The ion channel function of polycystin-1 in the polycystin-1/polycystin-2 complex

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THE ION CHANNEL FUNCTION OF THE POLYCYSTIN-1 IN THE POLYCYSTIN-1/POLYCYSTIN-2 COMPLEX

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ABSTRACT

THE ION CHANNEL FUNCTION OF THE POLYCYSTIN-1 IN THE POLYCYSTIN-1/POLYCYSTIN-2 COMPLEX

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Autosomal dominant polycystic kidney disease is caused by mutations in polycystin-1 (also known as PKD1), a polycystic kidney disease protein, or polycystin-2 (also known as PKD2 or TRPP2), a transient receptor potential channel. Polycystin-1 and polycystin-2 form a receptor-ion channel complex located in primary cilia. The function of this complex, especially the role of polycystin-1, is largely unknown due to the lack of a reliable function assay. In this study, we dissect the role of polycystin-1 by directly recording current from a gain-of-function polycystin-1/polycystin-2 channel. Our data show that this channel has distinct properties from that of the homomeric polycystin-2 channel. The polycystin-1 subunit directly contributes to the channel pore, and its eleven transmembrane domains are sufficient for its channel activity. We also show that the cleavage of polycystin-1 at the N-terminal G protein-coupled receptor proteolytic site is not required for the activity of the GOF polycystin-1/ polycystin-2 channel. These results demonstrate the ion channel function of polycystin-1 in the polycystin-1/ polycystin-2 complex, enriching our understanding of this channel and its role in ADPKD.
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INTRODUCTION

1. Ion channels

Bio-membranes, such as the cell plasma membrane and endoplasmic reticulum (ER) membrane, are formed by two layers of phospholipid and are hydrophobic. Hydrophilic and big molecules, such as water, ions, organic molecules, and hormones, have great difficulty crossing the bio-membrane (Bruce Alberts, 2014). Ion channels are a large group of membrane proteins that act as pores on the bio-membrane, conducting ions through the lipid-bilayer membrane (or bio-membrane). Ion channels contain few transmembrane (TM) helices that compose channel pores allowing ions across the bio-membrane. Different peptide element domains constructing with TM domains mediate and regulate channel function. With the lipid-bilayer membrane working as a parallel plate capacitor, the ion channels work as a conditional conductor, building an electric circuit across the bio-membrane. The concentration difference of molecules, especially ions, between the intra and extracellular side of bio-membrane generates voltage difference and chemical potential, which is the main kinetic regulator of the electric circuit. Ion channels, responding to the change in the circuit, initiate many ion-related bio-functions, such as osmolarity maintenance, action potential, calcium regulation, et al. (Hille, 2001). Therefore, Ion channels are a very critical target in many fields, such as pathology, drug development, cell signaling pathways, et al.

The diversity of the ion channel is chromatic. During long-term evolution, few ancestor genes developed numberless ion channels and their isoforms, varying in structure and function. It is common that ion channels from different gene families conduct the same ions, such as Na⁺, K⁺, or Ca²⁺, and share functional similarities, such as voltage-sensitivity and ligand-binding activation (Hille, 2001). Therefore, ion channels can be classified in many ways. For classification by gating mechanism, there are voltage-gated, ligand-gated, lipid-
gated, and others. For classification by ion conduction, there are sodium channels, potassium channels, calcium channels, cation channels, chloride channels, and others. For classification by cellular location, there are plasma membrane channels, mitochondrial channels, and others. Some other channels are classified by genetic homologs, such as the transient receptor potential (TRP) channel family. Proper classification can help investigators quickly describe the major feature and function of a certain group of ion channels.

### 1.1. Ion channel classification by gating mechanism

The gating mechanism is crucial for understanding the function and regulation of an ion channel. The term ‘gating’ refers to the ion channel conformation changes between the open (activation) stage and the closed (inactivation) stage. The open stage allows ions across the bio-membrane, whereas the closed stage does not. Ion channels respond to stimulus, such as membrane potential, chemical stimulus, signal ligand stimulus, thermic changing, or mechanical force. Consequently, protein conformations changing under response results in channel gating (Hille, 2001). In general, many ion channels share similar gating mechanisms that have similar functions and regulations. Thus, classifying ion channels by their gating mechanism groups channels by their function. It is one of the most common ways in the field to classify ion channels.

#### 1.1.1. Voltage-gated ion channels

Voltage-gated ion channels are a group of ion channels that open and close responding to the change in membrane potential (MP). MP is an electrical voltage difference across the bio-membrane. It is generated by ion diffusion equilibrium across the membrane and is essential to cell function. The ion distributions between the two sides of a membrane are similar in most cell types. For example, K⁺ concentration is higher in the intracellular side, while Na⁺ and Ca^{2+} concentrations are higher in the extracellular side. The major anion
in the extracellular side is Cl\(^-\), and in the intracellular side is phosphate (PO\(_4^{3-}\)) (Bruce Alberts, 2014). As per balanced osmolarity, if no diffusion occurs between the bio-membrane, it is electrically neutral for both sides of the bio-membrane. When K\(^+\)-specific ion channels exist on the membrane, with the chemical gradient, K\(^+\) will tend to diffuse from inside to outside, resulting in more cations in the extracellular side and superfluous anions in the intracellular side. This causes the intracellular side to become more electrical negative and a negative potential occurs. This attracts the K\(^+\) ions, which flow back. Therefore, a balance will finally reach between K\(^+\) efflux following chemical gradient and K\(^+\) influx following negative electrical potential, and the net flow of K\(^+\) becomes zero. The MP at this point is called potassium equilibrium potential, which can be calculated by the Nernst equation (Hille, 2001). In most cell types, only some K\(^+\) channels constitutively open in the plasma membrane. Therefore, the plasma membrane potential of these cells is majorly contributed by the diffusion equilibrium of K\(^+\), and the membrane potential of these cells is usually negative and numerically close to the potassium equilibrium potential. In other cells that have other channels open at the same time, the membrane potential is then determined by the equilibrium of all ions’ flow. The membrane potential of a neuron cell at rest is called the resting membrane potential that particular neuron. It is achieved by the opening of several different types of ion channels, mainly potassium channels, at rest. When a neuron is activated, the membrane potential will shift toward positive (depolarization), which will then activate voltage-gated sodium, potassium, and calcium ion channels, and lead to more dramatic depolarization followed by repolarization. This process in neurons is called an action potential, which is the major electrical signal conducted in neurons.

Voltage-gated ion channels “sense” the change of membrane potential with their voltage sensing (VS) domains. As the common regulator element in voltage-gated ion channels, VS domain is formed by four helices, and one helix of them, commonly the last
one, contains multiple positive-charge side chains, extending and twisting under MP change. Each subunit or identical homologous domain of voltage-gated channels has one VS domain, surrounding the central ion pathway formed by all subunits. When the MP changes, the VS domains slide up and down in the bio-membrane, triggering conformational change and mediating channel gating. Within the ion pathway, there are ion selectivity filters located above the channel gate, which help to select specific ions passing the pore. Voltage-gated channels play a crucial role in the nerve system by triggering depolarization and repolarization in axons, firing action potentials, and transmitting electrical signals (Purves, 2018). Meanwhile, in the sensory generation, pH-related biology function, and other fields, Voltage-gated channels are key and fundamental components.

1.1.1.1. Voltage-gated sodium channels

Voltage-gated sodium channels (Na\textsubscript{v}) are one of the few groups of ion channels that have been studied earliest. Sodium current was firstly described by Hodgkin and Huxley in 1952 in their study of action potential in neurons (Hodgkin & Huxley, 1952). With the voltage clamp technique, they recorded sodium-sensitive, voltage-dependent, and fast inactivated current during neuron depolarization. Following their work, the idea was established that sodium and potassium currents are conducted by specific ion channels. In the 1970s, studies found that some neurotoxins target the Na\textsubscript{v} channel in mammalian neurons (Catterall, 1980). Voltage-gated Na\textsuperscript{+} (Na\textsubscript{v}) channels comprise a macromolecular complex whose components regulate channel function. In the 1980s, scorpion toxins were utilized to identify the protein subunits in Na\textsubscript{v}, revealing one large α subunit (Na\textsubscript{v}α), around 260kDa, and two small β subunits (Na\textsubscript{v}β), around 30-40kDa (Catterall, 1980, 1984). In the following decade, about 10 Na\textsubscript{v}α subunits and 4 Na\textsubscript{v}β subunits were discovered and identified (Goldin, 2001). The Na\textsubscript{v}αs, named Na\textsubscript{v}1.1 through Na\textsubscript{v}1.9, are sufficient to form functional ion
channels and are composed of one single peptide each. Every Na,α subunit has four homologous transmembranes (TM) domains, which each contains 6 TM helices (Catterall, 1984, 1986). The peripheral Na,β subunits, structurally similar to the cell adhesion molecular family (Isom et al., 1995), associate with Na,α subunits and regulate their kinetics such as activation and inactivation. The association between Na,β and Na,α varies. β1 and β3 are associated non-covalently with α subunits, whereas β2 and β4 form disulfide bonds with α subunits (O’Malley & Isom, 2015).

The recent structural studies have significantly enhanced our understanding of the molecular structures of both prokaryotic and eukaryotic Na, channels. In contrast to the long four-domain containing Na,α subunit in eukaryotic Na, the bacterial sodium channel NaChBac and several prokaryotic relatives were composed of four homologous subunits. Each subunit has around 270 amino acids, containing 6 TM helices that are highly conserved among eukaryotic Na, channels (Koishi et al., 2004; Ren et al., 2001). The structures of several bacterial Na, Channels were solved by X-ray crystallography (McCusker et al., 2012; Payandeh, Scheuer, Zheng, & Catterall, 2011; Shaya et al., 2014). The structures demonstrate that the last two TM α-helixes (S5-S6) from each subunit array into a circle, forming the channel pore. The first 4 TM α-helices (S1-S4) form the voltage sensing (VS) domain, which response to the change in voltage across the bio-membrane to control the opening and closing of the channel pore. A C-terminal coiled-coil domain from each subunit binds together and is involved in the assembly. Recently, the structures of few eukaryotic Na, channel complexes were solved by cryo-EM (H. Shen, Liu, Wu, Lei, & Yan, 2019; Yan et al., 2017). The structure of the 24 TMs, six in each homologous domain, shows a similar arrangement as that of the prokaryotic Na, tetramer. However, unlike the symmetric assembling of the latter, the asymmetric topologic structure of the eukaryotic Na, channel indicates different and more complicated functions and regulation.
Na\textsubscript{v} channels play essential roles in neuron function as the opening of Na\textsubscript{v} conducts Na\textsuperscript{+} influx, which is the major cause of the rising phase of an action potential. Consequently, Na\textsubscript{v} channel complexes are directly involved in pain sensory, epilepsy, neuromuscular disorders, and cardiovascular diseases (de Lera Ruiz & Kraus, 2015). Therefore, they are important drug targets for local anesthesia and many neurological diseases.

### 1.1.1.2. Voltage-gated potassium channels

Voltage-gated potassium channels (K\textsubscript{v}) are the most diverse family in voltage-gated ion channel superfamily. After Na\textsubscript{v} was well studied, in 1987, the first voltage-gated potassium channel was discovered and cloned from the shaker mutant of Drosophila (Papazian, Schwarz, Tempel, Jan, & Jan, 1987; Pongs et al., 1988). Mutations in the shaker gene cause shaking of the fly’s legs under ether anesthesia. Afterward, the shaker gene was expressed in Xenopus oocytes, allowing the first gating current to be recorded from K\textsubscript{v} (Bezanilla, Perozo, Papazian, & Stefani, 1991). In the following decades, a large number of channels that are highly specific to potassium are discovered, including K\textsubscript{v}, Ca-activated potassium channels (K\textsubscript{Ca}), inwardly rectifying potassium channels (K\textsubscript{ir}), and tandem pore domain (two-pore-domain) potassium channels (K2P). K\textsubscript{v} family can be classified into 12 subfamilies according to amino acid sequence alignments of the hydrophobic pore-forming domain. Two phylogenetic trees, K\textsubscript{v}1-9, and K\textsubscript{v}10-12, were reconstructed from those subfamilies (Gutman et al., 2005; F. H. Yu & Catterall, 2004). In this classification, the K\textsubscript{v} channel encoded by the shaker gene is renamed as K\textsubscript{v}1.3 and belongs to K\textsubscript{v}1, shaker like K\textsubscript{v} family.

Compared to that of Na\textsubscript{v} and Ca\textsubscript{v} channels, the structures of K\textsubscript{v} channels are very well studied. Including K\textsubscript{v}, most of the potassium channels share similar fourfold symmetric stoichiometry (MacKinnon, 1991). In early stages, some basic channel elements, such as VS
domain, selective filter, and channel pore, were identified by electrophysiology experiments. At the same time, many gating kinetic models were established (Bezanilla, Perozo, & Stefani, 1994; Hoshi, Zagotta, & Aldrich, 1994). However, in lacking the structure, many functions and regulations of Kv channels are yet to be understood and explained. Until 1998, the structure of the pore domain and selectivity filter of KcsA (K channel of streptomyces A), a pH activated prokaryotic potassium channel, was solved by x-ray crystallography, allowing first direct observation and study of the functional elements of a potassium channel (Doyle et al., 1998). The tetramer structure of KcsA confirmed sequence-based structure prediction and the conformation of the selective filter explained potassium specification. In the present day, many full-length structures of other potassium channels are solved by similar methods and the structure of Kv1.2 is the first eukaryotic voltage-gated potassium channel structure to be solved (Jiang et al., 2002; Long, Campbell, & Mackinnon, 2005). With recently improved cryo-EM technology, more and more structures of Kv and potassium channels are being solved.

According to the structures, the relationship between Kv channels’ regulator elements and channels’ function becomes clear. Same as Na, the side chains containing positive charge on the S4 helix in VS domain sense the membrane potential (MP) difference. With the MP shifts from negative to positive, charged side chains change the conformation of the S4 helix from $3.6_{13}$ helix to $3_{10}$ helix, which triggers channel pore formation becoming wider and conducting potassium (Catacuzzeno, Sforna, & Franciolini, 2020). Meanwhile, the structures display that the selective filters of potassium channels, including Kv, are formed by the carbonyl oxygens of a conserved amino acid chain of TVGYG (MacKinnon, 2003). By showing potassium ions accompanying with the potassium channel, it is illustrated that the potassium ions will be dehydrated, when they pass the selective filter (Kuang, Purhonen, & Hebert, 2015). In contrast, the selective filters of Na associate with hydrated sodium and the
filters are wider than that of the potassium channels. As a result, other cations, such as guanidinium, ammonium, can also be conducted by Na (Hille, 2001). Hence, the narrow filter domain of Kv can only binds with dehydrated K+, which explains why potassium channels are highly specific to K+, but not other cations.

Kv play a critical role in many physiological functions. The potassium ion is a fundamental ion component in the cytoplasmic matrix and majorly contributes to the plasma membrane potential in its resting stage. The intracellular K concentration is around 25 to 45-fold higher than that of extracellular concentration in most of the cell types (155mM intro: 4mM extra in skeletal muscle), maintained by some leaky potassium channels or Na+/K+ pump. As stimulus appears and activates sodium, calcium, or other cation channels, Na+ and Ca2+ start to diffuse across the membrane, resulting the MP change, raising or dropping, to reach a new potential equilibrium. Meanwhile, voltage-gated potassium channels will be activated by the MP raising (depolarization) and pump K+ out to adjust the MP back (repolarization), which is critical for action potential generation in neurons (Purves, 2018). For this reason, Kv channel mutations lead to many neuron-disorder diseases, making Kv as essential targets for therapeutic treatments.

1.1.1.3. Voltage-gated calcium channels

voltage-gated calcium channels (Ca) are a group of voltage-gated channels conducting Ca2+. When the bio-membrane becomes permeable to Ca2+ by ion channels, a very low amount of Ca2+ influx is enough to dramatically change the membrane potential (Hille, 2001). Meanwhile, Ca2+ is also known as a secondary messenger, mediating many signal pathways that play critical roles in cells, such as the phospholipase C Pathway (PLC) (Bruce Alberts, 2014). Hence the ion channels conducting Ca2+, including Ca, are deeply involved in cell function, especially in neurons. At the end of the 1970s, the first Ca2+ current
was recorded from cardiac myocytes (Reuter, 1979). In the following decade, based on electrophysiological studies, a few types of calcium current were distinguished and defined. According to calcium current activation, inactivation feature and single channel conductance, L-type, T-type, N-type calcium currents were designated and these three types of calcium current are specifically inhibited by different Ca\(^{2+}\) antagonists (Nowycky, Fox, & Tsien, 1985). In contrast to L-type, T-type calcium currents are wide-spreading in many types of cells, while P-type, Q-type, and R-type calcium currents were identified and only recorded in some types of neurons (Llinás, Sugimori, Hillman, & Cherksey, 1992; Randall & Tsien, 1995).

Ca\(_v\) channels share some structural similarity with Na\(_v\) channels. Same as Na\(_v\), the pore-forming subunit, named as \(\alpha1\) subunit, of Ca\(_v\) is composed by a single-long peptide including 4 homologous domains which contain four VS helices and two pore-forming helices in each (Tanabe et al., 1987). Since Ca\(_v\) \(\alpha1\) subunit is sufficient to conduct Ca\(^{2+}\) current, by analyzing the Ca\(_v\) \(\alpha1\) subunit amino acid sequence, Ca\(_v\) can be classified into three classes, Ca\(_v\)1, Ca\(_v\)2, and Ca\(_v\)3. Ca\(_v\)1 mediates L-type calcium currents, and Ca\(_v\)3 mediates T-type calcium currents. N-, P/Q-, and R-type calcium currents are mediated by the Ca\(_v\)2 family (Ertel et al., 2000). Unlike Na\(_v\) and K\(_v\), besides the \(\alpha1\) subunit, Ca\(_v\) are composed by many other subunits, including \(\alpha2\), \(\beta\), \(\gamma\), \(\delta\) subunits. With the development of the cryo-EM technique, the high-resolution structure of the Ca\(_v\)1.1 complex was solved recently, allowing the association of the subunits in Ca\(_v\) complex to be clearly observed (Zhao et al., 2019). According to the structure model, the \(\alpha2\) subunit locates in the extracellular side and associates with the extracellular structures of the \(\alpha1\) subunit. The \(\delta\) subunit contains one TM helix and connects the \(\alpha1\) and \(\alpha2\) subunit. The \(\beta\) subunit is inside the intracellular site which associates with the intracellular loops of the \(\alpha1\) subunit. The \(\gamma\) subunit contains 4 TM helices and associates with the TM domain of the \(\alpha1\) subunit. Interestingly, many evidences show the
Ca\textsubscript{v} in skeletal muscle contains \(\gamma\) subunit, while the Ca\textsubscript{v} in the neuron of the brain does not. Instead, some \(\gamma\)-subunit-like proteins are involved in Ca\textsubscript{v} complex in the brain (Catterall, 2011). The combination diversity of subunit association in the Ca\textsubscript{v} complex indicates the delicate functions and regulations of Ca\textsubscript{v} in different neurons. In other words, a specific calcium regulation in a certain type of neuron may require a specific Ca\textsubscript{v} subunit combination.

