

# A conserved gating element in TRPV6 channels

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## ABSTRACT

The Ca<sup>2+</sup>-selective tetrameric Transient Receptor Potential Vanilloid 6 (TRPV6) channel is an inwardly rectifying ion channel. The constitutive current endures Ca<sup>2+</sup>-induced inactivation as a result of the activation of phospholipase C followed depletion of phosphatidylinositol 4,5-bisphosphate, and calmodulin binding. Replacing a glycine residue within the cytosolic S4-S5 linker of the human TRPV6 protein, glycine 516, which is conserved in all TRP channel proteins, by a serine residue forces the channels into an open conformation thereby enhancing constitutive Ca<sup>2+</sup> entry and preventing inactivation. Introduction of a second mutation (T621A) into TRPV6<sub>G516S</sub> reduces constitutive activity and partially rescues the TRPV6 function. According to the recently revealed crystal structure of the rat TRPV6 the T621 is adjacent to the distal end of the transmembrane segment 6 (S6) within a short linker between S6 and the helix formed by the TRP domain. These results indicate that the S4-S5 linker and the S6-TRP-domain linker are critical constituents of TRPV6 channel gating and that disturbance of their sequences foster constitutive Ca<sup>2+</sup> entry.

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## Contents

1. Introduction.....	24
2. Material and methods.....	26
2.1. Cells, transfected cDNA, transfection and Western blot.....	26
2.2. Ca <sup>2+</sup> -imaging.....	26
3. Results and discussion.....	26
Acknowledgements.....	27
References.....	27

