1 **TRPML1 gating modulation by allosteric mutations and lipids**

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Abstract:

Transient Receptor Potential Mucolipin 1 (TRPML1) is a lysosomal cation channel whose loss-of-function mutations directly cause the lysosomal storage disorder mucolipidosis type IV (MLIV). TRPML1 can be allosterically regulated by various ligands including natural lipids and small synthetic molecules and the channel undergoes a global movement propagated from ligand-induced local conformational changes upon activation. In this study, we identified a functionally critical residue, Tyr404, at the C-terminus of the S4 helix, whose mutations to tryptophan and alanine yield gain- and loss-of-function channels, respectively. These allosteric mutations mimic the ligand activation or inhibition of the TRPML1 channel without interfering with ligand binding and both mutant channels are susceptible to agonist or antagonist modulation, making them better targets for screening potent TRPML1 activators and inhibitors. We also determined the high-resolution structure of TRPML1 in complex with the PI(4,5)P$_2$ inhibitor, revealing the structural basis underlying this lipid inhibition. In addition, an endogenous phospholipid likely from sphingomyelin is identified in the PI(4,5)P$_2$-bound TRPML1 structure at the same hotspot for agonists and antagonists, providing a plausible structural explanation for the inhibitory effect of sphingomyelin on agonist activation.

Introduction:

Transient Receptor Potential Mucolipin 1 (TRPML1) is a Ca^{2+}-permeable, non-selective, lysosomal cation channel ubiquitously expressed in mammalian cells (Dong *et al*, 2008; LaPlante *et al*, 2002; Sun *et al*, 2000). TRPML1 plays critical roles in many important cellular activities including lipid accumulation (Shen *et al*, 2012), signaling transduction (Kilpatrick *et al*, 2016), lysosome trafficking (Venkatachalam *et al*, 2015), and autophagy (Scotto Rosato *et al*, 2019). The loss-of-function mutations in TRPML1 directly cause the lysosomal storage disorder mucolipidosis type IV (MLIV), a neurodegenerative disease characterized by abnormal neurodevelopment, retinal degeneration, and iron-deficiency anemia (Bargal *et al*, 2000; Bassi *et al*, 2000; Gan & Jiang, 2022; Nilius *et al*, 2007). Because of its physiological importance and direct disease association, TRPML1 has been extensively studied and is a potential target for drug development.

TRPML1 can be regulated by various ligands including both natural lipids and small synthetic molecules. The channel can be activated by the lysosome-specific phosphatidylinositol

62   3,5-bisphosphate (PI(3,5)P$_2$) (Dong *et al*, 2010), but inhibited by the plasma membrane-enriched

63   PI(4,5)P$_2$ (Zhang *et al*, 2012). Given its pharmacological importance, many synthetic agonists

64   and antagonists have been developed for TRPML1 activation and inhibition (Chen *et al*, 2014;

65   Grimm *et al*, 2010; Samie *et al*, 2013; Shen *et al.*, 2012). Interestingly, the mTOR (Mammalian

66   target of rapamycin) inhibitor rapamycin and its derivatives can also synergistically activate

67   TRPML1 with PI(3,5)P$_2$ (Gan *et al*, 2022; Zhang *et al*, 2019). Recent studies also suggest that

68   sphingomyelin, a major membrane component, can also modulate the TRPML1 activation (Prat

69   Castro *et al*, 2022; Shen *et al.*, 2012).

70   Several TRPML1 channel structures in both open and closed conformations with various

71   ligands have been determined (Chen *et al*, 2017; Fine *et al*, 2018; Gan *et al.*, 2022; Schmiege *et*

72   *al*, 2017; Schmiege *et al*, 2021), revealing some unique features of the TRPML1 channel. Firstly,

73   all ligand-binding sites in the structures converge to two hot spots: The N-terminal poly-basic

74   pocket for PIP$_2$ and the inter-subunit interface in the middle of the membrane between S5 and S6

75   for agonists, antagonists, and rapamycin (Fine *et al.*, 2018; Gan *et al.*, 2022; Schmiege *et al.*,

76   2017; Schmiege *et al.*, 2021) (Figure 1a). Secondly, all open TRPML1 structures are almost

77   identical regardless of the activation stimuli. Thirdly, structural comparison between the open

78   and closed conformation illustrates that TRPML1 gating is not merely a local conformational

79   change but involves the global movement of almost the entire channel mediated by tight inter-

80   and intra-subunit packing within the channel tetramer (Movie supplement 1). Finally, the

81   necessity of global movement for channel activation underlies the allosteric regulation of

82   TRPML1 by two distantly bound ligands - that is, the ligand-induced local conformational

83   change at one site can propagate to the other site and thereby affect the binding of the other

84   ligand (Gan *et al.*, 2022). The high allostery of TRPML1 gating would allow us to design

85   allosteric mutations that are remote from the channel pore but can still stabilize the channel in an

86   open or closed state, mimicking the ligand activation or inhibition of the channel. To this end,

87   we identified Tyr404 on the S4 helix as an allosteric site whose mutation can promote or inhibit

88   TRPML1 gating. Furthermore, we also determined a high-resolution structure of PI(4,5)P$_2$-

89   inhibited TRPML1 and demonstrated that in addition to competing against PI(3,5)P$_2$ activator for

90   the same site, PI(4,5)P$_2$ also allosteric inhibits small molecule agonist by stabilize the channel in

91   the closed conformation. Furthermore, the high-resolution PI(4,5)P$_2$-bound TRPML1 structure

92 also revealed a bound phospholipid likely from sphingomyelin at the agonist/antagonist site,

93 providing a plausible explanation for sphingomyelin inhibition of TRPML1.

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95 **Results:**

96 **Allosteric mutations at Tyr404 recapitulate TRPML1 gating**

97   Our previous study on the allosteric activation of TRPML1 by PI(3,5)P_2 and rapamycin

98 demonstrated that ligand-induced local conformational changes can propagate to distal parts of

99 the channel through tight inter- and intra-subunit packing within the channel tetramer, allowing

100 the channel to integrate the stimuli from these two distantly bound ligands. The PI(3,5)P_2 and

101 rapamycin-induced local conformational changes converge to the same driving force on S4 helix,

102 resulting in a slight bend of the C-terminal half of the S4 that facilitates the channel opening

103 (Gan *et al.*, 2022) (Figure 1b). A key interaction coupled to the S4 bending movement is the

104 insertion of Tyr404 side chain into a pocket surrounded by S1, S3, and S4 helices where its

105 aromatic ring is sandwiched between the side chains of Leu66 and Arg403. We hypothesized

106 that mutations at Tyr404 that stabilize its sidechain in the pocket would facilitate channel

107 activation; conversely, mutations that destabilize its sidechain in the pocket would negatively

108 modulate the channel activation. To test this, we replaced Tyr404 with tryptophan and alanine,

109 respectively, and measured the effect of these mutations on channel activity. As illustrated in the

110 electrophysiological recordings using whole-cell patches, the Y404W mutant elicits large

111 inward-rectifying currents without any ligands, indicating that Y404W is a gain-of-function

112 (GOF) mutant (Figure 1c and Figure supplement 1a). Adding extra activation ligands such as

113 PI(3,5)P_2, rapamycin, or small molecule agonist ML-SA1 only marginally increases the currents.

114 The Y404W GOF mutant mimics a ligand-activated channel, yet its mutation site is remote from

115 the pore domain and the channel can still be allosterically inhibited by small molecule

116 antagonists (ML-SI1 and ML-SI3) (Figure 1d and Figure supplement 1b). This is distinct from

117 other gain-of-function mutants in which proline substitutions on the S5 helix lock the pore in an

118 open state and the channels are no longer susceptible to antagonist inhibition (Dong *et al*, 2009;

119 Grimm *et al*, 2007; Kim *et al*, 2007; Nagata *et al*, 2008; Xu *et al*, 2007).

120   Y404A, on the other hand, represents a loss-of-function mutant and elicits much lower

121 currents even in the presence of potent agonist ML-SA1 (Figure 1e and Figure supplement 1c).

122   While ML-SA1 can potently activate the wild-type TRPML1 channel, the Y404A mutation
123   mimics PI(4,5)P$_2$ inhibition and allosterically inhibits ML-SA1 binding, significantly decreasing
124   the efficacy of ML-SA1 activation (Figure 1f & g).

125   **Structure of GOF Y404W mutant**

126   To reveal the structural basis underlying the channel activation of the Y404W mutant, we
127   determined its structure in the absence of any ligands to 2.86 Å resolution (Figure supplement 2-
128   3 and Methods). As expected, the Y404W mutant adopts an open conformation with a structure
129   almost identical to other ligand-activated open TRPML1, consistent with its GOF property
130   (Figure 2a and Figure supplement 3b). Like Tyr404 in the wide-type open TRPML1, the side
131   chain of W404 in the mutant is inserted into the pocket surrounded by S1, S3, and S4 helices and
132   sandwiched between Leu66 and Arg403 (Figure 2b). However, the larger indole ring of Trp404
133   provides a better spatial fitting into the pocket than the phenol ring of Tyr404 and several
134   surrounding residues (Lys65, Gln69, and Leu358) provide extra van der Waals contacts to the
135   Trp404 side chain. Thus, by enhancing the stability of the aromatic side chain inside the pocket,
136   Y404W mutation facilitates the bending of S4 which in turn propagates to the pore through the
137   S4-S5 linker and activates the channel (Gan *et al.*, 2022). The Y404W mutant structure
138   demonstrates that the sidechain packing in the pocket is essential for stabilizing the open channel
139   and the lack of such packing capacity in the Y404A mutant with a small side-chain likely
140   destabilizes the open conformation, yielding a loss-of-function channel. It is worth noting that
141   Arg403 plays two essential roles in TRPML1 gating: its side chain is part of the pocket that
142   stabilizes Tyr404 in the open state; its guanidinium group forms a salt bridge with the C3
143   phosphate group of PI(3,5)P$_2$ upon ligand activation (Gan *et al.*, 2022). As expected, Arg403 is
144   highly conserved in the TRPML channel family, and its R403C variant identified in an MLIV
145   patient is a loss-of-function mutant (Chen *et al.*, 2014).

