

# Trafficking of TRPP2 by PACS proteins represents a novel mechanism of ion channel regulation

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**The trafficking of ion channels to the plasma membrane is tightly controlled to ensure the proper regulation of intracellular ion homeostasis and signal transduction. Mutations of polycystin-2, a member of the TRP family of cation channels, cause autosomal dominant polycystic kidney disease, a disorder characterized by renal cysts and progressive renal failure. Polycystin-2 functions as a calcium-permeable nonselective cation channel; however, it is disputed whether polycystin-2 resides and acts at the plasma membrane or endoplasmic reticulum (ER). We show that the subcellular localization and function of polycystin-2 are directed by phosphofurin acidic cluster sorting protein (PACS)-1 and PACS-2, two adaptor proteins that recognize an acidic cluster in the carboxy-terminal domain of polycystin-2. Binding to these adaptor proteins is regulated by the phosphorylation of polycystin-2 by the protein kinase casein kinase 2, required for the routing of polycystin-2 between ER, Golgi and plasma membrane compartments. Our paradigm that polycystin-2 is sorted to and active at both ER and plasma membrane reconciles the previously incongruent views of its localization and function. Furthermore, PACS proteins may represent a novel molecular mechanism for ion channel trafficking, directing acidic cluster-containing ion channels to distinct subcellular compartments.**

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## Introduction

Mammalian cells express a diverse repertoire of ion channels that broadly affect cellular homeostasis, mandating tight regulation. Many ion channels carry specific trafficking motifs that regulate their exit from the endoplasmic reticulum (ER) to the *trans*-Golgi network (TGN), where they are directed to discrete plasma membrane domains to assume a highly compartmentalized distribution (Deutsch, 2003). Several ion channels are retained in the ER by a di-arginine motif until the assembly of a multimeric protein complex masks this retention signal (Ma and Jan, 2002). Other ion channels undergo rapid endocytosis and degradation, allowing only a small number of channel proteins to accumulate at the plasma membrane (Staub *et al*, 1997). Genetic mutations that disturb the subcellular trafficking of ion channels are often associated with human disease, underlining the importance of spatial regulation (Cheng *et al*, 1990; Bertrand and Frizzell, 2003; Hebert, 2003; Thomas *et al*, 2003).

