Interaction between the S4–S5 linker and C-terminus of TRPP3 channel: functional importance and regulation by PIP2

by

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Abstract

As one of the most common genetic renal diseases affecting over 12.5 million people worldwide, the autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in either the *PKD1* or *PKD2* gene. Transient receptor potential polycystin-3 (TRPP3), a member of the polycystin protein family, also called polycystic kidney disease (PKD) protein 2 Like 1 (PKD2L1), is a homologue of PKD2 but itself is not involved in ADPKD. A cation channel activated by calcium and protons, TRPP3 regulates calcium-dependent hedgehog signaling in primary cilia, intestinal development and sour tasting. It is an integral membrane protein featured with six transmembrane segments (S1-S6), a pore loop between S5 and S6, and intracellularly localized N- and C-termini. How TRPP3 channel function is regulated remains poorly understood. In this study, using the two-electrode voltage clamp (TEVC) electrophysiology in *Xenopus laevis* oocytes, Western blotting (WB), and immunofluorescence, I revealed three TRPP3 C-terminal domains critical for channel function: Ser581-Leu592, Arg596-Lys607 and Gly611-Thr622. We then identified conserved cationic residue (Lys 461) in the S4-S5

linker and aromatic residue (Tyr 564) in the C-terminal TRP-like domain of TRPP3 and studied their functional roles. While the physical proximity between the S4-S5 linker and TRP (-like) domain has been reported in many resolved TRP structures, it remains unknown whether there is physical and/or functional interaction. By co-immunoprecipitation (co-IP), *in vitro* pull-down, and blocking peptide strategy, we first revealed the physical and functional interaction between the S4-S5 linker and C-terminal TRP-like domain (L-C) of TRPP3 and then identified that it is mediated by a cationic-aromatic (K461-Y564) residue pair and seems to be inhibited by phosphatidylinositol 4,5- bisphosphate (PIP2). In summary, our studies constitute valuable contributions to the understanding of TRPP3 function and regulation.

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List of abbreviations

2-ME	2-mercaptoethanol
2BP	2-bromopalmitate
aa	Amino acid
ADPKD	Autosomal dominant polycystic kidney disease
BSA	Bovine serum albumin
CCD	Coiled-coil domain
Co-IP	Co-immunoprecipitation
CRD	C-terminus regulatory domain
cryo-EM	Cryo-electron microscopy
Ctrl	Control
CT	C-terminus
DTT	Dithiothreitol
EGF	Epidermal growth factor
GOF	Gain of function
hTRPP3	Human TRPP3
HEK	Human embryonic kidney
hr	Hour
Kv7.1	Voltage-gated K^+ channels
mTRPP3	Mouse TRPP3
mTRPM3	Mouse TRPM3
min	Minute
NT	N-terminus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde

PH	Plextrin homology
PIP2	Phosphatidylinositol (4,5) biphosphate
PKD1	Polycystic kidney disease protein 1
PKD1L1	Polycystic kidney disease protein 1 like 1
PKD1L3	Polycystic kidney disease protein 1 like 3
PKD2	Polycystic kidney disease protein 2
PKD2L1	Polycystic kidney disease protein 2 like 1
PKD2L2	Polycystic kidney disease protein 2 like 2
P3CP	TRPP3 C-terminal peptide
P3LP	TRPP3 S4-S5 linker peptide
RT	Room temperature
TM	Transmembrane domain
TEVC	Two-electrode voltage clamp
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPL	Transient receptor potential like protein
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPN	Transient receptor potential NOMPC
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid
UPR	Unfolded protein response
WB	Western blotting
WT	Wild-type

CHAPTER 1
Introduction

1. TRPP subfamily

Transient receptor potential polycystins (TRPP) are membrane proteins of the TRP superfamily which consists of a diverse group of cation channel proteins primarily featured with six transmembrane domains (S1-S6) and a putative pore domain between S5 and S6 and both N- and C-termini located intracellularly [1,2]. Based on gene sequences and functionality, the cation channel proteins of the TRP superfamily are further classified into seven subfamilies including TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPN (no mechanoreceptor potential C), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) [3]. In mammals, many TRP proteins are expressed predominantly in the nervous systems to form non-selective ion channels which are involved in various important sensory physiological processes including vision, taste, smell, hearing, mechanosensation, thermosensation, pain perception, vasorelaxation, and even male fertility [4,3,5]. Appropriate maintenance and regulation of TRP channel protein functionalities are extremely important in humans health since mutations in many of the 27 human TRPs are associated with diseases ranging from stationary night blindness to

neurodegenerative disease, kidney diseases, chronic pain, and skeletal abnormalities [6,5].

The polycystins are grouped in the TRPP subfamily which consists of three cation channel proteins including TRPP2 (PKD2, polycystin-2), TRPP3 (PKD2L1, polycystin-L), and TRPP5 (PKD2L2) [3]. TRPP2 is responsible for 15% of the autosomal dominant polycystic kidney disease (ADPKD), one of the most common genetic disorder of the kidney, affecting 1 in 400 to 1000 individuals worldwide [7]. Mutations in the *PKD1* gene account for the remaining ADPKD. The physiological roles of the other two TRPP members TRPP3 and TRPP5 are even less clear. While TRPP3 is a homologue of TRPP2 with 50% identity in protein sequence, it is not involved in ADPKD. TRPP3 is expressed in bipolar neurons in the tongue taste buds where it plays an important role in sour tastes. As shown in TRPP3 knockout studies, TRPP3 absence is not lethal but with a mild signaling and developmental defect [8]. In one study, absence of *TRPP3* (and other genes) from the tongue was associated with loss of sour taste in two patients [9] and in another, with retinal detachment [9,10]; in some others, it is linked to circulating phospholipid levels [11,12]. Little is known about the pathological role of TRPP5. Table 1 summarizes some

properties of mammalian TRPP2, -P3 and -P5 proteins.

Gene name	Sequence homology	Tissue expression	Location in the cell	Modulation of activity	Disease
TRPP2		Kidney, liver, heart, lung, brain, intestine	Primary cilia of the renal tubule, ER membrane of cardiomyocytes, apical surface of bronchial epithelial	Ca^{2+}, translocation, TRPP1, EGF, PIP2, fluid flow, actin cytoskeleton	ADPKD
TRPP3	50% identity and 71% homology to TRPP2	Spleen, lung, testis, kidney, heart, brain	Epithelial cells in the pancreatic ducts, reticular cells in the spleen, endothelial cells of the major blood vessels and the epicardium in the heart	Ca^{2+}	Sour taste
TRPP5	59% homology with TRPP2, 58% homology with TRPP3	Testis, heart, brain, kidney	Ganglion cells in the retina, spermatocytes in the testis, and the epicardium in the heart	--	--

* ER, Endoplasmic reticulum; EGF, epidermal growth factor; PIP2, phosphoinositide-4,5-bisphosphate

Table 1. Properties of mammalian TRPP proteins

1.1 TRPP3

The full length of transcripts of the *TRPP3* gene is found highly expressed in fetal tissues including kidney and liver, but down-regulated in adult tissues, and thus may be involved in cystic diseases in humans and animals given the fact that TRPP3 protein has 50% amino acid sequence identity and 71% homology to TRPP2 [13]. TRPP3 is a calcium-modulated nonselective cation channel protein that is permeable to small mono-

and divalent cations like, Ca^{2+}, Ba^{2+}, Sr^{2+} , K^{+}, Rb^{+}, Li^{+} and Na^{+} [14]. It was estimated that TRPP3 is 5 times more permeable to Ca^{2+} than Na^{+} [14] . For monovalent cations, TRPP3 exhibits similar permeabilities (P) to Na^{+}, K^{+} and Rb^{+}, but lower permeability to Li^{+} (with PNa : PK : PRb : PLi = 1 : 0:98 : 0:97 : 0:87), and very low permeabilities to N-methyl-D-glucamine, tetraethylammonium and choline [14]. TRPP3 is encoded by the *TRPP3* gene expressed in adult heart and skeletal muscle, brain, spleen, testis, and retina [15]. TRPP3 is expressed in taste receptor cells of the tongue and identified as a candidate of mammalian sour taste sensor, as supported by the fact that mice lacking TRPP3 cells are completely devoid of taste responses to sour stimuli [16]. Expression of TRPP3 occurs in neurons surrounding the central canal of spinal cord where it may be implicated in modulating pH-dependent action potential characteristics [16,17,18]. TRPP3 is also expressed in brain, kidney, and heart with unknown function [13]. Partially depending on the cell type, TRPP3 is localized on the surface membrane and/or endoplasmic reticulum membrane. TRPP3 in *Xenopus laevis* oocytes targets the plasma membrane when expressed alone and acts as a Ca^{2+}-activated channel permeable to Na^{+}, K^{+}, and Ca^{2+} [14]. When

over-expressed in human embryonic kidney (HEK) cell lines TRPP3 is mainly localized on the endoplasmic reticulum membrane when expressed alone but traffics to the plasma membrane when co-expressed with PKD1 [19]. Another two independent studies indicate that polycystic kidney disease protein 1-like 3 (PKD1L3) and TRPP3 heteromers may function as sour taste receptors [20,21].

1.2. Other TRPP members

1.2.1 TRPP2

TRPP2 is a Ca^{2+}-permeable nonselective cation channel protein of 968-amino acid residues encoded by the *TRPP2* gene, and contains six transmembrane domains with intracellular amino- and carboxyl-termini and a calcium-binding domain [7,22]. The autosomal dominant polycystic kidney disease (ADPKD) characterized by the growth of numerous fluid filled cysts in the kidney, one of the most common genetic renal diseases, affects 7-10% of patients needing renal replacement [23]. TRPP2 is account for 15-20% of the ADPKD. TRPP2 interacts with polycystic kidney disease 1 protein (PKD1), the other ADPKD protein responsible for 80-85% of the disease. The exact mechanism of the

disease is not well known, with interrupted intracellular Ca^{2+} levels pointed out by several studies [23,24]. Potential mediators of the disease include a reported unfolded protein response (UPR), increased ER stress and disrupted cytoskeletal structural connections of the channels [25,26]. TRPP2 is involved in cell proliferation and has an important role in placenta [27]. *TRPP2* knockout mice fail to survive and die in utero [27]. The interaction of TRPP2 and polycystic kidney disease protein 1-like 1 (PKD1L1) is a prerequisite for the coordination of three-dimensional renal tubular organization during its embryonic development [28].

1.2.2. TRPP5

TRPP5 is a protein of 624 amino acid residues encoded by the *TRPP5* gene on chromosome 5q31 in humans, which has 59% homology with TRPP2 and 58% homology with TRPP3 [29]. Seventeen exons are found within the 50 kb genomic DNA of *TRPP5* gene, which are expressed in human brain, kidney, testis, and HepG2 cells with three alternatively spliced variants of differential expression [30]. Another study indicates that *TRPP5* gene encodes a protein of 613 amino acid sequence which has 45% amino acid

sequence identity and 68% homology to TRPP2 [31]. Given its striking homology to *TRPP2* gene, *TRPP5* gene may have similar roles or may contribute to the function of polycystin-2 in autosomal dominant polycystic kidney disease [31].

2. TRPP3 structure and topology model

TRPP3 is a protein of 805 amino acids with six hydrophobic transmembrane segments, a pore region between S5 and S6, and intracellular locations of both N- and C-termini [13,29]. Figure 1 shows TRPP3 membrane topology and highlights the major domains and posttranslational modifications as described previously [14].

TRPP3 is different from TRPP2 in the N-terminal cytoplasmic domain where it lacks a 100-amino acid segment [13]. Like TRPP2, TRPP3 has putative EF-hand and coiled coil structures in its C-terminal cytoplasmic domain. In addition, TRPP 3 has several putative phosphorylation sites: one cyclic nucleotide, two protein kinase C, and four casein kinase II phosphorylation sites with strong motif sequences in the C-terminal cytoplasmic domain, and two other putative protein kinase C phosphorylation sites in the N-terminal cytoplasmic domain [13].

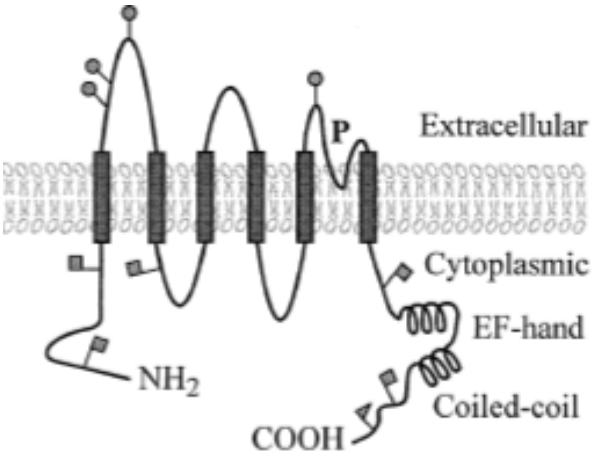


Figure 1. Schematic membrane topology of TRPP3.

Predicted N-glycosylation, PKA phosphorylation and PKC-phosphorylation sites are indicated with circles, triangles and squares, respectively. EF-hand and coiled-coil domains in C-terminal are shown [14].

2.1 The polycystin domain

The polycystin domain was first described by Shen and associates in a study on the cryo-EM structure of TRPP2 in lipid nanodiscs at a resolution of 3Å [32]. In the cryo-EM structure of TRPP2, an extracellular domain ($_{242}$Met-Tyr$_{465}$) covalently connected to S1 and S2 helices appears to define PKD2s (TRPP2, TRPP3, and TRPP5) as a unique subtype within the TRP channel family, which is referred as polycystin domain [32]. The polycystin domain has a novel three-layered folding only restricted to PKD2s, PKD1, and

PKD1-like proteins, contributes to channel assembly through extensive homotypic interactions with adjacent polycystin domains and regulates channel gating in TRPP2 [32]. Given the fact that the extracellular polycystin domain and pore helix 1 are two mutation hotspots in TRPP2 with 15 and 3 pathogenic missense mutations respectively, the extracellular polycystin domain plays critical roles in channel assembly and/or gating [32]. In contrast, no pathogenic missense mutations have been reported in the N terminus of TRPP2 to date. Shen *et al.* speculated that the polycystin domain may serve as a physical ‘‘lid’’ through which extracellular physical and/or chemical stimuli can allosterically modulate pore gating [32].

