Structural basis of dual Ca$^{2+}$/pH regulation of the endolysosomal TRPML1 channel

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The activities of organelar ion channels are often regulated by Ca$^{2+}$ and H$^+$, which are present in high concentrations in many organelles. Here we report a structural element critical for dual Ca$^{2+}$/pH regulation of TRPML1, a Ca$^{2+}$-release channel crucial for endolysosomal function. TRPML1 mutations cause mucolipidosis type IV (MLIV), a severe lysosomal storage disorder characterized by neurodegeneration, mental retardation and blindness. We obtained crystal structures of the 213-residue luminal domain of human TRPML1 containing three missense MLIV-causing mutations. This domain forms a tetramer with a highly electronegative central pore formed by a novel luminal pore loop. Cysteine cross-linking and cryo-EM analyses confirmed that this architecture is also found in the full-length channel. Structure–function studies demonstrated that Ca$^{2+}$ and H$^+$ interact with the luminal pore and exert physiologically important regulation. The MLIV-causing mutations disrupt the luminal-domain structure and cause TRPML1 mislocalization. Our study reveals the structural underpinnings of TRPML1’s regulation, assembly and pathogenesis.

Many types of ion channels are present in the membranes of intracellular organelles, such as the endoplasmic reticulum, Golgi apparatus, mitochondria, endosomes and lysosomes$^{1-6}$. These channels help to establish and maintain ionic-concentration gradients across organelar membranes, and/or serve as release channels for key signaling ions, including Ca$^{2+}$ and H$^+$. Many organelles have high concentrations of Ca$^{2+}$ and H$^+$ in their lumens$^{1,3-5,7}$. These ions in turn regulate the activities of numerous organelar ion channels$^{8-12}$. Elucidating the molecular mechanisms underlying this universal regulation is instrumental in understanding the physiological and pathophysiological functions of organelar ion channels.

TRPML1 is a member of the transient receptor potential mucolipin (TRPML) channel subfamily, which was first identified as a genetic determinant of mucolipidosis type IV (MLIV)$^{13-15}$, one of ~50 lysosomal storage disorders. Young children with MLIV often show cognitive, linguistic and motor deficits and are sometimes blind$^{6,16-18}$. TRPML1 is located primarily in lysosomes, where its main role is to conduct Ca$^{2+}$ from the lysosome lumen to the cytoplasm$^{5,6,19-23}$. This Ca$^{2+}$-release step is important in many lysosome-dependent cellular events, including exocytosis$^{5,6,21,24,25}$, membrane trafficking$^{5,6,25-27}$ and autophagy$^{5,6,24,25,28-30}$. TRPML1 activity is regulated by luminal and extracellular Ca$^{2+}$ and H$^+$ (refs. 6,19–23,31,32). In the presence of divalent ions, TRPML1 currents are greatly potentiated by H$^+$, via unknown mechanisms$^{6,19-23,31,32}$. The dual regulation by Ca$^{2+}$ and H$^+$ has clear physiological relevance: TRPML1 exists primarily in lysosomes, and during biogenesis and lysosomal exocytosis, it may also be present in other organelles, including late endosomes and the plasma membrane$^{23-25,33}$. These distinct subcellular compartments have different pH levels and Ca$^{2+}$ concentrations$^{1,5,6,23}$. The extracellular side of the plasma membrane has 1.8–2 mM Ca$^{2+}$ and a near-neutral pH of 7.2–7.4, whereas late endosomes have 0.5 mM Ca$^{2+}$ and an acidic pH of 5.5–6.0. Lysosomes have 0.5–0.6 mM Ca$^{2+}$ and an even more acidic pH of 4.5–5.0, a condition crucial for the activity of their native hydrolases$^{23,34}$. Thus, depending on its subcellular location, TRPML1 may be regulated to different extents by Ca$^{2+}$ and pH and may consequently exhibit different activities. The molecular mechanism of this dual regulation is unknown.

The crucial role of TRPML1 in cellular processes is further demonstrated by the existence of >20 mutations in the TRPML1 gene that have been linked to MLIV$^{6,18,35}$. Among these mutations, some (such as frameshift or nonsense mutations) completely abolish TRPML1 expression, but many (>12) are single–amino acid missense mutations$^{6}$. How these missense mutations alter the structure and function of TRPML1 is largely unclear.

We used a structural approach to investigate the molecular mechanism of dual Ca$^{2+}$/pH regulation of TRPML1 and MLIV pathogenesis, focusing on the linker between the first two transmembrane segments (S1 and S2) of TRPML1, which we refer to as the I–II linker. Like other TRP-channel subunits, TRPML1 contains six transmembrane segments (S1–S6; Supplementary Fig. 1a). The I–II linker accounts for...
more than one-third of the channel's length (Supplementary Fig. 1b). In lysosomes and endosomes, this linker faces the lumen (and is therefore also referred to as the "luminal linker"); on the plasma membrane, it faces the extracellular solution (Supplementary Fig. 1a). Moreover, it is the site of three single–amino acid missense mutations that cause MLIV6,17,18,36,37 (Supplementary Fig. 1b). We solved the crystal structures of the luminal linker at three different pH conditions (corresponding to the pH in lysosomes, endosomes and the extracellular milieu) and elucidated its role in the dual regulation of TRPML1 by luminal Ca\(^{2+}\) and pH, TRPML1-channel assembly and MLIV pathogenesis.

RESULTS

TRPML1-channel activity is regulated by luminal Ca\(^{2+}\) and pH

To obtain human TRPML1 currents, we followed a well-established method by expressing a constitutively active mutant channel designated TRPML1VP (tagged with EGFP on its N terminus) in HEK 293T cells20,38. TRPML1VP contains the V432P mutation, which mimics a spontaneous gain-of-function mutation (A419P) in mouse TRPML3 that causes the varitint-waddler (Va) phenotype39–41. The V432P mutation enables TRPML1 to traffic to the plasma membrane and to become spontaneously active20,38. In whole-cell patch-clamp recordings, TRPML1VP produced a strong inward-rectifying current in a nominal divalent-ion-free (NDF) extracellular/luminal solution at pH 7.4 (Fig. 1a,b). Addition of Ca\(^{2+}\) to the extracellular/luminal solution inhibited the current in a dose-dependent manner. A dose–response curve yielded an apparent half-maximal inhibitory concentration (IC\(_{50}\)) of 0.27 mM, with a Hill coefficient of 1 (Fig. 1c), thus suggesting a one-to-one blocking mechanism. Ca\(^{2+}\) inhibition of TRPML1 was strongly dependent on extracellular/luminal pH, and became much weaker when the pH of the extracellular/luminal solution was lowered from 7.4 to 4.6, the pH of the lysosome lumen. The dose–response curve was shifted to the right at pH 4.6 (Fig. 1c), thereby increasing the apparent IC\(_{50}\) by 14-fold, to 3.8 mM. Intriguingly, the shape of the dose–response curve was different, and the Hill coefficient was changed from 1 to 0.5 (Fig. 1c), thus suggesting negative cooperativity between extracellular/luminal Ca\(^{2+}\) and H\(^{+}\). Owing to attenuated Ca\(^{2+}\) inhibition at pH 4.6, TRPML1VP currents were augmented when the extracellular/luminal pH was lowered from 7.4 to 4.6 (Fig. 1d).

