

TRPML2 in distinct states reveals the activation and modulation principles of the TRPML family

Corresponding Author: Dr Xiaochun Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

TRPML channels play critical roles in cellular physiology and are associated with various human diseases. While the structures of these channels have previously been resolved in both closed and open conformations and in complexes with ligands, the molecular mechanisms underlying TRPML channel gating remain incompletely understood. In particular, structural insights into TRPML2, especially regarding how ligand binding induces gating, are still limited.

The current study reports six human TRPML2 structures with different modulators and four TRPML1 structures in the presence of distinct agonists. While the authors note that further structural and dynamic studies are necessary to fully elucidate how these agonists trigger the opening of the lower gate in these channels, the reported structures offer valuable insights into the activation mechanisms of TRPML and related channels.

Overall, the reviewer finds this manuscript to be well-written. Below are some comments for consideration:

1. Based on these structures, the π -bulge in S6 transitions to an α -helix in the pre-open conformation induced by (+)ML-SI3 or ML2-SA1 binding, while it reverts back to the π -bulge in the open conformation. Additionally, the residues forming the lower gate in the pre-open conformation return to those forming the gate in the closed conformation. Interestingly, the shift of the S4-S5 linker is smaller in the open conformation compared to the pre-open conformation. Why does the π -to- α transition in S6 occur only in this intermediate state during ligand-induced gating, and how does the lower gate open in these conformations, given that their overall structures more closely resemble the closed conformation? Further discussion and a proposed mechanism are essential to explain this unique feature.
2. The recordings of TRPML2 L414A were performed using the inside-out configuration, while the WT and other channels were recorded using whole endolysosomal patch-clamp. Since the inside-out configuration captures current from a much smaller membrane patch, the current for L414A will naturally be much smaller than for other channels. It is therefore inappropriate to directly compare the current sizes of WT and L414A in Fig. 4A. Can WT channels traffic to the plasma membrane for direct comparison with the L414A mutant using the same inside-out configuration? Additionally, is there another method to confirm that the L414A mutant channel is functional and can still be activated?
3. The lysosomal lumen is typically acidic. Could this low pH affect ligand binding, including PIP2, or influence channel gating after ligand binding? If resolving structures under low-pH conditions is not feasible, adding a related discussion would enhance the manuscript.
4. When PI(3,5)P2 was not added, was any endogenous ligand, such as a lipid molecule, observed to bind at the PI(3,5)P2-binding site?
5. On page 2, lines 30–32, the sentence should be rewritten for clarity, such as: "...of TRPML2 enables the mutated TRPML1 to be activated by...".
6. The expression level and pattern of L414A and Y496A need to be shown.
7. Previous studies have reported a rotation of the luminal linker domain during gating in other TRPML channels. Was a similar movement observed when comparing the closed and open structures of TRPML2 in this study?
8. Most recording data in Fig. 4 are based on only 3–4 data points, which is a very low sample size for a t-test. Additional data points are necessary to ensure statistical validity.
9. There are two typos in the name "ML2-SA1" in Fig. 4C. Please correct them.
10. Lines 296–297 require a citation.
11. In Fig. 4, the holding voltage used for the bar graph is not indicated. Please include this information in the figure or its legend.

Reviewer #2

(Remarks to the Author)

Remarks to the Author:

The manuscript by Schmiede et al. presents high-resolution cryo-EM structures of human TRPML2 in multiple functional states bound to distinct modulators, revealing diverse regulatory mechanisms. The structural analysis demonstrates an α -helical S6 segment in the closed state that undergoes a distinctive α -to- π transition during channel activation. This comprehensive work significantly advances our understanding of TRPML channel activation mechanisms and provides a valuable foundation for developing selective TRPML modulators. While this represents a solid and well-executed study, several important issues should be addressed prior to publication.

