Structural insights into the voltage and phospholipid activation of the mammalian TPC1 channel

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The organellar two-pore channel (TPC) functions as a homodimer, in which each subunit contains two homologous Shaker-like six-transmembrane (6-TM)-domain repeats1. TPCs belong to the voltage-gated ion channel superfamily2 and are ubiquitously expressed in animals and plants3,4. Mammalian TPC1 and TPC2 are localized at the endolysosomal membrane, and have critical roles in regulating the physiological functions of these acidic organelles5-7. Here we present electron cryo-microscopy structures of mouse TPC1 (MmTPC1)—a voltage-dependent, phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2)-activated Na+-selective channel—in both the apo closed state and ligand-bound open state. Combined with functional analysis, these structures provide comprehensive structural insights into the selectivity and gating mechanisms of mammalian TPC channels. The channel has a coin-slot-shaped ion pathway in the filter that defines the selectivity of mammalian TPCs. Only the voltage-sensing domain from the second 6-TM domain confers voltage dependence on MmTPC1. Endolysosome-specific PtdIns(3,5)P2 binds to the first 6-TM domain and activates the channel under conditions of depolarizing membrane potential. Structural comparisons between the apo and PtdIns(3,5)P2-bound structures show the interplay between voltage and ligand in channel activation. These MmTPC1 structures reveal lipid binding and regulation in a 6-TM voltage-gated channel, which is of interest in light of the emerging recognition of the importance of phosphoinositide regulation of ion channels.

TPC1 and TPC2 represent two major subfamilies of mammalian TPC channels and their functions are associated with various physiological processes, including hair pigmentation8-10, autophagy regulation11,12, blood vessel formation13, acrosome reaction in sperm14, mTOR-dependent nutrient sensing15, lipid metabolism16 and Ebola virus infection17, to name a few. Mammalian TPCs were initially suggested to mediate nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent calcium release from endolysosomes18-20. However, several recent studies have demonstrated that mammalian TPCs are Na+-selective channels activated by endolysosome-specific PtdIns(3,5)P2 rather than NAADP18,19. The dual regulation of TPC2 by PtdIns(3,5)P2 and NAADP has also been reported20. Distinct from TPC2, mammalian TPC1 activation is voltage-dependent, conferring electrical excitability to the endolysosome20. The atomic structure of a plant TPC from Arabidopsis thaliana (AtTPC1) was recently determined by X-ray crystallography, revealing the overall architecture of the TPC family21,22. However, mammalian TPCs share low sequence identity with their plant counterparts (Extended Data Fig. 1) and exhibit different gating and selectivity properties. Here we present the structural and functional analysis of MmTPC1.

When overexpressed in HEK293 cells, some MmTPC1 channels are trafficked to the plasma membrane, enabling us to directly measure channel activity by patching the plasma membrane (Extended Data Fig. 2 and Methods). In brief, MmTPC1 activation requires both membrane depolarization and the PtdIns(3,5)P2 ligand (Extended Data Fig. 2b, c). The voltage activation of MmTPC1 is modulated by endolysosomal luminal pH23, and a lower pH shifts voltage activation towards a more positive potential (Extended Data Fig. 2d, e). In our recordings, MmTPC1 exhibits higher selectivity for Na+ than K+ and Ca2+ (Extended Data Fig. 2f, g); this is different from plant TPC1, which is non-selective24,25.

MmTPC1 structures were determined in the presence and absence of PtdIns(3,5)P2 to a resolution of 3.2 and 3.4 Å, respectively, using single particle electron cryo-microscopy (cryo-EM) (Fig. 1, Extended Data Figs 3, 4 and Extended Data Table 1). The cryo-EM density maps of both structures are of sufficient quality for de novo model building of major parts of the protein (Extended Data Fig. 5). Here we use the higher-resolution PtdIns(3,5)P2-bound structure for the initial description of the overall structural features. Similar to AtTPC1, each MmTPC1 subunit contains two homologous 6-TM domains (6-TMI and 6-TMII) and two subunits that assemble into a rectangle-shaped functional channel, which is equivalent to a tetrameric Shaker-like channel (Fig. 1a, b and Extended Data Fig. 6). Following the same nomenclature as other voltage-gated channels, we labelled the six transmembrane α-helices and two β-strands and tightly wraps around the EF-hand domain (Fig. 1a, c). The transmembrane region of MmTPC1 is domain-swapped; the S1–S4 voltage-sensing domain (VSD) from one 6-TM interacts with the S5–S6 pore domain from the neighbouring 6-TM (Fig. 1b). The pore domain of the second 6-TM contains a luminal loop between IIS5 and pore helix 1 (IIP1) that forms an upright antenna-like β-hairpin; Asn600 and Asn612 on this luminal loop are glycosylated with visible density for the covalently linked N-acetylglucosamine moiety of the sugar26-28 (Fig. 1b, d and Extended Data Fig. 5c).