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147   **Structure of TRPML1 in PI(4,5)P$_2$-bound closed state**

148   While PI(4,5)P$_2$ inhibits PI(3,5)P$_2$ activation of TRPML1 by directly competing for the
149   same binding site, it also allosterically inhibits the agonist-activated channel (Chen *et al.*, 2017),
150   suggesting that PI(4,5)P$_2$ binding stabilizes the TRPML1 channel in a closed conformation. A
151   previous low-resolution structure of TRPML1 in complex with PI(4,5)P$_2$ revealed the

152 approximate location of $PI(4,5)P_2$ binding but failed to explain how its binding stabilizes the

153 channel in the closed state and allosterically inhibits the agonist-activated channel (Fine *et al.*,

154 2018). To address this, we determined the structure of $PI(4,5)P_2$-bound TRPML1 at 2.46 Å

155 (Figure 3a, Figure supplement 3-5 and Methods). The density from the IP3 head group of

156 $PI(4,5)P_2$, especially the phosphate groups on C4 and C5 of the inositol, can be clearly defined in

157 the EM map (Figure 3a-3c). The phosphatidyl group, however, is flexible and could not be

158 resolved in the structure. While $PI(4,5)P_2$ binding overlaps with that of $PI(3,5)P_2$, their IP3 head

159 group positions are quite different (Figure 3b-3e). In the $PI(3,5)P_2$-bound structure (Figure 3d),

160 the head group protrudes deep into the N-terminal PIP_2-binding pocket enclosed by two short

161 clamp-shaped helices of H1 and H2, and the cytosolic ends of S1 and S2 helices, allowing its C3

162 phosphate to engage in direct interactions with Arg403 and Tyr355 to facilitate channel

163 activation (Gan *et al.*, 2022). These C3 phosphate-mediated interactions are absent in $PI(4,5)P_2$-

164 bound structure. Instead, the head group of $PI(4,5)P_2$ is trapped at the entrance of the pocket and

165 forms a bridge between S1 and S2 with its phosphate groups stabilized by positively charged

166 residues from H2, S1, and S2 (Figure 3b & 3c). A major conformational change between the

167 open and closed states is an upward movement of the S1 helix, a prerequisite for Tyr404

168 insertion between Leu66 and Arg403 and the subsequent bending of S4 (Figure 3e). Therefore,

169 the $PI(4,5)P_2$-mediated bridging interaction between S1 and S2 would hinder the S1 movement

170 and stabilize the channel in the closed conformation, exerting allosteric inhibition on agonist

171 activation.

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173 **Endogenous sphingomyelin lipid at the agonist- and antagonist-binding site**

174 The high-resolution structure of $PI(4,5)P_2$-bound closed TRPML1 also reveals a well-defined

175 density from an endogenous lipid molecule at the inter-subunit interface between S5 and S6

176 (Figure 4a). The lipid contains a choline head group and is likely a phosphatidylcholine (PC) or

177 sphingomyelin (SM), the two main choline-containing phospholipid components of the outer

178 leaflet of the plasma membrane. The tail from one of the lipid alkyl chains penetrates deep into

179 an inter-subunit pocket in the middle of the membrane, overlapping with the hotspot for both

180 channel agonist and antagonist (Figure supplement 6). This alkyl chain has to be displaced upon

181 agonist or antagonist binding, suggesting that the lipid occupation would compete against agonist

182 or antagonist binding. We suspect this bound lipid is sphingomyelin which is also enriched in the

183   endocytic recycling compartment and has been shown to inhibit TRPML1 activity (Prat Castro *et*

184   *al.*, 2022; Schuchman, 2010; Shen *et al.*, 2012; Slotte, 2013). Key evidence to support SM

185   inhibition is that its enrichment can reduce the agonist (i.e. SF-51 and ML-SA1) activation of

186   TRPML1 (Shen *et al.*, 2012). Indeed, we did observe the reduction of SF-51-activated TRPML1

187   current upon SM enrichment (Figure 4b and Figure supplement 7a). However, based on our

188   structure, we hypothesize that the role of sphingomyelin is to stabilize rather than directly inhibit

189   the channel; the SM inhibition upon enrichment is an indirect effect attributable to its

190   competition against agonist binding that reduces the apparent efficacy of agonist activation. This

191   hypothesis would imply that SM can also function as an indirect activator by competing against

192   antagonists and reducing their effectiveness in channel inhibition. The gain-of-function Y404W

193   mutant, which is still susceptible to antagonist inhibition, provides a good system to test that. As

194   shown in Figure 4c and Figure supplement 7b, SM shows no obvious inhibition to the mutant

195   channel activity whereas antagonist ML-SI1 markedly reduces the mutant channel current; upon

196   SM enrichment, ML-SI1 inhibition is mitigated resulting in a recovery of the channel current.

197   This observation confirms the competitive binding of SM at the hot spot for both agonists and

198   antagonists.

199

Summary

201   In this study, we designed and analyzed the allosteric mutations at Tyr404 that recapitulate the

202   gating of TRPML1. Replacing this tyrosine with tryptophan or alanine stabilizes or destabilizes

203   the channel in the open state, yielding a gain- or loss-of-function mutant. The structure of the

204   Y404W mutant adopts the same open structure as ligand-activated TRPML1, once again

205   highlighting the global conformational change for TRPML1 channel activation. As Tyr404 is

206   distant from the hot spots for ligand binding, the two gain- and loss-of-function mutants can still

207   be allosterically modulated by antagonists and agonists. Thus, these allosteric mutants can mimic

208   ligand-activated or inhibited TRPML1 without interfering with ligand binding, making them

209   better targets for screening potent small molecule TRPML1 inhibitors and activators. We also

210   investigated the structural basis of $PI(4,5)P_2$ inhibition of TRPML1 by determining the $PI(4,5)P_2$-

211   bound structure, revealing a different binding mode by its head group at the N-terminal polybasic

212   site than that of $PI(3,5)P_2$. The head group of $PI(4,5)P_2$ mediates a bridging interaction between

213   S1 and S2 and stabilizes TRPML1 in a closed conformation. In the high-resolution PI(4,5)P$_2$-

214   bound TRPML1 structure, we also visualize clear density from a choline-containing

215   phospholipid at the same site for agonists or antagonists. In light of its high membrane

216   abundance and competing effect on agonist activation and antagonist inhibition, this bound lipid

217   is likely from sphingomyelin.

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221 **Methods:**

222 **Protein expression and purification**

223 Protein expression and purification were performed as previously described (Gan *et al.*, 2022).

224 The *Mus musculus* TRPML1 gene with a C-terminal thrombin cleavage site and a 10× His tag

225 was cloned into a pEZTBM vector (Morales-Perez *et al*, 2016) and heterologously expressed in

226 HEK293F cells using the BacMam system. The baculovirus was produced in Sf9 cells and used

227 to transduce the HEK293F cells at a ratio of 1:40 (virus:HEK293F, v/v) and supplemented with

228 1 mM sodium butyrate to boost the protein expression. Cells were cultured in suspension at

229 37 °C for 48 h and harvested by centrifugation at 3,000g. All purification procedures were

230 carried out at 4 °C unless specified otherwise. The cell pellet was re-suspended in buffer A

231 (20 mM Tris pH 8.0, 150mM NaCl) supplemented with a pro0tease inhibitor cocktail (containing

232 1 mg ml−1 each of DNase, pepstatin, leupeptin, and aprotinin and 1 mM PMSF) and

233 homogenized by sonication on ice. Protein was extracted with 1% (w/v) n-dodecyl-β-D-

234 maltopyranoside (DDM; Anatrace) supplemented with 0.2% (w/v) cholesteryl hemisuccinate

235 (CHS; Sigma-Aldrich) by gentle agitation for 2 h. After extraction, the supernatant was collected

236 after a 1 h centrifugation at 48,000g and incubated with Ni-NTA resin and 20 mM imidazole

237 with gentle agitation. After 1 h, the resin was collected on a disposable gravity column (Bio-Rad),

238 washed with buffer B (buffer A + 0.04% glyco-diosgenin (GDN; Anatrace)) with 20 mM

239 imidazole. The washed resin was left on-column in buffer B and digested with thrombin

240 overnight. After digestion, the flow-through was concentrated, and purified by size-exclusion

241 chromatography on a Superose 6 10/300 GL column (GE Heathcare) pre-equilibrated with buffer

242 B. The protein peak was collected and concentrated. For PI(4,5)P_2-bound structure, purified

243 protein was incubated with 0.5mM PI(4,5)P_2 on ice for 4 h. The lipid ligand used in this study is

244 PI(4,5)P_2 diC8 (Echelon)

245 **Electron microscopy data acquisition**

246 Electron microscopy data acquisition followed the protocol previously described (Gan *et al.*,

247 2022). The cryo-EM grids were prepared by applying 3.5 μl protein (3.5 mg/mL) to a glow-

248 discharged Quantifoil R1.2/1.3 200-mesh copper holey carbon grid (Quantifoil, Micro Tools

249 GmbH) and blotted for 3.0 s under 100% humidity at 4 °C before being plunged into liquid

250 ethane using a Mark IV Vitrobot (FEI). For the dataset of Y404W, micrographs were acquired

251   on a Titan Krios microscope (FEI) operated at 300 kV with a K3 Summit direct electron detector

252   (Gatan), using a slit width of 20 eV on a GIF-Quantum energy filter. Data were collected using

253   CDS (Correlated Double Sampling) mode of the K3 camera with a super resolution pixel size of

254   0.413 Å. The defocus range was set from −0.9 to −2.2 μm. Each movie was dose-fractionated to

255   60 frames with a dose rate of 1e-/Å^2/frame for a total dose of 60e-/Å^2. The total exposure time

256   was between 5 to 6 s. For the $PI(4,5)P_2$ bound dataset, micrographs were acquired on a Titan

257   Krios microscope (FEI) operated at 300 kV with a Falcon4 electron detector (Thermo Fisher),

258   using a slit width of 20 eV on a post-column Selectris X energy filter (Thermo Fisher Scientific).