The cryo-EM structure of TRPP3 at a 3.4 Å resolution revealed the polycystin domains separating S1 and S2 helices, and locking onto each other on the extracellular side to stabilize the homotetrameric ion channels [33]. The polycystin domains of TRPP3 also share similar structural features to TRPP2 [33].

2.2 The voltage-sensing domain

In voltage-gated ion channels (VGICs), the voltage-sensing domain (VSD) harbors

the positively charged S4 voltage sensor [34] and consists of the S1 to S4 helices. A channel with voltage-dependent properties, TRPP3 has a representative VGIC fold, with its first four transmembrane (TM) helices forming the VSD [35]. Unlike TRPV1 and TRPA1 but similar to TRPP2, TRPP3 harbors two lysines in S4 (K452 and K455), which could help explain the voltage-dependent properties [33]. Also similar to TRPP2, TRPP3 VSDs could couple to the pore gates. When the closed- and open-states of TRPP3 are superimposed, the potential coupled motions arise from the VSD that are associated with pore domains' movements. The S4 helix seems to drive the neighboring S5 helix from another protomer moving outwardly, due to the π stacking interactions in VSD/pore domain. Additionally, multiple aromatic residues could form extensive π–π interactions within the upper gate region. While TRPP3 highly resembles TRPP2 both sequentially and topologically, TRPP3 TM domains still differ structurally from TRPP2 in terms of conformational details. Depiction of the molecular mechanism of TRPP3 function relies on these details of conformational changes in the VSD [33].

2.3 S4-S5 linker

The S4-S5 linker, a cytoplasmic segment that connects the S1-S4 helices, the S5-pore-S6 segment, the distal part of helix S6, and the TRP domain, is believed to be involved in the regulation and modulation of channel function and trafficking. The S4-S5 linker is a highly conserved peptide sequence consisting of 15~30 amino acid residues with some variations among the TRP channels [36]. Mutations frequently spotted in the cytoplasmic peptide sequence of the S4-S5 linker in TRPV3 are related to the congenital Olmsted syndrome [37,38], in TRPV4 are related to skeletal dysplasias and motor/sensory neuropathies [39,40], and in TRPA1 are related to the familial episodic pain syndrome [41]. These pathogenic mutations in the S4-S5 linker region are influential in the regulation of channel functions and are often referred to gain-of-function mutations since they are frequently associated with constitutive channel activation and insensitive to regulation [36].

The amino acid residues of the S4-S5 linker in TRPP3 have 62.5% identity to TRPP2 and 87.5% identity to TRPP5 [32]. In human TRPP2, the S4-S5 linker is presumably coupled to the S6 helix through a hydrogen bond formed between the glutamine 585 in the S4-S5 linker and the lysine 688 within S6, with both glutamine 585

and lysine 688 being conserved in TRPP2, TRPP3, and TRPP5 at corresponding positions but not in other TRPs [36]. Mutation studies on the S4-S5 linker have been reported in TRPV3, TRPV4, TRPA1, and TRPM4 in relation to the regulation and modulation of channel function, e.g., in TRPV4, a hydrogen bond between the indole of W733 of the TRP helix and the backbone oxygen of L596 secures the helix/linker contact, which acts as a latch maintaining channel closure [42]. However, mutations of S4-S5 linker in relation to channel function have not been reported in TRPP2, TRPP3, and TRPP5.

2.4 TRP-like domain

TRP domain is a short (about 25 aa) conserved amino acid sequences in the C-terminal domain of the TRP subfamilies: TRPN, TRPV, TRPC and TRPM [43] , the function of TRP domain was unclear until 2005 when Rohacs and colleagues discovered that the TRPM8 TRP box binds phosphatidylinositol (4,5)-bisphosphate (PIP2) [44]. In their C terminus almost immediately after S6, TRPC, TRPV, and TRPM proteins contain a TRP domain characterized by a signature motif WKxxR (also called a TRP box) important for channel activation and PIP2 binding [45,46]. A TRP-like domain is the

corresponding fragment in TRPA1, which contains a WxxxK motif instead of WKxxR [47]. Parallel to the plasma membrane, a helical configuration adopted by all TRP and TRP-like domains in structurally resolved TRPs [48,49,50,51,47] suggests that they may have similar functional roles. It was discovered that the TRP box of TRPV1 bind PIPs thereby regulating the channel gating and desensitization [52]. Other studies revealed the role of conserved residues in the TRP box of TRPV1 and TRPV4, and highlighted the role of the tryptophan (W) residue within the box [42,53]. For example, W733 in the TRPV4 TRP box interacts with L596 in the S4-S5 linker [42]. While reported to influence the channel open probability and gating [42], such interaction does not dismiss the important role of other residues that mediates binding to lipid messengers and modulates channel functions [52].

2.5 Oligomerization domain

It is evident that the structure of functional TRP channels is tetrameric [54,55]. While able to form homotetramers, TRP channels can still be capable of heterotetramerization. TRPP3 can form homotetramers as shown by Huber A. *et al* [56], and is still able to form heterotetramer with PKD1L3 in a unique alternating subunit assembly and 1:3

stoichiometry [57]. Domains in TRP channel for tetramer formation and proper channel assembly were identified in the N-termini [58], C-termini [59,60], as well as the TM domains [61]. For example, TRPP3 N-terminus is essential to dimerization [62]. The C-terminal regulatory domains (CRDs) of TRPP3 play a vital role in channel oligomerization, and C-terminal coiled-coil-2 (CC2) domain (G699-W743) was reported to be central for its trimerization. Locking onto each other on the extracellular side, the polycystin domains stabilize the homotetrameric ion channels [33]. Some of the dimerization and trimerization domains identified in many TRPs are proven non-essential for channel function and others are essential to proper channel tetramerization and function. Although coiled coil domain in TRPP3 constitutes a trimerization domain [57,63] as shown in crystal structure and chromatography experiments, it was reported as a non-essential domain for channel function [64,61]. Cysteine 632 (C632) in the TRPP2 protein was found important for dimerization along with residues in the N- and C-termini [58]. Another example is TRPM6 and -7, which form functional homo- and heterodimers with kinase activity [65].

2.6 EF hand

EF hand is a putative Ca^{2+} binding motif that can be functionally either stimulatory or inhibitory. The deletion of the EF hand domain (E637- L665) in the C-terminus of TRPP3 leads to an increase in channel activity and a decrease in channel inactivation [64]. Interestingly, the ankyrin repeat motif present in the N-terminus of TRPA1 has characteristics similar to the known EF hand [66] and is capable of binding Ca^{2+} and activating the TRPA1 channel [67]

TRPP3 features a Ca^{2+} binding EF hand motif followed by an endoplasmic reticulum (ER)-retention signal sequence and a casein kinase 2-dependent phosphorylation site at S812 [68]. TRPP3 has a helix-loop-helix secondary structure or an EF hand [69] that allows calcium binding. The presence of the putative EF-hand structure in both TRPP3 and TRPP2 suggests that their functions may be affected by cytoplasmic calcium concentration [13], since binding of calcium to the helix-loop-helix EF-hand structures tends to inactivate certain type of calcium channels [70].

2.7 Coiled-coil domain

Coiled-coil domain (CCD) is a regulatory motif composed of α-helices that keeps the protein in a particularly stable state. CCD serves as an oligomerization domain for several TRP members as well as for other ion channels, structural proteins and transcription factors [71]. The CCD of TRPPs is implicated in formation of polymodal multiprotein/ion channels complexes [72]. The function of the CCD is determined by factors such as amino acid composition, length and how distal from TM6. Accordingly, the roles of CCDs in different TRP members are not the same. It is vital for oligomerization and trafficking in some melastatin (TRPM) [73,74] and vanilloid (TRPV) [73] TRP members but not important for other members [71,19]. Some studies have identified the interesting role of a CCD in channel gating [75,64] and proposed a mechanism where CCD is involved in maintaining a closed channel state [71,75,64].

3. Close proximity of S4-S5 linker to TRP-like domain in TRPP3

The cryo-EM structure of TRPV1 at a 3.4 Å resolution first revealed an intramolecular proximity of the S4-S5 linker and TRP domains [51,76,77]. This observation was later confirmed in the structures of TRPA1 [47], TRPV2 [78,48], TRPV6 [49], TRPP2 [79,80,32],

and NOMPC [81]. During the past 6 years, more and more TRP structures have been resolved, with 17 structures resolved so far. In this year alone, 9 TRP structures including TRPP3 were resolved (Figure 2). All these structural information has confirmed the past predicting topology of TRP channels, and ligand binding sites, gate residues, potential intermolecular and intramolecular interaction have bee revealed.

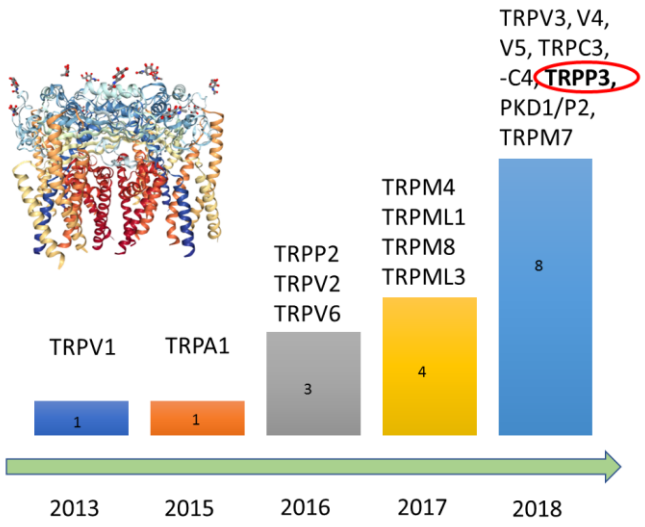


Figure 2. Tendency chart of resolved high-resolution structures of TRP channels

Figure 3 shows the proximity of the S4-S5 linker and TRP domain of different TRP channels. In TRPV1, the strategic location of TRP domain enables the interaction with both the S4–S5 linker and pre-S1 helix (E416-R428), and potentially a physical substrate through which stimuli allosterically affect pore conformation [51]. In TRPP2, a hydrogen

bonding interaction couples the S4-S5 linker with the pore-lining S6 helix through Gln585 and Lys688 [32]. In TRPA1, the TRP-like domain forms hydrophobic interactions with the second helix turn-helix motif and S4–S5 linker [47] . In TRPN subfamily NOMPC, the transmembrane domain and the ankyrin repeats (ARs) domain sandwich a series of linker helices in between, and create a network of interactions connecting the S4–S5 linker via the TRP domain to the ARs [50]. All these TRP structures provide a theoretical basis that S4-S5 linker is spatially close to TRP (TRP-like) domains, although the S4–S5 linker and TRP-like domain in TRPP3 are invisible at near-atomic resolutions, indicating the flexibility of the linker and TRP-like domain [33].

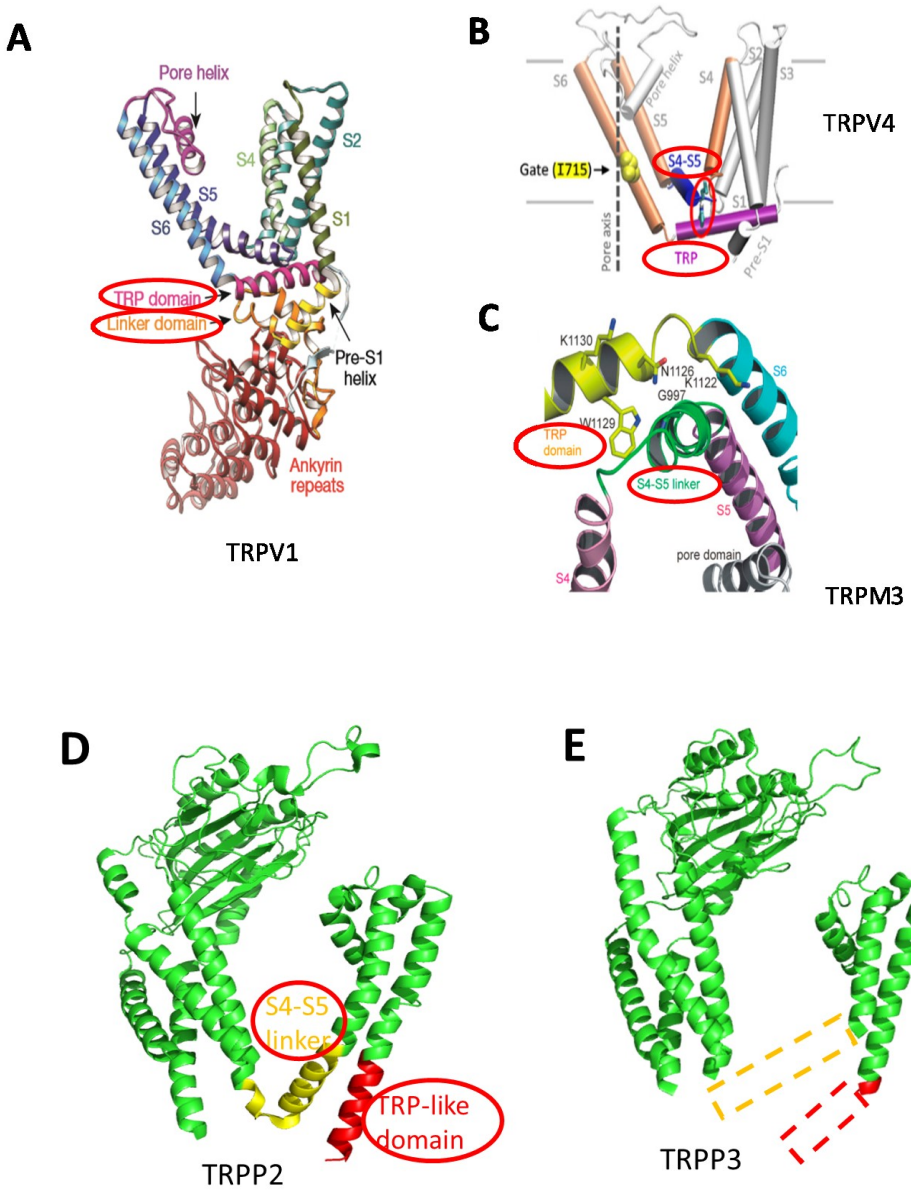


Figure 3. Diagram of the interactions between the TRP domain and S4-S5 linker of rat TRPV1, rat TRPV4, mouse TRPM3, human TRPP2 and mouse TRPP3 structures [76,42,36,32,33]. (Homology models of TRPV4 and TRPM3 are based on the cryo-EM structure of TRPV1)