Multiple cytosolic components within each TPC1 subunit—including the N-terminal H1 helix, the linker between the two 6-TMs and the C-terminal post IIS6 region—assemble into a tightly packed cytosolic domain (Fig. 1d). Despite low sequence homology, the linker between the two 6-TMs adopts the EF-hand domain structure with two EF-hand motifs (EF-1 and EF-2), similar to plant TPC1, and the C-terminal portion of the exceptionally long IIS6 serves as the E1 helix (Fig. 1d and Extended Data Fig. 6d). Ca2+-unlikelihood to bind to the EF motifs of MmTPC1 as these motifs lack essential Ca2+-chelating acidic residues (Extended Data Fig. 1). The N-terminal H1 helix is tightly packed with the EF-1 motif and becomes an integral part of the EF-hand domain (Fig. 1d). Compared with plant TPC1 and mammalian TPC2, MmTPC1 has a much longer C-terminal region, which adopts a horseshoe-shaped structure with four α-helices and two β-strands and tightly wraps around the EF-hand domain (Fig. 1d and Extended Data Figs 1, 6d).

The MmTPC1 ion conduction pore, which consists of S5, S6 and two pore helices, adopts a closed conformation in the apo structure and an open conformation in the PtdIns(3,5)P2-bound structure (Fig. 2a–d). In the apo structure, the four pore-lining S6 helices form...
and have atom-to-atom cross distances of about 8 Å (Fig. 2e). The residues of filter II use side chains to generate a much narrower pathway, with two constriction points formed by the Asn648 and Asn649 residues (Fig. 2e). Positioned at the centre of the filter and stabilized by hydrogen-bonding interactions with the filter I backbone carbonyls of Thr280 and Ala281, the Asn648 side chain forms the narrowest point along the filter pathway; it has a cross distance of about 3.7 Å and has the central role in defining the Na\(^{+}\) selectivity of MmTPC1 (Fig. 2e, f). Asn648Ala mutation results in a complete loss of Na\(^{+}\) selectivity (Fig. 2g and Extended Data Fig. 7). The Asn649 residues are positioned at the luminal entrance of the channel at a wider distance from one another, and Asn649Ala mutation reduces but does not abolish Na\(^{+}\) selectivity (Fig. 2g and Extended Data Fig. 7). With an elongated coin-slot-like ion pathway at the filter, Na\(^{+}\) ions probably pass through the MmTPC1 filter in a partially hydrated form. The two Asn648 side chains are positioned to provide optimal coordination to stabilize the permeating Na\(^{+}\) ion, but are too close to permit the passage of K\(^{+}\) or larger ions.

The two VSDs (VSD1 and VSD2) have virtually the same structures as their respective counterparts between the apo and ligand-bound states and, therefore, the higher-resolution PtdIns(3,5)P\(_2\)-bound structure will be used in the discussion (Extended Data Fig. 8a, b). Figure 3a provides the number of the S4 gating charge residues (R1–R5) from TPCs and other canonical voltage-gated channels for comparison. Although VSD1 contains three arginine residues in IS4 (Arg200, Arg203 and Arg206, at positions R2, R3 and R4, respectively) (Fig. 3b), it lacks some key features of canonical voltage sensors (see Extended Data Fig. 8a legend) and does not contribute to voltage-dependent gating—similar to the VSD1 of plant TPC1 as shown by the fact that replacing these arginines individually with a neutral residue does not affect the voltage activation of MmTPC1 (Fig. 3c and Extended Data Fig. 8c).

VSD2 contains only two S4 arginines (Arg540 at position R3 and Arg546 at position R5), preserves the key features of a canonical voltage sensor—including the 30° helix in IS4 and the conserved gating-charge transfer centre (Fig. 3d)—and is responsible for the voltage gating of MmTPC1. Mutations of Arg540 and Arg546 have a profound but opposite effect on the voltage dependence of the channel. The Arg540Gln mutation stabilizes VSD2 in an activated state and yields a voltage-independent channel that has a linear current–voltage relationship between −100 and 50 mV and can be activated by PtdIns(3,5)P\(_2\) even at hyperpolarization (Fig. 3e). The Arg546Gln mutant, by contrast, can barely be activated by voltage even at high concentrations of PtdIns(3,5)P\(_2\) as if the voltage sensor is trapped in the resting state (Extended Data Fig. 8d).

The MmTPC1 VSD2 adopts an activated conformation with its final voltage-sensing arginine (Arg546) positioned in the gating-charge transfer centre formed by Tyr487 and Glu490 from IIS2 and Asp512 from IIS3, and the other voltage-sensing residues (Arg540 and Gln543) facing the external, luminal side (Fig. 3d). The VSD2 of AtTPC1, with its S4 arginines residing at positions R3–R5, is also responsible for the voltage-gating of the channel, and its structure is in the resting state as shown by the fact that the Arg546Gln mutant, by contrast, can barely be activated by voltage even at high concentrations of PtdIns(3,5)P\(_2\) as if the voltage sensor is trapped in the resting state (Extended Data Fig. 8d).

The MmTPC1 VSD2 from the activated to the resting state by comparing its structure with that of AtTPC1 (Fig. 3f, g). Except for the S4 helix, the two structures superimpose well. This suggests that upon hyperpolarization the IS4 of MmTPC1 would slide down by about two helical turns without undergoing structural change in the rest of VSD2, and position its R3 arginine (Arg540) at the gating-charge transfer centre (Fig. 3g). Concurrent with the IS4 sliding, the IS4–S5 linker would swing downward and move closer to IIS6. Notably, VSD2 is in the activated state in both the apo and PtdIns(3,5)P\(_2\)-bound structures, indicating that the voltage sensor can be activated without opening the channel in MmTPC1.