259   Data was collected using Falcon 4 camera with a pixel size of 0.738 Å. The defocus range was

260   set from -0.9 to -2.2 μm. Each movie was dose-fractionated to 60 frames with a dose rate of 1e-

261   /Å^2/frame for a total dose of 60e-/Å^2. The total exposure time was between 3.5 to 4 s.

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Image processing

264   Images were processed as previously described (Gan *et al.*, 2022). Movie frames were motion

265   corrected and binned two times and dose-weighted using MotionCor2 (Zheng *et al*, 2017). The

266   CTF parameters of the micrographs were estimated using the GCTF program (Zhang, 2016). The

267   rest of the image processing steps were carried out using RELION 3.1 (Nakane *et al*, 2020;

268   Scheres, 2012; Zivanov *et al*, 2018). All resolution was reported according to the gold-standard

269   Fourier shell correlation (FSC) using the 0.143 criterion (Henderson *et al*, 2012). Local

270   resolution was estimated using Relion. Aligned micrographs were manually inspected to remove

271   those with ice contamination and bad defocus. Particles were selected using Gautomatch (K.

272   Zhang, MRC LMB, https://www2.mrc-lmb.cam.ac.uk/research/locally-developed-

273   software/zhang-software/) and extracted using a binning factor of 3. 2D classification was

274   performed in Relion 3.1. Selected particles after 2D classification were subjected to one around

275   3D classification. The mouse TRPML1 map (EMD-8883 (Chen *et al.*, 2017)) low-pass filtered to

276   30 Å was used as the initial reference. Classes that showed clear features of the TRPML1

277   channel were combined and subjected to 3D auto-refinement and another round of 3D

278   classification without performing particle alignment using a soft mask around the protein portion

279   of the density. The best resolving classes were then re-extracted with the original pixel size and

280   further refined. Beam tilt, anisotropic magnification, and per-particle CTF estimations and

281 Bayesian polishing were performed in Relion 3.1 to improve the resolution of the final
282 reconstruction.

283 For the Y404W structure dataset, a total of 4,724 movies were collected and 4,505 were selected
284 after motion correction and CTF estimation. A total number of 864,698 particles were extracted
285 from the selected micrographs and were subjected to one round of 2D classification, from which
286 87,846 particles were selected. After the initial 3D classification, 35,460 particles were selected
287 and subjected to a 3D auto-refinement job and further ctf refinements, yielding a map at 2.86Å
288 overall resolution (Figure supplement 2).

289 For the PI(4,5)P$_2$-bound dataset, a total of 8,164 movies were collected and 7,895 were selected
290 after motion correction and CTF estimation. A total number of 1,065,778 particles were
291 extracted from the selected micrographs and were subjected to one round of 2D classification,
292 from which 555,281 particles were selected. After the initial 3D classification, 359,441 particles
293 were selected and subjected to a 3D auto-refinement job. Next, a soft mask excluding the micelle
294 density was applied and particles were sorted into 5 classes without performing alignment. From
295 this, one classe with a total number of 60,597 particles were selected and further refined. In the
296 postprocess step, a B-factor of -60 was manually given, yielding a map at 2.46Å overall
297 resolution (Figure supplement 4).

Model building, refinement and validation

299 Model building, refinement and validation followed the previously described protocol (Gan *et al.*,
300 2022). The structure of mouse TRPML1 (PDB code: 5WPV) was used as the initial model and
301 was manually adjusted in Coot (Emsley *et al*, 2010) and refined against the map by using the real
302 space refinement module with secondary structure and non-crystallographic symmetry restraints
303 in the Phenix package (Adams *et al*, 2010). The final structure model of Y404W includes
304 residues 40-200, 216-527. The final structure model of the PI(4,5)P$_2$-bound includes residues 39-
305 200, 216-285, 296-527. About 40 residues at the amino terminus and 50 residues at the carboxy
306 terminus are disordered and not modeled. The statistics of the geometries of the models were
307 generated using MolProbity (Chen *et al*, 2010). All the figures were prepared in PyMol
308 (Schrödinger, LLC.), UCSF Chimera (Pettersen *et al*, 2004). Pore radii were calculated using the
309 HOLE program(Smart *et al*, 1996).

Electrophysiology

310

311   Electrophysiology was carried out following a previously described protocol with minor
312   modifications (Gan *et al.*, 2022). For electrophysiological analysis, the two di-leucine motifs
313   (15_{LL} and 577_{LL}) of mouse TRPML1 responsible for lysosomal targeting were replaced with
314   alanines to facilitate the trafficking of the channel to the plasma membrane (Grimm *et al.*, 2010;
315   Vergarajauregui & Puertollano, 2006). The N-terminal GFP tagged, plasma membrane-targeting
316   TRPML1 mutant (TRPML1-4A) and derived point mutations were overexpressed in HEK293
317   cells and the channel activities were directly measured by patching the plasma membrane. In this
318   setting, the extracellular side is equivalent to the luminal side of TRPML1 in endosomes or
319   lysosomes. 48 h after transfection, cells were dissociated by trypsin treatment and kept in
320   complete serum-containing medium; the cells were re-plated onto 35 mm tissue culture dishes
321   and kept in a tissue culture incubator until recording. Patch clamp in the whole-cell or inside-out
322   configuration was used to measure TRPML1 activity on the HEK plasma membrane. The
323   standard bath solution for whole cell current recording contained (in mM): 145 sodium
324   methanesulfonate, 5 NaCl, 1 $MgCl_2$, 10 HEPES buffered with Tris, pH 7.4; and the pipette
325   solution contained (in mM): 140 caesium methanesulfonate, 5 NaCl, 5 $MgCl_2$, 10 EGTA, 10
326   HEPES buffered with Tris, pH 7.4. The bath solution for inside-out configuration contained (in
327   mM): 140 potassium methanesulfonate, 5 NaCl, 2 $MgCl_2$, 0.4 $CaCl_2$, 1 EGTA, 10 HEPES
328   buffered with Tris, pH 7.4; and the pipette solution contained (in mM): 145 sodium
329   methanesulfonate, 5 NaCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES buffered with Tris, pH 7.4. For
330   whole cell recording of $PI(3,5)P_2$-activated channel, we had to include high concentration of
331    $PI(3,5)P_2$ (100 μM) in the pipette solution (cytosolic side) in order to quickly obtain stable
332    $PI(3,5)P_2$-evoked current, likely because of the slow diffusion of this lipid ligand. $PI(4,5)P_2$ was
333   added in the cytosolic side, Tem, ML-SA1, ML-SI3, ML-SI1, SM were added in the bath
334   solution. SM competition assays with SF-51 and ML-SI1 were conducted under pH 4.6. The
335   patch pipettes were pulled from Borosilicate glassand heat polished to a resistance of 2–5 MΩ
336   (2–3 MΩ for inside-out patch, and 3–5 MΩ for whole-cell current recoding). Data were acquired
337   using an AxoPatch 200B amplifier (Molecular Devices) and a low-pass analogue filter set to
338   1 kHz. The current signal was sampled at a rate of 20 kHz using a Digidata 1550B digitizer
339   (Molecular Devices) and further analyzed with pClamp 11 software (Molecular Devices). After
340   the patch pipette attached to the cell membrane, the giga seal (>10 GΩ) was formed by gentle

341 suction. The inside-out configuration was formed by pulling the pipette away from the cell, and

342 the pipette tip was exposed to the air for 2 seconds. The whole-cell configuration was formed by

343 short zap or suction to rupture the patch. The holding potential was set to $0\,mV$. The whole-cell

344 and inside-out macroscopic current recordings were obtained using voltage pulses ramped from

345 $-140\,mV$ to $+50\,mV$ over a duration of $800\,ms$. The sample traces for the I–V curves of

346 macroscopic currents shown in each figure were obtained from recordings on the same patch. All

347 data points are mean $\pm$ s.e.m. (n $\geq$ 5).

348 **Data availability.** The cryo-EM density maps of mouse TRPML1 have been deposited in the

349 Electron Microscopy Data Bank (EMDB) under accession numbers 45429 (Y404W), 45432

350 $(PI(4,5)P_2$-bound). Atomic coordinates have been deposited in the Protein Data Bank (PDB)

351 under accession numbers 9CBZ (Y404W), 9CC2 $(PI(4,5)P_2$-bound).

361

362 **Author contributions:** N.G. prepared the samples; Y.H. and N.G. performed data acquisition,

363 image processing, and structure determination; W.Z. performed electrophysiology recording; All

364 authors participated in research design, data analysis, discussion, and manuscript preparation.

