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Evidence that TMEM67 causes polycystic kidney disease through activation of JNK/ERK-dependent pathways

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Abstract

TMEM67 mutations are associated with severe autosomal recessive polycystic kidney disease (ARPKD) in both humans and animals. However, the molecular mechanisms underlying the pathogenesis of PKD caused by TMEM67 mutations remain to be determined. We have investigated the possible signaling pathways involved in the pathogenesis of PKD. Overexpression of TMEM67 in human embryonic kidney (HEK293) cells triggered the activation of overall tyrosine phosphorylated proteins, Extracellular Signal-Regulated Kinase (ERK) and c-Jun N-terminal kinase (JNK). Activation was suppressed by pharmacological inhibitors of ERK or JNK. Activation of the mammalian target of rapamycin (mTOR) or p70s kinase (S6K) did not occur, although elevated phosphorylation of eIF4E-binding protein 1 (4E-BP1), a target of S6K, was seen. In animal studies, activation of a variety of signaling molecules was linked to ERK, JNK and 4E-BP1. Significant induction of phosphorylation of tyrosine phosphorylated proteins, ERK and 4E-BP1, at different postnatal ages was detected in mutant kidneys of B6C3Fe a/a-*bpck* mice, a cystic renal disease mouse model caused by TMEM67 loss of function mutation. Based on these *in vitro* and *in vivo* observations, we propose that TMEM67 mutations cause PKD through ERK- and JNK-dependent signaling pathways, which may provide novel insight into the therapy of polycystic kidney diseases.

Keywords

TMEM67; polycystic kidney disease; cell signaling pathway; *bpck* mice

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Author contributions

R.H, and M.Q designed and supervised the experiments and contributed equally in grant funding to the study. E.D and H.L generated most of the data for the manuscript. S.J performed data analysis. X.H maintained mouse lines and genotyped mutant animals. H.L wrote and M.Q revised the manuscript.

Competing interests

The authors declare that they have no competing interests.

Introduction

Polycystic kidney disease (PKD) is one of the most common disorders in humans caused by mutations in a single gene. There are two types of PKD: Autosomal Dominant Polycystic Kidney Disease (ADPKD) and the less-common Autosomal Recessive Polycystic Kidney (ARPKD). TMEM67 encodes a 995 amino acid transmembrane receptor protein, which is composed of a signal peptide, at least 2 cysteine-rich repeats, and a 490-residue extracellular region with 4 N-linked glycosylated sites, followed by 7 transmembrane domains and a 30-residue cytoplasmic tail (Smith et al., 2006). The mutations of TMEM67 are a cause of Meckel syndrome type 3 (MKS3) (Smith et al., 2006) and Joubert syndrome type 6 (JBTS6) (Baala et al, 2007). Both are autosomal recessive diseases and display a common and overlapping clinical phenotype of cystic dysplasia within the kidneys.

Signaling mechanisms underlying the pathogenesis of PKD have been under intensive investigation as intervention may slow cyst growth and thereby delay the onset of renal failure. Activation of the mammalian target of rapamycin (mTOR, a serine/threonine protein kinase) is a common feature of PKD (Ibraghimov-Beskrovnaya and Natoli, 2011). Upregulation of mTOR signaling has been detected both in mice and in human with ADPKD (Shillingford et al., 2006) or ARPKD (Fischer et al., 2009; Becker et al., 2010). ERK is activated in primary cultured cyst epithelial cells from autosomal-dominant polycystic kidneys (Yamaguchi et al., 2003) and in PKD animal models (Nagao et al, 2003). A role for meckelin, TMEM67 gene product is involved in Wnt/PCP signaling (Leitch et al., 2008), but another report linked meckelin to the RhoA signaling pathway (Dawe et al., 2009). However, the precise mechanisms underlying TMEM67-associated ARPKD remain largely unknown. We have investigated the potential signaling mechanisms involved in the pathogenesis of PKD, and propose that TMEM67 mutations cause PKD through ERK- and JNK-dependent signaling pathways. This may provide new insight into the selection of pharmacological targets in the therapy of polycystic kidney disease.

Materials and Methods

Animal handling and Genotyping

B6C3Fe a/a-*bpck* mice were purchased from the Jackson Laboratory and maintained at the Research and Resource Center at University of Louisville. Animal care and experimental procedures conformed to National Institutes of Health guidelines, approved by the Institutional Animal Care and Use Committee at the University of Louisville (protocol # 09014), Louisville, KY, USA. Genotyping was done in accordance with the protocol of Jackson Laboratory.

RNA extraction and construct of TMEM67expression vector

Total RNA was extracted from kidneys of postnatal days 3 (P3) mice using a monophasic solution of phenol/guanidine isothiocyanate and TRIzol reagent (Invitrogen, Carlsbad, CA), and the samples were incubated with RNase-free DNase I (Ambion). The quality and concentration of each sample were confirmed by spectrophotometry (NanoDrop ND-1000; Asahi Glass, Tokyo, Japan). Reverse transcription was done with the SuperScript First-

Strand System for RT-PCR (Invitrogen). TMEM67 was retrieved using a pair of primers : forward 5'-tataagcttggtaccatggtgacgcgtaca-3' and reverse 5'-cgcggatccttagatcagaaatctttcatc-3', using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The full-length of TMEM67 cDNA was inserted into HindIII and BamHI sites of the pFlag-CMV2 expression vector (Sigma).

