Cryo-EM structures of the human endolysosomal TRPML3 channel in three distinct states

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TRPML3 channels are mainly localized to endolysosomes and play a critical role in the endocytic pathway. Their dysfunction causes deafness and pigmentation defects in mice. TRPML3 activity is inhibited by low endolysosomal pH. Here we present cryo-electron microscopy (cryo-EM) structures of human TRPML3 in the closed, agonist-activated, and low-pH-inhibited states, with resolutions of 4.06, 3.62, and 4.65 Å, respectively. The agonist ML-SA1 lodges between S5 and S6 and opens an S6 gate. A polycystin-mucolipin domain (PMD) forms a luminal cap. S1 extends into this cap, forming a ‘gating rod’ that connects directly to a luminal pore loop, which undergoes dramatic conformational changes in response to low pH. S2 extends intracellularly and interacts with several intracellular regions to form a ‘gating knob’. These unique structural features, combined with the results of electrophysiological studies, indicate a new mechanism by which luminal pH and other physiological modulators such as PIP2 regulate TRPML3 by changing S1 and S2 conformations.

The endocytic pathway is essential for cell signaling and physiology. It is regulated by a wide array of ion channels that are present in the endocytic vesicles. Members of the mucolipin subfamily of transient receptor potential channels (collectively referred to as TRPML channels) are primarily localized in endosomes and lysosomes. TRPML channels are tetramers. Each subunit has six transmembrane segments (S1–S6) and a pore loop between S5 and S6, which form a voltage-sensor-like domain (VSLD) and a pore domain. A long luminal/extracellular linker exists between S1 and S2 (the S1–S2 linker). This linker, the PMD, is a unique but shared feature of the TRPML and TRPP subfamilies of transient receptor potential channels. TRPML channels are nonselective cation channels that mainly conduct Ca2+ and monovalent cation currents from the lumen to the cytoplasm and extracellular milieu. Late endosomes have an acidic pH of 5.5–6.0, and lysosomes have a more acidic pH of 4.5–5.0 (refs. 2, 5.40–42), a condition necessary to maintain the activity of lysosomal hydrolases. In accordance with their endolysosomal localization, TRPML1 and TRPML3 have adopted common strategies to fine-tune their activities in the endocytic pathway. Thus, TRPML1 and TRPML3 are activated by phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2)16, which is enriched in the endolysosomal membrane17,44. In contrast, these channels are suppressed by PI(4,5)P2 (ref. 45) (H. Xu, personal communication), which is more abundant in the plasma membrane. Two separate clusters of positively charged amino acids in the N terminus of TRPML1 are critical for these effects45. Another common strategy is to use the low endolysosomal pH to regulate their activities. In the presence of Ca2+, TRPML1 currents are greatly enhanced by H+ (refs. 4, 13–17, 46, 47). Three aspartic residues in a luminal pore loop are crucial for this enhancement. In contrast, TRPML3 currents are inhibited by H+, and a histidine residue in the PMD (H283) is crucial for this inhibition46. A unique property of TRPML3 is that it is inhibited by Na+ (refs. 37, 48, 49), whose concentration in lysosomes may be as high as that in the extracellular solution50. Therefore, under physiological conditions, endolysosomal TRPML1 channels are active, whereas TRPML3 channels are inactive. However, after

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lysosomal damage or breakdown of lysosomal Na\(^+\) and H\(^+\) gradients, TRPML3 channels become activated. The ensuing Ca\(^2+\) influx triggers the exocytosis of neutralized lysosomes. This mechanism plays a role in the expulsion of pathogens from infected cells\(^5\). A large number of synthetic small-molecule agonists of TRPML channels have been identified\(^52,53\). Many of these molecules, such as ML-SA1, activate all three TRPML channel subtypes but show specificity toward other types of ion channels\(^53,54\). These molecules are useful tools for studying the physiological functions of TRPML channels.

To better understand the molecular mechanisms of TRPML channel function and regulation, we determined the structures of full-length human TRPML3 in the apo, ML-SA1-bound, and low-pH conditions corresponding to the pH in lysosomes, endo-
somes, and the extracellular milieu, respectively. Our new structures show striking differences as well as expected similarities between TRPML3 and TRPML1 PMDs and reveal novel structural features and conformational changes that shed light on TRPML3 activation and regulation.

RESULTS

Structure determination and general architecture

Full-length human TRPML3 was expressed in Tni insect cells. We used purified TRPML3 protein in the apo and ML-SA1-bound forms at pH 7.4 for single-particle cryo-EM analysis (Supplementary Figs. 2–4). With C\(_4\) symmetry imposed, we determined the structures of the two forms to overall resolutions of 4.06 Å and 3.62 Å, respectively. The central regions were better resolved than the peripheries (Supplementary Figs. 2, 3, and 5a). Most of the 553 amino acids were modeled in both structures, but some amino acids were not modeled owing to poor density, including 32 residues in the distal N terminus, 27 residues in the distal C terminus, and 18 residues in the PMD (Fig. 1, Supplementary Fig. 1, and Table 1).

