METHODS

Construct. The full-length human TRPV6 (residues 1–725) and rat TRPV6 (rTRPV6, residues 1–727) were each introduced into a pEG BacMam vector[30], with the C-terminal thrombin cleavage site (LVPRG) followed by the streptavidin affinity tag (WSHPQFEK). The R470E mutation in hTRPV6 was introduced by conventional mutagenesis. The rTRPV6 construct previously used for crystallographic studies, rTRPV6* (ref. 10), was also introduced into a pEG BacMam vector but with eGFP inserted between the thrombin site and the streptavidin tag. Compared to wild-type rTRPV6, the rTRPV6* construct is C-terminally truncated by 59 residues and contains three point mutations in the ankyrin repeat domain (I62Y, L92N and M96Q).

Expression and purification. All constructs were expressed and purified similarly to $TRPV6_{cryst}$[11]. Bacmids and baculoviruses were made using a standard method[30]. The P2 baculovirus, produced in Sf9 cells (Thermo Fisher Scientific, mycoplasma negative, GIBCO #12659017), was added to HEK 293S cells lacking *N*-acetylglucosaminyltransferase I ($GnTI^-$) and grown in suspension (mycoplasma test negative, ATCC #CRL-3022) in Freestyle 293 medium (GIBCO-Life Technologies #12338-018) supplemented with 2% FBS at 37 °C and 5% CO_2. Eight to twelve hours after transduction, 10 mM sodium butyrate was added to enhance protein expression and the temperature was reduced to 30 °C. At 48–72 h post-transduction, cells were harvested by low-speed centrifugation in a Sorvall Evolution RC Centrifuge (Thermo Scientific) at 5,471*g* for 15 min, washed in phosphate-buffered saline (PBS) pH 8.0, and pelleted in an Eppendorf Centrifuge 5810 at 3,202*g* for 10 min. The cell pellet was resuspended and subjected to sonication with a Misonix sonicator (12 × 15 s, power level 8) in a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM βME (β-mercaptoethanol) and protease inhibitors (0.8 μM aprotinin, 2 μg/ml leupeptin, 2 μM pepstatin A and 1 mM phenylmethysulfonyl fluoride); 50 ml was used per 800 ml of HEK 293 cell culture. Subsequently, the lysate was clarified after centrifugation using a Sorvall RC-5C Plus centrifuge at 9,900*g* for 15 min, and the membranes were collected by ultracentrifugation in a Beckman Coulter ultracentrifuge equipped with a Beckman Coulter Type 45 Ti Rotor at 186,000*g* for one hour. The membranes were then mechanically homogenized, and solubilized for 1–2 h in 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% DDM (n-dodecyl-β-D-maltopyranoside), 0.1% CHS, and 1 mM βME. Insoluble material was removed by ultracentrifugation for 40 min in a Beckman Coulter Type 45 Ti Rotor at 186,000*g* and the supernatant was added to streptavidin-linked resin and rotated for 10–14 h at 4 °C. Next, the resin was washed with 10 column volumes of wash buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 0.1% DDM, and 0.01% CHS. The bound protein was eluted in wash buffer to which 2.5 mM D-desthiobiotin was added. All constructs were purified by size exclusion chromatography using a Superose 6 column equilibrated in 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1mM βME, 0.1% DDM, and 0.01% CHS. Tris(2-carboxyethyl)phosphine (TCEP; 10 mM) was added to the peak fractions, which were pooled and concentrated for channel reconstitution in nanodiscs or amphipols. The rTRPV6 and rTRPV6* constructs were expressed and purified similarly to the hTRPV6 constructs but without the addition of CHS to any buffer. Additionally, after elution from the streptavidin-linked resin, the rTRPV6* fusion protein was concentrated to ~1.0 mg/ml and subjected to thrombin digestion at a mass ratio of 1:100 (thrombin:protein) for one hour at 22 °C with rocking, before size exclusion chromatography. Prior to reconstitution in nanodiscs or amphipols, the concentration of each construct was adjusted to approximately 1.2 mg/ml.

Reconstitution of TRPV6 protein into nanodiscs and amphipols. Both hTRPV6 and rTRPV6 were incorporated into conventional MSP2N2 lipid nanodiscs as described previously[18]. In brief, soybean polar lipid extract (Avanti #541602) was solubilized in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM TCEP, and 15 mM DDM to create a 10-mM stock. Purified sample was mixed with the soybean polar lipid extract stock (~7.6 mg/ml) and MSP2N2 (~5.3 mg/ml) at a molar ratio of approximately 1:3:166 for both hTRPV6 (monomer:MSP2N2:lipid) and rTRPV6 (monomer:MSP2N2:lipid) and rocked at room temperature for one hour. Subsequently, 10 mg of Bio-beads SM2 (Bio-rad) pre-wet in buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1mM βME) was added to 0.5 ml of mixture and the mixture was rotated at 4 °C. After one hour, an additional 10 mg of Bio-beads SM2 was added and the resulting mixture was rotated at 4 °C for ~20 h. The Bio-beads SM2 were removed by pipetting and TRPV6 reconstituted in nanodiscs was isolated from empty nanodiscs by size exclusion chromatography using a Superose 6 column equilibrated in 150 mM NaCl, 20 mM Tris-HCl pH 8.0, and 1 mM βME.

cNW11 circularized nanodiscs were prepared as described previously[31]. Purified rTRPV6* was incorporated into cNW11 (2.0 mg/ml) circularized nanodiscs using the procedure described above for the MSP2N2 nanodiscs but with a molar ratio of 1:10:267 (rTRPV6* monomer:cNW11:lipid).

