

TRANSMEMBRANE PROTEIN 2: A NOVEL PROTEIN IN POLYCYSTIC KIDNEY
DISEASE

BY

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TRANSMEMBRANE PROTEIN 2: A NOVEL PROTEIN IN POLYCYSTIC KIDNEY DISEASE

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ABSTRACT

Background: Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common hereditary cause of end stage renal disease. Affected individuals have mutations in *PKD1* or *PKD2* genes, generating defective polycystin -1 (PC1) or polycystin -2 (PC2) protein, respectively. Transmembrane protein 2 (TMEM2) is a novel protein with extensive homology to fibrocystin, the product of the polycystic the polycystic kidney and hepatic disease (*PKHD1*) gene. Mutations in *PKHD1* cause Autosomal Recessive Polycystic Kidney Disease. It has been shown that urinary exosomes from individuals with *PKD1* mutations have decreased levels of PC1 and increased levels of TMEM2 when compared with individuals with no mutations in the PKD genes. We sought to determine the expression status of TMEM2 in ADPKD and whether TMEM2 interacts with PC1.

Methods: Immunohistochemistry (IHC): Sections from ADPKD human kidney tissues and normal human kidney (NHK) tissues were probed for presence of TMEM2 and PC1.

Immunofluorescence: Cells from ADPKD and normal human kidneys were grown until confluent and probed with TMEM2 and tubulin.

Immunoprecipitations (IP): HEK293T cells were co-transfected with full length V5 tagged TMEM2 construct, C terminus FLAG tagged PC1 construct and various FLAG tagged N terminus PC1 constructs. Co-IPs were performed on membrane preparations derived from the transfectants.

Results: Increased TMEM2 expression was detected on the apical aspect of cyst epithelial cells in ADPKD kidneys when compared to normal human kidneys. There was an increase in cellular expression and colocalization of TMEM2 to ciliary structures in ADPKD cells compared to NHK cells. Co-IPs showed that there was an interaction between PC1 and TMEM2. The interaction was mapped to the N-terminal extra-cellular portion of PC1 but not the C terminus. TMEM2 appeared to have high affinity for the PKD and REJ domains within N terminal portion of PC1.

Conclusion: Increased TMEM2 expression in ADPKD kidneys and urinary exosomes combined with the interaction of TMEM2 with PC1 suggests that TMEM2 is a novel protein implicated in the pathogenesis of ADPKD. The exosomal PC1/TMEM2 ratio may have utility in the diagnosis of pre-cystic disease as well as in monitoring the disease progression and response to treatment.

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INTRODUCTION

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common hereditary cause of end stage renal disease (ESRD). The prevalence of the disease is 1:400 to in 1:1000 causing 5-7% of ESRD in North America^{1,2}. The disease is characterized by progressive cyst growth in the kidney causing compression of surrounding tissue and an inflammatory response. Slowly the cyst and the secondary response to the cyst will destroy all of the normal renal parenchyma. 77% of patients with ADPKD will reach end stage renal disease or death by the age of 70¹.

While the mutations are identified, the exact mechanism for cystogenesis is unknown. Affected individuals have mutations in *PKD1* or *PKD2* genes, generating defective polycystin -1 (PC1) or polycystin -2 (PC2) protein³. Mutations in *PKD1/PC1* are more common and accounting for 85% of patients with ADPKD. Mutations in *PKD2/PC2* cause a milder form of the disease than mutations in *PKD1/PC1*³. It is thought that PC1 and PC2 form a complex with calcium channel activity¹. Though most mutations and implicated proteins are known in ADPKD, there is still a significant knowledge gap about mechanism and progression of the disease.

Recent data has shown that the protein Transmembrane Protein 2 (TMEM-2) may play a role in ADPKD. TMEM2 is increased on urinary exosomes from individuals with *PKD1* mutations when compared with individuals who had no mutations in the *PKD1*⁴. TMEM2 has extensive homology to fibrocystin's extracellular domain. Fibrocystin, the product of the polycystic kidney and hepatic disease (PKHD1) gene, is found to cause Autosomal Recessive Polycystic Kidney Disease (ARPKD) when mutated; a rare but severe form of neonatal polycystic kidney disease⁵. Mutations in the G8 domain in PKHD1 will cause ARPKD. The G8 domain is a parallel β helix region, which TMEM2 and fibrocystin happen to share⁶.

TMEM2 is a novel membrane protein comprised of 1383 amino acid (154.4 kDa) in size⁷. It has a large extracellular C-terminal domain (1280 amino acids) and small N-terminal intracellular domain (82 amino acids), a type II membrane protein (Figure 1). TMEM2 is

necessary for cardiac development. Mutations in the zebrafish TMEM2 gene can cause 'frozen ventricle' or 'wickham' phenotypes where there is malformation of the AV cords⁸. In humans a single nucleotide polymorphism (SNP) in TMEM2 p.Ser1254Asn increases a person's chance to develop chronic hepatitis B (CHB) in the Chinese population⁹. This association was investigated further using cell lines and liver tissue, and researchers found that CHB liver tissue and hepatitis-infected cell lines had reduced expression of TMEM2 when compared to uninfected controls⁹.