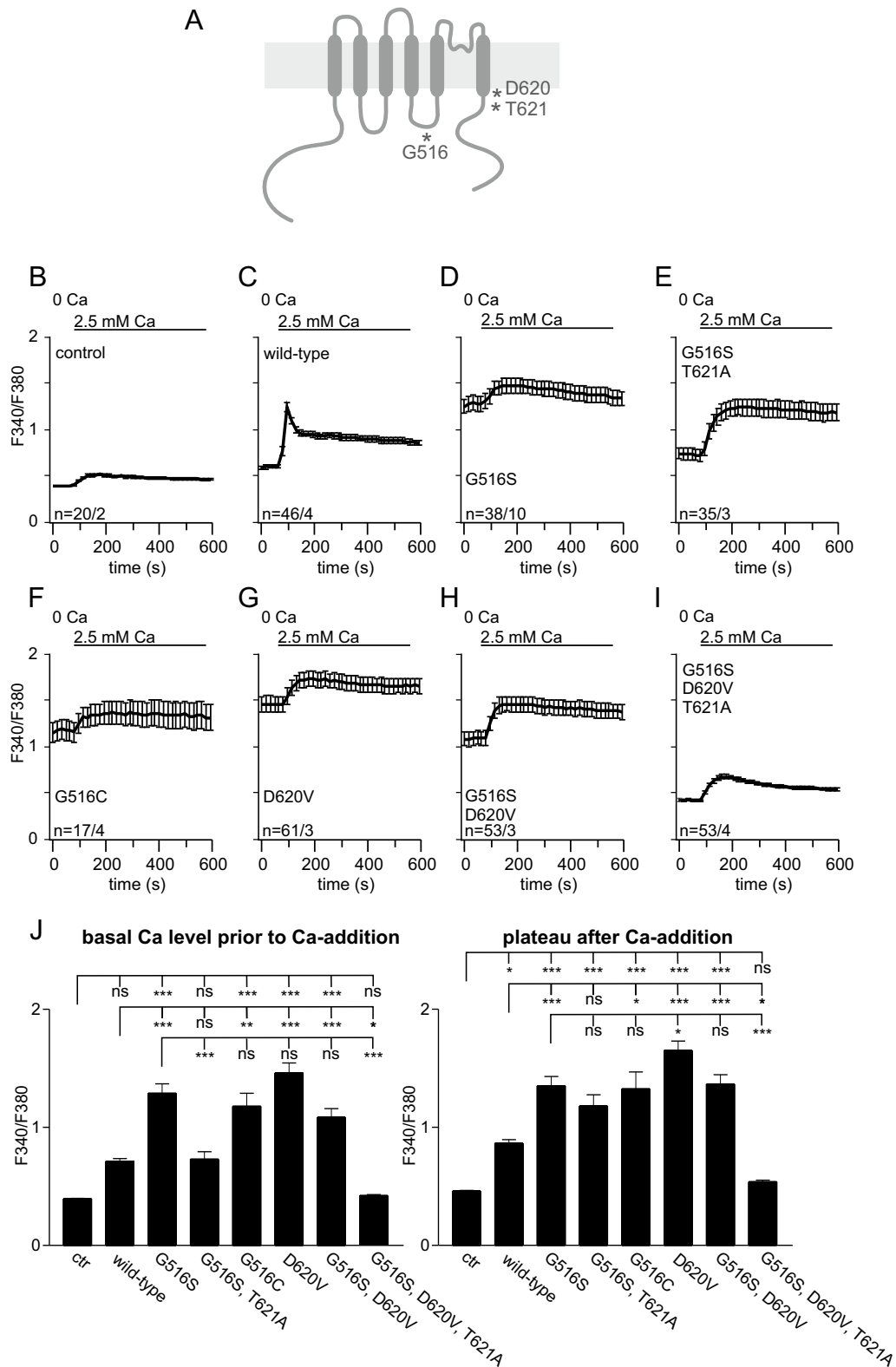
## 1. Introduction

The high Ca<sup>2+</sup> selectivity makes the TRPV5 and TRPV6 channels unique among the transient receptor potential channels. This selectivity is only matched by voltage-gated Ca<sup>2+</sup> channels and ORAI channels/Ca<sup>2+</sup>-release activated Ca<sup>2+</sup> channels but not by any other TRP channel [1,2]. A ring of aspartates within the pore loop (one aspartate per subunit of the presumed tetrameric channel) constitute the Ca<sup>2+</sup> binding site underlying this high Ca<sup>2+</sup> selectivity [3,4]. The recent crystal structure of the rat TRPV6 channel confirms these previous results [5]. Both channels, TRPV5 and TRPV6 support Ca<sup>2+</sup> retention in cells and in the mammalian body. TRPV5 is involved

in Ca<sup>2+</sup> re-uptake in the kidney and thereby prevents loss of Ca<sup>2+</sup> through the urine [6]. TRPV6 is supposed to underlie Ca<sup>2+</sup> uptake in the intestine [7,8]. Although this latter function might depend on the species under study [8], the TRPV6 channel also accomplishes Ca<sup>2+</sup> uptake by the epididymal epithelial cells [9]. Thereby the luminal Ca<sup>2+</sup> concentration in the epididymal duct is lowered and this in turn is required to keep sperms alive. The TRPV6 gene is also expressed in the prostate, in the exocrine pancreas and in the placenta and in these tissues TRPV6 might be required to retain the intracellular Ca<sup>2+</sup> concentration [8]. Recently, we have shown that translation of the TRPV6 mRNA is initiated at a non-AUG codon 120 nucleotides upstream of the first in frame AUG codon which is not used for translation initiation [10]. Accordingly, the TRPV6 protein is elongated by 40 amino acid residues [10] compared to the predicted TRPV6 protein initiated at the first in frame AUG codon.

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**Fig. 1.** (A) Scheme of the TRPV6 protein with transmembrane domains S1–S6 and the amino acid residues G516 within the S4–S5 linker, D620 and T621 within the distal S6 and the linker between S6 and the TRP domain. (B–I) Cytoplasmic Ca<sup>2+</sup> changes (Fura-2 ratios F340/F380) monitored in non-transfected HEK293 cells and cells transiently transfected with wild-type or mutant hTRPV6 cDNAs as indicated. Cells were loaded with Fura-2-AM and kept in nominally Ca<sup>2+</sup>-free bath solution. At 100 s 2.5 mM Ca<sup>2+</sup> was added. Data represent means  $\pm$  S.E.M. from n measured cells from x independent experiments (n/x). (J) Summary of basal Ca<sup>2+</sup> level (left) after 40 min in nominally Ca<sup>2+</sup>-free bath solution (F340/F380 ratios averaged from 1 to 100 s) and the plateau Ca<sup>2+</sup> level (right, averaged from 500 to 600 s). One-way ANOVA with Bonferroni correction was applied to test for significant differences, with  $p > 0.05$  (ns, non-significant),  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*) and  $p \leq 0.001$  (\*\*\*).

