Crystal structure of the epithelial calcium channel TRPV6

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Precise regulation of calcium homeostasis is essential for many physiological functions. The Ca²⁺-selective transient receptor potential (TRP) channels TRPV5 and TRPV6 play vital roles in calcium homeostasis as Ca²⁺ uptake channels in epithelial tissues. Detailed structural bases for their assembly and Ca²⁺ permeation remain obscure. Here we report the crystal structure of rat TRPV6 at 3.25 Å resolution. The overall architecture of TRPV6 reveals shared and unique features compared with other TRP channels. Intracellular domains engage in extensive interactions to form an intracellular ‘skirt’ involved in allosteric modulation. In the K⁺ channel-like transmembrane domain, Ca²⁺ selectivity is determined by direct coordination of Ca²⁺ by a ring of aspartate side chains in the selectivity filter. On the basis of crystallographically identified cation–binding sites at the pore axis and extracellular vestibule, we propose a Ca²⁺ permeation mechanism. Our results provide a structural foundation for understanding the regulation of epithelial Ca²⁺ uptake and its role in pathophysiology.

The TRP channels are a superfamily of cation-permeable ion channels that are widely known for their role as transducers of sensory modalities. TRPV5 and TRPV6 are TRP channels that are uniquely selective for Ca²⁺ (permeability ratio P_Ca/P_Na > 100) (ref. 2). They have not been reported to be responsive to temperature, tastants or odours, but the mechanosensitive properties of TRPV6 appear to be important for the formation of microvilli. TRPV5 and TRPV6 belong to the vanilloid subfamily of TRP channels, share ~75% sequence identity and are involved in the transport of calcium through epithelial cell membranes. Knockout of TRPV6 in mice leads to various phenotypes linked to impaired Ca²⁺ homeostasis, including defective intestinal Ca²⁺ absorption, lower body weight, impaired fertility and dermatitis. Altered TRPV6 expression has also been shown in various transgenic mouse models of human diseases, including Crohn’s and kidney stone diseases. In addition, TRPV6 is implicated in the development of various carcinomas and is linked to impaired Ca²⁺ homeostasis, including defective intestinal Ca²⁺ absorption, lower body weight, impaired fertility and dermatitis.

The best crystals of TRPV6 crystall diffracted to 3.25 Å resolution. We solved the TRPV6 structure by molecular replacement, and the electron density map (Extended Data Fig. 2) was readily interpretable for most of the polypeptide (see Methods). Sequence registry was aided by anomalous difference Fourier maps highlighting natural sulfur atoms of cysteines and methionines, and selenium atoms in protein with selenomethionines substituted for methionines (Extended Data Fig. 3). The resulting model of TRPV6 was refined to good crystallographic statistics and stereochemistry (Extended Data Table 1).

Architecture and domain organization

The four-fold symmetrical structure of TRPV6 (Fig. 1) contains two main components: a transmembrane domain with a central ion channel pore, and a ~70 Å-tall and ~110 Å-wide intracellular skirt where four subunits constitute walls enclosing a ~50 Å × 50 Å cavity underneath the ion channel. Like TRPV1 (ref. 14) (Extended Data Fig. 4) and TRPV2 (ref. 16), the intracellular domains of a single TRPV6 crystall subunit contain an ankyrin repeat domain with six ankyrin repeats, followed by a linker domain that includes a β-hairpin (composed of β-strands) and a helix–turn–helix motif resembling a seventh ankyrin repeat, and the pre-S1 helix, which connects the linker domain to the transmembrane domain (Fig. 1d–f and Extended Data Fig. 4). In addition, the conserved domains, TRPV6 also includes an N-terminal helix and C-terminal hook, which pack against each other to form an intersubunit interface along the corners of the intracellular skirt.

Similar to other TRP channels, the transmembrane domain of TRPV6 crystall crudely resembles voltage-gated K⁺ (ref. 18) or Na⁺ (ref. 19) channels and includes six transmembrane helices (S1–S6) and a pore loop (P-loop) between S5 and S6. The first four transmembrane helices form a bundle to constitute the S1–S4 domain. The packing of aromatic side chains in S1–S4 rigidifies the helical bundle conformation (Extended Data Fig. 4c), suggesting that this domain remains relatively static during gating. The linker between the S1–S4 domain and pore

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domain is unstructured, which is a marked contrast from other TRP channels, in which it assumes a helical conformation and mediates interdomain interactions14,16,17. Following S6 is the amphipathic TRP helix, which runs parallel to the membrane and interacts with intracellular soluble domains in a manner analogous to TRPV1, TRPV2 and TRPA1 (refs 14, 16, 17).

Although the overall domain organization of TRPV6cryst resembles TRPV1/2 (refs 14, 16) and, to a lesser degree, TRPA1 (ref. 17), electron density for the linker between S6 and TRP helix (Extended Data Fig. 2f) and disulfide crosslink experiments (Extended Data Fig. 5a–c) imply a unique non-swapped transmembrane domain arrangement in which the S1–S4 domain and pore domain of the same protomer are packed against each other. While this unique domain arrangement could have profound implications, we present this aspect of the TRPV6cryst model cautiously because of the absence of interpretable density for the S4–S5 linker.

**Assembly and subunit interfaces**

Assembly of TRPV6cryst is mediated by multiple interdomain interfaces (Fig. 2). Close packing of S5 against S4 and S1 of the adjacent S1–S4 domain immobilizes the pore module with respect to the S1–S4 domain (Fig. 2a), a trait that is reminiscent of the Slo2.2 K+ channel14,16,17 and distinct from voltage-gated channels18. Further, the S1–S2 extracellular loop contacts the S5–P and P–S6 loops (Fig. 2a). This interaction hints at a structural basis for the regulation of TRPV5 and TRPV6 function by the β-glucuronidase klotho, which modulates channel activity by modifying the conserved N-linked glycosylation site21 located in the middle of this loop (N357 in TRPV6cryst).