As a little studied protein, the role of TMEM2 and its role in polycystic kidney disease is not known. We sought to determine the expression status of TMEM2 in ADPKD as well as evaluate whether TMEM2 interacts with PC1.

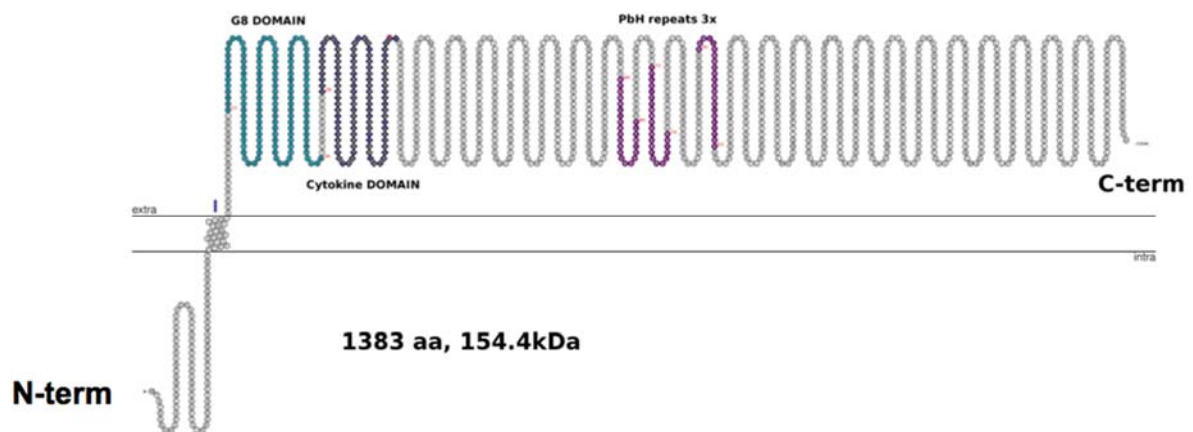


Figure 1. Diagram of TMEM2

METHODS

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tissue from normal and ADPKD human kidneys obtained from the PKD repository at University of Kansas Medical Center. Antigen retrieval was done after de-paraffinization. The slides were steamed in citrate buffer (10mM sodium citrate, 0.05% tween 20, pH: 6.0) and cooled to room temperature. 3% hydrogen peroxide was added to the tissues for 30 minutes then washed 2 times in phosphate buffered solution (PBS) to avoid endogenous peroxidase. The slides were blocked with 10% serum from the species that the secondary antibody was made in for one hour at room temperature and then probed with a polyclonal rabbit anti-human TMEM2 antibody (Sigma) at a dilution ranging from 1:25-1:100 dilution at 4^o C overnight. Slides were washed three times in PBS for 5 minutes each followed by a one hour incubation in biotinylated anti rabbit antibody (1:400 dilution). Slides were again washed and ABC substrate (25 μ lA+25 μ lB in 1.25ml PBS) from Vector Labs was added on to the tissues for 30 minutes. The slides were next washed again and then developed by using DAB (3,3'-Diaminobenzidine) (Sigma). Slides were coverslipped with paramount after dehydrating for 2 min each with increasing concentrations of ethanol followed by 2 xylene steps (30%, 60%, 80%, 95%, 100%, Xylene). Negative controls were done using phosphate buffered saline (PBS) replacing the first antibody preparation. Lieca microscope was used to take pictures under 20X magnification.

Immunofluorescence

Normal human kidney (NHK) cells and ADPKD cells from the PKD repository at University of Kansas Medical Center that is run by Dr. Darren Wallace were grown to confluency on slides. The cells were fixed in 4% paraformaldehyde (made in PBS) for fifteen minutes, washed in 100mM ammonium chloride for five minutes twice, and then permeabilized in 0.05% Triton in PBS for five minutes. The slides were blocked with for one hour with 10% serum from the species that the secondary was made in. Slides were incubated overnight at 4^o C in primary

antibody (Anti-TMEM2 1:50 (Sigma) and anti-acetylated tubulin 1:3000 (Sigma)). The slides were then washed three times in PBS. Secondary Alexa Fluor® (Life Technologies) antibodies were used at 1:400 dilution and incubated for one hour at room temperature and then washed three times with PBS for five minutes each. The mounting was done with Vectashield reagent with DAPI (Vector Labs.). Confocal microscopy (Leica) was used to view cells.

Cell lysates

Normal human kidney cells and ADPKD cells from the PKD repository at University of Kansas Medical Center were grown until visibly confluent. The cells were washed with PBS three times. The cells were then scraped off the plates and pelleted by centrifuging at 1500xg for fifteen minutes. Cell lysis was done using RIPA buffer (50 mM Tris HCl pH7.5, 137 mM NaCl, 1% IGEPAL, 2mM EDTA, and Complete Protease Inhibitor) and samples were analyzed on a Western blot.