It should be mentioned that the dual regulation by luminal Ca\(^{2+}\) and pH described above was observed in a gain-of-function mutant rather than the wild-type (WT) channel. Because the V432P mutation enables the channel to open constitutively, any effects of luminal Ca\(^{2+}\) and H\(^{+}\) on gating might not be revealed.

Crystal structure of the luminal linker

Because the TRPML1 luminal linker faces the endolysosomal lumen and constitutes a large part (>1/3) of the channel, we investigated its role in the Ca\(^{2+}\)/pH regulation of TRPML1. We first determined the X-ray crystal structure of the luminal linker (G84–S296) of human TRPML1 at a 2.3-Å resolution at pH 6.0 (Fig. 2 and Table 1). The structure shows that the linker forms a tetramer with four-fold rotational symmetry (Fig. 2a). The structure is ~90 Å wide (outer diameter) and ~30 Å tall. At the center of the tetramer exists a highly electronnegative pore, termed the luminal pore (Fig. 2b). Each protomer has a structural fold consisting of two long α-helices (α1 and α2, with 22 and 17 residues, respectively), two short α-helices (α3 and α4), eight β-strands and three loops with >15 residues (between α1 and α2, α3 and β1, and β4 and β5, respectively) (Fig. 2c). The four α-helices are packed tightly against a five-stranded β-sheet formed by β1, β4, β5, β6 and β7. One side of this β-sheet faces the luminal pore, and the other side faces the outside of the tetramer (Fig. 2a,c). Two intrasubunit disulfide bonds are present, one between C166 in β1 and C192 in β4, and another between C253 in β6 and C284 in the short loop between β7 and β8. The structure of E199–K219 (which constitutes part of the loop between β4 and β5) was unresolved, presumably because of its flexibility.

The TRPML1 I–II linker structure has a number of unique and notable features. (i) The luminal pore is lined by a novel 16–amino-acid, reentrant luminal pore loop (L106–T121) that connects to helices α1 and α2 (Fig. 2a,d). The narrowest region of the luminal pore is 14 Å, a diameter wide enough to allow fully hydrated Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\) ions to pass through. (ii) The luminal pore contains 12 aspartate residues (D111, D114 and D115 in each linker) (Fig. 2a,d), thus making this region highly electronegative at neutral pH (Fig. 2b). (iii) The first 22 amino acids of the luminal linker (G84–F105) form an α-helix (α1) that may be of strategic importance for allosteric modulation: it connects directly to S1 at the N terminus and to the luminal pore loop at the C terminus (Fig. 2a). When the TRPML1 I–II linker is placed on top of the transmembrane segments of the TRPV1 structure42, α1 aligns reasonably well with S1 (Supplementary Fig. 2). Indeed, α1 appears to form a continuous α-helix with S1, thereby anchoring the I–II linker to the rest of the channel. We speculate that in the full-length channel, sequential movements of cytoplasmic and transmembrane regions may propagate to the luminal pore through α1.
Another α-helix (α2, R122–A138), whose N terminus is connected to the luminal pore loop, forms a flat perimeter on the top of the luminal pore (Fig. 2a).

Confirming the luminal-linker structure in full-length TRPML1

The structure described above was obtained from an isolated channel domain. Is this tetrameric structure preserved in the full-length channel? We used two approaches to address this question. First, the crystal structure shows that each luminal linker interacts with two neighboring linkers (Fig. 2a); therefore, we performed biochemical experiments to examine cross-linking among them. In this experiment, we used *Caenorhabditis elegans* instead of human TRPML1 because, for unknown reasons, the human TRPML1 protein remained tetrameric even in reducing SDS–PAGE (data not shown). On the basis of the intersubunit interface of the human TRPML1 I–II linker, we engineered three complementary pairs of cysteines in full-length TRPML1.

| Table 1 Data collection and refinement statistics |
|--------------------------------------------------|
| **Data collection** | **TRPML1 I–II linker, pH 6.0 (PDB 5TJA)** | **TRPML1 I–II linker, pH 6.0, K2Pt(CN)4 derivative (PDB 5TJB)** | **TRPML1 I–II linker, pH 4.5 (PDB 5TJC)** | **TRPML1 I–II linker, pH 7.5 (PDB 5TJD)** |
| Space group | I422 | I422 | P4212 | F432 |
| Cell dimensions | 125.3, 125.3, 76.7 | 125.3, 125.3, 76.5 | 94.4, 94.4, 50.7 | 182.8, 182.8, 182.8 |
| a, b, c (Å) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Wavelength | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Resolution (Å) | 20.0–2.3 (2.38–2.30) | 20.0–2.6 (2.69–2.60) | 20.0–2.4 (2.49–2.40) | 20.0–2.4 (2.49–2.40) |
| Rwork (%) | 9.9 (37.7) | 13.8 (64.4) | 6.8 (43.4) | 5.9 (34.0) |
| Rfree (%) | 20.0 (4.6) | 14.6 (2.8) | 19.0 (2.8) | 22.3 (3.7) |
| Rwork (%) | 0.982 (0.944) | 0.961 (0.866) | 0.968 (0.872) | 0.969 (0.882) |
| Completeness (%) | 99.9 (99.6) | 99.9 (99.8) | 99.3 (98.3) | 98.0 (98.6) |
| Redundancy | 7.2 (6.1) | 6.9 (6.4) | 4.7 (3.8) | 4.2 (4.1) |
| Refinement | 20–2.3 | 20–2.4 | 20–2.4 |
| No. reflections | 13,298 (1,337) | 8,777 (927) | 9,088 (1,033) |
| Rwork / Rfree | 0.228 / 0.253 | 0.212 / 0.252 | 0.216 / 0.253 |
| No. atoms | 1,518 | 1,268 | 1,426 |
| Protein | 1,518 | 1,268 | 1,426 |
| Water | 111 | 104 | 66 |
| B factors | 33.0 | 42.1 | 50.3 |
| Protein | 33.0 | 42.1 | 50.3 |
| Water | 45.5 | 45.5 | 47.6 |
| R.m.s. deviations | 0.010 | 0.007 | 0.006 |
| Bond lengths (Å) | 1.7 | 1.5 | 1.4 |

