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Cryo-EM structure of the human NKCC1 transporter reveals mechanisms of ion coupling and specificity

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **18th Nov 2021**

Dear Dr Nissen,

Thank you for submitting your study, "Cryo-EM structure of the human NKCC1 transporter reveals mechanisms of ion coupling and specificity " (EMBOJ-2021-110169), to The EMBO Journal. I have now read and discussed your manuscript with my editorial colleagues and we have also consulted an external expert closer to the field. Overall, I regret to say that we have decided that we cannot offer publication.

Given the general relevance of cation:chloride co-transporters, we agree that mechanistic insights into transporter activity in this family are generally of interest to the readership of The EMBO Journal. However, the editorial team and I have reluctantly decided that, despite the insights afforded by the high resolution of your structure, the similarity of the inward-occluded state of the protein to the MhsT structures you published with us recently precludes findings with the level of novelty necessary for publication here. This concern was shared by our expert advisor, who further stated:

'The NKCC1 structure is in an inward-occluded state and to a higher resolution and the authors have been able to reach some conclusions regarding the likely pathway for Na+- intracellular release, which follows on from their work on a LeuT homologue MhsT (published in EMBO J. fairly recently). With the higher resolution achieved here, that can make some "firmer" statements regarding Cl- release compared to Na+ release. Unfortunately, however, it's the same conformation as previously published structures and the ion-binding site locations are the same as what could be seen in published structures. To analyse the ordering of ion-binding events we really need a structure in the opposite, outward-facing conformation. Even then, I think one must be careful to draw too much conclusions based solely on a static crystal structure and one must combine the structure with other methods to assess ion-binding, such as fluorescent-sensitive probes'

While we realize that the study may likely be a valuable contribution to a more structural journal at the present stage, you may also consider transfer to our broad sister venue Life Science Alliance.
Life Science Alliance is in principle interested in publication of the manuscript and would be pleased to have your manuscript in its current form assessed by referees. Please use the following link for transfer; no reformatting is required.

I am sorry not to be able to give you more positive news.

Kind regards,

William Teale

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Dear William
I'm sorry to hear of this decision and I will actually challenge it (what I rarely do)
I do not agree with the arguments.
The earlier structures do not include Na+ (too low resolution or poor maps to see, apparently) and they are therefore concluding wrong things (or being inconclusive) regarding the functional state and mechanistic coupling. Furthermore, the structures are poorly analysed (for some) or missing key points regarding the functional implications that can be drawn from it. We point out very important aspects of the Na+ driver site and the Na+ release pathway in Na+ dependent SLC12 transporters that are very important in molecular cell biology. Furthermore, we point to very important aspects of the Cl2 site in the SLC12 family as integral to funtion (conserved in all members and with analogy to a conserved Glu in SLC6 transporters) - this invokes important models of chloride feedback on function, also in the members that only transport one Cl- (from the Cl1 site).
So yes, earlier structures probably represent the same state, but they missed it, and I think the field clearly needs a proper and visible report on the NKCC1 structure and what it tells us on SLC12 function in view of that. I'm sure it will be received with great interest, so I urge you to reconsider this fine work for a review at EMBO J.

As a PS: our work is obviously not based on 'a static crystal structure', but cryoEM maps. Furthermore, we are not discussing ion binding events (which would of course require studies of an outward-oriented state), but the exact opposite, namely the cytoplasmic ion releasing events. I hope you will reconsider in light of such misunderstandings in the advise.
Furthermore, our MhsT work represents a different transporter family (SLC6 - no sequence similarity to NKCC1, only fold), and our analysis properly and elegantly combines the two, which was also missed in the earlier reports

Poul

Dear Poul,

Thank you again for the submission of your manuscript (EMBOJ-2021-110169) entitled "Cryo-EM structure of the human NKCC1 transporter reveals mechanisms of ion coupling and specificity". We have now received the reports from three referees, which I have copied to the bottom of this message.

As you can see, the reports consistently state that the work is based on a technically accomplished collection of experiments and is addressing a relevant question for the field. However, the reviewers also point to major concerns regarding the advance of the structural insights provided in light of preceding reports on the matter. Secondly, the experts state that the data will need to be given a more detailed mechanistic context before they can be published in EMBO Journal.