The features of calcium currents conducted by the three classes of Ca\textsubscript{v} are different, demonstrating they are responsible for different neuron functions. For instance, The Cav1 subfamily initiates skeletal muscle contraction and regulates the calcium-dependent signaling pathway in the cell. The Cav2 subfamily is meanly involved in synaptic transmission at fast synapses. The Cav3 subfamily plays a critical role in rhythmical cardiac myocytic contraction (Catterall, 2011). Seeing the massive neuron functions which Ca\textsubscript{v} are involved in, the understanding of Ca\textsubscript{v} is not yet profound enough. With more discoveries on voltage-gated calcium channels, more neuron functions can be explained, and the mechanism of the neuron network can be further understood.

1.1.1.4. Voltage-gated proton channels

Voltage-gated proton channels (H\textsubscript{v}) are a group of channels that conduct protons out by responding to membrane potential and pH difference between the bio-membrane. H\textsubscript{v} is identified and conserved in many species varied from single-celled organisms to invertebrates to mammals. Although several isoforms were found in certain species, only one gene, the HVCN1 gene which encodes H\textsubscript{v}1, has been confirmed (DeCoursey & Hosler, 2014). In mammals and many other species, the H\textsubscript{v} is formed as a dimer associating by the coiled-coiled domain on the C-terminus. Based on the structure revealed by nuclear magnetic resonance (NMR) spectroscopy, each H\textsubscript{v} subunit is composed by 4 TM helices (S1-S4) which
share amino acid sequence and structural similarity with the VS domain of \( K_v \), \( Na_v \) and \( Ca_v \) (Bayrhuber et al., 2019). In contrast to other voltage-gated ion channels which contain a pore as the ion pathway in the center and four VS domains surrounded the pore, each \( H_v \) subunit contains its own proton pathway formed by the 4 TM helices and the S4 helix, which contain several positive charged side chains that are responsible to transmit protons across the biomembrane. \( H_v \) conducts outward proton current, which is activated at depolarizing voltages and is sensitive to \( Zn^{2+} \) and transmembrane pH gradient (Ramsey, Moran, Chong, & Clapham, 2006; Sasaki, Takagi, & Okamura, 2006).

\( H_v \) is involved in many biological functions in humans. It is expressed in the flagellum of the sperm, triggering the movement of sperm (Lishko, Botchkina, Fedorenko, & Kirichok, 2010). It also was detected in immune cells, such as granulocytes, to play a role in clearing organisms such as bacterial pathogens (DeCoursey & Hosler, 2014). Furthermore, it was reported that \( H_v \) specifically express in highly metastatic breast cancer cells, where it may be involved in tumor progress (Y. Wang et al., 2011). With more discoveries of \( H_v \) in humans and other species, more developmental clues, and fundamental roles of \( H_v \) will be revealed.

1.1.1.5. **Hyperpolarization-activated cyclic nucleotide-gated channels**

Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) are a group of channels activated by hyperpolarized voltage and regulated by cyclic nucleotides binding. Unlike \( Na_v \) or \( K_v \), which are specific to \( Na^+ \) or \( K^+ \), HCN conducts both \( Na^+ \) and \( K^+ \). Four genes (HCNC1-4) are reported to encode HCN channels and their expressions were detected in the heart and central nervous system. Based on TM domain amino acid sequence alignment, HCN together with cyclic nucleotide-gated (CNG) channels and \( K_v 10-12 \) (the second phylogenetic tree of \( K_v \), also known as Eag-like \( K^+ \) channels) are belongs to a
structural related subgroup (F. H. Yu & Catterall, 2004). According to the structure solved by cryo-EM, the architecture of the TM core of HCN is the same as that of K_v (Lee & MacKinnon, 2017). Four identical HCN subunits form a 4-fold symmetrical TM core, including a central ion pathway and 4 VS domains surrounding the ion-conducting pore. Same as CNG, the cyclic nucleotide binding domains (CNBD) of the HCN subunits are in the C-terminus and the four CNBD located in the intracellular side sense cyclic nucleotide binding and regulate HCN current.

The currents conducted by HCN are designated as hyperpolarization activated currents (I_h) which initiate and regulate the action potential firing within groups of heart and brain cells. These currents are known as the “pacemaker current” due to their roles in generating rhythmic activity in these organs. At hyperpolarized MP, Na^+ influx through the activated HCN depolarizes the cell and trigger the activation of other voltage-gated ion channels, firing action potential. Neuron cAMP level regulates the activation range of HCN channels, determining the action potential firing frequency. Besides cAMP, HCN channels are regulated by many other molecules, such as PIP2, Cl^-, protons, and are involved in several signal pathways, such as Mitogen-activated protein (MAP) kinases pathway (Wahl-Schott & Biel, 2008). I_h was firstly recorded in cardiac pacemaker cells and was also found in many types of neurons (Pape, 1996). Included in cardiac myocytes, I_h in primary sensory neurons is regulated by HCNs, which play a key role in inflammatory, neuropathic pain, and other sensory (Momin, Cadiou, Mason, & McNaughton, 2008). Therefore, HCN channels are important drug targets in cardiac and nerve pathology.

### 1.1.2. Ligand-gated ion channels

As the name “ligand-gated” states, this group of ion channels are activated by the binding of ligands. Ligand-gated channels contain specific ligand-binding domains as a
receptor, responding to signaling ligand molecules, such as neurotransmitters, cyclic nucleotides, or G-proteins. As a result of the ligand binding, channel proteins undergo conformational changes and switch to the open stage and conduct ions across the membrane (Purves, 2018). Also, this group of ion channels is known as ionotropic receptors.

Since ligand-gated channels receive the molecule signal and change it to electrical or ion-related signals, this group of channels plays crucial roles as signal converters in many biological functions. For example, nicotinic acetylcholine (nAChR) receptors are found in the neuromuscular junction, gating by acetylcholine (Ach)-binding, and conducting Na\(^+\) in neurons (Itier & Bertrand, 2001). Na\(^+\) influx increases the membrane potential and depolarizes the neuron, which is the initiation of action potential firing. Therefore, the investigation of ligand-gated channels is extremely meaningful to understand neuron function and diseases. Meanwhile, the agonists and antagonists of ligand-gated channels are valuable candidates for drug development.

Since channel function is dependent on the signaling ligand, ligand-gated channels are commonly classified by their activating ligand. Typically, there are nicotinic acetylcholine (nAChR) receptor families, 5-hydroxytryptamine3 (5-HT3) receptor families, \(\gamma\)-aminobutyric acid A (GABAA) receptor families, glycine receptor families, ionotropic glutamate receptor families, and ATP-gated purinergic (P2X) receptor families and others (Collingridge, Olsen, Peters, & Spedding, 2009). Unlike voltage-gated channels family sharing conserved structure, ligand-gated channels have more diversity in structure. Ionotropic glutamate receptors, such as N-methyl-D-aspartate (NMDA) receptor and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, are tetramers. Whereas GABAA, 5-HT3, nAChR are pentamers and P2X receptors are trimers.
1.1.3. Lipid-gated ion channels

The concept of “lipid gated” classification was established only a couple of decades ago and has garnered much attention. In terms of lipid, it refers to the phosphatidylinositol bisphosphate (PIP2) which is a minor constituent of bio-membrane. PIP2, as a substrate, can be catalyzed by phospholipase C (PLC) and produce inositol trisphosphate (IP3) and diacylglycerol (DAG), which are known as second messengers and are involved in many cell signal pathways (Berridge & Irvine, 1984). In 1998, PIP2 was firstly found to bind on the C-terminal of inward rectifier potassium channels (K\textsubscript{ir}) and activate it (Huang, Feng, & Hilgemann, 1998). Although PIP2 is considered as activator or signaling ligand after that, the gating mode of K\textsubscript{ir} was still elusive for many years. The structure of K\textsubscript{ir}2.2 was revealed by X-ray crystallography and demonstrated that the binding of inner layer PIP2 with the TM helices of the channel causes conformation change of channel C-terminal tetramer which further opens the channel pore (H. Zheng, Liu, Anderson, & Jiang, 2011). The gating mechanism of many other classes of channels can most likely be explained similarly. The bio-membrane is dynamic. The tensions, thickness, and components of the bio-membrane are changing every second with the change of diffusion intensity, temperature, mechanical force, and other factors. Hence, more and more classes of channels, such as temperature-sensitive channels, mechanosensitive channels, may also potentially be lipid-gated channels.

1.1.4. Other ion channels

Apart from these major gating classes, there are several other groups of channels that have been discovered and studied.

Temperature gating channels: This group of channels is defined as ion channels gating under temperature change. This gating mechanism is critical in thermosensation. Some members in transient receptor potential (TRP) channel superfamily are known as temperature
gating channels, such as TRPV1 and TRPM8. Recently, some two-pore-domain potassium ion channels (K2P), such as TWIK-related potassium (TREK) channels, are reported as thermosensitive channels (Lamas, Rueda-Ruzafa, & Herrera-Pérez, 2019).

**Mechanosensitive channels (or stretch-activated channels):** This is a group of channels gated by mechanical force or stretch. They adhere to cytoskeleton or membrane mechanosensitive peptide, gated by those peptides’ movement. Typical mechanosensitive channels are TRPA and TRPV family in TRP superfamily, K2P families, and degenerin (Deg)/epithelial sodium channel (ENaC) superfamily (Valle, Cobo, Cobo, & Vega, 2012).

**Cyclic nucleotide-gated (CNG) ion channels:** Similar to the lipid-gated ion channel, here cyclic nucleotides, such as cAMP and cGMP, binds on C-terminal of CNG channels, resulting in channel gating. CNG channels are nonselective cation channels deeply involved in sensory transduction as well as cellular development (Kaupp & Seifert, 2002).

Besides these channels, **calcium-gated channels, light-gated channels**, and many other classes are identified. With more and more discoveries, new classes will be established. Likewise, existing classes might be reclassified with further understanding.

1.2. **Transient receptor potential (TRP) channel superfamily**

Transient receptor potential (TRP) channels are a large group of cation channels, widely spread in many species. The TRP channel was firstly found in Drosophila. The mutant strain, named "transient receptor potential", generates a transient instead of sustained receptor potential in its photo receptor responding to light stimuli and lacks the ability to react high ambient light (Cosens & Manning, 1969). Subsequently, numerous TRP channels are discovered and identified in mammals and invertebrates. TRP family channels share similar four-fold symmetrical conformation, formed by homo- or hetero- subunits. Each subunit contains 6 TM and the last two TM (S5-S6) form the center ion pathway, including the
selective filter at the top and ion gate at the bottom. The amino (N) and carboxyl (C) termini of TRP channels, located at the intracellular side, contain many recognized domains and motifs, such as α-kinase, ankyrin repeats, EF-hand, and coiled-coil, that are involved in channel assembly, activation and regulation (M. Li, Yu, & Yang, 2011). A common activation mechanism of TRPs is that the S4-S5 linker and TRP domains, an intracellular helix after S6 typically found in group 1 TRPs, transmit the conformation change from the activated S1-S4 and N- and C- termini under the stimulus and pull the S6. As the result, the diameter of the center ion pathway becomes wider and the channel is opened (Myers, Saimi, Julius, & Kung, 2008).

The TRP superfamily is divided into two groups. Group 1 contains TRPC ("C" for canonical), TRPN ("N" for no mechanoreceptor potential C), TRPV ("V" for vanilloid), TRPA ("A" for ankyrin), and TRPM ("M" for melastatin). Group 2 includes TRPP ("P" for polycystic) and TRPML ("ML" for mucolipin) (Fig. 1) (Venkatachalam & Montell, 2007). TRP channels play a critical role in animal sensories, such as light, sound, taste, pressure, temperature, pH, osmolarity, pain, and inflammation (Vriens, Nilius, & Voets, 2014). Therefore, TRP channels are essential to human health.
Figure 1. The TRP channel superfamily.

(adopted from Venkatachalam & Montell, 2007)

A The transmembrane topology of group 1 TRP channels

B The transmembrane topology of group 2 TRP channels

C Composition of the TRP superfamily in worms, flies, mice, and humans.
1.2.1. TRPC channels

Transient receptor potential canonical (TRPC) channels are a group of nonselective cation channels, conducting monovalent cation and Ca\(^{2+}\) and are widespread in many species (Clapham, 2003). TRPC1 is the first TRP channel homolog identified in mammalian (Wes et al., 1995). In the following decade, seven TRPC channels were identified in mammalian and divided in four subsets: TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7 (Venkatachalam & Montell, 2007). TRPCs can form a homomeric tetramer as a functional ion channel, and also can interact with other TRPCs, even other TRPs, forming a hetero-tetramer, such as TRPC1 interacts with TRPC4/5 (Clapham, 2003). TRPCs contain ankyrin repeats domains (ARD) on their intracellular N-terminus, and TRP, coiled-coil, and calmodulin- and IP3R-binding (CIRB) domains on their intracellular C-terminus (M. Li et al., 2011). ARD has a helix-turn-helix conformation, presenting in numerous proteins and commonly mediate intra- and inter-protein interactions (J. Li, Mahajan, & Tsai, 2006). The TRP domain is an intracellular helix specifically in TRP proteins and is commonly located downstream of the last TM helix, coupling with S4-S5 linker and controlling TRP channel gating (García-Sanz et al., 2007). Recently, many full-length structures of TRPC channels were solved by cryo-EM, providing further integral understanding in TRPC channels (J. Li et al., 2019). TRPC channels are widely expressed in many tissues and highly expressed in the brain. Some of them, such as TRPC3 and TRPC5, are specifically expressed in the brain (Venkatachalam & Montell, 2007). TRPC channel members can be activated by Phospholipase C (PLC) and are sensitive to intracellular Ca\(^{2+}\) (Montell, 2005; Pedersen, Owsianik, & Nilius, 2005). In addition, TRPC3/6/7 can be activated by DAG binding. Therefore, TRPC families in the cell are contributing to calcium influx by reacting to G protein signaling mediated by G-protein coupled receptors.
1.2.2. TRPV channels

Transient receptor potential vanilloid (TRPV) channels are a group of nonselective cation channels involved in heat and pain sensory, conducting $\text{Ca}^{2+}$ and are widespread in many species (Venkatachalam & Montell, 2007). TRPV1 is the first TRPV member identified in mammalian, as a capsaicin receptor activated by heat and involved in the pain pathway (Caterina et al., 1997). In mammal, six TRPV proteins are identified and they can be divided into two groups: TRPV1/V2/V3/V4 and TRPV5/V6. Like other TRP channels, TRPV channels are mainly formed by homomeric tetramers although TRPV5 and TRPV6 can form heteromeric tetramers with each other (Clapham, 2003). Each TRPV subunit contains various copies of ARD on the intracellular N-terminal tail, and TRP domain and calmodulin-binding side on the intracellular C-terminal tail (M. Li et al., 2011).

TRPV channels are widely expressed in many tissues, especially in neurons, and can be activated by many stimuli (Clapham, 2003; Montell, 2005; Venkatachalam & Montell, 2007). For example, TRPV1 is voltage-gated and can also be activated by spicy flavor-causing small molecules such as capsaicin and vanilloid, as well as ethanol, nicotine, PIP2, proton, and other molecules. Group one TRPV members, including TRPV1, can respond and be activated by temperature changing (heat), while group two TRPV members cannot. TRPV5 and V6 are constitutively opened and highly selective to $\text{Ca}^{2+}$ ($P_{\text{Ca}} : P_{\text{Na}} = 100:1$). An important function of TRPV channels is responding to heat and nociception stimulus, converting these signals to $\text{Ca}^{2+}$ influx in the cell, and initiating the pain and inflammation pathway in mammal.

1.2.3. TRPA channels

Transient receptor potential ankyrin (TRPA) channels are distinguished by 14 copies of ARD on its intracellular N-termini (Clapham, 2003). TRPA channels are widespread in
many species and have more diversity in arthropod species (Peng, Shi, & Kadowaki, 2015). In mammal, only one TRPA channel has been identified: TRPA1, as called ANKTM1, a Ca$^{2+}$-permeable nonselective cation channel, activated by cool temperature (<18°C) and some cool feeling-causing small molecules, such as icilin and isothiocyanates (Story et al., 2003; Venkatachalam & Montell, 2007). The full-length structure of TRPA1 has been solved by cryo-EM (Paulsen, Armache, Gao, Cheng, & Julius, 2015). The spatial distribution of the intracellular domain can be clearly observed in these structures. TRPA1 is expressed in sensory neurons and is activated by cool temperature, indicating that TRPA1 is involved in mammalian cool temperature sensory.

1.2.4. TRPM channels

Transient receptor potential melastatin (TRPM) channels conducting Ca$^{2+}$, Mg$^{2+}$ and some other divalent ion. The first TRPM member, TRPM1, is identified from a cancer, Melanoma (Duncan et al., 1998). It is encoded by the Melastatin gene, which is down-regulated in Melanoma. TRPM1 is also widely expressed in normal tissues and its function in normal tissues is unknown. In mammal, eight TRPM members have been identified and they can be divided into several subsets: TRPM1/M3, TRPM4/M5, TRPM6/M7, TRPM2, and TRPM8 (Venkatachalam & Montell, 2007). TRPM members contain many well-recognized protein motifs located in the intracellular C-termini, including the TRP domain, coiled-coil domain, and CIRB domain. Several TRPM membranes have protein kinase domain in their C-terminal tail, i.e. α-kinase in TRPM6, M7, and ADP ribose hydrolase in TRPM2 (M. Li et al., 2011).