The bound PtdIns(3,5)P\(_2\) can be unambiguously identified from the electron microscopy density map of the ligand-bound...
structure (Extended Data Figs 5d, 9a). PtdIns(3,5)P2 is situated at the junction formed by IS3, IS4 and the IS4–S5 linker of 6-TMI; its inositol 1,3,5-trisphosphate head group is positioned on the cytosolic side and its acyl chains are inserted upright into the membrane (Fig. 4a and Extended Data Fig. 9a). Figure 4b summarizes the protein–ligand interactions, which involve predominantly basic residues from the C terminus of H1, the N terminus of IS3, the IS4–S5 linker and the C-terminal part of IS6. Buried in the protein, the two phosphate groups on the C1 and C3 positions of the inositol muster the majority of protein–ligand interactions and probably define the ligand specificity. The C5 phosphate protrudes outwardly away from the ligand-binding pocket, and forms salt bridges with Lys87 and Lys331; the interaction with Lys331 participates in the coupling between the ligand and IS6, and has an important role in the ligand activation of the channel. Among all the ligand-interacting residues at the PtdIns(3,5)P2-binding site, mutations of the residues that predominantly interact with the C3 phosphate—including the three arginines (Arg220, Arg221 and Arg224) on the IS4–S5 linker and Lys331 on IS6—appear to have the most profound effect on PtdIns(3,5)P2 activation, which illustrates the central role of the C3 phosphate (Extended Data Fig. 9b). In a recent study, the three linker arginines have also been reported to be important for NAADP-mediated Ca2+ release30.

To investigate the affinity and specificity of the ligand, we measured the activity of the ligand-dependent channel in excised patches (obtained by applying a voltage pulse ramp from –100 to 100 mV). Currents were recorded with 2μM PtdIns(3,5)P2 in the pipette and repeated five times independently with similar results. f. Structural comparison of the cytosolic structure (Extended Data Figs 5d, 9a). PtdIns(3,5)P2 is situated at the junction formed by IS3, IS4 and the IS4–S5 linker of 6-TMI; its inositol 1,3,5-trisphosphate head group is positioned on the cytosolic side and its acyl chains are inserted upright into the membrane (Fig. 4a and Extended Data Fig. 9a). Figure 4b summarizes the protein–ligand interactions, which involve predominantly basic residues from the C terminus of H1, the N terminus of IS3, the IS4–S5 linker and the C-terminal part of IS6. Buried in the protein, the two phosphate groups on the C1 and C3 positions of the inositol muster the majority of protein–ligand interactions and probably define the ligand specificity. The C5 phosphate protrudes outwardly away from the ligand-binding pocket, and forms salt bridges with Lys87 and Lys331; the interaction with Lys331 participates in the coupling between the ligand and IS6, and has an important role in the ligand activation of the channel. Among all the ligand-interacting residues at the PtdIns(3,5)P2-binding site, mutations of the residues that predominantly interact with the C3 phosphate—including the three arginines (Arg220, Arg221 and Arg224) on the IS4–S5 linker and Lys331 on IS6—appear to have the most profound effect on PtdIns(3,5)P2 activation, which illustrates the central role of the C3 phosphate (Extended Data Fig. 9b). In a recent study, the three linker arginines have also been reported to be important for NAADP-mediated Ca2+ release30.

To investigate the affinity and specificity of the ligand, we measured the activity of the ligand-dependent channel in excised patches.
by using the voltage-independent Arg540Gln mutant, which simplifies ligand-dependent gating by eliminating the voltage effect. The mutant also elicits much larger currents, which makes it suitable for inside-out patches. The PtdIns(3,5)P$_2$-dependent activation of the mutant yielded a half-maximal effective concentration (EC$_{50}$) of about 145 nM (Fig. 4c and Extended Data Fig. 9c), similar to that of human TPC1 measured in whole lysosome patch.$^{23}$ The PtdIns(4,5)P$_2$ isoform cannot activate the channel or inhibit PtdIns(3,5)P$_2$ activation (Fig. 4d), indicating high lipid specificity of MmTPC1. The lack of PtdIns(4,5)P$_2$-binding can be explained by the missing C3 phosphate and the close proximity of Asn85 and Lys87 to the C4 hydroxyl group, which sterically excludes the C4 phosphate and thereby prevents the binding of the lipid (Fig. 4e).

Compared to the apo structure, PtdIns(3,5)P$_2$-binding does not introduce major structural changes around the ligand-binding pocket (Extended Data Fig. 9d), except for one key conformational change on IS6 mediated by Lys331 (Fig. 4f). In the apo state, the Lys331 side chain points away from the ligand-binding pocket. In the presence of PtdIns(3,5)P$_2$, the Lys331 side chain adopts an extended configuration to form salt bridges with both the C3 and C5 phosphates as well as a hydrogen bond with the C4 hydroxyl, pulling IS6 towards the ligand-binding pocket (Fig. 4f). This movement propagates to the other part of IS6, as well as IIS6, and opens the gate. Lys331 appears to be the only residue that couples IS6 to the bound PtdIns(3,5)P$_2$ and its mutation to Ala completely abolishes PtdIns(3,5)P$_2$ activation (Extended Data Fig. 9b).