Beck et al. [11] identified a glycine residue within the S4-S5 linker of TRPC4 and TRPC5 channels at which mutations to a serine, a cysteine, a methionine, an alanine or a leucine forced the channel into an open conformation. The glycine is conserved in all members of the TRPV channels. The spontaneous mutation of the corresponding glycine in TRPV3 causes hairless phenotypes in mice (*Nh* (no hair)) and rat (*Ht* (hypotrichosis)) [12] as well as the Olmsted Syndrome in humans [13,14], a rare disorder characterized by palmoplantar and periorificial keratoderma, alopecia and severe itching. The mutant TRPV3 mouse and rat channels are constitutively active [15]. A spontaneous mutation of the corresponding glycine (G600W) in human TRPV4 has been linked to skeletal dysplasia syndromes [16,17].

A corresponding mutation has not been identified in the TRPV6 gene and it is not known whether the mutation of the glycine within the S4-S5 linker has an influence on TRPV6 activity. We therefore replaced the glycine residue at position 516 by a serine or a cysteine residue (G516S or G516C) in the human TRPV6. After transfection of the mutant cDNAs in HEK293 cells we monitored cytoplasmic  $\text{Ca}^{2+}$  concentrations. Both mutations yield constitutive  $\text{Ca}^{2+}$  entry, which was counteracted by replacing a threonine residue within the linker between S6 and the TRP domain by an alanine residue (T621A). In the rat TRPV6 crystal structure [5] most parts of the S4 and S5 linker including the glycine residue (corresponding to residues 512–520 in the human TRPV6) were not included because of the absence of interpretable density. We come up with a model by which the mutant serine residue within the TRPV6 S4-S5 linker favors an active channel conformation. This model is different from the predicted one for the corresponding mutants in TRPC4 and TRPC5 [11] and affirms the role of the S4-S5 linkers of TRP channels as conserved gating elements, demonstrated by the high-resolution structures of TRPV1 [18–20], TRPV2 [21,22], and TRPA1 [23].

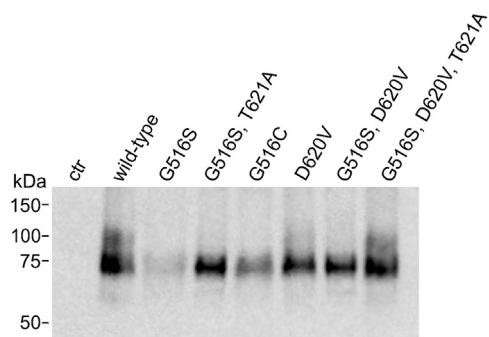
## 2. Material and methods

### 2.1. Cells, transfected cDNA, transfection and Western blot

Site directed mutagenesis was performed on the full length human TRPV6 cDNA (GenBank accession number NM\_018646.5) subcloned in the pcDNA3 vector as template and primers covering the mutated part of the sequence. After PCR had been performed the parental plasmid was restricted by DpnI and SphI-MfeI fragments containing the mutations were isolated. They were subcloned into the pcAGGS-TRPV6-IRES-GFP vector, cut by SphI and MfeI, to replace the corresponding non mutated SphI-MfeI TRPV6 fragment. HEK-293 cells (ATCC, CRL 1573) were transfected as described [10,11]. For Western blot, Cos-7 cells with 80% confluency grown on 3.5 cm dishes were transfected with the TRPV6 cDNAs using **Cos-7 Cell Avalanche Transfection Reagent (EZ Biosystems, College Park, USA) following manufacturer's instructions**. After 24 h, cells were washed with PBS and lysed in the presence of 75, 100 or 150  $\mu\text{l}$  Laemmli buffer, depending on cell densities of green cells/cell viability post transfection. Lysates were denatured for 20 min at 60 °C and subjected to 10% SDS-PAGE (Mini-PROTEAN TGX Pre-cast Gels, BIORAD). Proteins were transferred onto a nitrocellulose membrane and incubated with the in-house generated affinity-purified anti-TRPV6 antibody 429 [10]. Detection of proteins was accomplished using horseradish peroxidase-coupled secondary antibody and the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences).