Cell culture and transfection

Human embryonic kidney 293T cells were grown in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). When cells had reached 70% confluence, they were transfected with empty vector of pFlag-CMV2 (-) or Flag-tagged-TMEM67 vector (+) using Lipofectamine™ 2000 (Invitrogen). Cells were collected after 4 8 h of transfection. For inhibitory investigation, HEK293 cells were treated as described in the text.

Immunostaining

Cells were seeded on 6-well plates at 37°C in air with 5% CO_2 incubator overnight and transfected with either empty vector or flag-tagged TMEM67 vector. After 48 h of transfection, cells were rinsed twice in cold PBS and fixed in 4% paraformaldehyde, pH 7.3 in PBS, for 10 min at room temperature. Cells were labeled with anti-flag antibody overnight at 4°C, washed thoroughly, incubated with an appropriate Alexa-labeled secondary antibody (Invitrogen) for 1 hour at room temperature and were visualized by fluorescence microscopy.

Antibodies and Inhibitors

Antibodies o f p-tyr-100 (#9411), p-JNK (Thr183/Tyr185)(#9912), JNK, p-ATF2 (Thr71) (#9221), p-c-jun (Ser 63)(#9261), p-mTOR (Ser 2448)(#2971), mTOR (#2972), p-4E-BP1 (Thr37/46)(#2855), p-S6K (Ser371)(#9208), p-p38 (Thr180/182)(#9211), p-Akt (Ser473) (#4060), Akt (#2920), p-GSK3b (Ser9)(#9336), GSK3b(#9315) were purchased from Cell Signaling Technology, Inc; p-ERK (Thr202/Tyr204) (sc-81492) and ERK (sc-135900) from Santa Cruz

Biotechnology Inc; 4G10 (05-1050X) from Millipore Corporation; β-catenin (610153) from BD

Biosciences; β-actin (A5316) and anti-Flag (F7425) from Sigma. Inhibitors, U0126 (U-6770) andSP600125 (S-7979) were purchased from LC Laboratories.

Protein extraction from cells and kidneys

The collected cells were washed twice with cold PBS and lysed in modified RIPA buffer (45 mM Tris -HCl, pH 7.4, 135mM sodium chloride, 0.5% Igepal CA-630, 0.9% triton X-100, 0.9% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 2.2mM EDTA) containing 1:100 proteinase inhibitor cocktails (P-8340, sigma), 25 mmol/L sodium fluoride, 5mmol/L sodium orthovanadate, and 100 g/mL phenylmethylsulfonyl chloride (Sigma-Aldrich products). Kidney tissues were homogenized and lysed in the lysis buffer. Tissue and cell debris were removed by centrifugation at 13,000 rpm for 10 min at 4°C. Protein

concentration was determined in each extract by a Bradford protein assay (Cat#500-0006, Bio-Rad) and spectroscopy at 600 nm.

Western blotting analysis

Twenty to 100μg of proteins were boiled for 5 min in 1 x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% SDS, 0.01% bromophenol blue) containing 5% β-mercaptoethanol and were separated on a 10 to 12% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose for antibody incubation. Protein bands were visualized by enhanced chemiluminescence (ECL). Western blots were statistically analyzed following the normalization by using ImageJ densitometry software.

Results

Increased overall phosphorylation of tyrosine phosphorylated proteins upon TMEM67 overexpression

Induction of protein phosphorylation is an important mechanism in regulating cellular processes. Aberrant phosphorylation of protein is commonly associated with a variety of diseases. To investigate the pathogenetic mechanisms of TMEM67, we examined the effect of elevated TMEM67 expression on the level of protein phosphorylation in human embryonic kidney (HEK 293) cells. Cells were transfected with an empty vector (-) or the vector containing a Flag-tagged full-length TMEM67 cDNA (+). Forty-eight h following transfection, immunofluorescence was done using anti-Flag antibody. Compared with the control, TMEM67 was overexpressed and mostly localized on the plasma membrane (Figure 1A). On the western blot, there was around a 1.6-fold increase in the overall level of protein tyrosine phosphorylation induced by TMEM67 overexpression, as visualized by 4G10 antibody (Figure 1B) or p-tyr-100 antibody (both detect tyrosine phosphorylated proteins) (Figure 1B, top-right panel) as compared to the paired controls. The western blots were normalized with β-actin, and the difference between the control and over-expressed samples was significant ($p<0.05$; Figure 1B).

Activation of signaling molecules upon TMEM67 overexpression

The increased level of overall protein phosphorylation suggested the activation of signaling components by the overexpression of TMEM67. Although several signaling molecules are activated in cyst epithelial cells from autosomal-dominant polycystic kidneys in different animal models caused by different gene mutations (Yamaguchi et al, 2003; Nagao et al, 2003), they were not shown in PKD caused by TMEM67 mutation. Thus, we examined the activation of several key molecules of signaling transduction pathways, including 3 MAPK members (JNK, ERK and p38MAPK), mTOR, and Akt following TMEM67 overexpression. Overall, transfection of TMEM67 in HEK293 cells activated JNK (Thr183/Tyr185) by 5.5-fold, ERK (Thr202/Tyr204) by 2-fold, and their downstream effectors such as ATF2 (Thr71) by 9-fold, and c-Jun (Ser 63) by 3-fold (Figure 2A left panel). For p38MAPK (Thr180/182), no significant change of phosphorylation was found (Figure 2A, right panel). In addition, phosphorylation of eukaryotic translation initiation factor 4E-bingding protein 1 (4E-BP1, Thr37/46) was significantly elevated in response to overexpression of TMEM67 (Figure 2A, left panel) compared to the empty vector (-) group. Phosphorylation on serine

and threonine residues can inhibit the binding of 4E-BP1 to eIF-4E, resulting in upregulation of protein translation (Brunn et al., 1997), which may be required or responsible for the rapid cell growth in PKD diseases.