Our structures demonstrate that PtdIns(3,5)P$_2$ only binds to the first 6-TM domain and directly introduces conformational changes in IS6 helix, whereas voltage influences only the VSD2 in the second 6-TM domain, the conformational change of which is likely to affect the movement of IIS6 helix (Figs 3, 4). A global structural comparison between the apo and PtdIns(3,5)P$_2$-bound structures explains the interplay between the two stimuli (Fig. 5). Despite having an activated voltage sensor, the MmTPC1 pore remains closed in the apo structure, implying that PtdIns(3,5)P$_2$-binding is required to trigger the opening of the gate. Upon PtdIns(3,5)P$_2$-binding, the ensuing tethering interaction between Lys331 and PtdIns(3,5)P$_2$ straightens the IS6 helices that are initially bent at the $\pi$-helix just below the filter region in the closed state, resulting in the outward dilation and rotation at the bundle crossing (Figs 2d, 5a). The five-residue $\pi$-helix is present only in IS6 and may facilitate the helix bending. To open the pore, the IIS6 helices also have to undergo concurrent outward and rotational movements to accommodate the PtdIns(3,5)P$_2$-induced conformational change in IS6 helices, particularly the rotation of the two IS6 gating residues with large hydrophobic side chains (Leu317 and Phe321). Consequently, the two IIS6 gating residues (Val684 and L688) also rotate away from the central axis and open the gate (Figs 2d, 5b). The IIS6 motion is hindered around the residue immediately below the filter region, and is propagated to a much larger movement at the C-terminal end of IIS6, which swings upward and makes direct contact with the IIS4–S5 linker. Such motion is permitted only when IIS4 of VSD2 is in the activated, up state. Under hyperpolarized membrane potential, IIS4 is expected to slide downward and push the IIS4–IIS5 linker along with it, occluding the

**Figure 4** | PtdIns(3,5)P$_2$ binding in MmTPC1. a, PtdIns(3,5)P$_2$ binding in 6-TM1 of MmTPC1. Inset: zoomed-in view of the PtdIns(3,5)P$_2$ site. b, Schematic of the protein–ligand interactions. c, Concentration-dependent PtdIns(3,5)P$_2$ activation of Arg540Gln mutant at $-100$ mV. Curve is least square fit to the Hill equation. Data points are mean ± s.e.m. ($n = 5$ independent experiments). Sample I–V curves are shown in Extended Data Fig. 9c. d, Ligand specificity of MmTPC1 measured using the Arg540Gln mutant. Sample I–V curves were recorded on the same patch with different PtdInsP$_2$ isoforms. The experiments were repeated five times independently with similar results. e, Close proximity between the C4 hydroxyl of PtdIns(3,5)P$_2$ and the surrounding residues. f, Structural comparison at the region around Lys331 between the apo (green) and PtdIns(3,5)P$_2$-bound MmTPC1 (salmon).
space necessary for upward IIS6 movement upon PtdIns(3,5)P2 activation (Fig. 3f, g). PtdIns(3,5)P2 can probably still bind MmTPC1 under hyperpolarization, but the resting VSD2 prevents channel opening by blocking the movement of IIS6. Thus, membrane potential modulates the TPC1 channel activity by imposing a voltage-dependent constraint on PtdIns(3,5)P2 activation and the upward movement of VSD2 under depolarization is a prerequisite for the PtdIns(3,5)P2-induced gate opening (Fig. 5c).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Protein expression and purification. Mouse TPC1 (MmTPC1, NCBI accession: NM_145853.2) containing a C-terminal thrombin cleavage site followed by a GFP tag and a Xho I site was cloned into pET28a vector and heterologously expressed in HEK293F cells (Life Technologies) using the BacMam system (Thermo Fisher Scientific). The baculovirus was infected into S9 cells (Life Technologies) following the standard protocol and used to infect HEK293F cells at a ratio of 1:40 (virus:HEK293F, v/v) and supplemented with 10 mM sodium butyrate to boost protein expression. Cells were cultured in suspension at 37 °C for 48 h and collected by centrifugation at 3,000g. All purification procedures were carried out at 4 °C. The cell pellet was re-suspended in buffer A (20 mM Tris, pH 8.0, 150 mM NaCl) supplemented with a protease inhibitor cocktail (containing 2 μg/ml DNase, 0.5 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 μg/ml aprotinin and 0.1 mM PMSF) and homogenized by sonication on ice. MmTPC1 was extracted with 1% (w/v) n-dodecyl-β-D-maltopyranoside (Anatrace) supplemented with 0.2% (w/v) cholesteryl hemisuccinate (Sigma Aldrich) by gentle agitation for 2 h. After extraction, the supernatant was collected after a 60-min centrifugation at 20,000g and incubated with Ni-NTA resin (Qiagen) using gentle agitation. After 2 h, the resin was collected on a disposable gravity column (Bio-Rad). The resin was washed with buffer B (20 mM Tris, pH 8.0, 150 mM NaCl and 0.06% glyco-diosgenin (Anatrace) supplemented with 20 mM imidazole. The washed resin was left on column in buffer B and digested with thrombin (Roche) overnight. After thrombin digestion, the flow-through containing untagged MmTPC1 was collected, concentrated and purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare) pre-equilibrated with buffer B. The peak fraction was pooled and concentrated to 4.7 mg/ml for cryo-EM analysis. To obtain PtdIns(3,5)P₂-bound structure, the protein sample was supplemented with 0.5 mM PtdIns(3,5)P₂, diC8 (Echelon Biosciences) for 30 min on ice before electron microscopy grid preparation.

