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Crystal Structure of the Epithelial Calcium Channel TRPV6

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Summary

Precise regulation of calcium homeostasis is essential for many physiological functions. The Ca^{2+}-selective TRP channels TRPV5 and TRPV6 play vital roles in calcium homeostasis as Ca^{2+} uptake channels in epithelial tissues. Detailed structural bases for their assembly and Ca^{2+} 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 to 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, Ca^{2+} selectivity is determined by direct coordination of Ca^{2+} by a ring of aspartate side chains in the selectivity filter. Based on crystallographically identified cation binding sites at the pore axis and extracellular vestibule, we propose a Ca^{2+} permeation mechanism. Our results provide a structural foundation to understand the regulation of epithelial Ca^{2+} uptake and its role in pathophysiology.

Introduction

The transient receptor potential (TRP) channels are a superfamily of cation permeable ion channels that are widely known for their role as transducers of sensory modalities[1]. TRPV5 and TRPV6 are TRP channels that are uniquely selective for Ca^{2+} ($P_{Ca}/P_{Na} > 100$)[2]. They have not been reported to be responsive to temperature, tastants or odors, but the mechanosensitive properties of TRPV6 appear to be important for the formation of microvilli[3]. TRPV5 and TRPV6 belong to the vanilloid (TRPV) subfamily of TRP channels, share ~75% sequence identity, and are involved in the transport of calcium through epithelial cell membranes[4]. Knockout of TRPV6 in mice leads to various phenotypes linked to impaired Ca^{2+} homeostasis, including defective intestinal Ca^{2+} absorption, lower body weight, impaired fertility and dermatitis[5–8]. Altered TRPV6 expression has also been shown in various transgenic mouse models of human diseases[8], including Crohn's-like and kidney

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Author Information The structure coordinates have been deposited to the Protein Data Bank under accession codes 5IWK, 5IWP, 5IWR and 5IWT (see Extended Data Table 1 for detail). Reprints and permissions information are available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper.

stone diseases. In addition, TRPV6 is implicated in the development and progression of numerous forms of cancer and its overexpression pattern correlates with the aggressiveness of the disease[9,10]. Accordingly, TRPV6 has emerged as a target for the diagnosis and treatment of various carcinomas[11,12].

Structurally, TRPV5 and TRPV6 represent homo- or heteromeric assemblies of four subunits[13], each containing a central K^+ channel-like transmembrane (TM) domain that is flanked by intracellular N- and C-terminal domains. The overall architecture and potential gating mechanisms of TRP channels have recently been illuminated by cryo-electron microscopy (cryo-EM) structures of TRPV1[14,15], TRPV2[16] and TRPA1[17]. However, the absence of structural bases for the unique physiological properties of TRPV5 and TRPV6 motivated us to study these epithelial Ca^{2+} channels.

Structure Determination

We screened various orthologs of TRPV5 and TRPV6 and discovered rat TRPV6 as a promising candidate for our structural studies. We modified the 727-residue wild type rat TRPV6 polypeptide to create the crystallization construct $TRPV6_{cryst}$ (see Methods). Experiments with the fluorescent Ca^{2+} indicator Fura 2-AM show that cells expressing $TRPV6_{cryst}$ exhibit Ca^{2+} permeability similar to wild type (Extended Data Fig. 1).

The best crystals of $TRPV6_{cryst}$ diffracted to 3.25 Å resolution. We solved the $TRPV6_{cryst}$ 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 $TRPV6_{cryst}$ was refined to good crystallographic statistics and stereochemistry (Extended Data Table 1).

Architecture and domain organization

The four-fold symmetrical structure of $TRPV6_{cryst}$ (Fig. 1) contains two main components: a TM domain with a central ion channel pore, and a ~70-Å tall and ~110 Å wide intracellular skirt where four subunits comprise walls enclosing a ~50 Å X 50 Å wide cavity underneath the ion channel. Like TRPV1[14] (Extended Data Fig. 4) and TRPV2[16], the intracellular domains of a single $TRPV6_{cryst}$ subunit contain an ankyrin repeat domain (ARD) with six ankyrin repeats, followed by a linker domain that includes a β hairpin (composed of β-strands β1 and β2) and a helix-turn-helix motif resembling a seventh ankyrin repeat, and the pre-S1 helix, which connects the linker domain to the TM domain (Fig. 1d–f, Extended Data Fig. 4). Also similar to TRPV1/2, a six-residue stretch at the C-terminus comprises a β-strand (β3) that tethers to the β hairpin in the linker domain to create a three-stranded β-sheet. In addition to the conserved domains, $TRPV6_{cryst}$ 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[14,16,17], the TM domain of $TRPV6_{cryst}$ crudely resembles voltage gated K^+[18] or Na^+[19] channels and includes six TM helices (S1-S6) and a pore loop (P-loop) between S5 and S6. The first four TM helices form a bundle to comprise 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 domain is unstructured, which is a marked contrast from other TRP channels, where it assumes a helical conformation and mediates interdomain interactions[14,16,17]. 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[14,16,17].

Although the overall domain organization of $TRPV6_{cryst}$ resembles TRPV1/2[14,16] and to a lesser degree, TRPA1[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 TM 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 $TRPV6_{cryst}$ model cautiously due to the absence of interpretable density for the S4-S5 linker.

Assembly and subunit interfaces

Assembly of $TRPV6_{cryst}$ 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^+ channel[20] and distinct from voltage-gated channels[18]. 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 beta glucuronidase klotho, which modulates channel activity by modifying the conserved N-linked glycosylation site[21] located in the middle of this loop (N357 in $TRPV6_{cryst}$).

The intracellular domains of TRPV5 and TRPV6 have been implicated in tetrameric assembly[22], trafficking[23], and regulation of channel activity by the Ca^{2+} sensor calmodulin[24–26]. The structure of $TRPV6_{cryst}$ reveals that numerous non-contiguous intracellular domains engage in extensive inter- and intrasubunit interactions (Fig. 2c). At the center 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 Ca^{2+} uptake function in TRPV5[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, ARD, 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 $TRPV6_{cryst}$ affinity purification procedure (see Methods).

Ion-conducting pore

The extracellular portion of the $TRPV6_{cryst}$ 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).

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 (^{538}TIID^{541}) (Figs 3d–f). The side chains of D541, which have previously been identified as critical for Ca^{2+} selectivity, permeation, and voltage-dependent Mg^{2+} block[2], protrude toward the pore central axis to produce a minimum interatomic distance of 4.6 Å (Fig. 3f–g) at the upper tip of the selectivity filter. Three phenylalanine residues (F530, F533 and F536) in the pore helix, which are conserved in TRPV5–6, but only one of which is conserved in TRPV1–4 (Extended Data Fig. 7), may restrict its dynamics. A relatively static outer pore domain could reflect a key difference between TRPV5/6 and other TRPV channels, which gate in response to various stimuli and thus should display a higher degree of structural plasticity, as exemplified by toxin- and capsaicin-induced conformational changes in TRPV1[15].

Below the selectivity filter, the pore widens into a large, mainly hydrophobic cavity (Fig. 3e). Lateral pore portals (Fig. 3a–b) may provide hydrophobic access to this cavity for small molecules or lipids, similar to voltage gated Na^{+} channels[19]. The large diameter of the hydrophobic cavity (~13 Å) can easily accommodate a fully hydrated calcium ion, which has an effective diameter of 8–10 Å. The S6 helices cross at the intracellular portion of the channel, where the M577 side chains form the narrow constriction (5.1 Å diameter) and define the lower gate (Figs 3d–f), similar to TRPV2[16]. Importantly, anomalous diffraction from crystals grown with selenomethionine-labeled protein showed a robust signal (Fig. 3h, Extended Data Fig. 3c), confirming that M577 side chains occlude the pore. Despite high sequence conservation in this region (Extended Data Fig. 7), in TRPV1, the equivalent residue to TRPV6 M577 points away from the pore axis (Extended Data Fig. 6a).

Cation binding sites

Previous research has proposed that TRPV5 and TRPV6 achieve their exceptional Ca^{2+} selectivity through binding of Ca^{2+} to the selectivity filter[2]. Indeed, we observed a strong $2F_O$-F_C density consistent with a bound ion at the central pore axis, surrounded closely by the carbonyl oxygens of D541 side chains (Extended Data Fig. 2e). Since the pore diameter here (4.6 Å, measured between centers of opposing oxygen atoms) is large enough to accommodate a dehydrated calcium ion (typical Ca^{2+}-oxygen distance is ~2.4 Å), we contend that the selectivity filter is captured in a Ca^{2+}-conducting state. To further resolve cation-binding sites in the pore, we co-crystallized $TRPV6_{cryst}$ with Ca^{2+}, Ba^{2+} or Gd^{3+}, which have various permeation and channel-blocking properties[27] (Extended Data Fig. 1) and collected X-ray diffraction data to locate anomalous difference peaks.

The anomalous difference peaks suggest the presence of four types of cation binding sites in the $TRPV6_{cryst}$ channel pore (Fig. 4). Notably, two of these sites (Sites 1 and 2) have

locations approximately equivalent to Ca^{2+} sites in the genetically engineered Ca^{2+}-selective channel Ca_VAb[28], but none of these sites overlap with the putative Ca^{2+} site in $Ca_V1.1$[29] (Extended Data Fig. 6m–o). For Ba^{2+} and Gd^{3+}, four symmetry-related peaks were observed in the $TRPV6_{cryst}$ outer vestibule, in the vicinity of D517, E518 and D547 (Fig. 4c–f). Interestingly, the Ba^{2+} and Gd^{3+} sites occupy distinct locations, probably due to difference in charge density. Although these signals were not observed for $TRPV6_{cryst}$ co-crystallized with Ca^{2+} (Fig. 4a–b), presumably due to lower affinity, reduced occupancy, or weaker anomalous signal, we speculate that the highly electronegative outer vestibule is involved in the general recruitment of cations toward the extracellular vestibule of the TRPV6 channel. Lower affinity of the recruitment sites compared to the main binding site in the center of the pore for Gd^{3+} is consistent with the results of isothermal titration calorimetry experiments (Extended Data Fig. 8a–b).

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 (Fig. 4a–b,e-f), indicating that this locus comprises 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 partially 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_VAb, 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 trivalents can bind at D541 as well (Fig. 4e–f).