On balance, and after detailed discussion in the editorial team, I would like to invite you to address the comments of the referees in a revised version of the manuscript. In line with the referees' comments (see ref#3 and cross-comments), a functional characterization of the chlolesterol binding sites would be particularly important to be included in a revised manuscript. As you will see, the referees also requested a substantial re-organisation of the manuscript to focus the readers' attention on the novel insights accessible by the increased resolution structure. I will be available for a direct exchange on this during the next weeks if you have any questions on the referees' comments further.

As you know, it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve these concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

William

William Teale, Ph.D.

Editor

The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:

http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable

online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

Referee #1:

The manuscript by Neumann et al. reports the high resolution cryo-EM structure of human NKCC1 along with associated cell-based functional assays and all atom molecular dynamics. The Na-K-Cl cotransporter (NKCC) is a protein that aids in the secondary active transport of sodium, potassium, and chloride into cells and as such is an important component of cellular ion homeostasis. In addition, alterations in the function of the cation-chloride cotransporters (CCCs) NKCC1 and KCC2, may contribute to the poor efficacy of standard antiepileptic drugs used in the treatment of neonatal seizures and are under study to supplement existing treatments for epilepsy.

This paper therefore reports the structure of an important membrane protein with wide ranging phsyiological and pharmacological significance.

As noted by the authors, this is not the first structure of human NKCC1, which was reported in 2020 (Nat Commun 11: 1016-1016) and 2021 (2021) Commun Biol 4: 226-226). However, the structure contained in this report is of much higher resolution. I couldn't find the PDB deposition report for the structure in the submission, so I can't comment on the statistics. This would have been useful, as it was unclear to me whether the current higher resolution structure contains the missing side chains from the previously reported structures. I assume they are now modelled. Additionally, the authors could identify the Na, K and Cl binding sites and the functional/MD work in the study supports the identification of these sites along with plausible routes for ion binding and release.

(I can't really comment on the MD part of the study).

Overall, the work is of a high standard and the improved insights into ion binding, coupled with the detailed comparison with other APC family transporters results in an important contribution to the literature. In my view this is work is very suitable for publication in EMBO.

I would have liked to see more analysis on the cholesterol binding sites detailed in Figure 1. This section of the paper is perhaps the weakest part of the study, and overly descriptive without providing any insight into NKCC1 biochemistry. Whilst the use of LMNG most likely precludes liposome reconstutition, which could be used to test the importance of cholesterol on NKCC1 activity, no attempt was made to study this. Mutation of F487 in the cholesterol 1 binding site might prove very informative, especially given the prominance of TM6 in the transport mechanism?

I don't think this needs undertaking, and the work is strong enough to be published in my opinion as is. However, as it stands, for me, this part of the study is little flat.

Having said that, this is a very impressive peice of research and one that will be well received in several fields.

Minor comments:

- No need for e.g. in Abstract.

- I'm not sure SiaT is a member of the SLC35 family?

- Figures don't contain any indication of membrane or orientation labels.

Referee #2:

EMBOJ-2021-110169R-Q Neumann et al.

The authors present a high resolution structure of the human sodium/potassium/chloride transporter (NKCC1) at 2.6 Å resolution determined by cryo-electronmicroscopy. This is interpreted as a substrate-loaded, inward facing state. Prior structures of hNKCC1 as well as related transporters are all also in the inward facing state (see recent Liang review JMB 433 (2021) 167056, a reference that the authors should consider including here as it summarizes much of the prior findings on this family as well as open questions).

It appears that the overall conformation is identical to that of the previously determined, lower resolution NKCC1 structure. (Fig. S1A). It is striking that this simple fact is not clearly stated in the text. Indeed the only comment on Fig. S1A is on p. 4 where it is just stated that the structure is an inward facing state in the four bound ions The clear message from S1A is that this structure looks like all the other NKCCs. On the initial read of this manuscript, I had a very hard time sorting what was new from what was known before. The lack of comments on direct comparison of this structure with the other report of the human NKCC1 is conspicuous by its absence. Undoubtedly the authors can see many more things because of the resolution. Providing a clear assessment of those, as well as what is clearly different or simal with the better resolution zebrafish NKCC seems essential for establishing what the real advance is here with these new data.

