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THE EFFECTS OF A PATHOGENIC MUTATION AND A BINDING PARTNER ON THE ACTIVITY OF POLYCYSTIN ION CHANNELS

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ABSTRACT

THE EFFECTS OF A PATHOGENIC MUTATION AND A BINDING PARTNER ON THE ACTIVITY OF POLYCYSTIN ION CHANNELS

Haroon Chaudhry

Mutations within polycystin-1 (PC1) and transcient receptor potential polycystin channel 2 (TRPP2) are the leading causes for development of the most common kidney disease, Autosomal Dominant Polycystic Kidney Disease (ADPKD). Although significant efforts have been made to investigate properties and characteristics of these proteins over the last three decades, their roles in ADPKD and how mutations in these proteins lead to cystogenesis is still largely unknown. In the current work, I have studied a novel TRPP2 mutation found in a patient family diagnosed with ADPKD that removes the stop codon and results in adding an additional twelve amino acids on the C-terminus of the translated protein. My focus is to explore the characteristics of this mutation by utilizing gain-of-function TRPP2 mutants to record channel activity. We found that the pathogenic mutation significantly reduced TRPP2 expression and trafficking, leading to reduced channel activity. Furthermore, this mutation also reduces the activity of the heteromeric PC1/TRPP2 complex channel. My results gain insight into the pathogenesis of ADPKD caused by a mutation on TRPP2. In the last part of this thesis, I have also studied the role of a potential PC1 binding partner C1orf95, the human ortholog of the STUM protein, in regulating the channel function of the PC1/TRPP2 complex.

ACKNOWLEDGEMENT

I would like to extend my sincere thanks to my mentor and advisor, Dr. Yong Yu, without whom this project would not be possible. I am deeply grateful for the opportunity to have been exposed to the field of research involved in molecular physiology and biophysics. I thank my fellow laboratory colleagues, past and present, for making my time spent in the lab very productive and wholesome. I thank my committee members, Dr. Ales Vancura and Dr. Yan Zhu, for their helpful comments and suggestions for my improvement. Lastly, I thank the entire St. John's community for creating an enriched academic environment, which has allowed me to pursue higher education.

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INTRODUCTION

1.1 Calcium Signaling

Calcium is a vital second messenger that plays a role in many physiological functions, and calcium signaling regulates many cell activities such as neurotransmission, cell motility, metabolism, apoptosis, and many more crucial aspects (Clapham, 2007). Cell signaling is activated by either release of calcium ions (Ca^{2+}) that are intracellularly stored, such as the endoplasmic reticulum, or they enter the cell via the plasma membrane ion channels, for example the voltage-gated Ca^{2+} channels or other Ca^{2+} permeable channels.

1.2 TRP Protein Family

Transient Receptor Potential (TRP) channels are a group of calcium permeable channels. They are a diverse set of proteins whose primary function is to regulate the plasma membrane permeability to a variety of ions. The first TRP channel gene was discovered in *Drosophila melanogaster* in an analysis of a mutant fly whose photoreceptors failed to retain a sustained response to maintained light stimuli (Montell and Rubin, 1989). TRP proteins belong to a superfamily of cation-permeable channels, comprised of seven subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastin), TRPV (vanilloid), TRPN (NOMPC), TRPML (mucolipin) and TRPP (polycystin). Members of this superfamily share the common features of six transmembrane segments, varying degrees of sequence homology, and permeability to cations (Venkatachalam and Montell, 2007). There has been a growing interest in TRP proteins since they are modulators of

intracellular Ca^{2+} signaling, and there is evidence that TRP channels act as Ca^{2+} entry and release channels (Gees, Colsoul, and Nilius, 2010).

1.3 ADPKD and Polycystins

Our focus has predominantly been to study the TRP channel polycystin (TRPP) subfamily and its relatedness to Autosomal Dominant Polycystic Kidney Disease (ADPKD). ADPKD is the most common inherited kidney disease, and is caused by mutations in either PKD1 or PKD2 genes, encoding polycystin proteins polycystin-1 (PC1, also called PKD1) and TRPP2 (also called polycystin-2 or PC2, or PKD2). It affects approximately 1:400 – 1:1000 individuals, and cause formation of fluid filled cysts within the kidney leading to end-stage renal failure (Harris and Torres, 2009; Wu and Somlo, 2000).

TRPP2 is expressed in a variety of tissues, such as epithelial cells, vascular smooth muscle, cardiac myocytes, adrenal glands, ovaries, etc. (Ong, 2008). TRPP2 has 968 amino acids (109,561 Da), six transmembrane domains and intracellular N- and C-termini. The carboxy-terminal of TRPP2 has been of particular interest due to its several sequence elements required for proper function, a calcium-binding EF hand, a coiled-coil domain, an ER retention motif, and an amino acid cluster. The EF hand has been implicated in ion channel gating and the acidic cluster in protein trafficking. The coiled-coil domain is required for homomerization and heteromerization of TRPP2 subunits (Semmo, Kottgen, Hofherr, 2014). TRPP2 has a large extracellular loop between the first and second transmembrane domains. Similar to other TRP proteins, TRPP2 can form a homomeric cation channel.

PC1 is a large membrane protein containing 4,302 amino acids, comprising of eleven transmembrane domains, a large extracellular N-terminus, a GPCR proteolytic site (GPS), and a shorter cytoplasmic C-terminus (Tsiokas, 2009). Its last six transmembrane domains share significant sequence and structural similarity with the six transmembrane domain of TRPP2. Because of the well-recognized motifs on the N-terminus of PC1 are involved in protein-protein, protein-saccharide, and protein-ligand interactions, PC1 is thought to function as a cell surface receptor.

How the mutations in these two proteins lead to ADPKD remains rather unclear, however, the two proteins likely participate in similar processes since their pathological implications are similar. Indeed, evidence shows that PC1 and TRPP2 can form a heteromeric receptor/ion channel complex in a 3:1 stoichiometric ratio with three TRPP2 subunits and one PKD1 subunit (**Fig. 1**) (Yu et al, 2009). A recently published cryo-EM structure of this complex has confirmed this stoichiometry (Su et al, 2018). The assembly is mediated by the C-terminal coil-coiled domains and the extracellular loops between the first and second transmembrane domains of TRPP2 and the sixth and seventh of PC1 (Yu et al., 2009; Salehi-Najafabadi et al, 2017). Mutations that disrupt the TRPP2 coiled-coil domain trimer abolish the full length TRPP2 and PC1/TRPP2 complex and diminish the surface expression of both proteins (Yu et al, 2009). The PC1/TRPP2 complex was also found to express in the primary cilia of renal epithelial cells, where they may play essential roles in controlling renal tubular differentiation. Hence, it is important to evaluate the molecular mechanisms underlying the structure and function of the assembly in order to understand its physiological relevance.