For Ca^{2+} and Ba^{2+}, an additional anomalous difference signal is observed at the center 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, Fig. 4a,c, Fig. 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 center of the hydrophobic cavity, at the level of M569 (Site 3)(Fig. 4a,c, Fig. 5a,d). For Ca^{2+}, the anomalous peak at Site 3 is less robust (Extended Data Fig. 8c), presumably due to 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 toward the center 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 unfavorable due to 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_VAb[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 toward Site 2, where it is coordinated through its hydration shell by the backbone carbonyls and sidechain hydroxyls of T538. In Ca_VAb[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 Ca^{2+} ions at Site 1 and Site 2 (and possibly, the aforementioned site between Site 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 $TRPV6_{cryst}$ 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 $Ca^{2+}>Sr^{2+}\approx Ba^{2+}>Mn^{2+}$)[4] and weakly to trivalents (La^{3+} and Gd^{3+})[27] 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 in comparison to Site 1. Thus, knock-off of Ba^{2+} from Site 2 to Site 3 may be slower and more rate-limiting than knockoff 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 likely 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[2] through an analogous mechanism. Further studies are necessary to further elucidate the intricate details of cation permeation and selectivity in epithelial Ca^{2+} channels.

Methods

Constructs

Using fluorescence-detection size exclusion chromatography (FSEC)[32], we screened numerous TRPV5 and TRPV6 orthologs fused to enhanced green fluorescent protein (eGFP)[33] at the C-terminus and identified rat TRPV6 (GenBank EDM15484.1) as the best candidate for crystallographic trials. The fortuitous spontaneous mutation L495Q generated during gene synthesis was found to increase expression level of rat TRPV6. C-terminal truncation mutants of rat TRPV6-L495Q produced crystals in the C222 space group that diffracted to ~6 Å resolution. Based on an initial low-resolution molecular replacement solution, we designed numerous mutations aimed at improving crystal packing, including individual substitutions of surface residues, fusions with soluble protein partners and flexible loop deletions. Incorporation of the surface residue mutations L92N and M96Q helped improve the resolution limit of crystals in the C222 space group to ~4.0 Å. Further screening of surface residue mutations yielded the amino acid substitution I62Y, which facilitated crystallization in the $P42_12$ space group and improved diffraction resolution to 3.25 Å. Inspection of protein-mediated crystal contacts in this crystal form shows that cation-π and/or hydrogen bonding interactions involving the side chains of I62Y, K63, K66 and F67 may have permitted crystallization in the $P42_12$ space group and contributed to the improved resolution (Extended Data Fig. 9). The final construct, $TRPV6_{cryst}$, comprises residues 1–668 and contains the point mutations I62Y, L92N, M96Q and L495Q.

Expression and Purification

$TRPV6_{cryst}$ was introduced into a pEG BacMam vector[34] with C-terminal thrombin cleavage site (LVPRG) followed by eGFP and streptavidin affinity tag (WSHPQFEK). Baculovirus was made in Sf9 cells. For large scale expression, suspension-adapted HEK 293S cells lacking N-acetyl-glucosaminyltransferase I (GnTI) were grown in Freestyle 293 media (Life Technologies) supplemented with 2% FBS at 37°C in the presence of 5% CO_2. The culture was transduced with P2 baculovirus once cells reached a density of 2.5–3.5 × 10^6 per ml. After 8–12 hours, 10 mM sodium butyrate was added and the temperature was changed to 30°C. Cells were harvested 48–72 hours post-transduction and resuspended in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol (βME), 0.8 μM aprotinin, 2 μg/ml leupeptin, 2 mM pepstatin A and 1 mM phenylmethysulfonyl fluoride (PMSF). The cells were disrupted using a Misonix Sonicator (12 × 15 sec, power level 7), and the resulting homogenate was clarified using a Sorval centrifuge at 7,500 rpm for 15 min. Crude membranes were collected by ultracentrifugation for 1 hour in a Beckman Ti45 rotor at 40,000 RPM. The membranes were mechanically homogenized and subsequently solubilized for 2–4 hours in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM βME, 20 mM *n*-dodecyl-β-D-maltopyranoside (DDM), 0.8 μM aprotinin, 2 μg/ml leupeptin, 2 mM 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 hours. Resin was washed with 10 column volumes of wash buffer containing 150 mM NaCl, 20 mM Tris pH 8.0, 1 mM βME, and 1 mM DDM, and the protein was eluted using wash buffer supplemented with 2.5 mM D-desthiobiotin. The eluted fusion protein was concentrated to ~1.0 mg/ml and digested with thrombin at a mass ratio of 1:100

(thrombin:protein) for 1.5 hours 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, and 0.5 mM DDM. 10 mM tris(2-carboxyethyl)phosphine (TCEP) was added to fractions with elution time corresponding to the tetrameric channel, and protein was concentrated to 2.5–3.0 mg/ml using 100 kDa MWCO concentrator. All purification steps were conducted on ice or at 4°C. Typical purifications yielded ~1 mg of purified protein per liter of transduced cells.

Protocols to express selenomethionine-labeled protein in HEK cells were adapted from literature[35]. 6–8 hours 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 hours at 37°C, 60 mg of L-selenomethionine was added per liter of cells. 36–48 hours 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-labeled protein per liter of transduced cells.

Crystallization and structure determination

Initial high throughput vapor diffusion crystallization screens showed that purified $TRPV6_{cryst}$ crystallizes in numerous conditions containing low molecular weight 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. 2.0 μl of protein was mixed with 1.0–1.2 μl of reservoir solution, and incubated at 20°C in hanging drop vapor diffusion trays. Crystals grew as thin plates and reached full size (~400 μm x ~120 μm x ~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 Ca^{2+}, Ba^{2+} or Gd^{3+}, protein was incubated with 10 mM $CaCl_2$, 10 mM $BaCl_2$ or 1 mM $GdCl_3$, respectively, for at least 1 hour at 4°C prior to crystallization. Crystals of selenomethionine-labeled 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) was processed using XDS[36] or HKL2000[37]. The initial structural solution was obtained by molecular replacement using Phaser[38] and the structure of mouse TRPV6 ankyrin domain (PDB ID: 2RFA)[39] as a search probe and the rest of the molecule was iteratively built using rat TRPV1 structure (PDB ID: 3J5P)[14] as a guide. The model encompasses most of the polypeptide (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 COOT[40] and automatic refinement in Phenix[41] or Refmac[42]. Correct sequence registry was aided by anomalous difference Fourier maps calculated from crystals grown in the presence of 10 mM Ca^{2+} to highlight sulfur atoms of cysteines and methionines, and from

crystals labeled 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-labeled 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 Ca^{2+} and Ba^{2+}, 1.56 Å for Gd^{3+} and 0.979 Å for selenium. All structural figures were prepared in PyMol[43]. Surface representation of the ion permeation pathway was generated using the PyMol plugin version of Caver[44]. The pore radius was calculated using HOLE[45].

Fura 2-AM measurements

Wild type rat TRPV6 or $TRPV6_{cryst}$ fused to C-terminal strep tag was expressed in HEK cells as described above. 48–72 hours after transduction, cells were harvested by centrifugation at 600 g for 5 minutes. The cells were resuspended in prewarmed modified HBS (118 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 10 mM HEPES pH 7.4) containing 5 μg/ml of Fura2-AM (Life Technologies) and incubated at 37°C for 45 minutes. The loaded cells were then centrifuged for 5 minutes at 600 g, and resuspended in prewarmed, modified HBS and incubated again at 37°C for 20–30 minutes 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 hours before fluorescence measurements, which were conducted using spectrofluorometer QuantaMaster™ 40 (Photon Technology International) at room temperature in a quartz cuvette under constant stirring. Intracellular Ca^{2+} was measured by taking the ratio of two excitation wavelengths (340 and 380 nm) at one emission wavelength (510 nm). The excitation wavelength was switched at 1-s intervals.

Isothermal titration calorimetry experiments

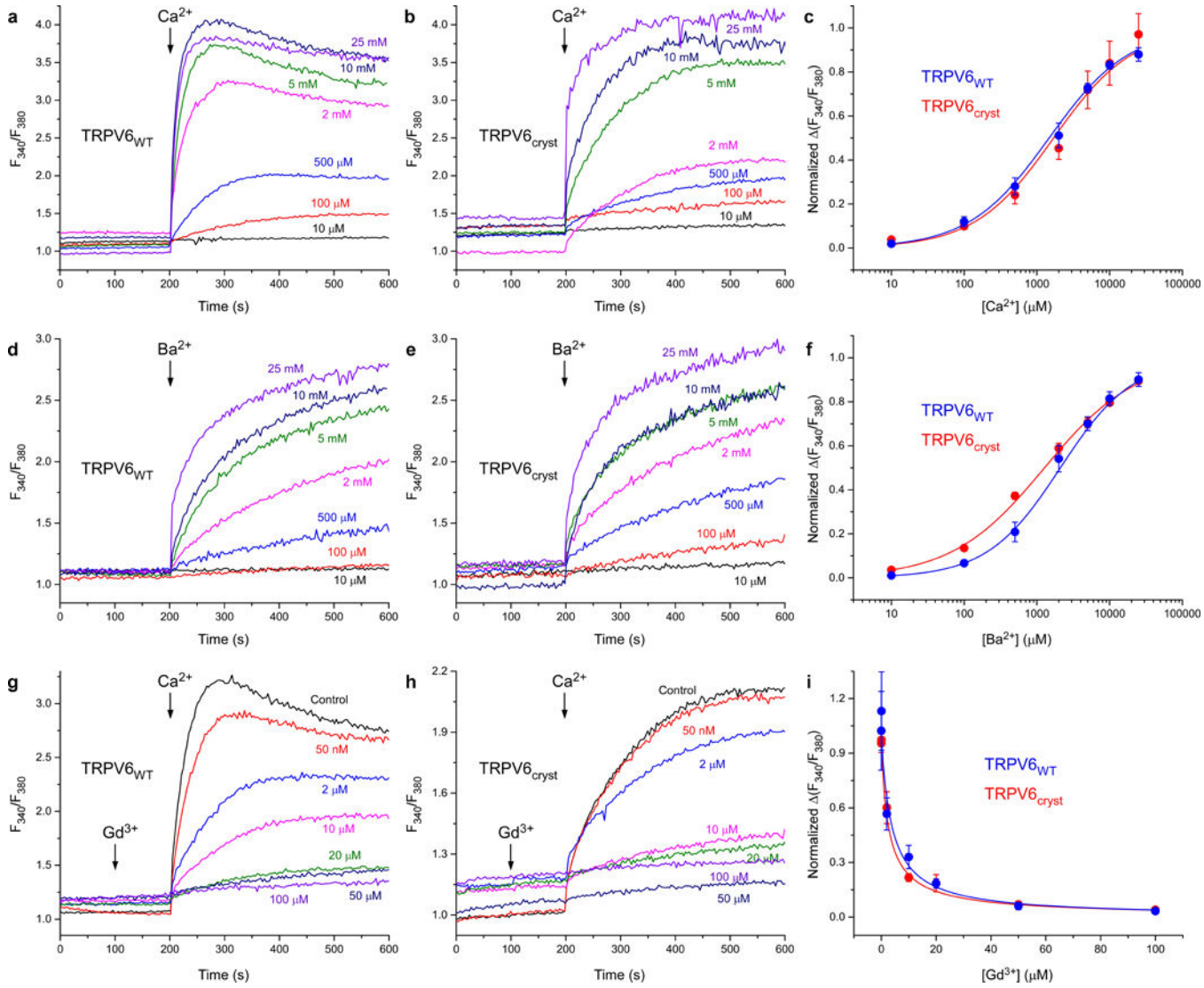
To study the energetics of Gd^{3+} block, we carried out isothermal titration calorimetry (ITC) experiments. For these experiments, we used a MicroCal Auto-iTC200 (Malvern Instruments Ltd, UK) instrument at the Columbia University ITC Facility. Wild type TRPV6 protein was purified in buffer containing 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 Gd^{3+} to avoid buffer mismatch. The experiments were carried out at 25°C using 2-μl volume injections for the titration and 700-rpm stirring speed for mixing the reactants. The experiments were carried out by titrating 700 μM Gd^{3+} (by robotically controlled syringe) to 6.38-μM TRPV6 (in cell) at 3-min intervals. The control experiments were performed to calculate the heat of dilution for each injection by injecting the same volumes of Gd^{3+} into buffer A. The data were analyzed using a specialized program in Origin (MicroCal ITC).