In general, any mechanistic advances in this manuscript are not clearly presented. The manuscript has much detail, but often it is presented without a clear context. Although I can appreciate the urge to go in to great detail given the resolution, there are few good anchor points for a reader (especially one who does not work on this class of proteins) to extract a clear, concise mechanistic message. This is certainly an important protein for human health. There is great interest in understanding how it works. The authors need to do a better job at presenting their new insights.

On p. 7. I do not understand what the authors are getting at with the reference to the 1998 KcsA structure (Doyle et al.). There is nothing in that paper about attracting sodium ions. Further, how the structure in a potassium channel relates to that in these transporters was completely unclear.

Referee #3:

The manuscript submitted by Neumann et al. describes a high-resolution structure of the transmembrane region of the human sodium-potassium-chloride transporter in the substrate-bound inward-facing conformation determined by single-particle cryo-EM. Compared to previously reported structures of this transporter, the improved resolution information allows to resolve bound ions and water molecules in the structure, but the C-terminal domain required for regulation of the transport is only poorly resolved and not discussed in the manuscript. Together with MD simulations, the structure of the transmembrane domain informs about the putative sequence of the ion binding and release steps and characterizes a putative Na release pathway near TM5. Through characterization of mutants in a cellular thallium flux assay, two glutamate residues (E429 and E431) conserved within the Na-transporting branch of SLC12 members are identified as key elements for intracellular sodium release.

Major Points:

A number of lipids and cholesterol molecules within the dimer interface are resolved by the improved EM map. However, the functional relevance of these lipids and cholesterol molecules remains speculative without further data from mutagenesis or MD simulations supporting that the lipids stabilize the inward-facing state. Hence, the putative role for cholesterol should be assessed experimentally by testing the activity of lipid binding site mutants in the thallium flux assay.

Bumetanide was added throughout purification of NKCC1, but the authors make no reference to this outside the methods section. The NKCC1-bumetanide complex structure would represent a major advance in the field. Could the authors comment on why they were unable to see bumetanide binding? Is there any unmodelled density in the NKCC1 maps at the VU0463271 site which was identified in a recent KCC1 structure (Zhao et al., 2020, BioRxiv)?

Whilst the manuscript is overall well-written, it reads more like a review due to extensive comparison to bacterial transporter structures in introduction, results and discussion. It would be appropriate to shorten these sections and describe this comparison in a more concise manner. Likewise, some of the comparisons to other structures would be more appropriate for an EV Figure rather than a main Figure. In its current form, the manuscript is therefore difficult to follow and not accessible to a broad readership. While similarities to SLC6 transporters are worth pointing out, the mechanistic comparisons are excessive and detract from the more interesting observations of ion specificity in the SLC12 family. The discussion in particular is far too long and requires a significant and more focused rewrite, with the use of subheadings between paragraphs.

Minor points:

-Rephrase line 1 in the last paragraph of the introduction. The presented structure is not full-length NKCC1 since the NTD is absent and the CTD is poorly resolved.

-Figure 2 legend, description of panel B: which coordinates of KCC1 have been used for preparing this panel ? (add the pdb code). Residue G517 highlighted in the text is also not labeled here. For panel E (page 24), the description of what the two green spheres represent (chloride ions ?) is missing.

-There is no space between the number and Angstrom at several places, eg. in the Figure legend and extended view figure legend descriptions at pages 23-25. Please correct 2.6A to 2.6 A (in Fig EV1 legend) etc.

-Figure 4 legend: panel a: "the main binding site of hNKCC..." correct to hNKCC1

-Figure S1 shows that the new "occluded" structure is almost identical in conformation to other CCC structures which the authors denote as "partially occluded". Can the authors clarify this use of terminology?

-Figure S2 is missing the PDB code for MhsT.

-Figure EV4 requires a description of how relative surface expression of the NKCC1 mutants was quantified (i.e., densitometry of the Western blot, number of replicates).

-There is duplication of (Chi et al., 2020, Cell Research) in the references but (Chi et al., 2021, EMBO J) is missing.

Cross-commenting session

William Teale

Thank you all very much for contributing with such informative and useful reports. As you will see, there are differences among your opinions and also among the courses of action I infer from them.
It seems that there are important data in the manuscript, but that there is a danger that the readership of EMBO J might well miss them altogether. I hope that this cross-consultation session will enable me to help the authors re-focus the manuscript on the key novel findings.