4. TRPP3 homomeric/heteromeric channel

Recently published TRPV1, -2, -6, TRPA1 and TRPP2 structures confirmed the prediction that similar to voltage-gated potassium channels, TRP channels function as tetramers [51,48,49,32]. TRPP2 and TRPP3 are also believed to assemble into homo-tetramers to be functional. Unlike other TRP members, TRPP2 and TRPP3 are also discovered to form hetero-tetramers with PKD1 and PKD1L3, respectively, with 3:1 stoichiometry [82,83]. Critical evidence to support the hypothesis that the homo- and hetero-tetramer of TRPP2 or TRPP3 share a trimer/monomer assembly model would be the existence of a trimerization domain. Indeed, a conserved trimerization domain, C-terminal coiled-coil-2 (CC2) domain (G699-W743), was identified later in C-terminus of TRPP2 and TRPP3 [57]. However, a previous study and our current study suggest that CC2 is not necessary for functional TRPP3 assembly as TRPP3 truncation mutants lacking CC2 exhibit similar channel function as WT TRPP3 [64]. We identified, for the first time, C1 domain (K575-T622), which does not overlap with CC2, to be critical for both TRPP3 trimerization and channel function. The involvement of C1 in assembly of

TRPP3/PKD1L3 channel complex is further indicated by C1 peptide expression's ability to block TRPP3/PKD1L3 channel function. Therefore, C1 would be the trimerzation domain for both TRPP3 homotetramers and TRPP3/PKD1L3 heterotetramers. It would be interesting to see whether TRPP2 contains a similar functionally important trimerization domain. Since recently published high-resolution TRPP2 structure lacks both N- and C-termini [32], it provides little insight into its oligomeric assembly. Another interesting question is whether the trimerization process is unique to TRPP3 and TRPP2 or also exists in other TRP members, which are generally speculated to form functional channels through dimerization of two homodimers.

4.1 Ca^{2+}-dependent on-response

Among the TRPP members, TRPP3 was the first shown to form an ion channel. In 1999, Chen *et al*. [14] demonstrated that, when expressed in *Xenopus* oocytes, human TRPP3 forms an ion channel activated by extracellular Ca^{2+} and is conducting to cations including Na^{+}, K^{+} and Ca^{2+}, and not negatively charged ions. This was followed by a reversible desensitization period that may last up to 10 min, of which the underlying

mechanism remains elusive. When oocytes were pre-injected with Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) to eliminate any free intracellular Ca^{2+}, extracellular Ca^{2+} can no longer induce TRPP3 activation, indicating that channel activation requires Ca^{2+} entry leading to increase of intracellular free Ca^{2+}. This Ca^{2+}-induced activation was not observed when expressed in HEK293T cells [84,85]. The latter was characterized by an outwardly rectifying current when expressing mTRPP3 [85,86] or hTRPP3 [84]. HEK expressing mTRPP3 was constitutively active, highly permeable to Ca^{2+} and its currents were inhibited by the high extracellular Ca^{2+} [85]. DeCaen *et al.* [84] reported that hTRPP3 expressed in HEK cells is not activated by high extracellular Ca^{2+}, but rather it forms homomeric channels that conduct large non-selective cation current with moderate Ca^{2+} selectivity. A Ca^{2+}-binding domain EF-hand in TRPP3 C-terminus was initially thought to mediate its activation/inactivation behavior. However, a truncation mutant lacking the EF-hand still exhibited a similar multi-phasic behavior as WT channels [64]. To date, the exact mechanisms underlying the TRPP3 multi-phasic activation and inactivation is still to be determined.

4.2 H^+-dependent off-response

In 2006, TRPP3 was proposed as a pH sensing channel [16,20,21]. While low extracellular pH inhibits the channel and decreases its basal conductance [14,85], the channel was proposed to have a 'delayed response to acids' [20]. Two years later, Inada *et al*. [87] proposed that this 'delayed response' is an off-response, where the activation is induced only when extracellular acid is removed [87]. Subsequent studies suggested TRPP3/PKD1L3 as the sour taste receptor [88,89]. Different from the PM targeting of TRPP3 in *Xenopus* oocytes, when heterologously expressed in mammalian cells, e.g., human embryonic kidney 293 (HEK293) cells, TRPP3 is mainly localized in the ER membrane with unknown channel function. However, co-expression with PKD1L3, a homologue of PKD1 protein, leads TRPP3 to substantially traffick to PM where the two form a heteromeric channel complex activated by acids in a so-called off-response manner. TRPP3/PKD1L3 channel off-response has some fundamental properties. TRPP3/PKD1L3 channel activity is dependent on the stimulating pH [16,20,87,90] and type of acid but independent of the duration of acid application time. TRPP3/PKD1L3 complex

off-response current amplitude was proportional to the difference between the stimulating pH and the washing pH [87]. Unlike TRPP3 on-response in *Xenopus* oocytes, TRPP3/PKD1L3 off-response can be repeated and does not exhibit inactivation [87]. TRPP3/PKD1L3 channel complex is Ca^{2+} permeable and two negatively charged residues D523 [91] and D525 [82] in the TRPP3 S5-S6 pore loop have been identified to affect the Ca^{2+} permeation, suggesting they may be the determinants of the channel complex selectivity. To date, the exact mechanism underlying the off-response still remains elusive.

5. TRPP3 research in our lab

In our previous study, we first identified the C1 domain (K575-T622), which is essential for both TRPP3 trimerization and channel function [19]. In TRPP3 N-termini, we determined a palmitoylation site C38 and a phosphorylation site T39, which independently regulate TRPP3 channel function [62]. We also revealed two intra-membrane residues critical for TRPP3 channel function: D349 and E356 in the third transmembrane domain [92]. We identified an intramolecular interaction of the TRPP3 N- and C-termini

(called N-C interaction) essential for TRPP3 channel function that is mediated by W81 in pre-S1 domain and K568 in TRP-like domain. Structure-function analyses revealed similar N-C interaction in TRPP2 as well as in TRPM8/-V1/-C4. In TRPP3, PIP2 binding site K568 and binding domain $_{594}$RLRLRK$_{599}$ (called a RRRK motif) have been revealed [93]. We systematically replaced the hydrophobic residues within the distal fragment of pore-lining helix S6 with hydrophilic residues and, based on *Xenopus* oocyte and mammalian cell electrophysiology and the hydrophobic gate theory, we identified a two consecutive-residue hydrophobic gate (L557-A558) in TRPP3 as well as in TRPV6 (A616-M617) and TRPV5 (A576-M577). Single hydrophobic gate in TRPV4/C4/M8 have been identified as well [94]. Figure 4 shows all the important residues described above.

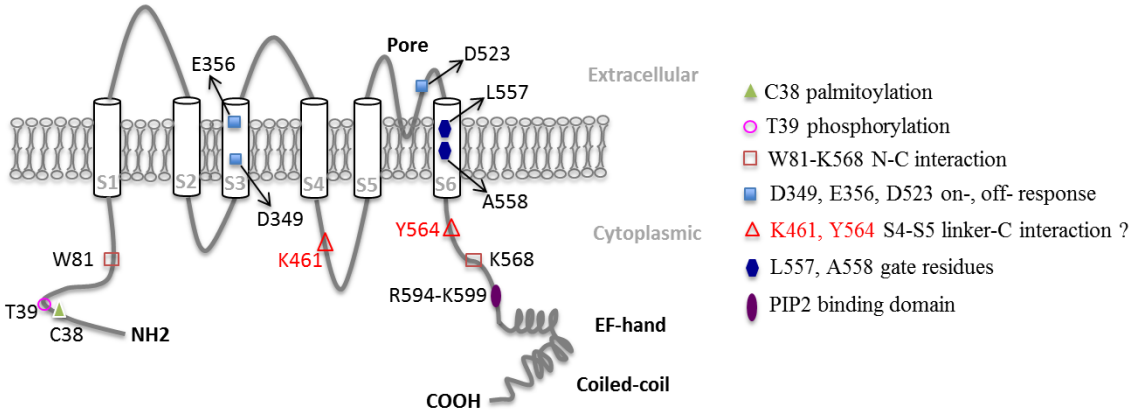


Figure 4. Topology model for TRPP3 with the indication of the locations of several functional amino acids being studied by our lab. The relative locations of the proposed interaction between amino acids K461 and Y564 are also displayed.

6. Thesis objectives

The main purpose of this study is to gain a better understanding of functional regulation of TRPP3 ion channel. Based on previous studies in our lab and TRP structural information from others' work, we propose that an intramolecular interaction between S4-S5 linker and C-terminal TRP-like domain (called L-C interaction) is functionally essential. The interaction is mediated by cationic residue K461 and aromatic residue Y564 through cation-π interaction. To be more specific, the hypothesis involves the following.

(1) TRPP3 S4-S5 linker contains an important cationic residue (K461) that is essential for channel function;

(2) TRPP3 C-terminus contains an important aromatic residue (Y564) that is essential for channel function;

(3) L-C interaction is mediated by K461 and Y564 through cation-π interaction;

(4) PIP2 negatively affects L-C interaction through binding cationic residues which are close to K461 or Y564, and then eventually breaking the interaction between the S4-S5 linker and C terminus.

CHAPTER 2
Materials and Methods

1. Plasmids, Mutagenesis, Antibodies, and Chemicals

Human Flag-TRPP3 cDNA (GenBank: NM_016112) was cloned into vector pCHGF [95] for *Xenopus laevis* oocyte expression. All mutations were made with QuikChange Lighting Site-Directed Mutagenesis kit (Agilent Technologies, CA) and confirmed by sequencing. Rabbit antibodies against Flag (D-8) and HA (Y-11) and mouse antibodies against GST (B-14), β-actin (C-4), and PIP2 (PIP2 2C11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibody against His (N144/14) for Western blotting was from NeuroMab (Davis, CA). Mouse antibody against His (27E8) and rabbit antibody against His (no. 2365) were purchased from Cell Signaling Technology (Danvers, MA) and were used in immunoprecipitation and immunofluorescence assays, respectively. Secondary antibodies were purchased from GE Healthcare (Waukesha, WI). diC8-PIP2 was purchased from Echelon Biosciences (Salt Lake City, UT).

2. *E. coli* transformation

Once the PCR products were obtained, they were digested with the *Dpn I* enzyme for 30 minutes (min) at 37 °C. The mixture of digested product (about 1 to 3 µL) and *E. coli* DH5αTM competent cells (50 µL) was placed on ice for 30 min, then put in 42 °C for 30-40 s. Cells were left to recover in 500 µL lysogeny broth (LB) medium through shaking for an hour at 37 °C. Finally, the transformed cells were placed on 0.15 µg/L LB agar plates (15 g of bacto agar in 1.0 L LB medium and 50 µg/mL ampicillin or kanamycin) and the plates were incubated overnight at 37 °C.

3. Plasmid isolation

E. coli colonies appeared on the LB plates after 14-16 hours (hr) of incubation at 37 °C. Single *E. coli* colonies were selected and cultured overnight in 3 mL LB at 37 °C with agitation. Plasmids were isolated by following DNA extraction protocols (Invitrogen Life Technologies, CA). Cells were collected by centrifugation at 12 000 x g for 2 min. Cell pellets were re-suspended in 200 µL resuspension buffer and mixed well. 200 µL of lysis buffer was added to the mixture and thoroughly mixed until the lysate turned viscous. 350 µL of neutralization buffer was added to precipitate the cell debris, briefly

mixed. Then the mixture was centrifuged at 12 000 x g for 5min. The pellet was discarded and the supernatant transferred to a new microfuge tube with column tube. The tube was centrifuged at 12 000 x g for 1 min and the flow-through liquid was discarded. 700 μL wash buffer was added to the column tube and centrifuged at 12 000 x g for 1 min and the flow-through liquid was discarded. Again, the tube was centrifuged at 12 000 x g for 1 min without any solution. 50 μL water or elution buffer was added to elute the plasmid DNA with centrifuging at 12 000 x g for 2 min.

4. Preparation of mRNAs and microinjection into oocytes

pCHGF plasmids containing TRPP3 WT or mutant cDNAs were linearized with *Mlu I*, followed by phenol/chloroform purification and ethanol precipitation. Linearized DNAs were then used to *in vitro* synthesize capped mRNAs using mMESSAGE mMACHINE kit (Ambion, Austin, TX). Stage V-VI oocytes were isolated from *Xenopus laevis*. Defolliculation of oocytes was performed through incubation in Ca^{2+}-free Barth's solution [96] containing collagenase (2 mg/ml) at RT for 2-2.5 hr. Oocytes were then incubated at 18˚C in the Barth's solution for at least 3 hr before injection of 50 nl

RNase-free water containing 50 ng mRNAs. An equal volume of water was injected into each control oocyte. The present study was approved by the Ethical Committee for Animal Experiments of the University of Alberta and was carried out in accordance with the Guidelines for Research with Experimental Animals of the University of Alberta and the Guide for the Care and Use of Laboratory Animals (NIH Guide) revised in 1996. Injected oocytes were incubated at 16-18˚C in the Barth's solution supplemented with antibiotics for 2-4 days prior to experiments.