The intracellular domains of TRPV5 and TRPV6 have been implicated in tetrameric assembly23, trafficking23 and regulation of channel activity by the Ca2+ sensor calmodulin24–26. The structure of TRPV6cryst reveals that numerous non-contiguous intracellular domains engage in extensive inter- and intrasubunit interactions (Fig. 2c). At the centre of these interactions is the N-terminal helix, which is positioned as a pillar along the corners of the intracellular skirt. Putative hydrogen bonds and salt bridges involving D34 stabilize the interaction between the N-terminal helix and three-stranded β-sheet. Notably, mutation of the equivalent D34 to alanine abolished Ca2+ uptake function in TRPV5 (ref. 23), suggesting this interaction’s functional importance.

The N-terminal helix also forms hydrophobic and hydrogen bonding interactions with the C-terminal hook and pre-S1 helix from an adjacent subunit. Since it is a hub for domain interactions, endogenous or exogenous factors could allosterically modulate channel activity by targeting the N-terminal helix. Interestingly, we observed a robust cylindrical density at the intersubunit interface formed by the N-terminal helix, ankyrin repeat domain and three-stranded β-sheet (Extended Data Fig. 5d–h). We have tentatively attributed this density to desthiobiotin (DTB), which was included as an eluent in the TRPV6cryst affinity purification procedure (see Methods).

**Ion-conducting pore**

The extracellular portion of the TRPV6cryst ion-conducting pore is formed by extracellular loops connecting the P-loop helix to S5 and S6, while the rest of the ion conduction pathway is formed entirely by the S6 helices (Fig. 3). Such pore architecture is conserved over the entire family of tetrameric ion channels (Extended Data Fig. 6).

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Figure 1 | Architecture and domain organization of TRPV6cryst. a–c, Side (a), bottom (b) and top (c) views of the TRPV6cryst tetramer, with each subunit shown in a different colour. d, Domain organization diagram of the TRPV6 subunit. e, f, Two views of the TRPV6cryst subunit, with domains coloured as in d.
The extracellular vestibule is a four-residue selectivity filter (538TIID541) with a highly electronegative ‘mouth’ to the pore (Fig. 3a–c). Below this, the region connecting S5 and S6 contains eight acidic residues per protomer, four of which face the ion conduction pathway to produce a highly electronegative ‘mouth’ to the pore (Fig. 3a–c). Below this extracellular vestibule is a four-residue selectivity filter (538TIID541).
Figure 3 | Permeation pathway.  

- **a–c**, Side (a), central slice (b) and top (c) views of TRPV6cryt structure in surface representation, coloured by electrostatic potential. 
- **d**, Ribbon diagram of the TRPV6cryt tetramer, with ion conduction pathway shown in cyan. 
- **e**, Expanded view of the TRPV6cryt pore, with front and back subunits excluded for clarity. Acidic side chains in the extracellular vestibule and pore-lining side chains are shown as sticks. 
- **f**, Radius of the pore calculated using HOLE. 

D541 and M577 form narrow constrictions at the selectivity filter and intracellular gate, respectively. 

- **g, h**, Top views of narrow constrictions formed by D541 (g) and M577 (h). In h, blue and pink shows electron density for M577 (2Fo − Fc, 45–3.25 Å, 1.0σ) and anomalous difference electron density from selenomethione-labelled crystal (30–5.00 Å, 3.0σ), respectively.

Figure 4 | Cation-binding sites in the TRPV6cryt pore.  

- **a, c, e** and top (b, d, f) views of the TRPV6cryt pore, with residues important for cation binding shown in stick representation. Front and back subunits in a, c and e are removed for clarity. Green, blue and pink mesh shows anomalous difference electron density for Ca2+ (a, b, 38–4.59 Å, 2.7σ), Ba2+ (c, d, 38–4.59 Å, 3.5σ) and Gd3+ (e, f, 38–4.59 Å, 7σ), and ions are shown as spheres of the corresponding colour. Purple mesh shows simulated-annealing Fo − Fc electron density maps contoured at 4σ for Ca2+ (50–3.65 Å), 3σ for Ba2+ (50–3.85 Å) and 3.5σ for Gd3+ (50–3.80 Å). The amplitudes of the anomalous peaks are listed in Extended Data Fig. 8c.

D547 and E518 side chains are apparently involved in coordination of Ba2+ ions at the recruitment sites. The Gd3+ recruitment sites are distinct from Ba2+ and apparently involve coordination by D517, E518 and D547 side chains.

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The strongest anomalous difference peaks for Ca$^{2+}$ and Gd$^{3+}$ were observed along the central pore axis at or near the same plane as D541 side chains (Figs 4a, b, e, f), indicating that this locus constitutes the main cation-binding site (site 1). The cation–oxygen distance of 2.4 Å (Fig. 5b) matches the reported average Ca$^{2+}$–oxygen distance calculated from crystal structures of various classes of Ca$^{2+}$-binding proteins\(^{30}\). This minimal interatomic distance suggests that the carboxylate oxygens of D541 directly coordinate an at least partly dehydrated Ca$^{2+}$ ion at this site. Similarly, structural studies of the hexameric Ca$^{2+}$-release-activated channel Orai suggest that Ca$^{2+}$ selectivity is achieved by direct coordination of Ca$^{2+}$ by a ring of glutamate residues at the extracellular entrance to the pore\(^{31}\). By contrast, in Ca$_V$Ab, the permeant Ca$^{2+}$ ion indirectly interacts with the pore through water molecules\(^{28}\). The presence of a robust Gd$^{3+}$ signal at site 1 shows that trivalent can bind at D541 as well (Fig. 4e, f).