For reconstitution in A8-35 amphipols (Anatrace #A835), we adapted the previously described procedure[32]. hTRPV6 or hTRPV6(R470E) was mixed with amphipols at a 1:3 mass ratio (protein:amphipols) and incubated for three hours with rotation at 4 °C. After three hours, 7–8 mg per 0.5 ml of mixture of Bio-beads SM2 pre-wet in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, 1mM βME was added to the protein–amphipols mixture to facilitate the reconstitution of TRPV6 into amphipols. The mixture was rotated for ~20 h at 4 °C and the amphipols-solubilized TRPV6 was purified as described above.

Cryo-EM sample preparation and data collection. Au/Au grids were prepared as described[33]. In brief, grids were prepared by first coating C-flat (Protochips) CF-1.2/1.3-2Au 200 mesh holey carbon grids with ~50 nm gold using an Edwards Auto 306 evaporator. Subsequently, an Ar/O_2 plasma treatment (6 min, 50 W, 35.0 s.c.c.m. Ar, 11.5 s.c.c.m. O_2) was used to remove the carbon with a Gatan Solarus (model 950) Advanced Plasma Cleaning System. The grids were again plasma treated (H_2/O_2, 20 s, 10 W, 6.4 s.c.c.m. H_2, 27.5 s.c.c.m. O_2) before sample application in order to make their surfaces hydrophilic. A Vitrobot Mark IV (FEI) was used to plunge-freeze the grids after the application of 3 μl protein solution with 100% humidity at 5 °C, a blot time of 2 or 3 s, blot force set to 3, and a wait time of 20 s. A concentration of 0.5 mg/ml was used for the nanodiscs-solubilized protein and 0.3 mg/ml for the amphipols-solubilized protein.

The hTRPV6 in nanodiscs data were collected on a Tecnai F30 Polara (C_s 2.26 mm) at 300 kV equipped with a Gatan K2 Summit direct electron detection (DED) camera (Gatan) using Leginon[34]. We collected 1,733 micrographs in super-resolution mode with a pixel size of 0.98 Å across a defocus range of −1.5 μm to −3.5 μm. The total dose, ~67 e^- $Å^{-2}$, was attained by using a dose rate of ~8.0 e^- $pixel^{-1}$ s^{-1} across 40 frames for 8 s total exposure time. We collected 1,538 hTRPV6 in amphipols micrographs and 1,301 rTRPV6* micrographs as described above. We collected 2,167 rTRPV6 micrographs as described above but in counting mode with a pixel size of 0.98 Å. The hTRPV6(R470E) data were collected on a C_s-corrected Titan Krios (FEI) equipped with a post-column GIF Quantum energy filter at 300 kV. We collected 3,540 micrographs in counting mode with a pixel size of 1.10 Å across a defocus range of −1.5 μm to −3.5 μm. The total dose, ~67 e^- $Å^{-2}$, was attained by using a dose rate of ~8.0 e^- $pixel^{-1}$ s^{-1} across 50 frames for 10 s total exposure time.

Image processing. Data were collected using the Gatan K2 Summit DED camera (Gatan) in super-resolution mode and binned 2 × 2. Frame alignment was done using MotionCor2[35]. CTF correction, using CTFFIND4[36] for the hTRPV6 in nanodiscs dataset and gCTF[37] for all other datasets, was performed on non-dose-weighted micrographs and subsequent data processing was done on dose-weighted micrographs. All other data processing, unless stated otherwise, was performed using Relion 2.0[38]. For each dataset, 1,000–2,000 particles were manually selected to generate 2D classes for use in auto-picking.

In processing the hTRPV6 in nanodiscs dataset, seven 2D classes were used for automatically picking 509,569 particles from the 1,733 collected micrographs. The particle images were binned to a pixel size of 1.96 Å per pixel and screened by 2D classification to remove aberrantly picked particles. The remaining 508,019 particles were subjected to 3D classification into 10 classes with no symmetry imposed. A density map was generated in Chimera from the crystal structure of rTRPV6 (PDB ID: 5IWK), low-pass filtered to 40 Å, and used as an initial reference. Five classes, comprising 313,369 particle images, exhibited structural features of a quality that warranted further processing. Of the five, one showed structural features of higher detail and comprised 71,582 particle images. The particle images composing this class were extracted without binning (0.98 Å per pixel), refined with C4 symmetry using the same reference (unbinned) as the prior round of classification, low-pass filtered to 40 Å, and post-processed. The resulting map was then used as a reference for the second round of 3D classification in which the particles composing the best five aforementioned classes were extracted with binning (1.98 Å per pixel) and split into ten classes with C4 symmetry imposed. Two new classes, comprising a total of 67,034 particles, exhibited structural features of a quality that warranted further processing. These 67,034 particle images were extracted without binning (0.98 Å per pixel), refined with C4 symmetry using the same reference (unbinned) as the prior round of classification low-pass filtered to 40 Å, and post-processed. The resulting map was used for the final round of 3D classification in which the two best classes from the prior round of 3D classification were without binning, and with C4 symmetry imposed, classified into 10. The four best classes, comprising 46,124 particle images, were refined together and post-processed to generate the final 3.6 Å map. This relatively small number of particles compared to the initial pool of picked up 509,569 particles indicates that the majority of picked up particles represent either artefacts or contaminants or TRPV6 molecules in alternative conformations including different gating states or unnatural conformations produced by the artificial environment of the cryo-EM grid.