5. Two-electrode voltage clamp (TEVC)

Current and voltage signals were measured with the conventional two-electrode voltage clamp (TMVC) technique with a commercial amplifier (TEV-200A, Dagan, Minneapolis MN, USA). Electrodes were fabricated from borosilicate glass (Warner Instruments, Hamden CT, USA) by a micropipette puller (P-87, Sutter Instruments, Novato, CA, USA) and filled with 3 M KCl with typical tip resistance of 0.5-3 MΩ. The standard extracellular solution (pH 7.5) containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 10 mM HEPES was used for extracellular perfusion (at approximately 1.5

ml/min). The solution containing extracellular Ca^{2+} was prepared from the standard solution with the addition of $CaCl_2$ to a final concentration of 5 mM. Duration of Ca^{2+} medium application was indicated in time course recordings. In experiments using a ramp or gapfree protocol [14], current/voltage signals were digitized at 200 ms/sample. Oocyte whole-cell currents were recorded using a Geneclamp 500B amplifier and Digidata 1322A AD/DA converter (Molecular Devices, Union City, CA). The pClamp 9 software (Axon Instruments, Union City, CA) was applied for data acquisition and analysis. SigmaPlot 12 (Systat Software, San Jose, CA) was used for data fitting and plotting.

6. Western blotting (WB)

Protein samples were prepared with CelLytic M lysis buffer (Sigma-Aldrich, Oakville, ON, Canada) from oocytes according to the manufacturer's instruction. This lysis buffer is an efficient reagent for the extraction of proteins. It consists of a dialyzable mild detergent, bicine, and 150 mM NaCl, resulting in minimal interference with protein interactions and biological activity. 50-80 g total proteins were then

separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes, which were then blocked for 1 hr at room temperature with 3% skim milk in PBS buffer supplemented with 1% Tween-20. This was followed by overnight incubation at 4 °C with diluted primary antibodies in blocking buffer according to suppliers' suggestions. Secondary horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were purchased from GE. Protein samples were separated on 8% (/15%) SDS-PAGE gels. For the reducing SDS-PAGE, samples were mixed with loading buffer supplemented with 0.5 M DTT or 5% 2-mercaptoethanol (2-ME), and subject to heating at 65˚C for 5 min. 30 μg of total protein was loaded per lane. Flag antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibody against TRPP3 (PR71) was custom made and used previously [97]. Secondary antibodies were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Band intensity was analyzed with the software ImageJ (NIH, Bethesda, MD).

7. Immunofluorescence

Xenopus oocytes were washed in PBS, fixed in 4% paraformaldehyde for 15 min, washed 3 times in PBS plus 50 mM NH_4Cl, and then permeabilized with 0.1% Triton X-100 for 4 min. Oocytes were then washed 3 times in PBS for 5 min each time, blocked in 3% skim milk in PBS for 30 min, and then incubated overnight with the rabbit anti-TRPP3 polyclonal antibody (cat# PAB5914, Abnova). This was followed by three 10-min washes in PBS. Oocytes were then incubated with a secondary AlexaFluor 488-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or Cy3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min, followed by three 10-min washes in PBS and mounting in Vectashield (Vector Labs, Burlington, ON). The slides were examined on an AIVI spinning disc confocal microscopy (Cell Imaging Facility, Faculty of Medicine and Dentistry, University of Alberta). Plasma membrane intensity of WT or mutant TRPP3 was assessed by quantifying the plasma membrane immunofluorescence using Volocity 6.2 (Perkin Elmer, Waltham, MA). Background

fluorescence was subtracted and data were normalized to the average TRPP3 WT intensity.

8. Oocyte surface protein biotinylation

Xenopus oocytes were washed three times with ice-cold PBS solution followed by incubation with 0.5 mg/mL sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) for 30 min at room temperature (RT). 1 M NH_4Cl was used to quench the non-reacted biotin. Oocytes were then washed with ice-cold PBS solution and harvested in ice-cold CelLytic M lysis buffer (Sigma-Aldrich, Oakville, ON, Canada) supplemented with proteinase inhibitor mixture (Thermo Scientific, Waltham, MA). Lysates were incubated at 4˚C overnight with gentle shaking upon addition of 100 µL streptavidin (Pierce, Rockford, IL). The surface protein absorbed by streptavidin was resuspended in a SDS loading buffer and subjected to SDS-PAGE.

9. Co-immunoprecipitation (Co-IP)

Co-IP experiments were performed using a modified protocol [98]. Briefly, a group of 30 oocytes expressing an indicated TRP channel and/or peptides was washed twice with

PBS and solubilized in ice-cold CelLytic-M lysis buffer (Sigma) supplemented with proteinase inhibitor mixture. Supernatants were collected after centrifugation at 16,000 x g for 15 min and precleared for 1 hr with protein G-Sepharose (GE Healthcare) and then incubated with indicated antibodies for 4 hr at 4˚C. After the addition of 100 μL of 50% protein G-Sepharose, the mixture was incubated overnight with gentle shaking at 4˚C. The immune complexes absorbed to protein G-Sepharose were washed five times with Nonidet P-40 lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, and 1% Nonidet P-40) and eluted by SDS loading buffer. Precipitated proteins were analyzed by WB using indicated antibodies.

10. Protein expression in and purification from *E. coli*

The cDNA fragments encoding the TRPP3 S4-S5 linker peptide F454-F480 (P3LP) and TRPP3 C-terminus peptide I560-K660 (P3CP) were inserted into pGEX5X vector (Pharmacia, Piscataway, NJ, USA) and pET28a(+) vector, respectively. BL21 (DE3) strain (Novagen, Darmstadt, Germany) was used for expression. Following growth at 37˚C to an optical density (OD) of 0.6, cultures were cooled to 30˚C, induced with 1 mM

isopropyl β -D-thiogalactoside (IPTG) and incubated for 3 hr. Cell lysates from 50 mL culture were prepared with CellLytic-B lysis buffer (Sigma) according to the manufacturer's instruction. P3CP and its mutant proteins were pulled down with Ni-NTA resin (Qiagen, Venlo, Netherlands) and eluted from beads with 250 mM imidazole by following the manufacturer's manual. P3LP and its mutant proteins were pulled down with glutathione-agarose beads (Sigma) and eluted from beads with elution buffer containing 10 mM glutathione, 50 mM Tris, and pH 8.0 by following the manufacturer's manual.

11. *In vitro* His pull-down

Purified GST-tagged P3LP (F454-F480; 2 mg) from *E. coli* was incubated with the same amount of purified His-tagged P3CP (I560-K660) from *E. coli* in the CelLytic-M lysis buffer (Sigma). The mixture was incubated at RT for 1 hr with gentle shaking, followed by another hour of incubation after addition of 10 uL 50% Ni-NTA agarose bead (QIAGEN, Hilden, Germany). The beads were then washed three times with PBS buffer supplemented with 1% Nonidet P-40, and the remaining proteins were eluted using

SDS loading buffer, resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then immunoblotted with His and GST antibodies.

12. Statistical analyses

Data were analyzed and plotted using Sigmaplot 13 and expressed as mean ± standard error of the mean (SEM). Student *t* tests were used to compare two sets of data, whereas one-way ANOVA for multiple comparisons. A probability value (p) of less than 0.05, 0.01, or 0.001 was considered statistically significant and indicated by *, **, and ***, respectively.

CHAPTER 3
Results

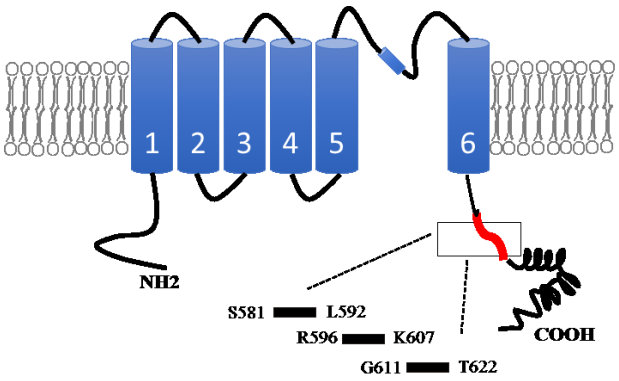
3.1. Domains S581-L592, R596-K607 and G611-T622 are critical for TRPP3 channel function

Previous work from our lab identified a novel C-terminal domain called C1 (K575-T622) which is essential for both TRPP3 trimerization and channel function [19]. However, this C1 domain contains 48 amino acids and is thus still fairly large. It is not certain which of the 48 amino acids play a crucial role in regulating TRPP3 channel function. Therefore, three deletion mutants, ΔS581-L592 (with fragment 581-592 deleted), ΔR596-K607, and ΔG611-T622 (Figure 5A), were created using site-specific mutagenesis and their channel function was tested.

When the whole-cell currents were measured at -50 mV using the TEVC technique, mutant ΔG611-T622 generated a large inward current which was 2-fold higher than the WT channel, indicating gain-of-function of channel activity by this mutant. However, the other mutants (i.e. ΔS581-L592 and ΔR596-K607) did not produce any appreciable currents (Figure 5B and 5C). Similar results were obtained when currents were measured

at other membrane potentials (i.e. -80 to 80 mV) using a ramp protocol (Figure 5D). To examine the protein expression and membrane localization of these mutants, further analyses with WB and immunofluorescence assays revealed that both expression and the surface targeting were not affected (Figure 5E and 5F). These data demonstrate that all three domains S581-L592, R596-K607 and G611-T622 are important for TRPP3 channel function. We next mutated every single residue in S581- L595 domain to alanine but no functionally impaired mutation was found (Figure 5G). Collectively, these data suggest the presence of a small domain (e.g. composed of 3-7 amino acids) localizes in S581-L592 that is critical for the function of the TRPP3 channel. In addition, important residues or motif needs to be further studied in the domain R596-K607 and G611-T622.

A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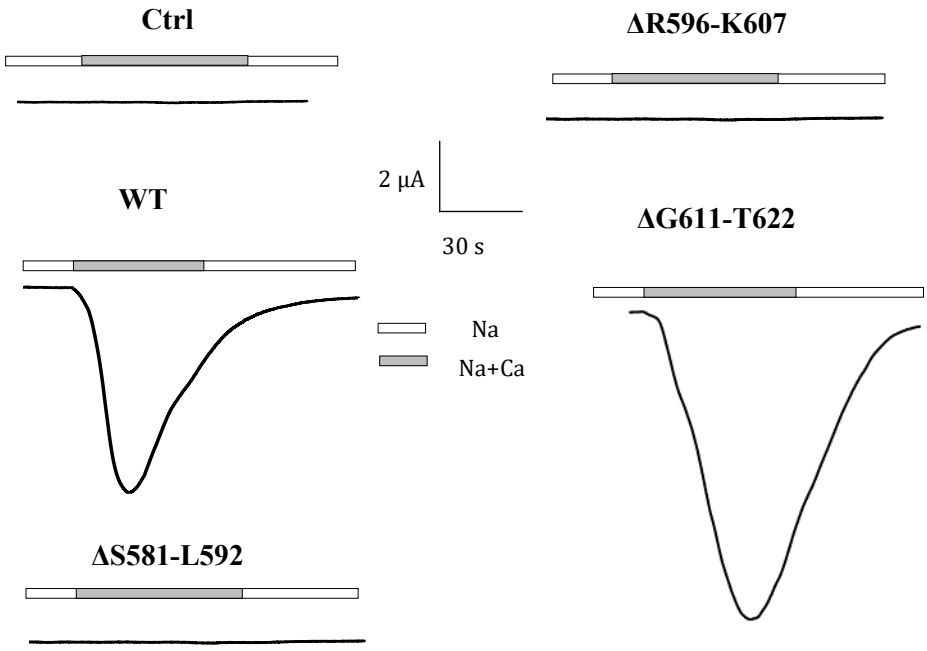
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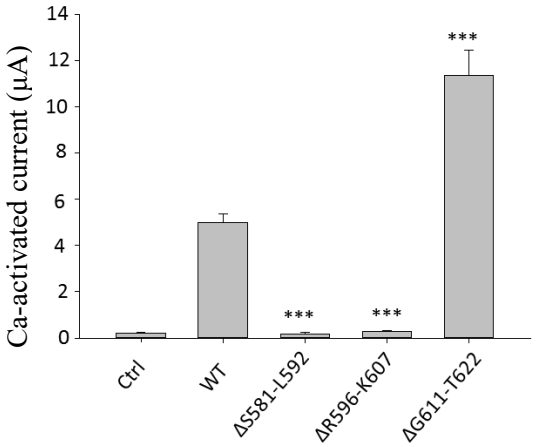
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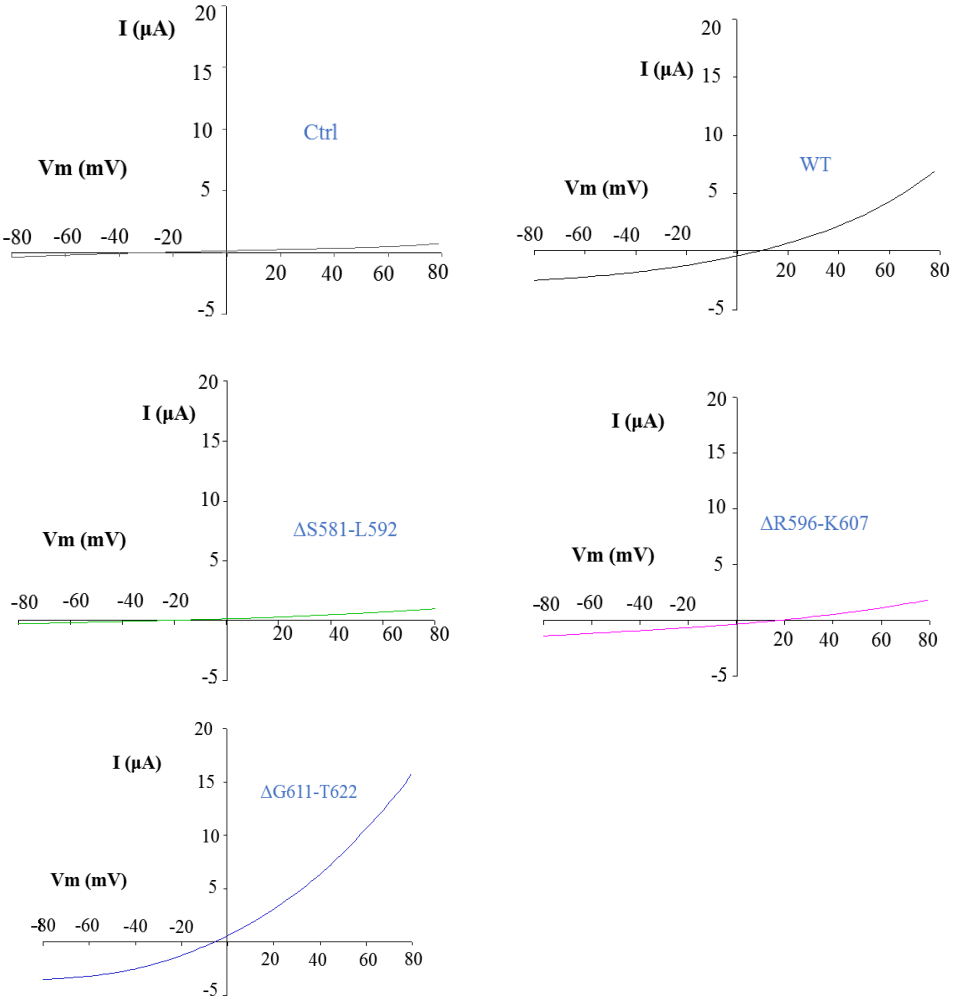
14
12
10
8
6
4
2
0
***
***
***
Ctrl
WT
ΔS581-L592
ΔR596-K607
ΔG611-T622