Cysteine crosslinking experiments

For SDS-PAGE and FSEC analysis, cysteine substitutions were introduced into the $TRPV6_{cryst}$ 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

a modified 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 leupeptin, 2 mM pepstatin A, 1 mM PMSF and stirred for 1–3 hours. After insoluble material was removed by ultracentrifugation, streptavidin-linked resin was added to the supernatant and rotated for 4–16 hours. Further steps were carried out 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 hours 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].

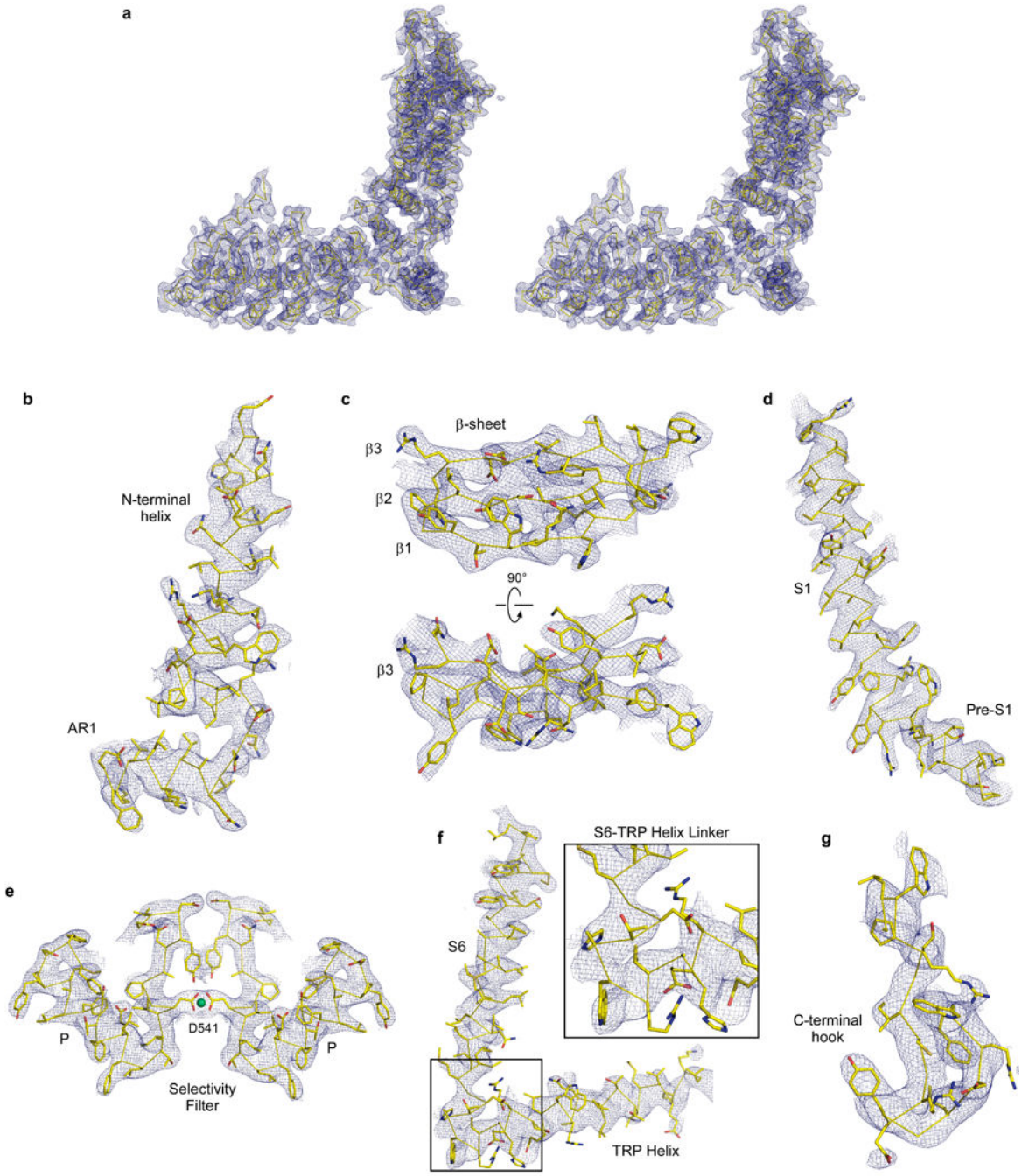
Extended Data



Extended Data Figure 1. Functional characterization of wild type rat TRPV6 and TRPV6$_{cryst}$

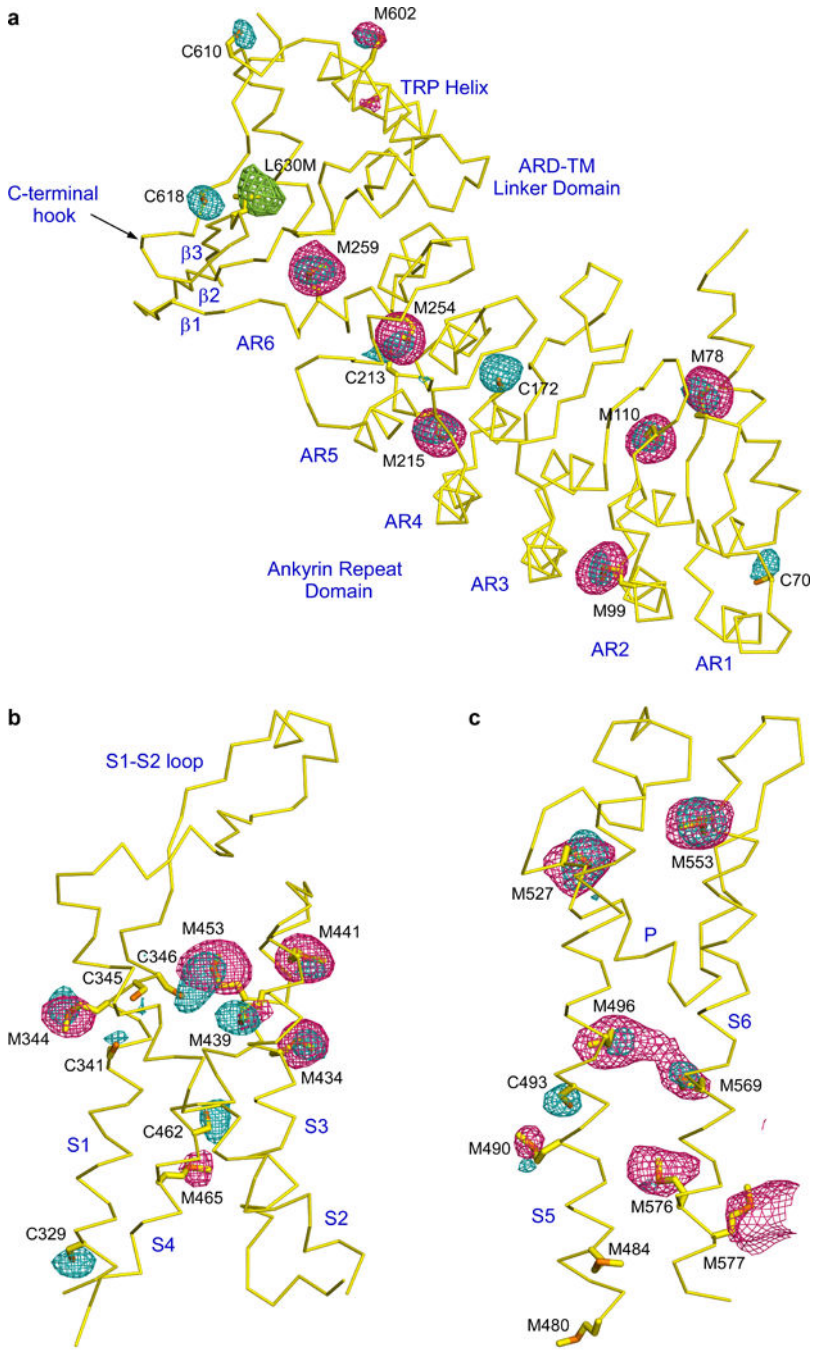
a,b,d,e,g,h, Representative ratiometric fluorescence measurements for HEK cells expressing wild type rat TRPV6 (**a,d,g**) or TRPV6$_{cryst}$ (**b,e,h**). Arrows indicate the time at which the

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 $TRPV6_{cryst}$. 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 (**g**) and $TRPV6_{cryst}$ (**h**), consistent with Gd^{3+} inhibition of wild type TRPV6 demonstrated previously using $^{45}Ca^{2+}$ uptake measurements[27] **c-f**, Dose-response curves for Ca^{2+} (**c**) and Ba^{2+} (**f**) permeation calculated for wild type (blue) and $TRPV6_{cryst}$ (red) (n=3 for all measurements). The changes in F_{340}/F_{380} were normalized to their approximated maximal values at saturating concentrations of Ca^{2+} or Ba^{2+}, respectively. The apparent values of EC_{50} for $TRPV6_{cryst}$ (1.70 ± 0.26 mM for Ca^{2+} and 1.27 ± 0.67 mM for Ba^{2+}) are similar to wild type (1.47 ± 0.80 mM for Ca^{2+} and 1.91 ± 0.74 mM for Ba^{2+}). **i**, Dose-response curves for Gd^{3+} inhibition calculated for wild type (blue) and $TRPV6_{cryst}$ (red) (n=3 for all measurements). The changes in F_{340}/F_{380} evoked by addition of 2 mM Ca^{2+} after preincubation 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 IC_{50} for wild type (3.87 ± 0.83 μM) are comparable with $TRPV6_{cryst}$ (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 Fura 2-AM signal in the case of $TRPV6_{cryst}$ is presumably due to its C-terminal truncation, which eliminated a calmodulin binding site involved in Ca^{2+}-dependent inactivation of TRPV6[46]. Error bars are for S.E.M.