Each data set was collected from a single crystal. aValues in parentheses are for the highest-resolution shell.
C. elegans TRPML1 that were predicted to form intersubunit disulfide bonds; these included D104C in linker A and T293C in linker B; H111C in linker A and Y136C in linker B; and D158C in linker A and D137C in linker B (Fig. 3a). We also engineered six noncomplementary pairs of cysteines in two neighboring linkers that were predicted not to form intersubunit disulfide bonds: D104C and Y136C; D104C and D137C; H111C and T293C; H111C and D137C; D158C and T293C; and D158C and Y136C. The results were as predicted: under nonreducing conditions (i.e., without DTT), purified full-length TRPML1 proteins containing complementary cysteine mutations formed oligomeric bands in SDS–PAGE at the expense of the monomeric bands (Fig. 3b, top, lanes 2, 6 and 10; uncropped images shown in Supplementary Data Set 1). However, those proteins containing noncomplementary cysteine mutations remained monomeric (Fig. 3b, top, lanes 1, 3, 4, 5, 7 and 8). (The D158C Y136C protein in lane 9 appeared to have been degraded or precipitated and did not yield a band.) Under reducing conditions (i.e., with DTT), all proteins existed as monomers (Fig. 3b, bottom). The existence of the oligomer bands only in the complementary cysteine pairs and only under nonreducing conditions was consistent with disulfide-bond formation between the engineered cysteines, even though the molecular weight of the oligomer bands was larger than that of a predicted tetramer, possibly because of tetramer aggregation.

Second, we carried out single-particle cryo-EM analysis of the purified full-length WT C. elegans TRPML1 protein (Supplementary Fig. 3). The cryo-EM density of the extracellular domain (ED) was clear and robust, but that of the transmembrane domain (TMD) was unclear and heterogeneous (Supplementary Fig. 3c,d). The ED and TMD were distinguishable and recognizable on the basis of their dimensions and the gross similarity of the TRPML1 and TRPV1 TMDs. We therefore used a masking procedure that allowed us to segregate the ED from the TMD during subsequent cryo-EM analysis (Supplementary Fig. 3c). A cryo-EM density map of the ED was obtained at an overall resolution of 5.28 Å with C4 symmetry imposed (Fig. 3c and Supplementary Fig. 3c). Even at this limited resolution, the ED, ~79% of which is made up of the luminal linker, is visible as a tetramer with a large pore in the center (Fig. 3c), thus confirming two of the most salient hallmarks of the crystal structure of the luminal linker. Given the limited resolution, we did not build an atomic model or identify secondary structures on this density map; however, the crystal structure of the luminal linker could be fitted with a correlation coefficient of 0.75, thus indicating a reasonably good fit (Fig. 3d).

The extra density not covered by the luminal-linker crystal structure in the composite map could come from the E199–K219 loop (which is unresolved in the crystal structure), other extracellular loops and/or glycosylation.

Together, the above biochemical and cryo-EM studies suggest that the TRPML1 luminal linker has the same structure in the full-length channel as in the isolated domain: it forms a tetramer and contains a large central pore. This conclusion was further supported by structure-guided mutagenesis studies described directly below.

The luminal-linker tetramer is crucial in TRPML1 assembly

The crystal structure of the luminal linker shows that it forms a tight tetramer through extensive intersubunit interactions. These interactions include hydrogen-bonding, formation of ion pairs, hydrophobic interactions and formation of an antiparallel, domain-swapped, three-stranded β-sheet between two neighboring subunits (Fig. 4a and Supplementary Fig. 1b). The interface between two adjacent linkers has a buried surface area of 1,430 Å². To examine whether the tetrameric assembly of the luminal linker is important in the tetrameric assembly of full-length TRPML1, we mutated L144 and R146, two residues that engage in intersubunit interactions (Fig. 4a), to lysine and serine, respectively, in both the isolated luminal linker and full-length human TRPML1. We then examined the oligomeric states of WT and mutant linker domains or full-length proteins by native gel electrophoresis. In a nondenaturing gel, the isolated luminal linker containing the L144K R146S mutation (Fig. 4b, top, lane 2) migrated at a much lower molecular weight than did the WT linker (Fig. 4b, top, lane 1), thus suggesting their being a monomer and a tetramer, respectively, and indicating that the double mutation disrupted the tetrameric assembly of the isolated linker.

**Figure 3** Verification of the I–II linker structure in the full-length protein. (a) Location of three pairs of amino acids in the I–II linker structure, with distances between the Cβ atoms indicated. Residues without or with parentheses correspond to human and C. elegans amino acids, respectively. (b) SDS–PAGE of full-length WT and mutant TRPML1 proteins under nonreducing (top) and reducing (bottom) conditions. The lanes are: 1, WT; 2, D104C T293C; 3, D104C Y136C; 4, D104C D137C; 5, H111C T293C; 6, H111C Y136C; 7, H111C D137C; 8, D158C T293C; 9, D158C Y136C; 10, D158C D137C. Uncropped images are shown in Supplementary Data Set 1. (c) Cryo-EM structure of the I–II linker in full-length TRPML1, without (c) or with (d) superposition of the I–II linker crystal structure. Top panels, top-down views from the extracellular/luminal side of the membrane. Bottom panels, side views parallel to the membrane.
In a blue native gel, full-length WT TRPML1 showed a distinct band, which presumably corresponded to tetramers (Fig. 4b, bottom, lane 1). This band was absent for mutant full-length TRPML1, which migrated as aggregated oligomers (Fig. 4b, bottom, lane 2), thus suggesting that the L144K R146S mutation disrupted the tetrameric assembly of full-length TRPML1.

In agreement with the above biochemical observations, a mutant TRPML1VP channel containing the L144K R146S mutation (termed TRPML1VP-LR) produced low whole-cell currents when it was expressed in HEK 293T cells, in an NDF solution at either pH 7.4 or 4.6 (Fig. 4c). We then examined the subcellular localization of TRPML1-LR, containing the L144K R146S mutation, by using confocal fluorescence microscopy. The WT and mutant channels tagged with EGFP were expressed in live HeLa cells, which were labeled with the lysosomal marker LysoTracker to identify lysosomes. WT TRPML1 had a punctuate distribution and localized to lysosomes, as previously observed43–46. TRPML1-LR, however, had a diffuse distribution throughout the cell and did not localize to lysosomes (Fig. 4d). Altogether, these results indicate that proper formation of the luminal linker tetramer is critical for the proper assembly and proper subcellular organelle targeting of TRPML1.

**MLIV mutations in the luminal linker disrupt TRPML1 assembly and localization**

Three MLIV-causing single–amino acid missense mutations (L106P, C166F and T232P) are present in the TRPML1 luminal linker (Fig. 5a). These mutations produce milder MLIV symptoms than do truncation or frame-shift mutations18,47,48, which generally result in a complete loss of the TRPML1 protein. The pathogenic mechanisms of these missense mutations at the channel level are unknown. The crystal structure of the luminal linker provides a structural context to investigate how these mutations affect TRPML1 structure, assembly, localization and activity.