Electron microscopy data acquisition. The cryo-EM grids were prepared by applying MmTPC1 (4.7 mg/ml, with or without 0.5 mM PtdIns(3,5)P₂) to a glow-discharged Quantifoil R1.2/1.3 500-mesh gold holey carbon grid. Grids were blottered for 4.0 s under 100% humidity at 4 °C before being plunged into liquid nitrogen. The whole-cell configuration was formed by gentle suction. The whole-cell configuration was formed by short vortexing and a low-pass analogue filter set to 1 kHz. The current signal was sampled at a rate of 50 kHz and analyzed using the software IMOD. In this analysis, the data were corrected for the background signal, which is the plateau level of the signal after the peak. The background signal was subtracted from the data, and the resulting signal was used to calculate the density. The resulting density maps were then used to calculate the orientation of the structure.

Electrophysiology. In human TPC2, the Leu11Ala and Leu12Ala mutations at the N-terminal targeting sequence have been shown to promote channel trafficking and to traffic the plasma membrane of the HEK293 cell, enabling channel activity measurement using patch clamp2,4. Therefore, we introduced the equivalent mutations (Leu11Ala and Ile12Ala) to MmTPC1. HEK293 cells overexpressed with the Leu11Ala/Ile12Ala mutant of MmTPC1 elicited much larger whole-cell currents than those expressed with wild-type MmTPC1 (Extended Data Fig. 2a). Therefore, the Leu11Ala/Ile12Ala mutant was used and considered as the wild-type channel in all our recordings. All other mutations in our experiments were generated on the background of this plasma-membrane-targeting MmTPC1. With the channels targeted to the plasma membrane, the extracellular side is equivalent to the lumenal side of TPC1 in endosomes or lysosomes. MmTPC1 and its mutants were cloned into pCGFP-EU vector4. After 2 μg of the plasmid containing the C-terminal GFP-tagged MmTPC1 or its mutant was transfected into HEK293 cells grown in a six-well tissue culture dish using Lipofectamine 2000 (Life Technologies). Forty-eight hours after transfection, cells were dissociated by trypsin treatment and kept in complete serum-containing medium and re-plated on 35-mm tissue culture dishes in a tissue culture incubator until recording.

Patch clamp in whole-cell configuration was used to measure channel activity in most of the experiments except the measurements of ligand affinity and specificity, which were recorded in excised patches (inside-out patches) using the voltage-independent Arg540Gln mutant. This mutant channel can be activated solely by PtdIns(3,5)P₂ and also yields much larger plasma membrane currents, which makes it more amenable for inside-out patches. The standard intracellular solution contained (in mM): 154 sodium methanesulfonate (Na-MS), 5 NaCl, 4 MgCl₂, 1 EGTA, 10 HEPES buffered with Tris, pH = 7.4. The extracellular solution contained (in mM): 145 Na-MS, 5 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES buffered with Tris, pH = 7.4. Various concentrations of PtdIns(3,5)P₂ as specified in each experiment were added to the intracellular solutions to activate the channel. For patches in whole-cell configuration, the intracellular solution was in the pipette and the extracellular solution was in the bath; the solution arrangement was reversed for the inside-out patches. The lipid ligands used in our studies are phosphatidylinositol-3,5-bisphosphate diC8 (PtdIns(3,5)P₂, diC8, Echelon) and phosphatidylinositol-4,5-bisphosphate diC8 (PtdIns(4,5)P₂, diC8, Echelon).

The data were acquired using an AxoPatch 200B amplifier (Molecular Devices) and a low-pass analogue filter set to 1 kHz. The current signal was sampled at a rate of 20 kHz using a Digidata 1200A digitizer (Molecular Devices) and further analyzed using Clampfit 9 software (Molecular Devices). Patch pipettes were pulled from borosilicate glass (Harvard Apparatus) and heat-polished to a resistance of 3−5 MΩ. After the patch pipette attached to the cell membrane, a gigaseal (>10 GΩ) was formed by gentle suction. The whole-cell configuration was formed by short zap or suction to rupture the patch. The inside-out configuration was formed by pulling the pipette away from the cell, and the pipette tip was exposed to the air for a short period in some cases. The holding potential was set to −70 mV. To generate G/Vₗ₁max versus V curves (G = I/V), the membrane was stepped from the holding potential (−70 mV) to various testing potentials (−100 mV to 100 mV) for 1 s and then stepped to −70 mV (Extended Data Fig. 2b). The peak tail currents were used to plot the G−V curve. G/Vₗ₁ was obtained from the peak tail current at 100 mV testing potential. V₁/₂ and Z values were obtained from the fits of data with Boltzmann equation, in which V₁/₂ is the voltage at which the channels have reached half of their maximum fraction open and Z is the apparent valence of voltage dependence. The same protocol was used to obtain current and voltage

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relationships (I–V curve) of the wild-type MmTPC1 (Fig. 3e, top trace), except that the peak current at each testing potential was used to generate the I–V curve. For voltage-independent Arg540Gln mutant, the holding potential was set to 0 mV, and the current and voltage relationship (I–V curve, Fig. 3e, bottom trace) was obtained directly by using voltage pulses ramp from −100 to 100 mV over 800-ms duration.