A 5.4-Å-resolution cryo-EM structure of mouse TRPML1 was described recently\(^55\). Our TRPML3 structures from the current study have a similar general architecture. The channel is a fourfold-symmetric homotetramer with a central ion-conducting pore and dimensions of 95 × 95 × 102 Å (Fig. 1a). The transmembrane domain (TMD) has a typical architecture, with S1–S4 and the S4-S5 linker forming a VSLD, and S5, S6, and the pore loop forming the pore domain (Fig. 1b). The pore loop contains the ion selectivity filter (SF), which is flanked by two pore helices (PH1 and PH2). The VSLD of one subunit interacts with the pore domain of a neighboring subunit in a canonical domain-swapped configuration (Fig. 1a). The PMD of each subunit interacts with both of its neighbors and forms a luminal (i.e., extracellular)

### Table 1 Cryo-EM data collection, refinement, and validation statistics

|                      | Apo-TRPML3, pH 7.4 | TRPML3–ML-SA1, pH 7.4 | Apo-TRPML3, pH 4.8 |
|----------------------|--------------------|-----------------------|--------------------|
| **Data collection and processing** |                    |                       |                    |
| Magnification        | 22,500             | 105,000               | 105,000            |
| Voltage (kV)         | 300                | 300                   | 300                |
| Electron exposure (e\(^-\)/Å\(^2\)) | 50                | 50                    | 50                 |
| Defocus range (µm)   | −1.5 to −3.0       | −1.0 to −2.5          | −1.0 to −2.5       |
| Pixel size (Å)       | 1.32               | 1.34                  | 1.34               |
| Symmetry imposed     | C4                 | C4                    | C4                 |
| Initial particle images | 1,059,673         | 697,805               | 171,031            |
| Final particle images | 43,542             | 50,726                | 42,559             |
| Map resolution (Å)   | 4.06               | 3.62                  | 4.65               |
| FSC threshold        | 0.143              | 0.143                 | 0.143              |
| **Refinement**       |                    |                       |                    |
| Model resolution (Å) | 4.06               | 3.62                  | 4.65               |
| FSC threshold        | 0.143              | 0.143                 | 0.143              |
| Model resolution range (Å) | 211.20–4.06 | 214.08–3.62          | 214.08–4.65        |
| Map sharpening B factor (Å\(^2\)) | −228               | −180                  | −244               |
| **Map composition**  |                    |                       |                    |
| Nonhydrogen atoms    | 15,664             | 15,780                | 10,456             |
| Protein residues     | 1,904              | 1,904                 | 1,756              |
| Ligands              | 0                  | 8                     | 0                  |
| B factors (Å\(^2\)) | 116                | 118                   | 153                |
| Protein             | —                  | 120                   | —                  |
| R.m.s. deviation     | Bond lengths (Å)   | 0.009                 | 0.005              | 0.013              |
| Bond angles (°)      | 1.44               | 1.11                  | 1.71               |
| **Validation**       |                    |                       |                    |
| MolProbity score     | 2.11               | 2.18                  | 2.62               |
| Clashscore           | 15.6               | 17.0                  | 17.4               |
| Poor rotamers (%)    | 0.9                | 0.7                   | 3.3                |
| Ramachandran plot    | Favored (%)        | 93.8                  | 93.0               | 91.9               |
| Allowed (%)          | 5.6                | 6.8                   | 7.6                |
| Disallowed (%)       | 0.6                | 0.2                   | 0.5                |

FSC, Fourier shell correlation.

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cap on top of the TMD (Fig. 1a). The N and C termini are located intracellularly.

The apo and ML-SA1-bound structures are very similar overall (Fig. 1c); the two structures superimpose well in the PMD (r.m.s. deviation of 0.63 for amino acids 81–275) and at S1–S4 (r.m.s. deviation of 0.60 for amino acids 61–80 and 284–395). But the two structures diverge substantially in the S4–S5 linker, S5, S6, and the pore loop.

The ML-SA1 binding pocket

Comparison of the electron density maps of the apo and ML-SA1-bound structures shows clear differences in a crevice surrounded by S5, S6, and PH1 of one subunit, and S6 of a neighboring subunit (Supplementary Fig. 5b). The normalized difference map between the apo and ML-SA1-bound structures is very similar overall (Fig. 2a). Supporting this proposition, ML-SA1 binding produces substantial conformational changes in the regions surrounding the crevice (Supplementary Video 1): S5 and S6 are pushed outward and the S4–S5 linker is pulled downward by ~2 Å, and S6 undergoes a 27° counterclockwise rotation (top-down view). Because of the limited resolution, we were unable to unambiguously define the orientation of ML-SA1 and its molecular interactions with the channel, but we identified two amino acids, Y423 in S5 and F497 in S6, as key residues participating in ML-SA1 binding (Fig. 2a).

To verify that ML-SA1 indeed binds in this crevice, we examined the effect of ML-SA1 on wild-type channels and mutant channels (Y423A or F497A mutation). Wild-type and mutant channels were expressed in HEK293T cells and studied by whole-cell recording, largely according to protocols from previous studies. We usually detected a basal current after we changed the bath solution from a Na+-free to a 160 mM Na+ solution (Fig. 2b). This knowledge could aid the development of selective TRPML activators that might ultimately yield clinical benefits for ML-IV and other LSDs.