In the structure, L106 is at the junction of the anchoring α-helix (ε1) and the luminal pore loop, C166 forms a disulfide bond with C192, and T232 is located on β5 of a core β-sheet (Fig. 5a and Supplementary Figs. 1b and 4a). The luminal-linker protein containing any one of the three pathogenic mutations was expressed but with low yields. CD measurements of the purified proteins revealed marked changes in the secondary structures of all three mutant linkers (Fig. 5b). In a blue native gel, the full-length WT protein migrated as a distinct putative tetramer band (Fig. 5c, lane 1), whereas the full-length mutant proteins migrated as aggregated oligomers (Fig. 5c, lanes 2, 3 and 4). Size-exclusion chromatography further confirmed increased aggregation of the mutant proteins (Fig. 5d). These results indicated that all three mutations disrupt the structure of the luminal pore and the tetrameric assembly of the full-length channel. These detrimental effects were expected, because two of the mutations (L106P and T232P) introduce a proline, and one of the mutations (C166F) disrupts an intrasubunit disulfide bond and replaces a small amino acid with a larger hydrophobic one.

In agreement with the biochemical characterizations, confocal imaging showed that the mutant channels expressed in HeLa cells were diffusely distributed in the cells and did not localize to lysosomes, where the WT channels were primarily found (Fig. 5e and Supplementary Fig. 4b). As expected, because of abnormal assembly, the mutant channels produced little or no current in HEK 293T cells when the mutations were introduced in TRPML1VP (Fig. 5f).

**The luminal pore is critical for Ca^{2+} and pH regulation of TRPML1**

The existence of a luminal pore is a distinct feature of TRPML1. The manual docking model (Supplementary Fig. 2) suggests that the luminal pore is a part of the ion-conduction pathway. To test this hypothesis, we simultaneously mutated three amino acids with relatively small side chains (S110, G112 and A113) in the luminal pore loop to cysteine in TRPML1VP, and examined the effect of MTSET...
The presence of 12 aspartate residues in proximity within the luminal pore (Fig. 2a,d) strongly suggests that this pore region plays a role in Ca\textsuperscript{2+} and pH regulation. We tested this possibility by simultaneously mutating all 12 aspartate residues (D111, D114 and D115 in each subunit) to glutamine in TRPML1\textsuperscript{VP} channels carrying a S110C G112C or a G112C A113C double mutation were not inhibited by 5 mM extracellular MTSET (data not shown).

The aspartate residues are important for Ca\textsuperscript{2+} and pH regulation of TRPML1. (A sulhydryl-specific modifying reagent with a head group 5.8 Å wide) on ion conduction in the mutant channel (termed TRPML1\textsuperscript{VP-3C}). The whole-cell current of TRPML1\textsuperscript{VP} did not change significantly after extracellular application of 5 mM MTSET, but the current of TRPML1\textsuperscript{VP-3C} was quickly and irreversibly inhibited (Supplementary Fig. 5b), thus supporting the hypothesis above. In agreement with the structural revelation of a wide luminal pore, the current reduction after MTSET modification was only partial (Supplementary Fig. 5b-d).

Further supporting the notion of a wide luminal pore and the specificity of thiold modification, the currents of TRPML1\textsuperscript{VP} channels carrying a S110C G112C or a G112C A113C double mutation were not inhibited by 5 mM extracellular MTSET (data not shown).

Effects of MLIV-causing mutations. (\(\theta\)) Locations of three MLIV-causing missense mutations in the structure of the I–II linker. (b) CD spectra of WT and mutant I–II linker proteins. (\(\theta\)) Mean residue ellipticity. (c) Native gel electrophoresis of WT and mutant full-length proteins. Uncropped images are shown in Supplementary Data Set 1. (d) Fluorescence-detection size-exclusion chromatography profiles of the indicated GFP-tagged proteins. (e) Confocal images of live HeLa cells expressing the indicated GFP-tagged channels. Red indicates Lysotracker-labeled lysosomes. Scale bar, 5 µm. (f) Average current density at ~80 mV of the indicated channels at pH 7.4. Numbers of recordings are indicated in above the bars. Error bars, s.e.m. Source data are available in Supplementary Data Set 2.
Conduction through TRPML1. However, the TRPML1<sup>VP-3DQ</sup> channel currents clearly still exhibited strong inward rectification, thus indicating that this property is not controlled by the luminal-pore aspartate residues (Supplementary Fig. 6).

The luminal-pore structure is not substantially altered by pH

There are two possibilities for how acidic pH attenuates Ca<sup>2+</sup> inhibition of TRPML1. First, low pH changes the structure of the luminal pore, either globally or locally. Second, low pH simply protonates the luminal-pore aspartates without causing substantial structural changes.

To distinguish between these possibilities, we obtained crystal structures of the TRPML1 luminal linker at pH 4.5, mimicking its native physiological state, and at pH 7.5, mimicking the pH at the plasma membrane. Both structures were determined at a 2.4-Å resolution (Fig. 7a and Table 1). Because residues E199–K219 were unresolved in the pH 6.0 structure, we removed R200–E213 in the constructs used for crystallization in the pH 6.0 structure. Figure 7 Structural models of TRPML1 I–II linker at different pH levels and demonstration of Ca<sup>2+</sup>/pH dual regulation. (a) Superposition of the crystal structure of the I–II linker obtained at pH 4.5, 6.0 and 7.5, viewed from the extracellular/luminal side of the membrane. (b) Side view of the superimposed luminal-pore-loop structures obtained at pH 4.5, 6.0 and 7.5. (c) Model of Ca<sup>2+</sup>/pH dual regulation of TRPML1. Two of the four subunits are schematized, with yellow representing the ED and green representing the TMD. The luminal-pore aspartates are red or pink, depending on luminal pH, and yellow when mutated to glutamine. Light-blue dots represent free Ca<sup>2+</sup> ions, and the dark-blue dot represents a bound Ca<sup>2+</sup> ion. Details in main text.
in the pH 4.5 and pH 7.5 structures. The different structures show some differences in the periphery; for example, at pH 4.5, the first nine amino acids of α1 become unstructured, and several β-strands (β2, β3 and β8’) could not be resolved. These differences might have been caused by different crystal packing in different pH conditions.

Remarkably, the structures are almost identical in most regions, including the luminal pore loop (Fig. 7a,b). Even the side chain orientation of the luminal-pore aspartates is virtually the same (Fig. 7b), although the crystals at different pH are in completely different crystal forms (Table 1). These structures indicate that the I–II linker exists in a highly stable state. Thus, the attenuated Ca2+ inhibition at the acidic pH (Fig. 1) is probably due to protonation of the luminal-pore aspartates rather than to large conformational changes in the luminal pore.