For Ca$^{2+}$ and Ba$^{2+}$, an additional anomalous difference signal is observed at the centre of the pore, 6–8 Å below site 1, between the backbone carbonyls and side-chain hydroxyl groups of T538 (site 2). The greater Ca$^{2+}$/Ba$^{2+}$–oxygen distance at site 2 (~4 Å, Figs 4a, c and 5c) indicates that the cation is equatorially hydrated at this location. Although the chemical environment of site 2 suggests that it binds cations at lower affinity than site 1, the Ba$^{2+}$ signal is stronger at this site (Extended Data Fig. 8c). The different relative anomalous peak intensities of sites 1 and 2 for Ca$^{2+}$ and Ba$^{2+}$, as well as their slightly different positions at site 1, may arise from the greater size of Ba$^{2+}$ (~3 Å diameter) than Ca$^{2+}$ (~2 Å diameter). This observation implies that the TRPV6 selectivity filter discriminates ions on the basis of size as well as charge.

Anomalous difference peaks were observed for Ca$^{2+}$ and Ba$^{2+}$ 6.8 Å below site 2 in the centre of the hydrophobic cavity, at the level of M569 (site 3) (Figs 4a, c and 5a, d). For Ca$^{2+}$, the anomalous peak at site 3 is less robust (Extended Data Fig. 8c), presumably because of weaker anomalous diffraction properties. The signal at site 3 suggests that cations bound here are ordered by water molecules, which can be held in place by weak hydrogen bonding interactions and pore helix dipoles pointing their partial negative charges towards the centre of the hydrophobic cavity.

**Mechanism of ion permeation**

The pore architecture and locations of cation-binding sites in the TRPV6$_{cryst}$ structure (Fig. 5a–d) illuminate a potential calcium permeation mechanism (Fig. 5e). The close proximity of carboxylate side chains at site 1 suggests that, in the present pore conformation, the absence of a bound Ca$^{2+}$ ion would be energetically unfavourable because of charge repulsion between D541 side chains. Thus, it is likely that a Ca$^{2+}$ ion is, in effect, constitutively bound at site 1 and removal of a Ca$^{2+}$ ion from site 1 would require immediate replacement with another Ca$^{2+}$ ion, necessitating a ‘knock-off’ mechanism of permeation similar to the genetically engineered Ca$^{2+}$-selective channel Ca$_V$Ab\(^{28}\). Given the large energetic barrier of displacing a Ca$^{2+}$ ion at site 1, a substantially high local concentration of Ca$^{2+}$ would be necessary for permeation to proceed at physiological membrane voltages. Recruitment sites in the highly electronegative extracellular vestibule might serve this purpose.

As direct coordination by aspartate side chains suggests that site 1 is the highest affinity site for Ca$^{2+}$ in TRPV6 channel pore, knock-off from site 1 is likely to be the rate-limiting step for Ca$^{2+}$ permeation. After the Ca$^{2+}$ ion is knocked off site 1, it moves towards site 2, where it is coordinated through its hydration shell by the backbone carbonyls and sidechain hydroxyls of T538. In Ca$_V$Ab\(^{28}\), Ca$^{2+}$ also binds in the middle of the selectivity filter, at a locus between site 1 and site 2 of TRPV6$_{cryst}$ (Extended Data Fig. 6m, n). Although we found no crystallographic evidence for Ca$^{2+}$ bound at an equivalent site in TRPV6$_{cryst}$, it is plausible that such a site is occupied transiently during stepwise Ca$^{2+}$ permeation. Whether a knock-off is necessary for the Ca$^{2+}$ ion to traverse from site 2 to site 3 is unclear, as electrostatic repulsion between sites 2 and 3 suggest that electrostatic repulsion between Ca$^{2+}$ ions does not preclude simultaneous binding at all three sites.
between Ca\(^{2+}\) ions at site 1 and site 2 (and possibly, the aforementioned site between sites 1 and 2) may contribute a driving force. At site 3, the Ca\(^{2+}\) ion is poised to enter the cell. Since the lower gate is closed in the current TRPV6	extsubscript{apo} structure, further studies are necessary to elucidate whether its opening affects cation binding in the pore.

Previous observations have suggested that, in addition to Ca\(^{2+}\), TRPV6 is permeable to other divalents (with ion permeation sequence Ca\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\) > Mn\(^{2+}\)) and weakly to trivalents (La\(^{3+}\) and Gd\(^{3+}\)) as well. The anomalous difference peaks for Ba\(^{2+}\) and Gd\(^{3+}\) indicate that the permeation mechanism of other cations differs from Ca\(^{2+}\) permeation to varying degrees. Ba\(^{2+}\), for example, apparently has a stronger anomalous electron density at site 2 (Extended Data Fig. 8c), which suggests a higher affinity for that site than site 1. Thus, knock-off of Ba\(^{2+}\) from site 2 to site 3 may be slower and more rate-limiting than knock-off from site 1 to site 2. Larger and more positively charged ions such as Gd\(^{3+}\) may permeate differently from divalent cations, since their high charge density may preclude simultaneous binding at sites 1 and 2. Nevertheless, trivalents probably block divalents from permeating by virtue of their strong positive charge, which results in higher affinity binding at site 1. Likewise, Ca\(^{2+}\) and Mg\(^{2+}\) probably block monovalent currents through an analogous mechanism. Further studies will be necessary to elucidate the intricate details of cation permeation and selectivity in epithelial Ca\(^{2+}\) channels.