Figure 2a

The ML-SA1-binding site is close to the A419P Va mutation site, and both ML-SA1 binding and the A419P mutation make the channel stay open. In addition, in the ML-SA1-binding site, there are electron densities in the apo structure that cannot be accounted for by the surrounding amino acid side chains (Supplementary Fig. 5b). Further investigation is needed to determine whether these densities represent endogenous molecules such as lipids or small ligands. Cheminformatics analysis categorizes TRPML3 agonists into 9 chemical scaffolds and 20 singletons. It may be interesting to determine where different classes of molecules bind in TRPML3. This knowledge could aid the development of selective TRPML activators that might ultimately yield clinical benefits for ML-IV and other LSDs.
Unique structures of S1 and S2

The TRPML3 structures show several striking and important features (Fig. 3). First, S1 extends extracellularly into the PMD by forming a continuous α-helix with the first 19 amino acids (designated as α1) of the PMD (Fig. 3a). This observation confirms our previous speculation about such a rigid connection based on the crystal structures of an isolated TRPML1 PMD.9 The S1 extension connects directly to the luminal pore loop, which has a divergent amino acid sequence among the three TRPML subunits (Supplementary Fig. 1) and contains three negatively charged amino acids (D108, D111, and D112 in TRPML3) (Fig. 3a). With this rigid physical connection, conformational changes that occur in the luminal pore loop are likely to propagate to S1 and, through it, to other transmembrane regions. We discuss this mechanism in more detail below. Because of its shape and its role in gating, we named the continuous S1-α1 helix the ‘gating rod’; we note that TRPML1 also has such a gating rod.5,5

Second, S2 extends intracellularly beyond the inner membrane boundary, forming a continuous α-helix with the first 21 amino acids of the S2-S3 linker (Fig. 3b). This S2 extension interacts with an N-terminal helix (NTH1), a C-terminal helix (CTH1), and another helix in the S2-S3 linker to form an irregular four-helix bundle (Fig. 3c). On the basis of its shape (Fig. 3b) and possible role in regulating channel gating,

**Figure 3** Unique structural features of TRPML3. (a) The structure of the S1 gating rod and its end connections, viewed parallel to the membrane. (b) The structure of the S2 gating knob, viewed parallel to the membrane. (c) A close-up view of the gating knob, illustrating the regions and hydrophobic interactions involved in the formation of the knob. (d) A comparison of selected regions of TRPML3 and TRPP2 (PDB 5T4D), highlighting the absence of a gating rod and gating knob in TRPP2.

**Figure 4** The pore and gate. (a) The solvent-accessible pathway in the TMD in the apo (left) and ML-SA1-bound (right) structures. Only two diagonally opposed subunits are shown (blue). The amino acids forming the selectivity filter and S6 gate (I498) are shown. (b) A pore-size profile of different sections of the solvent-accessible pathway, generated with the HOLE program. The origin of the pore axis is set at the cytoplasmic end of S6. (c) A close-up view of the selectivity filter in the apo (left) and ML-SA1-bound (right) structures. Only two diagonally opposed subunits are shown. The cryo-EM density map and modeled amino acids are represented by mesh and sticks, respectively. (d) Superposition of S6 near I498 in the apo and ML-SA1-bound structures. The I498 side chains are shown in both ribbon and space-filling representations. In c and d, the distances between the atoms (dashed lines) are measured as the center-to-center distance between two diagonally opposed atoms.
we named this helix bundle the ‘gating knob’. Conformational changes that occur in any part of the gating knob are likely to be transmitted to S2 and hence to other parts of the VSLD. The gating knob is formed through extensive hydrophobic interactions (Fig. 3c), and the involved hydrophobic amino acids are well conserved in the TRPML subfamily (Supplementary Fig. 1), thus suggesting a conserved structural configuration and functional mechanism. H283 is situated at the luminal end of S2 (Fig. 3b); its protonation could trigger conformational changes that would be transmitted to the VSLD and gating knob through S2.

Third, the amino acids important for PI(3,5)P2 activation of TRPML1 are R61 and K62 (ref. 45); the corresponding residues in TRPML3 are R43, and R44 (ref. 45); the analogous residues in TRPML3 are R39, R40, and K41 (Supplementary Fig. 1). They are located at the intracellular end of S1 and near the inner membrane surface (Fig. 3a). The amino acids important for PI(4,5)P2 inhibition of TRPML1 are R42, R43, and R44 (ref. 45); the analogous residues in TRPML3 are R39, R40, and K41 (Supplementary Fig. 1). They are located on NTH1, which is a part of the gating knob (Fig. 3b). We speculate that PI(3,5)P2 activates TRPML3 by pulling on S1 and that PI(4,5)P2 inhibits TRPML3 by pulling on the gating knob and consequently on S2. Precisely how PI(3,5)P2 and PI(4,5)P2 interact with TRPML3 to alter its activity remains to be elucidated structurally.

The direct connections of the VSLD to both the luminal (extracellular) and the intracellular domains through continuous rigid α-helices revealed in TRPML3 are a unique feature in the voltage-gated ion channel superfamily. Through such connections, luminal and intracellular modulators can change the conformation of the VSLD and thus change channel activity. These connections are not observed even in the closely related TRPP2. In TRPP2 structures10–12, S1 and α1 are interrupted by a loop, and the distal C-terminal end of S2 and the S2–S3 linker are not well resolved (Fig. 3d), which suggests that these regions are flexible.