Major comments:

1. The authors should provide PDB files and EM density maps for all reported structures. A supplemental figure demonstrating the fit of modulators within their corresponding density maps is essential. We recommend including wall-eyed stereo representations (with left and right views separated by $\pm 90^\circ$) to facilitate proper 3D visualization of the ligand densities.
2. The manuscript would benefit from a more detailed analysis of ligand-protein interactions. Specific intermolecular forces (e.g., hydrogen bonds, π - π interactions, hydrophobic contacts) between each modulator and its binding pocket should be explicitly described. Furthermore, additional mutational studies are needed to validate the functional significance of key residues identified in ligand binding, particularly those interacting with both agonists and antagonists.
3. The dramatic conformational difference observed for (+)-ML-SI3 between the open state and pre-open conformations requires further clarification. Molecular dynamics simulations could provide valuable insights into the transition pathway between these distinct binding poses.
4. The author should use a separate paragraph to explain whether they can elucidate the molecular mechanism behind this unique isomer preference by comparing the binding conformations of (-)-ML-SI3 and (+)-ML-SI3.
5. The authors have also resolved the structure of TRPML1. Could they explain why (+)-ML-SI3 acts as an inhibitor for TRPML1 but as an agonist for TRPML2?
6. Functional characterization of the TRPML2-A424V/G425A double mutant's response to (+)-ML-SI3 is crucial to validate the proposed activation mechanism. Electrophysiological analysis of this mutant should be included to confirm the structural predictions.
7. In the discussion, the authors should clarify how A424/G425 contribute to the recognition of the ML-SA2 agonist and why ML-SA2 selectively activates TRPML2. This discussion will aid in future structure-based drug development.

Minor comments:

1. Added purification chromatograms and SDS-PAGE analysis of purified proteins.
2. (-)-ML-SI3 \rightarrow (-)-trans-ML-SI3 or (1R,2R)-ML-SI3; (+)-ML-SI3 \rightarrow (+)-trans-ML-SI3 or (1S,2S)-ML-SI3.
3. Correct labeling in Figure 4c: "ML2-SA1" instead of "ML-2SA1" or "ML2SA1".
4. Provide clear color-coding explanations for all structural representations in Figure S6 legends.
5. Update Supplementary Table 1 with PDB IDs for initial models used in refinement.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed my concerns. I would like to see a discussion added on how gating might potentially be regulated by low pH, as well as the efforts made to obtain the structures under lower pH conditions.

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed all of my previous concerns. I have no further comments, and I believe the manuscript is now suitable for publication.

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Responses to Reviewers

Reviewer #1 (Remarks to the Author):

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Overall, the reviewer finds this manuscript to be well-written. Below are some comments for consideration:

We thank the reviewer for their thoughts and their time in reviewing our manuscript. Please find our responses to their comments below.

1. Based on these structures, the π -bulge in S6 transitions to an α -helix in the pre-open conformation induced by (+)ML-SI3 or ML2-SA1 binding, while it reverts back to the π -bulge in the open conformation. Additionally, the residues forming the lower gate in the pre-open conformation return to those forming the gate in the closed conformation. Interestingly, the shift of the S4-S5 linker is smaller in the open conformation compared to the pre-open conformation. Why does the π -to- α transition in S6 occur only in this intermediate state during ligand-induced gating, and how does the lower gate open in these conformations, given that their overall structures more closely resemble the closed conformation? Further discussion and a proposed mechanism are essential to explain this unique feature.

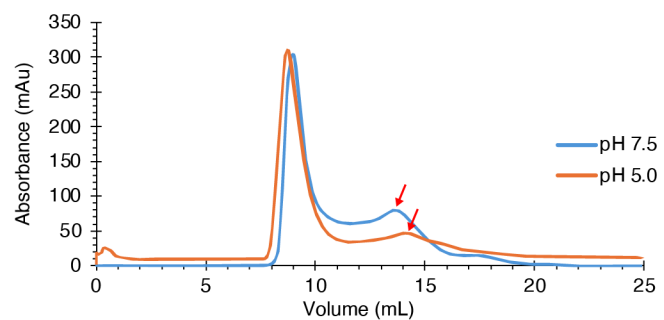
Response: This reviewer brings attention to an interesting point: the fact that the S6 in the open conformation is much closer aligned to the Apo (closed) conformation than the pre-open one. From our data, we hypothesize that there are two possible mechanisms for the opening of the lower gate: 1) the S6 helix rotates upon its transition from the apo to pre-open to open states; or 2) the lower gate can open when the S6 contains a π -bulge or when a π -to- α transition occurs. We performed the molecular dynamic simulations using the transmembrane domain of the (+)ML-SI3 bound pre-open and open states individually. After 200 ns simulations, the results from the open state showed that when S6 contains a π -bulge, the lower gate remains stable with a symmetric central pore. In contrast, simulations from the pre-open state revealed that when the π -to- α transition in S6 occurs, the channel becomes more dynamic and the central pore becomes more asymmetric, with an opening only in one direction (Fig. S11). Based on these results, we favor the second mechanism. Since we are currently not able to capture the open state after a π -to- α transition, it is possible that this state may need additional factors (e.g., lipid or other ligands) in the local environment to be stabilized *in vitro*. We have added this discussion into the manuscript (lines 323-335).

