved in hydrogen bond interactions with isoform specific residues of L7/8 (Glu895 and Asp861 in SERCA1a and Tyr894 and Gly860 in SERCA2a) with different interaction times (72% and 26%, respectively)", but readers cannot judge how much this difference could affect dynamics.

We modified this part of the manuscript and replaced the old Fig. 5 with a new Figure 6, which depicts the predicted interactions between isoform specific L7/8 residues and other luminal loops. For each salt bridge or hydrogen bond, the interaction time is depicted as % of the total simulation time, which illustrates the dynamics. Instead of Q965/Q966, we now focused on K958 in SERCA1a/Q957 in SERCA2a, because functional information of this residue is already available

(Xu et al, 2008). The mutation of K958 leads to a partial loss of function (Xu et al, 2008), showing its functional relevance.
In addition, we also compared the RMSDs of the luminal loops and observed isoform specific differences in the dynamic behaviour of some loops. We included this information in a new Figure 5.

Minor comments

13. Crystal packing of the [Ca2]E1-AMPPCP form should be shown, as the authors describe "In the [Ca2]E1-AMPPCP crystals, the TM regions of neighboring proteins interact in an antiparallel packing (i.e. not reflecting physiological contacts)". Only from this description, readers cannot understand how molecules are packed in the crystals.

An additional figure panel displaying the crystal packing of the SERCA2a E1 form is now included (Appendix Figure S1B). The antiparallel interactions are observed in all SERCA1a crystal forms.

14. Labeling for A, N and P-domains should be included in Fig.2 and also in Supplemental Figure 1 or its legend, to help better understanding for the non-specialists of P-type ATPase.

These labels are now indicated in Fig. 2C, D; while in Suppl Fig 1 we used different colours to depict the different domains (the colour code is explained in the legend).

15. I do not understand the sentence, "We cannot exclude that specific interactions at the N-domain are Other SERCA2a regulators have been described (Vandecaetsbeek et al, 2009, 2011; Nelson et al, 2016; Anderson et al, 2015), but their interaction site are less well characterized", which is probably a typo.

This is indeed a typo. It should say: “We cannot exclude that specific interactions with other SERCA2a regulators may be different for both isoforms (Vandecaetsbeek et al. 2009, 2011; Nelson et al. 2016; Anderson et al. 2015), but their interaction sites are less well characterized.”

16. In the abstract, authors describe "we present the first crystal structures of SERCA2a that were determined in the CPA-stabilized and H+-occluded [H2-3]E2-AlF4 - (3.3 Å) form, arranged as parallel dimers, and the Ca^{2+}-occluded [Ca2]E1-ATP (4.0 Å) form", but they solved the structure in complex with AMPPCP, not with ATP. This should be described correctly, although its complex structure would be referred as the ATP-bound form.

We apologize for this confusion and corrected the mistake in the abstract.

17. Two different color codes of dotted lines (cyan and red) in Fig. 5 are not explained in the corresponding legend. What do these different lines mean?

This Figure (now Figure 6) has been significantly modified and all the labelling information is now provided.

18. Add appropriate citation for the description, "In addition, only in SERCA2 isoforms L7/8 serves as an acceptor site for the luminal extension of the SERCA2b C-terminus, indicating that L7/8 is important for the isoform specific properties".

This part of the discussion was revised, but references on the role of L7/8 for the interaction with the luminal extension of the SERCA2b C-terminus are now included (Clausen et al, 2012; Vandecaetsbeek et al, 2009b).

References

Chen J, De Raeymaecker J, Hovgaard JB, Smaardijk S, Vandecaetsbeek I, Wuytack F, Møller JV, Eggermont J, De Maeyer M, Christensen SB, Vangheluwe P (2017) Structure/activity relationship of thapsigargin inhibition on the purified Golgi/secretory pathway Ca^{2+}/Mn^{2+}-transport ATPase (SPCA1a). *J. Biol. Chem.* **292:** 6938-51.