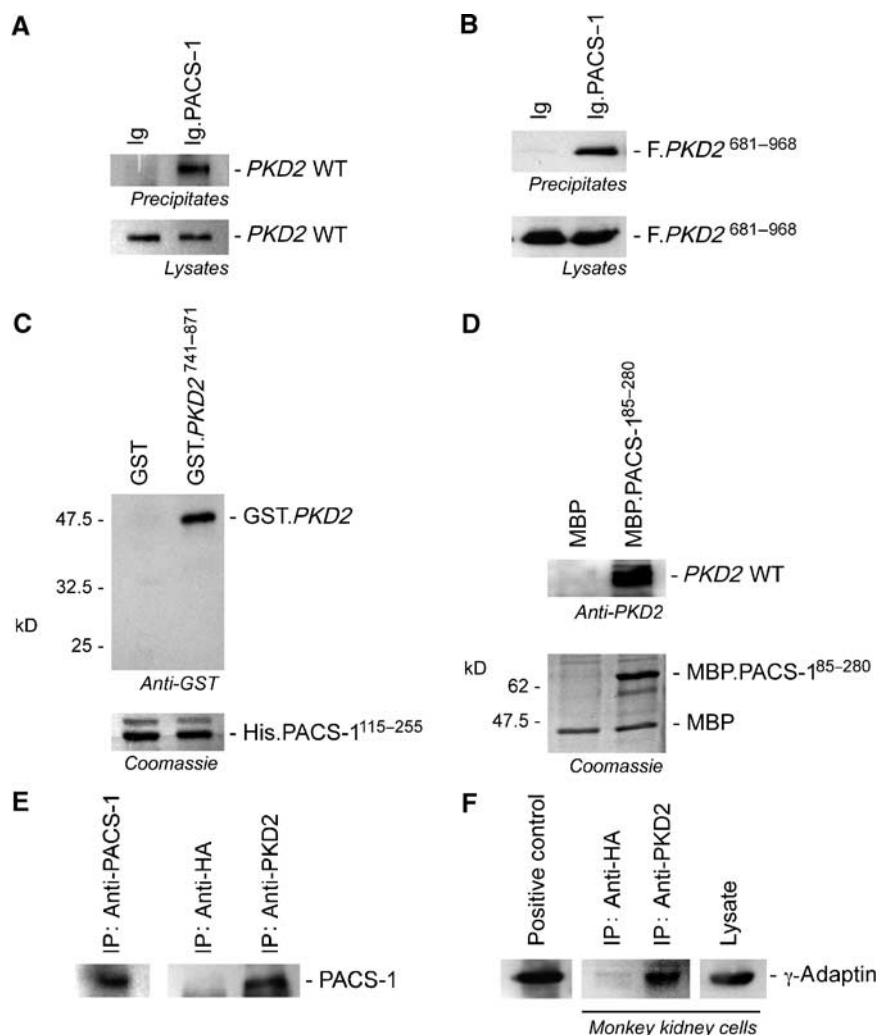
Autosomal dominant polycystic kidney disease (ADPKD) is caused by the mutation of either *PKD1* or *PKD2*, two genes encoding for the integral membrane proteins polycystin-1 and polycystin-2 (reviewed in Watnick and Germino, 2003). Polycystin-2, a member of the TRP family of ion channels (TRPP2) (reviewed in Montell, 2001; Clapham, 2003), functions as a calcium-permeable, nonselective cation channel (Hanaoka *et al*, 2000; Gonzalez-Perrett *et al*, 2001; Luo *et al*, 2003). Both polycystins are present in the primary cilia of kidney epithelial cells (Pazour *et al*, 2002; Yoder *et al*, 2002) and increase intracellular Ca<sup>2+</sup> in response to ciliary bending (Nauli *et al*, 2003). Polycystin-2 is present at the basolateral plasma membrane of developing renal tubules (Foggensteiner *et al*, 2000) and on the surface of several cell lines (Luo *et al*, 2003). Opposing these findings are reports that polycystin-2 is a Ca<sup>2+</sup>-activated Ca<sup>2+</sup> release channel in the ER, as a short motif in the carboxy-terminus of polycystin-2 localizes this protein to the ER (Cai *et al*, 1999; Koulen *et al*, 2002; Cai *et al*, 2004). Here we report that the subcellular localization and function of polycystin-2 is regulated by phosphofurin acidic cluster sorting protein (PACS)-1 and PACS-2, two sorting proteins that recognize an acidic cluster in the carboxy-terminal domain of polycystin-2. Blocking the binding to both PACS-1 and PACS-2 localizes a functional polycystin-2 to the plasma membrane. Interaction with these adaptor proteins is regulated by the phosphorylation of polycystin-2 by the protein kinase casein kinase 2 (CK2). Our observations indicate that polycystin-2 is not exclusive to ER or plasma membrane; it traffics to both. We therefore propose a dual role for polycystin-2 at both ER and plasma membranes. Lastly, the presence of acidic clusters within many ion channels, including other TRP channels such as TRPV4, suggests a broad and important role for PACS proteins in the sorting of ion channels to cellular compartments.

## Results

### Polycystin-2 interacts with the connector protein PACS-1

Analysis of the carboxy-terminal domain of polycystin-2 revealed a highly conserved acidic cluster in all mammalian polycystin-2 proteins, but not in the polycystin-2-like proteins. Acidic clusters act as sorting motifs that direct the localization of membrane proteins in the TGN/endosomal system. Sorting is directed by connector proteins that recognize and bind phosphorylated acidic clusters (Gu *et al*, 2001; Tekirian, 2002). A phosphorylatable residue within the acid cluster, typically a CK2 site, facilitates such cargo trafficking. Since a CK2 site is present in the mammalian polycystin-2 proteins, we decided to investigate whether the subcellular localization of mammalian polycystin-2 is directed by connector proteins that recognize phosphorylated acidic clusters.

Presently, two types of connector proteins are known to recognize phosphorylatable acidic clusters, the gamma-ear containing ARF binding (GGA) and the PACS adaptor proteins (reviewed in Gu *et al*, 2001; Tekirian, 2002). The lack of an adjacent di-leucine motif, typical for GGA recognition (Kato *et al*, 2002a), was evidence that polycystin-2 interacts with PACS-1 rather than with GGA-type proteins. PACS-1 is a cytosolic furin-sorting molecule that associates with the adaptor protein complex-1 (AP-1) and forms a ternary complex between furin and AP-1 (Wan *et al*, 1998; Crump *et al*, 2001). As shown in Figure 1A, PACS-1 fused to an immunoglobulin tag (Ig.PACS-1) precipitated either wild-type (WT) polycystin-2 or F.PKD2<sup>681-968</sup>, the Flag-tagged carboxy-terminal domain of polycystin-2 containing the acidic cluster (Figure 1B). The interaction between PACS-1 and the carboxy-terminus of polycystin-2 was direct, as demonstrated by the *in vitro* interaction of recombinant PACS-1 and