2. The recordings of TRPML2 L414A were performed using the inside-out configuration, while the WT and other channels were recorded using whole endolysosomal patch-clamp. Since the inside-out configuration captures current from a much smaller membrane patch, the current for L414A will naturally be much smaller than for other channels. It is therefore inappropriate to directly compare the current sizes of WT and L414A in Fig. 4A. Can WT channels traffic to the plasma membrane for direct comparison with the L414A mutant using the same inside-out configuration? Additionally, is there another method to confirm that the L414A mutant channel is functional and can still be activated?

Response: The WT in Fig. 4a is done in an inside-out manner like what we did on L414A. We have provided the fluorescence images and western blots in the revision to show that the WT and L414A can distribute on the cell surface. We also added PI(3,5)P₂ during the electrophysiological experiments to show that L414A can be activated by a lipid agonist (Fig. S8a-c).

3. The lysosomal lumen is typically acidic. Could this low pH affect ligand binding, including PIP₂, or influence channel gating after ligand binding? If resolving structures under low-pH conditions is not feasible, adding a related discussion would enhance the manuscript.

Response: This reviewer makes a good point that pH might affect both ligand and PI(3,5)P₂ binding to the channel, especially given that it is a lysosomal channel. Our previous study along with other groups' findings showed that TRPML1 can interact with compounds both at basic and acidic pHs without any difference in binding (PMID: 29019983 and 35131932). With TRPML2, we attempted to purify the protein at both pH 7.5 and pH 5; however, while the protein was moderately well behaved at a more neutral pH, which allowed us to obtain enough sample for cryo-EM analysis, the protein was less well behaved leading to a much lower yield at acidic pHs (please see the gel filtration curve of some of our preliminary screening purifications, the fractions containing TRPML2 are indicated by red arrows). This is supported by the paper by Viet et al. (2019) which demonstrated that the luminal domain was more stable at higher pHs (PMID: 31178222).



4. When PI(3,5)P₂ was not added, was any endogenous ligand, such as a lipid molecule, observed to bind at the PI(3,5)P₂-binding site?

Response: We have carefully checked all the maps. There was no PI(3,5)P₂ density observed in any of the other maps other than the dataset collected with supplemented PI(3,5)P₂. Moreover, this binding site is consistent with the PI(3,5)P₂ binding site in TRPML1 (PMID: 30305615 and 35131932). Thus, we are confident in assigning this density as the lipid in this site.

5. On page 2, lines 30–32, the sentence should be rewritten for clarity, such as: “...of TRPML2 enables the mutated TRPML1 to be activated by...”.

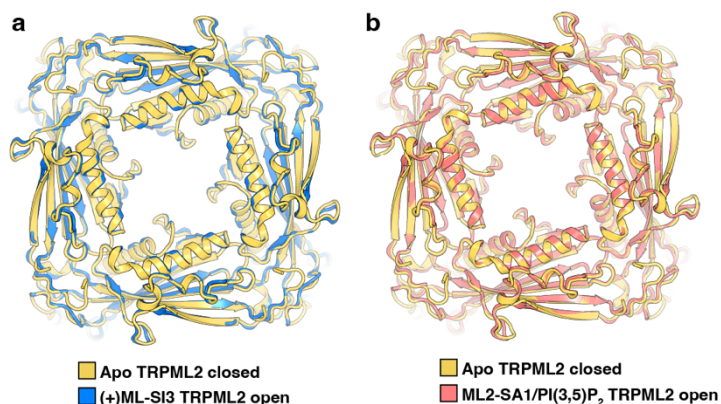
Response: We thank the reviewer for this edit and have changed the text accordingly.