Transfections

HEK293T cells were grown until 70% confluent. The media was replaced with serum free media for at least two hours prior to transfection. Constructs were transfected using Polyethylenimine (PEI) and the 2:15 ratio of DNA: PEI (8µg of TMEM2-V5 and 16µg of FLAG tag PC1 construct per 15 cm plate of cells). DNA and PEI were diluted with OPTIMEM media resulting in a final volume of 1mL per each 15 cm plate of cells. The mixture incubated at room temperature for 15 minutes then was added to the cells.

Membrane preparation

Transfected cells were washed three times using pre-chilled PBS eighteen hours after transfection. The cells were then scraped off the plates and pelleted by centrifuging at 1500xg for fifteen minutes. The pellet was taken up in a cocktail of 10 mM Tris-HCl pH 7.5, 0.5 mM MgCl₂, and protease inhibitor. The suspension was frozen at -80°C overnight, thawed on ice, and then homogenized with forty strokes of a dounce homogenizer. Sample was centrifuged at 5000xg for 10 minutes. The supernatant from that sample was again centrifuged at 18,000xG

for one hour. The membrane pellet was taken up in IP buffer composed of 50 mM Tris HCl pH7.5, 137 mM NaCl, 1% IGEPAL, 2mM EDTA, and protease inhibitor.

Co-immunoprecipitation

Membrane preparation, as described above, was used for the CO-IP. Each sample constituted lysate from 15 cm plate. Samples were pre-cleared by adding 2 µg IgG1 isotype (Thermo Scientific) for two hours on ice. Pierce Protein A/G Agarose Resin (Thermo Scientific) followed; 25 µl of resin slurry added to each sample and rocked at 4°C for thirty minutes. The resin was quickly pelleted and supernatant divided into CO-IP and negative controls. 2µg monoclonal antibody was used (V5 (IgG2a Isotype)(Bio-Rad), FLAG M2 (IgG1a isotype) (Sigma) IgG1 isotype control (Thermo Scientific), and IgG2a Isotype Control (Sigma). The sample was incubated at 4°C overnight while rocking. A/G resin were washed with 1ml IP buffer (50 mM Tris HCl pH7.5, 137 mM NaCl , 1% IGEPAL, 2mM EDTA, and Protease Inhibitor) three times. Then 60 µl slurry/IP was then added to the sample and rocked at 4°C for two hours. The sample was washed three times with wash buffer (50 mM Tris HCl pH 7.5, 500 mM NaCl , 1% IGEPAL, 2 mM EDTA, Protease inhibitor, and 0.2% SDS). After the last wash all of the supernatant was removed and samples were prepared for Western blotting.

Western Blotting

Samples from CO-IP were mixed with 4x NuPage (Novex) sample buffer (containing 25% tris(2-carboxyethyl) phosphine (TCEP)) heated to 65°C for ten minutes and loaded on a denaturing 4-12% MOPS gel (Invitrogen). The gel was allowed to run at 200 V for 50 minutes. The blots were transferred at 4°C using thirty volts for seven hours on Immobilon-P polyvinylidene difluoride (PVDF) 0.45 mm pore size (EMD Millipore) membrane. The blots were blocked in TBST (20 mM TRIS, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) with 5% milk for one hour. Blots were probed with primary antibody at 1:2000 dilution in TBST with 2.5% milk overnight. A monoclonal mouse anti-human PC1 mAb (7e12 IgG1k) was used for PC1, V5 tagged antibody directly conjugated with horseradish peroxidase (HRP) antibody (BioRad), and

FLAG tagged antibody directly conjugated with HRP antibodies were used (Sigma). The blots were washed with TBST three times a minimum of 5 minutes each. Secondary antibody, if used, was diluted (1:5000) human absorbed anti-mouse IgG1-HRP (Southern Biotech) for one hour at room temperature. The blot was imaged on GE Life Science AI600 Imager using West Femto Maximum Sensitivity Substrate (Thermo Scientific).

RESULTS

Localization of TMEM2

Since TMEM2 was shown to be increased in urinary exosomes from ADPKD patients in mass spectrometry studies we first determined the tissue localization of TMEM2⁴. Immunohistochemistry (IHC) was done on at least three different normal human kidney (NHK) and ADPKD tissues. IHC results showed an increased amount of TMEM2 in ADPKD (Figure 2). Western blot analysis of cell lysates confirmed higher expression of TMEM2 from NHK and ADPKD cells. The lysates show noticeable increase in TMEM2 in the ADPKD cells compared to the NHK cells (Figure 3). Light microscopy of cystic areas showed that there was locally enhanced expression in the cystic epithelial cells in the ADPKD kidney tissues (Figure 4). To further confirm the increase TMEM2 in ADPKD, we performed immunofluorescence on isolated cells from NHK and cystic cells from kidneys from patients with ADPKD. These cells were allowed to grow to confluency in culture so that they formed a monolayer before fixing them for labeling. Immunofluorescence showed increased expression of TMEM2 in ADPKD cells compared to NHK cells. Most of the staining was cytoplasmic in distribution in both cell types. NHK cells had two distinct distributions of TMEM2; most cells having minimal staining for TMEM2 but there were rare clusters of cells with increased signal (Figure 5). Cells were also stained for tubulin and TMEM2 to evaluate if TMEM2 localized to the primary cilium, the primary location of PC1. In NHK cells there was sporadic localization of TMEM2 to the cilia with most cilia not having any detectable TMEM2 on them. The ADPKD cells had significant localization of TMEM2 to the cilia (Figure 6).