### 2.2. $\text{Ca}^{2+}$ -imaging

Intracellular  $\text{Ca}^{2+}$  imaging experiments were performed using a Polychrome V and CCD camera (TILL Imago)-based imaging sys-

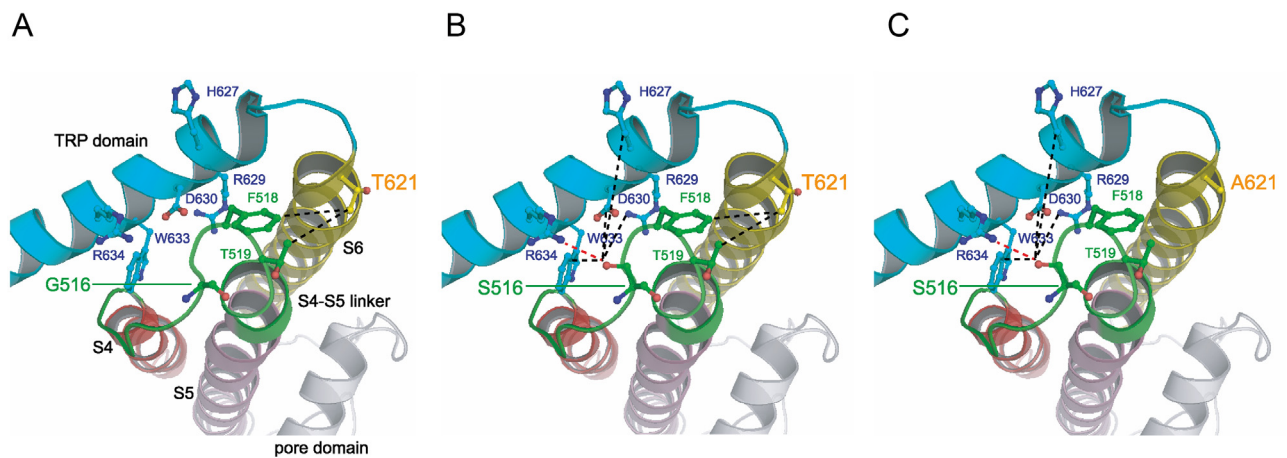


**Fig. 2.** Western blot of non-transfected Cos-7 cells (ctr, control) or Cos-7 cells expressing hTRPV6 wild-type or mutant cDNAs as indicated.

tem (TILL Photonics, Martinsried, Germany) at a Zeiss Axiovert S100 fluorescence microscope equipped with a Zeiss Fluor 20 $\times$ /0.75 objective. Data acquisition was accomplished with the imaging software TILLvision (T.I.L.L. Photonics). Cells were seeded on 2.5 cm round glass coverslips and transiently transfected with hTRPV6 wild-type or mutant cDNA in the pcAGGS-IRES-GFP expression vector [10,11]. Prior to the experiments, which were performed 24 h post transfection, cells were incubated in nominally  $\text{Ca}^{2+}$ -free solution (containing in mM: 115 NaCl, 5 KCl, 2 MgCl<sub>2</sub>, 10 HEPES, pH adjusted to 7.4 with NaOH) supplemented with 4  $\mu\text{M}$  of the  $\text{Ca}^{2+}$ -sensitive fluorescent dye Fura-2-AM for 30 min in the dark at room temperature, and washed four times with nominally  $\text{Ca}^{2+}$ -free solution to remove excess Fura-2-AM. The Fura2 ratio obtained from some mutants might be close to Fura2 saturation resulting in underestimation of  $\text{Ca}^{2+}$  influx. The coverslips with the Fura-2-loaded cells were transferred to a bath chamber containing nominally  $\text{Ca}^{2+}$ -free solution, and Fura-2 fluorescence (>510 nm) was monitored after excitation at 340 and 380 nm for 30 ms each at a rate of 1 Hz for 600 s. Cells were marked and the ratios of the background-corrected Fura-2 fluorescences at 340 and 380 nm (F340/F380) were plotted versus time. 2.5 mM CaCl<sub>2</sub> was added to the bath solution as indicated.