**DISCUSSION**

The long luminal/extracellular linker between the first two transmembrane segments is a unique but shared feature of the TRPML and TRPP subfamilies of TRP channels. Our crystal structure of the TRPML1 I–II linker is, to our knowledge, the first high-resolution structure of a TRPML channel. Because the I–II linkers of TRPML2 and TRPML3 share high amino acid sequence homology with TRPML1 (Supplementary Fig. 1b), they probably have the same or similar structures. Recently, a 3.0-Å-resolution cryo-EM structure of a human TRPP2 construct containing the ED and TMD has been obtained49. Comparisons of the structures of the TRPML1 and TRPP2 I–II linkers show that despite divergent primary sequences (Supplementary Fig. 7a), the two linkers have a very similar overall structural fold (Supplementary Fig. 7b) and form tightly bound tetramers (Supplementary Fig. 7c,d). We suggest that, given its unique presence in both TRP and TRPML channels, the I–II linker domain be named the ‘polycystin–mucolipin domain’ instead of the ‘polycystin domain’, as has been proposed recently49. However, structural comparisons also reveal that the highly electronegative luminal pore loop is a unique hallmark of TRPML1 (Supplementary Fig. 7e).

In TRPML1, this loop extends downward toward the ion-selectivity filter, whereas in TRPP2 it bends upward toward the luminal/extracellular entryway.

Our studies show that the novel luminal pore of TRPML1 has an important physiological function. Because of TRPML1’s crucial role in numerous cellular processes, especially lysosome-dependent events, the subcellular localization and activity of TRPML1 must be tightly regulated. Although TRPML1 localizes mainly to lysosomes, it can be inserted into the plasma membrane under certain circumstances, such as during lysosomal exocytosis23,25,33. Cells appear to use multiple regulatory mechanisms to ensure proper levels of TRPML1 activity in different subcellular compartments—high activity in lysosomes and low activity in the plasma membrane. One mechanism is subcellular-compartment–dependent regulation by phosphoinositides. Thus, TRPML1 is activated by phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) (ref. 22), which is enriched on the lysosome membrane23,50. However, TRPML1 is inhibited by PI(4,5)P2 (ref. 33), which is abundant on the plasma membrane. The Ca2+/PI(3,5)P2 dual regulation is another subcellular compartmentalized regulatory mechanism. The acidic environment of the lysosome ensures high TRPML1 conductance in lysosomes by diminishing TRPML1 inhibition by luminal Ca2+. In contrast, the neutral extracellular pH ensures low TRPML1 conductance on the cell surface by enhancing TRPML1 inhibition by extracellular Ca2+.

Notably, the dual regulation by luminal Ca2+ and pH described here was observed in a gain-of-function mutant rather than the WT channel.

Because the V432P mutation enables the channel to open constitutively, any allosteric effect of luminal Ca2+ and H+ on channel gating might not have been revealed. Our studies suggest, however, that the dual Ca2+/pH regulation is due to Ca2+ block of the luminal pore and modulation of this block by protonation. This dual regulation is largely conferred by the luminal-pore aspartate residues, which either form a low-affinity Ca2+-binding site or attract extracellular/luminal Ca2+ through electrostatic interactions, thus resulting in Ca2+ binding at a site in the transmembrane pore. From our structural and functional studies, we propose a molecular model for the dual regulation of TRPML1 by extracellular/luminal Ca2+ and pH. At pH 7.4, virtually all luminal-pore aspartates carry negative charges (Fig. 7c, top left). These negative charges attract and bind extracellular/luminal Ca2+, thereby blocking Ca2+ as well as monovalent-cation conduction (Fig. 7c, top right). Lowering the pH to 4.6, which is close to the pK of the aspartate side chain, results in protonation of the aspartates, thereby decreasing the net negative charges and attenuating the Ca2+ block (Fig. 7c, bottom left).

A docking model (Supplementary Fig. 2) suggests that the TRPML1 I–II linker constitutes virtually all of the exposed surface of TRPML1 in the endolysosomal lumen and thus may contain all the interaction sites between TRPML1 and the lipids and proteins in the lumen. Notably, the structure of E199–K219 in the TRPML1 I–II linker was unresolved (Fig. 2 and Supplementary Fig. 1b), probably because of its flexibility. This 21–amino acid loop may protrude into the endolysosomal lumen, beyond an otherwise rigid structure, and is thus a candidate interaction site with endolysosomal lumen molecules. Finally, the architecture and strong electronegativity of the luminal pore suggest that it may be a suitable site for structure-based design of TRPML1-specific blockers.

**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. M.L. obtained the first crystal version of the paper. M.L. and J.Y. wrote the paper. M.L. and J.Y. conceived and initiated the project. M.L. obtained the first crystal version of the paper. Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
Mutagenesis and cloning. The human TRPML1 clone (GenBank BC005149) was purchased from OpenBiosystems. The C. elegans TRPML1 (CUP-5) was cloned from a cDNA library. Site-directed mutagenesis was carried out with overlapping PCR or a QuickChange Site-Directed Mutagenesis Kit (TransGen Biotech) to produce all the mutations. The DNA sequences of all constructs were verified by DNA sequencing. Depending on the experiment, WT and mutant constructs were cloned into different expression vectors, as detailed in the respective sections.

Expression and purification of the TRPML1 I–II linker protein. A DNA fragment encoding the I–II linker (residues 84–296) of human TRPML1 was obtained by PCR and cloned into a modified pET26b(+) vector. A maltose-binding protein (MBP) tag was added to the N terminus of the I–II linker, and a DNA fragment encoding the recognition site of thrombin was inserted between the MBP tag and I–II linker. The resulting construct thus encoded a fusion protein consisting of an N-terminal MBP tag followed by the thrombin-recognition site, the I–II linker and a C-terminal hexahistidine tag.

Rosetta-gami 2 (DE3) cells were used to express the I–II linker to allow proper disulfide-bond formation. Transformed cells were selected and grown at 37 °C in 1 l of LB medium containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol in an incubator shaker at 250 r.p.m. When the optical density at 600 nm reached 1.0, the culture was cooled to 22 °C, and 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added. The bacterial cells were incubated for 12 h in the shaker at 22 °C before they were harvested by centrifugation at 3,000 g for 15 min. The cells were resuspended in a solution containing 50 mM sodium phosphate, pH 8.0, 300 mM NaCl and 2.5% (w/w) glycerol (solution A). Then 5 mM imidazole, 0.5 mg/ml lysozyme, 25 μg/ml DNase and 2 mM PMSF were added, and the cells were disrupted by sonication. Insoluble cell debris was removed by centrifugation at 17,000 g for 30 min. The supernatant was incubated with Ni–NTA His-Bind resin (Novagen) with gentle agitation at 4 °C for 1 h. The beads were spun down at 800 g for 1 min and transferred to a gravity-flow chromatography column. After the resin was washed with ten volumes of solution A containing 5 mM imidazole, the bound protein was eluted with 500 mM imidazole in solution A. The eluted protein was incubated with amylose resin (NEB) at 4 °C for 2.5 h. The resin was collected by centrifugation at 800 g for 1 min, transferred to a column, and washed with solution A. The MBP-tagged protein was eluted with 20 mM maltose in solution A. Thrombin (Sigma–Aldrich) was added at 4 U per milligram protein to cleave the MBP tag. After incubation at 16 °C overnight, the protein was purified and purified on a Superdex 200 column (GE Healthcare) to remove the MBP. The gel-filtration solution contained 10 mM HEPES and 150 mM NaCl, pH 7.5. The peak fractions corresponding to the tetrameric I–II linker protein were collected and concentrated to 4 mg/ml for crystallization.