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

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METHODS

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

Constructs. Using fluorescence-detection size-exclusion chromatography (FSEC)35, we screened numerous TRPV5 and TRPV6 orthologues fused to enhanced green fluorescent protein (eGFP)33 at the C terminus and identified rat TRPV6 (GenBank EMD15841.1) as the best candidate for crystallization trials. The final construct was fused to maltose binding protein (MBP) at the N terminus and to Strep tag (WSHPQFEK) at the C terminus. Expression and purification of MBP-TRPV6 was performed as described previously.23

Expression and purification. TRPV6cryst was introduced into a pEG BacMam vector26 with C-terminal thrombin cleavage site (LVPRG) followed by eGFP and streptavidin affinity tag (WSHPQFEK). Baculovirus was made in Sf9 cells (Thermo Fisher Scientific, mycoplasma test negative). For large-scale expression, suspension-adapted HEK 293S cells lacking N-acetyl-glucosaminyltransferase I (GnTI) (ATCC, mycoplasma test negative) were grown in Freestyle 293 media (Life Technologies) supplemented with 2% FBS at 37 °C in the presence of 5% CO2. The culture was transduced with P2 baculovirus once cells reached a density of 2.5 × 10^6 to 3 × 10^6 per millilitre. After 8–12 h, 10 mM sodium butyrate was added and the temperature was changed to 30 °C. Cells were harvested 48–72 h after transduction and resuspended in buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol (βME), 0.8 μM aprotinin, 2 μg ml-1 leupeptin, 2 μM pepstatin A and 1 mM phenylmethysulfonyl fluoride (PMSF). The cells were disrupted using a Misonix Sonicator (12 × 15 s, power level 7), and the resulting homogenate was clarified using a Sorval centrifuge at 9,900 g for 15 min. Crude membranes were collected by ultracentrifugation for 1 h in a Beckman Ti45 rotor at 86,000 g. The membranes were mechanically homogenized and subsequently solubilized for 2–4 h in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 20 mM n-dodecyl-β-maltopyranoside (DDM), 0.8 μM aprotinin, 2 μg ml-1 leupeptin, 2 μM pepstatin A and 1 mM PMSF. After insoluble material was removed by ultracentrifugation, streptavidin-linked resin was added to the supernatant and rotated for 4–16 h. Resin was washed with 10 column volumes of wash buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 1 and 1 mM DDM, and the protein was eluted using wash buffer supplemented with 2.5 mM β-d-desthiobiotin. The eluted fusion protein was concentrated to ~1.0 mg ml-1 and digested with thrombin at a mass ratio of 1:100 (thrombin:protein) for 1.5 h at 22 °C. The digested protein was concentrated and injected into a Superose 6 column equilibrated in a buffer composed of 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 0.5 mM DDM, Tris(2-carboxyethyl)phosphine (TCEP, 10 mM) was added to fractions with elution time corresponding to the tetrameric channel, and protein was concentrated to 2.5–3.0 mg ml-1 using a 100kDa MWCO concentrator. All purification steps were conducted on ice or at 4 °C. Typical purifications yield (by Bradford assay) and ion current per litre of transduction buffer were 500–1000 μg and 50–100 μA, respectively.

Protocols to express selenomethionine-labelled protein in HEK cells were adapted from literature37. Six to 8 h after transduction, cells were pelleted and resuspended in DMEM (Life Technologies) supplemented with 10% FBS and lacking l-methionine. After shaking methionine-depleted cells for 6 h at 37 °C, 60 mg of l-selenomethionine was added per litre of cells. Thirty-six to 48 h after transduction, cells were harvested and protein was purified using the same protocol as described above, except for the addition of 4 mM l-methionine to all purification buffers, excluding the final gel filtration buffer. This procedure yielded ~0.4 mg of selenomethionine-labelled protein per litre of transduced cells.

Crystallization and structure determination. Initial high-throughput vapour diffusion crystallization screens showed that purified TRPV6cryst crystallizes in numerous conditions containing low molecular mass polyethylene glycols (PEG 300, PEG 350 monomethyl ether (MME), PEG 400 or PEG 550 MME). The best crystals were grown using a reservoir solution consisting of 20–24% PEG 350 MME, 100 mM NaCl and 100 mM Tris-HCl pH 8.0–8.5. To increase crystal size, 50 mM ammonium formate was added to the protein immediately before crystallization. Two microlitres of protein were mixed with 1.0–1.2 μl of reservoir solution, and incubated at 20 °C in hanging-drop vapour diffusion trays. Crystals grew as thin plates and reached full size (~400 μm × ~120 μm × ~20 μm) within 2 weeks. Crystals were cryoprotected by incubating for a short time in a solution containing 33–36% PEG 350 MME, 100 mM NaCl, 100 mM Tris-HCl pH 8.2, 0.5 mM DDM and 50 mM ammonium formate, and flash frozen in liquid nitrogen. To obtain crystals with native protein, crystals were incubated with 10 mM CaCl2, 10 mM BaCl2 or 1 mM GdCl3, respectively, for at least 1 h at 4 °C before crystallization.

Crystals of selenomethionine-labelled protein were grown and cryoprotected using the same procedure as crystals of native protein. Diffraction data collected at APS (beamlines 24-ID-C/E), NSLS (beamlines X25 or X29) or ALS (beamlines 5.0.1 or 5.0.2) were processed using XDSS30 or HKL2000 (ref. 37). The initial structural solution was obtained by molecular replacement using Phaser31 and the structure of mouse TRPV6 ankyrin domain (PDB accession number 2ZA7)32 as a search probe and the rest of the molecule was iteratively built using rat TRPV1 structure (PDB accession number 3SJP)33 as a guide. The model encompasses most of the polyepptide (residues 27–637), excluding parts of the S2–S3 linker (residues 409–416) and S4–S5 linker (residues 471–479), which were not clearly visible in the electron density map. The model was refined by alternating cycles of building in COOT40 and automatic refinement in Phenix41 or Refmac42. Correct sequence registry was aided by anomalous difference Fourier maps calculated from crystals grown in the presence of 10 mM Ca2+ to highlight sulfur atoms of cysteines and methionines, and from crystals labelled with selenomethionine to highlight selenium atoms (Extended Data Fig. 3). To confirm sequence registry in the C-terminal region, where native methionines are absent, selenomethionine-labelled crystals were produced for protein containing a methionine substitution at L630 (L630M). The anomalous difference Fourier maps were calculated from X-ray diffraction data collected at 1.75 Å for Ca2+ and Ba2+, 1.56 Å for Gd3+ and 0.97 Å for selenium. All structural figures were prepared in PyMol43. Surface representation of the ion permeation pathway was generated using the PyMol plugin version of Caver44. The pore radius was calculated using HOLE45.