**Figure 1** The carboxy-terminal domain of polycystin-2 interacts with the PACS-1/ $\gamma$ -adaptein protein complex. (A) PACS-1, fused to the immunoglobulin tag (Ig.PACS-1), precipitates WT polycystin-2 (PKD2 WT) from transiently transfected HEK 293T cells. (B) The same PACS-1 fusion protein interacts with a Flag-tagged polycystin-2 truncation that contains the carboxy-terminal domain of polycystin-2 (F.PKD2<sup>681-968</sup>), demonstrating that PACS-1 recognizes a motif in the carboxy-terminal domain of polycystin-2. (C) The recombinant HIS-tagged furin-binding region of PACS-1 (HIS.PACS-1<sup>115-255</sup>) binds a recombinant GST-tagged polycystin-2 fragment containing amino acids 741-871 (GST.PKD2<sup>741-871</sup>) *in vitro* (upper panel). Equal loading of the HIS.PACS-1<sup>115-255</sup> is shown in the lower panel by Coomassie blue staining. (D) A recombinant PACS-1 protein (amino acids 85-280) containing the maltose-binding protein (MBP) (MBP.PACS-1<sup>85-280</sup>) immobilizes polycystin-2 from mouse kidney lysates, demonstrating that native polycystin-2 recognizes and binds PACS-1. (E) Immunoprecipitation of endogenous polycystin-2 from COS M6 kidney cells immobilizes PACS-1. (F) Endogenous  $\gamma$ -adaptein can be co-immunoprecipitated from COS M6 kidney cells using a polycystin-2-specific antiserum, indicating that polycystin-2 associates with the AP1 adaptor complex.

polycystin-2 proteins (Figure 1C). Recombinant PACS-1 also recognized endogenous polycystin-2 from mouse kidneys (Figure 1D), an interaction that was unaffected by a recombinant polycystin-1 containing the polycystin-2-binding domain (data not shown). Furthermore, polycystin-2 formed complexes with both endogenous PACS-1 and the  $\gamma$  subunit of AP-1 (Figure 1E and F), indicating that PACS-1 forms a ternary complex between polycystin-2 and AP-1, independent of polycystin-1.

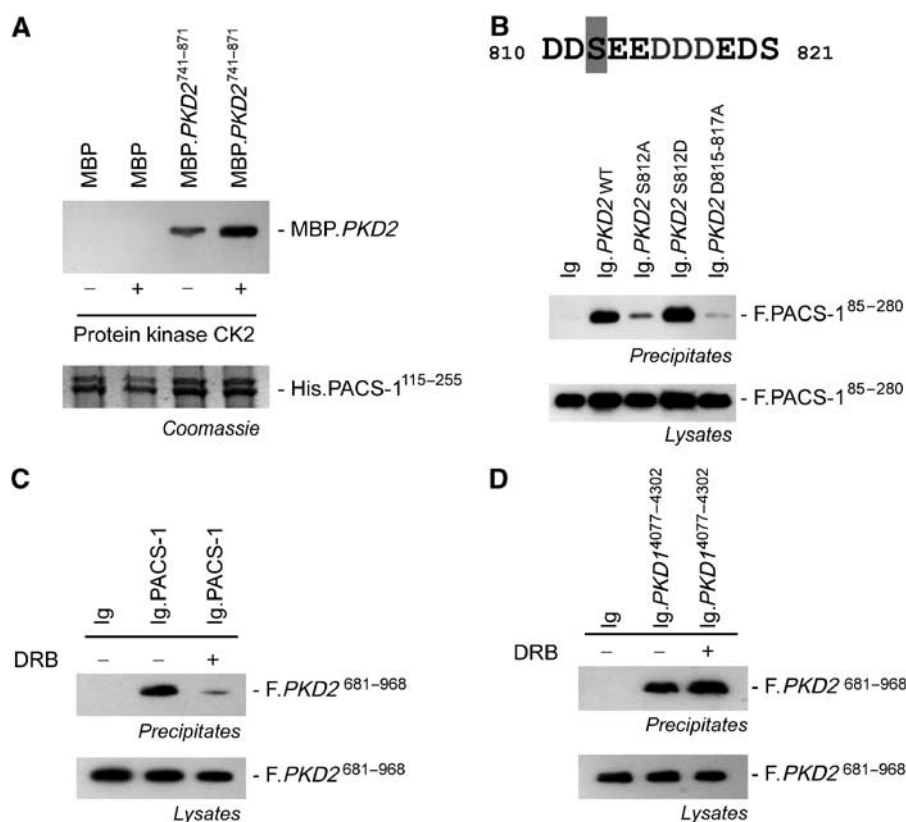
### Interaction between PACS-1 and polycystin-2 is phosphorylation-dependent