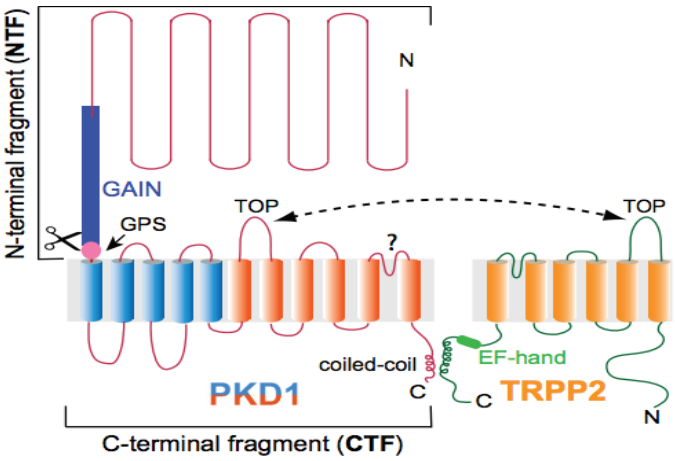

Figure 1. The PC1/TRPP2 receptor-ion channel complex. The PC1 and TRPP2 proteins share sequence homology over the transmembrane segments and contain a large loop separating the first two transmembrane domains. TRPP2 can form an ion channel complex with PC1 via the intracellular C-terminus at the coiled-coil domain in a three TRPP2: one PC1 stoichiometric ratio. PC1 is cleaved after being synthesized at the N-terminal GPS site, which generates the N-terminal fragment (NTF) and the 11 transmembrane C-terminal fragment (CTF). The GPS cleavage is essential for the function of PC1.

1.4 PC1 and TRPP2 mutations

ADPKD patients carry germ-line mutations in one allele of either PC1 or TRPP2. Mutations in PC1 are responsible for approximately 80 percent of cases of ADPKD, and TRPP2 mutations account for approximately 15 percent (Bergmann et al, 2018), while the remaining 5-10 percent are due to rare mutations within other loci (Gall, Torres, and Harris, 2018). However, an additional factor besides the germ-line mutation may be required for the phenotype of ADPKD. A somatic inactivation of the remaining allele associated with this disease, which suggest a two-hit model for this type leads to cystogenesis in ADPKD patients (Pei et al, 1999). From the ADPKD mutation database,

there are 2,303 germ-line mutations of PC1, of which 1,273 are pathogenic. For TRPP2, there are 278 germ-line mutations, of which 202 are pathogenic. These mutations range from frame-shift, missense, substitution, nonsense mutations, etc. Although, many are truncation or deletion mutations.

Our collaborator at the University of Toronto, Dr. York Pei, treated a patient family which has been diagnosed with ADPKD, and found a novel single point mutation at the stop codon of the TRPP2 gene. The mutation adds an additional twelve amino acids (NIHTKSYTQRLF) on the C-terminal fragment of the translated protein. We refer to this unique mutation as TRPP2-12aa (for twelve amino acids). Studying this pathogenic mutation may shed light on how this mutation affects the channel complex and more insight to understanding the molecular mechanisms underlying ADPKD.

1.5 STUM proteins

Animal locomotion depends on proprioceptive feedback (Desai et al, 2014), and is generated by mechanosensory neurons. In *Drosophila*, the mechanosesnation is mediated by multi-dendritic neurons, however, it is not clear how those neurons can be tuned to a joint angle modality. Performance of a genetic screening for impaired walking in *Drosophila* mapped to a gene, CG30263, which encodes a protein with 1870 or 1959 amino acids (Desai et al, 2014). This gene is named STUM, in short for stumble, due to a walking impairment. The human ortholog of the STUM gene is a member of the SPEC3 family, named C1orf95 (Chromosome 1 open reading frame 95). Mutations within this gene underlie un-coordination (Desai et al, 2014). The STUM protein is highly conserved among vertebrates and is predicted to contain two transmembrane domains. Propioceptive defects in adult flies have been attributed to malfunction of type-1

mechanoreceptor (ciliated) neurons (Avidor-Reiss et al, 2004). Electrophysiological recordings of mechanical responses from this type of neuron of the anterior notopleural bristle were taken to test whether the same defects underlie the STUM mutant. STUM expression within in the legs was localized to three neurons, at the femur-tibia joint, the tibia-tarsus joint, and the second tarsal segment. The dendrites of these neurons terminated at the former joints. These joint angles induced dendritic stretching and elicited an elevation of cellular calcium levels, these are not seen in STUM mutants (Desai et al, 2014).

We find the human ortholog of STUM (C1orf95) of particular interest, because this protein is found decreased in urinary exosomes from ADPKD patients. Biogenesis of exosomes involves the inward budding of endosomes to form multi-vesicular bodies (MVB). MVB coordinates transport of cargo, this process is facilitated by different mechanisms that involves ESCRT machinery or lipid rafts. C1orf95 exists in two isoforms within the human kidney, Exon 3- and Exon 3+. The third exon is a microexon of nine base pairs that is differentially spliced. C1orf95 is expressed and co-localized in the plasma membrane, MVB, and cilia, and co undergo palmitoylation. Both isoforms can elicit current in electrophysiological settings. C1orf95 is involved with the generation of giant vesicles up to two-micron size, and it is a subunit of stretch gated Ca^{2+} channel complex involved in proprioception in *Drosophila*. Our collaborator, Dr. Christopher Ward at University of Kansas Medical Center found that C1orf95 has interaction with PC1/TRPP2 complex in kidney exosomes. Thus, it is reasonable to test if C1orf95 may be associated with the PC1/TRPP2 complex channel activity.

CHAPTER 2

MATERIALS AND METHODS

2.1 Polymerase Chain Reaction

Template: HA-tagged TRPP2-F604P and TRPP2_L677A_N681A gain-of-function mutants (previously generated by Yong Yu's laboratory) were inserted into pGEMHE2-Yu vector. Q5 Forward and Reverse primers for adding 12aa to the C-terminus of TRPP2 were used. Q5 Polymerase (Invitrogen) was used. Denaturation 98°C (10 sec). Annealing 60°C (3 min). Extension 72°C (30 sec).