Although TRPM members share homologs in amino acid sequence and structure, the function of different subsets are very different (Montell, 2005; Venkatachalam & Montell, 2007). TRPM2 is gated by H$_2$O$_2$ and binding with the ADP ribose (ADPR) and nicotinamide
adenine dinucleotide (NAD) on its C-terminal ADPR hydrolase motif, conducting Ca\(^{2+}\) influx and leading to cell death (Hara et al., 2002; Perraud et al., 2001). TRPM3 forms a constitutively activated Ca\(^{2+}\)-permeable, nonselective cation channel, specifically expressed in the kidney and its activity is facilitated by osmolarity increasing (Grimm, Kraft, Sauerbruch, Schultz, & Harteneck, 2003). TRPM4 and M5 are Ca-activated monovalent cation selective channel, which is unique in the TRP family. They are also regulated by voltage (Hofmann, Chubanov, Gudermann, & Montell, 2003; Launay et al., 2002). In contrast, TRPM6 and M7 are also unique in the TRP family for their divalent cation selective channel function (Nadler et al., 2001). They conduct Ca\(^{2+}\) and majorly Mg\(^{2+}\) and can be inhibited by intracellular Mg\(^{2+}\) and PIP2 (Nadler et al., 2001; Runnels, Yue, & Clapham, 2002). Meanwhile, TRPM6 and M7 contain the functional α-kinase motif, a group of protein kinase transferring the phosphate from ATP to protein or lipid, in their intracellular C-termini, which is unique in ion channel superfamily (Runnels, Yue, & Clapham, 2001).

TRPM8 was identified from the genes up-regulated in prostate cancer (Tsavaler, Shapero, Morkowski, & Laus, 2001). Interestingly, it is a cold temperature (8-28°C) activated, non-selective, outwardly rectifying cation channel, involved in cold sensory (McKemy, Neuhausser, & Julius, 2002). However, the role of TRPM8 in prostate cancer is still largely unknown. In summary, more effect is necessary to reveal the potential linkage of the structure homologs to the function diversity between TRPM members and to demonstrate the roles that TRPM members play in cancer.

1.2.5. TRPN, TRPML, and other TRP channels

**TRPN**: Transient receptor potential no mechanoreceptor potential C (TRPN) channels were firstly identified from *Drosophila melanogaster*. They are nonselective cation channels and have over 20 copies of ARD on its intracellular N-terminal tail (Walker,
Willingham, & Zuker, 2000). Loss of function mutants of TRPNs in fruit flies led to loss of the flies’ mechanical sensory. None of TRPN homologs has been found in mammals.

**TRPML:** Transient receptor potential Mucolipin (TRPML) channels, as a member of group two TRP channels, are similar to TRPPs, containing a large extracellular loop between first two TM helices (M. Li et al., 2011). TRPML1 was firstly found due to its link to Mucolipidosis type IV, an autosomal recessive lysosomal storage disorder (Sun et al., 2000). Three members have been well described in mammal: TRPML1, TRPML2, and TRPML3.

Besides mammalian TRP channels, numerous TRP channels have been identified from the gene scanning of other species, such as insects, worm, and yeast (Peng et al., 2015). Based on those non-mammalian TRP channels, many other TRP subfamilies were established, such TRPS (“S” for Soromelastatin, a sister group of TRPM), TRPVL (“VL” for Vanilloid Like, a sister group of TRPV), TRPY (“Y” for yeast), and TRP-like (TRPL) family. As more genes are discovered in different species, more TRP channels will be identified and the evolution tree of TRP channel family can be filled and complete.

### 1.3. **Transient receptor potential polycystin (TRPP) channels**

Transient receptor potential polycystin (TRPP) channels are non-selective cation channels. In contrast to group 1 TRP channels, TRPP and TRPML subfamilies are distinguished by a large extracellular loop domain between the first two TM helices (S1 and S2). Three genes are identified encoding the channels in TRPP subfamily, including *polycystin kidney disease 2 (PKD2, encode TRPP2 or Polycystin-2), PKD 2-like 1 (PKD2L1, encode TRPP3 or polycystin-L), PKD 2-like 2 (PKD2L2, encode TRPP5 or polycystin-L2)* (Venkatachalam & Montell, 2007). TRPPs are consistent with each other on the TM domain core but have various intracellular N-terminal and C-terminal (M. Li et al., 2011).
1.3.1. TRPP2 (Polycystin-2)

Transient receptor potential polycystin-2 (TRPP2), also called polycystin kidney disease 2 (PKD2), TRPP1 in IUPHAR Database, or Polycystin-2 (PC2) which is used below, was firstly discovered from a gene involved in autosomal dominant polycystic kidney disease (ADPKD) (Mochizuki et al., 1996). PC2 forms a homomorphic cation channel and can also associate with other proteins to form heteromeric complexes. The most famous binding partner of PC2 is polycystin-1 (PC1), which is also called Polycystic kidney disease 1 (PKD1).

1.3.1.1. The localization and function of PC2

Homomeric PC2 channel functions as a nonselective cation channel with controversial reports on its Ca$^{2+}$-permeability. Unlike other TRP channels, the activation mechanism of PC2 is still unknown and none of the agonists of PC2 were reported. As the result, only a few limited methods, such as single channel patch clamp and intracellular Ca$^{2+}$ fluorescence, can be applied to study PC2 channel function (Gonzalez-Perrett et al., 2001; Koulen et al., 2002; Luo, Vassilev, Li, Kawanabe, & Zhou, 2003; Vassilev et al., 2001). It causes many limitations and difficulties in the PC2 functional study. Recently, a reliable platform for PC2 functional study was established by inducing a Gain-Of-Function (GOF) mutation, F604P, in PC2 (Arif Pavel et al., 2016). PC2_F604P mutant can be easily expressed in Xenopus oocytes and Zebrafish and work as an activated PC2, which significantly facilitated the study of PC2.

PC2 can be detected in many human tissues and have high expression levels in the heart, placenta, kidney, testis, and ovary (Mochizuki et al., 1996). Meanwhile, depending on the cell type or binding partners, PC2 has been found to be located on many subcellular compartments, such as the endoplasmic reticulum (ER) membrane, plasma membrane, and primary cilium (Giamarchi et al., 2006). For example, PC2 has been shown to be expressed
in ER as a calcium release channel (Koulen et al., 2002), as well as forming an ion channel complex with PC1 in primary cilia of kidney epithelial cells involved in ADPKD (Nauli et al., 2003). PC2 has also been shown to be involved in left-right asymmetry establishment in early embryonic development and systemic blood pressure (MacKay et al., 2020; Yuan, Zhao, Brueckner, & Sun, 2015).

### 1.3.1.2. The structure of PC2

PC2 protein has six TM domains (S1-S6) and intracellular N- and C-termini which contain many functional elements, such as ciliary trafficking motif, EF-hand motif, and coiled-coil domain (Fig. 2A) (Semmo, Kottgen, & Hofherr, 2014). Between the S1-S2, PC2 contains a large extracellular loop called Tetragonal Opening for Polycystins (TOP) domain. PC2 forms a homo-tetramer in the absence of PC1 and four subunits associate with each other at multiple sites, including the extracellular TOP domain and intracellular N-terminus and the coiled-coil domain on the C-terminus (Feng et al., 2008; Salehi-Najafabadi et al., 2017; Y. Yu et al., 2009). The structure of several PC2 structural elements, such as EF-hand and coiled-coil, were solved by X-ray crystallography or NMR previously (Petri et al., 2010; Y. Yu et al., 2009). Recently, the structure of the PC2 TM domain core was solved by three groups by the cryo-EM method (Fig. 2B) (Grieben et al., 2016; P. S. Shen et al., 2016; Wilkes et al., 2017). According to these structures, the TM domain core of PC2 is similar to other TRP channels as well as Kv and Na. In the cryo-EM structures, the S5-S6 helices from each subunit compose a 4-fold symmetrical ion pathway. The S4-S5 linker connects to the center pathway and S1-S4 helices stay surrounding it. The TOP domain from each subunit associates with others to form a doughnut-shaped structure at the extracellular side of PC2. The structure of PC2 TM core and the functional evidence of PC2 can support each other and provide a further understanding of this channel. For instance, D643 in PC2 was reported as a
critical amino acid that is involved in determining the ion selectivity of PC2 in the functional study (Arif Pavel et al., 2016) and was also shown to be located in the selectivity filter in the cryo-EM structures (P. S. Shen et al., 2016). Meanwhile, L677 in PC2 forms the narrowest region in the center ion pathway in cryo-EM structure (P. S. Shen et al., 2016) and this has been approved in our recent functional study (Z. Wang et al., 2019; W. Zheng et al., 2018). However, without the PC2 full-length structure, it is hard to imagine how the functional elements in N- and C-termini work with TM core in regulating channel function. Thus, more efforts are needed on solving the full-length structure of PC2.

1.3.1.3. The GOF PC2-F604P mutant

The amino acid phenylalanine (Phe) 604 in human PC2 is on the S4-S5 linker, a critical region involved in TRP channels activation (Myers et al., 2008). It is reported that a single proline (Pro) mutation in the S4-S5 linker of TRPML1 active this channel (Dong et al., 2009). After a series test of proline (Pro)-scanning mutagenesis, F604P was found as a GOF mutation (Arif Pavel et al., 2016). PC2_F604P mutant gave rise to robust whole-cell currents when expressed in Xenopus oocytes (Arif Pavel et al., 2016; Liu et al., 2018; W. Zheng et al., 2018). The cryo-EM structure shows that the F604P mutation leads to twisting and bending of the distal S6 helix. As a result, the S6 helix, which contains a π-helix turn in the middle, converts to an entire α-helix and the lower gate opens up (W. Zheng et al., 2018).

Interestingly, the structure of PC2_F604P is very similar to an open structure of TRPP3 (Q. Su, F. Hu, Y. Liu, et al., 2018), suggesting that the F604P mutation results in a conformational change reflecting the natural gating of PC2.
Figure 2. PC2 homomeric channel tetramer and PC1/PC2 ion channel complex

A Transmembrane topology of PC2 proteins. The TOP domain and EF-hand motif in PC2 are indicated.

B Side (middle) and bottom-up (right) views of the cryo-EM structure of PC2 homomeric tetramer. The structure shown here is previously reported with Protein Data Bank (PDB) code 5t4d (P. S. Shen et al., 2016). Each subunit is color coded.

C Transmembrane topology of PC1 and PC2 proteins. The two proteins associate at the C-terminus through the coiled-coil domains and the extracellular side via the TOP domains. The GAIN domain, the GPS site and PLAT domain in PC1 and the EF-hand motif in PC2 are indicated. The last six transmembrane domains (TLD) of PC1 (shown in blue) share sequence similarity with PC2.

D Side (middle) and bottom-up (right) views of the overall cryo-EM structure of PC1/PC2 ion channel complex. (PDB code 6A70) (Q. Su, F. Hu, X. Ge, et al., 2018). PC1-NTMD is
shown in yellow. PLAT domain in PC1 is shown in cyan. PC1-TLD is shown in blue. Three PC2 subunits are colored in green, magenta, and gray.
1.3.2. TRPP3 (polycystin-L)

Transient receptor potential polycystin-3 (TRPP3), also called polycystin kidney disease 2-like 1 (PKD2L1 or polycystin-L, PCL, which is used below) or TRPP2 in IUPHAR Database, is a Ca\(^{2+}\)-permeable nonselective cation channel (Chen et al., 1999). When expressed in *Xenopus* oocytes, the channel activity of PCL is voltage dependent and can be increased by either the extracellular or intracellular Ca\(^{2+}\) and decreased by low extracellular pH level (Shimizu, Janssens, Voets, & Nilius, 2009). Meanwhile, PCL and PKD1L3 form an ion channel complex with a 3 (PCL) : 1 (PKD1L3) subunit stoichiometry, involving soul taste sensory in a currently unknown way (Chaudhari & Roper, 2010; Y. Yu et al., 2012). Both PCL and PKD1L3 subunits are involved in ion channel pore-formation through association at the TOP domain and the coiled-coil domain (Salehi-Najafabadi et al., 2017; Y. Yu et al., 2012). Expression of both PCL and PKD1L3 in *Xenopus* oocytes gives rise to robust acid-induced “off-response” currents which only can be recorded when oocytes are neutralized after a low pH treatment. The TM core structure of PCL was solved by cryo-EM and has a high similarity with that of PC2 (Qiang Su et al., 2018).

1.3.3. TRPP5 (polycystin-L2)

Transient receptor potential polycystin-5 [TRPP5, also called polycystin kidney disease 2-like 2 (PKD2L2)], is the third member in TRPP families to be identified (Guo et al., 2000). The tissue distribution of TRPP5 is restricted and it is only detected in heart and testis (Guo et al., 2000). Comparing to other TRPPs, TRPP5 has very short N- and C-termini, as if only contain a TM core. Not many studies focus on TRPP5 function and no spontaneous TRPP5 activity has been recorded during whole-cell recordings (Sutton, Jungnickel, Ward, Harris, & Florman, 2006). TRPP5 can associate with TRPC1 and TRPC5 and none of the report shows that TRPP5 has any PKD family binding partner (Sutton et al., 2006). Limited
information is available on TRPP5 and further discovery is necessary to demonstrate the role of TRPP5 plays.

2. **Autosomal dominant polycystic kidney disease (ADPKD)**

Autosomal dominant polycystic kidney disease (ADPKD) and Autosomal Recessive PKD (ARPKD) are two types of polycystic kidney diseases, which are caused by a group of monogenic mutations, resulting the fluid-filled cysts developing in organs, majorly in the kidney (Harris & Torres, 2009).

2.1. **General information**

ADPKD is one of the most common inherited human diseases, affecting one in every 400-1000 individuals (Chapin & Caplan, 2010; Wu & Somlo, 2000; Zhou, 2009). ADPKD is characterized by fluid-filled cysts continually growing in kidneys, resulting in gradient renal function abnormality, with more than half of patients progressing to renal failure. The common renal manifestations of ADPKD include acute loin pain and renal pain, anemia, hypertension, renal colic, nephrolithiasis, hematuria, and urinary tract infections (Harris PC, 2018). ADPKD also has some extrarenal manifestations, such as polycystic liver disease (PLD), and vascular and cardiac disable. With the long progress of ADPKD, patients completely lose their kidney function at the end, resulting in end-stage renal disease (ESRD). ADPKD can be diagnosed by computed tomography scan (CT) and Magnetic resonance imaging (MRI), displaying a renal image with multiple cysts in the kidney. So far, there is no direct therapy to disrupt the developing progress of ADPKD. At the middle stage of ADPKD, ultrasound and surgery can be utilized to clear the cysts in the kidney. Until the ESTD, renal replacement therapy (dialysis or transplant) is necessary to maintain patients’ lives (Torres, Harris, & Pirson, 2007).
2.2. **Pathophysiology**

PC1 and PC2 form a protein complex and mutations in either PC1 or PC2 protein cause ADPKD (Cornec-Le Gall, Torres, & Harris, 2018). How mutations in PC1 and PC2 lead to ADPKD is unclear, It is generally believed that in the PC1/PC2 complex, PC1 functions as a receptor to sense unknown extracellular stimuli, such as mechanical force or chemical ligands, and couples it with intracellular signaling through Ca\(^{2+}\) influx conducted by the PC2 channel (Delling et al., 2016; Gonzalez-Perrett et al., 2001; Liu et al., 2018; Luo et al., 2003; Yuan et al., 2015) or PC1/PC2 channel (S. Kim et al., 2016; Nauli et al., 2003; Zhou, 2009). Missing the PC1/PC2 protein complex signal leads to cell proliferation that randomly occurs in any position on the renal ducts, which form the fluid-filled cysts (Torres et al., 2007). The random occurrence and continuous cell proliferation progress cause cysts to become progressively swollen and appear everywhere in the kidney, resulting in the patients’ kidneys exhibiting renal enlargement and polycystic morphology, as per the name of the disease. The development and enlargement of the fluid-filled cysts lead to a gradual functional decrease in the patients’ kidney, resulting in the renal manifestation of ADPKD, progressing renal insufficiency. Statistically, the whole process takes averagely 53 years for the patient containing the mutation of PC1 and 69 years for that of PC2, starting from the infant age (Grantham, 2008; Torres et al., 2007). The cysts in the kidney of an ADPKD patient are large enough to be detected at around 15-30 years old. With subsequent 20 to 30 years progression of the cyst’s formation, patients completely lose their kidney function and suffer renal failure.

2.3. **Genetics**

ADPKD is genetically heterozygous, which is caused by either mutation in *polycystin kidney disease 1 (PKD1)* gene, encoding PC1, or mutations in *PKD2* gene, encoding PC2
(Torres et al., 2007; Wu & Somlo, 2000). *PKD1* gene account for ~80% of clinically identified cases, which are usually more severe than those caused by mutations in *PKD2* gene, which factor for around 15% of clinically identified cases. Homozygous or compound heterozygous genotypes exhibit embryonic lethality (Zhou, 2009).

One of a wide accepted theory to explain the relationship from the genotypes to ADPKD is called the two-hit hypothesis (Zhou, 2009). The germline mutation inherited from the family in one of the two alleles is the first hit. When a somatic mutation occurs on the remaining normal allele, abolishing its expression, the second hit appears, resulting in neither allele being active and no functional polycystin protein remaining. This theory well explained the embryonic lethality, random cysts appearance, and long disease progression. Several lines of evidence support this theory. First, the somatic PKD1 or PKD2 mutations have been detected in patients’ kidneys (Qian, Watnick, Onuchic, & Germino, 1996). Moreover, conditional knockout of somatic *PKD1* in the specific life stage of germline heterozygous *PKD1* mutant mice cause cyst formation in adult kidneys (Starremans et al., 2008; Takakura, Contrino, Beck, & Zhou, 2008). However, this theory cannot explain the region progression difference in mature organ and Third-hit for rapid cysts formation was induced in this theory (Takakura et al., 2008).

**Human polycystin kidney disease 1 (PKD1)** gene, encoding PC1, is a large gene and locates in the chromosome 16p13.3, containing 46 exons and encoding a 14 kb mRNA (Consortium, 1995). Two mRNA isoform was discovered: PC1 isoform-1 and isoform-2 (NP_001009944, NP_000287). Furthermore, six *PKD1 pseudo*-genes have been reported in 16q13.11–16q13.13 (Semmo et al., 2014). Currently, 2322 germline mutations have been reported in the *PKD1* gene and half of the mutations are highly likely or definitely pathogenic. Meanwhile, 9 somatic mutations have been reported and all of them are highly likely or definitely pathogenic (Liu et al., 2018). Different types of mutations in *PKD1* gene
have been identified including missense mutations, nonsense mutations, splice site, in-frame, and out-of-frame deletions and insertions, and others. No correlations have been established between specific clinical manifestations and specific mutations.