Fura-2 AM measurements. Wild-type rat TRPV6 or TRPV6cryst fused to C-terminal strep tag was expressed in HEK cells as described above. Forty-eight to 72 h after transduction, cells were harvested by centrifugation at 600 g for 5 min. The cells were resuspended in pre-warmed modified HBS (118 mM NaCl, 4.8 mM KCl, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES pH 7.4) containing 5 μl ml-1 of Fura-2 AM (Life Technologies) and incubated at 37 °C for 45 min. The loaded cells were then centrifuged for 5 min at 600 g, and resuspended in pre-warmed, modified HBS and incubated again at 37 °C for 20–30 min in the dark. The cells were subsequently pelleted and washed twice, then resuspended in modified HBS for experiments. The cells were kept on ice in the dark for maximum of ~2 h before fluorescence measurements, which were conducted using a QuantMaster 40 spectrophotofluorometer (Photon Technology International) at ~25 °C in a quartz cuvette under constant stirring. Intracellular Ca2+ was measured by taking the ratio of two excitation wavelengths (340 and 380 nm) at one emission wavelength (510 nm). The PAM channel was switched at 1-s intervals.

Isotothermal titration calorimetry experiments. To study the energetics of Gd3+ binding, we performed ITC experiments. For these, we used a MicroCal Auto-ITC200 (Malvern Instruments) instrument at the Columbia University ITC Facility. Wild-type TRPV6 protein was purified in buffer containing 20 mM Tris, 150 mM NaCl, 1 mM DDM and 1 mM βME (buffer A), and the same buffer A was also used to dissolve the desired concentrations of Gd3+ to avoid buffer mismatch. The experiments were performed at 25 °C using 2-μl volume injections for the titration and 700 rpm stirring speed for mixing the reactants. The experiments were performed by titrating 700 μM Gd3+ (by robotically controlled syringe) to 60 μM TRPV6 protein per litre of transduction buffer. The complete run was performed to calculate the heat of dilution for each injection by injecting the same volumes of Gd3+ into buffer A. The data were analysed using a specialized program in Origin (MicroCal ITC).

Cystine crosslinking experiments. For SDS–PAGE and FSEC analysis, cysteine substitutions were introduced into the TRPV6cryst background with five exposed cysteines mutated to alanine or serine (C14S, C20S, C70A, C610A and C618A), and the surface mutation I62Y was reverted to the native isoleucine. Cysteine-substituted mutants with C-terminal EGFP and streptavidin affinity tag were expressed in HEK cells in the same way as protein for crystallization and purified with the same protocol. Crude cell pellets were resuspended in buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 20 mM DDM, 0.8 μM aprotinin, 2 μg ml-1 leupeptin, 2 μM pepstatin A, 1 mM PMSF and stirred for 1–3 h. After insoluble material was removed by ultracentrifugation, streptavidin-linked resin was added to the supernatant and rotated for 4–16 h. Further steps were...
performed in an identical manner to protein purification for crystallization as described above, with the exceptions that the final gel filtration buffer lacked βME, and TCEP was not added to purified protein. Within 24 h of purification, the protein samples were run on a 4–20% SDS–PAGE and visualized by Coomassie blue staining. A small portion of protein was subjected to FSEC analysis.32

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Extended Data Figure 1 | Functional characterization of wild-type rat TRPV6 and TRPV6cryst. a, b, d, e, g, h. Representative ratiometric fluorescence measurements for HEK cells expressing wild-type rat TRPV6 (a, d, g) or TRPV6cryst (b, e, h). Arrows indicate the time at which the corresponding ion was added. After resuspending the cells in nominally calcium-free buffer, addition of Ca\(^{2+}\) (a, b) or Ba\(^{2+}\) (d, e) resulted in robust concentration-dependent increase in Fura-2 signal for both wild-type rat TRPV6 and TRPV6cryst. In contrast, pre-incubation of cells with increasing concentrations of Gd\(^{3+}\) resulted in concentration-dependent reduction in Fura-2 signal for both wild-type rat TRPV6 and TRPV6cryst. 

Representative ratiometric fluorescence measurements for HEK cells expressing wild-type rat TRPV6 (a, d, g) or TRPV6cryst (b, e, h). Arrows indicate the time at which the corresponding ion was added. After resuspending the cells in nominally calcium-free buffer, addition of Ca\(^{2+}\) (a, b) or Ba\(^{2+}\) (d, e) resulted in robust concentration-dependent increase in Fura-2 signal for both wild-type rat TRPV6 and TRPV6cryst. In contrast, pre-incubation of cells with increasing concentrations of Gd\(^{3+}\) resulted in concentration-dependent reduction in Fura-2 signal for both wild-type rat TRPV6 and TRPV6cryst.