Referee 2
I don't have anything more to add. It seems Rev #3 and I are in agreement about accessibility. This was a hard read and I think that it needs a serous rewrite so that the key take home points are clarified.

Referee 1
Given the comments from Referees 2 and 3, I would certainly ask the authors to clarify the text to make it clear what is novel in this work and what is confirmatory. I certainly agree the discussion was rambling and overly focussed on the bacterial APC members. Also, following up on the points raised by referee 2, especially the BioRxve paper on human KCC1 from Zhao et al (https://www.biorxiv.org/content/10.1101/2020.07.26.221770v1) I would also like to suggest the authors experimentally test their hypothesis on the importance of those cholesterol sites. Originally I was sceptical that KCC1 could be reconstituted into

liposomes from LMNG (this is certainly not possible for the proteins I work with), and so I didn't think this was possible without a great deal of additional work. Clearly, I was wrong, and it is possible to reconstitute functional KCC1 into liposomes for in vitro analysis. As such, it should be 'relatively' straightforward to test whether the cholesterol sites identified from these structures are functionally important. I think this would substantially strengthen this paper.

Dear William Teale

Thanks for the opportunity to revise our manuscript **"Cryo-electron microscopy structure of the human NKCC1 transporter reveals mechanisms of ion coupling and substrate specificity"** for publication as an article in *EMBO Journal*.

Compared to current state of the art, our cryo-EM map of the occluded and inward-facing human NKCC1 is at a significantly improved resolution and reveals the presence of lipid molecules at the dimeric interface, cholesterol molecules associated with this conformational state of the transporter, and bound Na^+, K^+ and $2Cl^-$ ions at their respective sites. We qualify and investigate further our observations by a combination of sequence analyses, structural comparisons to other transporters, functional studies (Tl+ uptake), and MD simulations.

To meet the constructive feedback from the reviewers, we have made a substantial rewriting of the manuscript with a clearer message of novel insights gained, and with clearer distinctions of background, results and discussion. Furthermore, we present a large body of new data on cholesterol site mutations and cholesterol depletion experiments that clearly support a possible effect of cholesterol on NKCC1 function, as indeed requested by the reviewers.

I must apologize for the quite long time it took us preparing experiments and submitting our revised version. This was due to a combination of many challenges and obstacles in the process. We hope you agree that our current, revised report presents conclusive work on important observations and analyses based on experimental data. We are happy to address further questions that may arise, but hope you and the reviewers will find the manuscript to have reached an acceptable form.

On behalf of all authors

Poul Nissen

Referee #1:

The manuscript by Neumann et al. reports the high resolution cryo-EM structure of human NKCC1 along with associated cell-based functional assays and all atom molecular dynamics. The Na-K-Cl cotransporter (NKCC) is a protein that aids in the secondary active transport of sodium, potassium, and chloride into cells and as such is an important component of cellular ion homeostasis. In addition, alterations in the function of the cation-chloride cotransporters (CCCs) NKCC1 and KCC2, may contribute to the poor efficacy of standard antiepileptic drugs used in the treatment of neonatal seizures and are under study to supplement existing treatments for epilepsy.

This paper therefore reports the structure of an important membrane protein with wide ranging phsyiological and pharmacological significance.

As noted by the authors, this is not the first structure of human NKCC1, which was reported in 2020 (Nat Commun 11: 1016-1016) and 2021 (2021) Commun Biol 4: 226-226). However, the structure contained in this report is of much higher resolution. I couldn't find the PDB deposition report for the structure in the submission, so I can't comment on the statistics. This would have been useful, as it was unclear to me whether the current higher resolution structure contains the missing side chains from the previously reported structures. I assume they are now modelled. Additionally, the authors could identify the Na, K and Cl binding sites and the functional/MD work in the study supports the identification of these sites along with plausible routes for ion binding and release.

(I can't really comment on the MD part of the study).

Overall, the work is of a high standard and the improved insights into ion binding, coupled with the detailed comparison with other APC family transporters results in an important contribution to the literature. In my view this is work is very suitable for publication in EMBO.