365

366 **Declaration of interests: The authors declare no competing financial interests.**

367

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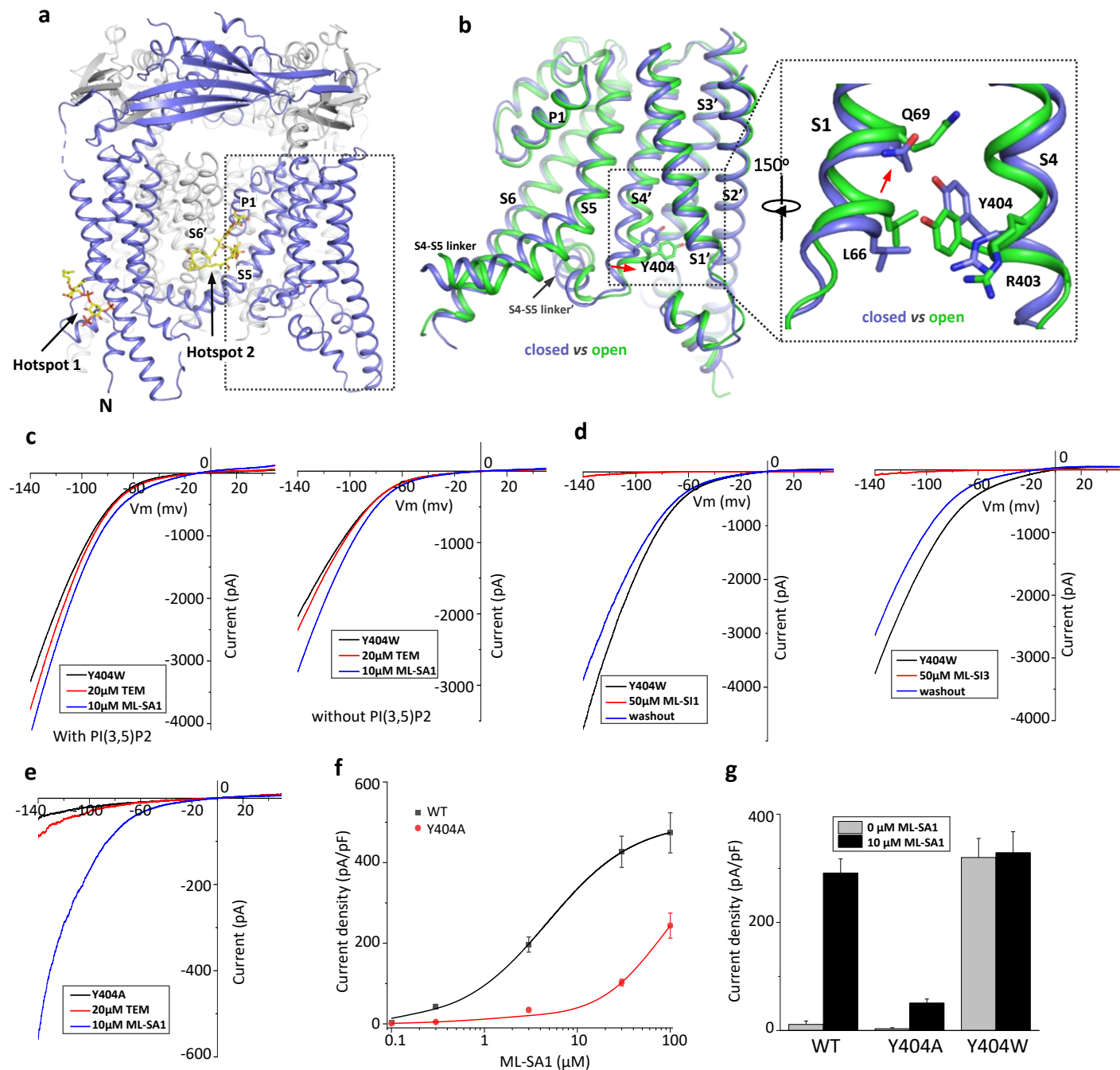
**Figure 1 Design and characterization of allosteric mutations at Tyr404 that recapitulate TRPML1 gating.** (a) The structure of PI(3,5)P$_2$/Temsirolimus-activated TRPML1 (PDB code:7SQ9) illustrating the two hot spots for ligand binding. Temsirolimus (Tem) is a rapamycin analog. (b) Ligand-induced conformational change and the zoomed-in view of the Y404 movement. Only the boxed region in (a) is shown in the structural comparison between the open (green) and closed (blue) structures. Red arrows mark the bending of S4 and upward movement of S1. (c) Sample traces of Y404W gain-of-function mutant recorded using patch clamp in whole-cell configuration with (left) or without (right) 100 μM PI(3,5)P$_2$ in the pipette (cytosolic). Tem or agonist ML-SA1 was introduced in the bath solution (extracellular/luminal). (d) Sample traces of Y404W inhibition by antagonists ML-SI1 (left) and ML-SI3 (right) recorded using patch clamp in whole-cell configuration. The antagonists were introduced in the bath solution (extracellular/luminal). (e) Sample traces of Y404A loss-of-function mutant with 100 μM PI(3,5)P$_2$ in the pipette (cytosolic). Tem or ML-SA1was introduced in the bath solution (extracellular/luminal). (f) ML-SA1 activation of TRPML1(WT) and Y404A mutant measured at -140 mV. Data for WT is least square fits to the Hill equation with EC$_{50}$=4.8±0.7 μM, n=0.93±0.10. Data points are mean ± SEM (n=5 independent experiments). (f) Current density of wild-type and mutant TRPML1 at -140mV with and without 10μM ML-SA1. Data points are mean ± SEM (n=5 independent experiments)
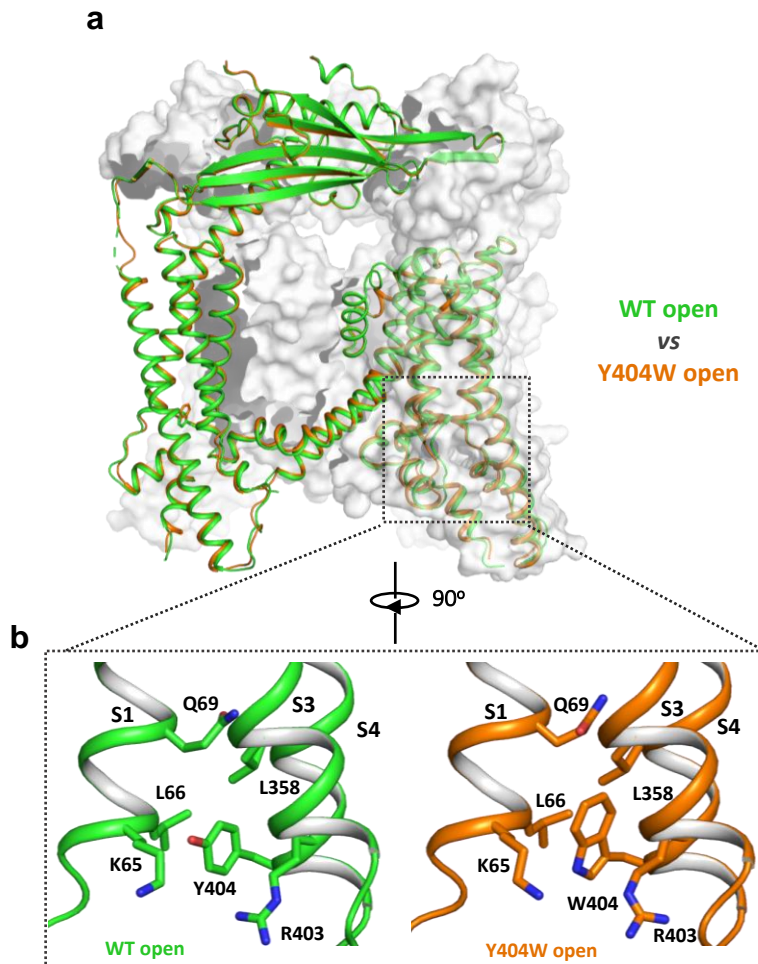