The ion conduction pathway and activation gate
The ion conduction pathway in the TMD consists of the ion SF, the central cavity, and the inner pore (Fig. 4a,b). The SF is formed by four conserved amino acids: N456, G457, D458, and D459 (Fig. 4c and Supplementary Fig. 1). Starting from the luminal entrance to the central cavity, it is lined by the side chains of D459 and D458 and the backbone carbonyls of G457 and N456 (Fig. 4c). The SF external entrance is thus highly electronegative and therefore attracts cations. The SF lumen is 5.4–7.3 Å wide in the apo state and 6.1–8.3 Å wide in the ML-SA1-bound state. These features are consistent with TRPML3 being a Ca2+-permeable, nonselective cation channel36–39. They also explain why the D458K and D458K/D459K mutations abolish channel activity39,56. A strong electron density is present in the center of the SF just above the central cavity in the ML-SA1-bound structure, but not in the apo structure (Fig. 4c). As Na+ was the only cation in the cryo-EM protein sample, this density may represent a Na+-binding site.

The luminal cap atop the TMD has multiple passageways for cations to flow to the SF, including an 11.4-Å-wide luminal pore in the center and four 10-Å-wide portals on the side (Supplementary Fig. 6a). These entryways are electronegative and sufficiently wide for hydrated monovalent and divalent cations to pass through.

The pH 7.4 apo and ML-SA1-bound structures probably represent closed and open states, respectively. In the apo structure, the narrowest constriction of the ion conduction pathway is formed by the side chains of I498 of S6 and is 5.3 Å wide (Fig. 4d). These side chains point to the center of the inner pore and form a hydrophobic seal that blocks monovalent and divalent cation conduction. In the ML-SA1-bound structure, S6 moves outward and rotates 27° counterclockwise, thereby placing the I498 side chains sideways. These movements dilate much of the inner pore (Fig. 4b and Supplementary Data Set 1).
Video 1) and expand the I498 hydrophobic seal to 10.4 Å (Fig. 4d and Supplementary Video 1), which is wide enough to allow the conduction of hydrated monovalent and divalent cations.

It was recently proposed that the closely related TRPP2 channel has two gates, one in the SF and one in S6; the latter is formed by L677 and N681 (refs. 10–12). We do not consider the SF to be a gate in TRPML3 because the SF has similar structures in the closed and open states and its physicochemical properties should allow partially hydrated monovalent and divalent cations to pass through in both states (Fig. 4b,c).

Our work suggests that TRPML3 has only one activation gate, namely, the S6 gate located at I498.

The polycystin-mucolipin domain
Each PMD protomer has a structural fold that consists of four α-helices (α1–α4) and eight β-strands (Fig. 1b and Supplementary Figs. 1 and 6b). The α1 and α2 helices are packed tightly against a five-stranded β-sheet (β1, β4, β5, β6, and β7) (Supplementary Fig. 6b). Between α1 and α2 is the luminal pore loop (Fig. 3a and Supplementary Fig. 6b). Although the overall structure of the TRPML3 PMD is similar to that of the TRPML1 PMD (Supplementary Fig. 6b), their luminal pore loops have different conformations (Supplementary Fig. 6c–e). Structural divergence in this 16-amino-acid loop is largest from position 5 to position 10, corresponding to MDRMDD in TRPML3 and SDGADD in TRPML1. As we discuss below, different luminal pore loop conformations correspond with vastly different functional consequences. Two glycosylation sites in the PMD (N138 and N172) were clearly glycosylated in the ML-SA1-bound structure (Fig. 1a).

Two modes of inhibition by low luminal pH
Previous studies showed that TRPML3 channels are inhibited by low luminal pH, with H283 in the PMD playing a critical role in this inhibition48. We reproduced these results but also found that low luminal pH actually produced two different modes of inhibition. In whole-cell recordings of wild-type TRPML3 channels, changing the bath solution from 160 mM Na+, pH 7.4, to 160 mM Na+, pH 4.6, fully inhibited the currents (Fig. 5). This inhibition was largely irreversible as long as the channels were continuously bathed with the high-Na+, neutral-pH solution. However, the currents were able to recover partially (Fig. 5a) or fully (data not shown) after the channels were bathed in a Na+-free, pH 7.4, solution. Surprisingly, the currents mediated by the H283A mutant channel were still inhibited by low pH (Fig. 5b). However, this inhibition was largely reversible even when the channels were continuously bathed in the high-Na+, neutral-pH solution (Fig. 5b). Thus, in the presence of a high luminal concentration of Na+, as is the case in lysosomes50, low luminal pH inhibits TRPML3 channels in a reversible mode (mode 1) and a long-lasting mode (mode 2). Only the long-lasting mode was abolished by the H283A mutation, which indicates that additional amino acids in TRPML3 can sense luminal pH.

The low-pH structure reveals a dynamic luminal pore loop
To elucidate the structural basis of low-pH inhibition, we obtained a cryo-EM structure of apo-TRPML3 at pH 4.8 at 4.65 Å resolution. The low-pH apo structure is markedly different from the pH 7.4 apo structure in both the PMD and the TMD (Figs. 5c and 6a,b). Strikingly, the luminal pore loop reorients and forms intersubunit bridging interactions that completely occlude the central luminal

Figure 6 Low-pH-induced conformational changes in the PMD and TMD. (a) Superposition of the PMD in the pH 4.8 (pink) and pH 7.4 (sky blue) apo structures, viewed from the luminal/extracellular side of the membrane. The former shows a 7° counterclockwise rotation relative to the latter. (b) Superposition of the TMD and selected key regions in the PMD in the pH 4.8 (gray) and pH 7.4 (color-coded) apo structures, viewed parallel to the membrane in four different orientations. (c) The location of H283 relative to the membrane. The boundary of the outer leaflet of the membrane is defined by the ends of S2, S4, S5, and S6. (d) A cryo-EM density map and atomic model (sticks) of the regions near H283.
pore; meanwhile, four new side luminal pores are formed, which are large enough to allow hydrated cations through (Fig. 5c).