We thank the reviewer for this positive comment

I would have liked to see more analysis on the cholesterol binding sites detailed in Figure 1. This section of the paper is perhaps the weakest part of the study, and overly descriptive without providing any insight into NKCC1 biochemistry. Whilst the use of LMNG most likely precludes liposome reconstutition, which could be used to test the importance of cholesterol on NKCC1 activity, no attempt was made to study this. Mutation of F487 in the cholesterol 1 binding site might prove very informative, especially given the prominance of TM6 in the transport mechanism?
I don't think this needs undertaking, and the work is strong enough to be published in my opinion as is. However, as it stands, for me, this part of the study is little flat.

This is a fair point. In response, we have performed additional MD simulations to investigate the stability of the cholesterol binding sites and we have peformed Tl^+ uptake experiments with NKCC1 constructs containing mutations within the cholesterol binding sites (Fig. 1) to investigate the function of cholesterol at different sites. Furthermore, we have added cholesterol depletion studies using methyl-beta-cyclodextrin. The added experiments clearly support that the cholesterol sites are of importance.

No need for e.g. in Abstract.

Agree, corrected.

I'm not sure SiaT is a member of the SLC35 family?

We rewrote/shortened our manuscript and deleted this paragraph.

Figures don't contain any indication of membrane or orientation labels.
We added indications of membrane orientation and labels to figures to strengthen the immediate readability.

Given the comments from Referees 2 and 3, I would certainly ask the authors to clarify the text to make it clear what is novel in this work and what is confirmatory. I certainly agree the discussion was rambling and overly focussed on the bacterial APC members. Also, following up on the points raised by referee 2, especially the BioRxve paper on human KCC1 from Zhao et al (https://www.biorxiv.org/content/10.1101/2020.07.26.221770v1) I would also like to suggest the authors experimentally test their hypothesis on the importance of those cholesterol sites. Originally I was sceptical that KCC1 could be reconstituted into liposomes from LMNG (this is certainly not possible for the proteins I work with), and so I didn't think this was possible without a great deal of additional work. Clearly, I was wrong, and it is possible to reconstitute functional KCC1 into liposomes for in vitro analysis. As such, it should be 'relatively' straightforward to test whether the cholesterol sites identified from these structures are functionally important. I think this would substantially strengthen this paper.

We are grateful for this feedback. We have simplified the text and background to make the novelty clearer and the manuscript more focused on new NKCC1 insight. We have not been able to establish a reconstitution method yet, but we performed additional Tl^+ uptake experiments in mammalian cells expressing NKCC1 with various mutations at the cholesterol sites (presented in Fig. 1), and we included also cholesterol depletion studies and MD simulations of cholesterol sites.

Referee #2:

The authors present a high resolution structure of the human sodium/potassium/chloride transporter (NKCC1) at 2.6 Å resolution determined by cryo-electronmicroscopy. This is interpreted as a substrate-loaded, inward facing state. Prior structures of hNKCC1 as well as related transporters are all also in the inward facing state (see recent Liang review JMB 433 (2021) 167056, a reference that the authors should consider including here as it summarizes much of the prior findings on this family as well as open questions).

This is indeed an informative review. The reference is now included in the text.

It appears that the overall conformation is identical to that of the previously determined, lower resolution NKCC1 structure. (Fig. S1A). It is striking that this simple fact is not clearly stated in the text. Indeed the only comment on Fig. S1A is on p. 4 where it is just stated that the structure is an inward facing state in the four bound ions The clear message from S1A is that this structure looks like all the other NKCCs. On the initial read of this manuscript, I had a very hard time sorting what was new from what was known before. The lack of comments on direct comparison of this structure with the other report of the human NKCC1 is conspicuous by its absence. Undoubtedly the authors can see many more things because of the resolution. Providing a clear assessment of those, as well as what is clearly different or simal with the better resolution zebrafish NKCC seems essential for establishing what the real advance is here with these new data.

We try making this point clear by stating that the structure is overall similar to earlier reports. However, with clear indications of Na+ sites in our maps, we interpret the functional state differently than those earlier reports. We have rewritten the introduction and more clearly indicated the similarity of our structure to the previously published structures and the differences in interpretation of functional state being analyzed.