Crystallization, data collection, structure determination and refinement. Crystallization of the TRPML1 I–II linker was carried out by using the hanging-drop vapor-diffusion method at 16 °C (for pH 6.0) or 20 °C (for pH 4.5 and 7.5). For the pH 6.0 crystal, the protein solution was mixed with reservoir solution at a 1:1 ratio. The reservoir solution contained 1.38 M sodium phosphate monobasic monohydrate and 0.42 M potassium phosphate dibasic, pH 6.0, and 5% pentaerythritol tetraethylene (3/4 EO/OH) (Hampton Research). The best crystals grew from drops formed with 4 mg/ml protein. For the pH 4.5 crystal, the protein concentration was 4 mg/ml, and the reservoir solution contained 200 mM magnesium sulfate, 5.3% PEG 3350 and 100 mM acetate, pH 4.5. Crystals at pH 7.5 were obtained by macroseeding in a reservoir solution of 100 mM magnesium sulfate, 4% PEG 3350 and 100 mM HEPES, pH 7.5, with a protein concentration of 4 mg/ml. Heavy atom–derivative crystals were prepared by soaking crystals in a solution containing 1.53 M sodium phosphate monobasic monohydrate and 0.47 M potassium phosphate dibasic, pH 6.0 and 1 mM K3[Pt(CN)6] for 24 h.

Single crystals were flash–cooled in liquid nitrogen with Paratone-N (Hampton Research) (for pH 4.5 and 6.0 crystals) or 30% glycerol as a cryoprotectant (for pH 7.5 crystals). X-ray diffraction data for native and derivative crystals (at pH 4.5 and 6.0) were collected at 100 K on a RAXIS-IV detector with Cu Kα radiation (λ= 1.5418 Å) from a Rigaku RuiH3R X-ray generator. X-ray diffraction data at pH 7.5 were collected at the National Synchrotron Light Source (NSLS) beamline X29 at Brookhaven National Laboratory.

The diffraction images were processed and scaled with the HKL package51. The structure of the I–II linker at pH 6.0 was solved with the single isomorphous replacement with anomalous scattering (SIRAS) method with native and platinum-derivative data sets. Two platinum sites were found with Shake and Bake52, and were input into SOLVE53 for calculating the initial phases. Density modification was done with RESOLVE55 to improve phase accuracy. The quality of the density map from RESOLVE was sufficiently good to locate most residues. The presence of two intrasubunit disulfide bonds aided in the tracing. The initial model was built with COOT54. Refinement with CNS55 and manual rebuilding were done iteratively. Structures of the I–II linker at pH 4.5 and 7.5 were solved by molecular replacement with PHASER56, with the structure at pH 6 used as the search model. Cycles of refinement with CNS and manual model correction with COOT were carried out. Crystallographic statistics are summarized in Table 1.

Expression and purification of the TRPML1 I–II linker for circular dichroism. TRPML1 I–II linkers were expressed through viral infection in Hi5 insect cells (Expression Systems, not tested for mycoplasma). The linkers were cloned in the pFastBac1 vector, which was modified by the insertion of a honeysbee melittin secretion signal peptide coding region followed by BamHI/Xhol restriction sites and a hexahistidine-tag coding region. DNA fragment encoding the WT or mutant I–II linkers were inserted into the BamHI/Xhol sites of the modified vector.

Recombinant baculovirus was generated with the Bac-to-Bac method (Invitrogen). The virus was amplified in Sf9 cells (Invitrogen, not tested for mycoplasma) and was used to infect Hi5 cells in ESF920 medium (Expression Systems). The expressed protein containing a hexahistidine tag on its C terminus was secreted into the extracellular medium, where the honeybee melittin secretion signal peptide was cleaved. Forty-eight hours after transfection, cells were removed by centrifugation, and the medium containing the desired protein was collected. The medium was concentrated, dialyzed against solution A containing 5 mM imidazole and incubated with TALON metal-affinity resin (Clontech) at 4 °C for 1 h. The resin was collected and washed with solution A containing 10 mM imidazole. The histidine-tagged protein was eluted with 300 mM imidazole in solution A. The protein was concentrated and changed to a solution containing 10 mM sodium phosphate, 150 mM NaCl, pH 7.0, by dialysis for CD analysis.

Circular dichroism spectroscopy. CD was performed with a J-815 CD spectrometer (Jasco). All proteins were adjusted to a concentration of 1 mg/ml and loaded into a quartz cuvette with a path length of 0.01 cm for measurement. A buffer containing no protein was used for subtraction of the baseline signal. Measurements were performed from 185 to 250 nm with a 0.1-nm interval, 1-nm bandwidth and scanning speed of 50 nm/min. For each protein, three samples were measured, and three accumulations were measured for each sample and averaged. The MRE was calculated from the raw CD signal, by the equation \[ \text{MRE} = \frac{\theta - (10 \times \text{Cr} \times l)}{1} \], where Cr is the protein concentration (M × residue number), and l is the cuvette path length (cm).

Cross-linking. The DNA fragment encoding C. elegans full-length TRPML1 was cloned into a modified pFastBac1 vector. An MBP tag was added before the N terminus of TRPML1, and a hexahistidine tag was added before the N terminus of MBP. A linker region containing a TEV protease–recognition sequence (underlined; NNNNNNNENLYFQGGGGS) was inserted between the MBP tag and TRPML1. Cysteine substitutions were subsequently introduced into this background construct.