To investigate whether luminal pore loop bridging plays a role in low-pH inhibition of TRPML3, we mutated to asparagine three aspartic residues (D108, D111, and D112) located in the luminal pore loop. This mutant channel, named TRPML3-3DN, did not produce significant basal currents (Fig. 5d). Thus, we were unable to examine whether the 3DN mutation affects low-pH inhibition. However, TRPML3-3DN was strongly activated by ML-SA1, which indicated that it folded properly and was expressed robustly on the plasma membrane (Fig. 5d). These results suggest that a proper conformation of the luminal pore loop is critical for TRPML3 activity. We speculate that the 3DN mutation causes a conformational change in the luminal pore loop, and that this conformational change transmits through α1 to S1 and stabilizes the closed state.

Low pH induces global conformational changes

In addition to the dramatic conformational change in the luminal pore loop, comparison of the pH 4.8 and pH 7.4 apo structures showed that the PMD undergoes a 7° counterclockwise rotation, whereas the VSLD undergoes a 3°–6° clockwise rotation, when viewed top-down from the luminal side (Fig. 6a and Supplementary Video 2). Further comparison of selected key regions of the PMD and TMD (including S1–S6, α1, α2, and the luminal pore loop) showed conformational changes in many regions (Fig. 6b and Supplementary Video 2): (1) the luminal pore loop extends into the center, as shown in Figure 5c; (2) α1 bends downward and toward the center; (3) S1, especially its intracellular end, bends outward; (4) S2 rotates upward and bends outward; (5) S3 also bends outward; (6) the S4-S5 linker moves upward; and (7) S5 and S6 move inward toward the pore. These concerted movements presumably lead to an energetically favorable, more stable closed state.

It should be noted that whereas the pH 7.4 apo structure was obtained from TRPML3 in the amphipathic surfactant amphipol, the pH 4.8 structure was obtained from TRPML3 in the detergent LMNG. The different environments could be responsible for some of the differences in the two structures. It is also possible that some of the conformational changes caused by the low pH were not triggered by the protonation of H283 and the luminal pore loop aspartates, as the entire TRPML3 channel was bathed in the pH 4.8 solution during protein-sample preparation for structure determination.

In contrast to the large conformational change of the luminal pore loop, the structural change of the luminal end of S2 was relatively small (Fig. 6b). H283 is located immediately above the luminal end of S2 (Fig. 6c) and does not interact with other amino acids in the pH 7.4 apo structure (Fig. 6c) or in the pH 4.8 structure. These observations contrast with functional data showing the importance of H283 in low-pH inhibition of TRPML3 (ref. 48) (Fig. 5a, b). The reason for
this discrepancy is probably related to the condition in which the low-pH structure was obtained—the cryo-EM protein sample was not in a lipid bilayer. A possibility is that in cells, protonation of H283 enables it to interact with certain lipids in the outer leaflet of the membrane, and these interactions change the conformation of S2. The changes in S2 in turn cause conformational changes in the VSLD and pore domain and produce a long-lasting inhibited state. Validation of this hypothesis requires further functional studies and determination of TRPML3 structures in the presence of lipids in nanodiscs.

DISCUSSION

Our study elucidates the structural basis of TRPML3 assembly, ion permeation, agonist activation, and low-pH inhibition. We find it interesting that TRPML3 and TRPML1 channels were both regulated by luminal pH, but with opposite effects. Low pH inhibited TRPML3, and this inhibition appears to have involved large, global conformational changes in the PMD and TMD (Fig. 6). In contrast, low pH potentiated TRPML1. Our previous work on an isolated TRPML1 PMD showed that changing pH does not change the structure of the PMD and that the conformation of the luminal pore loop is identical at pH 7.4, 6.0, and 4.6 (ref. 9). We proposed that H+ potentiates TRPML1 currents by protonating the luminal pore loop aspartates and attenuating blocking by Ca2+. According to this proposal, the effect of low pH in TRPML1 is local and does not involve conformational changes in the PMD or TMD. Verification of this view awaits the determination of full-length TRPML1 structures at different pH values.

Our structural and electrophysiological data suggest that low luminal pH inhibits TRPML3 through two different and independent mechanisms. In mode 1 (Fig. 7a), H+ ions protonate the three aspartic residues in the luminal pore loop, thereby causing a conformational change that propagates through α1 to S1 and subsequently to S2, S3, the S4–S5 linker, S5, and S6 (Figs. 5c and 6a,b, and Supplementary Video 2). These conformational changes produce an inhibited state that is readily reversible when the luminal pH becomes neutral. Although we have not functionally confirmed that it is the protonation of the luminal pore loop aspartates that produces mode 1 inhibition, the reversible low-pH inhibition of the H283A channel (Fig. 5b) indicates that there is indeed another pH sensor in addition to H283. Two other histidines (H252 and H273) are reported to play a role in the low-pH inhibition of TRPML3 (ref. 48), but functional studies suggest that role is mainly to affect the protonation of H283 (ref. 48).