In general, any mechanistic advances in this manuscript are not clearly presented. The manuscript has much detail, but often it is presented without a clear context. Although I can appreciate the urge to go in to great detail given the resolution, there are few good anchor points for a reader (especially one who does not work on this class of proteins) to extract a clear, concise mechanistic message. This is certainly an important protein for human health. There is great interest in understanding how it works. The authors need to do a better job at presenting their new insights.

We agree and rewrote and shortened the manuscript to highlight the new insights better.

On p. 7. I do not understand what the authors are getting at with the reference to the 1998 KcsA structure (Doyle et al.). There is nothing in that paper about attracting sodium ions. Further, how the structure in a potassium channel relates to that in these transporters was completely unclear.

The sodium ion release seems to work in a similar way as potassium ions release in KcsA potassium channel by attraction of the ion by negatively charged residues at the exit pathway (Doyle et al.). However, we acknowledge that it might also be confusing for a reader, as the two proteins do not relate and we deleted the sentence from the manuscript, and focus the discussion on other transporters, not channels.

Referee #3:

The manuscript submitted by Neumann et al. describes a high-resolution structure of the transmembrane region of the human sodium-potassium-chloride transporter in the substrate-bound inward-facing conformation determined by single-particle cryo-EM. Compared to previously reported structures of this transporter, the improved resolution information allows to resolve bound ions and water molecules in the structure, but the C-terminal domain required for regulation of the transport is only poorly resolved and not discussed in the manuscript. Together with MD simulations, the structure of the transmembrane domain informs about the putative sequence of the ion binding and release steps and characterizes a putative Na release pathway near TM5. Through characterization of mutants in a cellular thallium flux assay, two glutamate residues (E429 and E431) conserved within the Na-transporting branch of SLC12 members are identified as key elements for intracellular sodium release.

Major Points:

A number of lipids and cholesterol molecules within the dimer interface are resolved by the improved EM map. However, the functional relevance of these lipids and cholesterol molecules remains speculative without further data from mutagenesis or MD simulations supporting that the lipids stabilize the inward-facing state. Hence, the putative role for cholesterol should be assessed experimentally by testing the activity of lipid binding site mutants in the thallium flux assay.

We now performed additional MD simulations and Tl^+ uptake assays in order to validate the cholesterol binding sites and qualify their importance. We find that in particular mutation at cholesterol site 1 has an effect on function. If we get to see new functional states (e.g. outward-oriented structures), it will motivate more focused studies on this point.

Bumetanide was added throughout purification of NKCC1, but the authors make no reference to this outside the methods section. The NKCC1-bumetanide complex structure would represent a major advance in the field. Could the authors comment on why they were unable to see bumetanide binding? Is there any unmodelled density in the NKCC1 maps at the VU0463271 site which was identified in a recent KCC1 structure (Zhao et al., 2020, BioRxiv)?

We aimed for a bumetanide-bound NKCC1 structure, however, we could not locate any density for the inhibitor in the maps (also not at the VU0463271 site identified in the KCC1). As bumetanide is a competitive inhibitor of NKCC1, we think that the high concentrations of Na^+, K^+ and Cl^- in the buffers outcompeted the inhibitor.
We now made a comment in the end of the text in the method section. During revision, a paper emerged on a bumetanide-bound structure that we briefly comment on. The findings and conclusions of that paper do not interfere with the novelty or discussion of our report.

Whilst the manuscript is overall well-written, it reads more like a review due to extensive comparison to bacterial transporter structures in introduction, results and discussion. It would be appropriate to shorten these sections and describe this comparison in a more concise manner. Likewise, some of the comparisons to other structures would be more appropriate for an EV Figure rather than a main Figure. In its current form, the manuscript is therefore difficult to follow and not accessible to a broad readership. While similarities to SLC6 transporters are worth pointing out, the mechanistic comparisons are excessive and detract from the more interesting observations of ion specificity in the SLC12 family. The discussion in particular is far too long and requires a significant and more focused rewrite, with the use of subheadings between paragraphs.

We agree and thank for this feedback. We have rewritten the manuscript and deleted excessive comparisons with the SLC6 transporters. SLC6 transporters are however also very informative on mechanisms discussed here, so some comparisons are important and maintained

Minor points:

-Rephrase line 1 in the last paragraph of the introduction. The presented structure is not full-length NKCC1 since the NTD is absent and the CTD is poorly resolved.