Human polycystin kidney disease 2 (PKD2) gene, encoding PC2, locates in the chromosome 4q21-23, containing 15 exons and encoding a 5.4 kb mRNA (Mochizuki et al., 1996). One mRNA was built (NM_000297). Currently, 278 germline mutations have been reported in the PKD2 gene and 167 mutations of them are definitely pathogenic. Most of these pathogenic mutations are frameshift or truncation caused by splice and nonsense mutations. Meanwhile, 27 somatic mutations have been reported and all of them are definitely pathogenic (Liu et al., 2018). Same as the PKD1 gene, no specific clinical manifestations are linked to specific mutations.

3. Polycystic kidney disease (PKD) proteins

Polycystic kidney disease (PKD) protein was first discovered through the genetic study from ADPKD patients (Reeders et al., 1985). In the following two decades, PKD protein family, including 5 members, was established: PC1 (PKD1), PKD1-like 1 (PKD1L1), PKD1-like 2 (PKD1L2), PKD1-like 3 (PKD1L3), and PKDREJ (REJ: receptor for egg jelly) (Hofherr & Köttgen, 2011). PKD proteins are a group of membrane proteins and share a similar structure, containing a large external N-terminal and 11 TM C-terminal, with the last 6 TM (S6-S11) share sequence similarity with TRPP channels (Clapham, 2003). PKD proteins can bind with their TRPP protein partners, forming receptor/ion channel complexes, such as PC1/PC2 complex and PKD1L3/PCL complex. It is not known whether PKD proteins can form a functional ion channel individually.
3.1. **PKD1 (Polycystin-1, PC1)**

Polycystic kidney disease protein, also called Polycystin-1 (PC1), is a large protein with 4304 amino acids, widely-expressing in many tissues, highly-expressed in the sperm, brain, and kidney (Ward et al., 1996). PC1 can associate with PC2 to form a receptor/ion channel complex in the plasma membrane and primary cilia of epithelial cells in the kidney. As a membrane of the PKD protein family, PC1 has eleven transmembrane domains, a short (around 200 aa) intracellular C-terminal tail, and a large (around 3000 aa) extracellular N-terminus (Fig. 2C) (Consortium, 1995; Hughes et al., 1995). The extracellular N-terminus of PC1 contains well-recognized motifs that are usually involved in protein-protein, protein-saccharide, and protein-ligand interactions. Following sequence order, there are cysteine-rich domain (CRD), leucine-rich repeats (LRR), cell wall integrity and stress response component (WSC), C-type Lectin, low-density lipoprotein A (LDL-A), PKD domain repeats, sperm receptor for egg jelly (REJ), and G protein-coupled receptors autoproteolysis-inducing (GAIN) domain (Delmas, 2005; Harris & Torres, 2009; Ong & Harris, 2005; Zhou, 2009). LRR is an α/β horseshoe fold, commonly flanked by CRD, which is a unique motif of PC1 comparing to other PKDs. These tandem repeats form a solenoid protein domain, responsible for the function of adhesion, extracellular matrix binding, and protein-protein binding in many proteins (Rothberg, Jacobs, Goodman, & Artavanis-Tsakonas, 1990). Recently study shows that Wnt/Ca²⁺ signal pathway-induced PC1/PC2 complex activation requires the LRR-WSC region on PC1 N-terminus (S. Kim et al., 2016). PKD domain, which is a common motif in PKD proteins, has a β-sandwich fold and is known as immunoglobulin-like fold (Bycroft et al., 1999). The 16 copies of PKD domain repeats, starting from residues V268 to Q2142, form the majority part of PC1 N-terminus. REJ domain was firstly found in the sperm of sea urchin (su), which plays an essential receptor role in fertilization through sperm
acrosome reaction, and a high homology was reported between PC1’s REJ domain and suREJ (Moy et al., 1996). In summary, most of these motifs have been reported to be involved in receptor function in other proteins. Therefore, PC1 is generally thought to function as a cell surface receptor.

PC1 has significant similarities to the adhesion G protein-coupled receptors (aGPCRs), a large group of proteins involved in many signaling pathways (Purcell & Hall, 2018). Like aGPCRs, the GAIN of PC1 locates upstream of the first transmembrane domain and undergoes autoproteolytic cleavage at a G protein-coupled receptor proteolytic site (GPS), formed by three residues “HL*T”, within the GAIN domain (Qian et al., 2002; S. Q. Yu et al., 2007). GPS cleavage splits the protein into an extracellular N-terminal fragment (NTF) and a C-terminal fragment (CTF); the latter contains the transmembrane domains and the intracellular C-terminal tail (Qian et al., 2002). The cleaved NTF and CTF tether together through a non-covalent interaction (Qian et al., 2002; Wei, Hackmann, Xu, Germino, & Qian, 2007), and GPS cleavage is essential for in vivo function of PC1 in mice (Cai et al., 2014; S. Q. Yu et al., 2007). Consistently, studies demonstrate that PC1 has a G protein binding site on the intracellular C-terminus (Parnell et al., 1998; Parnell et al., 2002) and the function of PC1 is linked to G protein signaling (Delmas et al., 2002; Hama & Park, 2016; Parnell et al., 2018; Zhang, Tran, & Wessely, 2018). Thus, PC1 may function as an atypical GPCR, although we do not know if this function is PC2-dependent.

PC1-CTF contain 11 TM and can be divided into two part: the N-terminal transmembrane domain (NTMD) containing five TMs (S1-S5) and the C-terminal transmembrane domain (CTMD) containing last six TMs (S6-S11), also called TRP-like domain (TLD) (Fig. 2C) (Zhou, 2009). A polycystin-1, lipoxygenase, and α-toxin (PLAT) signature domain, one of well-recognized protein motif, was identified in the intracellular loop between S1-S2 in NTMD (Bateman & Sandford, 1999; Q. Su, F. Hu, X. Ge, et al.,
Previous study suggested that the PLAT domain regulates PC1 trafficking (Xu et al., 2016), indicating it may also play a key role in PC1/PC2 complex trafficking to primary cilia, similar to the functional of the \(\beta\) subunit in \(\text{Ca}_v\) complex. The amino acid sequence alignment shows that TLD is a TRP type-2 channel homologous domain, and it contains a TOP domain between S6-S7, corresponding to the TOP domain in PC2 (Qian & Noben-Trauth, 2005). The significant residue difference in pore-forming helices between PC1 and PC2 indicates the center ion pathway is asymmetrical in the PC1/PC2 ion channel complex, like that in the \(\alpha\) subunit of \(\text{Na}_v\) and \(\text{Ca}_v\). On the intracellular C-terminal tail, besides the G protein binding site, a coiled-coil domain was identified (Consortium, 1995), which plays a key role in the assembly with PC2 (Y. Yu et al., 2009).

### 3.2. PKD1L1

Human polycystic kidney disease 1-like 1 (PKD1L1) protein contains 2849 amino acids and specifically expressed in the testis and heart (Yuasa et al., 2002). The human \(PKD1L1\) gene is located in chromosome 7p12.3 and contains 58 exons in a 187-kb genomic region. One mRNA was established (NM_138295) and no other mRNA isoform was reported. Like other PKD proteins, PKD1L1 is constituted by eleven TMs, a short intracellular C-terminal tail with a coiled-coil domain on it, and a large extracellular N-terminus, containing two copies of PKD domain, a short REJ domain and the GAIN domain. The residues at the equal position of PDK1L1 GPS cleavage site is “RSV”, which is not conserved to that of PC1, indicating PKD1L1 may not undergo the GPS cleavage. As in PC1, the PLAT domain in PKD1L1 is localized in the intracellular loop between S1-S2 helices and the last 6-TM is the TRP-like domain (Yuasa et al., 2002). It has been directly recorded that transfected PKD1L1 with PCL generate a calcium current in primary cilia of human retina pigmented epithelium cell, indicating PKD1L1 might form an ion channel complex with PCL.
Furthermore, the study shows that PKD1L1 knockout mice present left-right transposition of visceral and thoracic organs, indicating PKD1L1 is possibly involved in the left-right organization during development (Vogel et al., 2010). Meanwhile, PC2 has also been reported that initiate vertebrate left-right asymmetry, indicating that PC2 could be a potential binding partner of PKD1L1, and the complex is involved in the vertebrate left-right organization (Yuan et al., 2015).

3.3. **PKD1L2**

Human polycystic kidney disease 1-like 2 (PKD1L2) protein contains 2460 amino acids and is widespread in many organs, such as skeleton muscle, and is highly expressed in liver and testis (A. Li, Tian, Sung, & Somlo, 2003; Yuasa, Takakura, Denker, Venugopal, & Zhou, 2004). Human *PKD1L2* gene is located in chromosome 16q23.2 and contains 43 exons. Two mRNA were established (NM_052892, NM_001076780). PKD1L2 protein is constituted by eleven TMs, a short intracellular C-terminal tail, and a large extracellular N-terminus. Similar to that of PKD1L1, the N-terminus of PKD1L2 contains two copies of the PKD domain, a short REJ domain and the GAIN domain (A. Li et al., 2003; Yuasa et al., 2004). The “HL*T” GPS cleavage site of PKD1L2 is conserved with PC1, indicating PKD1L2 might undergo the GPS cleavage. The PLAT domain of PKD1L2 locates in the intracellular loop between S1-S2 helices and the last 6-TM is the TRP-like domain, while the coiled-coil domain is not detected in the intracellular C-terminal tail of PKD1L2. The function of PKD1L2 is largely unknown. One study shows that PKD1L2 up-regulate in the *ostes* mouse model causing widespread muscle atrophy, small body size, and shortened life (Mackenzie et al., 2009). It is also demonstrated that two isoforms of zebrafish *PKD1L2* gene express alternatively during zebrafish developing (England, Campbell, Banerjee, Swanson, & Lewis, 2017).
3.4. **PKD1L3 and PKD1L3/PCL complex**

Human polycystic kidney disease 1-like 3 (PKD1L3) protein contains 1731 amino acids and is widespread in many tissues while highly expressed in liver and testis (A. Li et al., 2003). Human *PKD1L3* gene is located in chromosome 16q22.3 and contains 30 exons. One mRNA was established (XM_001133467). PKD1L3 protein is constituted by eleven TMs, a short intracellular C-terminal tail, and a huge extracellular N-terminus, containing a C-type Lectin domain and GAIN domain (A. Li et al., 2003). PKD domain and REJ domain are not found in human PKD1L3, while mouse PKD1L3 protein contains two copies of the PKD domain. The “HL*T” GPS cleavage site is conserved in PKD1L3 is conserved cleaved NTF and CTF of PKD1L3 were reported (Kashyap et al., 2019). The PLAT domain of PKD1L3 locates in the intracellular loop between S1-S2 helices and the last 6-TMs is the TRP-like domain. Like PLD1L2, the coiled-coil domain cannot be identified in the PKD1L3 intracellular C-terminal tail.

Two independent studies report that PKD1L3 expresses and co-localizes with PCL in mice taste cell, and may be involved in mammalian sour taste sensation (Ishimaru et al., 2006; LopezJimenez et al., 2006). Further studies show that PKD1L3 and PCL can form a protein complex, associating with each other at their extracellular TOP domain and coiled-coil domain in intracellular C-terminal tails (Salehi-Najafabadi et al., 2017; Y. Yu et al., 2012). The PKD1L3/PCL ion channel complex has a 1 PKD1L3: 3 PCL stoichiometry and the PKD1L3 subunit is directly involved in forming the ion channel pore (Y. Yu et al., 2012). Function studies show that PKD1L3/PCL complex is a cation channel and expressing PKD1L3/PCL complex gives rise to an acid off-response current, which is present after acid solution treatment followed by neutralization (Inada et al., 2008; Y. Yu et al., 2012). The evidences above indicate that the PKD1L3/PCL ion channel complex may be a candidate of
sour taste receptor. However, this hypothesis has met some challenges. First, it is hard to explain the relationship between acid off-response to sour taste sensation. Second, the splice variant of PKD1L3 in mice taste circumvallate papillae does not undergo GPS cleavage, which is crucial for the trafficking of PKD1L3/PCL complex, indicating non-cleavable PKD1L3 may have other potential function in vivo (Kashyap et al., 2019; LopezJimenez et al., 2006). Lastly, sour taste response still exists in PKD1L3 or PCL knockout mutant mice (Horio et al., 2011). Therefore, more effects are required to demonstrate the role of PKD1L3/PCL complex in sour sensation.

3.5. **PKDREJ**

Human polycystic kidney disease and receptor for egg jelly (PKDREJ) protein contains 2,253 amino acids and is specifically expressed in testis (Hughes, Ward, Aspinwall, Butler, & Harris, 1999). Human **PKDREJ** gene locates in chromosome 22q13.31 and contains only 1 exon. One mRNA was established (NM_006071). PKDREJ protein is constituted by eleven TMs, a short intracellular C-terminal tail, and a huge extracellular N-terminus, containing the REJ domain (Hughes et al., 1999). The PLAT domain of PKDREJ locates in the intracellular loop between S1-S2 helices and the last 6-TM is the TRP-like domain. Like PDK1L1, the GPS cleavage site of PKDREJ is not conserved with other PKD proteins. The specific expression in the male germ cell of PKDREJ suggests a receptor function similar to suREJ. Some data show that the fertilizing timing becomes significantly longer in PKDREJ knockout mice, supporting that PKDREJ plays a role in mammalian fertilization (Sutton, Jungnickel, & Florman, 2008). Furthermore, the TRP-like domain in PKDREJ, which is not existent in suREJ, provide a possibility that PKDREJ can associate with TRPP protein and form a protein complex to combine the receptor and ion channel role together during mammalian fertilization. It has been found that TRPP5 also specifically
expressed in testis, while PKDREJ can associate with PC2 and TRPP3, but not with TRPP5, which drives more questions about the potential candidates of PKDREJ’s binding partners (Sutton et al., 2006).

4. Polycystin-1/Polycystin-2 (PC1/PC2) complex

Polycystin-1 proteins and Polycystin-2 proteins form receptor/ion channel protein complexes, which are localized on the primary cilia of renal epithelial cells and are essential for renal tubular differentiation and other potential diverse roles in sensory systems (Semmo et al., 2014; Zhou, 2009).

4.1. The assembly of the PC1/PC2 complex

The assembly of the PC2 and PC1 occurs at multiple sites, including the N-termini, TOP domain, TMs, and coiled-coil domain in C-termini. It has been found that the PC2 coiled-coil domain tends to form a trimer and bind with one copy of the coiled-coil domain of PC1 (Y. Yu et al., 2009). The disruption of PC2 coiled-coil domain trimer formation will abolish its association with that of PC1 (Y. Yu et al., 2009). Another study shows that the TOP domains of PC2 and PC1 associate with each other, indicating the TOP domain association is involved in the assembly of the PC1/PC2 complex (Salehi-Najafabadi et al., 2017). In addition, single molecular photo bleaching experiment shows that each PC1/PC2 complex contains 1 PC1 subunit and 3 PC2 subunits, showing the 1:3 stoichiometry of PC1/PC2 complex (Y. Yu et al., 2009).

4.2. The structure of the PC1/PC2 complex

The structure of the PC1/PC2 complex TM core was solved by cryo-EM recently, which has greatly enhanced our understanding of the PC1/PC2 complex (Fig. 2D) (Q. Su, F. Hu, X. Ge, et al., 2018). Compared to the homotetramer of PC2, in the PC1/PC2 cryo-EM structure, one PC1 CTF replaces one of four PC2 subunit and forms the complex with the
other three PC2 subunits. The TRP-like domain of PC1, which structure is very similar to that of the PC2 subunit, associates with the other three PC2 subunits through the TOP domain and TM association, forming the ion channel core. The first five TMs of PC1 stays on the side of the channel core, and the first TM helix (S1) locates in the middle between other four TM (S2-S5) and S6-S11, and the loop between S1 and S2, which is the PLAT domain, is localized in the intracellular side and solved in the structure. This structure revealed a lot of information about how PC1 assembles with PC2, although a few questions still exist. First, the N- and C- termini of PC1 and PC2 were truncated in order to be utilized in this study. Hence, the structure information of N- and C- termini of the PC1/PC2 complex is missing. Second, in this structure, there is a gap around 15 degrees deviating from the expected symmetric position between the PC1 subunit and one of the PC2 subunits. There is no evidence of whether this naturally happens in complex assembly or it happened due to the lack of N- and C- termini.

With more study on the Ca\textsubscript{v} complex and PC1/PC2 complex, more linkage and similarity has arisen between these two Ca permeable ion channel complexes. Besides of the \(\alpha1\) subunit of Ca\textsubscript{v} as a functional ion channel core, Ca\textsubscript{v} complex also includes a large extracellular \(\alpha2\) subunit, an intracellular \(\beta\) subunit, a \(\gamma\) subunit containing 4 TM helices, and a \(\delta\) subunit as a transmembrane helix which links \(\alpha1\) and \(\alpha2\) subunits (see details in 1.1.1.3). PC1-CTF, containing 11 TM helices (6 + 1 + 4), is like a combination of one pore-forming \(\alpha1\) homologous domain and the \(\beta\), \(\gamma\), \(\delta\) subunits in Ca\textsubscript{v} (Fig. 2D). Including the huge external PC1-NTF, the PC1/PC2 complex contains all the structural elements that exist in a Ca\textsubscript{v} complex. This clue indicates the possibility that PC1/PC2 complex and the Ca\textsubscript{v} complex share some similarity in subunit-elements coordinated conformation change and ion conducting mechanism, which is conserved in ion channels mediating delicate Ca\textsuperscript{2+} signal. At the same time, the differences in their complex assembly may indicate the specific
requirement of biological function including the gating process and \( \text{Ca}^{2+} \) signal regulation in different cell types or tissues.