Clausen JD, Vandecaetsbeek I, Wuytack F, Vangheluwe P & Andersen JP (2012) Distinct roles of the C-terminal 11th transmembrane helix and luminal extension in the partial reactions

determining the high Ca^{2+} affinity of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2b (SERCA2b). *J. Biol. Chem.* **287:** 39460–39469

Dode L, Andersen JP, Leslie N, Dhitavat J, Vilsen B & Hovnanian A (2003) Dissection of the functional differences between sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) 1 and 2 isoforms and characterization of Darier disease (SERCA2) mutants by steady-state and transient kinetic analyses. *J. Biol. Chem.* **278:** 47877–89

Dode L, Vilsen B, Van Baelen K, Wuytack F, Clausen JD & Andersen JP (2002) Dissection of the functional differences between sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) 1 and 3 isoforms by steady-state and transient kinetic analyses. *J. Biol. Chem.* **277:** 45579–91

Gorski PA, Trieber CA, Larivière E, Schuermans M, Wuytack F, Young HS, Vangheluwe P (2012) Transmembrane helix 11 is a genuine regulator of the endoplasmic reticulum Ca^{2+} pump and acts as a functional parallel of β-subunit on α-Na^+,K^+-ATPase. *J. Biol. Chem.* **287:** 19876-85.

Vandecaetsbeek I, Trekels M, Maeyer M De, Ceulemans H, Lescrinier E, Raeymaekers L, Wuytack F & Vangheluwe P (2009b) Structural basis for the high Ca^{2+} affinity of the ubiquitous SERCA2b Ca^{2+} pump. *PNAS* **106:** 18533–18538

Xu C, Prasad AM, Inesi G & Toyoshima C (2008) Critical role of Val-304 in conformational transitions that allow Ca^{2+} occlusion and phosphoenzyme turnover in the Ca^{2+} transport ATPase. *J. Biol. Chem.* **283:** 3297–304

2nd Editorial Decision 5th Dec 2018

As you know Anne is leaving and she asked me to take over on your paper. We have now heard back from the two referees who both are happy with the revised version - their comments are provided below. They have a few suggestions for how to improve the presentations of the findings that should be easy enough to resolve. I am therefore very happy to let you know that we will accept your manuscript for publication here.

Before I can send you the formal acceptance letter would you please in addition to addressing the comments of the referees also resolve the issues below.

- We are missing a COI statement

- We are also missing ORCID ID for Claus Olsen

- I think figure callouts to 6AB, 7AB are missing

- The Appendix figures1-4 are uploaded as EV figures 1-4. If you wish to keep them as appendix figures you will have to create a separate appendix file with a TOC, figures and figure legends. If you wish to have EV figures - you can keep as is but change callouts to EV figure etc. See also our guideline to authors http://emboj.embopress.org/authorguide#expandedview.

- Please rename "Experimental Procedures" as "Materials & Methods"

- Please take a look at Figure 1A - is there a "cut" between E2 and E3? If so you should mark that with a white line.

- The appendix tables are very long and I think it might be best to use Excel to display this data. If you agree please label them Table XYZ and upload the files as datasets.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

- Our publisher has also done their pre-publication check on the manuscript and has made some

comments (see figure legends). Please see attached word document. Would you please incorporate their changes when you submit the revised version.

You can use the link below to upload the revised files.

That should be all - congratulations on a nice study!

REFEREE REPORTS:

Referee #1:

In the previous version I expressed a number of concerns related to the conclusions made regarding the kinetic differences between SERC1a and SERCA1a pumps based on a comparison of structures and MD simulations.

Whilst the modified version still cannot fully explain these differences, I think the authors have done a thorough revision to get the balance right between clear conclusions and conjecture. I think the paper is of high quality and will be well appreciated.

I have no further technical concerns.

Referee #3:

The authors have addressed a large part of my comments, and the manuscript has been substantially improved in the revised version. Especially, Fig. 5 would provide valuable information for readers. I have a few minor comments.