The structure is of full-length NKCC1, but clearly large parts are too flexible to be modelled. We rephrased the use of 'full-length' from the sentence.

-Figure 2 legend, description of panel B: which coordinates of KCC1 have been used for preparing this panel ? (add the pdb code). Residue G517 highlighted in the text is also not labeled here. For panel E (page 24), the description of what the two green spheres represent (chloride ions ?) is missing.

We now corrected the figure description and the figure to be more readable.

-There is no space between the number and Angstrom at several places, eg. in the Figure legend

and extended view figure legend descriptions at pages 23-25. Please correct 2.6A to 2.6 A (in Fig EV1 legend) etc.

Corrected

-Figure 4 legend: panel a: "the main binding site of hNKCC..." correct to hNKCC1

Corrected

-Figure S1 shows that the new "occluded" structure is almost identical in conformation to other CCC structures which the authors denote as "partially occluded". Can the authors clarify this use of terminology?

We now clarified the terminology in the text. The “partially occluded” annotation represents the earlier interpretations without Na+ (probably due to lower resolution in the maps)

-Figure S2 is missing the PDB code for MhsT.

Corrected.

-Figure EV4 requires a description of how relative surface expression of the NKCC1 mutants was quantified (i.e., densitometry of the Western blot, number of replicates).

We now corrected the figure description.

-There is duplication of (Chi et al., 2020, Cell Research) in the references but (Chi et al., 2021, EMBO J) is missing.

Indeed, a mistake! We now corrected the references.

Dear Poul,

We have now received re-review reports from two referees; both are pasted at the bottom of this email. You have addressed the concerns of the referees satisfactorily. However, before we can proceed towards publication I have a list of minor editorial points that need addressing.

Would you please:
Remove one key word, as our limit is five.
Include a Data Availability Section which explains where readers can access your datasets. The instructions for this are on our website in the "Instructions for Authors" section.
Update the Conflict of Interest statement according to these instructions.
Remove the Author Credit section from the manuscript. We have replaced this with an online AC/CrediT section which will be more searchable in the future.
ZIP both movies with their legends.

We encourage the publication of source data. Any PDF files will be published online with the article as supplementary "Source Data" files. Source Data can also include Excel tables to accompany your graphs. We anticipate that their inclusion will make your work more discoverable and useable to scientists in the future.

I would like to request you pay particular attention to the following figures:
Please fix the following issues with your figure callouts:
Fig 2C+D, 3C are called out after Fig 5.
Fig 5C is called out after 5E.
Fig EV3 panel callouts are missing.
Fig EV4A callout is missing.
The Appendix figure and table callouts need correcting to 'Appendix Fig. S#' and 'Appendix Table S1'.

Would you also please:
Move the figure legends to after the Reference section.
Remove the appendix figure & table legends and movie legends from the main manuscript file.
Datasets 1 & 2 are in Figure EV1 and Dataset 3 is in Figure EV2. Please consider uploading separately, or renaming the files.

We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper. We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

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Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.

- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (30th Oct 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

The authors have addressed my concerns. The revision of the manuscript has resulted in a clearer presentation of which findings are new as well as their overall context.

Referee #3:

The revised version of the manuscript by Neumann et al. addresses all points raised by the reviewers and presents additional functional transport activity data for several mutants located in the putative cholesterol binding sites identified in the cryo-EM structures. Furthermore, the results from depletion experiments using non-cell permeable methyl-beta-cyclodextrin to extract cholesterol from cell membranes and MD simulations were added to this work, which strengthen the conclusions about the potential role of cholesterol for NKCC1 transport activity. In addition, some sections in the introduction and discussion which lacked focus in the previous version have been re-written in a more concise way which improved the overall readability of the manuscript significantly. The authors also discuss important differences to the bumetanide-bound NKCC1 structure reported in a recent publication which required construct engineering to trap the transporter in the bumetanide-bound outward-facing conformation. This may also explain why bumetanide added to the purification buffers is not visible in the maps from the current study by Neumann et al. All in all, the revised version is substantially improved compared to the original submission and brings sufficient new insights to merit publication in The EMBO Journal.