Activation of mTOR was detected both in mice and humans with ADPKD (Shillingford et al., 2006) and ARPKD (Fischer et al., 2009; Becker et al., 2010; Ibraghimov-Beskrovnaya and Natoli, review, 2011). mTOR (Ser2448) and its downstream substrate, S6K (Thr371), however, failed to be activated in response to overexpression of TMEM67 (Figure 2A, right panel), suggesting that mTOR pathway may not be linked to the signaling mechanisms of TMEM67 function, at least in the HEK293 cell line. Consistently, overexpression of TMEM67 did not significantly alter the phosphorylation of Akt (Ser473) (Figure 2A, right panel), an upstream regulator of mTOR.

Pharmacological Inhibitory effects on TMEM67-induced phosphorylation

Based on the data above, we hypothesized that TMEM67 causes cystic pathogenesis through activation of the ERK and JNK signaling pathways. We therefore examined the effects of ERK and JNK inhibition on TMEM67-induced phosphorylation. Pharmacological inhibitors of ERK and JNK, including U0126, a highly selective inhibitor of ERK (Favata, et al, 1998) and SP600125, an inhibitor of JNK (Bennett et al., 2001), were applied to HEK293 cells transfected with either control vector (-) or Flag-tagged TMEM67 vector (+). Forty-eight h after transfections, cells were treated with either 1% DMSO (DMSO), 20μm U0126 (U), or 30μm of SP600125 (SP) for 6 h before harvesting for Western blotting (Figure 3). U0126 completely inhibited ERK phosphorylation (Figure 3), but somehow caused a small increase of ATF2 and c-Jun background phosphorylation. Importantly, inhibition of ERK phosphorylation by U0126 greatly reduced the induction of ATF2 as well as c-Jun phosphorylation induced, by TMEM67 overexpression as expected, since both ATF2 and c-Jun can be regulated by ERKs (Pulverer et al., 1991; Ouwens et al., 2002). Phosphorylation of JNK in response to overexpression of TMEM67 was significantly inhibited by SP600125 compared to the controls, providing further support for activation of JNK signaling pathway by TMEM67 overexpression. Inhibition of JNK by SP600125 completely cancelled the induction of ATF2 phosphorylation and significantly blocked c-Jun phosphorylation in response to TMEM67 overexpression, consistent with the report that gene transcription can be activated by JNK through c-Jun and ATF2 (Min et al., 2008).

ERK signaling pathway regulates the phosphorylation of 4E-BP1 (Herbert et al., 2002). JNK is involved in regulating the initiation of cap-dependent translation (Patel et al., 2012). In our pharmacological investigation, 4E-BP1 phosphorylation induced by TMEM67 overexpression produced a greater reduction by inhibition of JNK by 600125 compared to ERK inhibition by U0126, suggesting that 4E-BP1 phosphorylation upon TMEM67 overexpression was largely attributed to JNK signaling pathway.

Activation of signaling molecules in TMEM67 mutant kidneys

Our *in vitro* studies established that overexpression of TMEM67 stimulated the ERK and JNK pathways. We determined whether TMEM67 affects ERK and JNK signaling *in vivo* during cystic pathogenesis. B6C3Fe a/a-*bpck* mouse line with TMEM67 mutation was

recently identified as an autosomal recessive model of PKD disease (Cook et al., 2009), which displays a severe cystic kidney phenotype and quickly enlarged kidney after birth (Fig 4). Consistent with the original description (Cook et al., 2009), hematoxylin and eosin staining of *bpck* kidneys showed that cysts were present as early as embryonic day 15.5 (n=3, Fig 4A), the earliest age examined. The number and size of renal cysts progressively increased in neonatal and postnatal mice (Fig 4B-4E). By P18, mutant kidneys were bilaterally polycystic and grossly enlarged (Fig 4F), and only a minimal amount of medulla parenchyma was seen (Fig 4E).

Western blotting of mouse kidneys at P0, P10 and P18 with a variety of antibodies against 4G10, p-ERK (Thr202/Tyr204), p-JNK (Thr183/Tyr185), p-4E-BP1 (Thr37/46), p-mTOR (Ser2448), p-S6K (Thr371) and p-Akt (Ser473) were carried out (Fig 5). Consistent with the *in vitro* observations in HEK293 cells, accumulation of phospho-ERK and p-JNK in P0, phospho-4E-BP1 in P0/P10 and high level of overall phosphorylation (4G10) in P10/P18 were noticeable in mutant kidneys of *bpck* mice compared to their wild-type littermates. The level of phosphorylation of mTOR, S6K, and Akt was not significantly elevated in mutant kidneys, consistent with the observations of *in vitro* HEK293 cells. Although investigation of inhibitory effects of pharmacological inhibitors on animal is further needed, the data of *in vitro* and *in vivo* supported our hypothesis that TMEM67 may function via an ERK- and JNK -dependent manner.