Extended Data Figure 2. Electron density

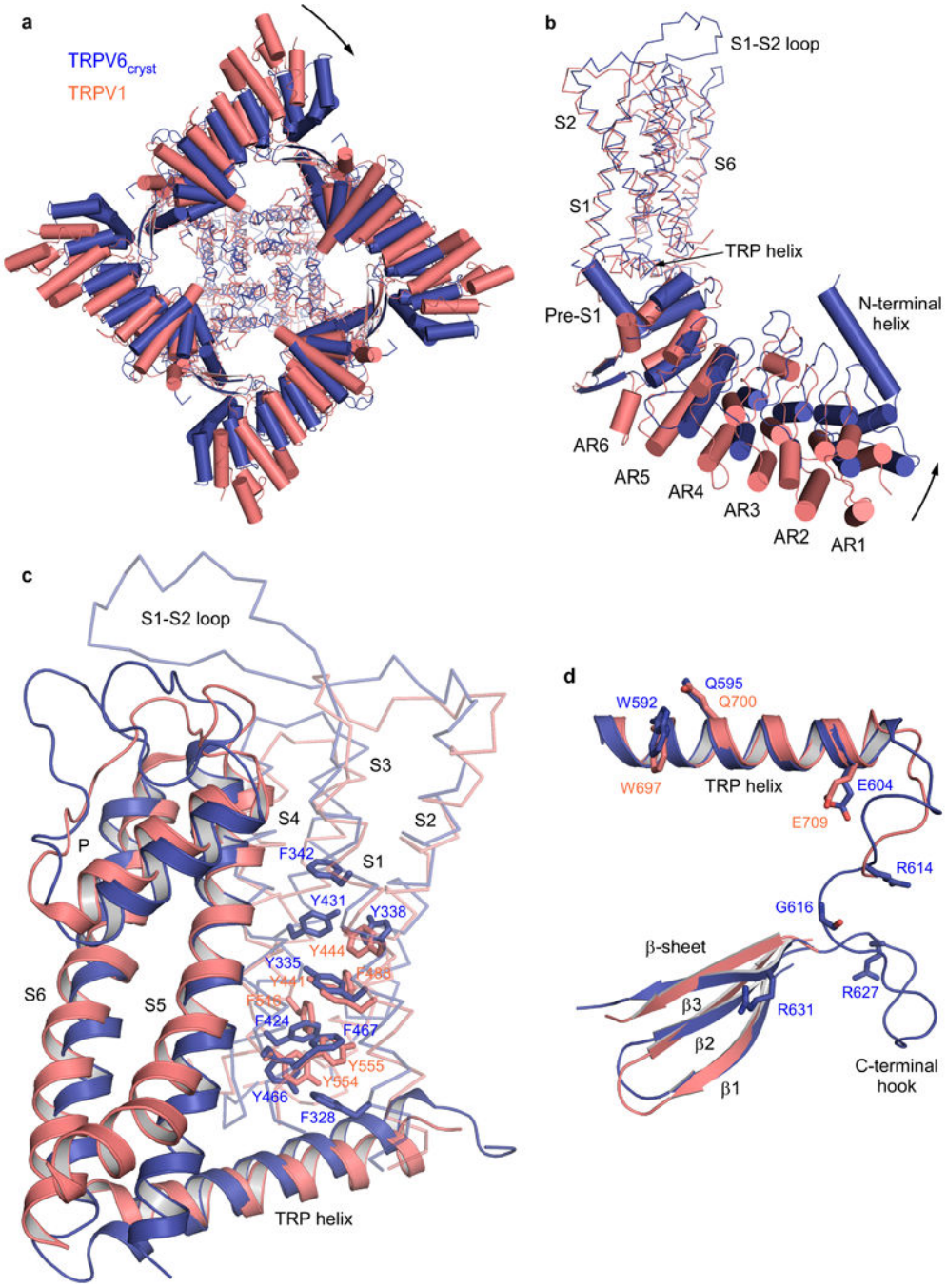
a, Stereo view of $2F_O$-F_C electron density map (blue mesh, 45–3.25 Å, 1.0σ) superimposed onto a ribbon model for the entire $TRPV6_{cryst}$ monomer. **b–g**, Close-up views of the $2F_O$-F_C map for various portions of $TRPV6_{cryst}$ model, with side chains shown in stick representation. In **e**, 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].



Extended Data Figure 3. Anomalous difference Fourier maps for sulfur and selenium

a–c, Fragments of the $TRPV6_{cryst}$ 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^{2+} (cyan mesh, 38–4.59 Å, 3.0σ) and at 0.979 Å from selenomethionine-labeled crystals (pink mesh, 30–5.00 Å, 3.2σ) of $TRPV6_{cryst}$. Anomalous signal collected from a selenomethionine-labeled crystal of $TRPV6_{cryst}$ 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 labeled in blue. Cysteine and methionine residues are shown as sticks and labeled. Sulfur anomalous

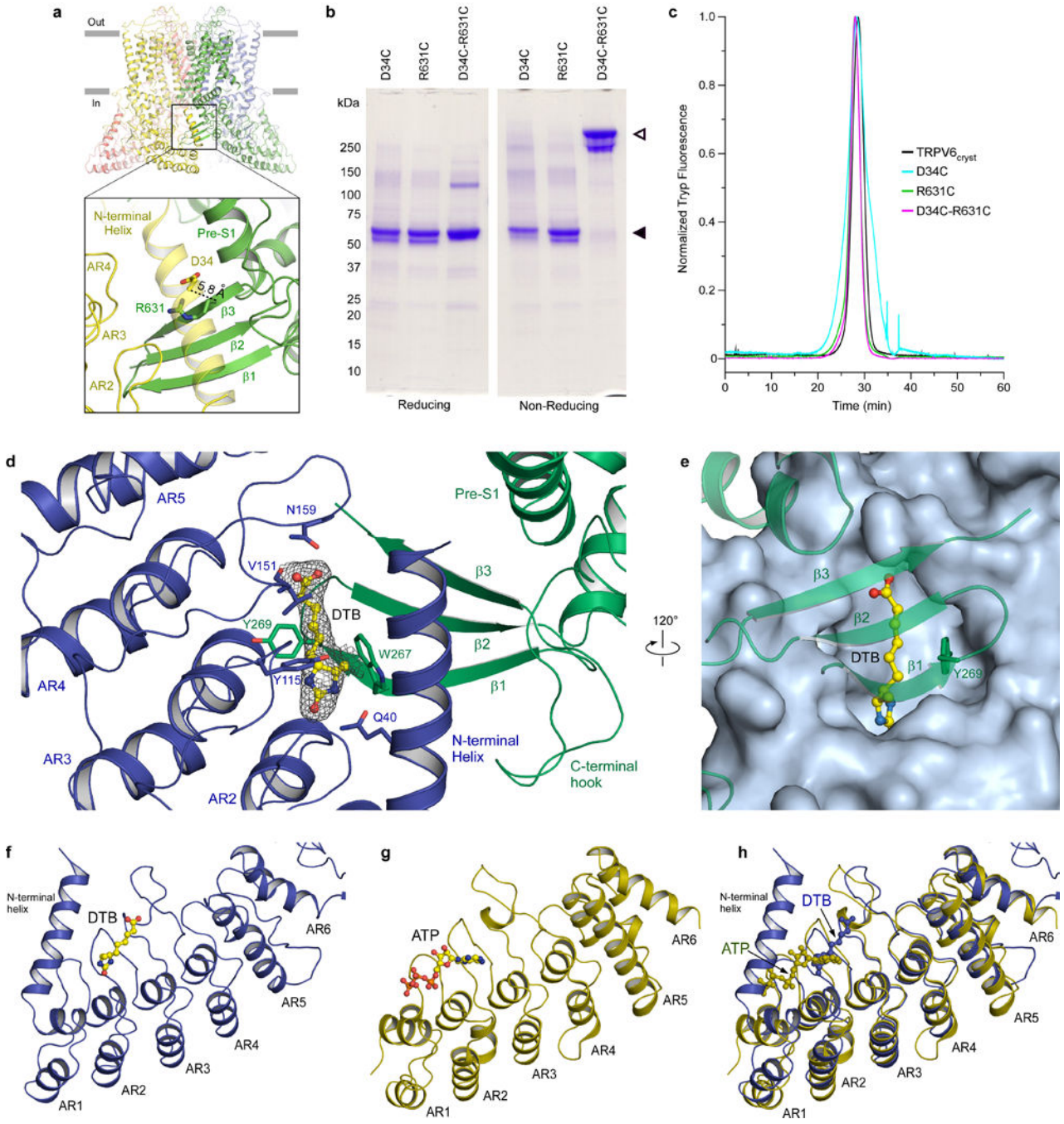
difference peaks were observed for all cysteines in the TRPV6$_{cryst}$ model. Selenium anomalous difference peaks were observed for all methionines in the model, except for M480 and M484 in S5, presumably due to flexibility.