The baculovirus of TRPML1 was generated in Sf9 cells with the standard Bac-to-Bac method and was used to infect Hi5 insect cells. Forty-eight hours after infection, cells were harvested by centrifugation at 4 °C and suspended in a buffer containing 50 mM HEPES–NaOH, pH 7.4, 500 mM NaCl and 5% glycerol, in the presence of a protease–inhibitor cocktail (Pierce). After cell disruption by sonication, the cell debris was removed by centrifugation at 4,000 r.p.m. for 10 min at 4 °C, and membranes were pelleted by ultracentrifugation at 45,000 r.p.m. at 4 °C for 1 h. The membranes were suspended in buffer A containing protease–inhibitor cocktail and homogenized with a glass Dounce homogenizer. TRPML1 proteins were extracted with 1% lauryl maltose neopentyl glycol (LMNG, Anatrace) for 1 h at 4 °C. The solubilized membranes were clarified by ultracentrifugation for 30 min and incubated with amylose resin (NEB) for 2 h at 4 °C with gentle agitation.
The resin was collected by low-speed centrifugation at 2,000 r.p.m., transferred into a gravity column, and washed with buffer A containing 0.5 mM LMNG and 0.1 mg/ml soybean lipids (Avanti Polar Lipids). This wash buffer with 20 mM maltose was used to elute the MBP-tagged TRPML1 proteins. TEV protease was added to the eluted proteins and incubated overnight at 4 °C to cleave the MBP tag. The proteins were mixed with 3× SDS loading buffer with or without DTT and analyzed with 10% SDS–PAGE.

**Native gel electrophoresis and fluorescence-detection size-exclusion chromatography.** The WT and L144K R146S mutant I–II linker proteins were obtained as described above in the CD experiment. The proteins were loaded on a 6% native acrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF membrane for western blot analysis. HisTag monoclonal antibody (EMD Millipore, cat. no. 70796) was used as the primary antibody. Alexa Fluor 680 goat anti-mouse IgG (Invitrogen, cat. no. A-21058) was used as the secondary antibody. Images were scanned and analyzed with an Odyssey Infrared Imaging System (Li-COR).

For native gel electrophoresis and fluorescence-detection size-exclusion chromatography (FSEC), full-length *C. elegans* TRPML1 was cloned in the pEGFP-C1 vector (Clontech), which was modified by replacing the multiple cloning site with a TEV protease–recognition-site coding region and SbfI/Ascl restriction sites. The DNA fragment encoding the full-length TRPML1 was inserted into the SbfI/Ascl site. The expressed protein thus had an EGFP tag on its N terminus and a TEV protease–recognition site in between the EGFP tag and the channel.

HEK 293T (ATCC) cells were transfected with the TRPML1 plasmid with Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were washed and resuspended in solution A containing Halt protease–inhibitor cocktail (Thermo). DDM was added to a final concentration of 2%, and lysates were incubated at 4 °C for 1 h. The solution was clarified by centrifugation at 16,000 r.p.m. for 10 min. This cell lysate was used for blue native PAGE and FSEC.

Blue native PAGE was performed with a NativePAGE Bis-Tris Gel System (Invitrogen). The solubilized cell lysate in DDM was mixed with NativePAGE sample buffer and Coomassie blue G-250. Electrophoresis was carried out on 3–12% Bis-Tris gel at 4 °C. The proteins were transferred to PVDF membranes for western blotting. Anti-EGFP monoclonal antibody (Santa Cruz, cat. no. 9996) and Alexa Fluor 680 goat anti-mouse IgG (Invitrogen, cat. no. A-21058) were used for detecting the GFP-tagged TRPML1 protein. Images were scanned and analyzed with an Odyssey Infrared Imaging System (Li-COR). All primary antibodies have been validated for their species and applications, per information on the manufacturers.

FSEC was performed with a spectrofluorometric detector RF-10AXL (Shimadzu) for fluorescence detection and a Superose 6 10/300 column (GE Healthcare) for size-exclusion chromatography.

**Electrophysiology.** All electrophysiology source data are presented in Supplementary Data Set 2.

**Constructs.** Human TRPML1 tagged with GFP on the N terminus and containing the V432P point mutation (TRPML1VP) was cloned in the pEGFP-C1 vector. TRPML1VP–SDQ, TRPML1VP–3C and TRPML1VP–LR were also cloned in this vector. TRPML1VP was also cloned in pDNA3.1, in which TRPML1VP–L106P, TRPML1VP–C166F and TRPML1VP–T232P were cloned.

**Cell culture and transfection.** HEK 293T cells were grown in DMEM (30–2002, ATCC) supplemented with 0.5% penicillin/streptomycin (Sigma, P-0781) and 10% FBS (Standard quality, PAA laboratory, 95025-534) with standard procedures. Transfections were performed with Lipofectamine (SignaGen Laboratories), per the manufacturer’s instructions.

**Whole-cell recording and data analysis.** All experiments were performed at room temperature (22–23 °C). Pipettes were fabricated from borosilicate glass (World Precision Instruments) and were fire-polished to resistances of 2–5 MΩ. Whole-cell currents were elicited by 150-ms voltage steps from −120 to +100 mV with 20-mV increments, with a holding potential of −30 or 0 mV. Currents were amplified with an Axopatch 200B and digitized with a Digidata 1440A system (Molecular Devices). Currents were low-pass filtered at 1 kHz and sampled at 10 kHz. PCLAMP software (Molecular Devices) was used for data acquisition and analysis. The pipette solution contained 120 mM cesium methanesulfonate, 4 mM NaCl, 10 mM EGTA, 2 mM MgCl2, 20 mM HEPES and 2 mM Na2-ATP, pH 7.2 with CsOH. The pH 7.4 NDF (nominal divalent ion-free) bath solution contained 160 mM NaCl, 5 mM KCl, 10 mM glucose and 20 mM HEPES, pH 7.4 with NaOH. The pH 4.6 NDF bath solution contained 160 mM Na-glucuronate, 5 mM KCl, 10 mM MES, 10 mM glucose and 10 mM HEPES, pH 4.6 with HCl.

To obtain different Ca2+ concentrations, an appropriate amount of 1 M CaCl2 stock solution was added to the NDF solutions to achieve a desired final concentration. In the cysteine-modification experiment, MTSET was freshly added to the pH 7.4 NDF solution and kept in the dark for <30 min before use.

Data points represent mean ± s.e.m. Dose–response relationships for calcium inhibition of TRPML1 channels were fitted to the Hill equation in the form of I(X) = I_{max} × X^n/(X^n + IC_{50^n}), where I_{max} is the current in the absence of calcium, I(X) is the normalized current in the presence of calcium, X is the calcium concentration, n is the Hill coefficient, and IC_{50} is the calcium concentration producing half maximal inhibition. Statistical differences were determined with Student’s t test.

**Intracellular Ca2+ imaging.** TRPML1VP and TRPML1VP–3DQ cloned in pEGFP-C1 were transfected into HEK 293T cells. Forty-eight hours after transfection, cells were loaded with Fura-2 AM (10 μM) and Phosphonic F-127 (0.02%) (Molecular Probes) at 37 °C for 1 h in a Ca2+-free solution. The fluorescence ratios of F340/F380 were measured with a fluorescence microscopic system. The Ca2+-free solution contained 150 mM NaCl, 1 mM MgCl2 and 10 mM HEPES, pH 7.4 with NaOH. In the Ca2+-containing solution, 3 mM CaCl2 was added to the solution above.