Moreover, both the open-state and closed-state structures show that they are either buried in the protein interior (H252) or exposed to the surface (H273), which suggests that they might not sense pH changes in the lumen.

In mode 2 (Fig. 7b), H+ ions protonate H283. The protonated, positively charged H283 side chain then interacts with the negatively charged head groups of certain outer leaflet lipids, thereby causing a conformational change in S2 and consequently in the VSLD and pore domain. Importantly, S2 movement also induces a conformational change in the gating knob. These conformational changes cause the channel to undergo an undefined modification and be in a long-lasting inhibited state that can recover only after the channels are pre-bathed in a Na+-free, neutral-pH luminal solution. The modification might be phosphorylation/dephosphorylation, sumoylation, or binding to a protein partner. How incubation in a Na+-free, neutral-pH luminal solution enables the channel to recover from the long-lasting inhibited state remains to be elucidated.

Structural studies of TRPML3 may aid the development of new treatment strategies for ML-IV. All ML-IV-causing mutations are loss-of-function mutations, and most of them are splicing, deletion, or frame-shift mutations that result in a total loss of functional TRPML1 protein in cells29–34. Gene therapy is one potential strategy for ML-IV treatment, as it is for several other LSDs58. However, the application of gene therapy in people with ML-IV is likely to be an arduous process. Another potential strategy is to develop means to functionally substitute TRPML1 with TRPML3 by taking advantage of the proteins’ endolysosomal colocalization and shared ion permeation properties4–6,52. The principal hypothesis here is that if one is able to selectively and judiciously activate the normally dormant TRPML3 in endosomes and lysosomes, one might be able to restore, at least partially, the functions of the endocytic pathway in people with ML-IV. Structural information about TRPML3 in different states and bound to different activators may be useful in this equally arduous endeavor.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.L. and J.Y. conceived and initiated the project. X.Z., M.L., J.Y., D.S., Q.J., H.L., X.L., and J.Y. designed the experiments, analyzed the results, and wrote the manuscript. M.L. and J.Y. performed all molecular biology and biochemical experiments and built the atomic models. X.Z. and X.L. performed all cryo-EM experiments, including data acquisition and processing. D.S., Q.J., and H.L. performed electrophysiology experiments. All authors contributed to manuscript discussion, preparation, and editing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Molecular biology. A DNA fragment encoding full-length human TRPML3 channel was cloned into a modified pFastBac1 vector. A maltose-binding protein (MBP) tag was added before the N terminus of TRPML3, and a linker sequence containing the tobacco etch virus (TEV) protease recognition sequence was inserted between the MBP tag and TRPML3.

For electrophysiology experiments, the full-length gene encoding TRPML3 was cloned into a pIRE52-EGFP vector. All site-specific mutants were subsequently generated in this TRPML3_pIRE52-EGFP construct by PCR-based overlapping extension.

Protein purification. The TRPML3-expressing baculovirus was generated with Sf9 cells via the standard Bac-to-Bac method (Invitrogen). Thin insect cells grown in suspension in ESF921 medium (Expression Systems) were infected with the TRPML3-expressing virus. Forty-eight hours after infection, cells were collected by centrifugation at 4 °C and suspended in buffer A, containing 50 mM NaH2PO4-Na2HPO4, pH 8.0, 500 mM NaCl and 5% glycerol, in the presence of Complete protease inhibitor cocktail (Roche). After cell disruption by sonication, cell debris was removed by centrifugation at 3,200g for 10 min at 4 °C, and cell membrane was pelleted from the supernatant by ultracentrifugation at 150,000g for 1 h at 4 °C. Membrane was suspended in buffer A containing protease inhibitor cocktail and homogenized with a glass Dounce homogenizer. TRPML3 protein was extracted with 1% lauryl maltose neopentyl glycol (LMNG; Anatrace) for 1 h at 4 °C. The solubilized membrane was clarified by ultracentrifugation at 150,000g for 30 min and incubated with amylase resin (NEB) for 2 h at 4 °C with gentle agitation. Subsequently, the resin was collected by low-speed spinning at 800g, transferred into a gravity column, and washed with wash buffer (buffer A containing 0.5 mM LMNG and 0.1 mg/ml soybean lipids (Avanti Polar Lipids)). MBP-tagged TRPML3 protein was eluted from amylase resin with 20 mM maltose in wash buffer.

For samples at pH 7.4, the eluted protein was mixed with amiphil A8-35 (Anatrace) at a 1:6 (w/v) ratio and incubated overnight at 4 °C with gentle agitation. Detergent was removed by incubation with Bio-Beads SM-2 resin (Bio-Rad) for 8 h. After removal of the Bio-Beads, the protein sample was mixed with TEV protease (100 µg/mg TRPML3) and incubated at 4 °C overnight to cleave the MBP tag. TRPML3 protein was concentrated and further purified on a Superose 6 column (GE Healthcare) in a buffer containing 20 mM HEPES-NaOH, pH 7.4, and 150 mM NaCl. Fractions containing TRPML3 protein were examined by negative staining electron microscopy and then pooled and concentrated. ML-SA1 was added to the complex before the cryo sample was prepared (see below).