Discussion

Activation of ERK and JNK signaling pathway are important regulatory mechanisms in a wide range of cellular processes, especially in growth and proliferation (Leppä et al., 1998; Kavurma MM and Khachigian, 2003). We have demonstrated that gain-of-function of TMEM67 stimulated overall phosphorylation of proteins, including ERK and JNK in HEK 293 cells (Fig 1-2), indicating hyperactivation of these 2 signaling pathways. Inhibition of ERK and JNK by pharmacological inhibitors in HEK293 cells significantly blocked the induction of phosphorylation of their downstream components including. Unexpectedly loss of TMEM67 in *bpck* mutant kidneys also stimulated phosphorylation of ERK and JNK. Thus, both gain and loss of function of TMEM67 can active ERK and JNK signaling pathways. This finding, however, may not be surprising, considering that loss of *pkd1*function caused PKD (Lu et al., 2001) and gain of *pkd1* or *pkd2* function also resulted in PKD (Thivierge et al., 2006; Park et al., 2009).

Enhanced mTOR signaling has been detected both in mice and humans with ADPKD, and may be responsible for quick cystic development after birth (Shillingford et al., 2006; Fischer et al., 2009; Becker et al., 2010). Unexpectedly, increased levels of phosphorylation of mTOR and S6K were observed neither *in vitro* nor *in vivo* (Fig 2), which suggests that TMEM67 may work in an mTOR-independent manner. Rapamycin, an inhibitor of mTOR, has been used to cure PKD. TMEM67 may cause pathogenesis of PKD through mTOR-independent, but a ERK- and JNK-dependent manner, suggesting that rapamycin may not be a good candidate for treatment of TMEM67-associated PKD.

Although we saw no increased phosphorylation of mTOR, 4E-BP1, a well-known substrate of mTOR, is highly phosphorylated upon overexpression of TMEM67 (Fig 2). Patel et al. (2012) demonstrated that JNK was involved in regulating the initiation of cap-dependent translation. 4E-BP1 phosphorylation induced by TMEM67 overexpression was completely blocked by JNK inhibitor (Fig 3), suggesting that the induction of 4E-BP1 phosphorylation by TMEM67 overexpression was highly attributed to JNK signaling pathway.

TMEM67 shares functional analogy with the Frizzled family of transmembrane Wnt receptors (Smith et al., 2006). Leitch et al. (2008) has provided evidence for its role in the non-canonical Wnt/PCP (Leitch et al., 2008). We found that TMEM67 activation of JNK (Fig 1), a downstream effector of the non-canonical Wnt/PCP pathway (Leitch et al., 2008), provided further support for these previous findings. In contrast, the canonical Wnt pathway known to be involved in cyst formation (Kim et al., 1999; Saadi-Kheddouci et al., 2001; Qian et al., 2004) did not respond to TMEM67. Overexpression of TMEM67 in HEK293 cells had no significant effects on the expression or phosphorylation of GSK3β (Supplementary Fig 1).

On the basis of these observations, we propose a pathogenesis model in which aberrant TMEM67 expression is linked to the activation of 2 signaling transduction pathways of ERK and JNK resulting in abnormal cell proliferation and cyst formation following the stimulation of gene transcription (Fig 6). Cross-talk is possible between the 2 pathways, and further studies are needed to determine the specific mechanisms of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinases
mTOR	mammalian target of rapamycin
S6K	p70S kinase
Akt	also known as protein kinase B (PKB), a serine/threonine-specific protein kinase
4E-BP1	eIF4E-binding protein 1

References

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukani Y. Genistein, a specific inhibitor of tyrosine specific protein kinases. J. Biol. Chem. 1987; 262:5592–5595. [PubMed: 3106339]

Baala L, Romano S, Khaddour R, Saunier S, Smith UM, Audollent S, et al. The Meckel-Gruber syndrome gene, MKS3, is mutated in Joubert syndrome. Am J Hum Genet. 2007; 80:186–94. [PubMed: 17160906]

Becker JU, Opazo Saez A, Zerres K, Witzke O, Hoyer PF, Schmid KW, Kribben A, Bergmann C, Nürnberger J. The mTOR pathway is activated in human autosomal-recessive polycystic kidney disease. Kidney Blood Press Res. 2010; 33(2):129–38. [PubMed: 20460933]

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu WM, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl. Acad. Sci. USA. 2001; 98:13681–13686. [PubMed: 11717429]

Brunn GJ, Hudson CC, Sekulić A, Williams JM, Hosoi H, Houghton PJ, Lawrence JC Jr, Abraham RT. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science. 1997; 277:99–101. [PubMed: 9204908]

Cook SA, Collin GB, Bronson RT, Naggert JK, Liu DP, Akeson EC, Davisson MT. A mouse model for Meckel syndrome type 3. J Amer Soc Nephrol. 2009; 20:753–764. [PubMed: 19211713]

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a Novel Inhibitor of Mitogen-activated Protein Kinase Kinase. J Biol. Chem. 1998; 273:18623–18632. [PubMed: 9660836]

Fischer DC, Jacoby U, Pape L, Ward CJ, Kuwertz-Broeking E, Renken C, Nizze H, Querfeld U, Rudolph B, Mueller-Wiefel DE, Bergmann C, Haffner D. Activation of the AKT/mTOR pathway in autosomal recessive polycystic kidney disease (ARPKD). Nephrol Dial Transplant. 2009; 24(6): 1819–1827. [PubMed: 19176689]

Herbert TP, Tee AR, Proud CG. The Extracellular Signal-regulated Kinase Pathway Regulates the Phosphorylation of 4E-BP1 at Multiple Sites. Journal of Biological Chemistry. 2002; 277:11591–11596. [PubMed: 11799119]

Ibraghimov-Beskrovnaya O, Natoli TA. mTOR signaling in polycystic kidney disease. Trends in Molecular Medicine. 2011; 17:625–633. [PubMed: 21775207]