The PACS-1-binding domain of furin (EECP<sub>S773</sub> DS<sub>S775</sub> EEDE) contains a pair of protein kinase CK2 sites (Molloy *et al*, 1994; Jones *et al*, 1995), two serines that when phosphorylated greatly enhance the interaction of PACS-1 with furin (Wan *et al*, 1998). Serine 812 within the carboxy-terminal acidic cluster of polycystin-2 is a candidate CK2 site (Figure 2); we postulated that the phosphorylation state of this site regulates the interaction between PACS-1 and polycystin-2. As seen in Figure 2A, phosphorylation of recombinant polycystin-2 by CK2 enhanced the binding of PACS-1. Similarly, an S-to-D

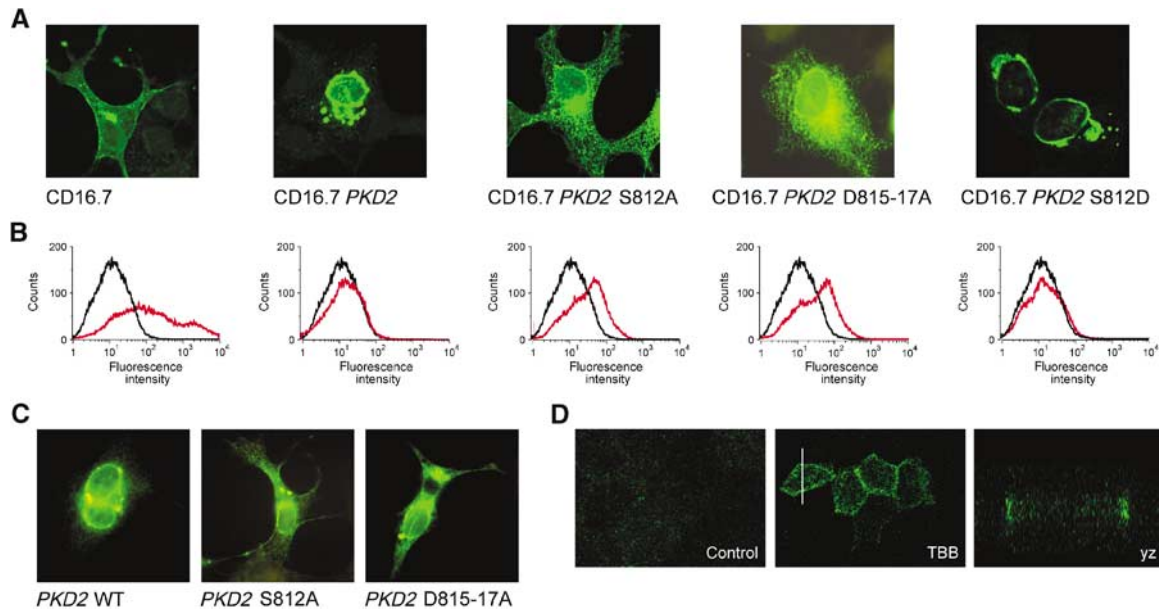
mutation at position 812, mimicking constitutive phosphorylation, maintained the interaction between PACS-1 and the carboxy-terminus of polycystin-2, whereas replacement of serine 812 by alanine or destruction of the acid cluster of polycystin-2 (PKD2 D815-17A mutation) greatly diminished their interaction (Figure 2B). The association between immunoglobulin-tagged PACS-1 and a Flag-tagged carboxy-terminus of polycystin-2 was also abrogated by the CK2 inhibitor 5,6-dichloro-1- $\beta$ -D-ribofuranosyl-benzimidazole (DRB) (Figure 2C) (Zandomeni *et al*, 1986), further demonstrating the requisite phosphorylation of serine 812 for efficient binding of PACS-1 to polycystin-2. In contrast, DRB had no effect on the interaction of polycystin-1 with polycystin-2 (Figure 2D). These results demonstrate that PACS-1 recognizes and binds to polycystin-2 in a phosphorylation-dependent manner.