## 3. Results and discussion

Two glycine residues within the S4-S5 linker are conserved among the six members of the transient receptor potential vanilloid (TRPV) subfamily of various species (graphical abstract and [2,24,25]). By site directed mutagenesis we replaced the glycine at position 516 of the human TRPV6 (Fig. 1A) by a serine residue (G516S). The cDNA was transfected in HEK 293 cells. As controls we used non-transfected HEK 293 cells and cells transfected with the wild-type human TRPV6 cDNA. During cell culture we noticed that in cells transfected with the mutant cDNA a considerable fraction of cells did not survive. To measure  $\text{Ca}^{2+}$  influx, we kept the cells in nominally  $\text{Ca}^{2+}$ -free solution, then added 2.5 mM  $\text{Ca}^{2+}$  to the bath solution, and monitored changes of the cytoplasmic  $\text{Ca}^{2+}$  concentration through  $\text{Ca}^{2+}$  influx (F340/F380). As shown in Fig. 1D already in nominally  $\text{Ca}^{2+}$ -free bath solution cytoplasmic  $\text{Ca}^{2+}$  (“basal  $\text{Ca}^{2+}$ ”) is increased in TRPV6<sub>G516S</sub> expressing cells compared to non-transfected cells (Fig. 1A and J, left) and cells expressing the TRPV6 wild-type cDNA (Fig. 1C). Upon addition of 2.5 mM  $\text{Ca}^{2+}$  to the bath solution cytoplasmic  $\text{Ca}^{2+}$  increased in the cells expressing the TRPV6<sub>G516S</sub> cDNA but in contrast to the more transient increase in cells expressing wild-type, the  $\text{Ca}^{2+}$  influx in TRPV6<sub>G516S</sub> persists over time (“plateau”, Fig. 1J, right). Likewise, replacing the glycine at position 516 of the human TRPV6 by a cysteine residue (G516C) behaves like the G516S mutant (Fig. 1F). Fig. 1J summarizes these results and demonstrates that both, basal  $\text{Ca}^{2+}$  as well as the plateau  $\text{Ca}^{2+}$  are significantly different in both



**Fig. 3.** Model for the interaction of the S4-S5 linker (green), S6 (yellow) and TRP-domain (blue) of the human TRPV6 based on the rat TRPV6 structure; S4 (red), S5 (pink), pore domain (grey); oxygen (red), nitrogen (blue). (A) Wild-type: T621 (orange) in the very distal S6 can interact with F518 and T519 of the S4-S5-linker as hydrophobic patch. (B) TRPV6<sub>G516S</sub>: the acidic patch of the S4-S5 linker (S516) interacting with TRP domain (R634) is indicated by the dashed red line. The hydrophobic patch of S516 interacting with H627, R629, D630, and W633 of TRP domain (blue) is indicated by the black dashed line. The side chain of S516 can interact with the adjacent residues. (C) TRPV6<sub>G516S/T621A</sub>. The figure was prepared using PyMOL Molecular Graphics System (Version 1.5.0.4, Schrödinger, LLC, New York, NY, USA) by Coot [30], based on the coordinates of rat TRPV6 (PDB: 5iwk).

G516-mutants compared to wild-type. Fig. 2 shows a Western blot of the wild-type and the various mutant TRPV6 proteins indicating that all TRPV6 cDNA constructs used in this study are efficiently expressed in HEK 293 cells.