For the sample at pH 4.8, the protein eluted from amylase resin in detergent solution was mixed with TEV protease (100 µg/mg TRPML3) and incubated overnight at 4 °C. Then the protein concentration was further purified on a Superose 6 column in a buffer containing 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, and 0.5 mM LMNG. Fractions containing TRPML3 protein were pooled and concentrated, and the pH was changed to 4.8 before cryo-sample preparation (see below). Proteins were concentrated by ultrafiltration with Amicon centrifugal filter units (EMD Millipore), and their concentrations were measured with a Bradford protein assay kit (Bio-Rad).

Cryo-EM sample preparation and data acquisition. For the apo-TRPML3 sample in pH 4.8 solution, a 4-µl drop of sample at a concentration of 1.9 mg/ml was loaded onto a glow-discharged Quantifoil holey carbon grid (R1.2/1.3, 300 mesh; Electron Microscopy Sciences). After waiting for 3 s, we blotted the grid for 4 s under 100% humidity at 8 °C with FEI Vitrobot (double-sided, blot force 1) and then immediately plunged it into liquid ethane cooled by liquid nitrogen. The cryo sample of the TRPML3–ML-SA1 complex preparation was similar to the apo sample, except that 100 mM ML–SA1 in DMSO was added to the protein sample to a final concentration of 100 µM before freezing. For the TRPML3 sample at pH 4.8, protein in 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, and 0.5 mM LMNG was concentrated to 2 mg/ml. 1 M Na acetate buffer, pH 4.6, was added to the protein solution to a final concentration of 100 mM. The pH of the mixed HEPES and acetate buffer was verified as 4.8.

We viewed the samples of TRPML3–ML-SA1 complex and TRPML3 at pH 4.8 with a Titan Krios microscope (FEI) operated at 300 kV, equipped with a K2 Summit direct electron detector (Gatan) working in super-resolution counting mode with a pixel size of 0.67 Å. The detector was placed at the end of a GIF Quantum energy filter (Gatan), operated in zero-energy-loss mode with a slit width of 20 eV. Etas (developed by Bo Shen in X.L.’s lab) was used for data collection under a defocus range from −1.0 µm to −2.5 µm. Each micrograph was dose-fractionated to 32 frames with a 0.25-s exposure time in each frame. The dose rate was 8.2 counts per physical pixel per second, which resulted in a total dose of −50 e−Å2. The data-acquisition condition for TRPML3 protein at pH 7.4 was mostly the same as described above except that the Titan Krios used was not equipped with a GIF Quantum energy filter, the pixel size was 0.66 Å, and the defocus ranged from −1.5 µm to −3.0 µm.

Image processing. The cryo-EM super-resolution micrographs were 2 × 2 binned. Motion correction was done with the MotionCorr2 program59 to generate integrated micrographs for further processing. CTFFIND3 was used to determine defocus values60. For the data on apo-TRPML3 at pH 7.4, the full data set of 2,721 micrographs was divided into two subsets with 726 and 1,995 micrographs, respectively. Each subset was subjected to two rounds of two-dimensional classification, and then the selected particles were classified into four classes in the three-dimensional classification procedure. The map of the CNG channel52 low-pass filtered to 60 Å was used as the initial model. Particle heterogeneity was observed among the resulting 3D classes, which presented two typical conformations: a near-perfect tetramer in the first subset and a defective tetramer with two missing/flexible domains in the second subset. So two rounds of two-reference 3D classification were carried out as described previously61. Finally, 43,542 particles corresponding to the near-perfect tetramer were gathered and subjected to final 3D refinement with C4 symmetry imposed, which resulted in a final map at 4.06-Å resolution. For TRPML3 in pH 4.8 buffer and TRPML3–ML-SA1 complex, the image-processing procedure was almost the same; after two rounds of 2D classification and one round 3D classification, the best one or two 3D classes were selected and gathered for final refinement. All the 2D classification, 3D classification, and refinement procedures were done by RELION 1.4 (ref. 62). The final refinement of the TRPML3–ML-SA1 complex was done with THUNDER software, developed by Mingxu Hu, Hongkun Yu, and colleagues in X.L.’s lab. The 3D reconstruction obtained by THUNDER had a 3.62-Å resolution, higher than the 3.82-Å resolution obtained by RELION 1.4. For the sample at pH 4.8, a 4.65-Å map was reconstructed. All resolutions were estimated by the gold-standard criterion of Fourier shell correlation (FSC) = 0.143. ResMap63 was used to calculate the local resolution map. The normalized difference map between the ML-SA1-bound pH 7.4 and apo pH 7.4 structures was calculated by THUNDER and was filtered to 6.0 Å.

Model building, refinement, and validation. Atomic models were built in Coot64. The 3.62-Å density map of the TRPML3–ML-SA1 complex was used for de novo manual model building of the transmembrane. Densities of the side chains for bulky residues (Trp, Tyr, and Phe) and predicted transmembrane ranges were used to guide amino acid assignment. The homologous crystal structure of the I-II linker of human TRPML1 (PDB 5TJA) was docked into the density map with UCSF Chimera65, mutated into human TRPML3 sequence, and rebuilt in Coot. The atomic model for membrane-proximal N and C termini and cytoplasmic II-III linker was built, although the densities of the side chains of some amino acids were missing. An atomic model was built for one subunit, and then the tetramer model of the channel was generated by a fourfold rotational symmetry operation. This model was then used as an initial model and was rebuilt into the density map of apo-TRPML3. Extra densities in the map of the TRPML3–ML-SA1 complex were found where an atomic model of the ligand ML-SA1 (coordinates downloaded from https://pubchem.ncbi.nlm.nih.gov) was tentatively put in. For TRPML3 at pH 4.8, individual transmembrane fragments and the PMD domain from the apo model were fitted into the density in UCSF Chimera. The model was then manually rebuilt in Coot. Residues missing side chain densities were mutated to alanine. All three models were refined in reciprocal space by Phenix66. Coordinates and individual B factors were refined via maximum-likelihood refinement, with secondary structure restraints and strict noncrystallographic symmetry constraints. Overfitting of the atomic model was checked via previously described methods67. The coordinates were randomly displaced up to 0.2 Å, and the new model was then refined against one of the two half-maps generated. FSC values were determined using a protocol implemented in IMAGIC68.
calculated between the resulting model and the two half-maps, as well as the averaged map of two half-maps. The quality of the models was evaluated with MolProbity\(^{68}\). PyMOL and UCSF Chimera were used to make figures. The pore radius was calculated with HOLE software\(^{69}\).

