Methods

afTMEM16 Expression and Purification

afTMEM16 was expressed and purified as described [1]. Briefly, *S. cerevisiae* transformed with the pDDGFP2 vector[2] containing the afTMEM16 gene were grown in yeast synthetic drop-out medium supplemented with Uracil (CSM-URA; MP Biomedicals) and expression was induced with 2% (w/v) galactose at 30°C for 22 hours. Cells were collected, snap frozen in liquid nitrogen, lysed by cryomilling (Retsch model MM400) in liquid nitrogen (3 × 3 min, 25 Hz), and resuspended 150 mM KCl, 10% (w/v) glycerol, 50 mM Tris-HCl, pH8 supplemented with 1 mM EDTA, 5 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin, 100 μM phenylmethane sulphonylfluoride and protease inhibitor cocktail tablets (Roche). Protein was extracted using 1% (w/v) digitonin (EMD biosciences) at 4 °C for 2 hours and the lysate was cleared by centrifugation at 40,000 g for 45 minutes. The supernatant was supplemented with 1 mM $MgCl_2$ and 10 mM Imidazole, loaded onto a column of Ni-NTA agarose resin (Qiagen), washed with 150 mM KCl, 10% (w/v) glycerol, 50 mM Tris-HCl, pH 8 + 30 mM Imidazole and 0.12% digitonin, and eluted with 150 mM KCl, 10% (w/v) glycerol, 50 mM Tris-HCl, pH8 + 300 mM Imidazole and 0.12% digitonin. The elution was treated with Tobacco Etch Virus protease overnight to remove the His tag and then further purified on a Superdex 200 10/300 GL column equilibrated with buffer A supplemented with 0.12% digitonin (GE Lifesciences). The afTMEM16 protein peak was collected and concentrated using a 50 kDa molecular weight cut off concentrator (Amicon Ultra, Millipore).

Liposome reconstitution

Liposomes were prepared as described [1]. Briefly lipids in chloroform (Avanti), including 0.4% w/w tail labeled NBD-PE, were dried under N_2, washed with pentane and resuspended at 20 mg ml^{-1} in 150 mM KCl, 50 mM HEPES pH 7.4 with 35 mM 3-[(3-cholamidopropyl)dimethylammonio]-1- propanesulfonate (CHAPS). All proteins were added at 5 μg protein/mg lipids and detergent was removed using five changes of 150 mg ml^{-1} Bio-Beads SM-2 (Bio-Rad) with rotation at 4 °C. Calcium or EGTA were introduced using sonicate, freeze-thaw cycles. Chain length experiments were done in the background of 7PC:3PG due to the availability of the long chain lipids. Lipids used were: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 14:0), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG, 14:0), 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine (16:1), 1,2-dipalmitoleoyl-sn-glycero-3-

(1'-rac-glycerol) (16:1) 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, 16:0-18:1), POPG (16:0-18:1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG, 18:1), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (20:1), 1,2-dieicosenoyl-sn-glycero-3-(1'-rac-glycerol) (20:1), 1,2-dierucoyl-sn-glycero-3-phosphocholine (DEPC, 22:1) and 1,2- dierucoyl-phosphatidylglycerol (DEPG, 22:1) .

Scrambling Assay

The scrambling assay was carried out as described [3]. Liposomes were extruded through a 400 nm membrane and 20 µl were added to a final volume of 2 mL of 50 mM HEPES pH 7.4, 300 mL KCl + 0.5 mM $Ca(NO_3)_2$ or 2 mM EGTA. The fluorescence intensity of the NBD (excitation-470 nm emission-530 nm) was monitored over time with mixing in a PTI spectrophotometer and after 100 s sodium dithionite was added at a final concentration of 40 mM. Data was collected using the FelixGX 4.1.0 software at a sampling rate of 3 Hz.