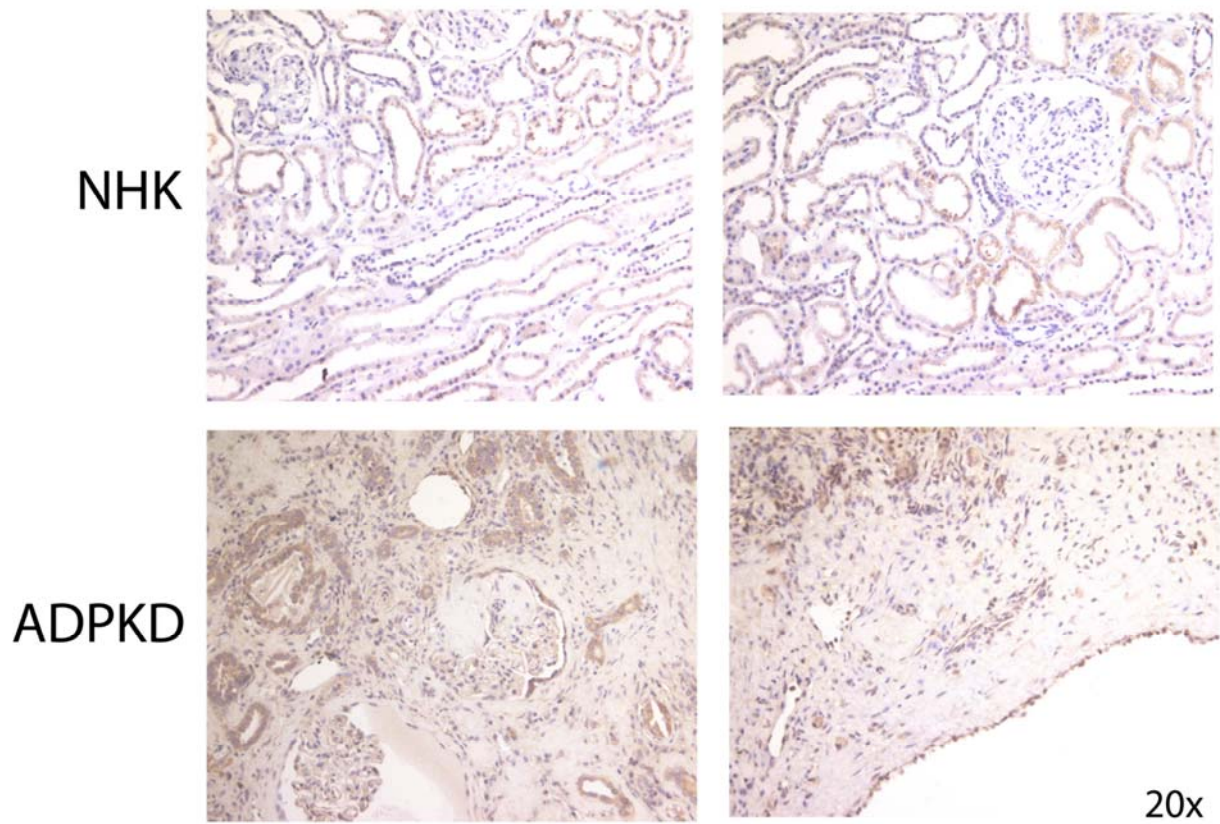


Figure 2. TMEM2 by immunohistochemistry showing increased diffuse staining of TMEM2 in the ADPKD kidney tissue compared to the NHK kidney tissues

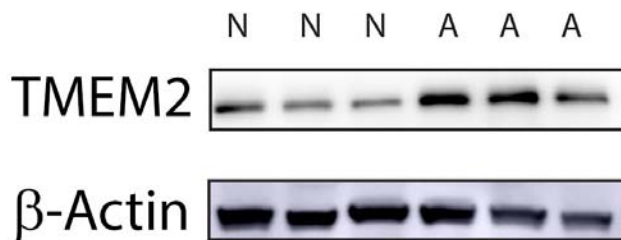


Figure 3. Western blot analysis of cell lysates from 3 normal (N) and 3 ADPKD (A) human kidneys showing that TMEM2 levels are higher in ADPKD than NHK.