### 4.3. The function of the PC1/PC2 complex in ADPKD

The molecular mechanism of the function of the PC1/PC2 complex in ADPKD is largely unknown. One hypothesis is that PC1/PC2 complex senses the shear stress generated by fluid flow in renal tubules to raise the cilium calcium, and activates the downstream signal pathway in the renal cell (Zhou, 2009). Several findings support this hypothesis. First, the flow-induced intracellular \( \text{Ca}^{2+} \) elevation observed in the renal epithelial cell requires both PC1 and PC2 located in primary cilia and is mediated by ryanodine receptors (Nauli et al., 2003). Second, PC2 channels in vascular endothelial cells contribute to flow-mediated vasodilation, indicating that the PC1/PC2 complex has similar shear stress sensing ability (MacKay et al., 2020). However, direct \( \text{Ca}^{2+} \) current could not be recorded from the PC1/PC2 complex and the role of \( \text{Ca}^{2+} \) elevation in cysts formation is still unclear, limiting this hypothesis.

Another hypothesis is that PC1 might function as GPCRs, raising intracellular cyclic adenosine monophosphate (cAMP) level and activating the cAMP dependent signal pathway. PC1 share structure similarity with G-protein receptors. Meanwhile, it is reported that PC1 functions as a constitutive activator of \( \text{G}_{\text{lo}} \)-type G-protein expressing in neurons, indicating PC1 plays the same role in renal epithelial cells (Delmas et al., 2004). The activity of adenylyl cyclase (AC), an enzyme catalyzing ATP to cAMP, is inhibited by the \( \text{G}_{i} \) protein signal pathway (Gilman, 1987). On the other hand, the high cAMP level was detected in ADPKD patients and some PKD mice models, matching the suggestion that the disfunction of PC1 leads to continuous activation of AC function and rise of the cAMP levels (Gattone, Wang, Harris, & Torres, 2003; Starremans et al., 2008). Also, the cAMP-dependent cell
proliferation happens in the renal cell from ADPKD patients, but not from the normal human kidney cortex (Gattone et al., 2003). In addition, cAMP-reducing treatment has a significant rescue effect in the PKD mice model (Torres et al., 2004). These results indicate that cAMP plays a critical role in ADPKD progression and it may be mediated by PC1-induced G-protein signaling. Nevertheless, no direct evidence shows that PC1-induced G-protein activation in renal epithelial cells increases the cell cAMP level. Also, how elevated cAMP level leads to cysts formation is still unknown. Furthermore, the role of PC2 in ADPKD in this hypothesis is elusive, which leave more blank to be filled in the future study.

4.4. The challenges in PC1/PC2 study

Although mutations in PC1 and PC2 have been linked to ADPKD for more than two decades, many challenges are present in the study of PC1/PC2 complex and the role of PC1 and PC2 in ADPKD pathogenesis. The major difficulty in the PC1/PC2 study is the lack of the knowledge on the activation mechanism of the PC2 homomeric channel and the PC1/PC2 complex channel. It has been very difficult to record currents from these channels, which has greatly delayed the study on their ion channel properties and their roles in ADPKD. Therefore, controversy still exists between many hypotheses and theories describing the function of the PC1/PC2 complex and its role in ADPKD. A common agreement among those theories is that PC2 plays an ion channel role in the complex. Concerning PC1, many ideas conflict with one another. Some investigators believe that PC2 subunits are enough for ion channel function and the PC1 subunit only works as a receptor, while other investigators consider that the PC1 subunit is directly involved in channel pore-formation.

There are several lines of evidence suggesting that PC1 functions as a channel pore-forming subunit in the PC1/PC2 complex. First, the last six transmembrane domains (S6-S11) of PC1 share sequence homology with TRPP channels (Fig. 3A) (Consortium, 1995;
Hughes et al., 1995). Second, our biochemical and biophysical studies found that the
PC1/PC2 complex contains one PC1 and three PC2 subunits (Y. Yu et al., 2009; Zhu et al.,
2011). Thus, in this complex, PC1 may take the position of the fourth PC2 subunit and form
the channel pore with the other three PC2 subunits. Third, a previous study showed that
another PKD/TRPP complex, PKD1L3/TRPP3, share the same 1:3 subunit stoichiometry and
that the single PKD1L3 subunit functions as a channel-forming component (Y. Yu et al.,
2012). PC1 may have a similar channel role in its complex with PC2. Fourth, the recently
published cryo-EM structure of a transmembrane fraction of the human PC1/PC2 complex
confirmed that this complex indeed has 1 (PC1): 3 (PC2) stoichiometry (Q. Su, F. Hu, X. Ge,
et al., 2018). More importantly, it shows that the last six transmembrane domains of the
single PC1 subunit assemble with three PC2 subunits into a channel-like complex. This
complex shares overall structural similarity to the homo-tetrameric PC2 channel, with the last
two transmembrane domains (S10-S11) of PC1 directly participating in forming the

Despite evidence indicating that PC1 functions as an ion channel subunit in the
PC1/PC2 complex, a solid conclusion could not be made since functional evidence is lacking.
This is mainly due to the lack of knowledge on the channel activation mechanism, making
functional analysis very challenging. To resolve this, we recently developed gain-of-function
(GOF) mutants of the PC2 channel, including the reported F604P mutant (Arif Pavel et al.,
2016). In the current study, taking advantage of another GOF PC2 mutant, we were able to
reliably record channel activity of PC1/PC2, study its ion permeability, and, for the first time,
dissect the function of PC1 in this channel. We further explored the core channel unit in PC1
and how GPS cleavage affects PC1/PC2 channel activity. This study sheds light on the
molecular mechanism of the PC1/PC2 function and ADPKD pathogenesis.
RESULT

Oocyte-expressed PC1 and PC2 retain their native characteristics.

To investigate the function of PC1/PC2, we coexpressed PC1 and PC2 proteins in *Xenopus* oocytes and evaluated their expression. Both proteins expressed very well as shown by Western blot (Fig. 3B, left images), and two important features were noted. First, oocyte-expressed PC1 was cleaved at the N-terminal GPS site, recapitulating *in vivo* and mammalian cell expression results (Qian et al., 2002; S. Q. Yu et al., 2007). When detected with an antibody recognizing the PC1 C-terminus, a full-length band (above the 250 kDa marker) and a cleaved CTF band (~130 kDa) of PC1 were detected (Fig. 3B, left top image), matching the pattern of PC1 cleaved at the GPS site (Qian et al., 2002). As observed *in vivo* (S. Q. Yu et al., 2007), the majority of oocyte-expressed PC1 was cleaved, since a greater amount of cleaved CTF than full-length protein was detected (Fig. 3B). Second, an additional PC2 band was identified at 130 kDa when PC1 was coexpressed, contrasting the single band at ~120 kDa when PC1 was absent (Fig. 3B, left middle image). It has been previously found that the 130 kDa PC2 is a higher-glycosylated (EndoH resistant) form of PC2 which stays in complex with PC1 in cilia of native tissues, while the 120 kDa PC2 has less and EndoH-sensitive glycosylation (H. Kim et al., 2014). Thus, both PC1 and PC2 retain their native features after being expressed in *Xenopus* oocytes, validating the feasibility of studying the function of PC1/PC2 complex in this system. Cell surface biotinylation experiment reveals that the PC1/PC2 complex traffics to the plasma membrane in oocytes (Fig. 3B). In the co-immunoprecipitation (co-IP) experiment, the association between PC1 and both forms of PC2 was detected (Fig. 3C). Proteins in our surface biotinylation or co-IP samples sometimes ran at a higher molecular weight than the same proteins in lysate samples, which is most likely
due to the high concentration of salt used in elution (we used 1.5x SDS loading buffer to elute).

Although the PC1/PC2 complex trafficked to the cell surface in oocytes, coexpression of the two proteins did not lead to a measurable current in recording with two-electrode voltage clamp (TEVC), while the previously described GOF PC2 mutant, PC2_F604P (Arif Pavel et al., 2016; W. Zheng et al., 2018), gave rise to robust currents in oocytes (Fig. 3D). This result suggests that the wild type (WT) complex channel stays in a closed state within the oocyte plasma membrane.

We hypothesized that coexpression of PC1 with PC2_F604 may lead to an open PC1/PC2_F604P complex channel. However, the coexpression of PC1 greatly reduced the current (Fig. 4). The current reduction may be due to lower activity of the complex channel formed by PC1 and PC2_F604P. A similar dominant negative effect was previously seen when coexpressing WT PC2 with PC2_F604P (Arif Pavel et al., 2016), which is likely due to that the presence of WT subunits in the pore obstructs the F604P-induced gating process. The effect of PC1 on complex channel activity suggests that association of the PC1 subunit also changes the channel pore of PC2_F604P. Since the much lower channel activity of PC1/PC2_F604P renders it unsuitable for studying channel function, we generated new GOF PC2 mutants intending to find one that leads to opening of the PC1/PC2 channel.
Figure 3. PC1 and PC2 express in *Xenopus* oocytes but yield no channel current.

A Transmembrane topology of PC1 and PC2 proteins. The two proteins associate at the C-terminus through the coiled-coil domains and the extracellular side via the TOP domains. The GAIN domain and the GPS site in PC1 and the EF-hand motif in PC2 are indicated. The last six transmembrane domains of PC1 (shown in orange) share sequence similarity with PC2.

B Western blot of oocyte lysate (left) and biotinylation-purified surface (right) samples showing the expression of PC1 and PC2 in *Xenopus* oocytes and enhanced surface trafficking of the PC1/PC2 complex compared to either protein expressed separately. Anti-PC1 C-terminus antibody (S. Q. Yu et al., 2007) recognized both full-length (asterisk) and GPS-
cleaved CTF (open circle) of PC1. A higher-glycosylated 130 kDa PC2 (star) band was only seen when PC1 is coexpressed.

C Co-IP followed by Western blot showing the association between PC1 and PC2 that were expressed in Xenopus oocytes. IP was done with an anti-FLAG antibody. Bands of full-length (asterisk) and GPS-cleaved CTF (open circle) of PC1 are indicated. Both 120 and 130 kDa bands of PC2 were seen in the IPed product.

D Representative current-voltage relationship (I-V) curves (left) and a scatter plot and bar graph (right) showing coexpression of WT PC1 and PC2 produced no current in TEVC recording. The current of the GOF PC2_F604P is included as a control. Currents at +60 mV are shown in the bar graph. Each point represents the recorded current from one oocyte. Oocyte numbers for scatter plot and bar graph are indicated in parentheses. Data are presented as mean ± SD (***P < 0.001, Student’s t-test).
Figure 4. Coexpression of PC1 greatly inhibited the current of PC2_F604P.

**Left:** representative current-voltage (I-V) curves of the indicated protein combinations.

**Right:** scatter plot and bar graph show the average currents at +60 mV. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (n.s.: not significant, *P < 0.05, ***P < 0.001, Student’s t-test).
Mutation at the lower gate generates a new GOF PC2 channel.

To generate new GOF mutants, we focused on the amino acids forming the lower gate of PC2. Structural study by cryo-EM and our recent functional study (W. Zheng et al., 2018) show that L677 and N681 are either physical or functional lower gate residues (Fig. 5A). Mutating both amino acids to alanine (L677A/N681A, named “AA”) produced significant currents in a bath solution containing 100 mM Na$^+$ and 2 mM Ca$^{2+}$ when the mutant channel was expressed in oocyte (Fig. 5B). The current size of PC2_AA is roughly double the size of that of PC2_F604P. Just like that of PC2_F604P (Arif Pavel et al., 2016), the current of PC2_AA is outwardly-rectifying. The inward current, presumably carried by Na$^+$, is inhibited when 2 mM extracellular Ca$^{2+}$ is present (compare the I-V curves in Fig. 5B and C). This suggests that at negative voltages, but to much less extents at depolarized voltages, extracellular Ca$^{2+}$ is attracted to the pore, which blocks Na$^+$ entry, a common phenomenon in cation channels. The majority of intracellular cations in oocytes are K$^+$ (Costa, Emilio, Fernandes, Ferreira, & Ferreira, 1989), thus the outward current of PC2_AA channel is mainly carried by K$^+$ efflux.

We further tested the channel’s Ca$^{2+}$ permeability by replacing all cations in the bath solution with 70 mM Ca$^{2+}$. In this solution, the PC2_F604P current was almost completely inhibited, indicating it has no or a very low Ca$^{2+}$ permeability that is not detectable with this method. However, PC2_AA gave rise to robust currents in this solution (Fig. 5D). The current-voltage relationship (I-V) curve of this current has an unusual trough at strong hyperpolarization, due to slow development of the currents upon application of the first strong negative voltage (Fig. 6). We postulate that the currents of PC2_AA in 70 mM Ca$^{2+}$ are combined PC2 current with Cl$^-$ currents conducted by endogenous calcium-activated chloride channels (CaCC) in oocytes (Barish, 1983; Caputo et al., 2008; Miledi, 1982;
Schroeder, Cheng, Jan, & Jan, 2008; Yang et al., 2008). CaCC can be activated by Ca$^{2+}$ influx and has been used as an amplifying system to monitor Ca$^{2+}$ influx such as store-operated Ca$^{2+}$ entry (SOCE) (Machaca & Hartzell, 1999). Indeed, including chloride channel blockers, niflumic acid (NFA) or N-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid (MONNA) (Oh et al., 2013; Wozniak, Phelps, Tembo, Lee, & Carlson, 2018), in the bath solution, dramatically reduced the current of PC2_AA in 70 mM Ca$^{2+}$ (Fig. 5E). These results confirm that a large portion of the currents in 70 mM Ca$^{2+}$ solution is conducted by CaCC. Since the CaCC current was not seen in WT and PC2_F604P-injected oocytes (Fig. 5D), the elevated CaCC activity in PC2_AA-injected oocytes is not produced by higher expression of the CaCC channel stimulated by PC2 expression. Thus, the results imply that PC2_AA is Ca$^{2+}$-permeable and that Ca$^{2+}$ influx through these channels led to CaCC activation. Our data suggest that the CaCC current can be used as a readout for measuring the relatively small Ca$^{2+}$ conductance of PC2 in Xenopus oocytes, which we have applied in the following experiments.
Figure 5. PC2_L677A/N681A (AA) mutant is a new GOF channel.

A Cryo-EM structure of PC2. Left: bottom view of PC2 homomeric tetramer showing the pore-lining S5-S6 from each subunit assemble into the channel pore. Right: side view of S5-S6 from two subunits showing the L677 and N681 (in red) contribute to the restriction at the lower gate. F604P (in cyan) on S5 and the three residues (in green) forming the selectivity filter are also indicated. (PDB code 5T4D) (P. S. Shen et al., 2016). A grey dashed line indicates the path of the ion flow.

B Representative I-V curves (left) and a bar graph (right) showing that the PC2_AA is a GOF mutant and gave rise to a larger current than PC2_F604P. Scatter plot and bar graph shows the average current sizes at +60 mV. The cations included in the bath solution, 100 mM Na+ and 2mM Ca^{2+} in this case, are indicated by the thick-lined boxes here and in all the following figures. Oocyte numbers for bar graph are indicated in parentheses. Data are presented as mean ± SD in bar graph (***P < 0.001, Student’s t-test).

C Representative currents of PC2_F604P and PC2_AA mutants in the divalent ion-free bath solution, which contains 100 mM Na+.
**D** Representative currents of indicated WT and mutants of PC2 in a bath solution containing 70 mM Ca\(^{2+}\).

**E** Calcium-activated chloride channel (CaCC) blocker MONNA (10 μM) or niflumic acid (NFA) (1 mM) partially blocked the current recorded from the PC2_AA-injected oocytes in the 70 mM Ca\(^{2+}\) bath solution.
Figure 6. The characteristic current of PC2_AA mutant in a bath solution containing 70 mM Ca$^{2+}$, showing how the trough of the I-V curve in strong negative voltage is generated.

A A typical voltage clamp protocol used for recording. The first several tested voltages, from -80 mV to -50 mV, were marked in colors.

B The corresponding currents of the PC2_AA channel. Colored traces indicated the currents at indicated voltages. Current at the first negative voltage application (-80 mV) is significantly smaller than that in the following several voltages. This result may be caused by the time delay between the start of the Ca$^{2+}$ influx and the activation of Ca-induced chloride channel. The portions of currents between the dashed lines were averaged for generating the I-V curve.

C I-V curve generated from the recording result. Thus, the channel seems to be slowly activated by hyperpolarizations, and data at -40 to -70 mV were acquired when channel is
more activated than at -80mV. To avoid it, we tried to record with longer protocol to increase holding time at -80 mV. However, it only led to slow development of larger currents (may be due to the increasing of the Cl⁻ channels activated by Ca²⁺ influx) without changing the final shape of I-V curve.
**PC1/PC2_AA is a GOF PC1/PC2 complex channel.**

We then coexpressed PC2_AA with full-length PC1 in *Xenopus* oocytes to determine whether they will form a GOF complex channel. The injected cRNA molar ratio of PC1 to PC2_AA was 1.5:1 to minimize the homomeric PC2_AA channel formation. Considering the 1:3 ratio of PC1 to PC2 in PC1/PC2 complex, the PC1 RNA we injected is 4.5 folds oversaturated. A later experiment confirmed that in this experimental condition, all detected PC2 are in the complex with PC1 (shown in Fig. 18D). In divalent ion-free solution, oocytes expressing both PC1 and PC2_AA gave rise to similar I-V curves, in terms of both the voltage dependence and current magnitude, to those expressing PC2_AA alone (Fig. 7A). The only difference we noticed is that the reversal potential (RP) was slightly shifted toward a positive voltage. However, when 2 mM Ca\(^{2+}\) was included in the bath solution, compared to the outwardly-rectifying current of PC2_AA, the current of PC1/PC2_AA shows almost no rectification, indicating that the PC1/PC2_AA complex has much less or no extracellular Ca\(^{2+}\) blocking (Fig. 7B). The inward current of PC1/PC2_AA is carried by Na\(^{+}\) influx and has no detectable CaCC component since adding MONNA led to no change of the current (Fig. 7C). Similar to Ca\(^{2+}\), 2 mM Mg\(^{2+}\) also abolished the inward current of the PC2-AA channel but not of the PC1/PC2-AA channel (Fig. 7D and E).