2.2 Gel Electrophoresis

PCR product was analyzed on 1% agarose gel at 130V for 20 minutes. Correct products were treated with KLD reaction (NEB). Ligation product was added to DH5α competent cell—fast transformation and plated onto a carbenicillin antibiotic plate. Incubation overnight at 37°C.

2.3 Plasmid Preparation

Colonies grown on antibiotic plate were picked and cultures grown for 18-20 hours at 37°C. Miniprep performed on bacterial cultures (Epoch Life Sciences). Test cut performed on purified plasmid with restriction enzyme HindIII (NEB) and Cutsmart Buffer (NEB) incubation at 37°C for 1 hour, cut site at 5kb and 0.8kb. Sample sent for sequencing (Eurofins Genomics) and blasted (NCBI blast). V5 tagged C1orf95_E3+ and E3- plasmids (provided by Chris Ward at KUMC), propagated on kanamycin bacterial

plate and purified during miniprep (Epoch).

2.4 RNA Transcription

DNA purification: linearize DNA using Buffer CS (NEB) and restriction enzyme Mlu1-HF (NEB), incubation overnight at 37°C. *In vitro* RNA transcription using T7 RNA Polymerase (incubation at 37°C for 3 hours). RNA was analyzed on RNA gel and concentration measured using a spectrophotometer. BsaI restriction enzyme (NEB) used to linearize the 3' end and T7 polymerase to run off the mRNA for C1orf95+E3 and C1orf95-E3.

2.5 Electrophysiology

Micro-injection of 1µl of RNA into needle (1.5µg/µl for HA-TRPP2-F604P and 1.8µg/µl and 5.4µg/µl for HA-TRPP2-F604P_12aa), (1.0 µg/µl for PC2_AA/TM11 and 1.0 µg/µl PC2_AA_12aa/TM11) injected into defolliculated *Xenopus laevis* oocytes (~50 ng of RNA per oocyte). Oocytes were incubated at 18°C for 2-3 days before measuring current using the two-electrode voltage clamp (TEVC) system. Currents were recorded at room temperature in divalent ion-free bath solution [100mM NaCl 99%, 2mM HEPES (pH 7.5 by NaOH/HCl)] and Ca^{2+} containing bath solutions [2mM $CaCl_2$, 100mM NaCl, 2mM HEPES (pH 7.5 by NaOH/HCl)] and [70mM $CaCl_2$, 2mM HEPES (pH 7.5 by NMDG)]. A standard TEVC protocol was used holding oocytes at 0 mV and measuring the current-voltage (I-V) relationship by applying steps from -80mV to +60mV. Micro-injection of 1µl RNA of C1orf95+/-E3 (1µg/µl) into *Xenopus* oocytes (~50 ng per oocyte). Same protocol as GOF TRPP2 mutations used for the human stum ortholog.

2.6 Cell Lysis

After 2-3 days post-injection and recording via TEVC system, oocytes were collected (8 oocytes per group) and homogenized in lysis solution [10µl/oocyte, 1X PBS, 10uM EDTA, 1% DDM, 10% glycerol, 1:50 protease inhibitor (Sigma Aldrich)]. Homogenized samples sonicated twice on ice [70% amplitude for 2 minutes (1 pulse/sec)]. Sonicated samples were incubated at 4°C for 1 hour and gently vortexed in 15 minute intervals. Lysates were pelleted by centrifugation at 13,000 rpm at 4°C for 30 minutes. The supernatants were collected on ice and treated with 3X SDS. SDS treated samples were incubated in 37°C for 30 minutes. Same protocol used for human stum ortholog.

2.7 SDS-PAGE and Western Blot

SDS treated samples were loaded onto Bolt 4-12% Bis-Tris Plus gels (Invitrogen by Thermo Fisher Scientific) and transferred onto PVDF (Bio-Rad). After transfer, the membrane was blotted with mouse monoclonal anti-P2 (YCE2) (Santa Cruz Biotechnology) or anti-HA (Biolegend) primary antibodies for 1 hour at room temperature or overnight at 4°C. The membrane was blotted with HRP-conjugated goat-anti-mouse secondary antibody for 1 hour at room temperature. Images were visualized using Odyssey CLx (LICOR) and Image Studio Ver 5.2. 10% Bis-Tris Plus (Invitrogen) gels used for c1orf95 samples. Anti-5183 (P1), anti-P2 (YCE2), and anti-V5 primary antibodies used. GaM 680 and GaR 800 secondary antibodies used. Film analyzed using LICOR system.

2.8 Surface Biotinylation

Approximately 30 oocytes were collected per group and washed with cold 1X OR2 solution, then incubated with Sulfo-NHS-SS-Biotin (Pierce) in 1X OR2 solution (6mg/10ml) for 30 minutes at 4°C with gentle agitation. The reaction was quenched with 500 ul of Quenching Solution (Pierce Surface Protein Isolation Kit) for 5 minutes and then washed off Tris-buffered saline (TBS), three times. The oocytes were lysed according to oocyte lysis protocol (see above), and the collected supernatant was partially saved as the whole cell lysate sample, and the remainder was combined with NeutrAvidin Agarose (Pierce Kit; 80 ul/sample washed with PBS-T, three times) in the provided column. The bottom plug and top cap were secured and the columns were incubated overnight at 4°C with rotary mixing. After centrifuging and discarding the supernatant, the NeutrAvidin Agarose were washed with Wash Buffer [provided or made (1X PBS, 1% DDM, 0.1% Tween-20, 1/100 protease inhibitor, 1M NaCl, and 1% TritonX-100)], three times. The protein was eluted by adding 75 ul of 1.5X SDS with 50 mM DTT to the agarose and incubated in 37°C for 30 minutes before centrifuging to collect the supernatant.

CHAPTER 3

THE EFFECT OF 12AA MUTATION ON POLYCYSTIN CHANNEL ACTIVITY

3.1 The extra C-terminal 12 aa reduces the activity of a GOF TRPP2 (TRPP2-F604P) channel

Due to the lack of a known activation mechanism of the TRPP2 homomeric channel and the PC1/TRPP2 heteromeric channel, it has been difficult to study the function and regulation of these channels and their roles in ADPKD. To facilitate the study of these channels, our laboratory have generated a series of gain-of-function (GOF) mutants of TRPP2. One of these mutants, TRPP2-F604P, has been used extensively in studying TRPP2 channel function (Pavel et al, 2016). TRPP2-F604P exhibits robust current when expressed in *Xenopus* oocytes and recorded using the two-electrode voltage clamp method following cRNA injection (Pavel et al, 2016). Recently, we have solved the cryo-EM structure of the TRPP2-F604P, which shows that the substitution of phenylalanine with proline disrupts the S4-S5 linker and the hydrophobic interactions between S5-S6, causing a conformational change of the S6 helix, opening the lower gate of the channel (Zhen et al, 2018). Similar channel gating mechanisms have been found before in other TRP channels (Cao et al, 2013; Schmeige et al, 2017, Zhen et al, 2018). The GOF TRPP2 provides a powerful tool for TRPP2 functional studies. In the current work, we have applied this GOF mutant to study the effect of the novel 12 amino acid mutation on TRPP2 channel function. By adding these 12 amino acids onto the C-terminus of TRPP2-F604P channel, we generated a new mutation named TRPP2-F604P-12aa (**Fig. 2**).