Kavurma MM, Khachigian LM. ERK, JNK, and p38 MAP kinases differentially regulate proliferation and migration of phenotypically distinct smooth muscle cell subtypes. J Cell Biochem. 2003; 89(2):289–300. [PubMed: 12704792]

Kim E, Arnould T, Sellin LK, Benzing T, Fan MJ, Grüning W, Sokol SY, Drummond I, Gerd Walz G. The Polycystic Kidney Disease 1 Gene Product Modulates Wnt Signaling. Journal of Biological Chemistry. 1999; 274:4947–4953. [PubMed: 9988738]

Leitch CC, Zaghloul NA, Davis EE, Stoetzel C, Diaz-Font A, Rix S, Alfadhel M, Lewis RA, Eyaid W, Banin E, Dollfus H, Beales PL, Badano JL, Katsanis N. Hypomorphic mutations in syndromic encephalocele genes are associated with Bardet-Biedl syndrome. Nat. Genet. 2008; 40:443–448. [PubMed: 18327255]

Leppä S, Saffrich R, Ansorge W, Bohmann D. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J. 1998; 17(15):4404–13. [PubMed: 9687508]

Lu W, Shen X, Pavlova A, Lakkis M, Ward C, Pritchard L, Harris P, Genest D, Perez-Atayde A, Zhou J. Comparison of Pkd1-targeted mutants reveals that loss of polycystin-1 causes cystogenesis and bone defects. Hum. Mol. Genet. 2001; 10:2385–2396. [PubMed: 11689485]

Min BW, Kim CG, Ko J, Lim Y, Lee YH, Shin SY. Transcription of the protein kinase C-delta gene is activated by JNK through c-Jun and ATF2 in response to the anticancer agent doxorubicin. Exp Mol Med. 2008; 40(6):699–708. [PubMed: 19116455]

Nagao S, Yamaguchi T, Kusaka M, Maser RL, Takahashi H, Cowley BD, Grantham JJ. Renal activation of extracellular signal-regulated kinase in rats with autosomal-dominant polycystic kidney disease. Kidney Int. 2003; 63:427–437. [PubMed: 12631108]

Ouwens DM, de Ruiter ND, van der Zon GC, Carter AP, Schouten J, van der Burgt C, Kooistra K, Bos JL, Maassen JA, van Dam H. Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras–MEK–ERK pathway and of Thr69 through RalGDS–Src–p38. EMBO J. 2002; 21(14):3782–93. [PubMed: 12110590]

Park EY, Sung YH, Yang MH, Noh JY, Park SY, Lee TY, et al. Cyst formation in kidney via B-Raf signaling in the PKD2 transgenic mice. J Biol Chem. 2009; 284:7214–7222. [PubMed: 19098310]

Patel MR, Sadiq AA, Jay-Dixon J, Jirakulaporn T, Jacobson BA, Farassati F, Bitterman PB, Kratzke RA. Novel role of c-jun N-terminal kinase in regulating the initiation of cap-dependent translation. Int J Oncol. 2012; 40:577–582. [PubMed: 22076560]

Pulverer BJ, Kyriakis JM, Aruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-Jun mediated by MAP kinase. Nature. 1991; 353:670–674. [PubMed: 1922387]

Qian CN, Jared Knol J, Igarashi P, Lin FM, Zylstra U, Teh BT, Williams BO. Cystic renal neoplasia following conditional inactivation of Apc in mouse renal tubular epithelium. J. Biol. Chem. 2004; 280:3938–3945. [PubMed: 15550389]

Saadi-Kheddouci S, Berrebi D, Romagnolo B, Cluzeaud F, Peuchmaur M, Kahn A, Vandewalle A, Perret C. Early development of polycystic kidney disease in transgenic mice expressing an activated mutant of the beta-catenin gene. Oncogene. 2001; 20:5972–5981. [PubMed: 11593404]

Shillingford JM, Murcia NS, Larson CH, Low SH, Hedgepeth R, Brown N, et al. The mTOR pathway is regulated by polycystin-1 and its inhibition reverses renal cystogenesis in polycystic kidney disease. Proceedings of the National Academy of Sciences of the USA. 2006; 103:5466–5471. [PubMed: 16567633]

Smith UM, Consugar M, Tee LJ, McKee BM, Maina EN, Whelan S, et al. The transmembrane protein meckelin (MKS3) is mutated in Meckel-Gruber syndrome and the wpk rat. Nat Genet. 2006; 38(2):191–6. [PubMed: 16415887]

Thivierge C, Kurbegovic A, Couillard M, Guillaume R, Coté O, Trudel M. Overexpression of PKD1 causes polycystic kidney disease. Mol Cell Biol. 2006; 26:1538–1548. [PubMed: 16449663]

Wahl PR, et al. Mitotic activation of Akt signalling pathway in Han:SPRD rats with polycystic kidney disease. Nephrology (Carlton). 2007; 12:357–363. [PubMed: 17635750]

Yamaguchi T, Nagao S, Wallace DP, Belibi FA, Cowley BD, Pelling JC, Grantham JJ. Cyclic AMP activates BRaf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. Kidney Int. 2003; 63:1983–1994. [PubMed: 12753285]