In 70 mM Ca\(^{2+}\) solution, the PC1/PC2-AA-expressing oocytes gave more than 3 times larger currents than that of the PC2-AA-expressing oocytes, although the same amount of PC2_AA RNA was injected in both groups (Fig. 7F). Similar to the PC2_AA current, addition of MONNA partially and significantly blocked the current of PC1/PC2_AA in 70 mM Ca\(^{2+}\) solution, indicating the current was conducted by both PC1/PC2_AA and CaCC (Fig. 7G). The latter was activated by Ca\(^{2+}\) entry through the PC1/PC2_AA channel. Compared to PC2-AA, PC1/PC2_AA produced a similar sized current in Na\(^{+}\) solution (Fig. 7H).
7A), but much larger current in 70 mM Ca\(^{2+}\) solution (Fig. 7F), indicating that PC1/PC2_AA channel has greater Ca\(^{2+}\): Na\(^{+}\) permeability ratio (P\(_{Ca}/P_{Na}\)) than PC2_AA channel. To further prove it, we compared the inward currents of PC2 and PC1/PC2 in solutions containing varying Ca\(^{2+}\) concentrations, in the presence of 20 μM of MONNA. No Na\(^{+}\) was included in these solutions, and the osmolarity was compensated by adding NMDG\(^{+}\), which is not permeable through these channels. In this condition, most of the inward currents should be carried by Ca\(^{2+}\) influx, although we could not rule out the presence of residual chloride current since MONNA may not completely inhibit all CaCC. The results show that decent amount of inward currents can be recorded from the PC1/PC2_AA channel and the reversal potential shifted to right when Ca\(^{2+}\) concentration increased in the solution, indicating Ca\(^{2+}\) permeability (Fig. 8). In contrast, almost no inward current was recorded from PC2_AA in all solutions, indicating a much smaller Ca\(^{2+}\) permeability of this channel (Fig. 8). Also, we noticed that increasing extracellular Ca\(^{2+}\) concentration also led to more blocking of outward current, which is carried mainly by potassium efflux, of PC2_AA, but not PC1/PC2_AA (Fig. 8). This is consistent with the fact that extracellular Ca\(^{2+}\) blocks more of the PC2_AA channel. Here although we have observed difference on Ca\(^{2+}\) influx between the two channels, we were not able to calculate the relative Ca\(^{2+}\) permeability since the apparent reversal potentials are largely affected by leak currents when channel’s inward currents are so small, especially in the case of PC2_AA.
Figure 7. The PC1/PC2_AA complex channel has more Ca$^{2+}$ permeability than the homomeric PC2_AA channel.

A, B, F Representative I-V curves (left) and scatter plot and bar graphs (right) showing the currents from oocytes expressing PC2_AA alone, PC1 with PC2_AA, and PC1 with WT PC2, in bath solutions containing 100 mM Na$^+$ (A), 100 mM Na$^+$ and 2 mM Ca$^{2+}$ (B), and 70 mM Ca$^{2+}$ (F). Scatter plot and bar graphs show currents at +80 mV and -80mV. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (n.s.: not significant, ***P < 0.001, Student’s t-test).
D, E Representative I-V curves of PC2_AA (D) and PC1/PC2_AA (E) channels in bath solutions containing the indicated ions (in mM), showing both 2 mM Ca\(^{2+}\) and 2 mM Mg\(^{2+}\) inhibit the inward current of PC2_AA channel, but not that of PC1/PC2_AA channel. Also, 2 mM Mg\(^{2+}\) inhibits more on the outward current of PC2_AA than 2 mM Ca\(^{2+}\).

C, G Representative I-V curves of PC1/PC2_AA recorded in a bath solution containing 100 mM Na\(^{+}\) and 2 mM Ca\(^{2+}\) (C) or 70 mM Ca\(^{2+}\) (G) solution in the absence or presence of 10 μM MONNA.
Figure 8. Representative I-V curves of PC2_AA and PC1/PC2_AA in solutions with varying Ca\textsuperscript{2+} concentration.

A Representative current of PC2_AA in solutions containing the indicated Ca\textsuperscript{2+} concentrations (left) and the currents of the full voltage scale (right) and the details of solutions used.
Representative currents of PC1/PC2-AA in solutions containing the indicated Ca^{2+} concentrations (left) and the currents of the full voltage scale (right) and the details of solutions used. Components of these solutions are shown in the table. 20 μM of MONNA was included in all solutions and the osmolarity was compensated by adding corresponding concentrations of NMDG^+, which is not permeable through these channels.
Since these results were obtained from using a GOF PC1/PC2 channel, we further confirmed the Ca\(^{2+}\) permeability of the WT PC1/PC2 channel by \(^{45}\)Ca radiotracer uptake experiments (Y. Yu et al., 2012) in oocytes expressing PC1 alone, PC2 alone, or both PC1 and PC2. Due to the high sensitivity of this method, we were able to trace the small Ca\(^{2+}\) influx through the WT channels even with their low open probability. Five times more PC2 cRNA was injected for the PC2 alone condition to boost the surface expression of PC2 homomeric channel. The results showed that the \(^{45}\)Ca uptake rate of the PC1 or PC2-alone-injected oocytes was only slightly higher than H\(_2\)O-injected oocytes, while that of oocytes expressing PC1/PC2 was 6.72±3.25 fold of that of the PC2 alone-expressing oocytes after background subtraction (Fig. 9A). Under the designed experimental conditions, the total amount of surface expressed PC2 in the PC1/PC2 sample is about 1.5 folds of that in the PC2 alone sample (Fig. 9B, measured with ImageJ). Therefore, our data from this experiment are in strong support that WT PC1/PC2 has higher Ca\(^{2+}\) permeability than PC2 channel. We noticed that expressing the channel proteins usually depolarize the membrane potential of oocytes to more positive than -25 mV. The driving force for cation influx will be bigger under more negative physiological membrane potential. Thus, the Ca\(^{2+}\) permeability difference between PC1/PC2 and PC2 channels under physiological conditions can be greater than what we observed in the \(^{45}\)Ca uptake experiment.
Figure 9. Wild-type PC1/PC2 complex has higher Ca\textsuperscript{2+} permeability than that of the PC2 homomeric channel

A Scatter plot and bar graph showing the higher radiolabeled \textsuperscript{45}Ca uptake rate of oocytes expressing the PC1/PC2_AA complex channel compared to that of oocytes expressing PC1 alone or PC2 alone. For oocytes injected with PC2 alone, five times more concentrated PC2 RNA was injected to increase its surface expression. The purple dashed line indicates the background \textsuperscript{45}Ca uptake set by the measurement with the water-injected oocytes. Data were averaged from three independent experiments. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (***P < 0.001, Student’s t-test).

B Surface biotinylation followed by Western blot showing the expression levels of the indicated proteins in lysate and plasma membrane of oocytes in \textsuperscript{45}Ca uptake experiments.
Both the CTF and the last six transmembrane domains are sufficient for ion channel function of PC1 in the PC1/PC2 complex.

Cleavage at the GPS site breaks down PC1 into an NTF (G24-L3040) and a CTF (T3041-T4293), and the latter contains all 11 transmembrane domains and the intracellular C-terminal tail (Fig. 10A) (Qian et al., 2002). A previous study reported a significant amount of NTF detaches from the CTF in vivo, indicating that each isolated fragment may function independently in this case (Kurbegovic et al., 2014). We tested whether PC1-CTF itself is sufficient for forming a channel complex with PC2 by coexpressing the PC1-CTF with PC2_AA in Xenopus oocytes. An Ig k-chain leader sequence was fused to the N-terminus of PC1-CTF to prompt its trafficking to plasma membrane. In solution containing 100 mM Na\(^+\), PC1-CTF/PC2_AA-injected oocytes yielded almost the same current as that of the full-length PC1/PC2_AA channel (Fig. 10B). Likewise, extracellular 2 mM Ca\(^{2+}\) did not block the inward current of PC1-CTF/PC2_AA, similar to that of the full-length channel (Fig. 10C). Furthermore, its current in solution containing 70 mM Ca\(^{2+}\) is also similar to that of the full-length channel (Fig. 10D). These results indicate that the channel function of the PC1-CTF/PC2_AA complex resembles that of full-length PC1/PC2_AA. Therefore, the CTF is sufficient for forming a functional channel with PC2 (shown with PC2_AA here).

Coexpressing PC1-CTF with WT PC2, or introducing a mutation in the putative pore region of PC1-CTF, R4090W, completely abolished the current of PC1-CTF/PC2_AA (Fig. 10E), which strongly supports the role of PC1-CTF in conducting currents together with PC2_AA and rule out the likelihood that currents be mediated by induced endogenous channels.

We then sought the PC1 core channel functional unit by further shortening the fragment. The similarity between PC2 and the S6-S11 of PC1 suggests a potential ion channel role of the latter. Indeed, the cryo-EM structure of PC1/PC2 indicates that the PC1
S6-S11 assembles with three PC2 proteins into a TRP channel-like structure (Q. Su, F. Hu, X. Ge, et al., 2018). Interestingly, a natural PC1 cleavage product, P100, has been identified and likely contains S6-S11 and the intracellular C-terminal tail (Woodward et al., 2010), suggesting a physiological role of this part of PC1, which we named PC1 TRP-Like Domain (TLD) here. We tested the channel function of PC1-TLD by generating a construct including the fragment from G3592 to T4293 of PC1, which is composed of the loop between S5 and S6, the transmembrane domains S6 to S11, and the intracellular C-terminal tail (Fig. 11A). Just like in the case of the PKD-CTF, we fused an Ig k-chain leader sequence to the N-terminus of PC1-TLD. The results show that coexpressing PC1-TLD with PC2_AA produced a functional channel. Although the currents of PC1-TLD/PC2 AA are much smaller, it resembles the current of the full-length channel in all tested solutions (Fig. 11B-D). PC1-TLD/PC2 AA channel is not blocked by 2 mM Ca^{2+} (Fig. 11B). In the 70 mM Ca^{2+} solution, the trough at strong hyperpolarization of the current of PC1-TLD/PC2 AA (Fig. 11D) and the inhibition of the current by MONNA (Fig. 11E) shows the activation of CaCC, indicating the Ca^{2+} permeability of this channel.
Figure 10. PC1-CTF is sufficient for ion channel function of PC1 in the PC1/PC2 complex.

A Transmembrane topology of PC1 and PC2 proteins, showing the NTF, CTF, and the TLD of PC1. The six transmembrane domains in TLD shares sequence homology with the transmembrane domains of PC2.

B, C, D Representative I-V curves (left) and scatter plot and bar graphs (right) showing the comparison between the currents of full-length PC1/PC2 with that of PC1-CTF/PC2_AA in bath solutions containing 100 mM Na\(^+\) (B), 100 mM Na\(^+\) and 2 mM Ca\(^{2+}\) (C), or 70 mM Ca\(^{2+}\) (D). Currents at both +80 mV and -80 mV are displayed in the scatter plot and bar graphs.
Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (n.s.: not significant, *P < 0.05; ***P < 0.001 Student’s t-test).

E Scatter plot and bar graph showing that the current of PC1-CTF/PC2_AA is completely abolished when WT PC2 was used, or after introducing R4090W mutation in the putative pore region of PC1-CTF. Inserted are Western blot images showing the expression of the corresponding proteins. Top: anti-PC1. Bottom: anti-PC2. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (***P < 0.001, Student’s t-test).
Figure 11. TRP-Like domain (TLD) is sufficient for ion channel function of PC1 in the PC1/PC2 complex.

A Transmembrane topology of PC1 and PC2 proteins, showing the NTF, CTF, and the TLD of PC1. The six transmembrane domains in TLD shares sequence homology with the transmembrane domains of PC2.

B, C, D Representative I-V curves of oocytes injected with the indicated RNAs in a bath solution containing 100 mM Na\(^+\) and 2 mM Ca\(^{2+}\) (B), 100 mM Na\(^+\) (C), or 70 mM Ca\(^{2+}\) (D),
showing PC1-TLD/PC2_AA gave rise to current with similar properties as full-length PC1/PC2_AA channel.

E MONNA inhibits the current of PC1-TLD/PC2_AA in 70 mM Ca^{2+}
Consistently, co-IP and surface biotinylation experiments showed that, as full-length PC1, PC1-CTF and PC1-TLD can associate with PC2_AA, and the resulting complexes traffic to the plasma membrane (Fig. 12). Thus, our data shows that PC1-CTF and even PC1-TLD are sufficient for ion channel function of PC1 in the PC1/PC2 complex. For example, in most of our experiences, we have noticed less surface PC2_AA when it was coexpressed with PC1-LTD than it was with the other two longer PC1 constructs (Fig. 12B). Thus, beside of the possibility that the S1-S5 transmembrane domain facilitate PC1 channel activity, the reduction of channel current of PC1-LTD/PC2AA may be caused by partial defect in channel folding or trafficking without S1-S5 of PC1. Further study is needed to investigate the roles of S1-S5 in the function of PC1. As negative controls, expressing PC1, PC1-CTF, or PC1-TLD alone did not give rise to significant current in solution containing 100 mM Na\(^+\) and 2 mM Ca\(^{2+}\) (Fig. 13). We occasionally noticed that expression of PC1-CTF alone, but not the full-length PC1 or PKD2-TLD, led to tiny whole-oocyte current which is much smaller than that when PC2_AA was coexpressed (Fig. 13). It will be interesting to figure out whether this current is from complex formed by PC1-CTF with endogenously expressed TRPP or other proteins.
Figure 12. The association and surface expression of complexes formed by PC2 with either full-length PC1, PC1-CTF, or PC1-TLD followed by Western blot.

A Co-IP followed by Western blot showing the association between PC2_AA and the indicated full-length or fragments of PC1. Bands of full-length (asterisk), GPS-cleaved CTF or expressed CTF fragment (open circle), and TLD (star) of PC1 are indicated.

B Surface biotinylation followed by Western blot showing the expression of the complexes formed between PC2_AA and full-length PC1, PC1-CTF, or PC1-TLD at the oocyte surface. Surface samples were also blotted with an anti-PC1 N-terminus antibody 7e12 and the cleaved NTF fragment was found associated to plasma membrane in full-length sample. Bands of full-length (asterisk), GPS-cleaved CTF or expressed CTF fragment (open circle), TLD (star), and cleaved NTF (arrow head) of PC1 are indicated.
Figure 13. Expressing full-length PC1, PC1-CTF, or PC1-TLD without PC2_AA in *Xenopus* oocytes did not give rise to significant current.

Scatter plot and bar graph shows the average current sizes of oocytes expressing indicated full-length or fragment PC1 proteins in a solution containing 100 mM Na$^+$ and 2 mM Ca$^{2+}$. Oocytes expressing both PC1-CTF and PC2_AA were used as positive control. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (n.s.: not significant, ***P < 0.001, Student’s t-test).
PC1/PC2 complex channel has different pore properties compared to the homomeric PC2 channel.

Both the fact that PC1/PC2_AA has higher Ca\textsuperscript{2+} permeability than PC2_AA (Fig. 7) and a mutation in the putative pore region of PC1 can abolish the PC1/PC2_AA current (Fig. 10E) suggest that PC1 contributes to the channel pore. Clearly, this indicates that the pore of the complex channel is different from that of the homomeric PC2 channel. To confirm this, we measured the permeability of the two channels to several inorganic and organic cations by analyzing the reversal potential of the I-V curves. Solutions containing 100 mM of the following ions were tested: Na\textsuperscript{+} [atomic mass (m\textsubscript{a}) = 23], Li\textsuperscript{+} (m\textsubscript{a} = 7), Cs\textsuperscript{+} (m\textsubscript{a} = 133), dimethylamine\textsuperscript{+} (DMA\textsuperscript{+}, molecular weight (MW)= 45.1), diethylamine\textsuperscript{+} (DEA\textsuperscript{+}, MW= 73.1), tetraethylammonium\textsuperscript{+} (TEA\textsuperscript{+}, MW= 130.3), and N-Methyl-D-glucamine\textsuperscript{+} (NMDG\textsuperscript{+}, MW=195.2). Since the majority of cations inside of the oocytes are K\textsuperscript{+}, the RP of the current recorded in a particular tested ion solution reflects the permeability ratio (selectivity) between the tested ions and K\textsuperscript{+}. A more positive RP means the channel tends to be more selective to the tested ion. The results show that the RPs of PC2_AA currents in these ions can be divided into two groups. All inorganic ions fall into the range from -15 mV to -25 mV, while all organic ions fall into a much more negative range from -55 to -80 mV (Fig. 14A, red I-V curves), indicating that the PC2_AA channel is much less permeable (or impermeable) to the large organic ions. Previously, PC2 has been shown to have good permeability to DMA, TEA and even larger organic ions (Anyatonwu & Ehrlich, 2005). Our results may reflect the difference between the WT and the GOF PC2_AA channels. When PC1-CTF/PC2_AA current was recorded with these ions, dramatic RP changes were observed. The RPs of all ions, except for NMDG, became much more positive (Fig. 14A, blue I-V curves, and B). The
RPs of the DMA$^+$ and DEA$^+$, as two extreme examples, shifted 80.2 mV and 63.2 mV respectively, in comparison to PC2_AA (Fig. 14A and B).

To gain more insight into the pore difference between these two channels, we calculated the permeability ratio (selectivity) between different tested ions. Since the exact intracellular K$^+$ concentration is unknown, we are not able to accurately calculate the permeability ratios of the tested ion to that of K$^+$. Instead, we used Na$^+$ as a reference ion and calculated the permeability ratios of the other tested ions (x) to it ($P_x/P_{Na}$) with the Goldman-Hodgkin-Katz equation (Hille 2001). The results show that compared to PC2-AA, the PC1/PC2_AA channel has greater $P_x/P_{Na}$ of Li$^+$, DMA$^+$, DEA$^+$, and TEA$^+$, and smaller $P_x/P_{Na}$ of Cs$^+$ and NMDG$^+$ (Fig. 14C). The largest ratio increase was seen for DMA$^+$ and DEA$^+$, indicating a significant greater permeability of the PC1/PC2_AA channel to these relatively large ions. Since the currents of the two channels have similar RPs in NMDG$^+$, compared to Na$^+$, NMDG$^+$ serves as a better reference ion to evaluate the difference between the pores of the two channels. We then calculated the permeability ratios of the other tested ions to NMDG$^+$ ($P_x/P_{NMDG}$). As expected, the $P_x/P_{NMDG}$ of all ions for PC1-CTF/PC2_AA are remarkably greater than that of PC2_AA (Fig. 14D). These results show that the PC1-CTF/PC2_AA channel has greater permeability to these ions, indicating it has a larger or more flexible ion conducting pore than the PC2_AA channel.
Figure 14. PC1-CTF/PC2_AA heteromeric channel has a different ion permeability to that of the PC2_AA homomeric channel.