Quantification of scrambling activity

Quantification of the scrambling rate constants by afTMEM16 was determined as described [3,4]. Briefly, the fluorescence time course was fit to the following equation

$$F_{tot}(t) = f_0(L_i^{PF} + (1 - L_i^{PF})e^{-\gamma t}) + \frac{(1-f_0)}{D(\alpha+\beta)}\{\alpha(\lambda_2+\gamma)(\lambda_1+\alpha+\beta)e^{\lambda_1 t} + \lambda_1\beta(\lambda_2+\alpha+\beta+\gamma)e^{\lambda_2 t}\} \quad (1)$$

Where $F_{tot}(t)$ is the total fluorescence at time t, L_i^{PF} is the fraction of NBD-labeled lipids in the inner leaflet of protein free liposomes, where γ is the rate constant of dithionite reduction, f_0 is the fraction of protein-free liposomes in the sample, α and β are respectively the forward and backward scrambling rate constants and

$$\lambda_1 = -\frac{(\alpha+\beta+\gamma)-\sqrt{(\alpha+\beta+\gamma)^2-4\alpha\gamma}}{2} \quad \lambda_2 = -\frac{(\alpha+\beta+\gamma)+\sqrt{(\alpha+\beta+\gamma)^2-4\alpha\gamma}}{2}$$

$$D = (\lambda_1+\alpha)(\lambda_2+\beta+\gamma) - \alpha\beta$$

The free parameters of the fit are f_0, α and β while L_i^{PF} and γ are experimentally determined from experiments on protein-free liposomes. In protein-free vesicles a very slow fluorescence decay is visible, likely reflecting a slow leakage of dithionite into the vesicles or the spontaneous flipping of the NBD-labeled lipids. A linear fit was used to estimate that the rate of this process is $L=(5.4\pm1.6)10^{-5}$ s^{-1} (n>160). For wiltype and mutant afTMEM16 the leak is >2 orders of

magnitude smaller than the rate constant of protein-mediated scrambling and therefore is negligible. All conditions were tested side by side with a control preparation. In some rare cases this control sample behaved anomalously, judged by scrambling fit parameters outside 3 times the standard deviation of the mean for the wildtype. In these cases the whole batch of experiments was disregarded.

MSP1E3/MSP2N2 Purification and Nanodisc Reconstitution

MSP1E3 and MSP2N2 was expressed and purified as described [5]. Briefly, MSP1E3 in a pET vector (Addgene #20064) was transformed into the BL21-Gold (DE3) strain (Stratagene). Transformed cells were grown in LB media supplemented with Kanamycin (50 mg l^{-1}) to an OD_{600} of 0.8 and expression was induced with 1 mM IPTG for 3 hrs. Cells were harvested and resuspended in 40 mM Tris-HCl pH 78.0, 300 mM NaCl supplemented with 1% Triton X-100, 5 µg ml^{-1} leupeptin, 2 µg ml^{-1} pepstatin, 100 µM phenylmethane sulphonylfluoride and protease inhibitor cocktail tablets (Roche). Cells were lysed by sonication and the lysate was cleared by centrifugation at 30,000 g for 45 min at 4° C. The lysate was incubated with Ni-NTA agarose resin for one hour at 4 °C followed by sequential washes with: 40 mM Tris-HCl pH 78.0, 300 mM NaCl + 1% triton-100, + 50 mM sodium cholate, + 20 mM imidazole and + 50 mM imidazole. The protein was eluted with buffer C + 400 mM imidazole, desalted using a PD-10 desalting column (GE life science) equilibrated with 150 mM KCl, 50 mM Tris pH 8.0 supplemented with 0.5 mM EDTA. The final protein was concentrated to ~8 mg ml^{-1} using a 30 kDa molecular weight cut off concentrator (Amicon Ultra, Millipore), flash frozen and stored at -80 °C.