Extended Data Figure 4. Comparison of TRPV6$_{cryst}$ and TRPV1

a, Bottom up view of TRPV6$_{cryst}$ (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

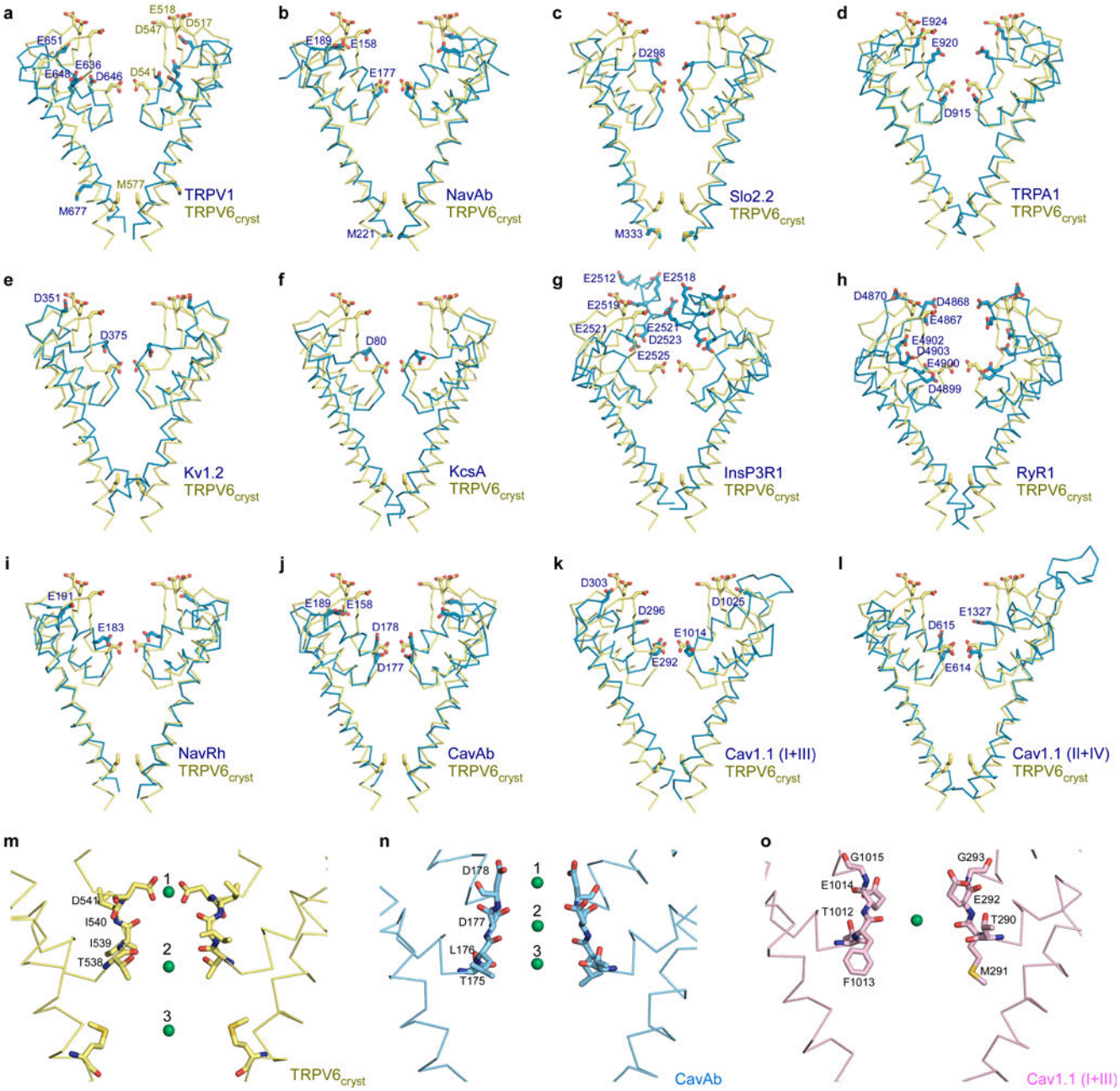
skirt of TRPV1. **b**, Side view of $TRPV6_{cryst}$ (blue) and TRPV1 (salmon) monomers with S1-S4 domain based alignment. The ankyrin repeat domain of TRPV1 extends slightly further into the cytoplasm than $TRPV6_{cryst}$**c**, Alignment of $TRPV6_{cryst}$ (blue) and TRPV1 (salmon) transmembrane domains. Adjacent S1-S4 and pore domains are shown for comparison. Similar to TRPV1, aromatic residues pack against each other to immobilize the $TRPV6_{cryst}$ S1-S4 domain core (shown as sticks). The absence of curvature in S5 and the long extracellular S1-S2 loop protruding toward the pore are distinct features of the $TRPV6_{cryst}$ TM domain. **d**, Alignment of the $TRPV6_{cryst}$ 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.



Extended Data Figure 5. Cysteine crosslinking at the intracellular skirt interface and putative desthiobiotin binding site at the intracellular intersubunit interface

a, The $TRPV6_{cryst}$ tetramer with each subunit colored differently (top panel) and expanded view of boxed region (bottom panel), with cysteine-substituted residues shown as sticks. Dashed line and label show C_α-C_α 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 ($TRPV6_{CysKO}$), in which exposed cysteines in $TRPV6_{cryst}$ were mutated to serine or alanine (C14S, C20S,

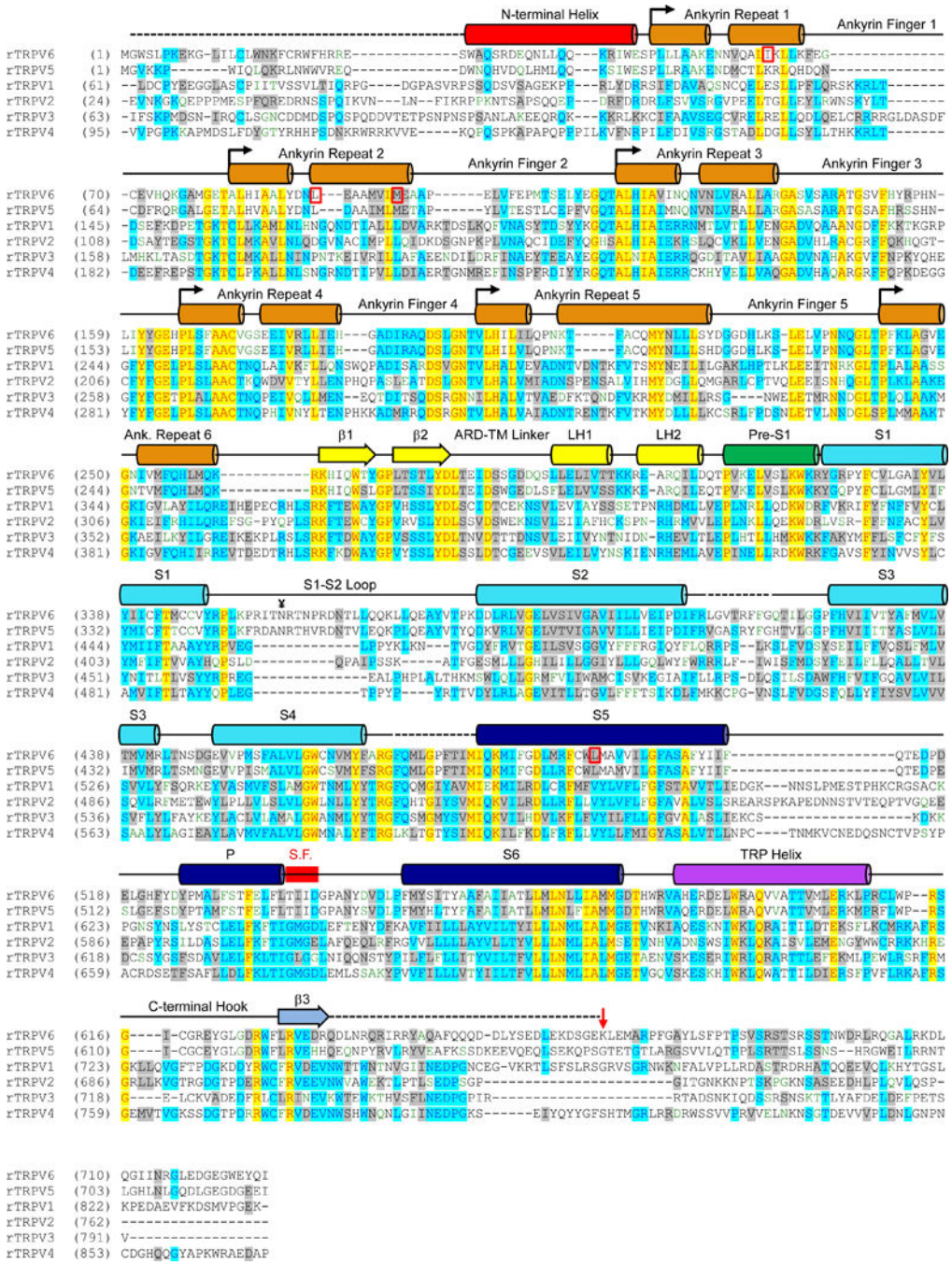
C70A, C610A and C618A) to prevent nonspecific aggregation. Positions corresponding to monomer and tetramer bands are indicated by filled and open triangles, respectively. The appearance of a robust band corresponding to covalently crosslinked tetramer in the D34C–R631C double mutant indicates that the 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[14,16] and TRPA1[17], this data suggests 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 weight 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 $TRPV6_{CysKO}$ crosslink mutants in the absence of reducing agent. Each trace shows a single major peak with elution time corresponding to the $TRPV6_{cryst}$ tetramer (black trace). **d-e**, The putative desthiobiotin (DTB) binding site is comprised of a pocket formed by the N-terminal helix and ankyrin repeats 2 through 4 of one subunit (blue) and the linker domain of an adjacent subunit (green). DTB is shown as ball and stick, with 2Fo-Fc density shown as grey mesh (45–3.25 Å, 1.0σ). 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 ankryin domain crystal structure of TRPV1[47], which was later demonstrated to be conserved in TRPV3 and TRPV4[48]. The presence of DTB close to this location in TRPV6 corroborates the assertion made by Liao et al.[14] that ligands bound in this region modulate activity by perturbing subunit interactions. Further work is necessary to establish any functional role, if any, of DTB like compounds on TRPV6 function. **f-h**, comparison of the putative DTB binding site in $TRPV6_{cryst}$ (**f**) and the ATP binding site in the crystal structure of the TRPV1 ankyrin domain (**g**, PDB entry code 2PNN). DTB and ATP are shown in ball and stick. While the ATP binding site in TRPV1 is shifted toward ankyrin repeat finger 1, both binding sites are located at intersubunit interfaces, as illustrated when the structures are superimposed (**h**).