A Representative I-V curves of the PC2_AA and PC1-CTF/PC2_AA channels in divalent ion-free bath solutions containing 100 mM of indicated ions, showing the differences in reversal potential.

B Bar graphs showing the markedly different reversal potentials of PC2_AA channel and the PC1-CTF/PC2_AA channel in bath solutions containing 100 mM of the indicated ions. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD (n.s.: not significant, ***P < 0.001, Student’s t-test).
C, D  Bar graphs showing the permeability ratios of the indicated ions to that of Na$^+$ ($P_x: P_{Na}$) (C) and NMDG$^+$ ($P_x: P_{NMDG}$) (D). These ratios are strikingly different between the two channels. The ratios of the particular ions, which are the values of the bars, are indicted on top of the bars.
Another piece of evidence showing the pore difference between the PC1/PC2 and PC2 channels came from measuring the effects of channel blockers. Previously, we found that the cation channel blockers Gd$^{3+}$, ruthenium red (RuR), and amiloride dramatically block the PC2_F604P channel (Arif Pavel et al., 2016). In the current experiments, these three blockers have significantly different blocking effects on the PC2_AA and PC1-CTF/PC2_AA channels. Gd$^{3+}$ at 0.5 mM, but not 0.1 mM, greatly blocked the inward current of PC2_AA channel (by ~93% at -80 mV) (Fig. 15). However, 0.5 mM Gd$^{3+}$ only blocked less than 50% of PC1-CTF/PC2_AA channel at -80 mV (Fig. 15). RuR at either 0.01 mM or 0.1 mM blocked ~70-75% of PC2_AA channel at -80 mV, but only around ~30-40% of complex channel (Fig. 15). In contrast, amiloride blocked the PC1-CTF/PC2_AA channel more than PC2_AA channel. Amiloride at 1 mM or 5 mM blocked only ~10% and ~45% of PC2_AA channel current at -80 mV respectively, while it blocked ~45% and ~80% of PC1-CTF/PC2_AA channel current at -80 mV respectively (Fig. 15). Since these blockers most likely function by binding to the channel pore, these results further supported that the pores of the two channels are quite different.

The above findings demonstrate that PC1-CTF/PC2_AA has a different channel pore from PC2_AA and suggest that PC1-CTF directly participates in forming the ion conducting pore of the complex. However, an alternative explanation would be that the differences found here reflect a conformational change in the pore of the homotetrameric PC2_AA channel caused by an association with PC1-CTF. We can rule out this possibility if pore mutations in both proteins can change ion permeability of the complex channel.
Figure 15. The different effects of three channel blockers on the currents of the PC2_AA and the PC1-CTF/PC2_AA channels.

A, B, C Left two charts: representative I-V curves of the indicated two channels in the bath solution containing 100 mM Na⁺ in the absence and presence of two concentrations of indicated three blockers: 0.1 and 0.5 mM Gd³⁺ (A), 0.01 and 0.1 mM ruthenium red (RuR) (B), and 1 and 5 mM Amiloride (C). Right: Scatter and bar graphs show the inhibition (%) of the channel currents at -80 mV caused by corresponding blockers in two different
concentrations as shown on the left. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD in bar graph (***P < 0.001, Student’s t-test).
Pore mutations of PC1 and PC2 change ion selectivity of the PC1-CTF/PC2_AA channel.

A widely held and extensively tested prediction of channel-forming proteins is that mutating residues lining the ion conduction pathway significantly alters ion selectivity (not simply current amplitude) of the channel. Based on the cryo-EM structures of PC2 and PC1/PC2 complex (P. S. Shen et al., 2016; Q. Su, F. Hu, X. Ge, et al., 2018), we selected several amino acids at the pore regions of both PC1 and PC2, and tested how mutations at these sites affect ion selectivity (Fig. 16A).

Three amino acid residues, L641, G642, and D643, form the ion selectivity filter of the homomeric PC2 channel (P. S. Shen et al., 2016). In the reported PC1/PC2 structure, they are also located at the putative ion selectivity filter position (Fig. 16A) (Q. Su, F. Hu, X. Ge, et al., 2018). Mutating the hydrophobic L641 to negatively-charged aspartic acid (D) showed that although there is no significant RP change in currents of two large ions, TEA+ and NMDG+, the RP in Na+, Li+, Cs+, DMA+, and DEA+ all dramatically shifted toward negative voltage, indicating the reduction of the permeability ratios between the tested ions and K+ (Fig. 16B for reversal potential changes and Fig. 17 for representative I-V curves). The D643N mutation also led to a significant, though mild, negative shift of RP in Li+, DMA+, and DEA+ (Figs. 16C and 17). We also mutated N674 on S6 of PC2, which is located directly above the lower gate in the PC2 homomeric channel (P. S. Shen et al., 2016), and its side chain faces into the pore of the PC1/PC2 channel (Fig. 16A) (Q. Su, F. Hu, X. Ge, et al., 2018). The N674C mutation, similar to L641D, led to dramatic negative RP shift for all ions tested except for NMDG+ (Figs. 16B and 17). Accordingly, for most tested ions, the permeability ratios $P_x/P_{Na}$ and $P_x/P_{NMDG}$ of these mutants, especially L641D and N674C, are dramatically changed (Fig. 16D and E). For example, for L641D, $P_{Li}/P_{Na}$, $P_{DMA}/P_{Na}$, and
$P_{\text{DEA}}/P_{\text{Na}}$ are all smaller, while $P_{\text{TEA}}/P_{\text{Na}}$ and $P_{\text{NMDG}}/P_{\text{Na}}$ are both greater than that of the pseudo WT channel (Fig. 16D). At the same time, $P_{i}/P_{\text{NMDG}}$ of all tested ions, except for TEA$^+$, are greatly smaller than that of the pseudo WT channel (Fig. 16E). These data confirm that PC2 directly participates in the formation of the channel pore of the PC1/PC2 channel.

In the published cryo-EM structure of PC1/PC2, the conformations of the pore region of PC1 are very different from that of PC2 (Q. Su, F. Hu, X. Ge, et al., 2018). The S6 of PC1 is bent in the middle, and the selectivity filter and canonical TRP pore helices are missing (Fig. 16A) (Q. Su, F. Hu, X. Ge, et al., 2018). Protein flexibility leads to low resolution in cryo-EM structures, which may have contributed to the poor resolution of the top of the PC1 pore region and the linker between S5 and S6 (Q. Su, F. Hu, X. Ge, et al., 2018). As a consequence, side chains of many residues in this region, including those aligned with the selectivity filter of PC2, L4083, R4084, and V4085 (L4093, R4094, and L4095 in human PC1), were not seen in the structure (Fig. 16A). Mutation of PC1 L4083 or V4085 to aspartic acid produced similarly drastic effects on ion selectivity as the L641D and N674C mutations in PC2. L4803D led to dramatic negative RP shift in all tested ions except for NMDG$^+$ (Figs. 16B and 17). The mutant channel has smaller $P_{\text{Li}}/P_{\text{Na}}$, $P_{\text{DMA}}/P_{\text{Na}}$, and $P_{\text{DEA}}/P_{\text{Na}}$, and larger $P_{\text{NMDG}}/P_{\text{Na}}$, as well as smaller $P_{i}/P_{\text{NMDG}}$ of all tested ions than pseudo WT channel (Fig. 16D and E). V4085D caused similar negatively shifts in RP except for that in TEA$^+$ and NMDG$^+$ (Figs. 16B and 17). The mutant channel has larger $P_{\text{Cs}}/P_{\text{Na}}$ and $P_{\text{TEA}}/P_{\text{Na}}$, and smaller $P_{i}/P_{\text{Na}}$ of other ions and smaller $P_{i}/P_{\text{NMDG}}$ of almost all ions than pseudo WT channel (Fig. 16D and E). Interestingly, the mutation R4084A, located between the two mutations above, only caused a small negative RP shift and relative permeability reduction in DEA$^+$ and did not significantly affect the permeability to the other ions (Figs. 16C-E, and 17). E4068, the closest negatively-charged amino acid to the putative selectivity filter in the PC1/PC2 structure, is located in the unsolved part of the S5-S6 linker of PC1 (Fig. 16A). When we
mutated it to hydrophobic leucine, the RP in Li\(^+\), Cs\(^+\), DMA\(^+\), DEA\(^+\), and TEA\(^+\) were all significantly shifted toward negative voltage, suggesting this amino acid contributes to ion permeability (Figs. 16C-E, and 17). These data strongly indicate that the PC1 subunit directly participates in forming the channel pore together with PC2, and that the region L4083-V4085 plays a crucial role in the ion selectivity, as suggested by the cryo-EM structure.
Figure 16. Pore mutations in PC1 or PC2 led to changes in ion permeability of the PC1-CTF/PC2-AA channel.

A TOP: a bottom view of a previously reported cryo-EM structure of PC1/PC2 channel (PDB code 6A70) (Q. Su, F. Hu, X. Ge, et al., 2018), which is missing the NTF of PC1 and the intracellular C-terminal tails from both proteins. S10-S11 of one PC1 subunit and S5-S6 from
three PC2 subunits assembled to form the pore. Bottom: the mutated amino acids in this experiment (in purple) are mapped on S10-S11 of one PC1 and S5-S6 of one PC2 subunit in the complex. The dashed grey line indicates the putative ion flow path. Since human PC1 was used for structure determination, mouse amino acids mutated in this study are indicated in parentheses. Due to the low resolution of the PC1 pore region in the structure, side chains are not seen for the three mutated amino acids at the putative selectivity filter region. E4068 (E4078 in human) is not solved in the structure, and it is labeled to indicate its approximate location in the structure.

**B, C** Bar graphs showing the reversal potentials of PC1-CTF/PC2_AA and the indicated mutants tested in bath solutions containing 100 mM of the indicated ions (top). Two PC1 and two PC2 mutations caused dramatic changes in the reversal potential for almost all tested ions (B), indicating that these amino acids are essential for ion permeability. Another two PC1 mutations and one PC2 mutation only led to relatively small changes of reversal potential for some ions but not others (C), indicating a less important role of these amino acids in ion permeability. Statistical significance between reversal potentials of all pore mutants and that of the PC1-CTF/PC2_AA channel are indicated. Representative I-V curves are shown in **Fig. 17**. Oocyte numbers are indicated in parentheses. Data are presented as mean ± SD (n.s.: not significant, *P < 0.01, ***P < 0.001, Student’s t-test).

**D, E** Bar graphs showing the permeability ratios of the indicated ions to that of Na⁺ \( (P_x: P_{Na}) \) (D) and NMDG⁺ \( (P_x: P_{NMDG}) \) (E). Pore mutations in both PC1 and PC2 lead to significant changes of the permeability ratios in most of cases.
Figure 17. Pore mutations change the ion permeability of the PC1/PC2_AA channel.

Representative I-V curves show the shifts of reversal potential caused by indicated mutations in bath solutions containing 100 mM of indicated ions. Compared to group 2 mutations, Group 1 mutations cause more dramatic reversal potential shifts.
GPS cleavage is not necessary for the GOF PC1/PC2_AA channel activity.

PC1/PC2_AA has many potential applications in studying the function and regulation of this channel. To illustrate this, we studied whether N-terminal GPS cleavage is necessary for the channel activity of the PC1/PC2_AA channel. GPS cleavage occurs at the HL*3041 tripeptide (*: scissile bond. Amino acids are numbered as in mouse PC1) (Wei et al., 2007) (Fig. 18A). Two mutants that abolish the cleavage of PC1, L3040H and T3041V, have been found to have defective PC1 in vivo functions in mice. PC1_L3040H appears to have complete loss of the function since PC1-L3040H-BAC transgenic mouse lines did not rescue the embryonic lethality of Pkd1−/− mice (Cai et al., 2014). However, PC1_T3041V exhibited partial PC1 function since the knock-in mice were alive and had normal appearing kidneys at birth, although they developed rapid renal cysts in distal nephron segments later (S. Q. Yu et al., 2007). In order to understand the effect of the GPS cleavage on the PC1/PC2 channel function, and the functional difference between the two non-cleavable mutants, we tested the mutants with the GOF PC1/PC2 channel.

Coexpression of PC1_T3041V and PC2_AA in oocytes gave similar channel currents to that of PC1/PC2_AA, although the current of PC1_T3041V/PC2_AA is slightly smaller in 70 mM Ca²⁺ (Fig. 18B and C). However, the channel current was completely abolished when PC1_L3040H was coexpressed with PC2_AA (Fig. 18B and C). We then checked the protein expression and plasma membrane trafficking. Both L3040H and T3041V mutants expressed well and were not cleaved since only full-length proteins were seen in Western blot (Fig. 18D, the image on the left). However, surface biotinylation revealed that PC1_T3041V/PC2_AA expressed nicely on oocyte plasma membrane, while PC1_L3040H/PC2_AA did not traffic to plasma membrane at all since coexpressed PC2 was not detectable in the surface sample (Fig. 18D). This result is consistent with the previous
finding that PC1_T3041V acquires EndoH resistance, meaning it can at least traffic to Golgi (H. Kim et al., 2014), while PC1_L3040H is stuck in the ER and remains EndoH sensitive (Cai et al., 2014). Consistently, the 130 kDa band of PC2 was not seen when PC1_L3040H was coexpressed (Fig. 18D). The fact that no PC2 was seen on surface when PC1_L3040H was coexpressed also suggests that in our experimental condition, the majority of PC2 are in the complex of PC1 and almost no homomeric PC2 channel exists.

Thus, our results suggest that GPS cleavage is not necessary for PC1 to play its channel role in complex with PC2_AA as long as the complex traffics to the plasma membrane. However, since the studied GOF channel is constitutively open, we could not test whether the GPS cleavage plays a role in channel gating.
Figure 18. GPS cleavage of PC1 is not necessary for channel activity of the PC1/PC2_AA channel.

A Transmembrane topology of PC1 and PC2 proteins, showing the cleavage at the N-terminal GPS site (in circles), the “HLT” tripeptide where the cleavage happens, and the two mutations, L3040H and T3041V, that abolish GPS cleavage.

B, C Representative currents and a scatter plot and bar graph showing the currents of the full-length WT and mutant PC1s associated with PC2_AA in bath solutions containing 100 mM Na\(^+\) (B) or 70 mM Ca\(^{2+}\) (C). Currents were normalized to the average current of PC1/PC2_AA recorded from the same batch of oocytes. Oocyte numbers in scatter plot and bar graph are indicated in parentheses. Data are presented as mean ± SD in bar graph (n.s.: not significant, **P < 0.01, ***P < 0.001, Student’s t-test).

D Surface biotinylation followed by Western blot showing the surface expression of the PC1_T3041V/PC2_AA complex, but not the PC1_L3040H/PC2_AA complex, in Xenopus oocytes. PC2_AA is completely absent from the surface sample when PC1_L3040H was
coexpressed, indicating that, in our experimental condition, all PC2_AA were in the complex with PC1_L3040H and trapped in the process of cell surface trafficking. Bands of full-length (asterisk) and GPS-cleaved CTF (open circle) of PC1, and 130 kDa PC2 are indicated.
DISCUSSION

In this study, for the first time, we were able to generate a GOF PC1/PC2 channel and dissect the role of PC1 in this complex. Although channel current mediated by the PC1/PC2 complex has been previously reported in several experimental systems (Delmas et al., 2004; Hanaoka et al., 2000; S. Kim et al., 2016), replication has been inconsistent (P. S. Shen et al., 2016; Y. Yu et al., 2009). In the current study, we generated several new GOF PC2 mutant channels by changing residues at the lower gate and found one double mutation, L677A/N681A (AA), that led to opening of the PC1/PC2 channel. The new heteromeric GOF channel has significantly different permeability to monovalent cations (Fig. 14) and different responses to divalent cations (Figs. 7, 8 and 9) and channel blockers (Fig. 15), compared with the PC2_AA homomeric channel. More importantly, mutations in either PC1 or PC2 pore region can change this channel’s ion permeability (Fig. 16). All these data confirm that the currents we recorded are conducted by PC1/PC2_AA heteromeric channel.

Association with PC1 leads to distinct effects on PC2 GOF mutants, F604P and AA. PC1/PC2_AA produced a robust current, while the PC1/PC2_F604P current was much smaller. This difference should be due, at least in part, to the fact that the two mutations open the channel pore in different ways. Mutation F604P abolishes the hydrophobic interaction between S5 and S6, which leads to twisting and bending of the four PC2 S6 helices to open the lower gate of the homomeric channel (Grieben et al., 2016; W. Zheng et al., 2018). When in complex with PC1, it is possible that either the F604P-induced S6 conformational changes are somehow blocked by the PC1 subunit, or the PC1 subunit directly clogs the pore, resulting in a smaller conductance of PC1/PC2_F604P in comparison with homomeric PC2_F604P (Fig. 4). In the case of PC2_AA, the alanine mutations at L677 and N681 shorten the side chains that form the restriction at the lower gate, directly widening the pore.
size and therefore, the channel’s overall conformation would remain in a closed state, as our recent data indicated (W. Zheng et al., 2018). Thus, PC2_AA is distinct from PC2_F604P with respect to the way by which the channel pore is opened and the conductance of the resulting opened pore, which is supported by current size difference between them (Fig. 5B and C) and the larger Ca\textsuperscript{2+} permeability of PC2_AA (Fig. 5D). These results also suggest that the Ca\textsuperscript{2+} permeability in PC2 is also defined by the lower gate of channel pore. Similarly, it was found that mutations at the inner pore/lower gate region in TRPA1 affect ion, including Ca\textsuperscript{2+}, permeability (Benedikt, Samad, Ettrich, Teisinger, & Vlachova, 2009). In the case of the PC1/PC2_AA complex, substitution of one of the four PC2_AA subunits with PC1 subunit presumably is not sufficient to block the heteromeric lower pore, allowing constitutive cation permeation. Indeed, in the cryo-EM structure of PC1/PC2 (Q. Su, F. Hu, X. Ge, et al., 2018), there is no bulky side chain from the PC1 subunit that lines up with PC2-L677 and N681 (Fig. 19). AA mutations on three PC2 subunits is sufficient to open up the restriction at this position.
Figure 19. Several key residues in the pore of PC1/PC2 channel may play roles in ion conductance.