"high percentage of the isoform specific residues participate in such interactions: 15.6% in SERCA1a and 12.5% in SERCA2a for salt bridges, and 36.9% of SERCA1a and 53.1% of SERCA2a for hydrogen bonds"
I cannot judge whether these percentages are high or not, only from this information.

The authors added hydrogen bond interactions during the MD simulation in Appendix Table S4, probably according to my comment, but such a long list is not kind to the readers. Is it possible to revise this table with a simple plotting representation on the structure (ex. only SERCA2a unique interactions are shown as the line representation on the structure)? The authors could leave this table as in the current form, but I strongly recommend further improvement. Otherwise, the readers cannot easily accept the authors' conclusion.

"Only the SERCA2a E2 crystal form is marked by an unusual parallel packing."
Crystals of P-type ATPases are usually obtained in the type-I form, namely with the HiLiDe method, in which proteins are in the lipid membrane-like environment. My own perspective is that the preferred anti-parallel packing of the P-type ATPases are probably due to their asymmetric molecular shapes. The N-, P-, and A-domains constitute a large portion of the molecule, which is larger as relative to the TM region, and thus prevents tight packing of the molecules if in the same orientations within a layer.
According to the method section, the authors did not add any lipids this time, and the obtained crystals are likely to be in the type-II form, in which proteins in the detergent micelles are packed by the interactions at the outer-membranous regions. This is also supported by the crystal packing of the [Ca2]E1-AMPPCP form. In this crystal, two molecules are included in one detergent micelle in an anti-parallel manner, and the transmembrane region of the dimer is discrete from that of the adjacent dimers. I agree that the crystal packing of the E2-AlF4-CPA form is unusual, but this is probably because this crystal also belongs to the type-II crystal. With the TM region covered by the detergent micelle, molecules do not prefer anti-parallel arrangement. Considering this situation, I still do not support the hypothesis that the observed interactions in part reflect dimeric interaction in the membrane. This is my personal perspective, and the authors could leave this discussion, but I feel that too speculative discussion might lead science to a wrong directions.

2nd Revision - authors' response 29th Dec 2018

Reviewer comments (just for Referee #3)

"high percentage of the isoform specific residues participate in such interactions: 15.6% in SERCA1a and 12.5% in SERCA2a for salt bridges, and 36.9% of SERCA1a and 53.1% of SERCA2a for hydrogen bonds" I cannot judge whether these percentages are high or not, only from this information. Left as is

The authors added hydrogen bond interactions during the MD simulation in Appendix Table S4, probably according to my comment, but such a long list is not kind to the readers. Is it possible to revise this table with a simple plotting representation on the structure (ex. only SERCA2a unique interactions are shown as the line representation on the structure)? The authors could leave this table as in the current form, but I strongly recommend further improvement. Otherwise, the readers cannot easily accept the authors' conclusion. We have transferred the material to excel sheet data sets, as suggested

"Only the SERCA2a E2 crystal form is marked by an unusual parallel packing." Crystals of P-type ATPases are usually obtained in the type-I form, namely with the HiLiDe method, in which proteins are in the lipid membrane-like environment. My own perspective is that the preferred anti-parallel packing of the P-type ATPases are probably due to their asymmetric molecular shapes. The N-, P-, and A-domains constitute a large portion of the molecule, which is larger as relative to the TM region, and thus prevents tight packing of the molecules if in the same orientations within a layer. According to the method section, the authors did not add any lipids this time, and the obtained crystals are likely to be in the type-II form, in which proteins in the detergent micelles are packed by the interactions at the outer-membranous regions.

Egg yolk PC lipids are added (see Materials & Methods) and we consider the crystal packing to be regular type I.

This is also supported by the crystal packing of the [Ca2]E1-AMPPCP form. In this crystal, two molecules are included in one detergent micelle in an anti-parallel manner, and the transmembrane region of the dimer is discrete from that of the adjacent dimers.