6. The expression level and pattern of L414A and Y496A need to be shown.

Response: We have added a western blot analysis to show the expression levels of the TRPML2 mutants used in this paper, as well as fluorescence images of the expression patterns of each mutant. These were added into Fig. S8a-b, d.

7. Previous studies have reported a rotation of the luminal linker domain during gating in other TRPML channels. Was a similar movement observed when comparing the closed and open structures of TRPML2 in this study?

Response: While other studies involving TRPML1 and TRPML3 demonstrate a rotation of the luminal domain, we did not observe an obvious rotation in the luminal domain of TRPML2 in our structures (see the right figure). We have mentioned this point in our revision (lines 305-307).



8. Most recording data in Fig. 4 are based on only 3–4 data points, which is a very low sample size for a t-test. Additional data points are necessary to ensure statistical validity.

Response: Point accepted. We have added more measurements to all the functional analyses. They all now have an $n \geq 4$.

9. There are two typos in the name "ML2-SA1" in Fig. 4C. Please correct them.

Response: Point accepted. We have corrected these typos in Fig. 5c.

10. Lines 296–297 require a citation.

Response: Point accepted. We have added the required citation to this section (line 312).

11. In Fig. 4, the holding voltage used for the bar graph is not indicated. Please include this information in the figure or its legend.

Response: The holding voltage used for bar graphs is indicated now in the figure legends and is consistently set to -100 mV.

Reviewer #2 (Remarks to the Author):

Remarks to the Author:

The manuscript by Schmiede et al. presents high-resolution cryo-EM structures of human TRPML2 in multiple functional states bound to distinct modulators, revealing diverse regulatory mechanisms. The structural analysis demonstrates an α -helical S6 segment in the closed state that undergoes a distinctive α -to- π transition during channel activation. This comprehensive work significantly advances our understanding of TRPML channel activation mechanisms and provides a valuable foundation for developing selective TRPML modulators. While this represents a solid and well-executed study, several important issues should be addressed prior to publication.

We thank the reviewer for their thoughts and their time in reviewing our manuscript. Please find our responses to their comments below.

Major comments:

1. The authors should provide PDB files and EM density maps for all reported structures. A supplemental figure demonstrating the fit of modulators within their corresponding density maps is essential. We recommend including wall-eyed stereo representations (with left and right views separated by $\pm 90^\circ$) to facilitate proper 3D visualization of the ligand densities.

Response: Point accepted. We have deposited the PDB and EM density files for the reported structures to PDB and EMDB and have uploaded them for this reviewer (they have been cropped due to the large size). We have also included a supplemental figure demonstrating the fit of all the ligands within their respective densities as the reviewer requested (Fig. S2).

2. The manuscript would benefit from a more detailed analysis of ligand-protein interactions. Specific intermolecular forces (e.g., hydrogen bonds, π - π interactions, hydrophobic contacts) between each modulator and its binding pocket should be explicitly described. Furthermore, additional mutational studies are needed to validate the functional significance of key residues identified in ligand binding, particularly those interacting with both agonists and antagonists.

Response: Point accepted. We appreciate this comment and have included a more detailed analysis of the intermolecular forces between the ligands and the residues of interest in the binding pocket. We have also added TRPML2^{F457A} to the revision. Our cellular expression and localization analysis showed that this mutant expressed on the cell surface (implying that it has been folded), however, it could not be activated by either (+)ML-SI3 or ML2-SA1. More interestingly, unlike TRPML2^{L414A}, this mutant could not be activated by PI(3,5)P₂ suggesting that F457A affects ligand binding as well as channel opening (see Fig. 4 and Fig. S8c)

3. The dramatic conformational difference observed for (+)-ML-SI3 between the open state and pre-open conformations requires further clarification. Molecular dynamics simulations could provide valuable insights into the transition pathway between these distinct binding poses.

Response: Point accepted. We have added more discussion of this difference in the text (lines 165-174). We also performed the molecular dynamic simulations using the transmembrane domain of the (+)ML-SI3 bound pre-open and open states individually. After 200 ns simulations, the results

from the open state showed that when S6 contains a π -bulge, the lower gate remains stable with a symmetric central pore. In contrast, simulations from the pre-open state revealed that when the π -to- α transition in S6 occurs, the channel becomes more dynamic and the central pore becomes more asymmetric, with an opening only in one direction (Fig. S11). Thus, it is possible that the lower gate can open when the S6 contains π -bulge or when a π -to- α transition occurs (MD simulation discussion was added in lines 325-331).