D


I (μA)
Vm (mV)
Ctrl
WT
ΔS581-L592
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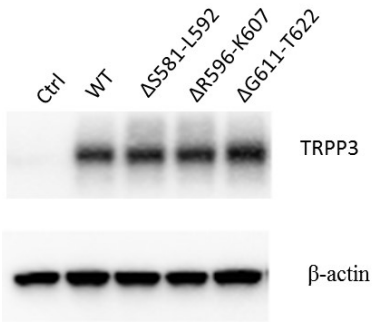
E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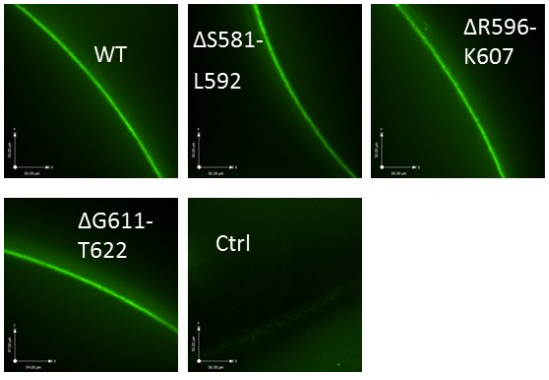
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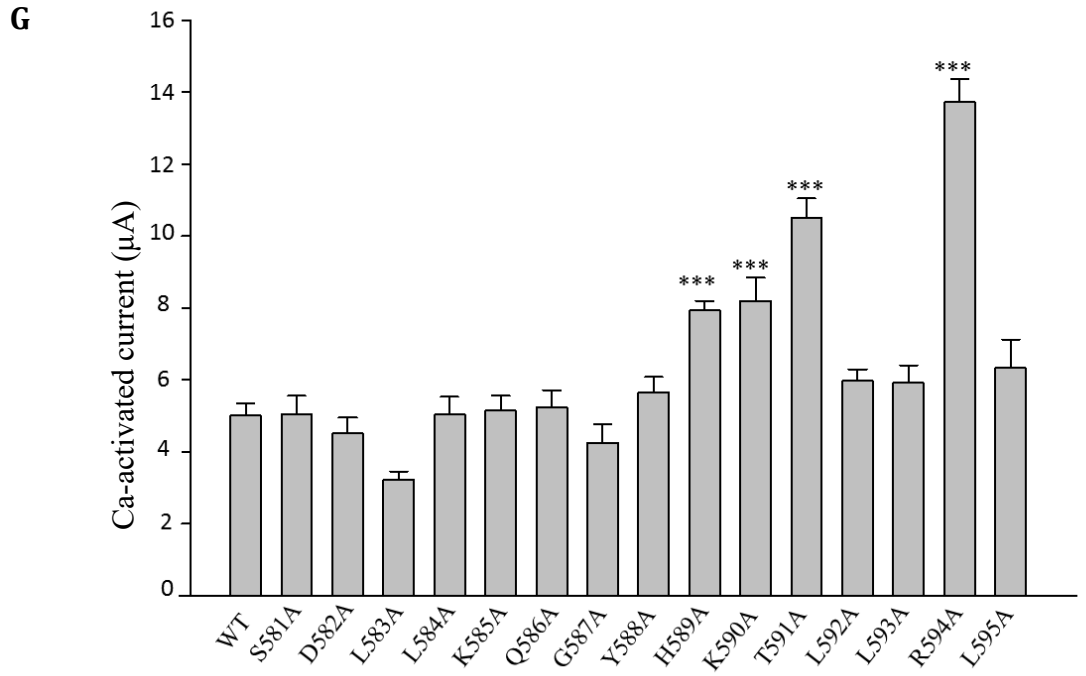


Figure 5. Location of three domains that are mutated in the human TRPP3 C-terminus and their roles in regulating channel function.

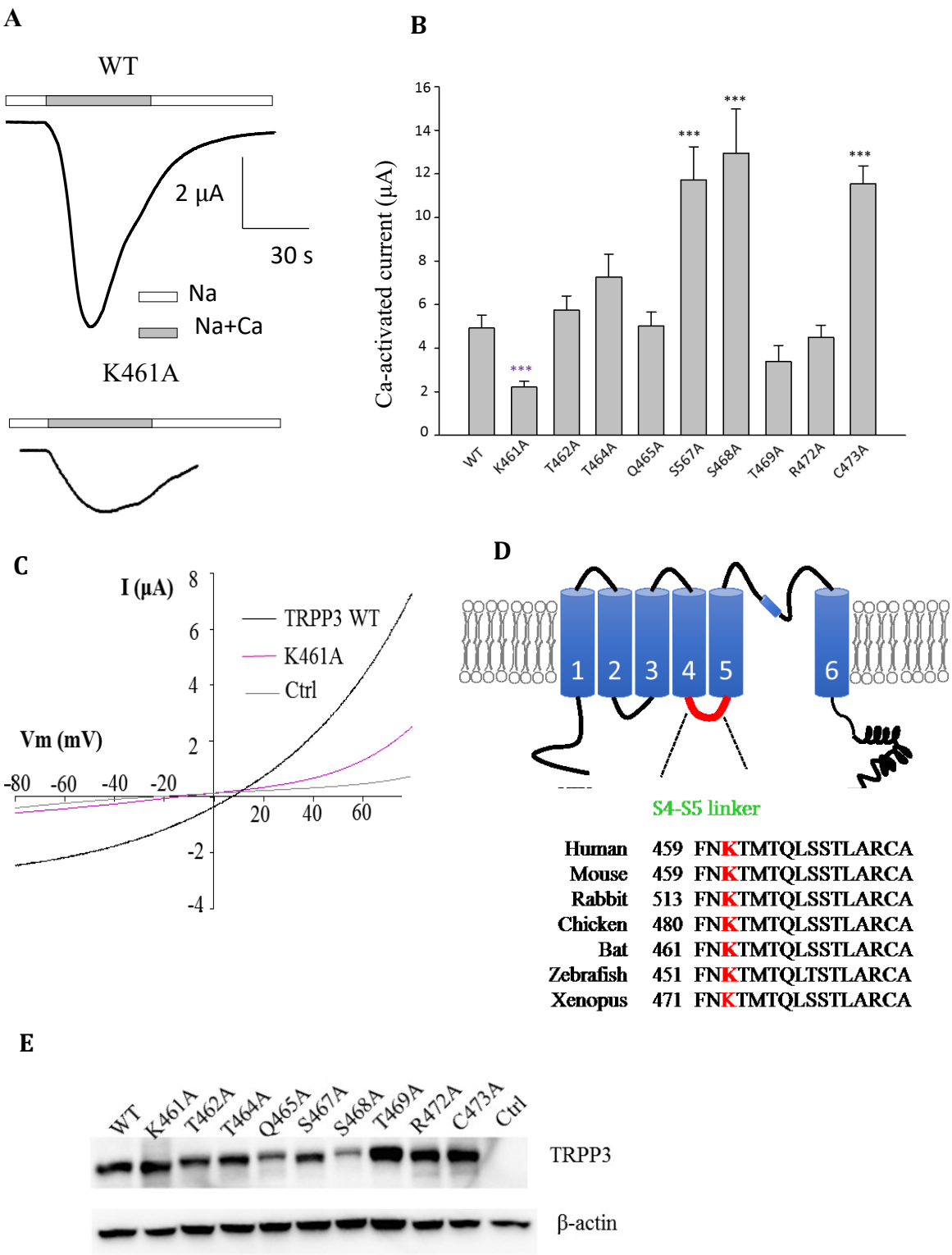
(A) Locations of three deletion mutants ΔS581-L592, ΔR596-K607, and ΔG611-T622 mapped onto the predicted membrane topology of a TRPP3 subunit. (B) Representative whole-cell current tracings obtained from *Xenopus oocytes* expressing TRPP3 WT, ΔS581-L592, ΔR596-K607, and ΔG611-T622 using the TEVC technique. Oocytes were voltage clamped at −50 mV. Data from a water-injected oocyte served as a negative control (Ctrl). Currents were measured using standard extracellular solution pH 7.5 (Na) (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES [pH 7.5]) or standard extracellular solution containing 5 mM $CaCl_2$ (Na+Ca). (C) Averaged currents obtained from oocytes expressing TRPP3 WT, ΔS581-L592, ΔR596-K607, and ΔG611-T622 or water (Ctrl) (n = 3, N = 15-21). n indicates the number of independent experiments and N refers to the total number of oocytes utilized. *** refers to highly significant at $p \leq 0.001$ using Student *t*-test when compared to the WT data. (D) Representative current-voltage (I-V) curves obtained from oocytes expressing human WT or a mutant TRPP3, as indicated, in the presence of an addition of 5 mM $CaCl_2$ (Na+Ca). (E) WB detection of Flag-tagged human TRPP3 WT, ΔS581-L592, ΔR596-K607, and ΔG611-T622 or water (Ctrl)

over-expressed in oocyte. (F) Representative immunofluorescence image showing the plasma membrane expression of TRPP3 WT, ΔS581-L592, ΔR596-K607, and ΔG611-T622 or water (Ctrl). (G) Averaged Ca-activated currents form oocytes expressing TRPP3 WT or an indicated point mutant. *** refers to highly significant at $p \leq 0.001$ using Student *t*-test when compared to the WT data. (n = 3, N = 13-19).

3.2 The cationic residue K461 of S4-S5 linker is critical for TRPP3 channel function

The S4-S5 linker recently became a hot spot for gain-of-function mutations within many TRP subfamily members, which cause different TRP channelopathies [36]. It appears that changes in one or more amino acids in the S4-S5 linker domain may cause dysfunctional channel activities and related diseases, and thus substitution of a particular amino acid residue may also potentially affect its interaction with other domains. To examine whether there is/are any critical residue(s) in S4-S5 linker important for TRPP3 channel function, we employed TEVC electrophysiology to scan some hydrophilic residues of fragment K461-C473 by applying alanine substitution and found that only the mutation at K461 remarkably reduces the channel function as indicated by a 50% decrease in inward current magnitude compared to the WT under -50 mV (Figure 6A and 6B). Similar results were observed when currents were measured at various membrane potentials (i.e. -80 to 80 mV) using a ramp protocol (Figure 6C). Sequence alignment also showed that K461 is highly conserved across different species including human, mouse, rabbit, chicken, bat, zebrafish, and frog (Figure 6D). Further verification by WB

and immunofluorescence revealed that all the mutants are expressed and targeted to the PM as the WT TRPP3 (Figure 6E and 6F). Further quantification of the surface expression needs to be done by biotinylation assays.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2 μA
30 s
Na
Na+Ca
K461A
B
Ca-activated current (μA)
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T462A
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S567A
S468A
T469A
R472A
C473A
C
I (μA)
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Ctrl
D
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2
3
4
5
6
S4-S5 linker
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S467A
S468A
T469A
R472A
C473A
Ctrl
TRPP3
β-actin

F

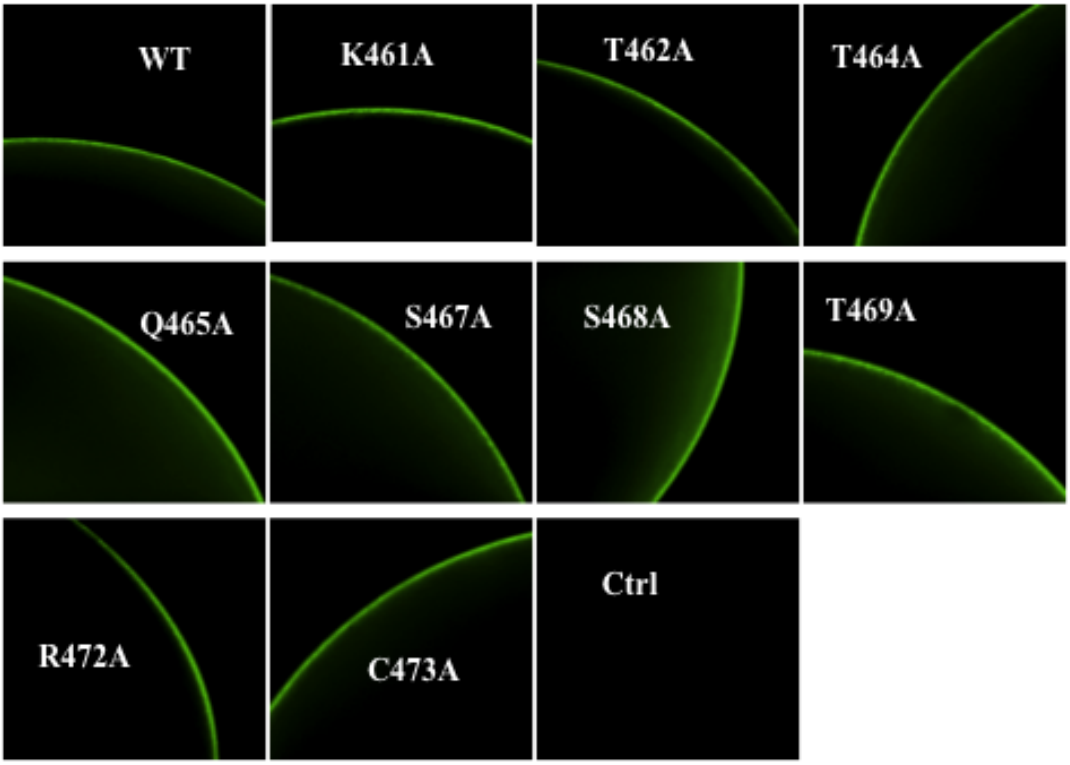


Figure 6. Roles of the TRPP3 residue K461 (S4-S5 linker) in the channel function.