### The acidic cluster regulates the subcellular localization of polycystin-2

Mutation of the CK2 phosphorylation site or the acidic cluster of polycystin-2 had profound effects on its localization. The carboxy-terminal cytoplasmic domain of polycystin-2 fused



**Figure 2** The interaction between PACS-1 and polycystin-2 is direct and requires phosphorylation of S812 of polycystin-2 for efficient binding. (A) The HIS-tagged furin-binding region of PACS-1 (amino acids 115–255) binds the carboxy-terminal amino acids 741–871 of polycystin-2 fused to the MBP. Phosphorylation of the recombinant MBP-polycystin-2 fusion protein (MBP.PKD2<sup>741–871</sup>) results in significantly increased binding *in vitro*. The Coomassie blue staining (lower panel) shows that equal amounts of HIS-PACS-1 were used to immobilize the polycystin-2 fusion protein. (B) Mutation of serine 812 to alanine (PKD2 S812A) or destruction of the acidic cluster (PKD2 D815-817A), but not the serine 812 to aspartic acid mutation abrogates the interaction between polycystin-2 and PACS-1. HEK 293T cells were transiently transfected as indicated, and the carboxy-terminal domain of WT or mutant polycystin-2 fused to the slg7 tag was precipitated to monitor binding of Flag-tagged PACS-1 (amino acids 85–280). (C) A specific inhibitor of protein kinase CK2, DRB, abrogated the interaction between immunoglobulin-tagged PACS-1 and the Flag-tagged carboxy-terminus of polycystin-2. DRB was added at a concentration of 30  $\mu$ M for 4 h to transiently transfected HEK 293T cells. Ig.PACS-1 was precipitated from lysates using protein G; the Flag-specific M2 antibody was used to detect immobilized polycystin-2. (D) DRB does not have an effect on the interaction between polycystin-1 and polycystin-2. The Flag-tagged carboxy-terminus of polycystin-2 (F.PKD2) interacts with the carboxy-terminus of polycystin-1 fused to the slg7 tag. Addition of DRB had no effect on this interaction.



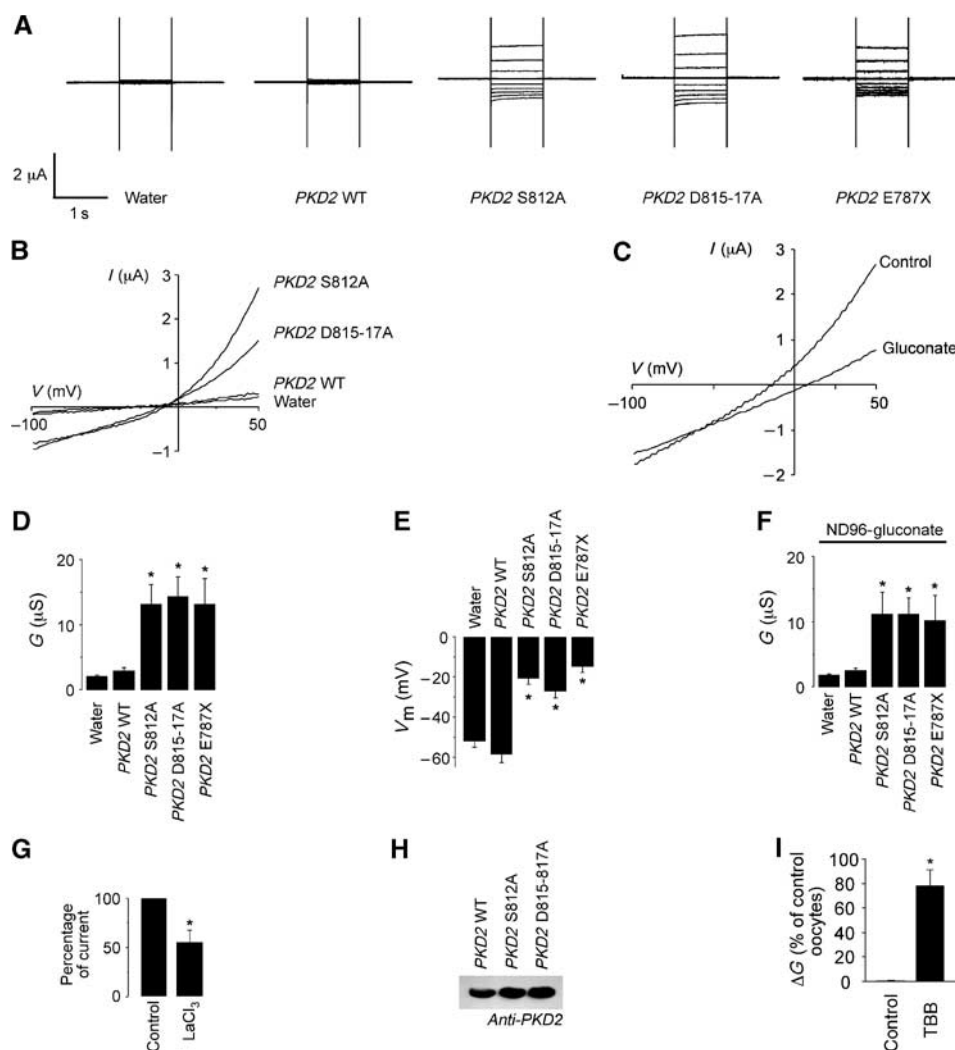
**Figure 3** Subcellular localization of polycystin-2. (A) The subcellular localization of the carboxy-terminal domain of polycystin-2, fused to the extracellular domain of CD16 and the transmembrane domain of CD7, was compared to either control protein (CD16.7) or the polycystin-2 mutants CD16.7 *PKD2* S812A, CD16.7 *PKD2* S812D and CD16.7 *PKD2* D815-17A in transiently transfected COS M6 cells labelled with a fluorescein isothiocyanate (FITC)-conjugated anti-CD16 antibody. (B) FACS analysis was performed with nonpermeabilized cells to determine the surface expression of the CD16.7 fusion proteins. In addition to the control protein CD16.7, only CD16.7 *PKD2* S812A and CD16.7 *PKD2* D815-17A were detectable at the cell surface (black curves, control; red curves, transfected cells). (C) The subcellular localization of full-length polycystin-2 or the S812A and D815-17A mutants of polycystin-2 in transiently transfected COS M6 cells was determined using a polycystin-2-specific rabbit antiserum followed by an FITC-labelled anti-rabbit antiserum. Polycystin-2 showed the typical perinuclear accumulation, whereas both the polycystin-2 S812A and the D815-17A mutations assumed a more dispersed punctate distribution and some membrane staining. (D) Surface labelling of nonpermeabilized, polarized MDCK cells. Polycystin-2<sup>702-968</sup> containing an extracellular Tac tag is translocated to the lateral membrane after treatment with a specific inhibitor of CK2 (TBB, 20  $\mu$ M, 4 h).