Reconstitution of afTMEM16 in nanodiscs was carried out as described [6]. Lipids in chloroform (Avanti) were dried under N_2, washed with pentane and resuspended in buffer D and 40 mM sodium cholate (Anatrace) at a final concentration of 20 mM. Molar ratios 1:0.8:50 MSP1E3:afTMEM16:lipids and 1:0.8:140 for MSP2N2:afTMEM16:lipids were mixed at a final lipid concentration of 7 mM and incubated at room temperature for 20 minutes. Detergent removal was carried out at 4ºC via incubation with Bio-Beads SM-2 (Bio-Rad) with agitation for two hours and then overnight with fresh Bio-Beads SM2 at a concentration of 200 mg ml^{-1}. The reconstitution mixture was purified using a Superose6 Increase 10/300 GL column (GE Lifesciences) pre-equilibrated with 50 mM HEPES pH 8.0 150 mM KCl plus 5 mM EDTA or 0.5 mM $CaCl_2$ and

the peak corresponding to afTMEM16-containing nanodiscs was collected for cryo electron microscopy analysis.

Atomic Force Microscopy

Sample preparation

Large unilamellar vesicles (LUVs) with concentration 0.01mg/ml were deposited on freshly cleaved mica for 20 mins, followed by careful rinsing with imaging buffer (50 mM HEPES, pH 7.4, 300 mM KCl). The sample was kept in imaging buffer for all AFM measurements.

Imaging and mechanical measurements

All AFM measurements were performed using a JPK Nanowizard 4 AFM (Bruker, Berlin, Germany). Images were acquired in tapping mode by using cantilevers (FastScan D, Bruker) with a normal spring constant of ~0.25 N/m, and a resonant frequency of ~110 kHZ in liquid. The sensitivity of cantilevers was measured on mica and the spring constant were calibrated by using thermal tune methods [7]. Force-distance curves were acquired at 500 nm/s.

Data processing

All the data analysis were performed using JPK data processing software. The Young's modulus of the supported lipid bilayers was derived by fitting the force distance curves with the following model [8, 9] $F = \frac{E}{1-\nu^2}\frac{2\tan\alpha}{\pi}\delta^2$ (1),

where F is the Force, E is the Young's modulus, n is the Poisson's ratio, d the indentation (vertical tip position), and a is the half cone angle or half face angle of the pyramidal tip shape.

The area stretch modulus (k_A) and bending stiffness (k_c) of the supported lipid bilayers were calculated based on the measured Young's modulus (E) and membrane thickness (h) using the following equations (thePoisson's ratio n is assumed 0.5 [10]):

$$k_A = \frac{Eh}{1-\nu^2} \quad (2)$$

$$k_c = \frac{Eh^3}{24(1-\nu^2)} \quad (3)$$

Grid preparation

3.5-5 uL of afTMEM16-containing nanodiscs (2-7mg mL^{-1}) supplemented with 1.5 or 3 mM Fos-Choline-8-Fluorinated (Anatrace) was applied to a glow-discharged UltrAuFoil R1.2/1.3 300-mesh gold grid (Quantifoil) and incubated for one minute under 100% humidity at 15°C. Following

incubation, grids were blotted for 2 s and plunge frozen in liquid ethane using a Vitrobot Mark IV (FEI).

Electron Microscopy Data Collection

	C18/Ca^{2+}	C18/0 Ca^{2+}	C14/0 Ca^{2+}	C22/Ca^{2+} (MSP1E3)	C22/Ca^{2+} (MSP2N2)	DE/AA C14/Ca^{2+}
Microscope/camera	Krios/K3	Krios/K3	Krios/K2	Krios/K3	Krios/K3	Krios/k3
Acquisition	SerialEM	Leginon	Leginon	SerialEM	SerialEM	SerialEM
Accelerating Voltage (kV)	300	300	300	300	300	300
Number of frames	30	40	50	30	30	30
Dose (e-/ $Å^2$)	42.8227	59.13	71.68	42.7884	44.4	42.18
Defocus range	-0.5-2.3	-1.5-2.0	-1.5 -2.3	-0.5-2.3	-0.5-2.3	-0.5-2.3
Exposure time	2.6964	2.80	10	2.3968	2.6964	1.65
Pixel Size	0.53 (super resolution)	0.426 (super resolution)	1.06 (counting)	0.53 (super resolution)	0.53 (super resolution)	0.53 (super resolution)
Energy Filter	Yes (20 eV)	N/A	Yes (20 eV)	Yes (20 eV)	Yes (20 eV)	Yes (20 eV)

Table 2: Electron Microscopy data collection parameters

Image Processing

Data collection was carried out for each structure using the parameters specified in Table 2. Image analysis was carried out using Relion 3.0 or 3.1 beta [11]. Motion correction was carried out using the Relion implementation of MotionCorr2 [11] and contrast transfer function (CTF) estimation was performed using CTFFIND4 [12] via Relion. For all datasets except the wildtype afTMEM16 in 50% 14:0 lipids, 2x binning was used for motion correction. All refinements were run in Relion initially without a mask, and the converged refinement was continued with a mask excluding the nanodisc or micelle. The final resolution of all maps was determined by applying a soft mask around the protein and the gold-standard Fourier shell correlation (FSC) = 0.143 criterion using Relion PostProccessing. Relion was used to estimate the local resolution for all final maps. Processing strategy for the various datasets differed; details are described below.

Processing of dataset for afTMEM16 in 50% C14 lipids in 0 Ca^{2+}

7,432 micrographs were collected and following manual inspection, 5,253 were included for analysis with Relion 3.0. Following motion correction and CTF estimation, auto-picking was carried out using a 3D volume of the afTMEM16/nanodisc complex low pass filtered to 30 Å and

yielded 1,728,771 particles. Particles were extracted using a box size of 271 Å with 2x binning and subjected to two rounds of 2D classification. 739,898 particles with structural features resembling the afTMEM16-nanodisc complex were selected and subjected to 3D classification without symmetry. 567,785 particles from well-defined 3D classes were extracted without binning and subjected 3D auto-refinement, which resulted in a 3.7 Å reconstruction. Several rounds of CTF refinement [11] and Bayesian polishing [13] followed by refinement and 3D classification with and without particle alignment were carried out, in which particles not resembling afTMEM16/nanodisc complex were discarded, resulting in a subset of 334,014 particles. This subset was used for extensive classification to identify alternate conformations described below. After no different conformations were identified, additional rounds of 3D classification without alignment and a mask excluding the nanodisc were used to improve the resolution, resulting in a final subset of 244,507 particles. Additional rounds of CTF refinement and Bayesian polishing led to a masked reconstruction with a resolution 3.4 Å, which was C2 symmetric. This initial map (without application of C2 symmetry) was used to analyze the effects on the membrane. Additional rounds of refinement were carried out with C2 symmetry applied resulting in a final reconstruction of 3.3 Å which was used for model building.

Processing of dataset for afTMEM16 in C22 lipids in MSP1E3 nanodiscs in 0.5 mM Ca^{2+}

9,395 micrographs were collected and after manual inspection 7,157 were included for analysis in Relion 3.0 and 3.1 beta. Following motion correction with 2x binning (pixel size 1.06) and CTF estimation, auto-picking was carried out using a 3D volume of TMEM16/nanodisc complex low pass filtered to 30 Å and picked 2,643,590 particles. These particles were extracted using a box size of 271 Å with 4x binning (pixel size 2.12) and subjected to two rounds of 2D classification. 1,923,936 particles with structural features resembling the afTMEM16-nanodisc complex were selected and subjected to 3D classification without symmetry. 1,094,546 particles from well-defined 3D classes were extracted with 2x binning (pixel size 1.06) and subjected to several rounds of CTF refinement [11] and Bayesian polishing [13] followed by refinement and 3D classification with and without particle alignment, in which particles not resembling afTMEM16/nanodisc complex were discarded, resulting in a subset of 538,468 particles. This subset was used for extensive classification to identify alternate conformations described below, with the exception that classification on monomers was carried out on the reduced subset of 245,824 particles (see below).

No attempts to find alternate conformations were successful. To improve the resolution, several additional rounds of 3D classification without alignment and CTF refinement and Bayesian polishing were carried out, leading to a subset of 245,824 particles. Using the Bayesian polishing job in Relion 3.1, these particles were further un-binned to a pixel size of 0.7066 (~1.3x binning from the micrographs). After additional rounds of 3D classification without alignment and CTF refinement and Bayesian polishing, a final subset of 132,332 particles were selected which resulted in a 2.76 Å reconstruction. The protein was not centered in the nanodisc and density for TM6 was missing from one monomer as previously described [6]. Signal subtraction of the nanodisc density resulted a symmetric reconstruction and additional rounds of refinements with imposed C2 symmetry were carried out, resulting in a final 2.7 Å reconstruction, which was used for model building. The C1 reconstruction prior to signal subtraction was used to analyze the effects on the membrane. Signal subtraction and symmetry expansion were also carried out on the 2x binned particles (pixel size 1.06) and the described classification of the monomers (below) was carried out on these particles.

Processing of dataset for afTMEM16 in C22 lipids in MSP2N2 nanodiscs in 0.5 Ca^{2+}

7,915 micrographs were collected and after manual inspection 5,805 were included for analysis in Relion3.0 and cryoSPARC [11,14]. Following motion correction with 2x binning (pixel size 1.06) and CTF estimation, Laplacian of gaussian auto-picking with conservative parameters was carried out [11]. 447,947 particles were extracted with a box size of 271 Å with 2x binning (pixel size 1.06) and subjected to two rounds of 2D classification to generate templates for auto-picking. 1,641,081 particles picked with Template-based auto-picking were extracted with a box size of 305 Å and 4x binning (pixel size 2.12) and subjected to three rounds of 2D classification. 1,168,291 particles with features of the afTMME16/nanodisc complex were extracted with a box size of 305 Å and 2x binning (pixel size 1.06) and were imported into cryoSPARC and subjected to heterogenous refinement with forced classification with one model resembling the afTMEM16/nanodisc complex and two models resembling empty nanodiscs [14]. 208,670 particles from the class resembling the afTMEM16/nanodisc complex were subjected to several rounds of *Ab-initio* reconstruction with two classes in which particles that did not lead to models resembling the afTMEM16/nanodisc complex were discarded. A final subset of 31,890 particles resulted in a 4 Å reconstruction via non-uniform refinement. These particles were brought to Relion 3.0 using the

csparc2star.py script [15] and subjected to auto-refinement with local angular searches without symmetry. After three rounds of CTF refinement [11] and Bayesian polishing [13], these particles refined to 3.7 Å. As in the structure of afTMEM16 in 22:1 lipids in MSP1E3 nanodiscs, the protein was not centered in the nanodisc and density for TM6 was missing from one monomer. Signal subtraction of the nanodisc density was performed in Relion and the subtracted particles were imported into cryoSPARC and subjected to non-uniform refinement with C2 symmetry enforced, resulting in a 3.5 Å reconstruction, which was used for model building. The C1 reconstruction prior to signal subtraction was used to analyze the effects on the membrane.

Processing of dataset for afTMEM16 in C18 lipids in MSP1E3 nanodiscs in 0.5 mM Ca^{2+}

8,891 micrographs were collected and after manual inspection 6,336 were included for analysis in Relion 3.0 and 3.1 beta. Following motion correction with 2x binning (pixel size 1.06) and CTF estimation, auto-picking was carried out using a 3D volume of the afTMEM16/nanodisc complex low pass filtered to 30 Å and picked 4,551,732 particles. These particles were extracted with a box size of 271 Å with 4x binning (pixel size 2.12) and subjected to two rounds of 2D classification from which 2,645,466 particles displayed afTMEM16/nanodisc complex features and were subjected to two rounds of 3D classification. 1,601,172 particles from classes resembling the afTMEM16/nanodisc complex were extracted with 2x binning (pixel size 1.06) and subjected an additional round of 3D classification. 1,335,468 particles were subjected to auto-refinement and yielded a 2.8 Å reconstruction. Following three rounds of CTF refinement [11] and Bayesian polishing [13], these particles were subjected to extensive classification to identify other conformations (see below). No attempts to identify alternate protein conformations were successful. Using the Bayesian polishing job in Relion 3.1, these particles were further un-binned to a pixel size of 0.7066 (*~1.3x binning from the micrographs). After three rounds of CTF refinement [11] and Bayesian polishing [13], these particles yielded a C2 symmetric 2.5 Å reconstruction. Additional rounds of refinement and classification without alignment were performed with C2 symmetry enforced, resulting in a final subset of 994,187 particles and a Å 2.28 reconstruction. To identify additional associated lipids, the 1,335,468 particles unbinned to 0.7066 Å/pix were symmetry expanded and further classified with a mask on one monomer, with and without local alignment with varying T values. One class with 1,058,829 monomers showed strong density for several additional lipids surrounding the permeation pathway. Local refinement

of these particles yielded a Å 2.28 reconstruction which was used to build additional lipids (Supp. Fig. 1-2).

Processing of dataset for afTMEM16 D511E/E514A in C14 lipids in 0.5 mM Ca^{2+}

9,853 micrographs were collected and following manual inspection, 7,693 were included for analysis with Relion 3.0. Following motion correction and CTF estimation, 3,136 particles were manually picked to generate 2D class averages that were subsequently used as templates to pick a total of 3,820,960 particles. Particles were extracted using a box size of 271 Å with 4x (pixel size 2.12) binning and subjected to two rounds of 2D classification. 2,151,574 particles with structural features resembling the afTMEM16-nanodisc complex were selected and subjected to 3D classification without symmetry. 886,967 particles from well-defined 3D classes were extracted with 2x binning (pixel size 1.06) and subjected 3D auto-refinement, which resulted in a 3.56 Å reconstruction. CTF refinement [11] and Bayesian polishing [13] followed by refinement and 3D classification with and without particle alignment were carried out, in which particles not resembling afTMEM16/nanodisc complex were discarded, resulting in a subset of 483,023 particles. This subset was used for extensive classification to identify alternate conformations described below. After no alternate conformations were identified, additional rounds of 3D classification without alignment and a mask excluding the nanodisc were used to improve the resolution, resulting in a final subset of 155,902 particles. Additional rounds of CTF refinement [11] and Bayesian polishing [13] led to a masked reconstruction with a resolution 3.3 Å, which was C2 symmetric. This initial map (without application of C2 symmetry) was used to analyze the effects on the membrane. Additional rounds of refinement were carried out with C2 symmetry applied resulting in a final reconstruction of 3.08 Å which was used for model building.

Processing of dataset for afTMEM16 in C18 lipids in 0 Ca^{2+}

6,400 micrographs were collected and included for analysis with Relion 3.0. Following motion correction and CTF estimation, 5,315 particles were manually picked to generate 2D class averages that were subsequently used as templates to automatically pick a total of 2,411,226 particles. Particles were extracted using a box size of 218 Å with 4x binning (pixel size 2.12) and subjected to 2D classification. 2,142,631 particles were selected and extracted with 2x binning (pixel size 0.85) for another round of 2D classification. 1,931,850 particles with structural features resembling

the afTMEM16-nanodisc complex were selected and subjected to 3D classification without symmetry. 525,407 particles from well-defined classes were subjected 3D auto-refinement, which resulted in a 3.46 Å reconstruction. CTF refinement [11] and Bayesian polishing [13] followed by refinement and 3D classification with and without particle alignment were carried out, in which particles not resembling afTMEM16/nanodisc complex were discarded, resulting in a subset of 461,108 particles. This subset was used for extensive classification to identify alternate conformations described below. After no alternate conformations were identified, additional rounds of 3D classification without alignment and a mask excluding the nanodisc were used to improve the resolution, resulting in a final subset of 151,197 particles. Additional rounds of CTF refinement [11] and Bayesian [13] polishing led to a masked reconstruction with a resolution 3.25 Å, which was C2 symmetric. This initial map (without application of C2 symmetry) was used to analyze the effects on the membrane. Additional rounds of refinement were carried out with C2 symmetry applied resulting in a final reconstruction of 3.07 Å which was used for model building.