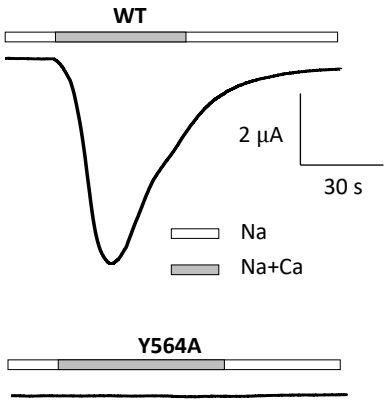
(A) Representative whole-cell current traces obtained from *Xenopus oocytes* expressing human TRPP3 WT or mutant K461A, using the TEVC technique. Oocytes were voltage clamped at -50 mV. (B) Averaged Ca-activated currents from oocytes expressing TRPP3 WT or an indicated point mutant. *** $P < 0.001$ (n = 3, N = 15-20). (C) Representative current-voltage (I-V) curves obtained from oocytes expressing TRPP3 WT or indicated K461 point mutant, in the presence of additional 5 mM $CaCl_2$ (Na+Ca). (D) Amino acid sequence alignment of the TRPP3 S4-S5 linker from indicated species, with the conserved residue K highlighted in red. (E) WB detection of Flag-tagged human TRPP3 WT or point mutants include K461A, T462A, Q465A, S467A, S468A, T469A, R472A, and C473A over-expressed in oocyte. (F) Representative immunofluorescence data showing the plasma membrane expression of TRPP3 WT, or point mutants in (E) or water (Ctrl).

3.3 The aromatic residue Y564 in TRP-like domain is critical for TRPP3 channel function

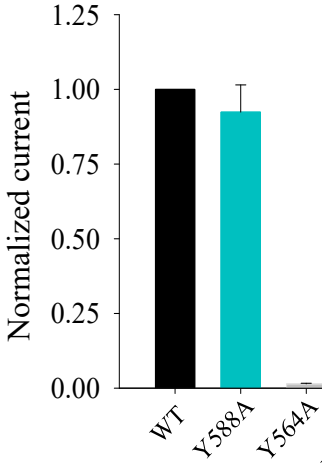
The structures of recently reported TRPs (TRPV1, -V2, -V6, -A1, and NOMPC) revealed intramolecular proximity among the S4-S5 linker, pre-S1 and TRP/TRP-like domain [51,48,49,47,50]. Zheng and associates proposed a mechanistic gating of TRPP2 by the F604P mutation involving interactions among S4-S5 linker, pre-S1 and TRP-like domain [99]. In addition, Zheng *et al.* [93] have proven that the interaction between N- and C-termini of TRPP3 is mediated through aromatic residues W81 and cationic residue K568 via a "π-cation" interaction, which is functionally important. So we hypothesize that an energetically favorable "cation-π" interaction may exist between S4-S5 linker and C-terminal TRP-like domain. Because TRPP3 K461 is located within the S4-S5 linker that is highly conserved across species (Figure 6D), we carried out alanine substitution in the TRP-like domain (N561-L593) to identify a functionally important aromatic residue(s). We found that only mutation Y564A corresponds to loss of function (Figure 7A-C). Sequence alignment also showed that Y564 is highly conserved across different

species including human, mouse, rabbit, chicken, bat, zebrafish, and frog (Figure 7D). Further analysis using WB revealed that Y564A is normally expressed, and immunofluorescence assay indicated that it is similarly localized on PM as the WT (Figure 7E and 7F). Taken together, our data suggest that TRPP3 channel function may require the presence of an interaction between the S4-S5 linker and TRP-like domains that is mediated by the K461-Y564 pair through a “cation-π” interaction.

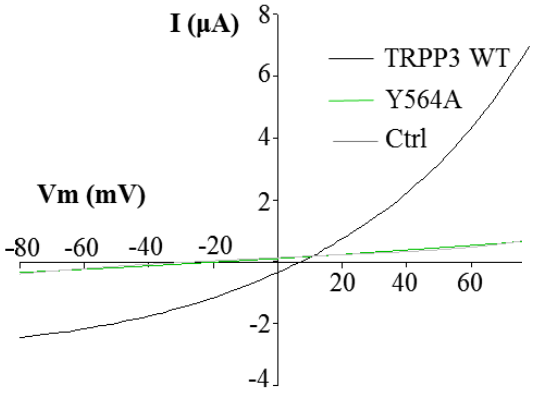
A



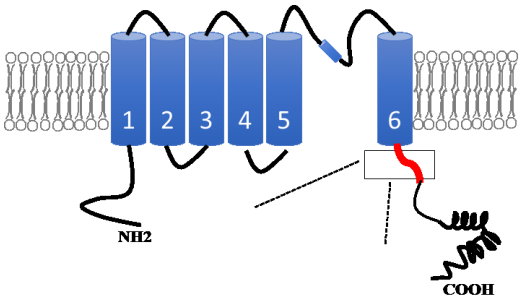
B



C



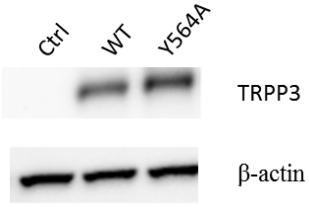
D



TRP-like domain

Human	561	NDTYSEVKEELAG
Mouse	561	NDTYSEVKEELAG
Rabbit	615	NDTYSEVKEELAG
Chicken	582	NDTYSEVKEELSS
Bat	563	NDTYSEVKEELAG
Zebrafish	553	NDTYSEVKSELAS
Xenopus	573	NDTYSEVKEELSN

E



F

Figure 7. Roles of the TRPP3 residue Y564 (TRP-like domain) in the channel function. (A) Representative whole-cell current traces obtained from *Xenopus oocytes* expressing human TRPP3 WT or mutant Y564A, using the TEVC technique. Oocytes were voltage clamped at -50 mV. (B) Averaged Ca-activated currents from oocytes expressing TRPP3 WT or an indicated point mutant. ***$p < 0.001$ ($n = 3$, $N = 17$-21). (C) Representative current-voltage (I-V) curves obtained from oocytes expressing TRPP3 WT or a mutant Y564A, in the presence of additional 5 mM CaCl2 (Na+Ca). (D) Amino acid sequence alignment of the TRPP3 TRP-like helix from indicated species, with the conserved residue Y highlighted blue. (E) WB detection of Flag-tagged human TRPP3 WT or Y564A mutant or water (Ctrl) over-expressed in oocyte. (F) Representative immunofluorescence data showing the plasma membrane expression of TRPP3 WT, or Y564A mutant or water (Ctrl).

3.4 Roles of the TRPP3 K461 (S4-S5 linker) and Y564 (TRP-like domain) residues in the L-C interaction and channel function

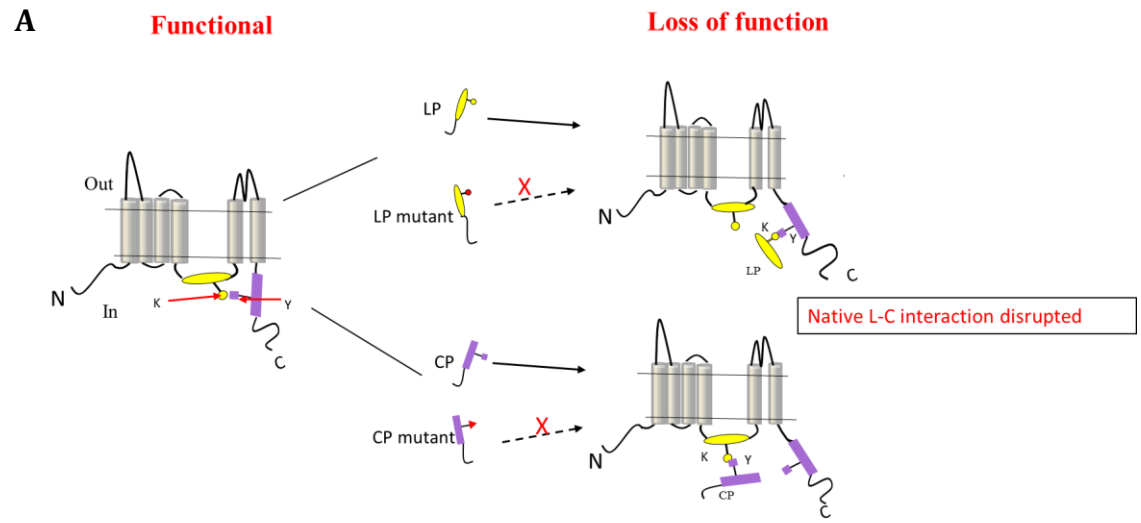
In order to test whether there is an interaction between the S4-S5 linker and TRP-like domains that is mediated by the K461-Y564 pair through a "cation-π" interaction, we used a blocking peptide strategy, as shown in Figure 8A. In the physiological condition, we assume that S4-S5 linker interacts with C-terminus, which maintains the TRPP3 channel function. When TRPP3 co-expressed with excess amount of TRPP3 S4-S5 linker peptide F454-F480 (P3LP), the P3LP will competitively bind with TRPP3 C-terminus, leading to the interruption of the native L-C interaction and loss of function of TRPP3. P3LP mutant can't bind with TRPP3 C-terminus, interrupt the native L-C interaction or cause loss of function. This also applies to TRPP3 C-terminal peptide I560-L584 (P3CP) and P3CP mutants. We next performed co-IP experiments and found that the P3LP and full-length (FL) TRPP3 are in the same complex and that this interaction is abolished if K461 in P3LP or Y564 in FL TRPP3 is substituted by alanine respectively (Figure 8B), indicating that the K461-Y564 pair

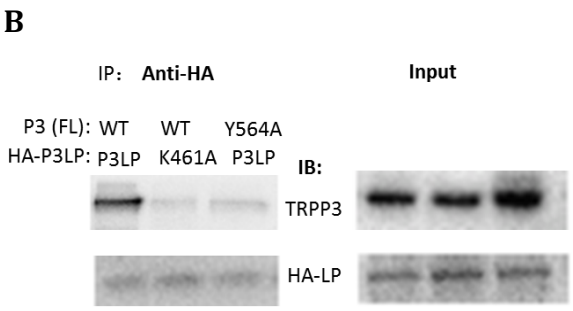
mediates the interaction. We next performed *in vitro* pull-down assays using the recombinant and purified glutathione S-transferase (GST)-tagged TRPP3 F454-F480 (GST-P3LP) and the His-tagged C-terminal peptide I560-K660 (His-P3CP). The two peptides directly bound each other, and the binding was abolished in the presence of the K461A mutation in the GST-P3LP peptide (Figure 8C), confirming that the K461-Y564 pair mediates the L-C binding. The L-C interaction was further supported by whole-oocyte co-immunofluorescence assays: after cRNA injection, peptide P3LP was distributed along the oocyte surface membrane if the FL TRPP3 cRNA was co-injected (Figure 8D). This attachment of P3LP to the surface membrane was not observed in the presence of the K461A mutation in P3LP or the Y564A mutation in FL TRPP3 or in the absence of FL TRPP3 expression (Figure 8D). These co-IP and immunofluorescence data together are in support of the concept that the K461-Y564 pair-mediated interaction between P3LP and FL TRPP3 allows P3LP to colocalize with FL TRPP3 along the surface membrane. We wondered whether the expressed peptide P3LP would compete with the P3LP fragment within the FL TRPP3 protein for binding with the Y564 in the

TRP-like domain of the FL TRPP3, thereby disrupting the L-C binding within FL TRPP3 and abolishing the channel function. Indeed, co-expression with P3LP substantially inhibited the FL TRPP3 channel function, whereas co-expression with mutant peptide P3LP-K461A had no effect (Figure 8E), indicating that P3LP acts as a blocking peptide that inhibits TRPP3 function through disrupting the L-C binding in the FL channel.

Similarly, we proposed that the expressed peptide P3CP would compete with the P3CP fragment within the FL TRPP3 protein for binding with the K461 residue in the S4-S5 linker of the FL TRPP3, thereby disrupting the L-C binding within FL TRPP3 and reducing the channel function. Meanwhile, this situation is much more complicated due to the presence of the N-C interaction, i.e., interaction between the pre-S1 and TRP-like domains that is mediated by the W81-K568 pair through a π-cation interaction [93]. We created a new HA-tagged C-terminal peptide I560-L584 (HA-P3CP), because previous His-P3CP (I560-K660) has a trimerization domain. Indeed, co-expression with HA-P3CP substantially inhibited the FL TRPP3 channel function, and it seems that co-expression with mutant peptide P3CP-Y564A, -K568A and P3CP-Y564A/K568A have no effect

(Figure 8F). However, biotinylation result showed that both TRPP3 membrane expression and total TRPP3 expression increase for those HA-P3CP mutants co-expression (Figure 8G). After normalizing surface expression bands, we further normalized each group's current again and found that co-expression with HA-tagged P3CP, P3CP-Y564A or P3CP-K568A significantly inhibits the FL TRPP3 channel function whereas co-expression with P3CP-Y564A/K568A has no effect on TRPP3 channel function (Figure 8H). Meanwhile, co-IP experiments showed that HA-P3CP and FL TRPP3 are in the same complex and that this interaction is still present if Y564 or K568 in FL TRPP3 is substituted by alanine. This is because both the N-C and L-C interactions are absent only if both Y564 and K568 in FL TRPP3 are substituted by alanine (Figure 8I). Together all these data indicate that P3CP acts as a blocking peptide that inhibits TRPP3 function through disrupting the L-C and N-C binding in the FL channel. In summary, our data demonstrated that direct L-C binding does occur in TRPP3 and that K461 of the S4-S5 linker and Y564 of the TRP-like domain, presumably through cation-π interaction, are crucial for this binding and channel activation.