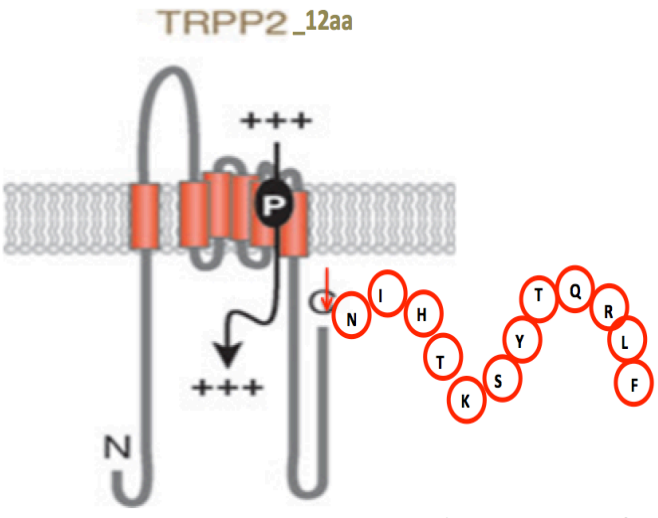

Figure 2. A novel pathogenic mutation of TRPP2. The mutation happens at the stop codon and leads to the additional twelve amino acids found at the TRPP2 C-terminus.

We first checked whether the 12 aa mutation affects the expression of TRPP2 in oocytes. To do that, we used a western blot method to compare the expression level of TRPP2-F604P and that of the TRPP2-F604P-12aa. When we injected the same RNA concentration (1.8μg/μl) for TRPP2-F604P and TRPP2-F604P-12aa into oocytes, we found that 12aa mutation gave much less expression than TRPP2-F604P (**Fig 3A**). By analyzing the band intensity with Image J, we figured out that the overall expression of the TRPP2-F604P was approximately 3-fold of that of the TRPP2-F604P-12aa. Consistently, when we increase the RNA concentration of TRPP2-F604P-12aa to 5.4 μg/μl, we found that the protein expression on our western blot was now similar to TRPP2-F604P with 3-fold less RNA concentration (1.8 μg/μl) (**Fig 3B**). On western blot, we usually can see several higher molecular weight oligomers of TRPP2, in addition to the monomer band at approximately 120 kDa. Occasionally, we see a different pattern of these bands between TRPP2-F604P and TRPP2-F604P-12aa (**Fig 3C**). We see the same

amount of monomer, but much more oligomers for the 12aa mutant. It seems the extra 12 aa may leads to some folding issues after protein synthesis that cause the oligomers more resistance to SDS treatment.

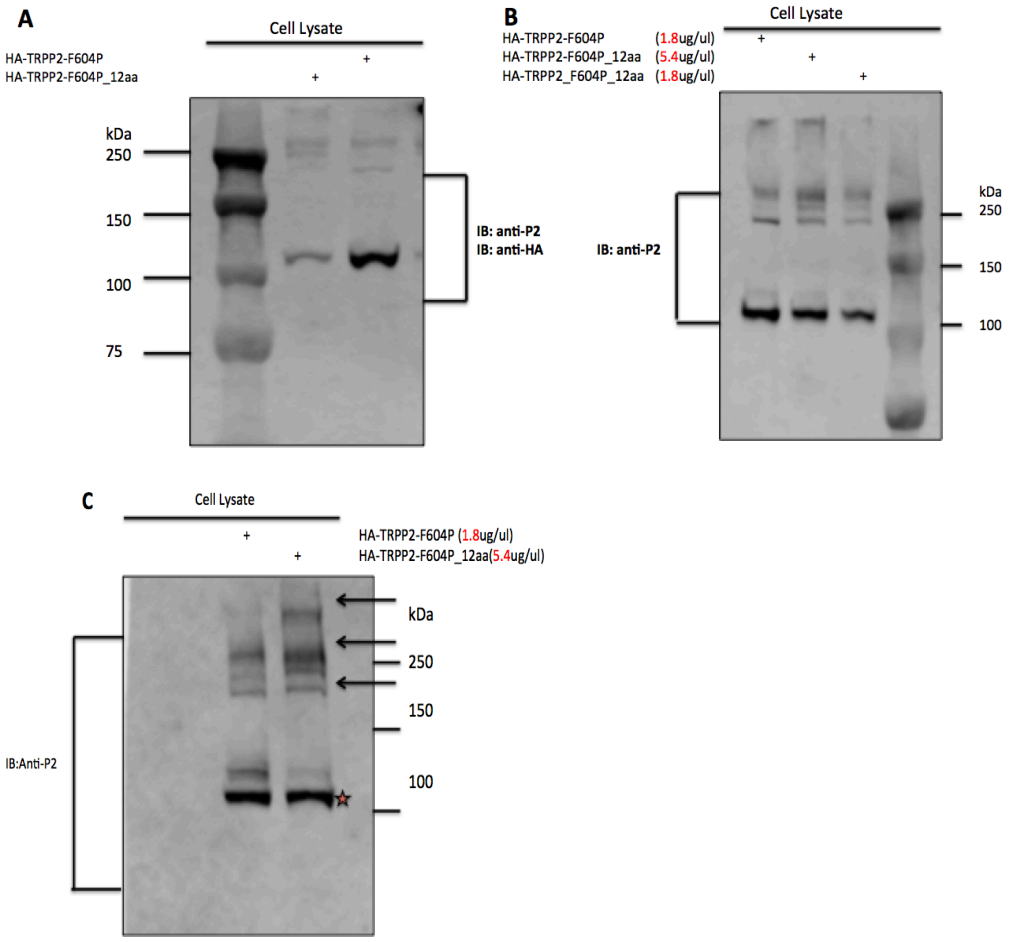

Figure 3. The 12 aa mutation reduces TRPP2 channel expression in *Xenopus* oocytes. Immunoblotting showing the expression of TRPP2-F604P and TRPP2-F604P-12aa in *Xenopus* oocytes. **(A)** TRPP2-F604P (1.8µg/µl) and TRPPP2-F604P_12aa (1.8µg/µl). **(B)** TRPP2-F604P (1.8µg/µl), TRPP2-F604P-12aa (1.8µg/µl), TRPP2-F604P-12aa (5.4µg/µl). **(C)** TRPP2-F604P (1.8µg/µl) and TRPP2-F604P-12aa (5.4µg/µl). "Star" represents the monomer band size corresponding to the expressed protein at approximately 120kDa. "Arrows" show the oligomers.