Lipid-detergent bilayers in type I crystal packing can be quite wavy as observed for the E1 form and also several SERCA1a crystal forms

I agree that the crystal packing of the E2-AlF4-CPA form is unusual, but this is probably because this crystal also belongs to the type-II crystal. With the TM region covered by the detergent micelle, molecules do not prefer anti-parallel arrangement. Considering this situation, I still do not support the hypothesis that the observed interactions in part reflect dimeric interaction in the membrane. This is my personal perspective, and the authors could leave this discussion, but I feel that too speculative discussion might lead science to a wrong directions.

We write:

However, the E2-AlF$_4$-CPA crystal form is marked by an unusual parallel packing of SERCA2a molecules involving contact points between the A- and N-domains and between the A- and P-domains, as well as N-domain and L7/8 (Figure EV1). These SERCA2a

specific regions may play a role in the formation of SERCA2a dimers, which also have been reported in cardiomyocytes (Blackwell et al, 2016). No such parallel packing modes have been observed for SERCA1a crystal forms, and many of the involved residues differ for SERCA2a and SERCA1a, and are conserved within one isoform.
– i.e. we merely mention what we observe and what other have observed earlier. We do not embark any extensive discussions on these observations, so we leave the text as is.

All requested editorial changes were made.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Nissen, Poul; Vangheluwe, Peter; Olesen, Claus
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2018-100020

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The purified SERCA2a protein used in this study represents a pooled sample obtained from a minimum of three individual pig hearts. A minimum of three independent measurements on separate cell fractions were performed for the assessment of SERCA activity in COS microsomes. Each biological replicate was measured three times as technical replicates. (final number: 3 biological replicates, with 3 technical replicates of each). The MD simulations were performed 3 times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For the COS microsomes, we only included samples that successfully expressed SERCA2a in equal amounts as determined by Western blotting (up to 30% deviation was tolerated). A few technical replicates were exluded from the final analysis due to pipetting errors. These were pre-established criteria.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The normal distribution of the data was confirmed by Q-Q plot analysis. Results of the Ca2+ dependent ATPase measurements were fitted with the Hill function using Origin 8.0. One-way ANOVA with a Bonferroni post-hoc test was used for establishing significance.
Is there an estimate of variation within each group of data?	Yes. Standard deviation was assessed for the technical repetitions of each biological replicate. Standard error of the means was used to estimate variation between biological replicates.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Expression levels of SERCA1a and SERCA2a constructs were tested by Western Blot with a non-isoform specific SERCA TRY2 antibody (Mountian, Irina; Baba-aı, Fawzia; Jonas, Jean-christophe; Smedt, Humbert De; Wuytack, Frank; Parys, Jan B, Expression of Ca(2+) Transport Genes in Platelets and Endothelial Cells in Hypertension; Hypertension, 2001. Jan;37(1):135-141. The presence of phospholamban in the sample was tested with the anti-PLB antibody (Merck, catalog number 05-205)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	COS-1 cells were from Sigma-Aldrich. Catalog number: 88031701, Lot number: 06B009. The cells were frequently tested for mycoplasma contamination in-house, and no mycoplasma contamination was observed.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No experiments were conducted on living animals, and no animals were sacrificied for this project. Isolated hearts from non-genetically modified and non-treated control pigs (Sus scrofa) were only obtained from animals that were sacrificied for other purposes: pig hearts were collected from the slaughter house (Danish Crown in Horsens, Denmark) or obtained from the department of cardiovascular sciences in the framework of other research projects for which ethical approval was obtained by Dr. K. Sipido (KU Leuven, Belgium, ECD N° P176/2014). Pigs were housed according to local legislation. Animal age and gender were random.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No experiments were conducted on living animals.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	rSERCA1a: Genbank, GeneID: 100037716, Uniprot: UniProtKB - P04191, PDB: 5MPM pSERCA2a: Genbank, GeneID: 11938, Uniprot: UniProtKB - P11607, PDB (6HXB).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Acknowledged.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Acknowledged.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	The computational model that was used is the Force field in the GROMACS program, which is publicly available. The results that originated from this model are the MD runs, which are provided in PDB format. These PDB files were used to analyze the salt/H-bridges and RMSDs. The PDB files will be provided upon request.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA