Methods

Expression and purification of human TMEM16E

The cDNA clone of human TMEM16E, isolated from the Saos-2 osteosarcoma cell line (kindly provided by Dr. G. Galietta, Istituto Giannina Gaslini, Genoa, Italy) [1], corresponding to the alternative splicing isoform of exon 4 and encoding an 898-amino-acid protein (XP_005252878.2), was subcloned into the pEG BacMam vector with a C-terminal 3C protease cleavage site followed by eGFP and a StrepII tag (a gift from Dr. Eric Gouaux, Addgene plasmid #160686; RRID:Addgene_160686) for baculovirus production and expression of the target protein. This clone contains the known human single nucleotide polymorphism rs1276900571 (chromosome 11: 22,250,301; C>T; transcript ENST00000324559.9) p.Leu307Phe that is predicted benign/tolerated by SIFT (SIFT - Predict effects of nonsynonmous / missense variants) and PolyPhen-2 (https://genetics.bwh.harvard.edu/pph2/). The vector was further modified by replacing the StrepII tag with a Twin-Strep-tag to enhance protein yield.

Recombinant hTMEM16E$_{898}$ protein was expressed in HEK-293F (ATCC) suspension cells, cultured in a 37°C incubator shaker with 5% CO2, following baculovirus mediated mammalian cell expression system [2]. Briefly, bacmids encoding hTMEM16E$_{898}$ WT, R582I or G503E were generated in DH10Bac competent cells and used to transfect Sf9 cells (Expression Systems) with Cellfectin II. Three generations of baculovirus were amplified and used to transduce 2–6 l of HEK293F cells at a density of 2.0×10^6 cells ml^{-1}at with 10-15% (v/v) baculovirus. After transduction, the cell culture temperature was reduced to 30 °C, and 16 h post-transduction the cultures were supplemented with 10 mM sodium butyrate. Cells were harvested 35–48 h post-transduction, washed with PBS, flash-frozen in liquid nitrogen and stored at −80 °C until further use.

Protein purification was carried out at 4°C and completed within 16 hours. Cell pellet was resuspended in 30% glycerol and then mixed with hypotonic lysis buffer containing 10mM Hepes pH 7.5, 20 mM NaCl, 0.5 mM MgCl2, 1mM dithiothreitol, 0.2 mg ml^{-1} DNase and protease inhibitor cocktail for 40 min. Cell membrane fractions were then isolated by high-speed ultracentrifugation at 186,000× *g* for 1 h at 4°C. The membrane pellet was mechanically homogenized and solubilized in the extraction buffer containing 150mM NaCl, 20mM HEPES pH7.5, 5mM EGTA, 0.5mM MgCl2, 2% digitonin, 0.2 mg/ml DNase

and protease inhibitor cocktail for 2 h. The insoluble fractions were discarded by centrifugation at 40,000× *g* for 1 h. The supernatant was then incubated with Strep-TactinXT 4Flow resin (IBA Lifesciences GmbH) for 1 h at 4 °C. The resin was subsequently washed with 20 column volumes of wash buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.5 and 2 mM EGTA, 0.1% digitonin(w/v). The protein was eluted with 5 volume of the same buffer supplemented with 50 mM biotin and the C-terminal tag was removed by 3C protease cleavage for 2 h at 4 °C after elution. The eluted protein was concentrated, filtered through a Spin-X column and injected into a Superose 6 Increase column (Cytiva) equilibrated with SEC buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.5 and 2 mM EGTA, 0.1% digitonin(w/v). For samples purified in the presence of ligand, 1 mM $CaCl_2$ was added instead of EGTA. Fractions containing TMEM16E were pooled and concentrated to ~1–2 mg ml^{-1}.

Liposome reconstitution

Proteoliposomes containing purified human TMEM16E were prepared by detergent-mediated reconstitution followed by detergent removal, as previously described [3]. Lipids dissolved in chloroform were mixed at the indicated ratios and supplemented with 0.4% (w/w) tail-labelled NBD-PE, 1-myristoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoethanolamine, for fluorescence-based lipid scrambling assays. Two lipid compositions were used: soybean polar lipid extract supplemented with 20% cholesterol, or a defined mixture of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, 18:1), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS, 16:0–18:1) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC,16:0-18:1) at a 2:1:1 molar ratio.

Lipids were from Avanti Polar Lipids. Lipid mixtures were dried under a stream of N_2, washed with pentane and dried again under N_2 to remove residual solvent. Dried lipids were resuspended at 20 mg ml^{-1} in reconstitution buffer containing 150 mM potassium chloride (KCl), 50 mM HEPES, pH 7.4, and 35 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). Purified human TMEM16E was added at 5 µg protein per mg lipid. Detergent was removed by five sequential exchanges with 150 mg ml^{-1} Bio-Beads SM-2 adsorbent resin (Bio-Rad) under rotation at 4 °C. Calcium

or EGTA was incorporated into the liposomes by sonication followed by freeze–thaw cycles. Protein-free liposomes were prepared in parallel using the same protocol but omitting protein. Before fluorescence measurements, liposomes were extruded through a 400-nm polycarbonate membrane (Whatman). Reconstitution efficiency was assessed by SDS–PAGE analysis of samples collected before and after detergent removal.

Scrambling assay

The scrambling assay was carried out as described previously [4]. TMEM16E activity was measured from the dithionite-induced quenching of NBD-labeled phospholipids incorporated into proteoliposomes. In protein-free liposomes, membrane-impermeant dithionite reduces only the outer-leaflet NBD fluorophores, causing an approximately 50% decrease in fluorescence. In the presence of active TMEM16E, lipid redistribution between leaflets exposes additional NBD-labeled lipids to dithionite, leading to a larger fluorescence decrease.

Before the assay, liposomes were extruded through a 400-nm membrane. For each measurement, 20 µl of liposomes were diluted into a final volume of 2 ml assay buffer containing 50 mM HEPES, pH 7.4, 300 mM KCl and either 0.5 mM $Ca(NO_3)_2$ or 2 mM EGTA. NBD fluorescence was monitored with continuous mixing in a PTI spectrophotometer using excitation and emission wavelengths of 470 and 530 nm, respectively. After recording the baseline for 100 s, sodium dithionite was added to a final concentration of 40 mM. Fluorescence traces were collected using FelixGX 4.1.0 software at a sampling rate of 3 Hz.

Quantification of scrambling activity

Scrambling activity was quantified by fitting the fluorescence decay after dithionite addition to a kinetic model that accounts for dithionite reduction, protein-free vesicles and bidirectional lipid scrambling. The total fluorescence at time *t* was fitted according to the equation:

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

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 the forward and reverse scrambling rate constants, respectively, 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 in the fit were f_0, α and β while L_i^{PF} and γ were determined experimentally from protein-free liposomes. Protein-free vesicles showed a very slow fluorescence decay, likely due to slow dithionite leakage into vesicles or spontaneous flipping of NBD-labelled lipids. A linear fit was used to estimate that the rate of this process is L = (5.4 ± 1.6)10^{-5} s^{-1} (n > 160) [5]. This contribution was negligible compared with TMEM16E-mediated scrambling under the conditions tested. All measurements were performed side by side with a control preparation of WT TMEM16E. Scrambling data were analyzed using Ana program Ana (available at http://users.ge.ibf.cnr.it/pusch/) and Prism 7.0 (GraphPad, San Diego, CA) or SigmaPlot 10.0 (SYSTAT Software).

Grid preparation and data collection

Purified human TMEM16E was concentrated to 1.7 mg ml^{-1} immediately before grid preparation. Cryo-EM grids were prepared using glow-discharged UltrAuFoil R1.2/1.3 300-mesh gold grids. A total of 3.5 µl of protein sample, supplemented with either 2 mM EGTA or 1 mM Ca^{2+}, was applied to each grid and incubated for 1 min at 100% humidity and 12 °C. Grids were blotted for 2–2.5 s with a blot force of 0 and plunge frozen in liquid ethane using a Vitrobot Mark IV at the Weill Cornell Medicine Cryo-EM Core Facility.

Sample grids were screened and data were collected either on a 300 kV Titan Krios microscope (Thermo Fisher Scientific) equipped with a K3 direct electron detector (Gatan) at New York University Langone Health's Cryo-Electron Microscopy Laboratory using Leginon [REF], or on a 200 kV Glacios microscope (Thermo Fisher Scientific) equipped with a Falcon4i direct electron detector (Thermo Fisher Scientific) at the Weill Cornell Medicine Cryo-EM Core Facility using EPU 3 software (Thermo Fisher Scientific). An energy filter, either from Gatan or Thermo Fisher Scientific, set to a slit width of 20 eV

was used during data collection. Additional data collection parameters are reported in Table 1.

Image processing

Cryo-EM image processing was performed in RELION 5.0 [6]. Movie frames were motion-corrected and dose-weighted using the RELION implementation of motion correction [7], and contrast transfer function parameters were estimated with CTFFIND4 [8]. Micrographs with poor CTF fits, excessive drift, crystalline ice or visible contamination were excluded from further processing.

Particles were picked using a combination of crYOLO [9] and template-based picking. Extracted particles were subjected to multiple rounds of reference-free 2D classification to remove false-positive picks, damaged particles and poorly resolved views. 2D classes displaying structural features consistent with TMEM16-family proteins were selected for subsequent 3D refinement and 3D classification.

The WT TMEM16E datasets in 2 mM EGTA and 1 mM Ca^{2+} were processed independently. Selected particles were subjected to iterative rounds of 3D refinement and 3D classification, with alignment, to remove residual low-quality particles and improve particle homogeneity. During early 3D classification of the Ca^{2+}-bound WT dataset, a subset of particles formed an asymmetric class characterized by weak or missing density for TM3 and TM4 in one protomer, consistent with increased local flexibility in this region. This asymmetric class represented approximately one-third of the classified particles, whereas the remaining well-resolved particles formed symmetric classes. Particles belonging to the asymmetric class were excluded from subsequent focused classification and final reconstruction**.** Particles from well-defined 3D classes were subjected to CTF refinement followed by Bayesian polishing in RELION 5.0 to correct for beam-induced particle motion and radiation damage [10, 11]. Polished particles were re-extracted and further processed by 3D refinement and 3D classification to obtain the final homogeneous particle subsets.

For the Ca^{2+}-bound WT TMEM16E dataset, symmetry expansion and signal subtraction were performed to analyze individual protomers independently. Focused 3D classification

and 3D refinement were carried out using masks encompassing the Ca^{2+}-binding region to assess local heterogeneity and improve interpretability of the Ca^{2+}-binding sites.

The mutant datasets were processed using the same general 2D and 3D classification workflow described for WT TMEM16E. The apo R582I and 1mM Ca^{2+}G503E datasets were collected on the 200 kV Glacios microscope, whereas the Ca^{2+}-bound R582I dataset was collected on the 300 kV Titan Krios microscope. After particle picking, 2D classification and 3D cleanup, particles from the mutant datasets were subjected to CTF refinement, Bayesian polishing, re-extraction and final 3D refinement. As observed for the Ca^{2+}-bound WT dataset, early 3D classification of the Ca^{2+}-bound R582I dataset also identified an asymmetric subset with weak or missing TM3–TM4 density in one protomer. This class represented a smaller fraction of the classified particles than in the WT dataset and was excluded from the subsequent focused analysis and final reconstruction**.** The Ca^{2+}-bound R582I dataset was additionally processed by symmetry expansion, signal subtraction and focused 3D classification/refinement to evaluate heterogeneity at the Ca^{2+}-binding sites, following the strategy used for the Ca^{2+}-bound WT dataset.

Final refinements were initially performed without a mask and then continued with a soft mask around the protein. C2 symmetry was imposed during final refinements when no major structural asymmetry between protomers was observed. Final map resolutions were determined using the gold-standard Fourier shell correlation criterion at FSC = 0.143 after application of the soft mask. Local resolution was estimated in RELION, and, for the representation of protein densities, unfiltered half maps and the final mask were used as input for sharpening by DeepEMhancer [12]. Sharpened maps were used for visualization and model interpretation, whereas unsharpened and locally filtered maps were inspected during model building, particularly in regions of weaker density, including extracellular loops, the TM3–TM6 groove region, Ca^{2+}-binding sites and non-protein densities. Cα r.m.s.d. calculations were performed using ChimeraX [13]. Final particle numbers, map resolutions and processing statistics are reported in Table 1, and the processing workflows are summarized in Supplementary Fig.2,3,6,7 .

Model building and refinement

An initial model of human TMEM16E was generated using SWISS-MODEL [14] and fitted into the cryo-EM maps using UCSF ChimeraX [13]. The model was then manually adjusted in Coot [15] and refined iteratively in PHENIX real-space refinement [16]. Refinement included secondary-structure restraints and non-crystallographic symmetry restraints, when appropriate, together with morphing and simulated annealing. Manual inspection was performed after each refinement cycle to correct local model-to-map mismatches, rotamer outliers and poorly fitted regions.

For the WT TMEM16E EGTA and Ca^{2+}-bound structures, the model was rebuilt according to the experimental density, with particular attention to the transmembrane helices, extracellular loops, the TM3–TM6 groove region and the Ca^{2+}-binding sites. DeepEMhancer sharpened maps and unsharpened and locally filtered maps were used to assist model building in regions where the sharpened maps showed weaker or discontinuous density. For the Ca^{2+}-bound structures, Ca^{2+} ions were modeled at the Ca^{2+}-binding sites only when supported by clear map density.

Models of the R582I and G503E mutants were generated from the refined WT TMEM16E model by introducing the corresponding amino-acid substitutions and rebuilding the surrounding regions according to the mutant cryo-EM densities. For R582I, model building focused on the extracellular loop region surrounding residue 582 and its interactions with neighboring residues. For G503E, local rebuilding focused on the TM3–TM4 region around residue 503.

Regions with weak or discontinuous density were left unmodeled or truncated to the last residue that could be assigned reliably. Final models were refined in PHENIX and inspected in Coot and ChimeraX to correct local geometry, rotamer conformations and model-to-map fit. Model refinement statistics are reported in Table 1.

Model validation

Model validation was performed using PHENIX [17] and MolProbity [18]. The agreement between each refined model and the corresponding cryo-EM map was assessed by calculating the Fourier shell correlation between the model and the final map (FSCsum). To evaluate potential overfitting, random shifts of up to 0.3 Å were introduced into the final model, and the displaced model was refined in PHENIX against one of the two unfiltered

half-maps. The FSC between this refined model and the half-map used for refinement (FSCwork) was then compared with the FSC between the same model and the independent half-map that was not used during refinement (FSCfree). Similarity between the FSCwork and FSCfree curves was used to assess the absence of substantial model overfitting.

Model geometry was evaluated using MolProbity, including analysis of bond lengths, bond angles, clashscore, rotamer outliers and Ramachandran statistics. Final validation and refinement statistics are reported in Table 1.

Flow cytometry assay for quantifying TMEM16E-mediated phospholipid scrambling

Cell-based phospholipid scrambling by TMEM16E was quantified by flow cytometry using fluorescent annexin V binding as a readout of phosphatidylserine exposure at the plasma membrane. TMEM16F-knockout HEK293T cells [19] were a kind gift from Dr. Huanghe Yang (Duke University). Cells were transiently transfected with plasmids encoding WT human TMEM16E or the indicated disease-associated mutants, including R582I and G503E. Constructs were expressed as fluorescently tagged proteins to identify the transfected cell population.

Cells were seeded and transfected using Lipofectamine 2000 (Thermo Fisher Scientific; 11668027). Six hours after transfection, the culture medium was replaced with calcium-free DMEM (Gibco, 21068028) to remove Lipofectamine–DNA complexes and minimize cell toxicity. Flow cytometry was performed 24 h after transfection using a BD Fortessa analyzer. Cells were harvested by collecting the medium, washing with Dulbecco's PBS (Gibco, 14190250) and detaching with TrypLE Express enzyme (Gibco, 12604013). Cells were then resuspended in 1× annexin-binding buffer (Thermo Fisher Scientific, V13246) at a final concentration of 1×10^6 cells ml^{-1}.

For each condition, 200 µl of cell suspension were mixed with 800 µl annexin-binding buffer containing 5 µM ionomycin (Alomone), 150 nM DAPI (Thermo Fisher Scientific, D1306) and Alexa Fluor 647–conjugated annexin V (Thermo Fisher Scientific, A23204) diluted 1:2000. For each construct, samples were analyzed in parallel under basal conditions and after ionomycin stimulation. Basal phosphatidylserine exposure was

measured at 40 s in the absence of ionomycin, while ionomycin-induced scrambling was quantified at 40 and 300 s after addition of 5 μM ionomycin. Cells were analyzed by gating on the fluorescent-protein-positive, DAPI-negative population, and TMEM16E-mediated scrambling was quantified as the percentage of annexin V-positive cells using FlowJo software.

Molecular systems for atomistic molecular dynamics simulations

The Ca^{2+}-bound closed-state cryo-EM structures of WT TMEM16E and the G503E TMEM16E were used as initial models for molecular dynamics (MD) simulations. Unresolved loop residues in the cryoEM structures (residues 144-156, 415-443, 481-499 in WT TMEM16E and 140-170, 416-417, 480-499 in G503E) were built as loops using the SWISS-MODEL server [20]. Unresolved residues in TM3 (444-446 and 476-480 in WT TMEM16E and 444-446 in G503E) and in the cytosolic region (418-443 in G503E) were modeled using coordinates obtained from an AlphaFold2-predicted structure [21]. Homo dimeric structures were generated which spanned protein residues 53-870 for WT TMEM16E and 53-868 for G503E. Using the CHARMM-GUI web server [22], the WT and G503E mutant Ca^{2+}-bound closed state models were then embedded into a lipid membrane composed of 7:3 POPC:POPG, solvated and ionized for electroneutrality with ~150 mM KCl. The periodic box dimensions of the WT system were ~18.1 x 18.1 x 15.3 nm^3 and contained 870 lipids, 6 calcium ions, 549 potassium ions, 290 chloride ions, and 108,712 water molecules. The periodic box dimensions of the G503E system were ~18.1 x 18.1 x 15.0 nm^3 and contained 880 lipids, 6 calcium ions, 558 potassium ions, 300 chloride ions, and 105,707 water molecules.

Atomistic molecular dynamics simulations

Adaptive ensemble molecular dynamics (aeMD) simulations were conducted in three sequential stages to adaptively sample the conformational space of the two molecular constructs. The molecular systems were first independently equilibrated 10 times by resetting initial velocities using the standard set of scripts provided by CHARMM-GUI. This produced a 10-replicate ensemble for each construct. Following equilibration, the simulations of the ensembles were run in 3 stages whereby each independent replicate

(10 per construct) was simulated for 250 ns per stage, resulting in a cumulative sampling of 7.5 μs per construct. At the end of each stage, frames were extracted at 25 ns intervals and their RMSD was calculated relative to TM4-TM7 residues of a homology model of TMEM16E based on an active structure of TMEM16F (PDBID: 9O1N) built using the SWISS -MODEL server). For each construct a frame with low RMSD relative to the stage was used to seed the next simulation stage. The simulations were carried out using the CHARMM36m [23] force field for protein and lipids and with Amber 24 [24] (University of California, San Francisco). Particle mesh Ewald was used to describe electrostatic interactions with a cutoff of 12 Å; for the van der Waals interactions, a 12-Å cutoff distance with a 10 Å switching distance was used. The simulations were performed under NPT ensemble using semi-isotropic pressure coupling, with an integration timestep of 2 fs using the SHAKE algorithm [25]. All simulations were run at 310 K, with the Langevin thermostat to maintain temperature; a Monte Carlo barostat was used to maintain a target external pressure of 1.0 bar.

Coarse-grained molecular dynamics (cgMD) system preparation

The cgMD systems were built from models obtained from the Stage 3 aeMD simulations with low Cα R.M.S.D. values relative to the target active homology model (2.3 Å for WT and 1.3 Å for G503E) using the CHARMM-GUI web server. Using the Martini bilayer maker with the ElNeDyn 3.0.0 model [26, 27], the aa protein structures were first transformed into cg representations (with calcium ions removed). Then, these cg protein structures were embedded into a cg 7:3 POPC–POPG membrane. The systems were solvated in a water box with ~150 mM NaCl. Once systems were built, calcium ions were reintroduced by adding CA beads (cg Ca^{2+}) into their binding sites and by correspondingly modifying simulation input files. To retain electroneutrality and account for the introduction of the six cg CA beads, six NA cg beads in the water solution were swapped into six cg CL beads. The periodic box dimensions of the cgMD WT and G503E systems were $18.1 \times 18.1 \times 16.2\ nm^3$ and $18.0 \times 18.0 \times 16.0\ nm^3$, respectively. The systems contained 900 CG lipids, 6 CA beads, 616 NA beads, 352 CL beads and 29,284 water beads and 890 CG lipids, 6 CA beads, 611 NA ions, 346 CL beads and 28,629 water beads, respectively.

cgMD simulations

The cgMD simulations were performed using the Martini 3 force field [26] and GROMACS (version 2023.4) [28]. Simulations used semi-isotropic pressure coupling with the Parrinello–Rahman barostat, using a reference pressure of 1.0 bar, a coupling time constant of 12.0 ps and a compressibility of 3×10^{-4} bar^{-1}. A 20 fs integration time was used. Long-range electrostatics were treated using the reaction-field method [29] and both electrostatic and van der Waals interactions were truncated at a cutoff distance of 1.1 nm (11 Å). The simulation temperature was set to 310 K to match the aaMD setup and was maintained using the velocity-rescaling thermostat with a coupling constant (τ_t) of 1.0 ps. The simulations employed ElNeDyn. Restraints parameters were set to the default 0.9 nm cut off and an elastic bond strength of 500 kJ mol^{-1} nm^{-2}. To ensure that the CA beads remained bound to their binding sites during simulation, we also included distance restraints with force constant 1,000 mol^{-1} nm^{-2} using flat-bottom potentials between bound CA beads and coordinating side-chain bead residues (residues 384, 614, 657, 660, 689, 693 and 841). For both WT and G503E mutant systems, 10 replicate ensembles were generated and then each system independently underwent the standard equilibration protocol provided by CHARMM-GUI. Following the equilibration, all ten replicates of each construct were simulated for 5 µs each, resulting in a total sampling time of 50 µs for the Wild-Type and G503E mutant cgMD ensembles.

Lipid Scrambling events

Lipid scrambling events were quantified from the cgMD simulations as we recently described [30]. Briefly, a dynamic ‘groove region slab’ was defined for each frame of the simulation with a lower boundary placed at the mean *z* coordinate of the Cα bead of residue 541 and the upper boundary placed at the mean *z* coordinate of the Cα bead of residue 560. Each lipid was classified on the basis of its PO4 bead *z* position relative to this slab; PO4 beads above the upper boundary were assigned a value of +1 (extracellular region), PO4 beads within the slab were set as 0 (groove/pore region) and PO4 beads below the lower boundary were labeled as −1 (intracellular region). These labels provided us with time-resolved positional tracking of all lipid molecules in our system. Scrambling

events were identified as specific triplets where [+1 → 0 → −1] denoted an extracellular to intracellular transition and [−1 → 0 → +1] indicated an intracellular to extracellular transition.

Lipid density volumetric maps

We generated volumetric maps using the volmap tool in VMD [31] to visualize the membrane thinning induced by rearrangements in the groove region during each simulation stage. To this end, the aeMD and cgMD trajectories were preprocessed by removing periodic boundary artifacts, centering the protein in the simulation box, aligning the protein to remove global translational and rotational motions, stripping water molecules and down sampling the trajectory by a factor of 10 to reduce file size and computational load. Preprocessed trajectories were loaded into VMD and the volmap tool was used to generate volumetric occupancy with the selection 'name P and within 10 of protein' for aeMD and 'name PO4 and within 10 of protein' for cgMD using a resolution of 1.0 Å. Occupancy was computed for all frames in each ensemble so that the resulting map represented the average occupancy. This protocol was performed separately for the Wild-Type and G503E simulation ensembles. This resulted in Volmaps of aaMD stage 1-3 and of the cgMD for each construct.

Water density volumetric maps

To visualize how hydration varied between the WT and G503E constructs we generated water volumetric maps using the volmap tool in VMD [31]. The aaMD and cgMD trajectories were preprocessed by removing periodic boundary artifacts, centering the protein in the simulation box, aligning the protein to remove global translational and rotational motions, and down sampling the trajectory by a factor of 10 to reduce file size and computational load. Preprocessed trajectories were loaded into VMD and the volmap tool was used to generate volumetric occupancy with the selection 'resname WAT and name O and within 10 of protein' for aaMD using a resolution of 1.0 Å. Occupancy was computed for all frames in each ensemble; therefore, the resulting map of ensemble was an average occupancy density. This protocol was performed separately for the aaMD stage 3 ensemble of Wild-Type and G503E.

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