Minor editorial points that need addressing:
Would you please:

- Remove one key word, as our limit is five - done
- Include a Data Availability Section which explains where readers can access your datasets - done
- Update the Conflict of Interest statement according to the instructions - done

- Remove the Author Credit section from the manuscript. We have replaced this with an online AC/CrediT section which will be more searchable in the future. - done.
- ZIP both movies with their legends. - done
- We encourage the publication of source data. Any PDF files will be published online with the article as supplementary "Source Data" files. Source Data can also include Excel tables to accompany your graphs. We anticipate that their inclusion will make your work more discoverable and useable to scientists in the future. – done

I would like to request you pay particular attention to the following figures:
Please fix the following issues with your figure callouts:

- Fig 2C+D, 3C are called out after Fig 5. – We have introduced a call for the entire Fig. 2 on page 6 ("*Defining the dynamics and functional properties of this pathway, and how it compares to the Na+ independent transporters (Fig. 2), therefore is important for understanding NKCC1 transport activity*"). Furthermore, we now call Fig. 3C on page 7: "*MD simulations also implicate these residues (Fig. 3C, see below).*"
-
- Fig 5C is called out after 5E. – We have interchanged panels in Fig. 5 to fit that.

- Fig EV3 panel callouts are missing. – We have included a call for EV3 on page 4, together with EV1 and 2

- Fig EV4A callout is missing.- We have added a call for the entire EV4 and later for EV4C-D specifically.

- The Appendix figure and table callouts need correcting to 'Appendix Fig. S#' and 'Appendix Table S1'. Done

Would you also please:

- Move the figure legends to after the Reference section. Done
- Remove the appendix figure & table legends and movie legends from the main manuscript file. Done

- Datasets 1 & 2 are in Figure EV1 and Dataset 3 is in Figure EV2. Please consider uploading separately, or renaming the files. We prefer to keep them

separate and named data sets 1, 2 and 3 as they have been collected separately and merged afterwards. The uploaded dataset is the combined dataset, and we think it will only confuse and impair users is we upload the individual datasets separately

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper. We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier – Synopsis and a summary figure are included

3rd Revision - Editorial Decision **16th Sep 2022**

Dear Poul,

There remain some minor editorial points to be addressed before I can finally accept your manuscript for publication. Would you please:

rename the conflict of interest statement the "Disclosure and competing interests statement",

rename movies Movie EV1 and Movie EV2, and

specify which figures the source data file support, arranging as one file/folder per ZIPped figure.

Regarding your figures, please correct the following:

the callout for Fig. 3A is before 2B, and callout for Fig. 3C should be after 3B,

Fig 5C is still called out after 5E, and a callout for Fig EV4A is still missing.

Callouts for Appendix Figure S5 and Appendix Table S1 are also missing.

Please upload Datasets 1, 2 and 3 separately.

Best wishes,

William

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

There remain some minor editorial points to be addressed before I can finally accept your manuscript for publication. Would you please:

rename the conflict of interest statement the "Disclosure and competing interests statement",- It is renamed now.

rename movies Movie EV1 and Movie EV2 – The movies are renamed now.

specify which figures the source data file support, arranging as one file/folder per ZIPped figure. Source data files provided for figures 1,3,4,5, S1

Regarding your figures, please correct the following:

the callout for Fig. 3A is before 2B, and callout for Fig. 3C should be after 3B- It is corrected now.

Fig 5C is still called out after 5E, and a callout for Fig EV4A is still missing.- It is corrected now (Fig. EV4A is renumbered as EV5A)

Callouts for Appendix Figure S5 and Appendix Table S1 are also missing. – The callouts are now added (Appendix Figure S5 is now renumbered as Appendix Figure S4).

Please upload Datasets 1, 2 and 3 separately. – Processing of the three datasets is now described in figures EV1, EV2 and EV3.

4th Revision - Editorial Decision **22nd Sep 2022**

Dear Poul,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Congratulations on a nice study. I'm really glad to see this one in print!

N.b. There is still a call-out missing for EV4A. If you let me know where you'd like it to go, I can add it to the text for you.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Best wishes,

William

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Per standard practice in the lab, pilot experiments were performed to identify requirements to accurately assess the test parameters. Following, transport experiments were performed a minimum of 3 times using different batches of
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	PDB (7ZGO) structure and cryo-EM maps deposited.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	