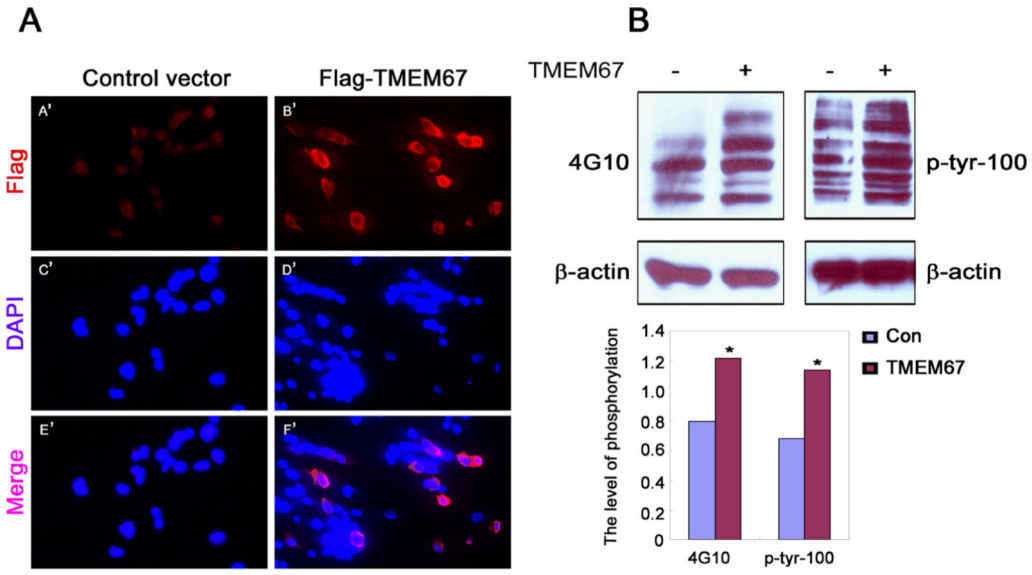

Figure 1.

Increased overall phosphorylation induced by overexpression of TMEM67. HEK 293 cells were transfected with control vector (-) or Flag-tagged-TMEM67 vector (+) for 48 h. A. TMEM67 overexpression was determined by immunofluoresence with anti-Flag antibody, and nuclei were stained with DAPI. B. Western blotting with 4G10 and p-tyr-100 antibodies (both against phospho-tyrosine proteins). β-actin was applied as a loading control. The bar graphs represent the level of phosphorylation (n=4, *$p<0.05$).

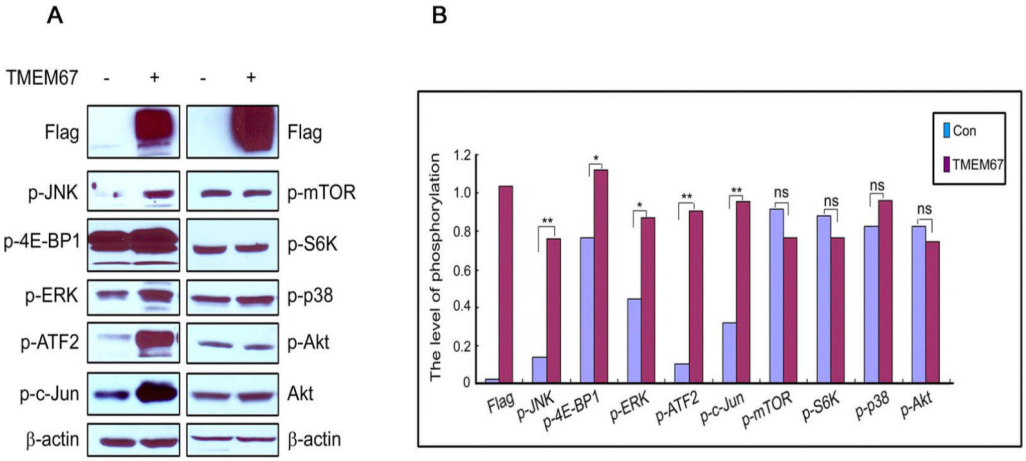

Figure 2.

Activation of signaling molecules upon TMEM67 overexpression. Cells were transfected with either empty vector (-) or Flag-tagged-TMEM67 vector (+) and subjected to Western blot (A) with the antibodies against Flag, p-JNK (Thr183/Tyo185), p-4E-BP1 (Thr37/46), p-ERK (Thr 202/Tyr 204), p-ATF2 (Thr71), p-c-Jun (Ser 63), p-mTOR (Ser 2448), p-S6K (Ser371), p-p38 (Thr180/182), p-Akt (Ser473) and Akt, respectively. β-actin was used as a loading control. B. The western blots were analyzed after normalization and quantification by using Image J densitometry software (*$p<0.05$, ** $p<0.01$, ns, no significant difference).