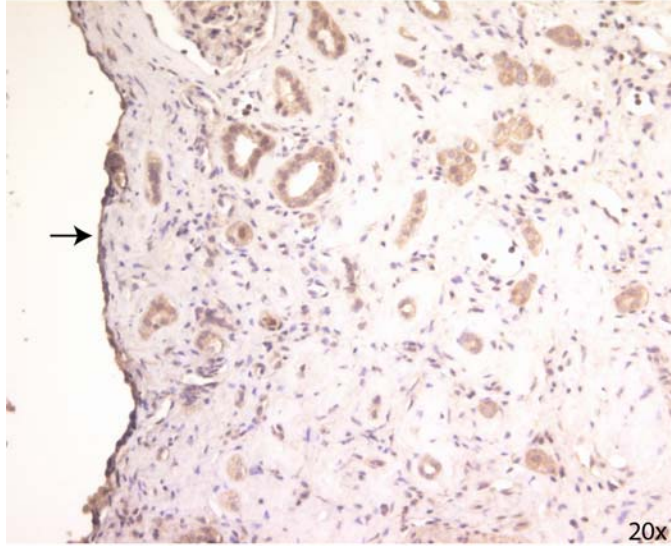


Figure 4. Immunohistochemistry done on ADPKD human kidney tissue showing locally enhanced staining seen in the cyst lining; demarcated by the arrow.

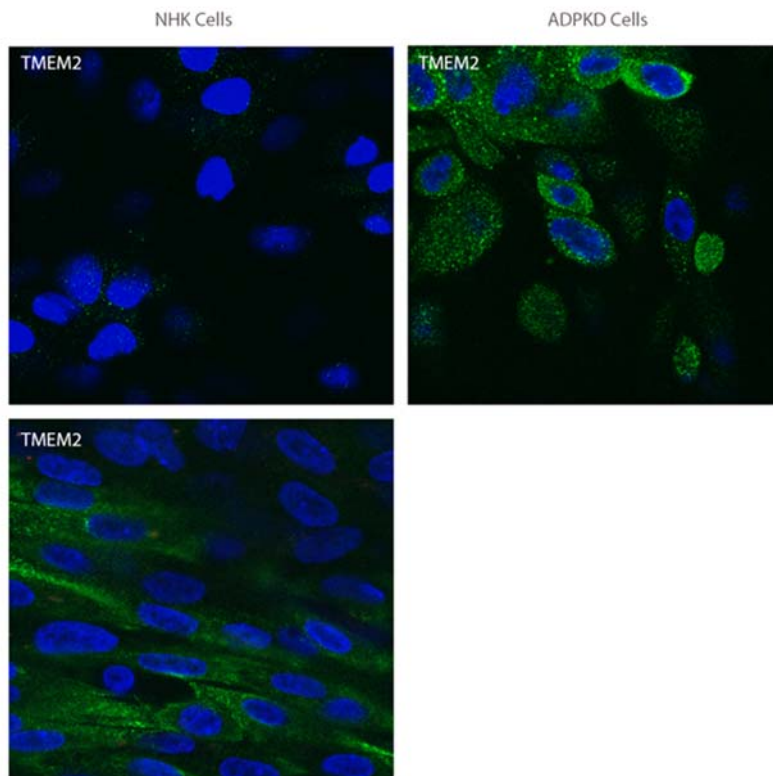


Figure 5. Immunocytochemistry on NHK and ADPKD cells shows an increase in TMEM2 in the ADPKD cells. There were two distinct staining patterns of TMEM2 in the NHK cells. The top image shows the pattern seen majority of the time. The bottom image shows the rare cluster of cell that had increased TMEM2 staining