Next, using the two-electrode voltage clamp (TEVC) system, we recorded ion channel activity of TRPP2-F604P and TRPP2-F604P-12aa. Three different extracellular solutions have been used in our recording. Since TRPP2 is a cation-permeable nonselective cation channel, we first use 100mM Na^+ solution to test the cation current. TRPP2 has been known to be blocked by extracellular Ca^{2+} (Arif Pavel et al., 2016). Thus, we also tested the solution with 100 mM Na^+ and 2 mM Ca^{2+}. Lastly, TRPP2-F604P has very low or no Ca^{2+} permeability (Arif Pavel et al., 2016; Wang et al, 2019). To test whether 12 aa mutant leads to any change on this property, we use the third solution in which all monovalent ions have been replaced by 70mM Ca^{2+}. Our results show that the 12 aa mutation leads to no change on Ca^{2+} blocking and the Ca^{2+} permeability. In low (2mM) and high (70mM) extracellular Ca^{2+} solution, there is blockage of inward current (**Fig 4A** and **4C**, respectively), and in Ca^{2+}-free solution (100mM Na^+), there is no inhibition (Fig **4B**). However, 12 aa mutant greatly affected the channel activity. TRPP2-F604P at 1.8μg/μl gives much bigger current compared to TRPP2-F604P-12aa at 1.8μg/μl and 5.4μg/μl. When TRPP2-F604P and TRPP2-F604P-12aa RNA concentration is kept the same, the latter gives a much lower current and expression, and when TRPP2-F604P-12aa RNA concentration is increased, it still gives a lower activity than TRPP2-F604P. This confirms that the 12aa mutation reduces TRPP2 ion channel activity.

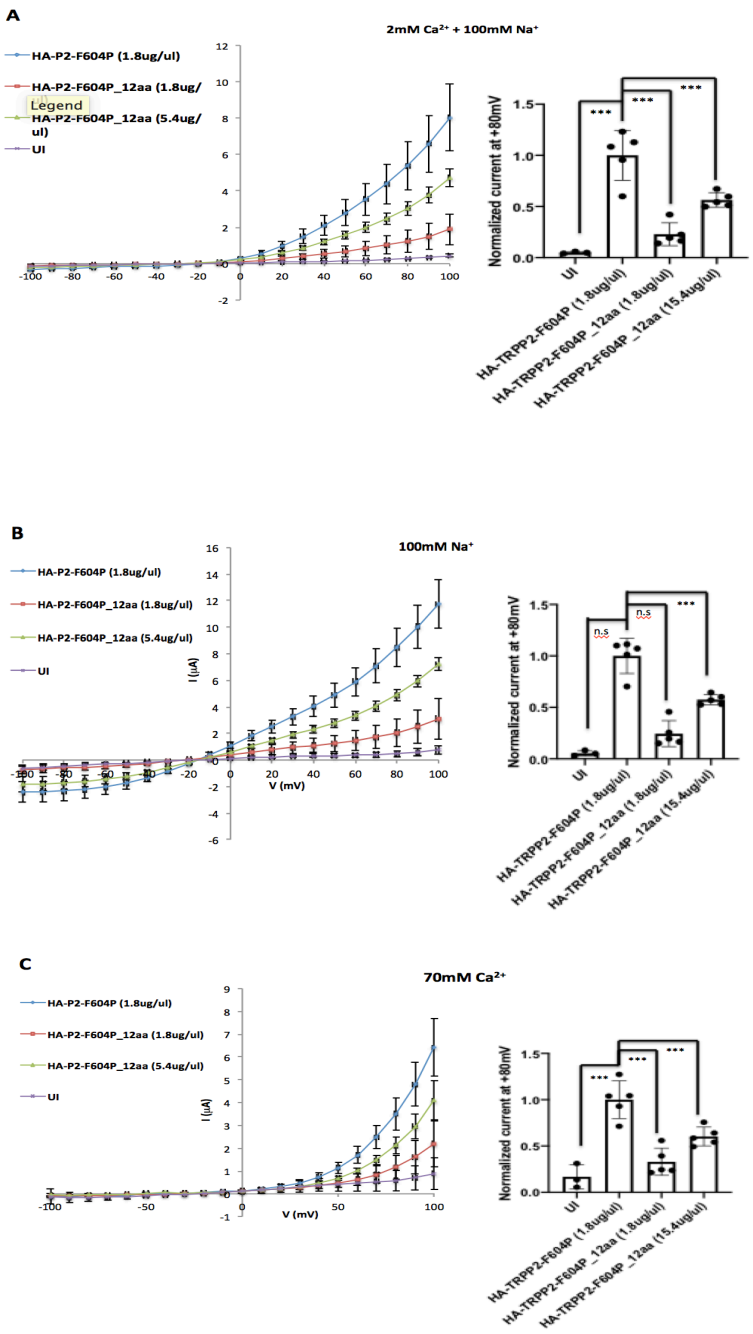

Figure 4. The 12 aa mutation reduces TRPP2 ion channel activity. Average I-V relationship (left) and normalized bar graph (right) showing the current sizes in solutions containing 2mM Ca^{2+} + 100mM Na^{+} **(A)** , 100mM Na^{+} **(B)**, and 70mM Ca^{2+} **(C)**. n = number of oocytes tested. TRPP2-F604P (1.8μg/μl) (blue), TRPP2-F604P_12aa (5.4μg/μl) (green),TRPP2-F604P_12aa (1.8μg/μl) (red). Un-injected oocytes (purple) as negative control. Bar graphs show the currents at +80mV. Error bars represent standard error. *** significant by t-test; n.s.= no significance.