Figure 2. Y404W mutant adopts an open conformation in the absence of ligands. (a) Structural comparison between PI(3,5)P$_2$/Tem-bound open structure (green) and the Y404W mutant structure (orange). Only the front subunit and the neighboring S1-S4 regions are highlighted in color for clarity. **(b)** Zoomed-in views of the regions surrounding Y404 (WT, green) and W404 (mutant, orange).
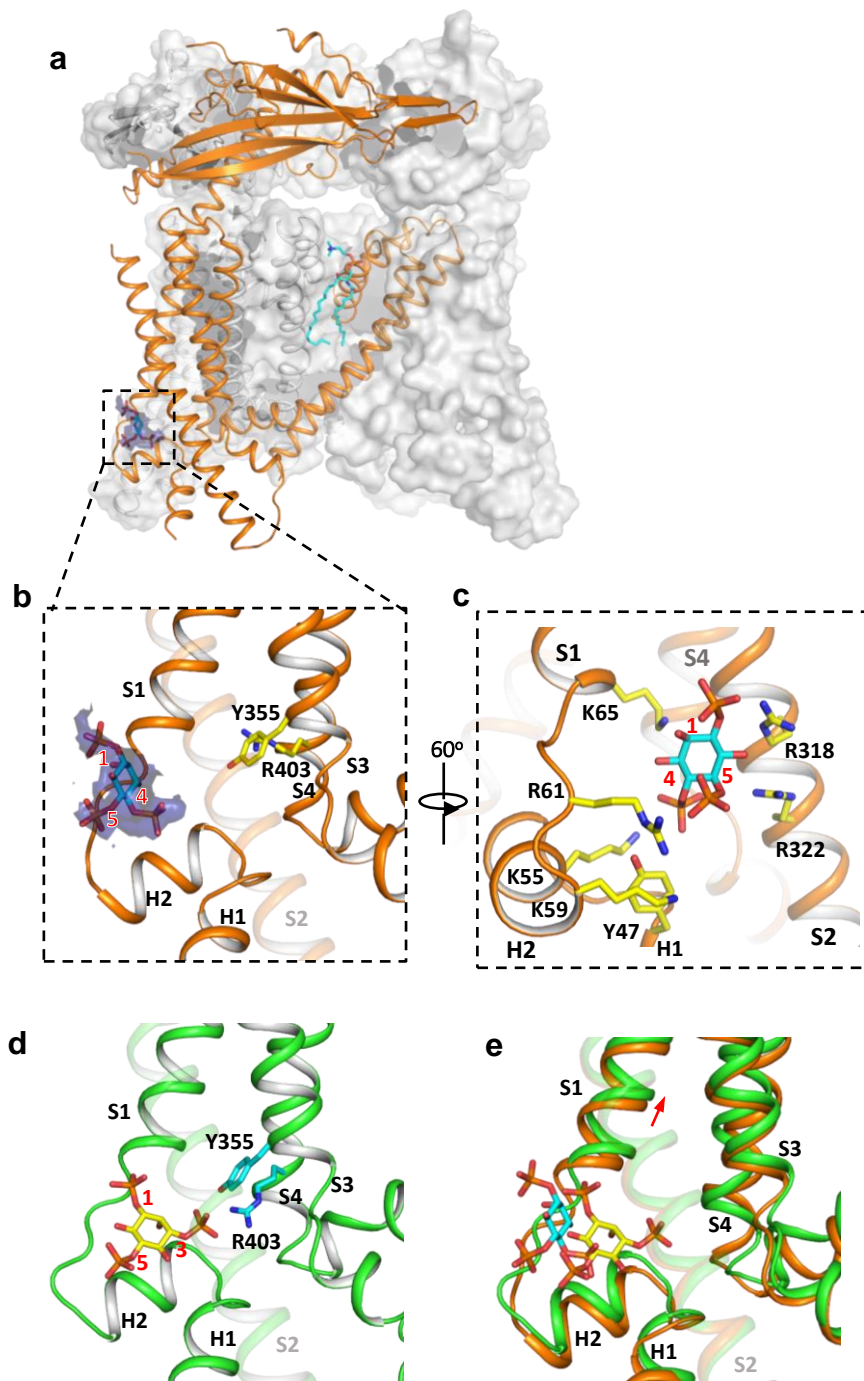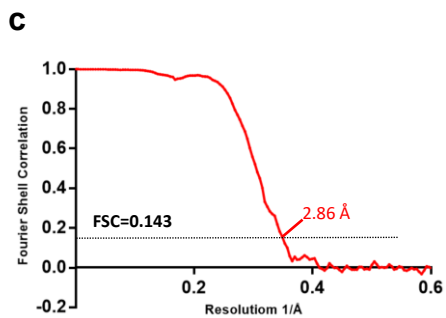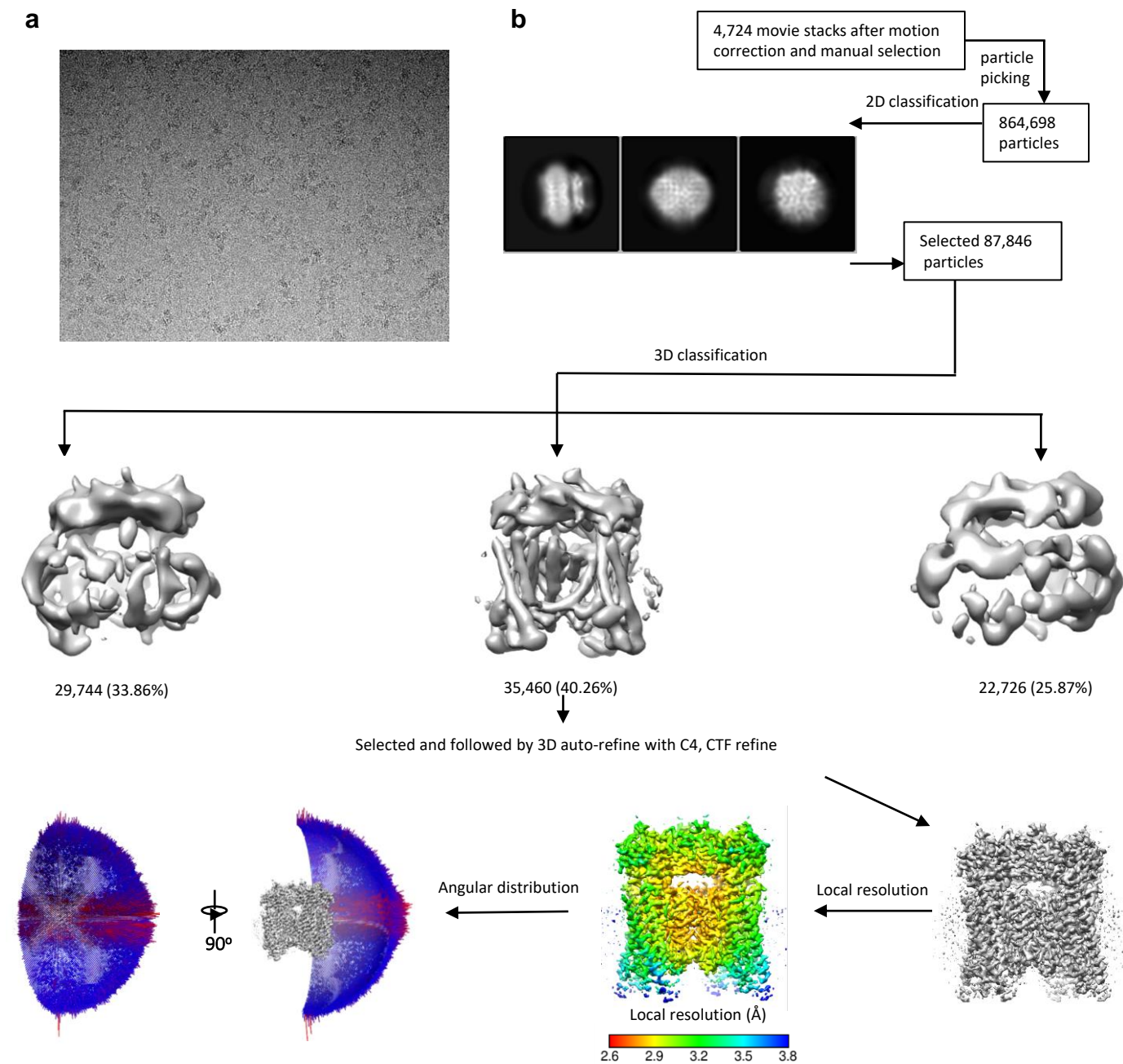
Figure 3. Structure of TRPML1 in complex with PI(4,5)P$_2$. (a) Overall structure of PI(4,5)P$_2$-bound TRPML1 with the front subunit shown in orange cartoon and the rest shown as grey surface representation. Density for PI(4,5)P$_2$ head group is shown in blue surface. (b) Zoomed-in view of the PI(4,5)P$_2$-binding pocket with the density of its IP3 head group shown in blue surface. (c) Zoomed-in view of the PI(4,5)P$_2$-binding pocket with side chains of IP3-interacting residues shown as yellow sticks. (d) Zoomed-in view of the IP3 position in the PI(3,5)P$_2$-bound open TRPML1 structure. The C3 phosphate group directly interacts with Y355 and R403. (e) Comparison of the head group positions in PI(3,5)P$_2$-bound open (green) and PI(4,5)P$_2$-bound closed (orange) structures. The inositol rings PI(3,5)P$_2$ and PI(4,5)P$_2$ are colored yellow and cyan, respectively. The red arrow marks the upward movement of S1 from closed to open conformation.