Classification to identify alternate conformations

On all datasets except for afTMEM16 in C22/Ca^{2+} in MSP2N2 nanodiscs, we carried out extensive 3D classification to identify potential alternate conformations. We tried classification with alignment with global searches and with local searches both with a without a mask excluding the nanodisc. The following approaches were tried: (*i*) varying the starting model (open and closed permeation pathways), the low pass filter (10 to 20 Å), the T parameter (4-10), and number of classes (6-20); *(ii)* 3D classification without alignment (using the angles from refinement) with masks excluding the nanodisc and varying the same parameters as classification with alignment; *(iii)* symmetry expansion and signal subtraction to isolate the monomers followed by subsequent focused classification/refinement on the permeation pathway (TM3-7) [16] to account for potential alternate conformations between two protomers; *(iv)* focused classification on the monomers on the expanded but not subtracted particles; *(v)* refinement using cryoSPARC [14] for all described structures and cisTEM [17]) for the C14/0 Ca^{2+} dataset using particles picked in Relion. In cryoSPARC, we sorted using one round of 2D classification followed by several rounds of *ab initio* model generation using 3-5 classes, discarding particles from each round that did not result in a reasonable reconstruction. Heterogeneous refinement with 3-5 classes was carried out following each round of *ab initio* model generation. We also tried 3D variability analysis on all

particles selected from 2D classification and on a reduced set of particles sorted using *ab initio* and heterogenous refinement, both of which revealed a single population and no movements of the protein. In cisTEM we imported particles picked in Relion and sorted using 2D classification and tried global and local refinement using 5-10 classes. None of these approaches led to the observation of a class with an open permeation pathway.

Model Building and Refinement

Previous apo or Ca^{2+}-bound afTMEM16 structures were used as a starting model and fit into the new maps using several rounds of PHENIX real space refinement [18] including morphing and simulated annealing every macrocycle. The improved resolution in these structures, particularly the C18/Ca^{2+} allowed us to correct errors in the earlier models and to build additional parts of the protein including the hairpin preceding TM3 and the extracellular region between TM5 and TM6 (Fig. 1A-B). Lipids were modeled initially as POPG (PGW) and truncated according to the observed density. In most cases the full headgroup could not be resolved so the lipids were truncated at the phosphate atom. The models were inspected and areas that were better resolved were build *de novo*. The model was improved iteratively by real space refinement in PHENIX imposing crystallographic symmetry and secondary structure restraints followed by manual inspection and removal of outliers. For all models, the unsharpened maps were used to aid in building. Residue ranges and positions with truncated side chains are listed in Table 3.

Residue ranges and side chain truncations

	C18/Ca^{2+}	C18/0 Ca^{2+}	C14/0 Ca^{2+}	C22/Ca^{2+} (MSP1E3)	C22/Ca^{2+} (MSP2N2)	DE/AA C14/Ca^{2+}
Included Residues	12-463, 487-660, 701-724	11-24,32-89, 103-255, 269–311, 318-401, 421–442, 491–593, 604–659 and 703–717	11-88, 101-400, 420-447, 489-594, 603-660, 701-728	11-260, 267-397, 419-452, 488-660, 701-727	13-260, 267-312, 317-396, 419-453, 468-660, 701-724	11-94, 102-258, 268–312, 317-398, 421–446, 489–593, 604–659 and 702–724
Side chain truncations	E45, K70, E121, E132, K134, R260, E262, E266, K409, E410, H411, K414, K460, E461, Y462, E488, D489, and R710	Q11,E12, D17, I33, K34, K35, E37, E42, E45, E51, D56, E57, N58, K69, K70, K73, R74, R89, E104, E106, K119, R129, E132, K134, D137, E145, E146, R149, Q150, R153, E154, S156, K157, E164, E232,	E12, D26, D29, K34, E37, E45, E57, K69, K70, H247, K254, K257, E258,	D28, K34, E45, E57, K100, E121, K134, E146, E232, R260, E488, R492, D489,E703, R710, and E717	K34, E37, E45, D56, E57, K69, K70, K73, Q102, K134, Y319, E488, D489, R642,	Q11, E12, D17, N23, D26, I27, D28, T29, P30, E31, I33, K34, K35, E37, V38, L41, E42, E45, E51, D56, E57, N58, S68, K69, K70, K71, K73, R74, D83, N90, T91,