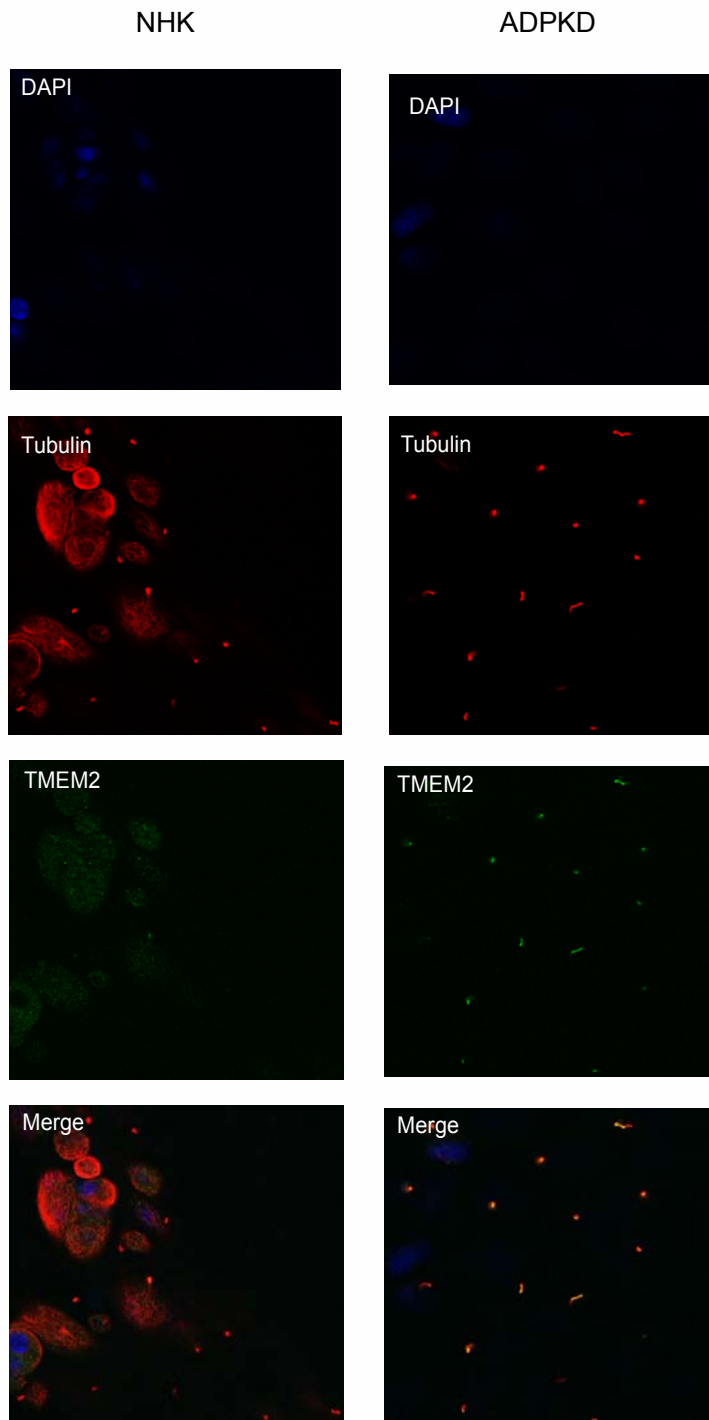


Figure 6. Immunocytochemistry and staining for tubulin and TMEM2 showed that there is sporadic TMEM2 and tubulin colocalization in NHK cells but significant TMEM2 and tubulin colocalization in ADPKD.

TMEM2 and PC1 interaction

The colocalization of PC1 and TMEM2 on the primary cilium led us to hypothesize that the two proteins may physically interact¹⁰. Transient expression of the V5:TMEM2 construct in HEK293T cells shows that TMEM2 was capable of interacting with endogenous Polycystin -1 (PC1), as shown by IP (Figure 7). To further narrow down the regions of PC1 that interacted with PC1, we made two FLAG tagged constructs of PC1, the NTF (extracellular) domain and the CTF (intracellular) domain (Figure 8). Each of these constructs was transiently co-transfected with TMEM2, again in HEK293T cells. The COIP showed that TMEM2 was interacting exclusively with the NTF region and not the CTF region (Figure 9). Short constructs were then made of the entire NTF region and FLAG tagged to further map out the interaction region, refer to Figure 10 to see which constructs were transfected and Figure 8 for the location of the construct on PC1. Interactions were found with PKD 2-3, 5-7, 7-8, 14-15, 15-17, and the REJ:GAIN domain. There was no interaction seen with LDL repeat or the N-terminal to the C-type lectin (Figure 10). This *in vitro* evidence shows that TMEM2 interacts with the PKD repeats and the REJ: GAIN domain. This multi-level interaction needs to be further investigated.

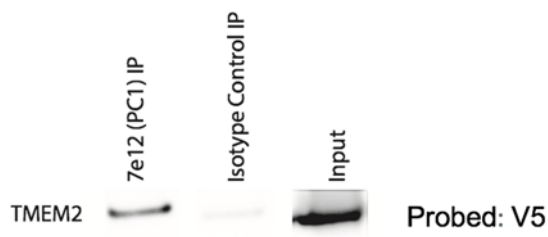


Figure 7. CO-IP done of transiently expressed V5:TMEM2 indicates an interaction with endogenous PC1.