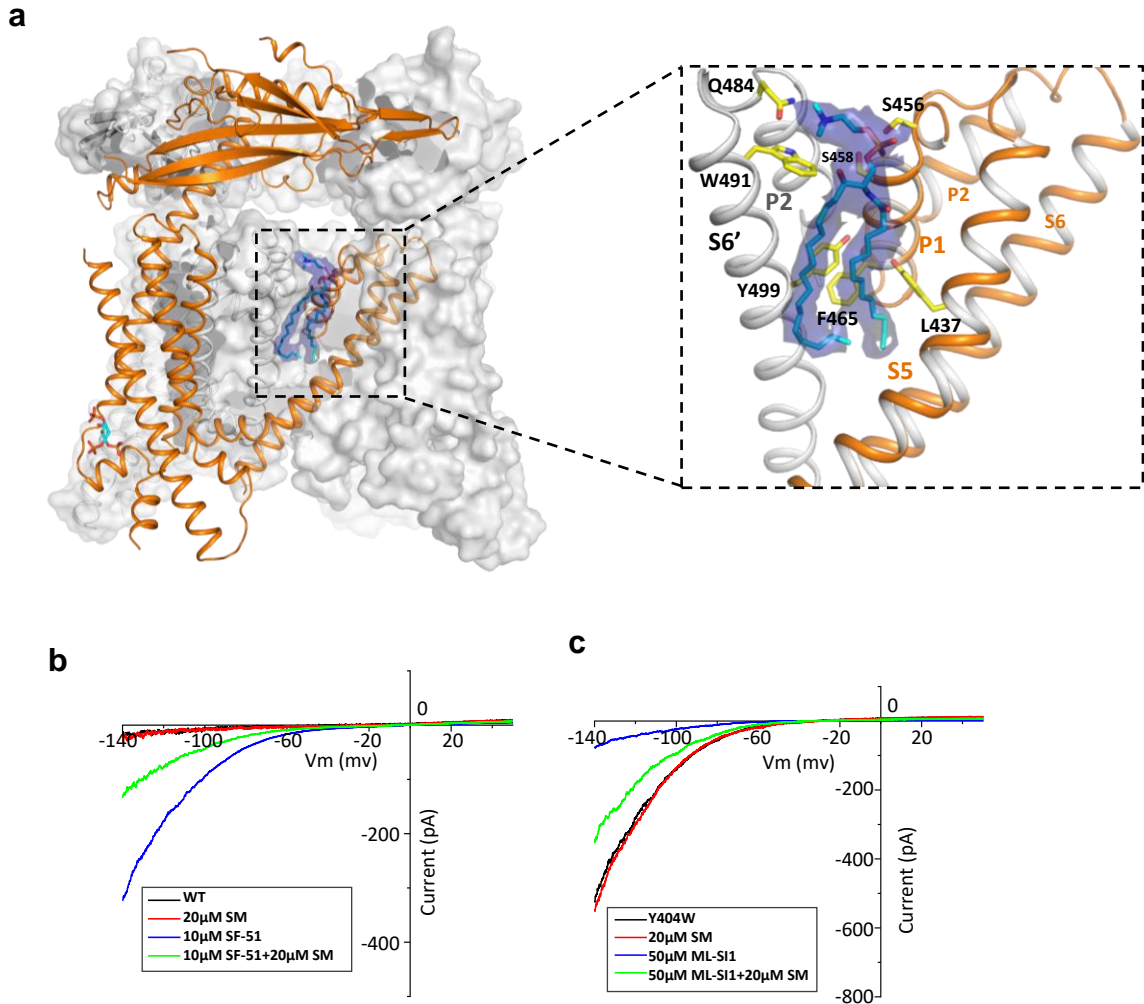
Figure 4. Sphingomyelin binding in TRPML1. (a) Overall structure of PI(4,5)P$_2$-bound TRPML1 and the zoomed-in view of the lipid-binding site. The lipid density is shown as blue surface and modeled as sphingomyelin (SM). The side chains of lipid-interacting residues are shown as yellow sticks. (b) SM inhibition effect on SF-51-activated wild-type TRPML1. (c) SM activation effect on ML-SI1-inhibited Y404W mutant. Currents shown in (b) and (c) were recorded using patch clamp in whole-cell configuration with pH 4.6 in the bath solution as the adverse effect of SM on agonist or antagonist is subtle and is measurable only at low luminal pH.
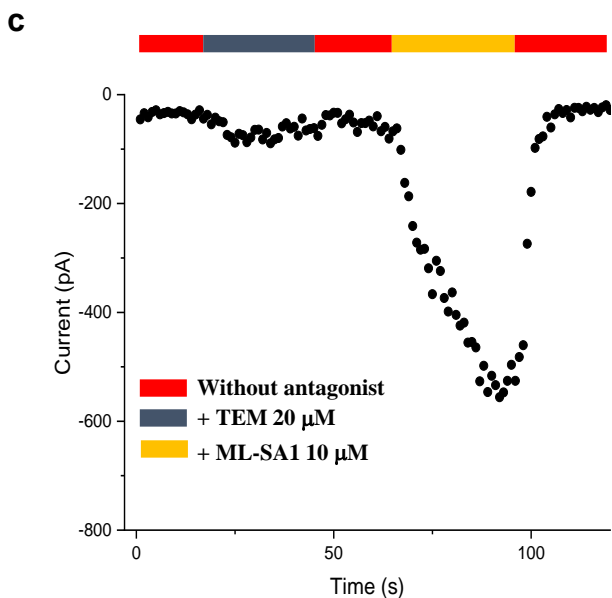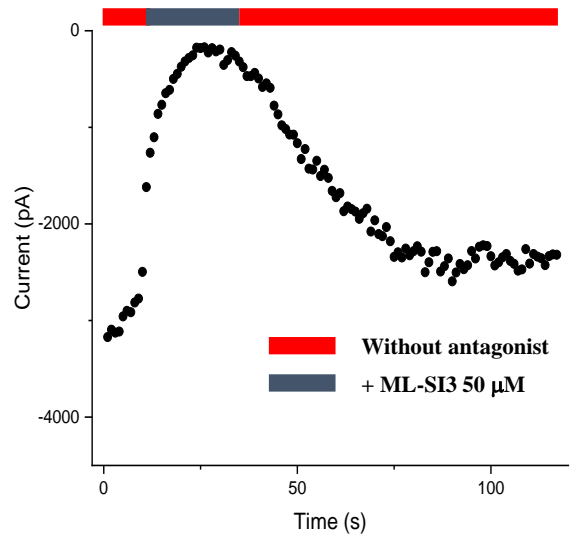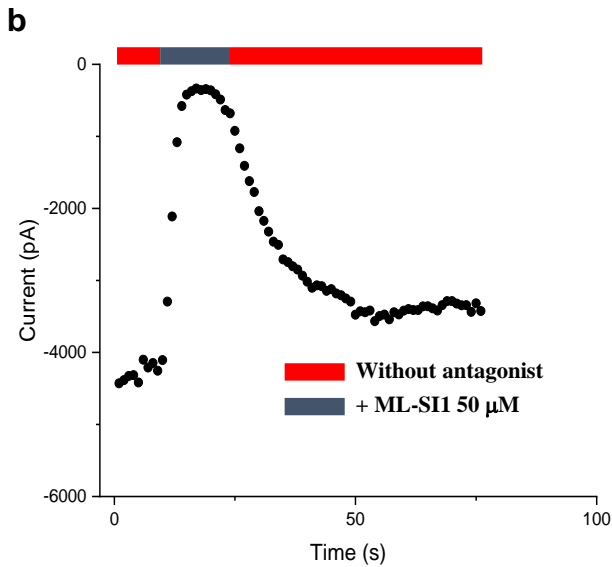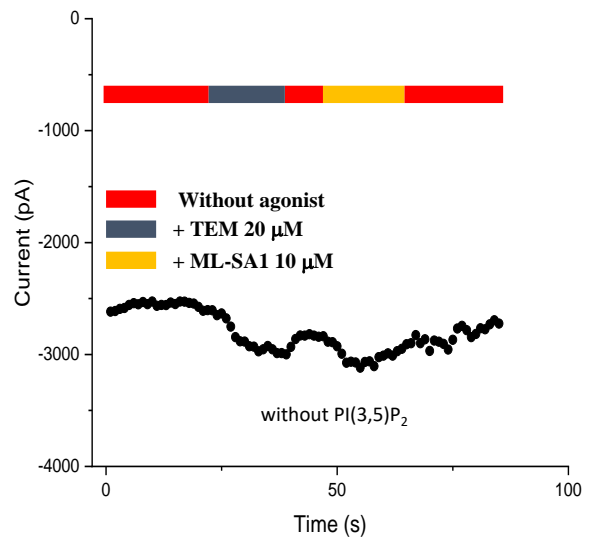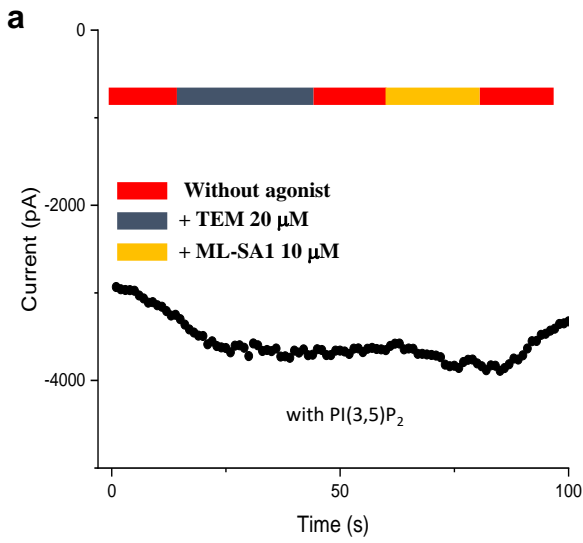
Figure supplement 1: Time course plots of current amplitudes of Y404 mutations recorded at -140mV with symmetrical pH of 7.4. (a) Time course plots of Y404W recorded using patch clamp in whole-cell configuration with (left) or without (right) 100 µM PI(3,5)P$_2$ in the pipette (cytosolic). Tem or agonist ML-SA1 was introduced in the bath solution (extracellular/luminal). (b) Time course plots of Y404W inhibition by antagonists ML-SI1 (left) and ML-SI3 (right) recorded using patch clamp in whole-cell configuration. The antagonists were introduced in the bath solution (extracellular/luminal). (c) Time course plots of Y404A with 100 µM PI(3,5)P$_2$ in the pipette (cytosolic). Tem or ML-SA1 was introduced in the bath solution (extracellular/luminal).