		S244, H247, E248, K249, E252, K254, M277, Q283, E305, F307, E310, Y319, L320, D348, E353, D356, K359, R391, D397, F399, H400, L401, N422, D424, F442, R492, E502, E504, Y506, D507, D510, D511, E514, R545, E636, R641, R642, E643, E646, R647, M650, R651, K652, R653, D656, T657, E703, R704, M707, R708, R710, K713, E714, and E717.	E310, and R704		E703, R710, and E714	E92, E94, Q102, E104, P118, H130, E132, K134, N135, D137, E145, E146, T147, R149, Q150, R153, E154, K157, E164, R168, E232, Q239, K241, H247, E248, K249, R250, E252, F253, K254, P255, E256, K257, E258, R268, V270, T274, K275, M277, Y278, Q283, L288, L289, E305, I306, S309, E310, Y312, K317, Y319, L320, V321, I323, D348, E353, D356, Q364, N370, S390, R391, D397, V398, I421, D424, F442, L444, E445, T446, D489, E490, R492, E502, E504, D505, D507, T509, D510, M515, E543, R545, S546, D547, K550, E554, D571, N593, T604, R606, L635, E636, R641, R642, E643, E646, R647, M650, R651, K652, R653, D656, T702, E703, R704, M707, R708, R710, K713, E714, E717, L720, S721, and L722.

Table 3: Residue ranges and side chain truncations

Model validation

To validate the refinement, the FSC between the refined model and the final map was calculated (FSCsum). To evaluate for over-fitting, random shifts of up to 0.3 Å were introduced in the final model and the modified model was refined using PHENIX [18] against one of the two unfiltered half maps. The FSC between this modified-refined model and the half map used in refinement (FSCwork) was determined and compared to the FSC between the modified-refined model and the other half map (FSCfree) which was not used in validation or refinement. The similarity in these

curves indicates that the model was not over-fit. The quality of all three models was assessed using MolProbity [19] which indicates that the models are of high quality. Statistics are found in Table 4.

Map Parameters	C18/Ca^{2+}	C18/0 Ca^{2+}	C14/0 Ca^{2+}	C22/Ca^{2+} (MSP1E3)	C22/Ca^{2+} (MSP2N2)	DE/AA C14/Ca^{2+}
Final Pixel Size (Å)	0.7066	0.85	1.06	0.7066	1.06	1.06
Symmetry	C2	C2	C2	C2	C2	C2
Initial particles	4,551,732	2,411,226	1,728,771	2,643,590	1,168,291	3,820,960
Final particles	994,187	151,197	244,507	132,332	31,890	155,902
Map resolution (Å)	2.28	3.07	3.30	2.71	3.51	3.08
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Map local resolution range (Å)	2.2-3.0	3.1-3.9	3.3-4.1	2.6-4.0	3.1-4.3	3.1-3.9
Map sharpening B factor ($Å^2$)	-70.964	-106.40272	-118.182	-67.7814	-81.7	-106.7626
Initial Model Used	6E0H	7RX3	6DZ7	7RXG	7RXG	7RX3
Model resolution (Å) (FSC_{model}=0.5)	2.4	3.21	3.5	2.9	3.7	3.33
Model Composition						
Nonhydrogen atoms	11,380	8,506	10,134	10,390	9,908	8,694
Protein residues	1,300	1,096	1,182	1,230	1,212	1,156
Ligands	26	8	16	18	8	8
r.m.s. deviations bond length (Å)	0.005	0.009	0.007	0.009	0.008	0.005
r.m.s. deviations bond length (Å)	0.630	0.922	0.948	0.849	1.066	0.836
Validation						
MolProbity Score	1.42	1.14	1.85	1.52	1.95	1.23
Clash Score	5.43	0.72	6.88	3.47	9.12	1.32
Poor Rotamers (%)	0.18	0	0.2	0.19	1.35	0
Ramachandran Plot						
Favored (%)	97.36	93.96	92.46	94.38	94.61	94.57
Allowed (%)	2.48	6.04	7.54	5.62	5.05	5.43
Disallowed (%)	0.26	0	0	0	0.34	0

Table 4: Map and model statistics for all structures.

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