The reduction of the TRPP2 current in the 12 aa mutant can be caused either by reduced channel function or by reduced plasma membrane expression. To distinguish the potential effects, we compared the plasma membrane expression of our samples in *Xenopus* oocytes by purifying the biotinylated surface proteins. Surface biotinylation was performed to monitor protein trafficking and in our result, the WT expressed better on surface than the 12 aa mutant. These results indicate that the 12 aa mutant also reduces channel trafficking besides its effect on protein folding.

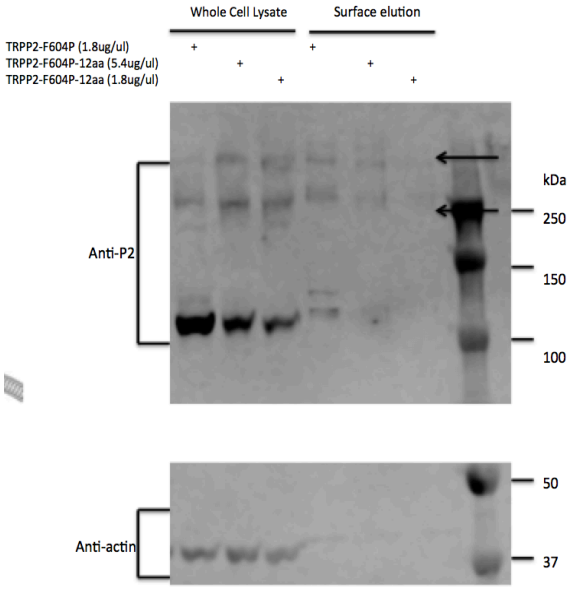

Figure 5. **The 12 aa mutant reduces TRPP2 surface expression.** TRPP2-F604P (1.8μg/μl), TRPP2-F604P-12aa (1.8μg/μl), and TRPP2-F604P-12aa (5.48μg/μl) are used in oocyte injection. Whole cell lysate (left three lanes) and surface expression (right three lanes) are shown. Arrows correspond to the oligomers on the TRPP2-F604P-12aa.

3.2 The extra 12 aa on TRPP2 reduces the activity of PC1-CTF/TRPP2-AA complex channel

Our laboratory has previously generated another TRPP2 GOF mutation, L677A_N681A (TRPP2_AA) (Wang et al., 2019). L677 and N681 form the lower gate constriction pore

of TRPP2 (Shen et al, 2016). By mutating both amino acids into alanine, this channel reduces the bulkiness of the side chain in the pore, and gives constitutive ion conductance of TRPP2 (Wang et al, 2019). It produced significant currents in a bath solution containing 2mM Ca^{2+} and 100mM Na^{+} when expressed in oocytes (Wang et al, 2019). This mutant channel gives current size roughly double that of TRPP2-F604P. More importantly, this mutation shows GOF effect in the complex channel formed by PC1 and TRPP2 (Wang et al, 2019), making it a very useful GOF mutant for studying the function of the PC1/TRPP2 complex channel.

Cleavage at the GPS site dissects PC1 into an NTF (PC1-NTF) and CTF (PC1-CTF) (**Fig. 1**) (Kurbegovic, et al 2014). CTF (sometimes we also call it TM11) has 11 transmembrane domains and an intracellular C-terminal tail. We have found that PC1-CTF is sufficient to form a functional channel with PC2 (Wang et al, 2019). We next tested whether the 12aa mutation affects the heteromeric PC1/PC2 channel function by co-expressing TRPP2-L677A-N681A_12aa (P2-AA-12aa) with PC1-CTF (TM11). Different from that of the TRPP2-F604P, the extracellular 2mM Ca^{2+} did not block the inward current of TRPP2-AA/TM11 (**Fig. 6A**). In 100mM Na^{+}, there are larger inward and outward current for TRPP2-AA/TM11 (**Fig 6B**). Importantly, the TRPP2-AA/TM11 channel is permeable to Ca^{2+}. By replacing the extracellular monovalent cations with 70mM Ca^{2+}, TRPP2-AA/TM11 results in robust current (**Fig. 6C**). This current is a mixture of TRPP2-AA/TM11 current and the Ca^{2+} induced chloride channel current (Wang et al., 2019). When comparing the P2-AA/TM11 with P2-AA-12aa/TM11, we observe a great reduction in current size of the complex with the 12aa pathogenic mutation, indicating that the 12 aa mutant may also affect the channel function of the

PC1/TRPP2 complex. In the future, we would need to check the surface expression and the protein expression via western blot to better understand its mechanism.