For measuring ion selectivity of MmTPC1 and its mutants in whole-cell patches, 10μM PtdIns(3,5)P₂ was included in intracellular (pipette) solution to fully activate the channel. The membrane potential was stepped from the holding potential (−70 mV) to 100 mV for 1 s to activate the channels, and then stepped to various testing potentials (−120 mV to 4 mV) for 1 s (Extended Data Fig. 2f). The peak tail currents at various testing potentials were plotted to determine the reversal potential (Vrev). To measure the relative permeability between Na⁺ and K⁺, the extracellular (bath) solution (in mM) was changed to 145 K-MS, 5 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES buffered with Tris, pH 7.4. The ion permeability ratios were calculated with the equations: $P_{Na}/P_{K} = [K]_o/[Na]_o \exp(V_{rev}/FRT) - [Na]_o$, and $P_{Na}/P_{Ca} = 4[Ca]_o/[Na]_o \exp(V_{rev}/FRT)/(1 + \exp(V_{rev}/FRT))$, in which $V_{rev}$ is the reverse potential, $F$ is Faraday’s constant, $R$ is the gas constant, $T$ is the absolute temperature, o is extracellular and i is intracellular.

All electrophysiological recording were repeated at least five times using different patches. Most data points shown are mean ± s.e.m. ($n=5$ independent experiments).

Data availability. The cryo-EM density maps of the MmTPC1 have been deposited in the Electron Microscopy Data Bank under accession number EMD-7434 for the apo state, and accession number EMD-7435 for the PtdIns(3,5)P₂-bound state. Atomic coordinates have been deposited in the RCSB Protein Data Bank under accession number 6C96 for the apo state, and accession number 6C9A for the PtdIns(3,5)P₂-bound state. Source Data for Fig. 3c and Extended Data Fig. 2c, e are available in the online version of the paper.
Extended Data Figure 1 | Sequence alignment of MmTPC1, HsTPC1, AtTPC1, MmTPC2 and HsTPC2. Secondary structure assignments are based on the structure of PtdIns(3,5)P$_2$-bound MmTPC1. Red dots mark the ligand-binding residues; black dots mark the S4 arginine residues and residues at the gating-charge transfer centre; cyan dots mark the key S6 gating residues; green dots mark the residues predicted to participate in Ca$^{2+}$ coordination in EF-hand domains of AtTPC1. MmTPC1 and AtTPC1 share about 25% sequence identity.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Gating and selectivity of MmTPC1.

a, Sample traces and current density (current/capacitance) of wild-type MmTPC1 and the L11A/I12A mutant of MmTPC1, recorded in the whole-cell configuration with 100 μM PtdIns(3,5)P₂ in the pipette (cytosolic). The experiments were repeated five times independently with similar results. Data points for current density are mean ± s.e.m. (n = 5 independent experiments). The L11A/I12A mutant elicited much larger whole-cell currents and was therefore used as the wild-type channel in all recordings. The extracellular side of MmTPC1 in plasma membrane is equivalent to the luminal side of MmTPC1 in lysosomes.

b, Sample traces of PtdIns(3,5)P₂-dependent voltage activation of MmTPC1. Whole-cell currents were recorded with varying PtdIns(3,5)P₂ concentrations in the pipette (cytosolic) at pH 7.4. The experiments were repeated five times independently with similar results. c, G/Gmax–V curves of MmTPC1 at various PtdIns(3,5)P₂ concentrations. Boltzmann fit yields $V_{1/2} = 16.2 ± 0.8 \text{ mV}$, $Z = 0.91 ± 0.02$ at pH 7.4, $V_{1/2} = 38.2 ± 1.2 \text{ mV}$, $Z = 0.95 ± 0.02$ at pH 6.0. All data points were normalized against $G_{\text{max}}$ obtained at 100 mV activation voltage and pH 7.4. All data points are mean ± s.e.m. (n = 5 independent experiments).

d, Luminal pH modulates the voltage activation of MmTPC1. Whole-cell currents of MmTPC1 recorded in the presence of 2 μM cytosolic PtdIns(3,5)P₂ with a varying luminal (bath) pH of 7.4, 6.0 or 4.6. Sample traces were obtained from the same patch. The experiments were repeated five times independently with similar results. e, G/Gmax–V curves of MmTPC1 at various luminal pH values. Boltzmann fit yields $V_{1/2} = 16.2 ± 0.8 \text{ mV}$, $Z = 0.91 ± 0.02$ at pH 7.4, $V_{1/2} = 38.2 ± 1.2 \text{ mV}$, $Z = 0.95 ± 0.02$ at pH 6.0. All data points were normalized against $G_{\text{max}}$ obtained at 100 mV activation voltage and pH 7.4. All data points are mean ± s.e.m. (n = 5 independent experiments).

f, Sample traces of whole-cell currents with 150 mM Na⁺ in the pipette solution, and either 150 mM Na⁺ or 145 mM K⁺ and 5 mM Na⁺ in the bath solution, and the I–V curves generated from the tail currents of the sample traces. g, Sample traces of whole-cell currents with 150 mM Na⁺ in the pipette solution and 150 mM Na⁺ or 100 mM Ca²⁺ in the bath solution, and the I–V curves generated from the tail currents of the sample traces. Data in f and g were recorded with 10 μM PtdIns(3,5)P₂ in the pipette at pH 7.4 and both experiments were repeated five times independently with similar results.