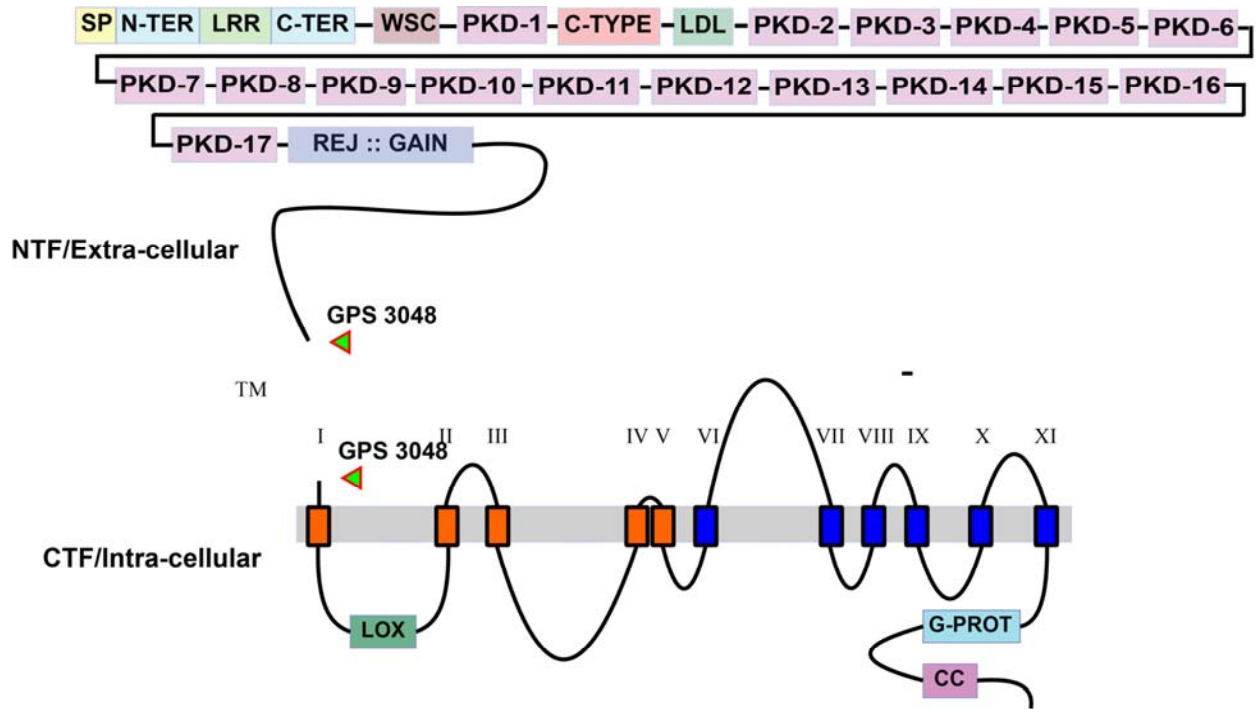


Figure 8. Diagram of Polycystin 1 separated in to two segments the NTF or extracellular and the CTF the intracellular segment.

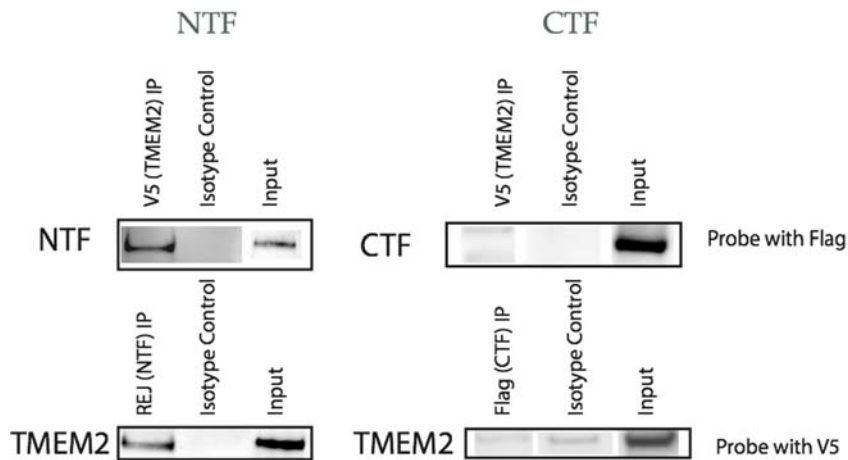


Figure 9. CO-IP of membranes from HEK293T cells transiently transfected with FLAG tagged PCI-1 construct and V5-TMEM2 that shows TMEM2 is interacting with the NTF segment of PC1 and not the CTF segment.