4. The author should use a separate paragraph to explain whether they can elucidate the molecular mechanism behind this unique isomer preference by comparing the binding conformations of (-)-ML-SI3 and (+)-ML-SI3.

Response: Point accepted. We have expanded our discussion of the difference in binding mechanisms of these two compounds (lines 165-174). In our structures, (+)ML-SI3 penetrates deeper into the binding pocket, establishing more contacts with key residues that facilitate further channel pore opening. This deeper penetration is driven by the compound's isomerism, as the geometry of (-)ML-SI3 prevents it from folding in a way that enables the same interactions.

5. The authors have also resolved the structure of TRPML1. Could they explain why (+)-ML-SI3 acts as an inhibitor for TRPML1 but as an agonist for TRPML2?

Response: We have compared the structure of ML-SI3 (mixture) bound TRPML1 with those of (+)ML-SI3 and (-)ML-SI3 bound TRPML2. The ML-SI3 in TRPML1 adopts the same conformation as that of (-)ML-SI3. In contrast, the piperazine ring of (+)ML-SI3 would clash with Val432 and Ala433 of TRPML1 (Fig. 5a). Thus, when we mutated V432A to A433G in TRPML1, the TRPML1 variant became sensitive to stimulation by (+)ML-SI3 and it could also bind (+)ML-SI3.

6. Functional characterization of the TRPML2-A424V/G425A double mutant's response to (+)-ML-SI3 is crucial to validate the proposed activation mechanism. Electrophysiological analysis of this mutant should be included to confirm the structural predictions.

Response: Our structural analysis revealed the interaction details between (+)ML-SI3 and TRPML2. While Ala424/Gly425 play important roles for the engagement of (+)ML-SI3, the other residues of TRPML2 which are not conserved in TRPML1 (e.g., Val460, Ile498) also contribute the recruitment of (+)ML-SI3. Despite the fact that TRPML1^{VA/AG} was able to be activated by (+)ML-SI3, the activation was still not as strong as it was for TRPML2 (Figs. 4 and 5c). Thus, it seems that TRPML2-A424V/G425A may not be a direct representative to validate our findings.

7. In the discussion, the authors should clarify how A424/G425 contribute to the recognition of the ML-SA2 agonist and why ML-SA2 selectively activates TRPML2. This discussion will aid in future structure-based drug development.

Response: Point accepted. When we docked the ML2-SA1 into the TRPML1 structure, the norbornane ring of ML2-SA1 is positioned close to V432 which would potentially clash with ML2-SA1. Additionally, residue Y507 prevents the accommodation of the 2,6-dichlorophenyl group of ML2-SA1 (Fig. 5b). The corresponding space in TRPML1 is relatively smaller and

therefore unable to accommodate ML2-SA1. We have addressed this aspect in the revision (lines 250-255).

Minor comments:

1. Added purification chromatograms and SDS-PAGE analysis of purified proteins.

Response: Point accepted. We have added the purification chromatographs and the accompanying SDS-PAGE of the peak fraction to Fig. S1c.

2. (-)ML-SI3 → (-)-trans-ML-SI3 or (1R,2R)-ML-SI3; (+)ML-SI3 → (+)-trans-ML-SI3 or (1S,2S)-ML-SI3

Response: Point accepted. We have added these clarifications in the beginning of the text (lines 50-51), and stated that we refer to them throughout the text as just (+)ML-SI3 or (-)ML-SI3. We hope that this helps the clarity.

3. Correct labeling in Figure 4c: "ML2-SA1" instead of "ML-2SA1" or "ML2SA1".

Response: Point accepted. These have been corrected in the figure.

4. Provide clear color-coding explanations for all structural representations in Figure S6 legends.

Response: Point accepted. We have added the yellow color code to this figure (which stands for the Apo TRPML2 model), which we hope will help the clarity of this figure.

5. Update Supplementary Table 1 with PDB IDs for initial models used in refinement.

Response: Point accepted. We apologize for any confusion. The initial models used for the refinement of the TRPML2 models was the Apo TRPML2 model from this paper, which was built using the AlphaFold model initially. We have added "(this manuscript)" to prevent further confusion.