i. Dose–response curves for Gd\(^{3+}\) inhibition calculated for wild type (blue) and TRPV6cryst (red) (n = 3 for all measurements). The changes in the fluorescence intensity ratio at 340 and 380 nm (F\(_{340}/F_{380}\)) evoked by addition of 2 mM Ca\(^{2+}\) after pre-incubation with various concentrations of Gd\(^{3+}\) were normalized to the maximal change in F\(_{340}/F_{380}\) after addition of 2 mM Ca\(^{2+}\) in the absence of Gd\(^{3+}\). The apparent values of half-maximum inhibitory concentration (IC\(_{50}\)) for wild type (3.87 ± 0.83 μM) are comparable to TRPV6cryst (2.57 ± 0.28 μM). Overall, the mutations introduced to crystallize TRPV6 did not significantly alter its cation permeation and inhibition properties. The absence of time-dependent decay of the Fura-2 AM signal in the case of TRPV6cryst is presumably due to its C-terminal truncation, which eliminated a calmodulin-binding site involved in Ca\(^{2+}\)-dependent inactivation of TRPV6 (ref. 46). Error bars, s.e.m.
**Extended Data Figure 2 | Electron density.**

a. Stereo view of $2F_o - F_c$ electron density map (blue mesh, 45–3.25 Å, 1.0$\sigma$) superimposed onto a ribbon model for the entire TRPV6$_{cryst}$ monomer. b–g. Close-up views of the $2F_o - F_c$ map for various portions of TRPV6$_{cryst}$ model, with side chains shown in stick representation. In e, two diagonally opposed subunits are shown to clarify the position of the central pore axis, and the bound Ca$^{2+}$ ion is shown as a green sphere. In f, inset shows expanded view of the boxed region, demonstrating electron density for connectivity in the S6-TRP helix linker that is distinct from other TRP channel structures$^{14,16,17}$.  

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Extended Data Figure 3 | Anomalous difference Fourier maps for sulfur and selenium. a–c, Fragments of the TRPV6\textsubscript{cryst} model (yellow ribbon) superimposed onto anomalous difference Fourier maps from X-ray diffraction data collected at 1.75 Å from crystals grown in 10 mM Ca\textsuperscript{2+} (cyan mesh, 38–4.59 Å, 3.0σ) and at 0.979 Å from selenomethionine-labelled crystals (pink mesh, 30–5.00 Å, 3.2σ) of TRPV6\textsubscript{cryst}. Anomalous signal collected from a selenomethionine–labelled crystal of TRPV6\textsubscript{cryst} with L630M substitution (a, green mesh, 30–7.20 Å, 3.2σ) was used to aid registry in the C-terminal β3 strand. Domains are labelled in blue. Cysteine and methionine residues are shown as sticks and labelled. Sulfur anomalous difference peaks were observed for all cysteines in the TRPV6\textsubscript{cryst} model. Selenium anomalous difference peaks were observed for all methionines in the model, except for M480 and M484 in S5, presumably because of flexibility.
Extended Data Figure 4 | Comparison of TRPV6\textsubscript{crys} and TRPV1.

a, Bottom-up view of TRPV6\textsubscript{crys} (blue) and TRPV1 (salmon) tetramers, with ankyrin repeat domain and linker domain helices shown as cylinders. When S1–S4 domains are aligned, as shown, the cytoplasmic skirt of TRPV6 is rotated clockwise with respect to the cytoplasmic skirt of TRPV1. b, Side view of TRPV6\textsubscript{crys} (blue) and TRPV1 (salmon) monomers with S1–S4 domain based alignment. The ankyrin repeat domain of TRPV1 extends slightly further into the cytoplasm than TRPV6\textsubscript{crys}. c, Alignment of TRPV6\textsubscript{crys} (blue) and TRPV1 (salmon) transmembrane domains. Adjacent S1–S4 and pore domains are shown for comparison. Similar to TRPV1, aromatic residues pack against each other to immobilize the TRPV6\textsubscript{crys} S1–S4 domain core (shown as sticks). The absence of curvature in S5 and the long extracellular S1–S2 loop protruding towards the pore are distinct features of the TRPV6\textsubscript{crys} transmembrane domain. d, Alignment of the TRPV6\textsubscript{crys} TRP helix, C-terminal hook and three stranded β-sheet with homologous domains in the TRPV1. Conserved residues (Extended Data Fig. 7) are shown in stick representation.