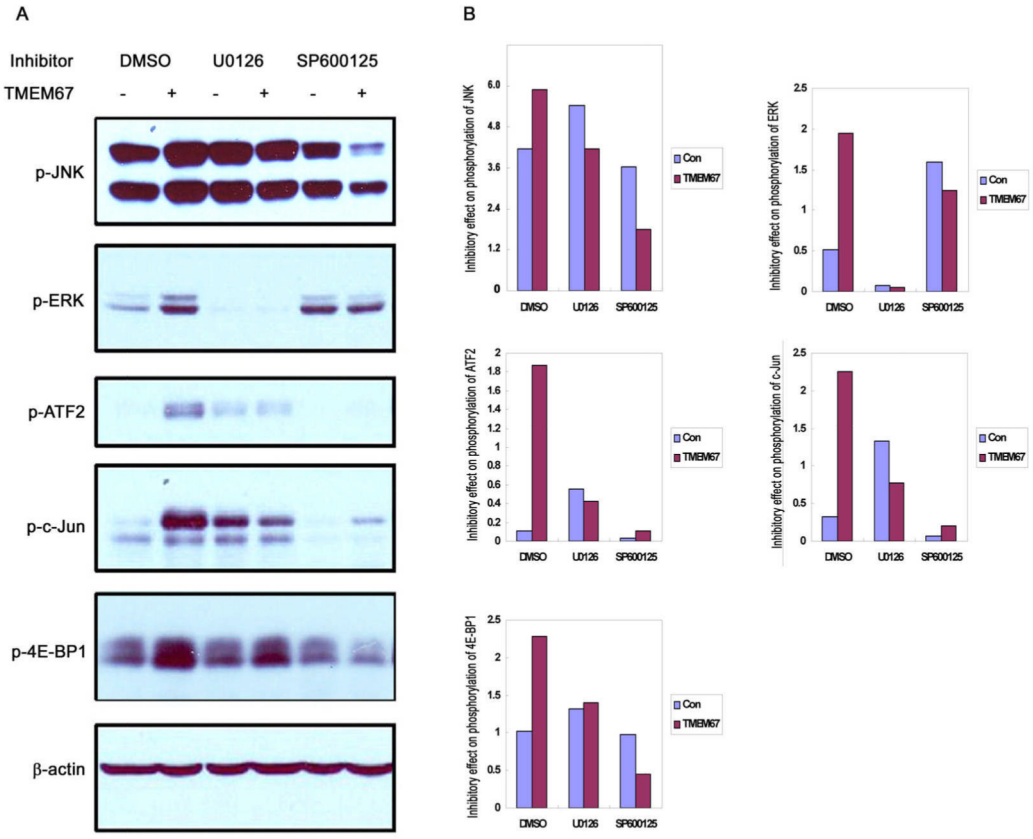

Figure 3.

Pharmacological inhibition of ERK and JNK prevented the activation of ATF2, c-Jun and 4E-BP1 by TMEM67 overexpression. HEK293 cells were transfected with either empty vector (-) or Flag-tagged TMEM67 vector (+). After 48 h of transfection, cells were treated with either 1% of DMSO (Con), 20μm of U0126 (U), or 30μm of SP600125 (SP) for 6 h. **A.** Cells were analyzed by western blotting with a variety of antibodies against p-JNK (Thr183/Tyr185), p-ERK (Thr202/Tyr204), p-ATF2 (Thr71), p-c-Jun (Ser63) and p-4E-BP1 (Thr37/46), respectively. β-actin was visualized as a loading control. **B.** The western blots were statistically analyzed following the normalization by using Image J densitometry (*$p<0.05$; ** $p<0.01$).

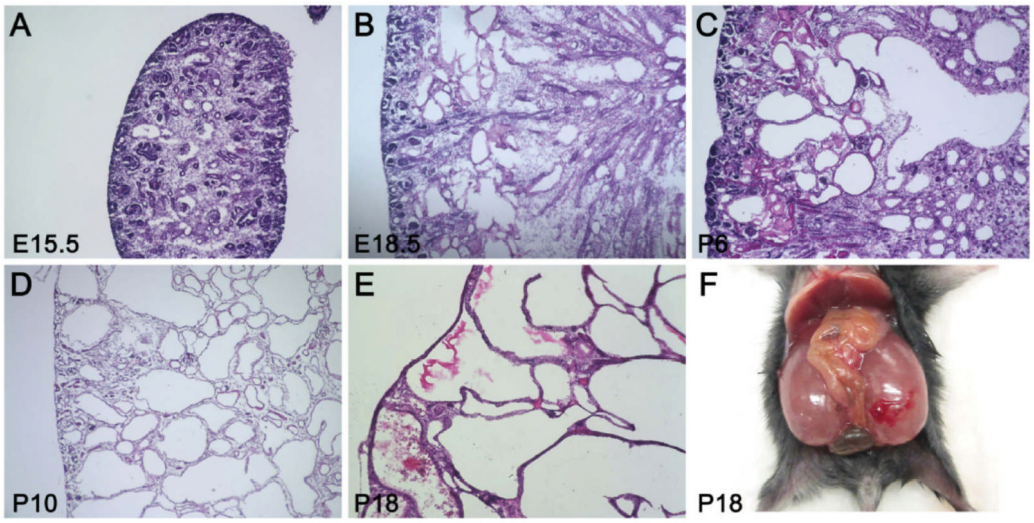

Figure 4.

Hematoxylin and eosin staining of *bpck* kidneys showing the progression of the bilateral polycystic kidney phenotype. (**A**) E15.5 (n = 3), (**B**) E18.5 (n = 3), (**C**) P6 (n = 3), (**D**) P10 (n = 3) and (**E**) P18 (n = 3). (F) Autopsy image of a P18 *bpck*/*bpck* with bilaterally enlarged polycystic kidneys.