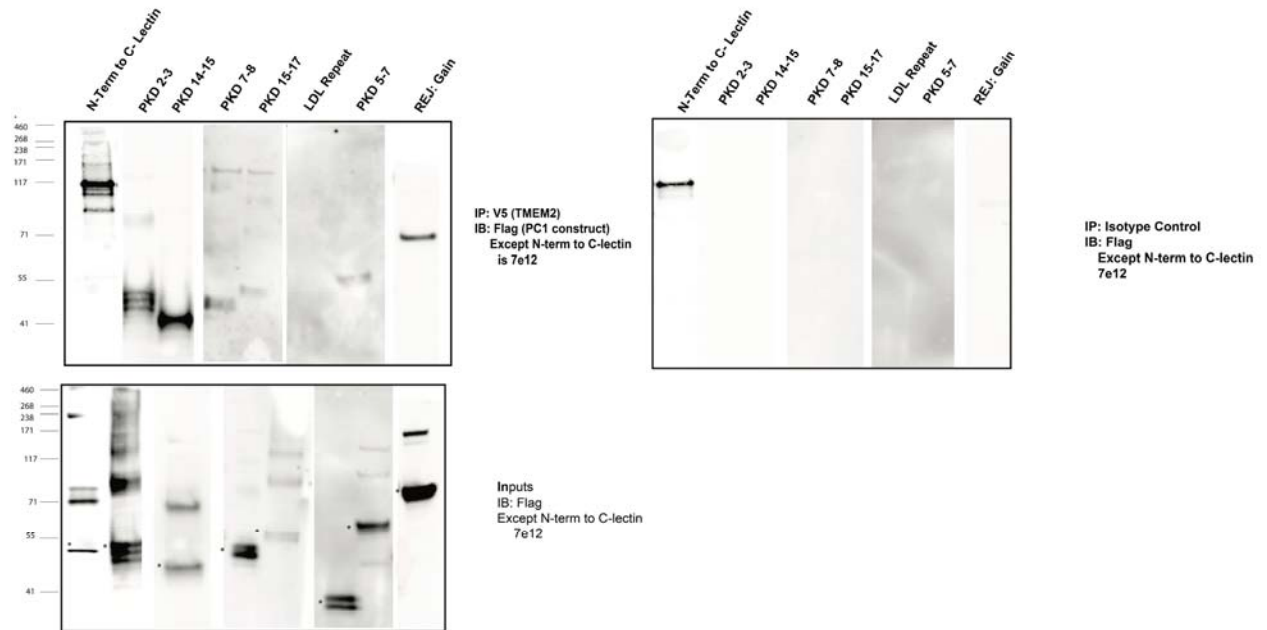


Figure 10. CO-IP from PC1:FLAG tag constructs and V5:TMEM2 transiently transfected HEK293T cell membranes that show TMEM2 is interacting with the PKD repeats. * Demarcates the predicted band from the input that the construct should be.

DISCUSSION

TMEM2 is increased on the urinary exosomes of patients with ADPKD when compared to non-disease controls⁴. Our experiments presented here support that patients with ADPKD have increased TMEM2. TMEM2 is more abundant in ADPKD cells compared to normal human kidney cells. The distribution also changes in ADPKD cells with TMEM2 localizing to the cilia at a higher frequency than in normal human kidney cells. In patient tissue samples, TMEM2 is detectable in the cystic lining. The mechanism for the observed increase in TMEM2 abundance in ADPKD is obscure. It is possible that the increase occurs as a protective physiological response to lower levels of PC1 protein and that TMEM2 can complement decreases in PC1 levels. Alternatively PC1 and TMEM2 may reciprocally model each other's function by direct interaction. For example, PC1 could target TMEM2 for degradation, therefore a lack of PC1 allows for a deleterious rise in TMEM2 activity.

There is limited knowledge about the whole polycystin complex and the role it plays in ADPKD is even less understood. It is known that PC1 and PC2 interact and form a calcium channel that plays a role in mechanosensing¹⁰, and while other functions of the proteins have been proposed, nothing is agreed upon in the field. Other proteins have been found to be part of this Polycystin complex, these include E-Cadherin, catenins¹¹, and importantly, fibrocystin. Wang et al showed that fibrocystin is a part of this large protein complex and interacts with PC1¹². There is homology between the extracellular domain of TMEM2 and fibrocystin⁵. This indicates that TMEM2 is most likely a protein component to the larger polycystin complex.

TMEM2 interacts with the PKD repeats of PC1. 17 copies of the PKD repeats are found in the NTF region of PC1, and these repeats are 80 amino acids long β sheets¹³. Not much is known about the function of the PKD repeats in the NTF segment of PC1. It has been shown that the PKD repeats can interact with themselves, but no other interaction has been demonstrated in the literature¹⁴. There appears to be multiple points of interaction between TMEM2 and PC1; the PKD and REJ:GAIN domain. Multiple interaction sites for the proteins

have been seen in other protein complexes; the senilin complex is one well-known example of this. γ -Secretase and Pen-2 two proteins in that complex have 2 different points of interaction¹⁵. PC1 and TMEM2 were found to be on the same exosome fraction, implying that they are on the same exosomes, which further supports that there is an interaction occurring between these proteins⁴. Further work will be required to see the sites on TMEM2 that are necessary and sufficient to bind PC1.

The localization of TMEM2 changes between ADPKD and NHK. There is not only increased expression in ADPKD, but also an increase in localization of TMEM2 to primary cilium. Primary cilia are thought to play an essential role in the development of ADPKD, as loss of cilia result in cystogenesis and many proteins of interest in PKD localize to the primary cilia. It is unclear if TMEM2 is being retained in the cilia or if there is increased trafficking of TMEM2 to the cilia. Though PC1 is a widely distributed protein, it is prominently expressed on the primary cilium¹⁰. Thus cilia seem a likely candidate for the location of the TMEM2 and PC1 interaction.

Overall we conclude that TMEM2 is increased in ADPKD and is interacting with the PKD repeats in PC1 and the REJ:GAIN domain. Further studies are required to know the function of TMEM2 and what possible role it may play in ADPKD disease progression and treatment.

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