Extended Data Figure 6. Comparison of the ion channel pore in TRPV6$_{cryst}$ with other tetrameric ion channels

a–l, The pore of TRPV6$_{cryst}$ (yellow ribbon) was aligned with TRPV1 (**a**, PDB entry code 3J5P), Na$_V$Ab (**b**, PDB entry code 3RVY), Slo2.2 (**c**, PDB entry code 5A6E), TRPA1 (**d**, PDB entry code 3J9P), K$_V$1.2 (**e**, PDB entry code 2R9R), KcsA (**f**, PDB entry code 1BL8), InsP$_3$R1 (**g**, PDB entry code 3JAV), RyR1 (**h**, PDB entry code 3J8H), Na$_V$Rh (**i**, PDB entry code 4DXW), Ca$_V$Ab (**j**, PDB entry code 4MVM), Ca$_V$1.1 domains I and III (**k**, PDB entry code 3JBR), and Ca$_V$1.1 domains II and IV (**l**, PDB entry code 3JBR). In each of the alignments, acidic residues located at or close to the selectivity filter region are shown as

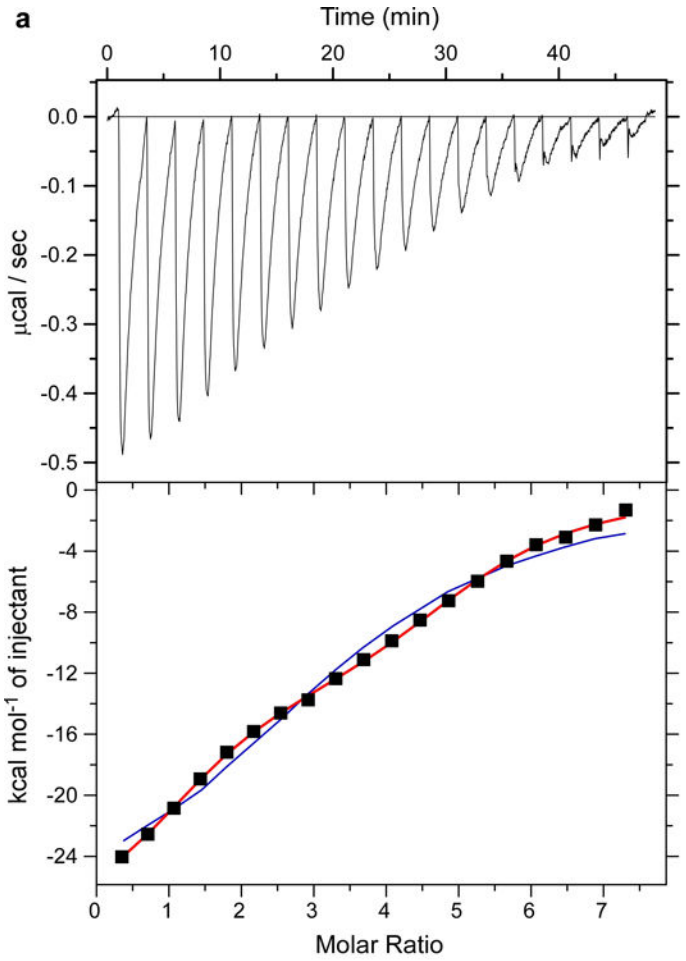
sticks for comparison. Notably, structures of Ca^{2+} permeable channels (**a, d, g, h, j, k, l**) display a high concentration of acidic residues in the outer pore region. In **a–c**, methionine residues close to the S6 bundle crossing are shown as sticks. Notably, the methionine at the lower gate points 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_VAb (**c**), methionine side chains occlude the lower gate as in $TRPV6_{cryst}$, indicating that the closed conformation of the lower gate can be chemically similar for Na^+, K^+, and Ca^{2+} selective channels. **m–o**, Comparison of calcium binding sites in $TRPV6_{cryst}$ (**m**), the engineered voltage gated Ca^{2+} channel Ca_VAb (**n**), and the putative Ca^{2+} site in Cav1.1 (**o**, domains I and III are shown). Residues comprising the selectivity filters are shown in stick representation. Ca^{2+} ions are shown as green spheres. Sites 1 and 2 from $TRPV6_{cryst}$ overlap with the positions of Sites 1 and 3 from Ca_VAb, respectively. While it has been proposed that due to electrostatic repulsion, Sites 1, 2 and 3 cannot be simultaneously occupied in Ca_VAb, distances between Ca^{2+} binding sites in $TRPV6_{cryst}$ are sufficiently large such that they can be simultaneously occupied. The putative Ca^{2+} site in $Ca_V1.1$ is near the equivalent location of Site 2 in Ca_VAb.



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 $TRPV6_{cryst}$ construct not included in the $TRPV6_{cryst}$ structural model. Red boxes and a red arrow highlight substitution mutations and the C-terminal truncation point in $TRPV6_{cryst}$, respectively (see methods). ¥ 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.

a



b

Parameter	Value
n_1	1.23 ± 0.08 sites
K_1	1.54 ± 0.04 µM^{-1}
ΔH_1	-29.3 ± 2.2 kcal/mol
ΔS_1	-70 cal/mol/deg
ΔG_1	-8.44 ± 0.16 kcal/mol
n_2	3.74 ± 0.09 sites
K_2	0.182 ± 0.022 µM^{-1}
ΔH_2	-13.8 ± 1.3 kcal/mol
ΔS_2	-22.2 cal/mol/deg
ΔG_2	-7.18 ± 0.07 kcal/mol

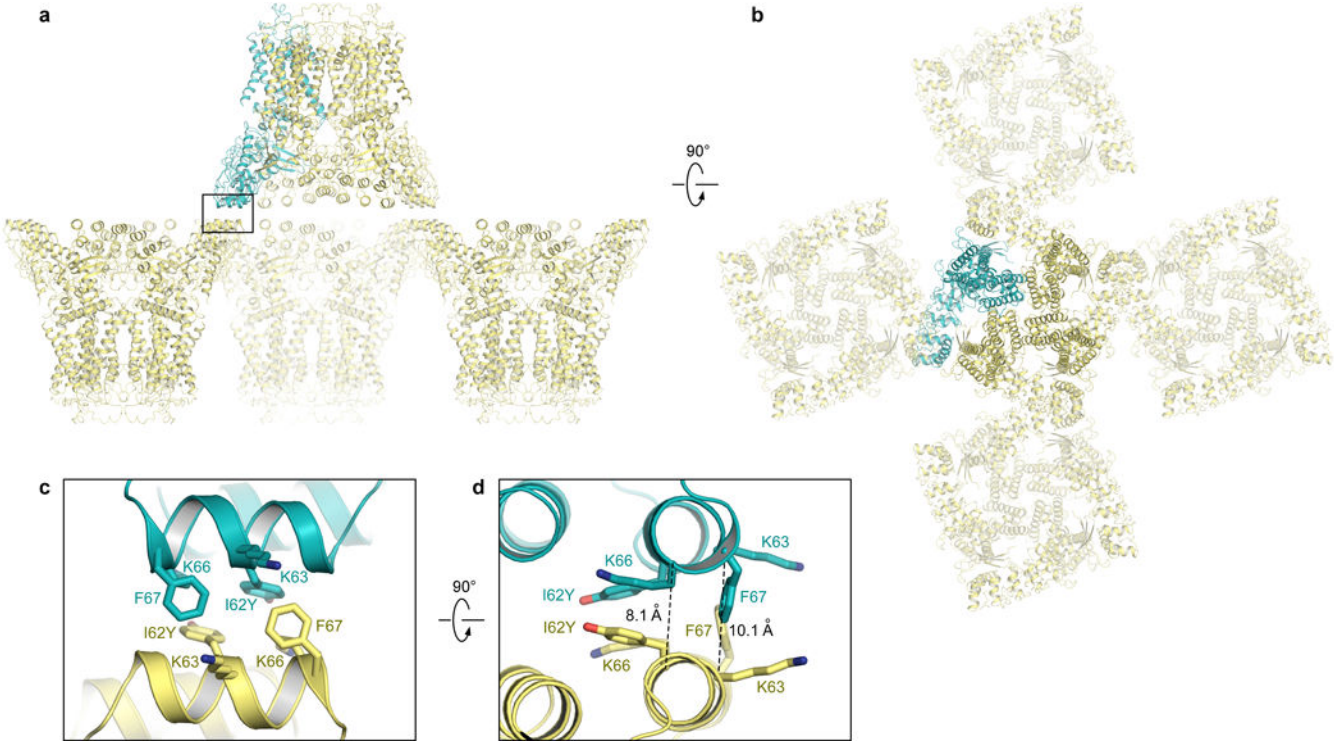
c

	Ca^{2+}	Ba^{2+}	Gd^{3+}
Sites			
Site 1	10.2	4.4	31.40
Site 2	7.5	14.6	–
Site 3	3.6	4.6	–
Recruitment Site	–	4.2	8.0

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. The upper panel shows 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. The bottom panel shows the integrated heat per injection versus molar ratio. Binding of Gd^{3+} to TRPV6 was analyzed using models with one and two types of binding sites. 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 sites, according to equation $Q_i^{tot} = V_0 M_{tot} ((n_1 \Delta H_1 K_1[X] / (1 + K_1[X])) + (n_2 \Delta H_2 K_2[X] / (1 + K_2[X])))$, where Q_i^{tot} is total heat after the i^{th} injection, V_0 is the volume of calorimetric cell, M_{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 ΔH_1 and ΔH_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 ΔG and TΔS 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 sites. The straightforward interpretation of the ITC results is that the ITC type 1 (n~1) and type 2 (n~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 ~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 are not observed.



Extended Data Figure 9. Crystal lattice contact of $TRPV6_{cryst}$

a–b, two views of $TRPV6_{cryst}$ crystal packing in the $P42_12$ space group. A single $TRPV6_{cryst}$ 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_α-C_α distances are labeled in **d**. The crystal contact is apparently mediated by cation-π and/or hydrogen bonding interactions between these residues. Crystals in the $P42_12$ 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	$P42_12$	$P42_12$	$P42_12$	$P42_12$	$P42_12$	$P42_12$
Cell dimensions	143.81	144.35	144.35	144.35	143.60	143.95
a, b, c, (Å)	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 (94.7)	99.5 (95.4)	99.9 (99.8)	99.5 (97.3)	99.9 (100.0)	100.0 (100.0)
Redundancy *	8.7 (9.2)	15.4 (13.9)	26.5 (17.2)	11.4 (6.4)	16.3 (17.5)	13.7 (13.9)
$\|I/\sigma\|$ *	16.9 (1.3)	15.2 (1.5)	25.0 (2.4)	19.4 (1.6)	27.3 (6.3)	21.8 (4.7)
R_{meas} (%) *	9.8 (132.6)	13.1 (228.5)	10.6 (143.1)	8.9 (120.7)	20.7 (86.4)	19.2 (93.5)
$CC_{1/2}$	99.8 (85.7)	98.0 (76.8)	99.5 (85.7)	99.9 (63.7)	98.5 (89.8)	98.3 (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 (93.8)	100 (99.9)	100 (99.9)	99 (96.9)		
Number of reflections	18531 (1724)	21705 (2187)	25439 (2521)	22443 (2170)		
R_{work}/R_{free}	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 ($Å^2$)						

	Native	Ba^{2+}	Ca^{2+}	Gd^{3+}	L630M–SeMet	SeMet
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.