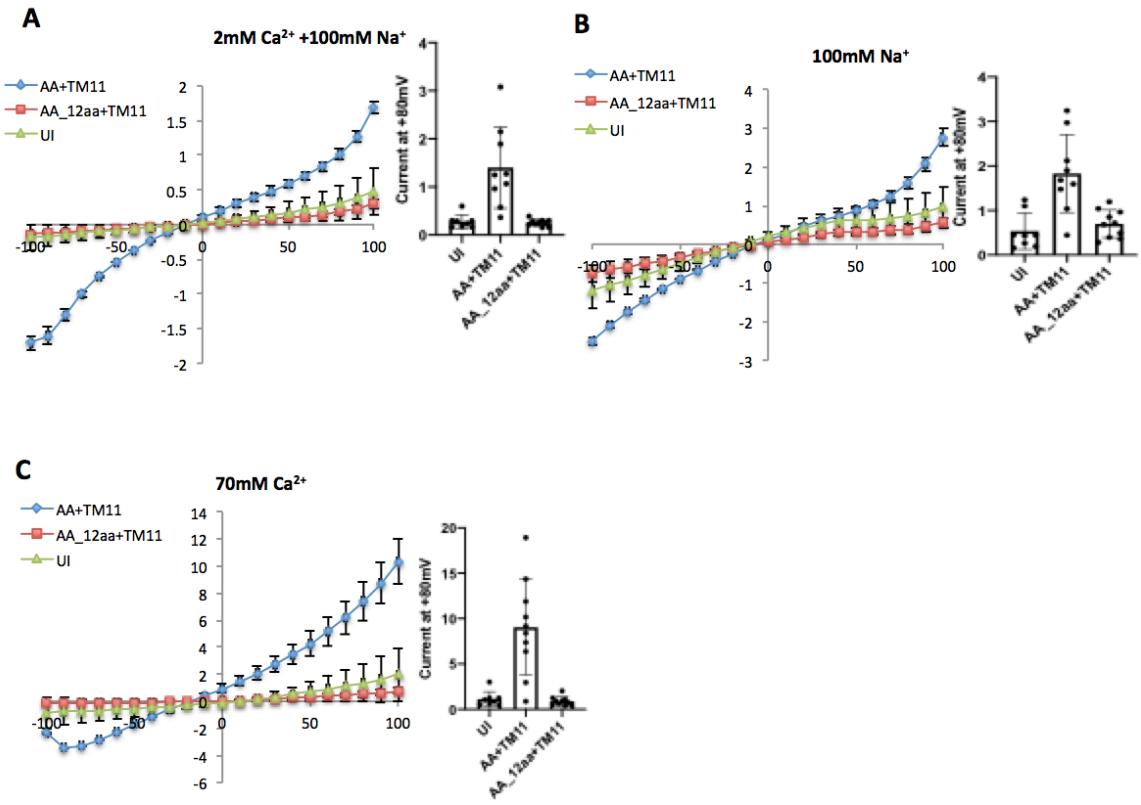

Figure 6. The 12 aa mutant reduces the channel function of PC1-CTF/TRPP2-AA. Representative I–V curve (left) and bar graph at +80mV (right) showing current sizes in bath solutions containing 2mM Ca^{2+}/100mM Na^{+}**(A),** 100mM Na^{+} **(B)** and 70mM Ca^{2+} **(C)** respectively.

3.3 Discussion and Future Direction

Our laboratory has generated GOF TRPP2 mutants to facilitate its study further.

It provides a tool to study the pathogenic mutations of ADPKD. In our results, the 12aa mutation led to a significant reduction on channel activity of TRPP2-F604P. Immunoblot analysis shows the 12 aa mutant reduces the expression of TRPP2. Also, we observe

more oligomers in TRPP2-F604P-12aa compared to the wild-type. The additional twelve amino acids on the C-terminus of the TRPP2 protein seems to exert a pathogenic effect reducing the TRPP2 ion channel activity and may also affect the folding and/or assembly of the protein. The additional twelve amino acids that have reduced the expression may be caused by the conformational change due to the higher molecular weight bands. This changed conformation leads to more degradation. Due to the high oligomers, it would be necessary to run a Blue Native-PAGE to analyze how the 12aa mutant affects protein assembly.

Additionally, we have shown that the 12 aa mutant reduces the surface expression of TRPP2, an effect may be related to its effect on protein folding/assembly. This information will provide more insight of these pathogenic mutations affecting the proteins underlying ADPKD.

Studying the PC1/TRPP2 channel would gain more insight into the proteins involved in the complex, since the complex is essential for renal tubular differentiation. The function of this complex is unknown due to the lack of a functional assay. Therefore, GOF mutants were created to dissect its role. However, the GOF F604P was unable to open the PC1/PC2 channel, hence, the intent to create a new GOF, TRPP2-L677A-N681A. Co-expressing the 12aa mutation with PC1-CTF/TRPP2-AA, allowed us to understand the affect of this mutation on this channel, and it exhibited a lower current size. However, a further investigation and surface and western expression is required to make a solidified conclusion of how the 12aa mutation affects the PC1/TRPP2 complex channel.

CHAPTER 4

THE EFFECT OF STUM PROTEIN ON PC1/TRPP2 CHANNEL FUNCTION

4.1 The effect of co-expressing C1orf95 on the activity of the PC1-CTF/TRPP2-AA complex channel

The STUM gene, short for stumble, is a mechanosensory transduction mediator homolog, first identified in *Drosophila*, due to a walking impairment. Diseases associated with STUM include, Shwartz-Jampel Sydrome, Type 1 and Charcot-Marie-Tooth Disease, Axonal, Type 2N. The human ortholog of the STUM gene is a part of the SPEC3 family, C1orf95 (Chromosome 1 Open Reading Frame 95). Two isoforms of C1orf95 exist within the human kidney with difference in exon 3 inclusion. Here we named them C1orf95+E3 (exon 3 present) and C1orf95-E3 (no exon 3 skipped). It has been reasoned that this protein co-localizes with PKD1 and ESCRT (data not shown). Our collaborator, Dr. Christopher Ward, at University of Kansas Medical Center found that both C1orf95 present in exosomes in human urine and can be co-IPed with PC1 (personal communication). In order to study the role of C1rof95 in regulating PC1/TRPP2 complex channel, we co-expressed V5-tagged C1orf95+E3 and C1orf95-E3 with the PC1/TRPP2 complex in oocytes and measured the functional change of the channel. In order to record the channel current, we have used a short PC1 C-terminal fragment (named TM11) and TRPP2-L677A-N681A (P2-AA) GOF mutant generated in our laboratory (Wang et al, 2019).

All three co-expressed proteins were measured as the same total RNA concentration, 1μg/μl. P2-AA /TM11 (1μg/μl), P2-AA/TM11/C1orf95-E3 (1μg/μl), and P2-AA/TM11/C1orf95-E3 (1μg/μl). Our expression resulted in the STUM protein at approximately 20 kDa and all three groups exhibited similar expression (**Fig 7A and 7B**). However, all three groups exhibit a similar current pattern and size in multiple bath solutions, including low Ca^{2+} (100 mM Na^{+}, 2mM Ca^{2+}) and high Ca^{2+} (70mM Ca^{2+}), and in Ca^{2+}-free (100mM Na^{+}) extracellular solutions (**Fig. 8 A-C**). The representative current shows no significant difference between the co-expressed groups.