Structure of the PC1/PC2 complex showing the side view of S10-S11 from the PC1 subunit and S5-S6 from one PC2 subunit in the cryo-EM structure of PC1/PC2 (PDB code 6A70) (Q. Su, F. Hu, X. Ge, et al., 2018). Side chains of PC2-L677A and N681 are shown in red and N674 in cyan. Side chains of amino acids on S11 of PC1 at this region were also shown in purple for the three positively-charged amino acids and in green for others. In PC1/PC2 complex, no bulky side chains from PC1 lines up with PC2-L677A and N681 to form the restriction at this position, while R4090 of PC1 and N674 of PC2 narrow down the channel pore in the middle.
The cryo-EM structure of PC1/PC2 shows that three positively-charged amino acids on S11 of PC1, R4100, R4107, and H4111 (align to R4090, R4097, and H4101 in mouse PC1) face to the pore and are proposed to disfavor cation penetration (Fig. 19) (Q. Su, F. Hu, Y. Liu, et al., 2018). This feature suggests a possibility that the solved structure may be in a closed state, and these residues will be moved out of the pore when the channel is gated. In the GOF PC1/PC2_AA, the channel was opened by removing the obstruction formed by the side chains of L677 and N681 on PC2. We believe the channel’s overall conformation would remain in a closed state. If this is true, then these three positively-charged residues on PC1 are most likely still in the pore. Since we can record robust current, at least in this GOF channel, ions can pass through in spite of having these positive charges in the pore. Among the three residues, only R4090 is located above the PC2-L677 and N681, which pairs up with N674 on the PC2 subunit at the opposite position and narrows down the pore (Fig. 19). Indeed, mutation R4090W, which adds an even bulkier side chain at this position, completely abolished the PC1-CTF/PC2 current (Fig. 10E). At the same time, mutation PC2-N674C greatly changed the ion permeability of the channel (Fig. 16B). These results indicate a crucial role of the R4090-N674 position in regulating ion permeability of PC1/PC2.

The presence of PC1 in the channel complex significantly alters channel properties, including ion selectivity. Firstly, common cation channel blockers inhibit the PC1/PC2_AA and PC2_AA channels differently (Fig. 15). Secondly, both 2 mM Ca\(^{2+}\) or Mg\(^{2+}\) extracellularly block the PC2_AA channel but not the PC1/PC2_AA channel (Fig. 7B, D and E), and PC1/PC2_AA has a relatively larger Ca\(^{2+}\) permeability than PC2_AA (Fig. 7F and 8). While these data, obtained through GOF mutants, may not reflect what is really happening in the WT channels, the \(^{45}\)Ca uptake experiment directly shows that the WT PC1/PC2 channel indeed has higher Ca\(^{2+}\) permeability than PC2 alone (Fig. 9). Thirdly, our results show that the PC1/PC2_AA channel has a larger or more flexible pore, and higher
permeability to most monovalent cations tested in this study (i.e. Na\(^+\), Li\(^+\), Cs\(^+\), DMA\(^+\), DEA\(^+\), and TEA\(^+\)) when compared to PC2_AA alone (Fig. 14). This is consistent with the structure revealed by the cryo-EM, where the upper pore region of PC1 is more flexible due to missing pore helices (Fig. 16A) (Q. Su, F. Hu, X. Ge, et al., 2018). Collectively, these results tell us that the heteromeric PC1/PC2 channel and the homomeric PC2 channel are two distinct channels that likely have their unique functions. The fact that the homomeric PC2 channel has low Ca\(^{2+}\) permeability (Arif Pavel et al., 2016; Liu et al., 2018) while the PC1/PC2 channel complex has much higher Ca\(^{2+}\) permeability indicates their very distinct physiological roles and thus contributions to ADPKD.

Recently, two groups have reported PC2-dependent channel activity on primary cilia by doing single channel recording directly from cilia (Kleene & Kleene, 2017; Liu et al., 2018). The currents recorded in the two studies are significantly different on Ca\(^{2+}\) permeability, which has been extensively discussed (Liu et al., 2018). Briefly, the Ca\(^{2+}\) permeability of the current reported in Kleene and Kleene (Kleene & Kleene, 2017) is more than 20 times higher than that of the current reported in Liu et al. (Liu et al., 2018). The latter found that their current is PC1-independent and it has similar ion permeability and extracellular Ca\(^{2+}\)-blocking as that of the GOF PC2_F604P (Liu et al., 2018), which indicates that their current is most likely conducted by homomeric PC2 channel. It will be very interesting to find out if the current recorded by Kleene and Kleene (Kleene & Kleene, 2017) is conducted by the higher Ca\(^{2+}\)-permeable PC1/PC2 complex instead. In our recording, to get significant Ca\(^{2+}\) influx, we need to clamp the oocytes at hyperpolarized negative membrane potential and apply high concentration Ca\(^{2+}\) at the extracellular side (Fig. 8).

Previous study has determined that the resting membrane potential of cilia is about -18 mV (Delling, DeCaen, Doerner, Febvay, & Clapham, 2013). If the GOF PC1/PC2_AA channel has a similar Ca\(^{2+}\) permeability as the naturally gated WT PC1/PC2 channel, then under this
voltage and relatively low physiological \( \text{Ca}^{2+} \) concentration, we will expect small \( \text{Ca}^{2+} \) influx. However, due to the extremely small volume of cilia (Delling et al., 2013), the \( \text{Ca}^{2+} \) influx through PC1/PC2 channel might be enough to trigger \( \text{Ca}^{2+} \) signaling in cilia. At the same time, we cannot rule out the possibility that the natural gated PC1/PC2 channel may have greater \( \text{Ca}^{2+} \) permeability than the GOF channel.

Our results showed that the PC1-CTF preserves almost the same channel function as that of the full-length protein in complex with PC2 (Fig. **10B-D**). The CTF is a naturally occurring PC1 fragment generated by GPS cleavage, and usually remains attached to the cleaved NTF through noncovalent interaction (Qian et al., 2002; Trudel, Yao, & Qian, 2016), however detachment does occur (Kurbegovic et al., 2014). In some aGPCRs, NTF and CTF were found to function as separate proteins in cell-surface reception and signaling (Volynski et al., 2004). In the case of PC1, the detached CTF may also have NTF-independent channel function when assembled with PC2 (Fig. **10**). The cryo-EM structure of PC1/PC2 was solved using a truncated PC1 fragment containing the CTF lacking the intracellular C-terminal tail (Q. Su, F. Hu, Y. Liu, et al., 2018). The structure shows that this PC1 fragment assembles with PC2 into a channel-like complex (Q. Su, F. Hu, Y. Liu, et al., 2018), consistent with our functional data. We further show that the shorter fragment, TLD, retains channel function when associated with PC2 (Fig. **11**). Together with the structural data (Q. Su, F. Hu, Y. Liu, et al., 2018), this result shows that TLD is the core structural component for PC1’s channel function. The cleaved P100 fragment of PC1, which was found in mouse kidney (Woodward et al., 2010), should be very similar to TLD in sequence. Our study therefore infers that this naturally cleaved fragment of PC1 may have a distinct physiological function.

Although essential for ciliary trafficking of PC1 (H. Kim et al., 2014), GPS cleavage is not necessary for PC1 channel function in the PC1/PC2_AA complex in our experimental system, since the non-cleavable PC1_T3041V contributed full channel activity (Fig. **18**). One
of the reasons that PC1_T3041V is pathogenic in mouse is that it cannot traffic to primary cilia (H. Kim et al., 2014). Our conclusion is consistent with the observation that PC1_T3041V retains function to some extent since knock-in mice exhibited milder cystic phenotypes than PC1 null mice (S. Q. Yu et al., 2007). Indeed, a significant portion of PC1 remains as non-cleaved full-length protein in the embryonic kidney, indicating a special function of this form in embryonic development (S. Q. Yu et al., 2007). We show that as long as this non-cleaved form reaches the plasma membrane in complex with PC2, it will contribute to channel function. Together with the function of PC1-CTF, these results suggest that the presence or absence of NTF has little influence on the GOF PC1/PC2 channel function. However, because we only examined the effect on the GOF channel, which is constitutively open, the potential roles of GPS cleavage and the NTF in ligand binding and channel gating in WT channel remain to be determined. In aGPCRs, cleaved NTF was found associated with the membrane-tethering CTF and may function as an endogenous ligand, or involved in ligand binding (Langenhan, Aust, & Hamann, 2013). It will be valuable to assess whether PC1-NTF also plays a role in modulating WT PC1/PC2 channel function.

Studying the role of PC1 in the PC1/PC2 complex is very challenging due to the lack of a known activation mechanism. The GOF channel generated here provides a platform for study of the function and regulation of this complex. With our GOF mutant, we were able to reliably record the ion channel activity of this complex and dissect the function of PC1. By making mutations in the pore region, we provide direct evidence that the PC1 protein functions as an ion channel pore-forming subunit in the PC1/PC2 complex. In combination, the results from this study with our previous finding of the 1:3 subunit stoichiometry (Y. Yu et al., 2009), and the recently published cryo-EM structure (Q. Su, F. Hu, Y. Liu, et al., 2018), have greatly advanced the understanding of the channel function of PC1/PC2.
MATERIAL AND METHODS

**cDNA constructs and cloning.** Full-length mouse PC1 cDNA (NCBI accession No. NM_013630) and human PC2 cDNA (NCBI accession No. U50928) were cloned into a modified pGEMHE vector for in vitro transcription. The signal peptide (first 23 amino acids in the N-terminus) of PC1 was replaced by an Ig k-chain leader sequence (from the pDisplay vector from Invitrogen), and a FLAG tag was added immediately after this sequence. The PC2 used in this study has an N-terminal HA tag. For CTF and TLD of PC1, the cDNAs encoding the protein fragment from T3041 to the C-terminal end (T4293) and the fragment G3592-T4293 respectively, were cloned into the modified pGEMHE vector. An Ig k-chain leader sequence was added to the N-terminus of the CTF construct, and both CTF and TLD contain an HA tag on the N-terminus. All mutations were generated with PCR and confirmed by sequencing.

**Electrophysiology.** Whole-cell currents of *Xenopus* oocytes were recorded with the two-electrode voltage clamp (TEVC) technique. All recordings have been repeated with at least two batches of oocytes, and most of them have been done three or more times. RNAs were synthesized *in vitro* and injected into follicle-membrane-free *Xenopus* oocytes. For every oocyte, 30 ng of PC2 was injected. When injecting the PC1 full-length and PC2 combination, the quantity of injected PC1 RNA is 1.5 times in the molar ratio of PC2. This is increased to 2 times in the case of PC1-CTF and PC1-TLD. The excess amount of PC1 protein is to eliminate the homomeric PC2 complex formation. For oocytes expressing PDK1-CTF alone, 100 ng of PC1-CTF was injected per oocyte.

After injection, the oocytes were incubated at 18°C before whole-oocyte currents were recorded. The incubation time is 2-3 days for oocytes injected with PC2 RNA alone, PC1_CTF RNA alone, combined PC1-CTF and PC2_AA RNAs, and combined PC1-TLD
and PC2_AA RNAs, and their corresponding mutants. The incubation time is 3-5 days for oocytes injected with combined full-length PC1 and PC2_AA RNAs and their corresponding GPS mutants. The longer incubation time is necessary for full-length PC1-including complex due to its slower trafficking to the plasma membrane.

**Electrophysiology solutions.** Unless otherwise indicated, oocytes were recorded at room temperature in standard divalent ion-free bath solution (100 mM NaCl and 2 mM HEPES, pH 7.5), 2mM Ca$^{2+}$-containing bath solution (100 mM NaCl, 2 mM CaCl$_2$, and 2 mM HEPES, pH 7.5), or 70 mM Ca$^{2+}$ bath solution (70 mM CaCl$_2$, and 2 mM HEPES, pH 7.5). For solutions used in the ion permeability assay, 100mM NaCl in the standard divalent ion-free bath solution was replaced with 100mM LiOH, CsCl, dimethylamine (DMA) hydrochloride, diethylamine (DEA) hydrochloride, tetaethylammonium (TEA) hydrochloride, or N-Methyl-D-glucamine (NMDG) hydrochloride. The pH of Li$^+$ and NMDG$^+$ solutions were adjusted to 7.5 with HCl. The pH of the Cs$^+$, DMA$^+$, DEA$^+$, and TEA$^+$ solutions were adjusted to 7.5 with NMDG·HCl. Before applying these ion permeability assay solutions to oocytes, 1 mM niflumic acid was added to inhibit unknown channel activity induced by some of these ions.

**Electrophysiology protocol and relative ion permeability ratio calculation.** A standard TEVC protocol includes holding oocytes at 0 mV and measuring the current-voltage (I-V) relationships by applying an I-V protocol which runs 80 ms voltage steps from −100 to +100 mV (or indicated ranges) in 10 mV increments. In the experiments for determining ion permeability, oocytes were perfused with 100 mM NMDG$^+$ bath solution first, and the voltage step protocol was applied in order to extract the I-V curve and the reversal potential of NMDG$^+$. Oocytes were then washed with solutions containing 100 mM of the other ions tested. The I-V was monitored with a voltage ramp protocol (from −80 to +60 mV in 170 ms) until the reversal potential shift stopped (usually takes about 20s). Finally, another voltage
step protocol was applied to extract the I-V curve and reversal potential for the tested ion. Relative permeability of the tested ions was calculated based on the difference between the reversal potentials of the tested ion and NMDG+. The permeability ratios, $P_x/P_{\text{NMDG}}$, were calculated using the following modified Goldman-Hodgkin-Katz (GHK) equation (Hille 2001):

$$\frac{P_x}{P_{\text{NMDG}}} = e^{\frac{\Delta E_{\text{rev}}}{RT}}$$

Where $E_{\text{rev}}$ is the reversal potential, and $\Delta E_{\text{rev}}$ is the shift of reversal potential when ion $x^+$ was switched to NMDG+. $\Delta E_{\text{rev}} = E_{\text{rev,x}} - E_{\text{rev,NMDG}}$. $F$ is Faraday's constant, $R$ is the universal gas constant, and $T$ is the absolute temperature.

Co-immunoprecipitation (co-IP). Twenty oocytes of each group were used for the co-IP experiment. After proper incubation, oocytes were washed with cold PBS solution three times and kept in a lysis solution containing 1x PBS, 1 mM EDTA, 10% glycerol, 1% n-Dodecyl b-D- maltoside (DDM) and 1/50 (v/v) Protease Inhibitor Cocktail (Sigma). They were homogenized by passing through a 25-G needle 10 times. Oocyte lysates were incubated by rotating at 4 °C for 1 h. Lysates were centrifuged for 30 min at 10,000 g, and supernatants were collected. The supernatants were mixed with 20 µl anti-HA (Pierce) or anti-FLAG (Sigma) antibodies coated magnetic beads and rotated at 4 °C for 2–5 h. The beads were collected and then washed with 400 ml wash solution (lysis solution plus 500 mM NaCl and 0.25% Triton X-100) three times by rotating at 4 °C for 5 min. Proteins were then eluted with 40 µl acid elution buffer (0.1 M glycine, pH 2.6) and neutralized with 1M Tris pH 8.5, or eluted with 1.5x SDS loading buffer by incubating at 37 °C for 30 min.

Surface biotinylation. Proteins on *Xenopus* oocyte plasma membrane were detected with Pierce Cell Surface Protein Isolation kit following a modified protocol described previously (Pavel et al., 2014). In brief, two or four days after cRNA injection, oocytes (20-
30 oocytes per group) were washed with cold OR2 solution (82.4 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.6). Oocytes were then incubated with 0.4 mg/ml sulfo-NHS-SS-Biotin in ice-cold OR2 at 4°C for 30 min. The reaction was quenched, and oocytes were washed following the manufacturer’s protocol. Oocytes were then homogenized and lysed. Lysates were mixed with NeutrAvidin agarose at 4°C overnight. After beads were washed, proteins were eluted with 1.5x SDS sample loading buffer with 50 mM DTT at 37°C for 30 min. Eluted samples were analyzed by SDS-PAGE and Western blot. When required, the intensity of Western blot bands was measured with ImageJ (NIH).

**SDS-PAGE, Western blot, and antibodies.** After TEVC recording, oocytes were collected, and protein expression was assessed by Western blot. Oocytes were lysed in the same way as in the co-IP experiment. Lysate samples were run on 4-12% Bolt Bis-Tris Plus gels (Life Technologies) and transferred to PVDF membrane. Rabbit polyclonal anti-PC1 antibody recognizing aa 4123-4291 on the C-terminus of mouse PC1 (S. Q. Yu et al., 2007), mouse monoclonal anti-PC1 N-terminus antibody 7e12 (Santa Cruz Biotechnology), mouse monoclonal anti-PC2 (YCE2, Santa Cruz Biotechnology), anti-HA (BioLegend), anti-FLAG (Sigma), or anti-β-actin antibody (GenScript) were used. Blot signals were visualized with the Molecular Imager Chemi Doc XRS+ imaging system (Bio-Rad) or LI-COR Odyssey.

The protein expression of all mutations tested in this study has been confirmed by Western blot. All Western blots were repeated at least twice.

**45Ca uptake measurements:** 45Ca uptake measurements on oocytes were adapted from the previous description (Y. Yu et al., 2012). Briefly, 45CaCl₂ (PerkinElmer, Catalog No. NEZ013) at 30 μM and non-radioactive CaCl₂ at 1 mM were added to the uptake solution (100mM NaCl, 2 mM KCl, 10 mM HEPES, pH 7.5). 10 oocytes per group were incubated with 0.3 mL uptake solution for 30 min. The uptake process was terminated by washing the oocytes with cold uptake solution without calcium 5 times. Individual oocytes
were then lysed with 300 μL of 10% SDS. Four ml of scintillation cocktail (MP Biochemicals LLC. Catalog No. 88245305) was added to the lysate and the radioactivity of each sample was measured with an LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA). Data were analyzed by SigmaPlot 13.0.

**Structural graphics.** The structural graphics were prepared with the software PyMOL (The PyMOL Molecular Graphics System).

**Statistical analysis.** Recording data were analyzed with Excel or GraphPad Prism, and statistical significance was calculated with Student’s t-test. Results of p< 0.05 were considered statistically significant (differences p< 0.05 denoted by *, p< 0.01 denoted by **, and p< 0.001 denoted by ***)). Results are presented as means ±SD.

**Animal use.** Frogs care and experimental protocols were conducted upon approval of IACUC at St. John’s University.
REFERENCES


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