a

b

4,724 movie stacks after motion correction and manual selection

particle picking

864,698 particles

2D classification

Selected 87,846 particles

3D classification

29,744 (33.86%)

35,460 (40.26%)

22,726 (25.87%)

Selected and followed by 3D auto-refine with C4, CTF refine

90º

Angular distribution

Local resolution (Å)

2.6   2.9   3.2   3.5   3.8

Local resolution

c

FSC=0.143

2.86 Å

Figure supplement 2: Cryo-EM data processing scheme of the TRPML1 Y404W. (a) Representative micrograph. (b) Flow chart of the cryo-EM data processing procedure and the Euler angle distribution of particles used in the final three-dimensional reconstruction. Selected 2D class averages are shown. The final structure represent an open state. (c) Fourier Shell Correlation curves showing the overall resolution at FSC=0.143.

a

H1&H2
(40-60)

S1
(63-88)

Luminal domain
(89-140, α1 α2)

S2 (296-337)

S3 (349-377)

S4
(383-407)

S4-S5 linker
(408-424)

S5
(425-448)

Pore helices and
filter (457-486)

S6 (487-527)

b

Distance along the pore (Å)

Pore radius (Å)

Filter

Central
cavity

Lower gate

G470

I514

T518

Apo closed
Y404W open
PI(3,5)P$_2$/Tem-bound open
PI(4,5)P$_2$-bound closed

c

S1
S3
S4
Q69
L66
L358
K65
W404
R403
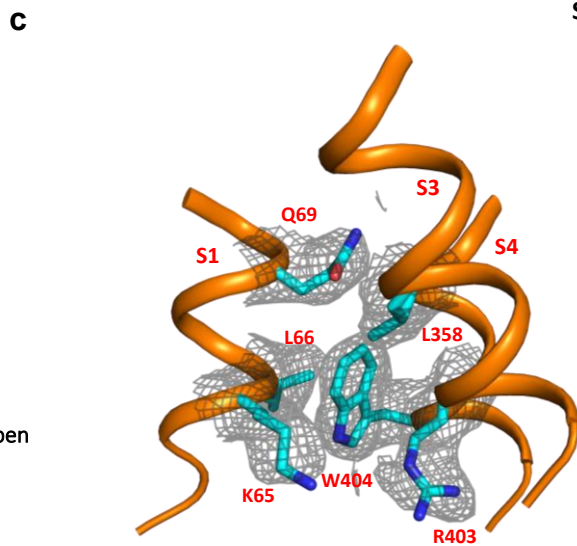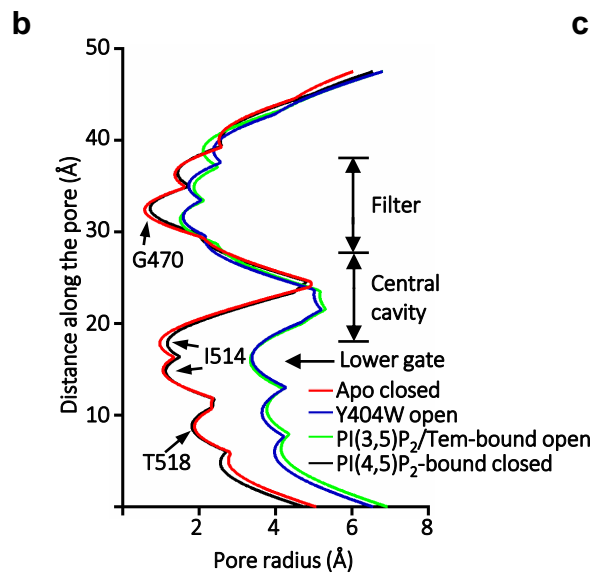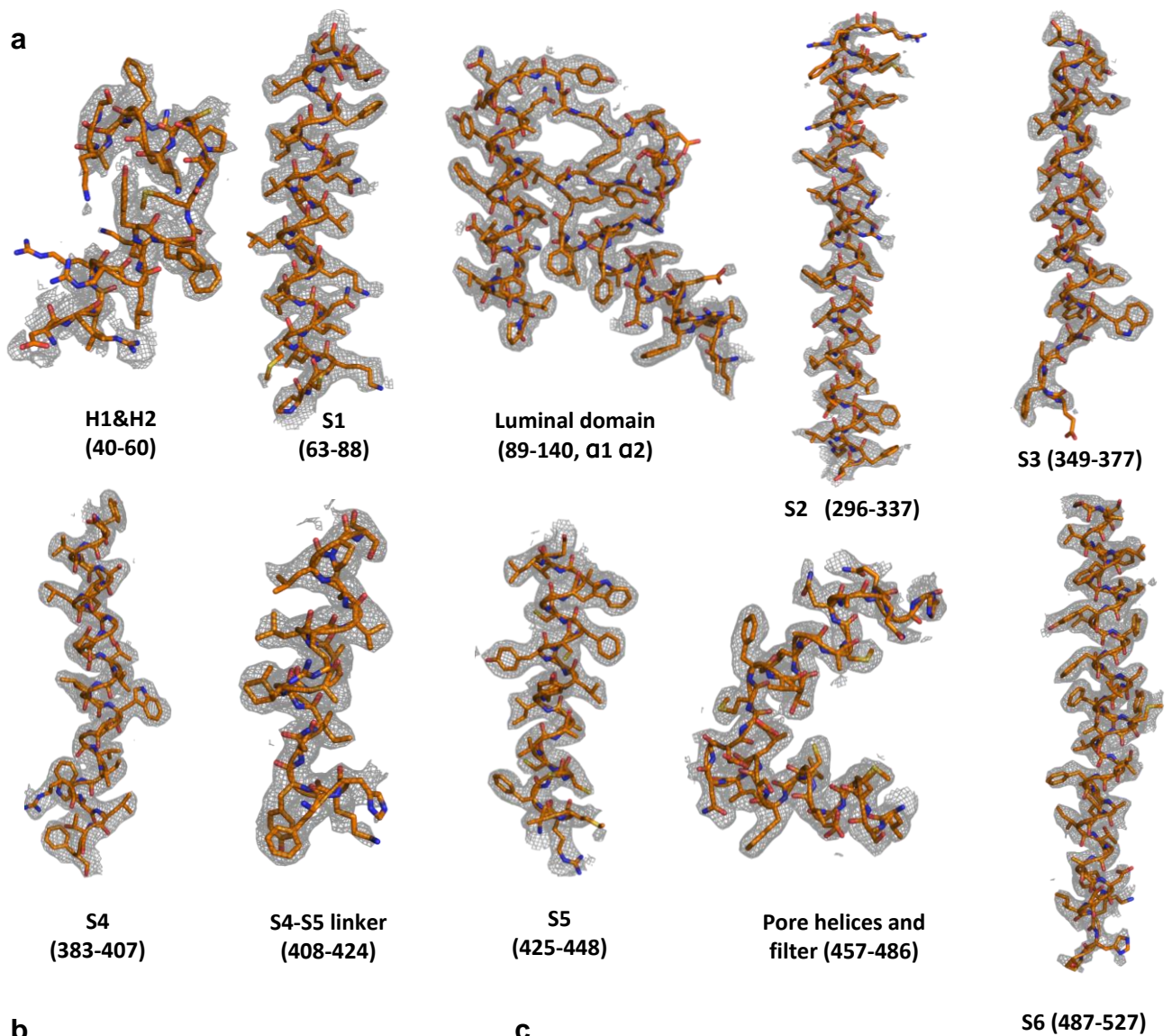
Figure supplement 3: Sample density maps of Y404W and pore radius: (a) Sample density maps of the Y404W open TRPML1 structure contoured at 4 σ. (b) Pore radius along the central axis in the open and closed states. PDB codes for apo closed and PI(3,5)P$_2$/Tem-bound open are 7SQ8 and 7SQ9, respectively. (c) EM density map surrounding W404 region shown in grey mesh and contoured at 4σ, key W404-interacting residues are shown in cyan. The local resolution of this region is 3.2 Å.

a

b

8,164 movie stacks after motion correction and manual selection

particle picking

1,065,778 particles

2D classification

Selected 555,281 particles

3D classification

135,321 (24.37%)   114,277 (20.58%)   164,307 (29.59%)   31,651 (5.70%)   109,667 (19.75%)

Selected and followed by 3D auto-refine with C4 and 3D classification without alignment

20,452 (5.69%)   18,151 (5.05%)   25,987 (7.23%)   60,597 (16.93%)   233,996 (65.10%)

Selected 3D auto-refine with C2, CTF refine and polish

Angular distribution

Local resolution

Local resolution (Å)