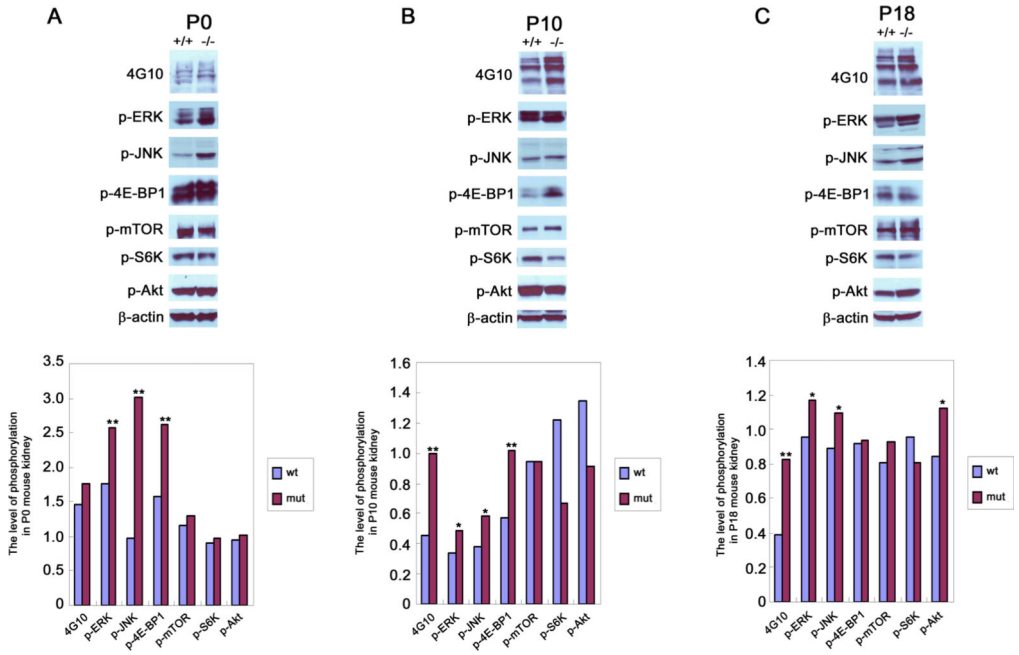

Figure 5.

Signaling pathways related to cystic pathogenesis in mutant kidneys. Kidney tissues from P0 (A), P10 (B) and P18 (C) wild-type (+/+) and *bpck* mutants (−/−) were immunoblotted with antibodies against 4G10, p-ERK (Thr202/Tyr204), p-JNK (Thr183/Tyr185), p-4E-BP1 (Thr37/46), p-mTOR (Ser 2448), p-S6K (Thr389) and p-Akt (Ser473), respectively. β-actin was visualized as a loading control. The western blots were statistically analyzed (n=3, *p<0.05) following the normalization by using Image J densitometry software. The bar graphs represent the level of phosphorylation.

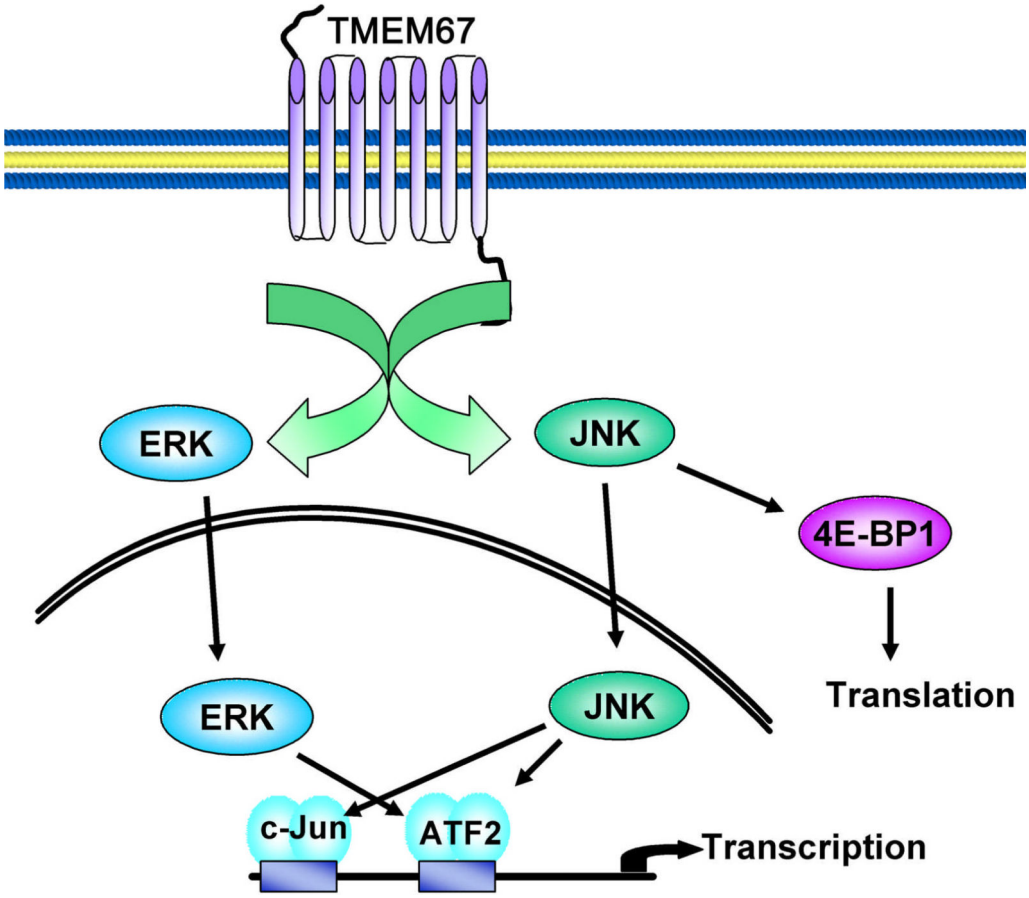

Figure 6.

A diagram of TMEM67 signaling pathways in cystic pathogenesis. 1) Aberrant expression of TMEM67 targets downstream signal cascade of ERK and JNK. 2) Activated ERK and JNK further regulate gene transcription and translation for increased cell proliferation.