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Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | Cysteine crosslinking at the intracellular skirt interface and putative desthiobiotin-binding site at the intracellular intersubunit interface. a, The TRPV6cryst tetramer with each subunit coloured differently (top) and expanded view of boxed region (bottom), with cysteine-substituted residues shown as sticks. Dashed line and label show Cα–Cα distance. b, SDS–PAGE (4–20% gradient gel) analysis of purified TRPV6 cysteine-substituted mutants in the presence (left) and absence (right) of reducing agent. Cysteines were introduced into a background construct (TRPV6CysKO), in which exposed cysteines in TRPV6cryst were mutated to serine or alanine (C145, C205, C70A, C610A and C618A) to prevent non-specific aggregation. Positions corresponding to monomer and tetramer bands are indicated by filled and open triangles, respectively. The appearance of a robust band corresponding to covalently crosslinked tetramer in the D34C–R631C double mutant indicates that the interacting N-terminal helix (which precedes the S1–S4 domain) and β3 strand (which follows the TRP helix) are from different protomers. Taken together with the S6–TRP helix linker connectivity (Extended Data Fig. 2f) that is different from TRPV1/2 (refs 14, 16) and TRPA1 (ref. 17), these data suggest a non-swapped arrangement of the pore and S1–S4 domains; if the canonical domain-swapped arrangement were true, the interacting N-terminal helix and β3 strand would be from the same monomer and no crosslinked high molecular mass species would form. However, in the absence of interpretable density for the S4–S5 linker, we suggest cautious interpretation of this domain arrangement. c, FSEC analysis of purified TRPV6CysKO crosslink mutants in the absence of reducing agent. Each trace shows a single major peak with elution time corresponding to the TRPV6cryst tetramer (black trace). d, e, The putative DTB-binding site is composed of a pocket formed by the N-terminal helix and ankyrin repeats 2–4 of one subunit (blue) and the linker domain of an adjacent subunit (green). DTB is shown as ball and stick, with 2Fo − Fo density shown as grey mesh (45–3.25 Å, 1.0 σ). In d, residues that contact DTB are shown as sticks. In e, the binding pocket is shown in surface representation. Interestingly, the DTB-binding site overlaps with the ATP-binding site revealed in the ankyrin domain crystal structure of TRPV1 (ref. 47), which was later demonstrated to be conserved in TRPV3 and TRPV4 (ref. 48). The presence of DTB close to this location in TRPV6 corroborates the assertion made in ref. 14 that ligands bound in this region modulate activity by perturbing subunit interactions. Further work is necessary to establish a functional role, if any, of DTB-like compounds on TRPV6 function. f–h, Comparison of the putative DTB-binding site in TRPV6cryst (f) and the ATP-binding site in the crystal structure of the TRPV1 ankyrin domain (g, PDB accession number 2PNN). DTB and ATP are shown in ball and stick. While the ATP-binding site in TRPV1 is shifted towards ankyrin repeat finger 1, both binding sites are located at intersubunit interfaces, as illustrated when the structures are superimposed (h).
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Extended Data Figure 6 | Comparison of the ion channel pore in TRPV6<sub>cryst</sub> with other tetrameric ion channels. a–l, The pore of TRPV6<sub>cryst</sub> (yellow ribbon) was aligned with TRPV1 (a, PDB accession number 3J5P), Na<sub>V</sub>Ab (b, PDB accession number 3RVY), Slo2.2 (c, PDB accession number 5A6E), TRPA1 (d, PDB accession number 3J9P), K<sub>v</sub>1.2 (e, PDB accession number 2R9R), KcsA (f, PDB accession number 1BL8), InsP3R1 (g, PDB accession number 3JAV), RyR1 (h, PDB accession number 3J8H), NaVRh (i, PDB accession number 4DXW), Ca<sub>v</sub>Ab (j, PDB accession number 4MVM), Ca<sub>v</sub>1.1 domains I and III (k, PDB accession number 3JBR) and Ca<sub>v</sub>1.1 domains II and IV (l, PDB accession number 3JBR). In each of the alignments, acidic residues located at or close to the selectivity filter region are shown as sticks for comparison. Notably, structures of Ca<sup>2+</sup>-permeable channels (a, d, g, h, j–l) display a high concentration of acidic residues in the outer pore region. In a–c, methionine residues close to the S6 bundle crossing are shown as sticks. Notably, the methionine at the lower gate points away from the pore in TRPV1 (a), despite high sequence conservation in this region among TRPV channels (Extended Data Fig. 7). In Slo2.2 (b) and Na<sub>V</sub>Ab (c), methionine side chains occlude the lower gate as in TRPV6<sub>cryst</sub>, indicating that the closed conformation of the lower gate can be chemically similar for Na<sup>+</sup>, K<sup>+</sup>- and Ca<sup>2+</sup>-selective channels. m–o, Comparison of calcium-binding sites in TRPV6<sub>cryst</sub> (m), the engineered voltage gated Ca<sup>2+</sup> channel Ca<sub>v</sub>Ab (n) and the putative Ca<sup>2+</sup> site in Cav1.1 (o, domains I and III are shown). Residues constituting the selectivity filters are shown in stick representation. Ca<sup>2+</sup> ions are shown as green spheres. Sites 1 and 2 from TRPV6<sub>cryst</sub> overlap with the positions of sites 1 and 3 from Ca<sub>v</sub>Ab, respectively. While it has been proposed that, owing to electrostatic repulsion, sites 1, 2 and 3 cannot be simultaneously occupied in CavAb, distances between Ca<sup>2+</sup>-binding sites in TRPV6<sub>cryst</sub> are sufficiently large such that they can be simultaneously occupied. The putative Ca<sup>2+</sup> site in Cav1.1 is near the equivalent location of site 2 in Ca<sub>v</sub>Ab.
Extended Data Figure 7 | Sequence alignment of rat TRPV subtypes. Secondary structure elements are depicted above the sequence as cylinders (α-helices), arrows (β-strands) and lines (loops). Dashed lines show residues in the TRPV6cryst construct not included in the TRPV6cryst structural model. Red boxes and a red arrow highlight substitution mutations and the C-terminal truncation point in TRPV6cryst, respectively (see Methods). The ¥ symbol marks the N-linked glycosylation site in the extracellular loop connecting S1 and S2 conserved in TRPV6 (and TRPV5) channels. The thick red line marks the location of the selectivity filter.
Extended Data Figure 8 | Isothermal titration calorimetry analysis of TRPV6 interaction with Gd$^{3+}$ and anomalous peak amplitudes. a, Gd$^{3+}$ in the syringe (700 μM) was titrated into TRPV6 (6.38 μM) loaded into the cell. Measurements were performed at 25 °C. Top, the raw data for nineteen 2-μl injections of Gd$^{3+}$. The area of each injection peak is equal to the total heat released from that injection. Bottom, the integrated heat per injection versus molar ratio. Binding of Gd$^{3+}$ to TRPV6 was analysed using models with one and two types of binding site. A model with one type of binding site was not sufficient to explain the binding isotherm (blue line). In contrast, analyses of the binding isotherm using the model with two types of binding site, according to equation $Q^\text{tot}_i = V_0M_\text{tot}((n_1\Delta H_1 K_1[X]/(1 + K_1[X])) + (n_2\Delta H_2 K_2[X]/(1 + K_2[X]))$, where $Q^\text{tot}_i$ is total heat after the $i$th injection, $V_0$ is the volume of calorimetric cell, $M_\text{tot}$ is the bulk concentration of protein, $[X]$ is the free concentration of Gd$^{3+}$, $n_1$ and $n_2$ are the numbers of type 1 and 2 sites, $K_1$ and $K_2$ are the observed equilibrium constants for each type of the sites and $\Delta H_1$ and $\Delta H_2$ are the corresponding enthalpy changes, satisfactorily described the data (red line), and the corresponding values of thermodynamic parameters are given in b. The values of $\Delta G$ and $T\Delta S$ were calculated using the following relationships: $\Delta G = -RT\ln K$ and $\Delta G = \Delta H - T\Delta S$. b, Table showing the parameters of experimental data fitting to the model with two types of Gd$^{3+}$-binding site. The straightforward interpretation of the ITC results is that the ITC type 1 ($n \approx 1$) and type 2 ($n \approx 4$) sites represent the main (site 1) and recruitment sites identified crystallographically (Fig. 4e, f). Correspondingly, the affinity to Gd$^{3+}$ for recruitment sites is $\sim$10 times lower than for site 1. c, Table showing anomalous peak amplitudes in σ calculated from data collected for Ca$^{2+}$ (38–4.59 Å), Ba$^{2+}$ (38–4.59 Å) and Gd$^{3+}$ (38–4.59 Å). No numbers are given if the peaks were not observed.
Extended Data Figure 9 | Crystal lattice contact of TRPV6\textsubscript{cryst}.