2.3   2.5   2.7   2.9   3.1

90°

c

FSC=0.143

2.46 Å

Fourier Shell Correlation

Resolutiom 1/A

Figure supplement 4: Cryo-EM data processing scheme of the TRPML1 sample prepared in the presence of $PI(4,5)P_2$. (a) Representative micrograph. (b) Flow chart of the cryo-EM data processing procedure and the Euler angle distribution of particles used in the final three-dimensional reconstruction. Selected 2D class averages are shown. The final structure represent an open state. (c) Fourier Shell Correlation curves showing the overall resolution at FSC=0.143.
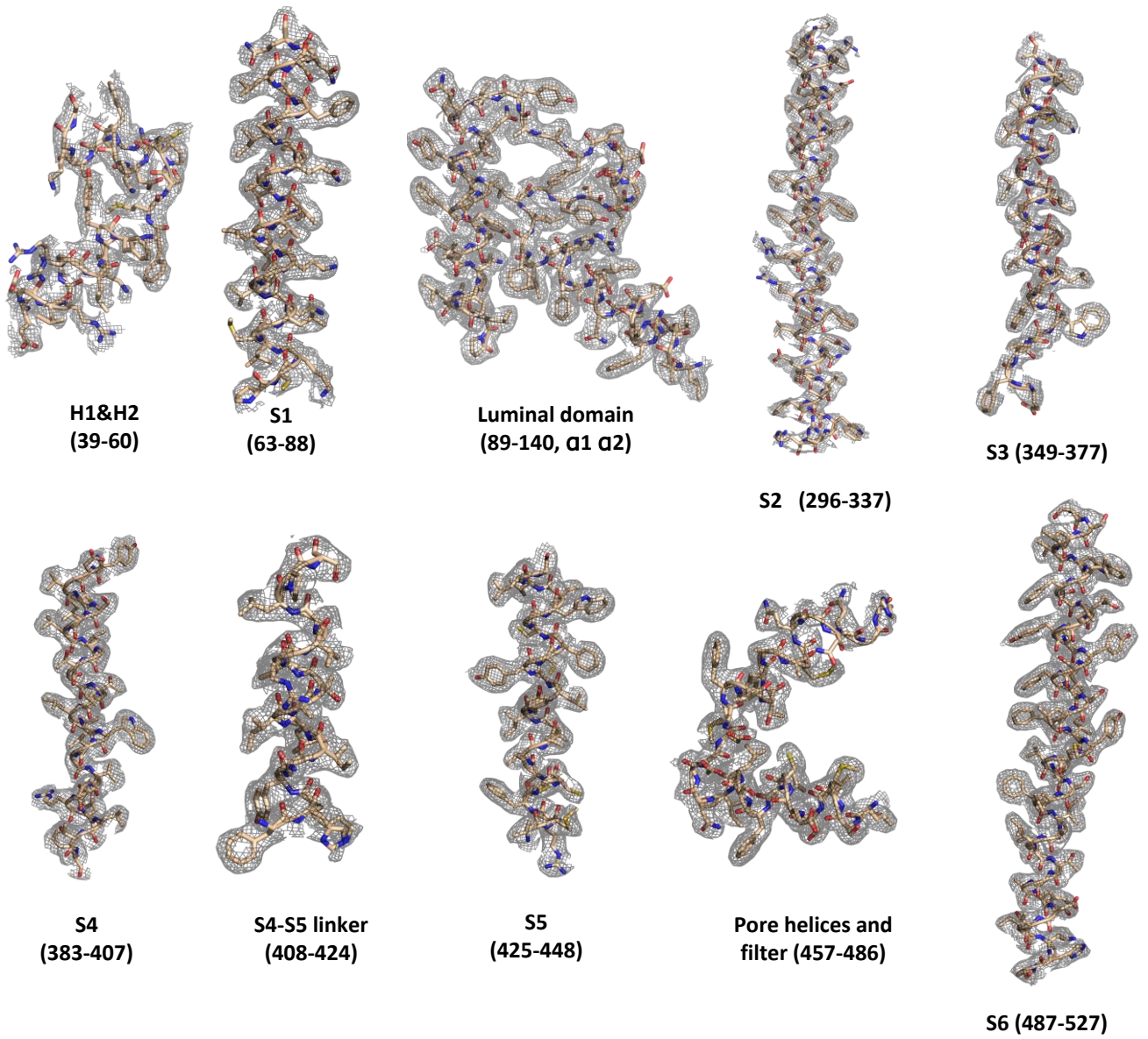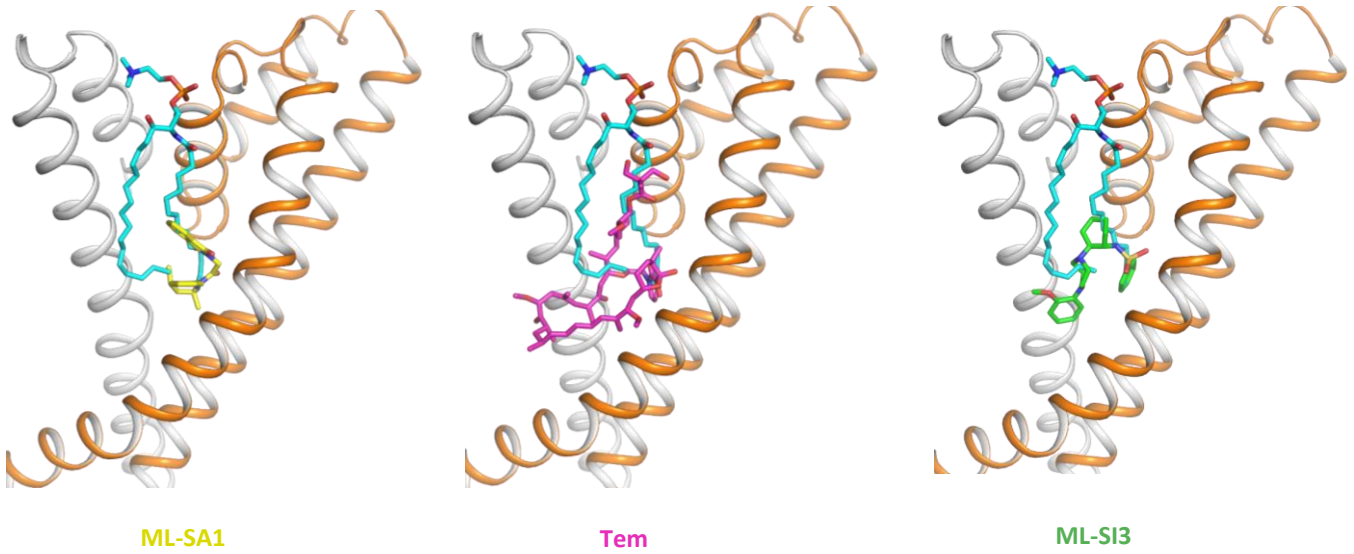
**H1&H2
(39-60)**

**S1
(63-88)**

**Luminal domain
(89-140, α1 α2)**

S2   (296-337)

S3 (349-377)

**S4
(383-407)**

**S4-S5 linker
(408-424)**

**S5
(425-448)**

**Pore helices and
filter (457-486)**

S6 (487-527)

Figure supplement 5 : Sample density maps of the PI(4,5)P$_2$-bound closed TRPML1 structure contoured at 4 σ.

Figure supplement 6: Sphingomyelin (cyan) binding overlaps with that of agonist ML-SA1 (yellow), rapamycin analog Tem (magenta), or antagonist ML-SI3 (green).
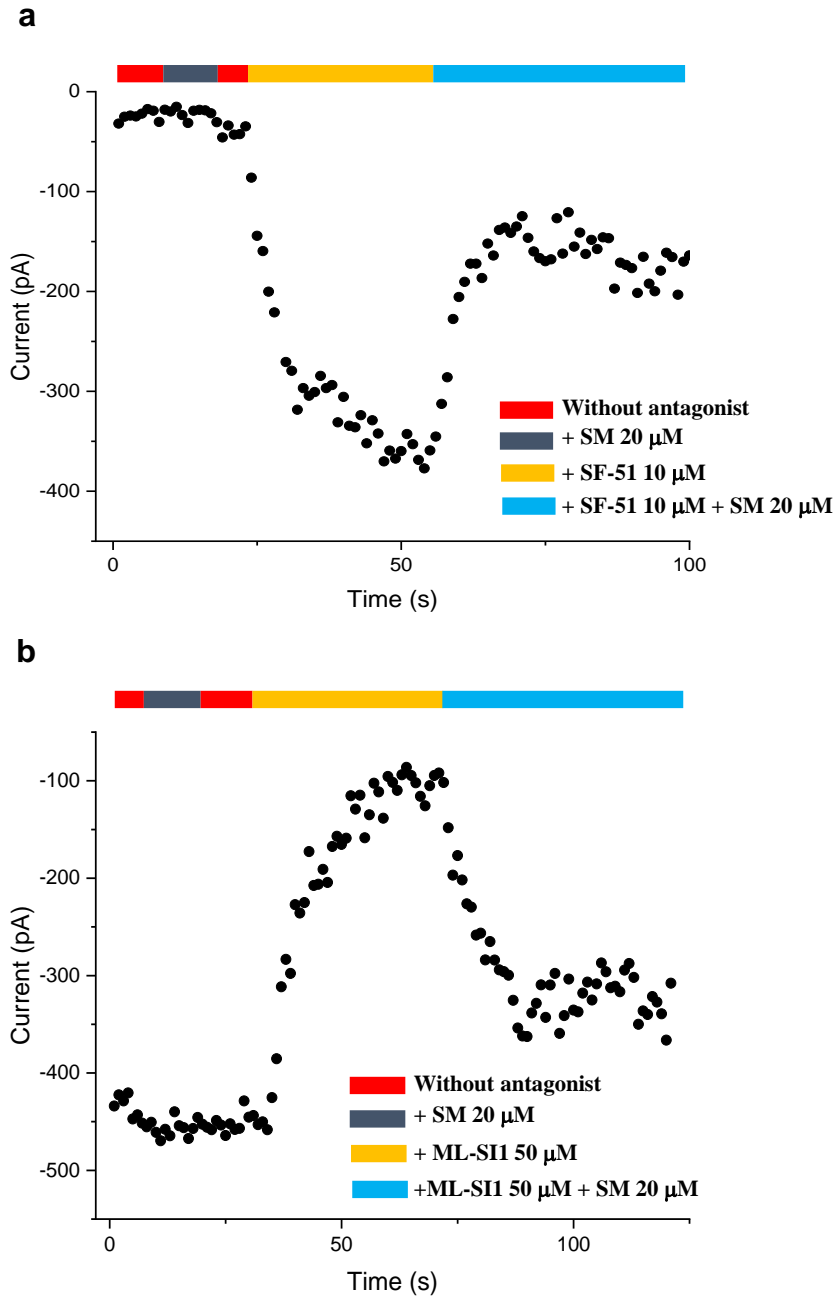
Figure supplement 7: Time course plots of sphingomyelin affected TRPML1 current amplitudes. (a) Sphingomyelin inhibition effect on SF-51-activated wild-type TRPML1. (b) SM activation effect on ML-SI1-inhibited Y404W mutant. Currents shown in (a) and (b) were recorded at -140mV using patch clamp in whole-cell configuration with pH 4.6 in the bath solution as the adverse effect of SM on agonist or antagonist is subtle and is measurable only at low luminal pH.

Movie supplement 1. Conformational changes between open and closed TRPML1