Z is apparent valence. All data points are mean ± s.e.m. (n = 5 independent experiments).
Extended Data Figure 3 | Structure determination of PtdIns(3,5)P2-bound MmTPC1. a, Representative electron micrograph of PtdIns(3,5) P2-bound MmTPC1; 2,348 micrographs were used for structure determination. b, 2D class averages. c, Euler angle distribution of particles used in the final 3D reconstruction, with the heights of the cylinders corresponding to the number of particles. d, Final density maps coloured by local resolution. e, Gold-standard FSC curves of the final 3D reconstructions. f, FSC curves for cross-validation between the models and the maps. Curves for model versus summed map in black (sum), for model versus half map in blue (work) and for model versus half map not used for refinement in red (free). g, Flowchart of electron microscopy data processing for PtdIns(3,5)P2-bound MmTPC1 particles.
Extended Data Figure 4 | Structure determination of apo MmTPC1. a, Representative electron micrograph of apo MmTPC1; 2,937 micrographs were used for structure determination. b, 2D class averages. c, Euler angle distribution of particles used in the final 3D reconstruction, with the heights of the cylinders corresponding to the number of particles. d, Final density maps coloured by local resolution. e, Gold-standard FSC curves of the final 3D reconstructions. f, FSC curves for cross-validation between the models and the maps. Curves for model versus summed map in black (sum), for model versus half map in blue (work) and for model versus half map not used for refinement in red (free). g, Flowchart of electron microscopy data processing for apo MmTPC1 particles.
Extended Data Figure 5 | Sample electron microscopy density maps (blue mesh) for MmTPC1. a–d, Sample electron microscopy density maps for various parts of PtdIns(3,5)P_2-bound MmTPC1: IS1–IS6 and filter I (a), IIS1–IIS6 and filter II (b), NAGs of Asn600 and Asn612 (c), and PtdIns(3,5)P_2-binding site (d). The maps are low-pass filtered to 3.2 Å and sharpened with a temperature factor of $-105 \, \text{Å}^2$. e, f, Sample electron microscopy density maps for the key parts of apo MmTPC1: ligand binding site (e) and S6 helices (f). The maps are low-pass filtered to 3.4 Å and sharpened with a temperature factor of $-98.5 \, \text{Å}^2$. Residues discussed in main text are labelled in red.
Extended Data Figure 6 | Structure comparison between MmTPC1 and AtTPC1. a, Superposition of the overall structures of MmTPC1 (blue) and AtTPC1 (salmon). b, Superposition of the pore regions. c, Superposition of VSD1 domains. The comparison of the VSD2 domains is shown in Fig. 3f. d, Superposition of cytosolic soluble domains.
Extended Data Figure 7 | Sample traces of whole-cell currents for Asn648Ala and Asn649Ala filter mutants. The pipette solution contained 150 mM Na\(^+\) and the bath solution contained 150 mM Na\(^+\), or 145 mM K\(^+\) and 5 mM Na\(^+\). The tail currents were used to generate the I–V curves shown in Fig. 2g. The experiments were repeated five times independently with similar results.
Extended Data Figure 8 | Voltage-sensing domains. a, Superimposition of MmTPC1 VSD1 structures in the PtdIns(3,5)P₂-bound (green) and apo (pink) states with S1 helices removed for clarity. The MmTPC1 VSD1 lacks some key features of canonical voltage sensors: the conserved aromatic residue on S2 and acidic residue on S3 that form the gating-charge transfer centre become Val152 and Lys177, respectively, in MmTPC1; the conserved basic residue at the R5 position becomes Phe209 in MmTPC1; no arginine from IS4 is positioned in the gating-charge transfer centre. b, Superimposition of MmTPC1 VSD2 structures in the PtdIns(3,5)P₂-bound (orange) and apo (cyan) states. c, Sample traces of voltage activation of MmTPC1 and its IS4 arginine mutations, recorded in whole-cell configuration with 2 μM PtdIns(3,5)P₂ in the pipette. Peak tail currents were used to generate the G/Gₘₐₓ–V curves shown in Fig. 3c. The experiments were repeated five times independently with similar results. d, Sample traces of voltage activation of Arg546Gln mutation, recorded in whole-cell configuration with 2 μM and 100 μM PtdIns(3,5)P₂ in the pipette. The experiments were repeated five times independently with similar results.
Extended Data Figure 9 | PtdIns(3,5)P₂-binding in MmTPC1. a, Model of bound PtdIns(3,5)P₂ (left) and its electron microscopy density (right). Density of two other membrane lipid molecules (blue mesh in left panel) was also observed near PtdIns(3,5)P₂ in the structure. b, Current density of mutations at the PtdIns(3,5)P₂-binding site measured at −100 mV in whole-cell recordings. All mutants were generated on the background of Arg540Gln mutant, which was used as control. All data points are mean ± s.e.m. with the number of independent experiments for each mutant shown in parentheses. c, Sample I–V curves of Arg540Gln mutant recorded in excised patches with varying concentrations of PtdIns(3,5)P₂ in the bath (cytosolic). The experiments were repeated five times independently with similar results. Currents at −100 mV were used to generate the concentration-dependent PtdIns(3,5)P₂ activation curve shown in Fig. 4c. Iₘₐₓ is the current recorded at −100 mV with 10 μM PtdIns(3,5)P₂ in the bath. d, Structural comparison at the ligand-binding site between the PtdIns(3,5)P₂-bound (green) and apo (salmon) states.
### Extended Data Table 1 | Cryo-EM data collection and model statistics