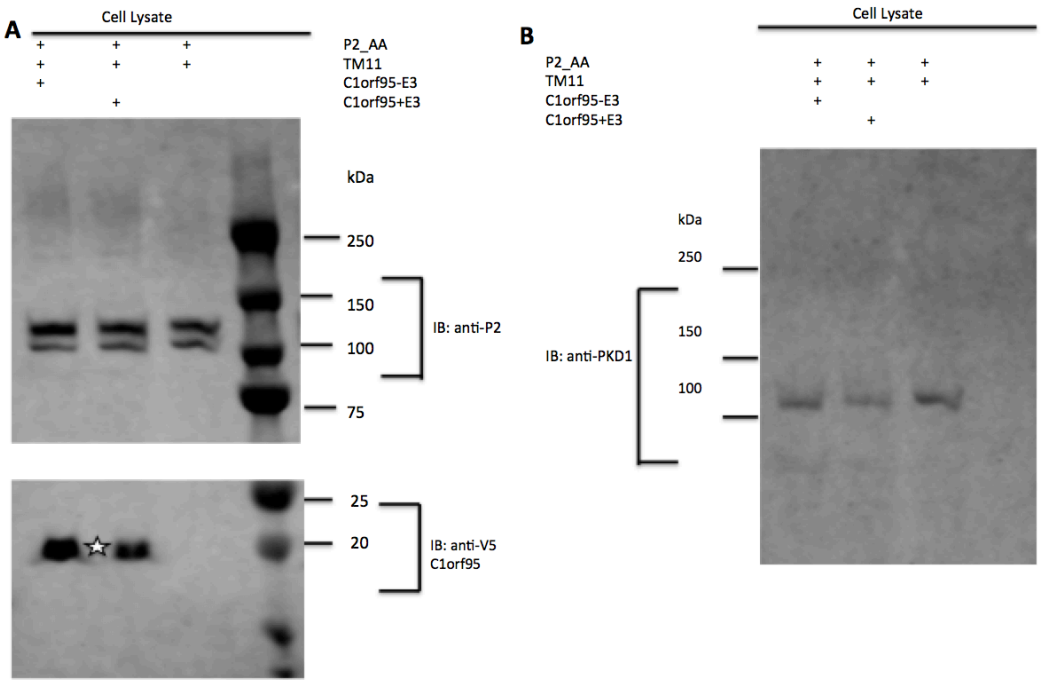

Figure 7. Co-expression of STUM with PC1-CTF/TRPP2-AA (A) Immunoblot analysis of co-expressed proteins with PKD2 and V5 antibody. P2 bands expressed between 100-130kDa and STUM bands expressed at 20kDa. **(B)** Immunoblot analysis of co-expressed proteins with PKD1 antibody. P1 bands expressed at 110kDa.

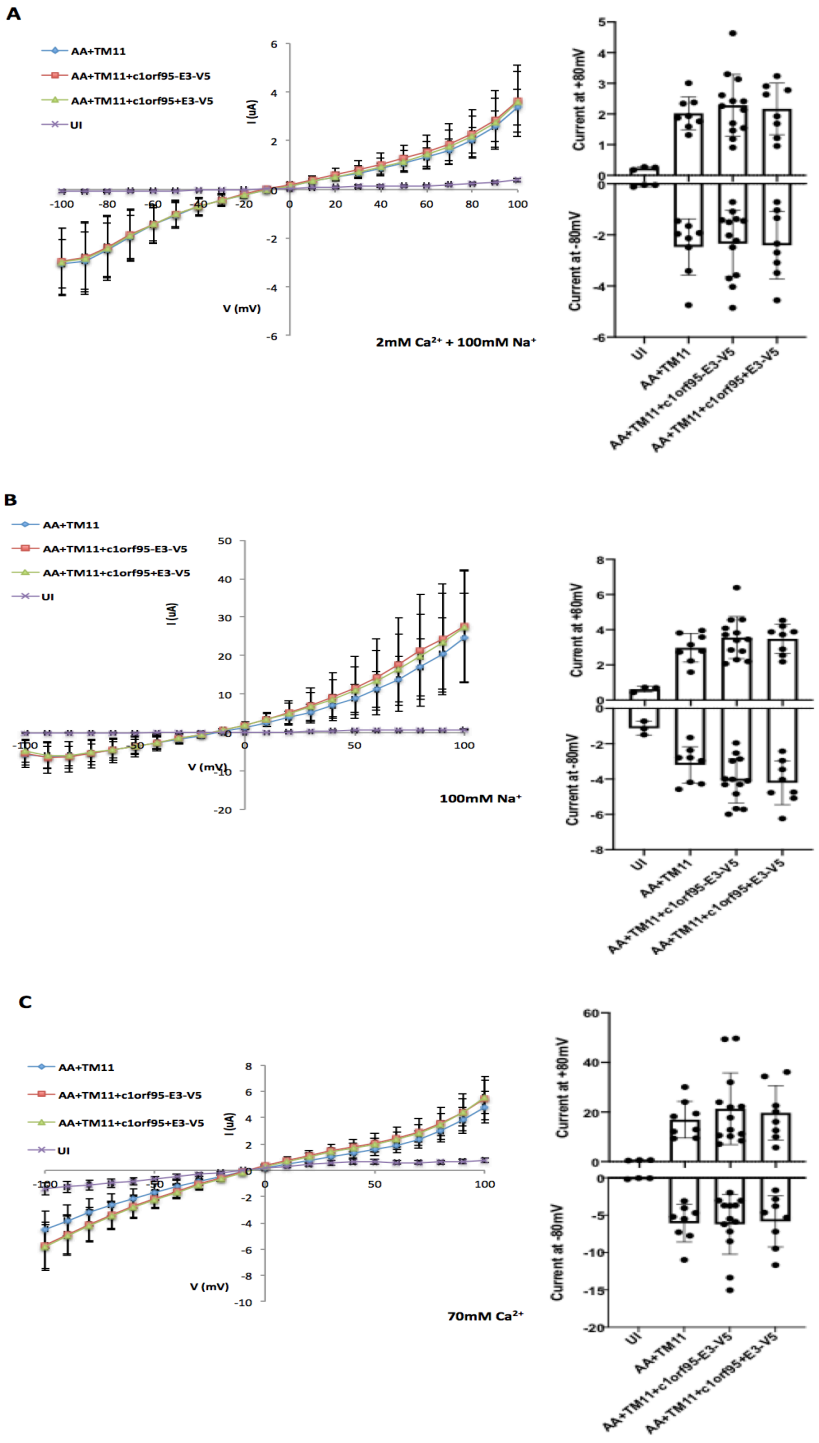

Figure 8. Co-expression of C1orf95 proteins does not affect the PC1-CTF/TRPP2-AA ion channel activity. Average I-V curves (left) and bar graphs (right) show the currents of P2-AA/TM11, n=8 (blue), P2-AA/TM11/C1orf95-E3, n=14 (red), P2-AA/TM11/C1orf95+E3, n=8 (green), and un-injected oocytes (purple) in 2mM Ca^{2+} + 100mM Na^{+} (**A**), 100mM Na^{+} (**B**), and 70mM Ca^{2+} (**C**) extracellular solutions**.**

4.2 Discussion and Future Direction

Our immunoblot analysis shows similar expression for P2-AA/TM11, P2-AA/TM11/C1orf95+E3 and P2-AA/TM11/C1orf95-E3 samples (**Fig. 7**). Although, our collaborator found an interaction with C1orf95 with the PC1/TRPP2 complex of kidney exosomes, we were not able to find an effect of co-expressing C1orf95 on channel activity. However, we cannot rule out the possibilities that C1orf 95 may be involved in signaling pathways induced by PC1/TRPP2 complex and play a role in ADPKD.

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