**Electrophysiology.** HEK293T cells (ATCC; not tested for mycoplasma) were grown in DMEM (Gibco) plus 10% FBS (VWR) and penicillin (100 U/ml)–streptomycin (0.1 mg/ml) (Sigma). HEK293T cells were transiently transfected with wild-type or mutant TRPML3 with LipoD293 (SignaGen Laboratories) and used within 48 h. All experiments were done at room temperature (~22 °C). Pipettes were fabricated from borosilicate glass (Corning Pyrex) with a micropipette puller (PC-10; Narishige) and were fire-polished to resistances of 2–3 MΩ for whole-cell recording. Whole-cell currents were elicited by 400-ms voltage ramps from −100 to +100 mV at a frequency of 0.5 Hz with a holding potential of 0 mV. Currents were amplified by Axopatch 200B and digitized by Digidata 1322A (Molecular Devices). Currents were low-pass filtered at 1 kHz and sampled at 1 kHz. pCLAMP 8.2 software (Molecular Devices) was used for data acquisition and analysis. The standard pipette solution contained 120 mM cesium methanesulfonate, 10 mM BAPTA, 2 mM MgCl\(_2\), 20 mM HEPES, pH 7.2, adjusted with CsOH. The pH 7.4 standard bath solution contained 160 mM NaCl, 5 mM KCl, 10 mM D-glucose, 20 mM HEPES, and the pH was adjusted with NaOH. The pH 4.6 standard bath solution contained 160 mM NaCl, 5 KCl, 10 mM D-glucose, 10 mM HEPES, 10 mM MES, and the pH was adjusted with NaOH. The pH 7.4 sodium-free solution contained 165 mM NMDG, 10 mM D-glucose, 10 mM HEPES, 10 mM MES, and the pH was adjusted with HCl.

**Data availability.** 3D density maps and atomic models have been deposited in the Electron Microscopy Data Bank and the Protein Data Bank under the following accession numbers: apo-TRPML3 at pH 7.4, EMD-7018 and PDB 6AYE; ML-SA1-bound TRPML3 at pH 7.4, EMD-7019 and PDB 6AYF; and apo-TRPML3 at pH 4.8, EMD-7020 and PDB 6AYG. The THUNDER software is being prepared for publication and is available from XL, upon request. Electrophysiology data for Figures 2b–d and 5a,b,d are provided in Supplementary Data Set 1. Other data supporting the findings of this study are available from the corresponding author(s) upon reasonable request. A Life Sciences Reporting Summary for this paper is available.

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## Experimental design

1. **Sample size**
   - Describe how sample size was determined.
     - For most functional studies, the sample sizes was >5 to ensure adequate power.

   **Data exclusions**
   - Describe any data exclusions.
     - No data were excluded from the analysis.

   **Replication**
   - Describe whether the experimental findings were reliably reproduced.
     - We did to replicate the structural experiments, which is standard practice. The electrophysiological experiments were readily and reliably reproduced.

   **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
     - Randomization is not relevant to the structural experiments. For electrophysiological recordings, experiments with different conditions (e.g., WT vs. mutant channels) were performed in an alternating order on the same day.

   **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
     - Blinding is not relevant to the structural experiments. Blinding was not done for the electrophysiological experiments because we needed to design specific protocols for each experiment and to record from different types of channels (i.e., WT vs. mutant) in an alternating fashion.

   **Note:** all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

   **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   - **Confirmed**
     - The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
     - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
     - A statement indicating how many times each experiment was replicated
     - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
     - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
     - The test results (e.g., P values) given as exact values whenever possible and with confidence intervals noted
     - A clear description of statistics including central tendency (e.g., median, mean) and variation (e.g., standard deviation, interquartile range)
     - Clearly defined error bars

   *See the web collection on statistics for biologists for further resources and guidance.*
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

- pCLAMP 8.2, MotionCorr2, CTFFIND3, RELION 1.4, ResMap, Coot, USCF chimera, Phenix, MolProbity, PyMOL, HOLE, and THUNDER (developed by Mingxu Hu, Hongkun Yu and colleagues at Xueming Li Lab)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

- All materials are available from the authors or commercial sources described in the paper.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- N/A

9. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

- HEK 293T from American Type Culture Collection (ATCC), Sf9 cells were from Invitrogen, and Tni cells were from Expression Systems.

b. Describe the method of cell line authentication used.

- None of the cell lines used have been authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

- The cells lines were not tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

- N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

- No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

- This study did not involve human subjects.