|                      | PtdIns(3,5)P$_2$-bound MmTPC1 (EMD-7435) | Apo MmTPC1 (EMD-7434) (PDB 6C9A) |
|----------------------|------------------------------------------|-----------------------------------|

#### Data collection and processing

|                          | PtdIns(3,5)P$_2$-bound MmTPC1 (EMD-7435) | Apo MmTPC1 (EMD-7434) (PDB 6C9A) |
|--------------------------|------------------------------------------|-----------------------------------|
| Magnification            | 46730                                    | 46730                             |
| Voltage (kV)             | 300                                      | 300                               |
| Electron exposure (e$^{-}$/Å$^2$) | ~50                                     | ~50                               |
| Defocus range (μm)       | -1.5 to -3.0                             | -1.5 to -3.0                      |
| Pixel size (Å)           | 1.07                                     | 1.07                              |
| Symmetry imposed         | C2                                       | C2                                |
| Initial particle images (no.) | 941,754                                 | 1,260,054                         |
| Final particle images (no.) | 82,819                                  | 42,870                            |
| Map resolution (Å)       | 3.2                                      | 3.4                               |
|                         |                                          | 0.143                             |
| FSC threshold           |                                          | 0.143                             |

#### Refinement

| Initial model used (PDB code) | PtdIns(3,5)P$_2$-bound MmTPC1 (EMD-7435) | Apo MmTPC1 (EMD-7434) (PDB 6C9A) |
|------------------------------|------------------------------------------|-----------------------------------|
| Model resolution (Å)         | 3.2                                      | 3.4                               |
| FSC threshold                | 0.143                                    | 0.143                             |
| Map sharpening B factor (Å$^2$) | -105.07                                 | -98.52                            |
| Model composition            |                                          |                                   |
| Non-hydrogen atoms           | 12182                                    | 12090                             |
| Protein residues             | 12004                                    | 12004                             |
| Ligands                      | 178                                      | 86                                |
| $B$ factors (Å$^2$)          |                                          |                                   |
| Protein                      | 80.93                                    | 98.57                             |
| Ligand                       | 67.59                                    | 81.03                             |
| R.m.s. deviations            |                                          |                                   |
| Bond lengths (Å)             | 0.009                                    | 0.010                             |
| Bond angles (°)              | 1.286                                    | 1.309                             |
| Validation                   |                                          |                                   |
| MolProbity score             | 1.41                                     | 1.5                               |
| Clashscore                   | 2.79                                     | 3.5                               |
| Poor rotamers (%)            | 0                                        | 0.3                               |
| Ramachandran plot            |                                          |                                   |
| Favored (%)                  | 94.99%                                   | 94.85%                            |
| Allowed (%)                  | 5.01%                                    | 5.15%                             |
| Disallowed (%)               | 0                                        | 0                                 |

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- Experimental design

1. Sample size
   Describe how sample size was determined.
   All electrophysiological recordings were repeated at least five times by patching different cells. The sample size was determined based on the reproducibility of the recordings. As all recordings yielded consistent results, the sample size was sufficient to provide accurate measurement.

2. Data exclusions
   Describe any data exclusions.
   3-D classification yielded multiple 3-D reconstruction maps. Only the particles that give rise to homogeneous density were selected, combined and used in the final reconstruction and refinement. Details were described in the flowcharts of Extended Data Fig. 3&4.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experimental findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   For structure refinement and model validation, all particles were randomly split into two groups, one group was used for refinement and the other for validation. For channel recording, cells with GFP fluorescence (proteins were GFP-tagged) were randomly selected for recording.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The investigators were blinded to group allocation during data collection and analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. 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).

| n/a | 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.*

7. Software

Policy information about availability of computer code

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

- EPU1.9, RELION 2.0, MotionCorr2.1, GCTF1.06, pClamp 9, Coot0.8.8, ResMap1.1.4, PHENIX1.11.1, MolProbity (part of PHENIX package), PyMol1.8.6.2, Chimera1.11.2

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.

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.

- No restrictions on availability of materials.

9. Antibodies

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

- No antibodies were used.

10. Eukaryotic cell lines

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

- Both HEK293F and sf9 cell lines were purchased from Life Technologies

b. Describe the method of cell line authentication used.

- Cell line authentication was not performed.

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

- Cell 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.

- No commonly misidentified cell lines were used.

9. 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 in this study.
12. Description of human research participants

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

This study did not involve human research participants.