to a transmembrane protein (CD16.7) was retained in the ER of transiently transfected COS M6 cells (Figure 3A and B). Mutation of the critical serine (S812A) of the candidate CK2 phosphorylation site within the acidic cluster altered the subcellular localization of the carboxy-terminus of polycystin-2 from ER to cell surface. A similar redistribution of the polycystin-2 carboxy-terminus was achieved by replacing three aspartic acids of the acidic cluster with alanine (D815-17A). As expected, the S812D mutation, a construct with an enhanced PACS-1-binding capacity, was retained in the ER. Changes in subcellular localization were less striking for full-length polycystin-2 mutants. Nevertheless, in a significant portion of transiently transfected cells, mutation of either S812A or D815-17A released polycystin-2 from the ER compartment and facilitated its translocation to the plasma membrane, whereas WT polycystin-2 was retained in the ER (Figure 3C). To determine whether CK2 regulates basolateral targeting of polycystin-2, we examined polarized MDCK cells by confocal microscopy after incubation with the specific CK2 inhibitor 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB, 20  $\mu$ M, 4 h). As shown in Figure 3D, polycystin-2 is scarcely present at the surface of nonpermeabilized MDCK cells stably expressing Tac-*PKD2*. After treatment with TBB, polycystin-2 appeared at the lateral membrane, but not the apical or basal membrane. No change in cilia expression was detected (data not shown).

#### Disruption of the acidic cluster increases ion channel activity of polycystin-2

Since WT polycystin-2 does not exit the ER nor produce whole cell currents in *Xenopus* oocytes and CHO cells

(Hanaoka *et al*, 2000; Chen *et al*, 2001), we investigated whether generating *PKD2* mutants defective in PACS-1 binding would result in polycystin-1-independent ion channel activity at the plasma membrane. Whole cell currents of *Xenopus laevis* oocytes, microinjected with water, WT *PKD2*, the S812A or the D815-17A mutant of *PKD2*, were recorded using a standard two electrode voltage clamp. Both the *PKD2* S812A and *PKD2* D815-17A mutations produced a significant increase in whole cell currents, characterized by a current-voltage relationship closely resembling that generated by the polycystin-1/polycystin-2 complex (Hanaoka *et al*, 2000; Figure 4A and B). Disruption of PACS-1 binding by the *PKD2* S812A and D815-17A mutations, or the *PKD2* E787X truncation increased whole cell conductance from  $3.1 \pm 0.52 \mu\text{S}$  ( $n = 21$ ) in oocytes expressing WT *PKD2* to  $13.5 \pm 3.2 \mu\text{S}$  ( $n = 25$ ),  $14.5 \pm 2.9 \mu\text{S}$  ( $n = 36$ ) and  $13.4 \pm 3.8 \mu\text{S}$  ( $n = 19$ ), respectively (Figure 4D). In contrast, the S812D mutation had no effect on whole cell conductance (data not shown). The membrane potential was depolarized from  $-58.6 \pm 3.7 \text{ mV}$  ( $n = 21$ ) in oocytes expressing WT *PKD2* to  $-21 \pm 3.2 \text{ mV}$  ( $n = 25$ , *PKD2* S812A),  $-27.4 \pm 3.3 \text{ mV}$  ( $n = 36$ , *PKD2* D815-17A) and  $-14.8 \pm 2.9 \text{ mV}$  ( $n = 19$ , *PKD2* E787X) (Figure 4E). The slight outward rectification of the current induced by the *PKD2* mutants was likely the result of the endogenous  $\text{Ca}^{2+}$ -activated chloride ( $\text{Cl}^-$ ) channel in *Xenopus* oocytes, a channel commonly used as a readout for increased  $\text{Ca}^{2+}$  permeability. Hence, the complete removal of extracellular  $\text{Cl}^-$  abolished outward rectification and shifted the reversal potential to positive values (Figure 4C). However, the whole cell conductance in the negative voltage range was still significantly increased even