Most of the residues of the S4-S5 linker are not included in the TRPV6<sub>cryst</sub> structural model but its glycine residue at position 516 (Fig. 3A) is well conserved throughout the various TRP proteins [24–26]. In TRPC4 and TRPC5 this conserved glycine is predicted to be in about 8 Å distance from a serine residue immediately adjacent to or at the cytoplasmic end of S6 of the TRPC proteins [11]. When the glycine at position 503 is replaced by a serine, a water-mediated contact may be formed between the hydroxyl groups of both amino acid residues or even a direct contact after some minor conformational rearrangements might occur. This interaction could elicit some constraint on the positions of the S4-S5 linker and the distal part of the S6, and as a result the channel may be forced into an open conformation. In TRPC4 (and TRPC5) replacement of this serine by an alanine counteracted the impact of the mutant serine residue within the S4-S5 linker yielding a channel that can be regulated again [11].

In TRPV6 the amino acid residue corresponding to the serine in TRPC4 is a threonine (T621). This threonine was replaced by an alanine. After expressing the resulting TRPV6<sub>G516S/T621A</sub> double mutant the Ca<sup>2+</sup> influx was reduced (Fig. 1E). In this respect the double mutant resembled the wild-type but not the mutated G516S channel (Fig. 1D). The S4-S5 linker is quite flexible, although in wild-type the hydrophobic patch of its F518 and T519 side chains apparently interacts with T621 (Fig. 3A). In the G516S mutant it is possible, that the polar serine residue, which replaces the non-polar glycine residue, interacts with polar amino acid residues such as H627, R629, D630, and W633 within the TRP domain and adjacent residues such as L515 and P517 (Fig. 3B). These interactions could elicit some additional constraint on the positions of the S4-S5 linker and the distal part of the TRP domain, and as a result, the channel is forced into an open conformation. Replacing the threonine residue at position 621 by an alanine residue (T621A) counteracts, in part, the impact of the mutant serine at position 516 in TRPV6 and yield a channel that recover part of its function compared to the TRPV6<sub>G516S</sub> channel (Figs. 3C and 1E, J).

Expression of the TRPV6<sub>D620V</sub> cDNA also yielded constitutively active Ca<sup>2+</sup> entry (Fig. 1G), thereby resembling the G516S and G516C mutants. The double mutant obtained by introducing the

D620V mutation into the G516S mutant behaves like the single mutants (Fig. 1H) whereas the triple mutant G516S/D620V/T621A behaves like non-transfected cells (Fig. 1I), although the protein is present (Fig. 2B).

Based on the available high-resolution structures of TRPV1 [18–20], TRPV2 [21,22], TRPV6 [5] and TRPA1 [23] the S4-S5 linker, the distal S6, the TRP domain and the short linker between S6 and the TRP domain are essential elements that contribute to the gating of these channels. In TRPV6, the linker between S6 and the TRP domain is distinct from TRPV1, TRPV2 and TRPA1 channel structures, but the absence of interpretable density for the S4-S5 linker of TRPV6 prevents direct comparison of the interactions within the four channels [5]. In TRPV1 the glutamate 571 in the S4-S5 linker is one of the critical residues for capsaicin binding [27,28]. The corresponding residue in TRPV2 and TRPV6 is a glutamine, which may be the reason that both channels are not sensitive to capsaicin [22]. Whereas in TRPV1 the TRP domain interacts with the S4-S5 linker via polar interactions [18,23] these interactions are exclusively hydrophobic in TRPA [23]. The glycine residue at position 516 within the human TRPV6 S4-S5 linker is not only conserved within the other TRPV channels but within all TRP channels including the unique yeast channel yvc1 [29]. Its replacement by a serine or cysteine residue in TRPV6 enhances constitutive Ca<sup>2+</sup> entry, most probably by adding some structural constraint on the positions of the S4-S5 and the S6-TRP domain linkers. Additionally, interactions of the S4-S5 linker of one subunit with the S5 and S6 of an adjacent subunit have been identified in the TRPV1 and TRPV2 structures [20,23], but similar intramolecular interactions have yet to be identified in TRPV6.

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