5% of reflections were used for calculation of R_{free}

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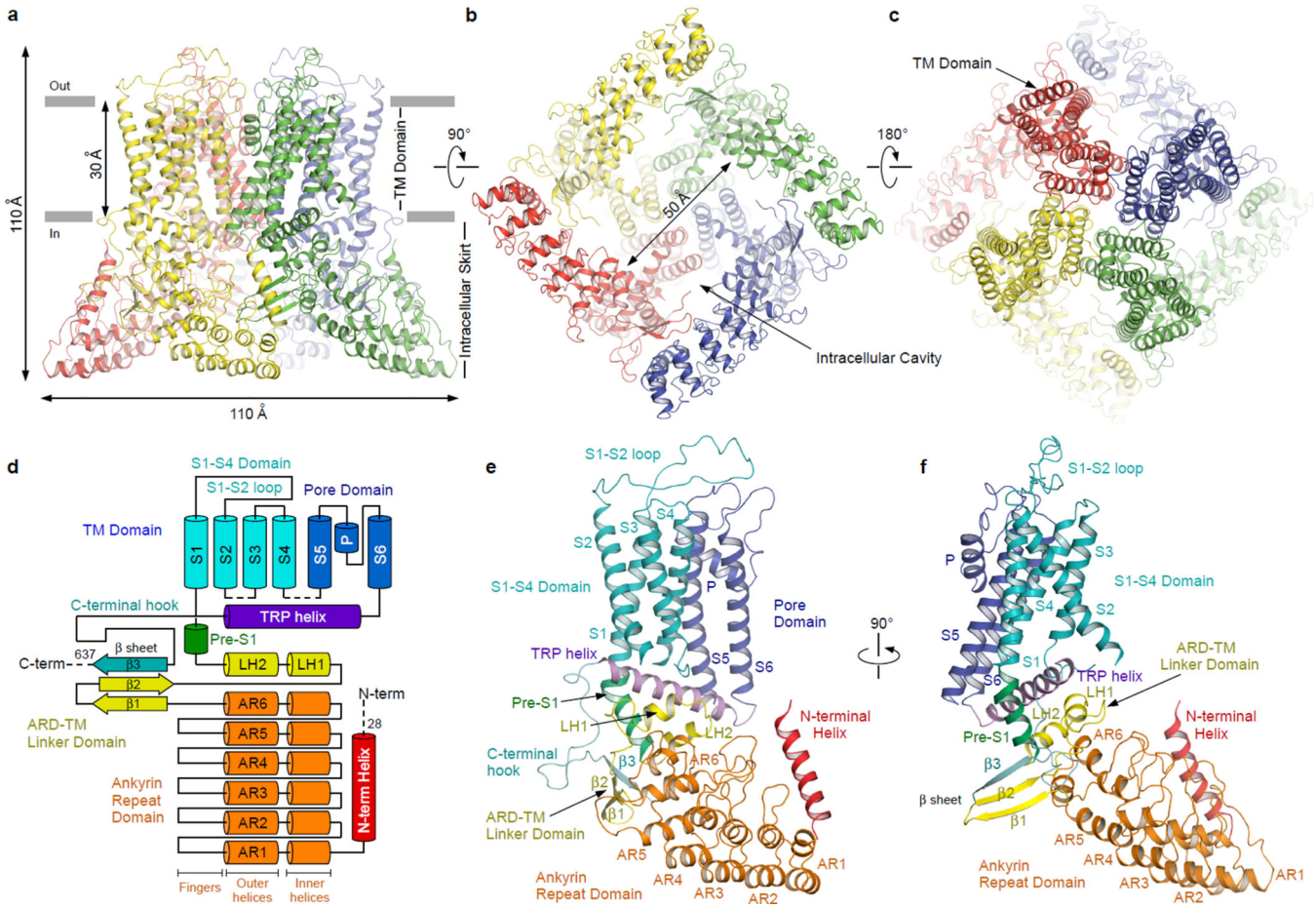

Figure 1. Architecture and domain organization of TRPV6$_{cryst}$

a–c, Side (**a**), bottom (**b**), and top (**c**) views of the TRPV6$_{cryst}$ tetramer, with each subunit shown in different color. **d**, Domain organization diagram of the TRPV6 subunit. **e–f**, Two views of the TRPV6$_{cryst}$ subunit, with domains colored as in **d**.

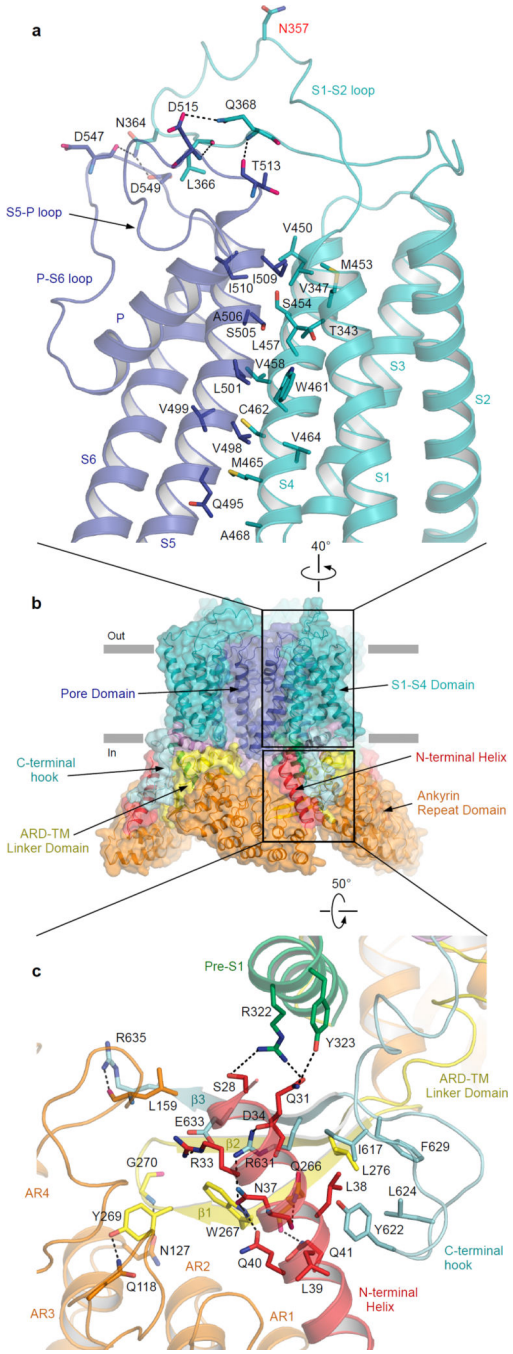

Figure 2. Domain interfaces

a, TM helices S4 and S5 and extracellular loops S1-S2, S5-P and P-S6 contribute to interfaces between the S1-S4 domain and pore domain. **b**, Side view of the $TRPV6_{cryst}$ tetramer with domains colored differently. Boxes indicate domain interfaces expanded in **a** and **c. c**, Interfaces between soluble domains. Residues at domain interfaces in **a** and **c** are shown in stick representation, with potential hydrogen bonds and electrostatic interactions shown as dashed lines. The predicted N-linked glycosylation site conserved in TRPV6 channels, N357 in $TRPV6_{cryst}$, is labeled red in **a**.