a, b, Two views of TRPV6\textsubscript{cryst} crystal packing in the $P4_212$ space group. A single TRPV6\textsubscript{cryst} protomer in the asymmetric unit is shown in blue.

c, d, Close-up views of boxed region in a. Contacting residues are shown in stick, and $C_{\alpha}$-$C_{\alpha}$ distances are labelled in d. The crystal contact is apparently mediated by cation-$\pi$ and/or hydrogen bonding interactions between these residues. Crystals in the $P4_212$ space group did not form when the native isoleucine was present at position 62.
### Extended Data Table 1 | Data collection and refinement statistics

|                  | Native | Ba\(^{2+}\) | Ca\(^{2+}\) | Gd\(^{3+}\) | L630M-SeMet | SeMet |
|------------------|--------|-------------|-------------|-------------|-------------|-------|
| **Data Collection** |        |             |             |             |             |       |
| Beamline         | APS-24ID-C | APS-24ID-C | APS-24ID-C | APS-24ID-C | APS-24ID-E | APS-24ID-E |
| Space group      | P42,2  | P42,2       | P42,2       | P42,2       | P42,2       | P42,2  |
| Cell dimensions  |        |             |             |             |             |       |
| a, b, c (Å)      | 143.81 | 144.35      | 144.35      | 144.35      | 143.60      | 143.95 |
|                  | 143.81 | 144.35      | 144.35      | 144.35      | 143.60      | 143.95 |
|                  | 113.22 | 113.37      | 113.37      | 113.37      | 114.44      | 113.04 |
| α, β, γ (°)      | 90 90 90 | 90 90 90 | 90 90 90 | 90 90 90 | 90 90 90 | 90 90 90 |
| Wavelength (Å)   | 0.9791 | 1.75        | 1.75        | 1.7101      | 0.9792      | 0.9792 |
| Resolution (Å)*  | 44.48 - 3.25 (3.36 - 3.25) | 49.56 - 3.85 (3.99 - 3.85) | 49.56 - 3.65 (3.78 - 3.65) | 50.00 - 3.80 (3.936 - 3.80) | 40.00 - 7.20 (7.46 - 7.20) | 40.00 - 5.00 (5.18 - 5.00) |
| Completeness (%)* | 96.0 | 99.5 | 99.9 | 99.5 | 99.9 | 100.0 |
| Redundancy*      | 8.7  | 15.4       | 26.5       | 11.4       | 16.3       | 13.7  |
|                  | (9.2) | (13.9)     | (17.2)     | (6.4)      | (17.5)     | (13.9) |
| ||l||l*          | 16.9  | 15.2       | 25.0       | 19.4       | 27.3       | 21.8  |
|                  | (1.3) | (1.5)      | (2.4)      | (1.6)      | (6.3)      | (4.7) |
| R<sub>max</sub> (%)* | 9.8 | 13.1 | 10.6 | 8.9 | 20.7 | 19.2 |
|                  | (132.6) | (228.5) | (143.1) | (120.7) | (86.4) | (93.5) |
| CC<sub>1/2</sub> | 99.8 | 98.0 | 99.5 | 99.9 | 98.5 | 98.3 |
|                  | (85.7) | (76.8) | (85.7) | (63.7) | (89.8) | (92.5) |
| **Refinement**   |        |             |             |             |             |       |
| Resolution (Å)*  | 44.48 - 3.25 (3.36 - 3.25) | 49.56 - 3.85 (3.99 - 3.85) | 50.00 - 3.65 (3.78 - 3.65) | 49.56 - 3.80 (3.94 - 3.80) |
| Completeness (%)  | 96 | 100 | 100 | 99 |           |       |
|                  | (93.8) | (99.9) | (99.9) | (96.9) |           |       |
| Number of reflections | 18531 | 21705 | 25439 | 22443 |           |       |
|                  | (1724) | (2187) | (2521) | (2170) |           |       |
| R<sub>free</sub>/R<sub>calc</sub> | 0.273/0.289 | 0.291/0.326 | 0.276/0.281 | 0.298/0.321 |
| Number of atoms  |        |             |             |             |             |       |
| Total            | 4747 | 4775 | 4735 | 4759 |           |       |
| Ligand           | 16 | 19 | 18 | 17 |           |       |
| B-factor (Å<sup>2</sup>) |        |             |             |             |             |       |
| Protein          | 120.5 | 143.8 | 135.1 | 144.23 |           |       |
| Ligand           | 77.27 | 136.75 | 24.14 | 178.86 |           |       |
| RMS deviations   |        |             |             |             |             |       |
| Bond length (Å)  | 0.003 | 0.002 | 0.003 | 0.002 |           |       |
| Bond angles (°)  | 0.7 | 0.62 | 0.62 | 0.63 |           |       |
| Ramachandran     |        |             |             |             |             |       |
| Favored (%)      | 93.6 | 92.9 | 93.7 | 92.8 |           |       |
| Allowed (%)      | 5.7 | 6.93 | 6.13 | 7.03 |           |       |
| Disallowed (%)   | 0.17 | 0.17 | 0.17 | 0.17 |           |       |

*Highest resolution shell in parentheses.
Five per cent of reflections were used for the calculation of R<sub>free</sub>.