**Confocal imaging.** HeLa cells (gift of R. Prywes, Columbia University) were maintained in DMEM (Gibco) containing 10% FBS (Atlanta Biologicals) and 100 μg/ml of penicillin/streptomycin (Invitrogen). Cells were cultured and plated onto poly-n-lysine hydrobromide (Sigma)-coated coverslips 18 to 24 h before transfection and grown on the coverslips in DMEM plus 10% FBS supplemented with 100 μg/ml of penicillin/streptomycin at 37 °C until cells became ~80% confluent. Cells were transfected with WT or mutant full-length TRPML1, all tagged with EGFP on the N terminus and cloned in the pcDNA3.1 vector, with Lipofectamine In vitro DNA Transfection Reagent Ver II (SignaGen Laboratories). Seventeen hours after transfection, the cells were loaded with 100 nM of the lysosomal marker LysoTracker Red DND-99 (Invitrogen) for 50 min at 37 °C. Cells were gently and briefly washed three times with PBS (Gibco), and live-cell images were acquired in PBS at 20 °C. Images were acquired from random cells in an unbiased manner with a Nikon Eclipse TE2000-S microscope with a 60× oil-immersion objective with a spinning-disc confocal system, a Hamamatsu C9100-13 back-thinned EM-CCD digital camera (Hamamatsu), and a spinning-disc head (Yokogawa). z-stacks were acquired at an optical slice thickness of 0.3 μm with a Pixo Focus Drive (Ludl). Images were analyzed with Volocity software (PerkinElmer). All z-stacks were deconvolved with the iterative restoration function in Volocity, with the confidence limit set to 99% and the iteration limit set to 40. All images displayed in the paper are derived from deconvolved z planes.

**Cryo-EM sample preparation, data acquisition and image processing.** *C. elegans* TRPML1 was expressed in Hi5 insect cells and purified with amylose resin with the same procedures as described above in the cross-linking experiment. Eluted TRPML1 protein from amylose resin was mixed with amphipol A8–35 (Anatrace) at 1:6 (w/w), and TEV protease was added at 1:20 (w/w) to cleave the MBP tag. The mixture was incubated with gentle agitation at 4 °C overnight, and detergent was removed with Bio-Beads SM-2 (Bio-Rad) at 4 °C. After Bio-Beads removal, the TRPML1 protein was concentrated and further purified on a Superose 6 10/300 column in a buffer composed of 20 mM HEPES–NaOH, 150 mM NaCl, pH 7.4. The peak corresponding to the tetrameric TRPML1 protein was collected for cryo-EM analysis.

A drop of 4 μl of the amphipol-solubilized protein at 0.6 mg/ml concentration was loaded on a glow-discharged Quantifoil R1.2/1.3 holey carbon grid, incubated for 3 s, blotted and then plunged into liquid ethane cooled with liquid nitrogen by using a Vitrobot device (FEI Company) at 100% humidity and 8 °C. The grids were imaged with a Titan Krios microscope operated at 300 kV and equipped with a K2 Summit electron-counting camera (Gatan Company). UCSFImage4 (ref. 58) was used for data collection with nominal magnification of 22,500×, corresponding to an image pixel size of 0.66 Å under super-resolution counting mode. Images were recorded with a defocus range from 2.1 to 3.1 μm. The dose rate was set to 8.2 counts/physical pixel/s on the camera.
plane. The total exposure time was 8 s, thus resulting in a total accumulated dose of ~50 e⁻/Å². Each micrograph was fractionated into 32 frames, each with an exposure time of 0.25 s.

All micrographs were first 2 × 2 binned, thus generating a pixel size of 1.32 Å. Motion correction was performed with MotionCorr59. The aligned frames were integrated and used for further processing. Defocus for all images was determined with CTFFIND3 (ref.60). Particle-picking and all 2D and 3D classification and refinement steps were performed with RELION61. Approximately 200,000 autopicked particles were screened with several rounds of 2D classifications to remove most of the ‘junk’ and bad particles. The particles were 4 × 4 binned to a pixel size of 2.64 Å for further image analysis. A star-shaped density map with C₄ symmetry was made and used as an initial reference for 3D classification. 71,052 good particles were selected by 3D classification and subjected to first-round 3D refinement with C₄ symmetry imposed, thus yielding a reconstruction at a resolution of 8.12 Å. The transmembrane region showed much poorer quality than the soluble region, in agreement with the asymmetric features shown in 2D class averages. To improve the resolution of the soluble region, we applied a soft-edged mask to remove the possible transmembrane region. Further 3D refinement of the soluble region generated a reconstruction at a resolution of 5.28 Å. The resolutions were estimated on the basis of the gold-standard FSC = 0.143 criterion. ResMap62 was used to calculate the local resolution map. The crystal structure of the human I–II linker at pH 6.0 was fit into this cryo-EM density map with Chimera63.

**Data availability.** 3D cryo-EM density maps of full-length *C. elegans* TRPML1 or the I–II linker, without and with low-pass filtering and amplitude modification, have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-6669 and EMD-6670, respectively. The coordinates of the atomic model of the human TRPML1 I–II linker at different pH values have been deposited in the Protein Data Bank under accession numbers PDB 5TJB (pH 4.5), PDB 5TJA (pH 6.0) and PDB 5TJC (pH 7.5). Data for electrophysiology experiments in Figures 1, 3, 4 and 5 and for Supplementary Figs. 5 and 6 are provided as Supplementary Data Set 2. Other data supporting the findings of this study are available from the corresponding author upon reasonable request.

51. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326 (1997).
52. Weeks, C.M. & Miller, R. The design and implementation of SnB version 2.0. *J. Appl. Crystallogr.* **32**, 120–124 (1999).
53. Terwilliger, T.C. SOLVE and RESOLVE: automated structure solution and density modification. *Methods Enzymol.* **374**, 22–37 (2003).
54. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).
55. Brünger, A.T. et al. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54**, 905–921 (1998).
56. McCoy, A.J. et al. Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674 (2007).
57. Kawate, T. & Gouaux, E. Fluorescence-detection size-exclusion chromatography for precrytalization screening of integral membrane proteins. *Structure* **14**, 673–681 (2006).
58. Li, X., Zheng, S., Agard, D.A. & Cheng, Y. Asynchronous data acquisition and on-the-fly analysis of dose fractionated cryoEM images by UCSFImage. *J. Struct. Biol.* **192**, 174–178 (2015).
59. Li, X. et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* **10**, 584–590 (2013).
60. Mindell, J.A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J. Struct. Biol.* **142**, 334–347 (2003).
61. Scheres, S.H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).
62. Kucukelbir, A., Sigworth, F.J. & Tagare, H.D. Quantifying the local resolution of cryo-EM density maps. *Nat. Methods* **11**, 63–65 (2014).
63. Petterson, E.F. et al. UCSF Chimera: a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).