**Figure 4** The acidic cluster of polycystin-2 affects whole cell currents in *Xenopus* oocytes. (A) Measurements of current ( $I$ ) in oocytes injected with water, *PKD2* WT, *PKD2* S812A, *PKD2* D812-817A and *PKD2* E787X. Voltage was clamped between  $-100$  and  $+60$  mV (increments of  $20$  mV for  $1$  s). (B) Current–voltage ( $I$ – $V$ ) relationship of *PKD2* WT and mutants. (C)  $I$ – $V$  relationship of an oocyte expressing *PKD2* S812A after replacement of extracellular  $\text{Cl}^-$  by gluconate. (D) Whole cell conductance ( $G$ ; determined from  $-100$  to  $0$  mV) of oocytes injected with water ( $n = 49$ ), *PKD2* WT ( $n = 21$ ), *PKD2* S812A ( $n = 25$ ), *PKD2* D815-817A ( $n = 36$ ) or *PKD2* E787X ( $n = 12$ ). In contrast to *PKD2* WT, expression of *PKD2* mutants with a disrupted PACS-1-binding motif significantly increased the whole cell conductance and (E) depolarized the membrane potential ( $V_m$ ) of injected oocytes (water  $n = 46$ , *PKD2* WT  $n = 19$ , *PKD2* S812A  $n = 22$ , *PKD2* D815-817A  $n = 35$ , *PKD2* E787X  $n = 12$ ). (F) The whole cell conductance remained significantly increased by the *PKD2* mutants in the absence of extracellular chloride ( $\text{Cl}^-$ ) (ND96-gluconate) (*PKD2* WT  $n = 23$ , *PKD2* S812A  $n = 25$ , *PKD2* D815-817A  $n = 35$ , *PKD2* E787X  $n = 10$ ). (G) Lanthanum ( $500 \mu\text{M}$ ) partially inhibited the whole cell conductance of oocytes injected with *PKD2* S812A ( $n = 10$ ). (H) Equal expression of different *PKD2* full-length constructs was confirmed by Western blot (five oocytes were pooled for each condition). (I) Preincubation of oocytes expressing *PKD2* WT with the specific CK2 inhibitor TBB ( $20 \mu\text{M}$ ,  $3$ – $6$  h) significantly increased the conductance ( $\Delta G$ ) compared to water-injected oocytes treated with TBB. Asterisks indicate statistical significance with  $P < 0.05$ .

in the absence of extracellular  $\text{Cl}^-$  (ND96-gluconate) (Figure 4F). The reduction of extracellular  $\text{Na}^+$  from  $96$  to  $10$  mM (ND96-NMDG solution) resulted in a significant decrease of whole cell conductance by  $1.54 \pm 0.4 \mu\text{S}$  ( $n = 21$ , *PKD2* S812A),  $2.44 \pm 0.63 \mu\text{S}$  ( $n = 28$ , *PKD2* D815-17A) and  $2.21 \pm 0.45 \mu\text{S}$  ( $n = 13$ , *PKD2* E787X). Lanthanum, an inhibitor of nonselective cation channels, inhibited the currents produced by the *PKD2* S812A mutant (Figure 4G). Western blot analysis revealed that the changes in whole cell currents were not the result of different protein levels (Figure 4H). To further substantiate the critical role of CK2-dependent phosphorylation, *Xenopus* oocytes were preincubated with the CK2 inhibitor TBB ( $20 \mu\text{M}$ ,  $3$ – $6$  h). TBB significantly increased