A
Functional
Loss of function
Out
In
N
C
K
Y
LP
LP mutant
X
CP
CP mutant
Native L-C interaction disrupted


B
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Input
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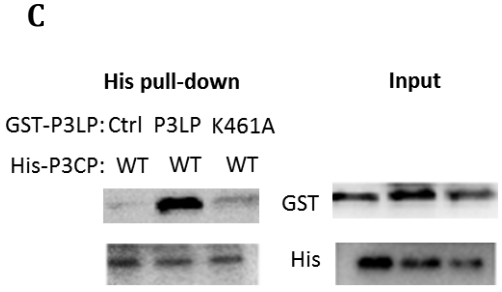
HA-P3LP: P3LP K461A P3LP
IB:
TRPP3
HA-LP


C
His pull-down
Input
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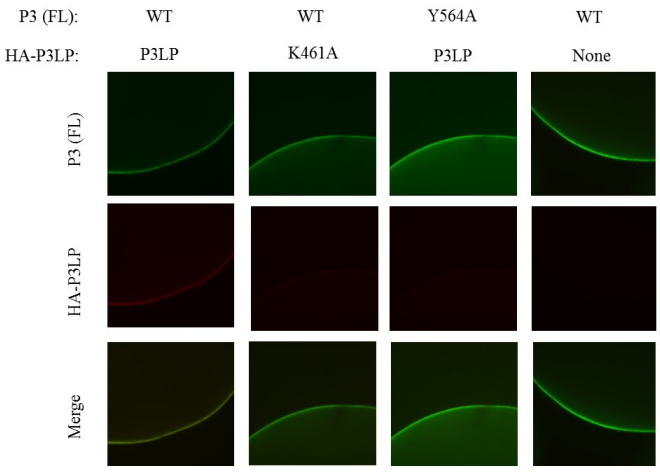
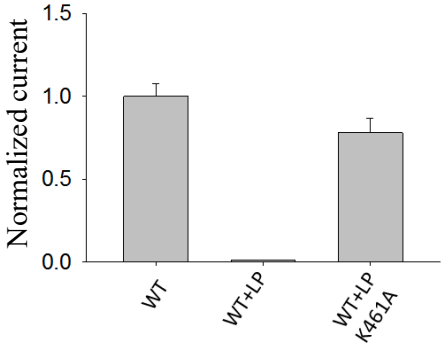
His-P3CP: WT WT WT
GST
His


D
P3 (FL): WT WT Y564A WT
HA-P3LP: P3LP K461A P3LP None
P3 (FL)
HA-P3LP
Merge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1.0
0.5
0.0
WT
WT+LP
WT+LP K461A

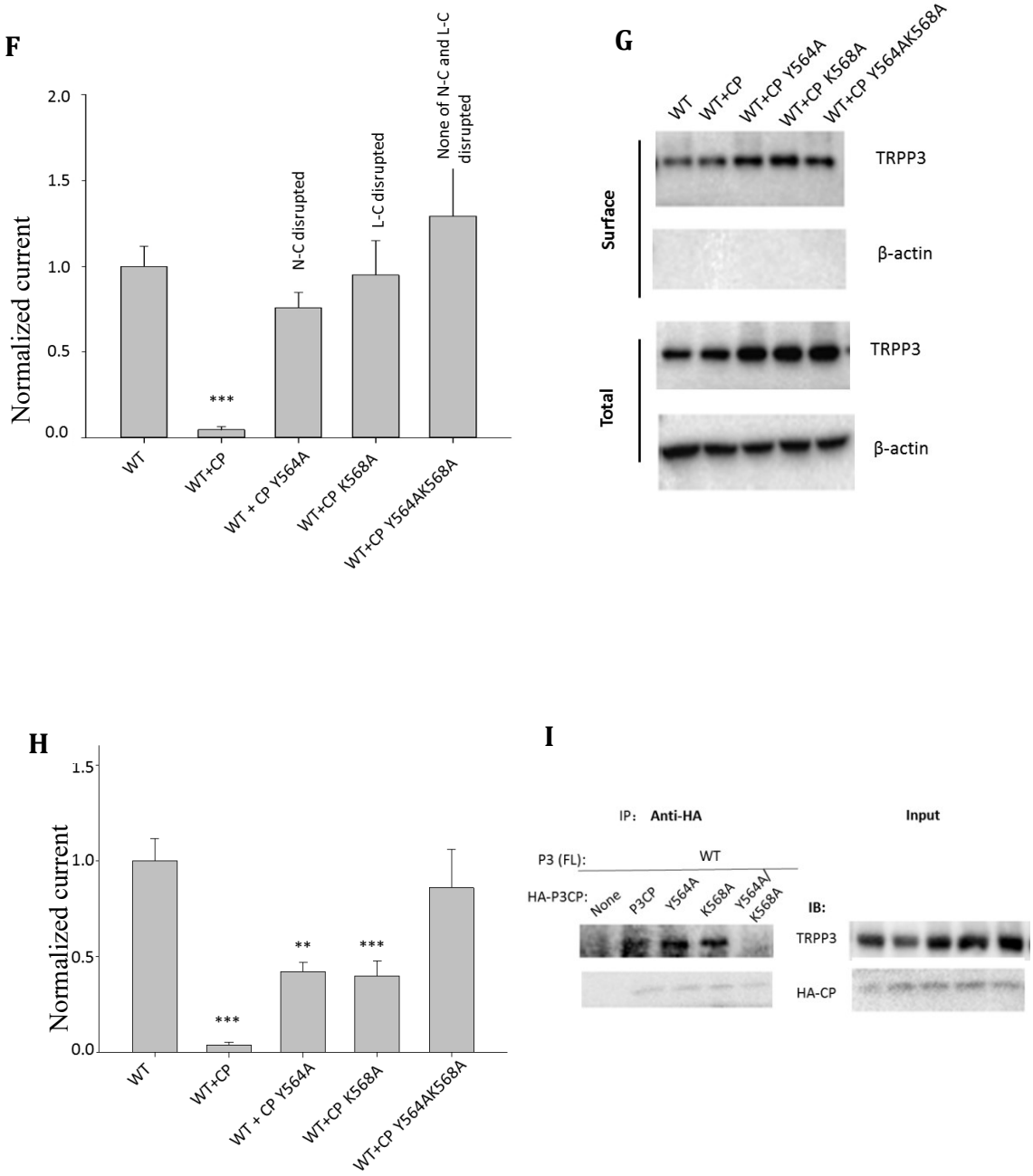
F
Normalized current
2.0
1.5
1.0
0.5
0.0
***
N-C disrupted
L-C disrupted
None of N-C and L-C disrupted
WT
WT+CP
WT + CP Y564A
WT+CP K568A
WT+CP Y564AK568A
G
WT
WT+CP
WT+CP Y564A
WT+CP K568A
WT+CP Y564AK568A
Surface
TRPP3
β-actin
Total
TRPP3
β-actin
H
Normalized current
1.5
1.0
0.5
0.0
***
**
***
WT
WT+CP
WT + CP Y564A
WT+CP K568A
WT+CP Y564AK568A
I
IP: Anti-HA
Input
P3 (FL):
WT
HA-P3CP:
None
P3CP
Y564A
K568A
Y564A/ K568A
IB:
TRPP3
HA-CP

Figure 8. Roles of the TRPP3 K461 (S4-S5 linker) and Y564 (TRP-like domain) residues in the L-C interaction and channel function.

(A) Blocking peptide working model. (B) K461-Y564 interaction examined with co-IP assays using oocytes co-expressing full-length (FL) TRPP3 and HA-tagged TRPP3 S4-S5 linker peptide (HA-P3LP; F454-F480). (C) K461-Y564 interaction examined with His pull-down assays using the purified GST-tagged human TRPP3 S4-S5 linker peptide (GST-P3LP; F454-F480) and His-tagged human TRPP3 C-terminal peptide (His-CP; I560-K660) from *E. coli*. Ctrl, purified GST-human FUBP1 M1-V112 from *E. coli*. (D) Colocalization of P3LP with FL TRPP3 examined with co-immunofluorescence assays using oocytes co-expressing FL TRPP3 and HA-P3LP. (E) Effects of co-expressed P3LP or its point mutants on TRPP3 Ca-activated currents. Currents were averaged from three independent experiments and normalized to that of WT (n = 3, N = 16–21). (F) Effects of co-expressed HA-P3CP (HA-P3CP; I560-L584) or its point mutants on TRPP3 Ca-activated currents. Shown are normalized and averaged currents from three independent experiments (n = 3, N = 14–19). (G) Representative immunoblots of the surface biotinylated (surface) and whole-cell (total) TRPP3 WT on the condition of co-injected of WT and HA-P3CP or its point mutants. (H) Effects of co-expressed HA-P3CP or its point mutants on TRPP3 Ca-activated currents after normalized with corresponding WB band. (E normalized by F). (I) K461-Y564 interaction examined with co-IP assays using oocytes co-expressing full-length (FL) TRPP3 and HA-P3CP or its point mutants.

3.5 Regulation of the TRPP3 L-C binding by PIP2

PIP2 is known to regulate almost all mammalian TRPs [100], in spite of low overall sequence resemblance. While some channels (e.g., TRPV5, TRPV6, TRPM4, TRPM5 and TRPM8) are positively modulated [100] and others (e.g., TRPC4 and TRPP2) are negatively regulated [101,102]; TRPV1 channel function, depending on the experimental conditions, is stimulated or inhibited [103,104]. Although PIP2 is known to bind to cationic residues in some TRPs *in vitro*, including TRPM4, TRPM8, and PTRV1 [105,44,52], it remains largely unclear as to the structural basis of the PIP2 regulation. To explore how the L-C interaction can be modulated, we examined the effect of the negatively charged, membrane- anchored phosphoinositide PIP2 on the L-C interaction. We looked into PIP2 because our previous study demonstrated that PIP2 binding to TRPP3 disrupts the W81-K568 pair-mediated N-C binding, which is essential for channel activation [93]. Additionally, our previous work identified that there are two PIP2 binding sites which are RRRK motif ($_{594}$RLRLRK$_{599}$) and K568 [93]. Thus, we next tested whether PIP2 can modulate TRPP3 channel function through affecting the L-C interaction. By co-IP

experiments, we found that addition of diC8-PIP2 (a dioctanoyl analog of PIP2) to the cell lysate weakens the TRPP3-P3LP binding in oocytes co-expressing FL TRPP3 and HA-P3LP (Figure 9A), suggesting that PIP2 has inhibitory effect on the L-C interaction, which is consistent with the reported negative effect of PIP2 on TRPP3 channel function [93]. As PIP2 disrupts the N-C interaction presumably by competitively binding to K568 [93], we tested whether it interrupts the L-C interaction by completely binding to K461 or Y564. However, the co-IP assays showed that neither K461 nor Y564 interacts with PIP2 (Figure 9B).

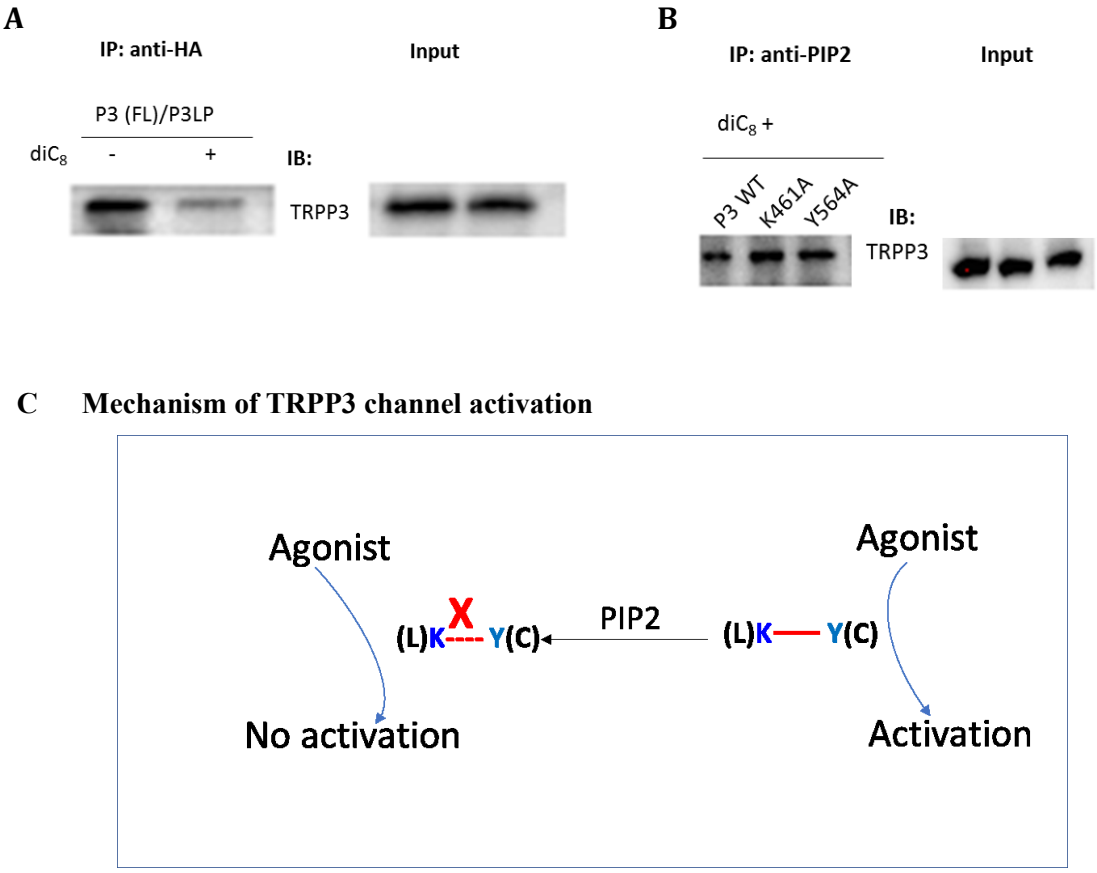

Figure 9. The inhibitory effect of PIP2 on the L-C binding of TRPP3 channel

(A) The effect of diC8-PIP2 on the interaction of P3LP with FL TRPP3 in oocytes. diC8-PIP2 was added in the cell lysis buffer to a final concentration of 15 mM. (B) Representative co-IP data showing the interaction of PIP2 with expressed WT or a TRPP3 mutant in oocytes.