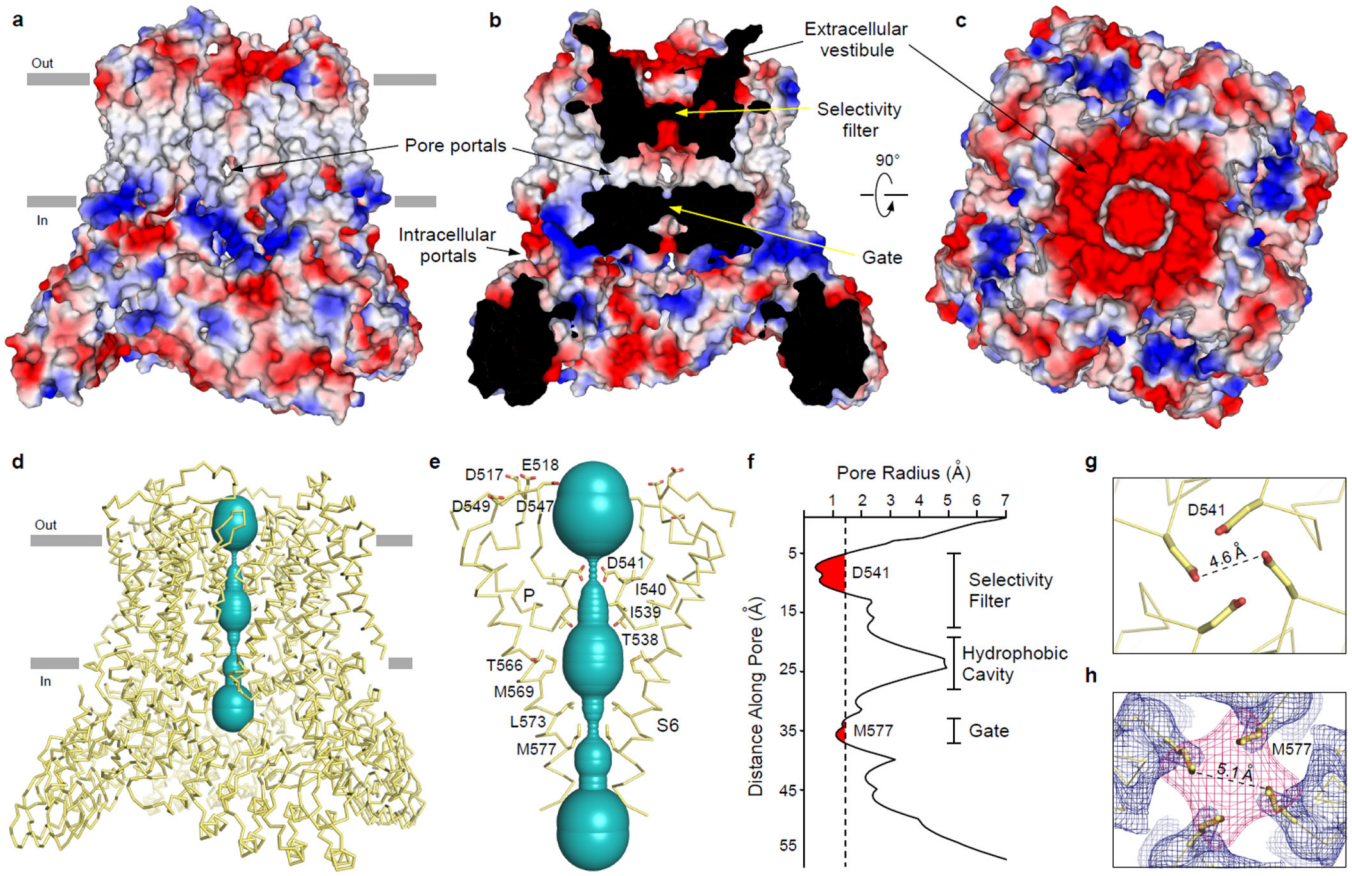

Figure 3. Permeation pathway

a–c, Side (**a**), central slice (**b**) and top (**c**) views of $TRPV6_{cryst}$ structure in surface representation, colored by electrostatic potential. **d**, Ribbon diagram of the $TRPV6_{cryst}$ tetramer, with ion conduction pathway shown in cyan. **e**, expanded view of the $TRPV6_{cryst}$ 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 mesh shows electron density for M577 ($2F_O$-F_C, 45–3.25 Å, 1.0σ) and anomalous difference electron density from selenomethione-labeled crystal (30–5.00 Å, 3.0σ), respectively.

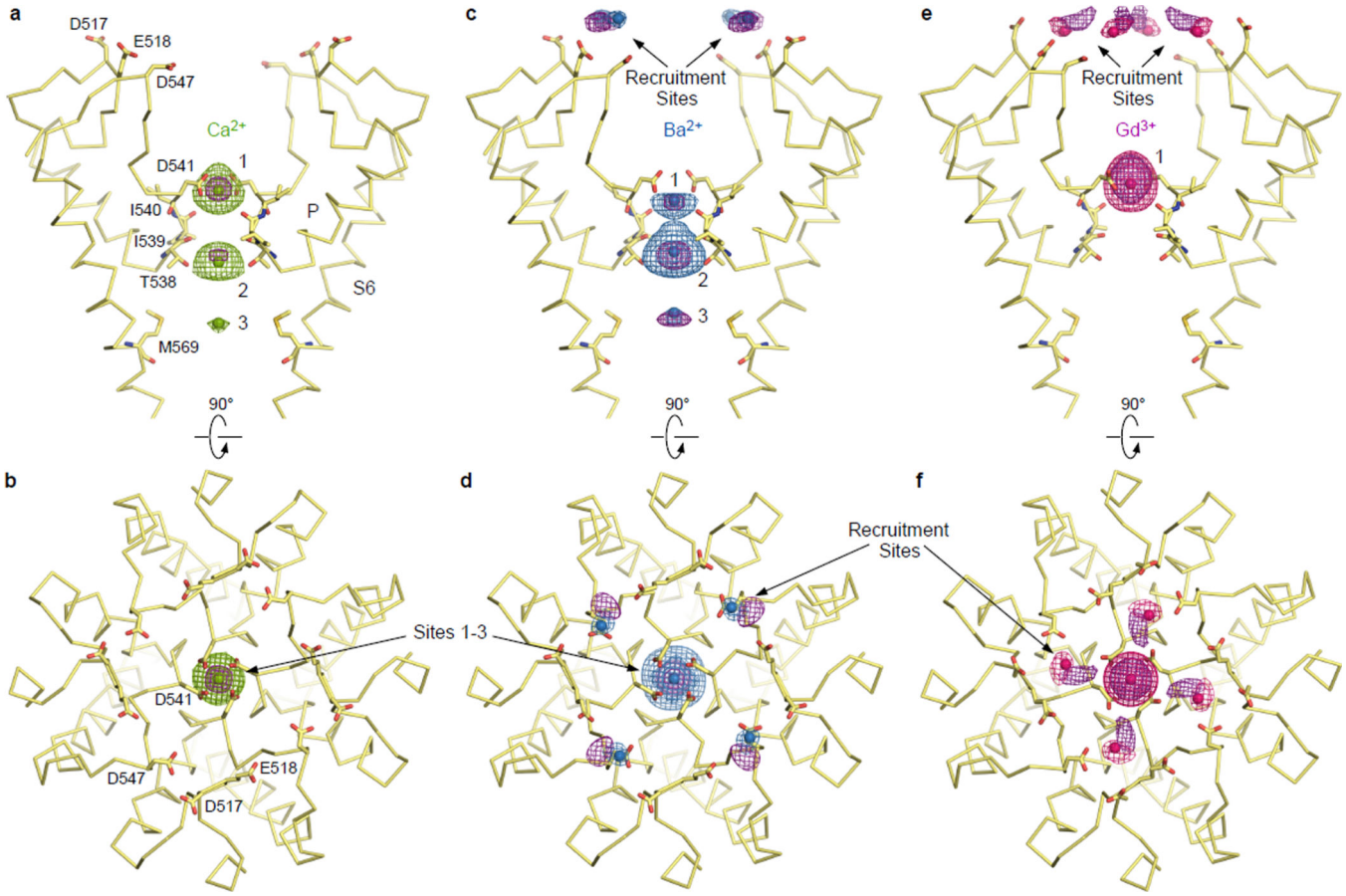

Figure 4. Cation binding sites in the TRPV6$_{cryst}$ pore

Side (**a,c,e**) and top (**b,d,f**) views of the TRPV6$_{cryst}$ 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 Ca^{2+} (**a–b**, 38–4.59 Å, 2.7σ), Ba^{2+} (**c–d**, 38–4.59 Å, 3.5σ) and Gd^{3+} (**e–f**, 38–4.59 Å, 7σ) and ions are shown as spheres of the corresponding color. Purple mesh shows simulated-annealing F_O-F_C electron density maps contoured at 4σ for Ca^{2+} (50–3.65 Å), 3σ for Ba^{2+} (50–3.85 Å) and 3.5σ for Gd^{3+} (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 Ba^{2+} ions at the recruitment sites. The Gd^{3+} recruitment sites are distinct from Ba^{2+} and apparently involve coordination by D517, E518, and D547 side chains.

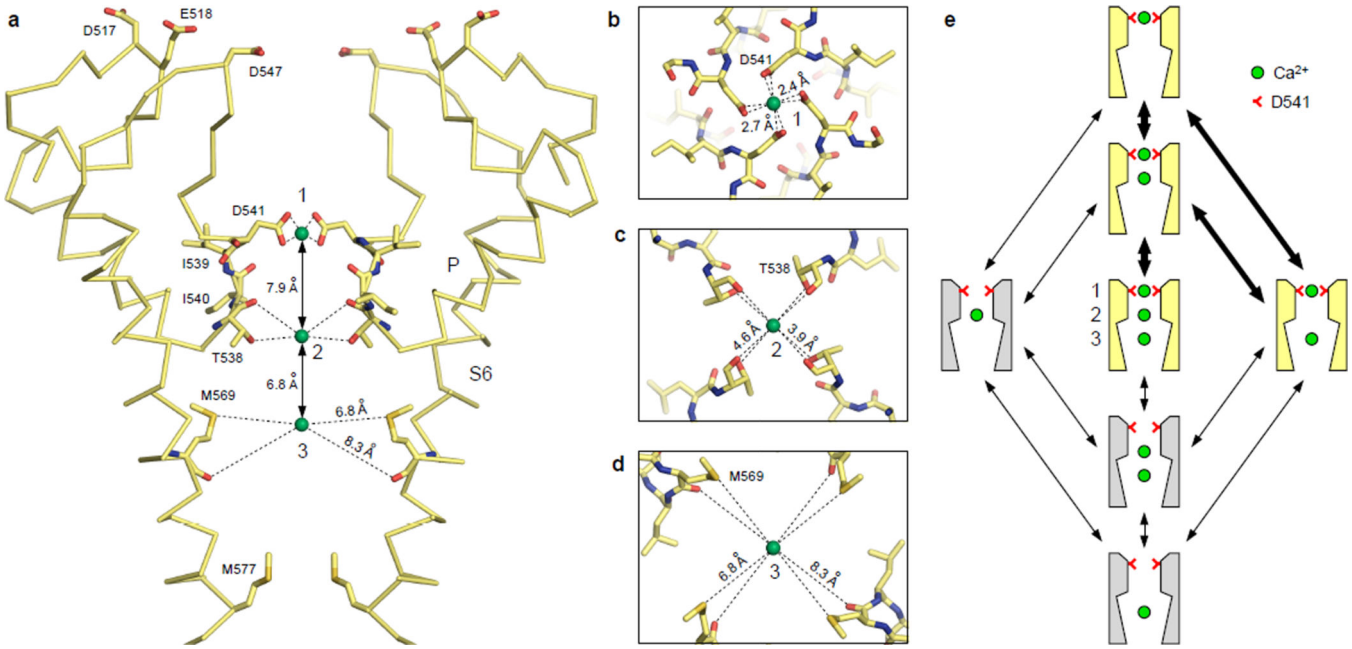

Figure 5. Calcium permeation mechanism

a, Side view of $TRPV6_{cryst}$ pore, with front and back subunits removed for clarity. Residues that surround or contribute to cation binding sites are shown as sticks, and Ca^{2+} ions at Sites 1, 2 and 3 are shown as green spheres. **b–d**, Top views of Ca^{2+} ions at Sites 1 (**b**), 2 (**c**) and 3 (**d**), with nearby residues shown as sticks. The interatomic distances illustrated by dashed lines suggest that Ca^{2+} is directly coordinated by D541 side chains at Site 1, while a hydrated Ca^{2+} ion indirectly interacts with the pore at Sites 2 and 3. **e**, Schematic representation of various Ca^{2+} occupancy states in TRPV6. Presumed lower energy states are shown in yellow, and most probable transitions are highlighted with bold arrows. Occupancy states in which Site 1 is vacant (shown in grey) are likely to only be transiently populated, due to electrostatic repulsion of D541 side chains. Sufficiently large distances between Sites 1, 2 and 3 suggest that electrostatic repulsion between Ca^{2+} ions does not preclude simultaneous binding at all three sites.