CHAPTER 4
Discussion and Conclusions

In the current study, we revealed S581-L592, R596-K607 and G611-T622 domains are important for TRPP3 channel function, and we are still testing a small motif (2 or more amino acids) among these two domains that are crucial for TRPP3 channel function and oligomerization. Meanwhile, we determined an activation mechanism involving a physical intramolecular interaction between the S4-S5 linker and C-termini in TRPP3 channel. This L-C binding is mediated by a cationic lysine residue in the S4-S5 linker and an aromatic tryptophan residue in the TRP-like domain. We further showed that PIP2 can modulate TRPP3 channel activity by directly interfering with the L-C interaction. Our previous study showed that PIP2 can modulate TRP channels activity by directly interfering (inhibiting or facilitating) with the N-C interaction. All these data suggest a connection between PIP2 and N-C or L-C interaction.

4.1 Important domain(s) for TRPP3 channel function

Deletions in an oligomerization domain of an ion channel would substantially affect the channel architecture and subsequently channel activities as the function of an ion

channel presumably relies on its intact oligomeric state. While CC2 domain (G699-W743) was reported to be important for its trimerization and the surface membrane expression of TRPP3 and complex TRPP3/PKD1L3 [82,57,63], it remains unknown whether it promotes the trafficking to or prevents the retrieval from the surface membrane. Independent studies by our group [64,19] showed that CC2 does not affect TRPP3 channel function. Zheng and associates found that a novel C1 domain (K575-T622) is essential for both TRPP3 homotrimerization and channel function [19]. To further characterize this domain, in this study we used the *Xenopus* oocytes expression system and identified that the S581-L592 and R596-K607 domains do not seem to affect the TRPP3 surface membrane expression (Figure 5F), but were found critical for the channel function in the current study (Figure 5C). However, no functionally important single mutation was found (Figure 5G). All these data suggest that some small domain(s) (including two or more amino acids) could be critical for the function of the TRPP3 channel, and the domain(s) may be important for a functional TRPP3 trimeric state. Future studies especially regarding double or triple mutants will be needed to identify the specific domain(s).

Two or more trimerization domains seem to collectively ensure proper surface membrane configuration, stability and channel function. TRPP2 and TRPP3 and other members of the TRP superfamily are assumed to assemble into homo- and/or hetero-tetramers with either a PKD1 homologue or another TRP protein; however, the molecular mechanisms underlying these assemblies are still uncertain. Similar to TRPP3, the TRPP2 C-terminus was found to form trimers [106,57]. The trimerization of the TRPP2 or TRPP3 C-termini appears to nicely explain the tetrameric organization of the TRPP2/PKD1 or TRPP3/PKD1L3 complex with a trimer/monomer assembly [106,63].

While TRPP3 is activated by Ca^{2+}, followed by channel inactivation, seemingly through an increase in the intracellular Ca^{2+}, concentration [14], it remains unclear whether Ca^{2+} regulates TRPP3 channel activity through direct binding. Ca^{2+}-binding EF hand motif (E637-L665) in C-terminus negatively regulates the ability of TRPP3 channel to be activated by Ca^{2+}, indicating that Ca^{2+} binds to this domain to prevent the channel from over-activation [64]. While direct binding of Ca^{2+} to C-terminus was indeed reported [63], it remains uncertain whether and how this binding is relevant to channel function. Our

current study shows that TRPP3 channel function has been significantly increased after deleting G611-T622 domain (Figure 5C), which suggest that this domain may act as the same function as EF hand that negatively regulates the ability of TRPP3 channel to be over-activated by Ca^{2+}. Further studies are needed for more direct evidence to address this issue.

4.2 The relationship between N-C and L-C bindings

An intramolecular proximity of the S4-S5 (or pre-S1) linker to TRP domains was first observed in the high resolution of the TRPV1 structure [51,76,77]. This observation was later confirmed in the other TRP structures [47,78,48,49,79,80,32,50], suggesting that residues in pre-S1, S4-S5 linker, and TRP domains may mediate intramolecular interactions that are important for structural stability, channel gating, or allosteric modulation. Wang and colleagues revealed a gating mechanism involving a physical intramolecular interaction between the N-terminal pre-S1 and C-terminal TRP-like domain (N-C) in TRPP3 channels [93]. In this study, we identified an activation mechanism involving a physical intramolecular interaction between the S4-S5 linker and C-termini (L-C) in TRPP3

channel. An interesting question would be whether and how N-C interaction is related to L-C interaction. There are two possibilities: 1) N-C and L-C interactions are independent from each other; 2) L-C interaction is related to N-C interaction. Our studies seem to support the latter. Through LP or CP blocking peptide strategy, breaking either L-C binding or N-C binding will influence TRPP3 channel function. Specifically, breaking L-C binding will influence N-C binding, and vice versa (Figure 8E and 8H). To further confirm this, *in vitro* pull down [NP+CP (Ctrl), NP+CP+LP, NP+CP+LP K461A] and co-IP [NP+FL P3 (Ctrl), NP+FL W81A, NP+FL W81A/K461A] need to be performed.

4.3 Effects of PIP2 on N-C and L-C bindings

We showed that PIP2 can modulate TRPP3 channel activity by directly interfering with the L-C interaction. The scheme in Figure 9C summarizes these findings, in which 1) the TRPP3 L-C binding is inhibited by PIP2 and 2) an agonist is able to activate the channel when the L-C binding is present, whereas it is not when the L-C binding is absent or weak. Our previous study has demonstrated that PIP2 binding to some TRP channels (e.g. TRPM8, TRPV1, TRPP3, TRPP2) may enhance or weaken the N-C interaction that

is mediated by the residues which located in the N- and C-terminus respectively[93]. In this study, we found that there is a negative effect of PIP2 on the L-C binding in TRPP3 channel (Figure 9A). PIP2 binding sites in TRP channels were identified in both N- and C-terminus [107,108,109], as well as in the S4-S5 linker [52], and all those PIP2 binding sites are cationic residues no matter where they are located in TRP channels. The binding of PIP2 to both N- and C-termini of TRP channels, especially if the binding sites/pockets are close to or overlap with the N-C interaction sites, may allow PIP2 to efficiently regulate the intramolecular N-C interaction [93]. In TRPM8/TRPP3, functionally identified PIP2 binding sites R998/K568 overlap with N (W682)-C (R998)/N (W81)-C (K568) respectively. In TRPV1, functionally identified PIP2 binding sites (Q561, R575, R579, and K688) were indeed close to N (W426)-C (R701), based on the resolved TRPV1 structures [110]. One of the key properties shared by TRPP3 and TRPM8 is that the cationic residue in the TRP domain, K568 of TRPP3 or R998 of TRPM8, mediates both the N-C interaction and binds to PIP2. Since our data indicated that PIP2 weakens the L-C binding of TRPP3, it is therefore reasonable to hypothesize that PIP2 may compete with Y564 for

binding K461. However, our result showed that K461 is not a PIP2 binding site (Figure 9B). Wang *et al.* [93] have proven that K568 and RRRK motif ($_{594}$RLRLRK$_{599}$) are PIP2 binding site/domain in TRPP3. There is therefore another possibility that PIP2 weakens the L-C binding of TRPP3 through binding K568 or RRRK motif.

In terms of the relationship among N-C, L-C and PIP2 bindings, we proposed that PIP2 could modulate the function of TRPP3 by breaking down these two intramolecular interactions, and those interactions could be related to each other. Significant conformational changes following PIP2 binding in the Kir2.2 K^+ channel has previously been reported [111], and similar structural change, induced by PIP2, may lead to a stronger or weaker interaction among TRP channels. This is demonstrated by Wang and colleagues' finding that PIP2 negatively regulates TRPP3/-P2 through inhibiting the N-C interaction. Interestingly, they also found that the positive effect of PIP2 on TRPM8/-V1 is mediated through stabilizing the interaction between the N and C termini of TRPM8/-V1 [93]. These findings are consistent with previous discovery of the positive

regulation of PIP2 on TRPM8/-V1 channel function [44,107,104,52] and inhibitory effect of PIP2 on TRPP3/-P2 channel function [102].

Furthermore, the TRP domain is reported to be bimodal. For example Doerner *et al*. [112] clearly demonstrate that TRPV3 channels are sensitized by depletion of PIP2 in a TRP domain-specific fashion. Additionally, the work of David Julius shows a desensitizing effect of PIP2 on TRPV1 activation [113,114] .

PIP2 interaction leads to a reconfiguration favoring channel activation. Although the cryo-EM structure of TRPP3 is insufficient to show the directly reconfiguration change among PIP2, N-C and L-C interactions, it is tempting to suggest that PIP2 would affect N-C and L-C interactions.

Meanwhile, especially for those TRP channels which are affected by two opposing effects (inhibited and stimulated) of PIP2 [103,104], interactions among different domains of TRPs including N-C and L-C interactions may contribute some reciprocal inhibition or synergistic effect to it.

While hydrogen bonds, salt bridges, and the hydrophobic effect all play roles in folding a protein and establishing its final structure, the cation-π interaction is increasingly recognized as an important noncovalent binding interaction relevant to structural biology [115,116,117]. Theoretical and experimental studies have shown that cation-π interactions can be quite strong that are intrinsic to a protein's structure and likely contribute to protein stability [118]. Wang and associates discovered that an intramolecular π-cation interaction of the TRPP3 N- and C-termini (N-C) is functionally essential. Subsequent structure-function analyses revealed similar N-C interaction in TRPP2 as well as TRPM8/-V1/-C4 via highly conserved tryptophan and lysine/arginine residues [93]. As the S4-S5 linker and TRP (and TRP-like) domain are highly conserved among different TRP channels, the cation-π interaction between S4-S5 linker and C-terminus seen in TRPP3 may also be present in other TRP channels. PIP2 is necessary and sufficient for activating many TRP channels; it would therefore be meaningful to explore whether PIP2 affects N-C and L-C interactions and regulates activation or inactivation of different TRP channels.

Thus, it is still an interesting matter of discussion on how PIP_2, N-C and L-C bindings promote potentiation or reduction of ionic currents derived from thermal, agonist, or voltage activation.

4.4 Breakdowns of N-C or L-C binding in other channels leads to 'gain of function'

Interactions such as N-C and L-C bindings also contribute to gating of other TRP channels. For example, L596-W733 binding between the S4-S5 linker and the TRP domain (L-C) stabilizes the closed state of TRPV4 channel [119]. It increases open probability [gain of function (GOF)] when this interaction is disrupted. As well as in TRPP2, mutation F604P induces a displacement among S4-S5 linker, pre-S1 and TRP-like domain, and therefore causes GOF [99].

In summary, this study identified that two conserved amino acid residues, K461 and Y564 in the S4-S5 linker and the C-terminal TRP-like domain respectively, underlie interaction of the two domains. This L-C binding in TRPP3 is critical for the channel activation and regulation by PIP2. This intracellular L-C binding may together with N-C

and PIP2 bindings serve as a shared molecular switch that transduces the conformational changes induced by diverse ligands to channel pore opening and closing.

CHAPTER 5
Future Directions

As a strong candidate for sour taste sensation [120], TRPP3 is an important protein that deserves more studies. Sour tasting is one of the basic animal senses and a fundamental part of human and animal physiology. The signalling pathway underlying how sour tasting is transduced is still poorly understood. It is therefore critical to investigate the mechanism underlying TRPP3 function and to examine how TRPP3 proteins operate in detail.

As for the S4-S5 linker of TRPP3, future studies are needed to examine the effects of those GOF mutants on TRPP3 channel function. From previous studies on TRPP3, more than one phosphorylation site is likely to be affected during the activation/inactivation pathway. Our previous work already proved that T39 is a phosphorylation site [62], and now we have found that S467 may be a candidate site for TRPP3 phosphorylation because a mutation S467E significantly affects (substantially reduces) the channel function (data not shown). More studies are required to further validate this assumption. Many channel proteins, such as the large conductance

voltage-gated potassium channel and TRPML1, have been reported to be post-translationally modified by palmitoylation at cysteine residues to regulate channel trafficking and/or function [121,122,123] . An interplay between palmitoylation and phosphorylation at a nearby site has also been reported [124,125,126]. It would therefore be interesting to further investigate if a palmitoylation site is present at the nearby S467. First, we will test palmitoylation score of those candidate palmitoylation residues by using palmitoylation prediction program CSS-Palm version 4.0, then we will treat TRPP3-expressing oocytes with widely used palmitoylation inhibitor 2-bromopalmitate (2BP) and examine the 2BP effect on the TRPP3 plasma membrane expression by using immunofluorescence assays. Meanwhile, we hypothesize that one (or more) of these GOF mutant(s) may together with GOF mutants in TRP-like domain have effect on TRPP3 gating. Further studies are required to elucidate the underlying mechanism.

In terms of L-C interaction of TRPP3 mediated by PIP2, it would be interesting to identify the potential role of PIP2 in TRPP3 channel function. To investigate the effect of PIP2 on TRPP3 expressed in oocytes, we are currently optimizing the use of diC8-PIP2

which act as a mimic of PIP2 and found that it has inhibitory effect on TRPP3 channel. Further scanning of potential PIP2 binding sites in S4-S5 linker or TRP-like domain might be of interest. Especially, to examine whether PIP2 affect the L-C binding of TRPP3 through binding K568 or RRRK motif, which are close to L-C interaction sites, we will do co-expression of FL TRPP3-K568A or FL TRPP3-4Q (substitution of $_{594}$RLRLRK$_{599}$ to QLQLQQ) with LP. Additionally, we have generated a few of potential PIP2 binding site mutants (locates in S4-S5 linker) that need further characterization.

There are tremendous opportunities in better understanding how TRPP3 is regulated and deciphering the steps by which the channel is gated. The enhanced understanding would help propose signaling transduction mechanism for TRPP3 acid sensing. In terms of the knowledge in the field, the current structural and functional information about TRPP3 is only the tip of an iceberg. More effort is required to pursue this curiosity-driven research.

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