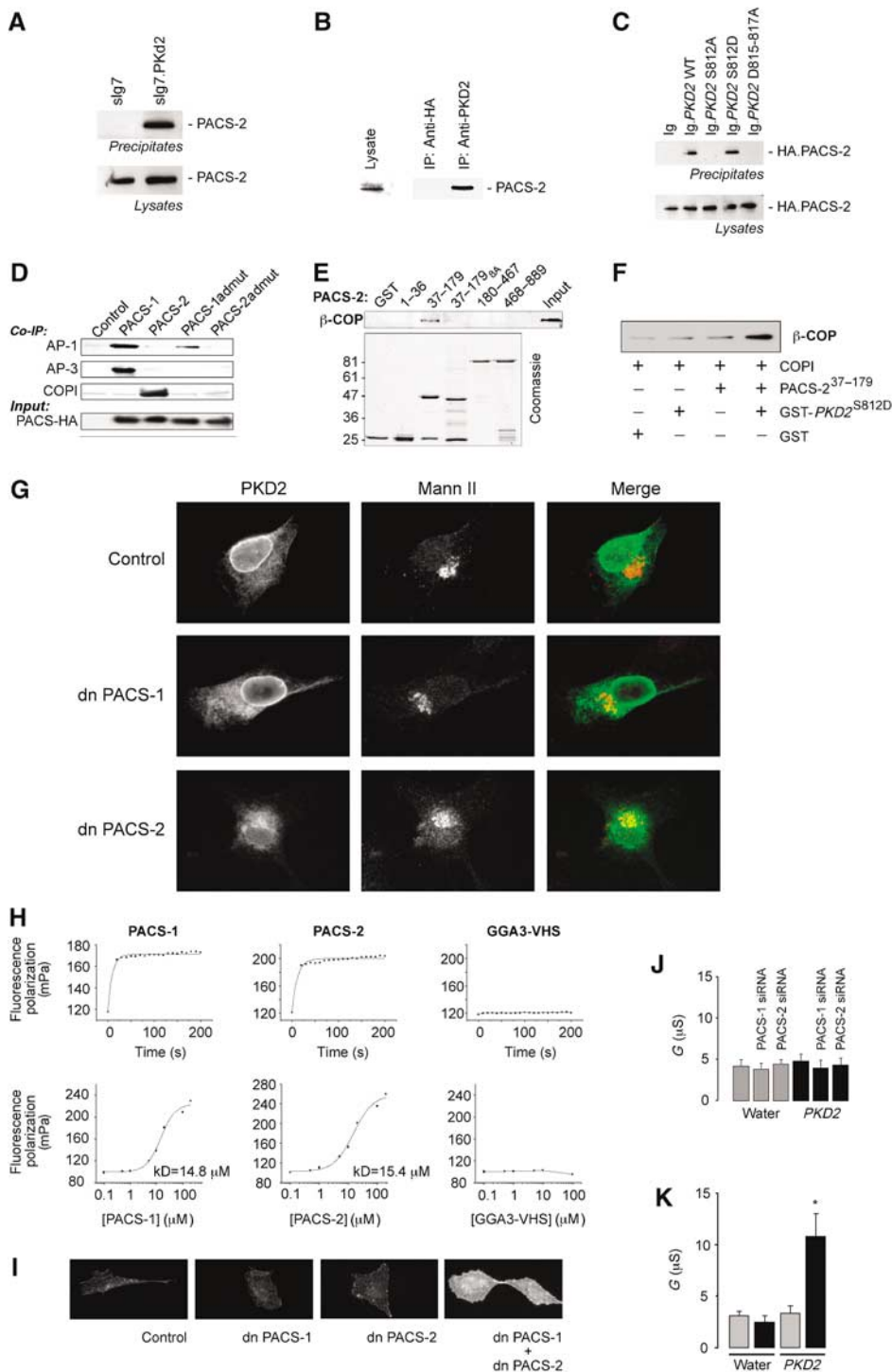
the whole cell conductance of oocytes expressing polycystin-2 (Figure 4I), controlling for the increased permeability of control oocytes. These results demonstrate that polycystin-2 functions as a  $\text{Ca}^{2+}$ -permeable nonselective cation channel in the plasma membrane upon release from its interaction with PACS-1.

#### **PACS-2, a novel PACS protein, localizes polycystin-2 to the endoplasmic reticulum**

Upon evaluating the role of PACS-1 in targeting polycystin-2 to the plasma membrane, we observed that PACS-1admut, a dominant-negative (dn) PACS-1 (Crump *et al*, 2001), failed to alter the subcellular localization of polycystin-2 (Figure 5),

demonstrating that PACS-1 alone was not decisive in polycystin-2 distribution. Blast searches with PACS-1 identified a novel adaptor protein, termed PACS-2, that is highly homologous to PACS-1, but localizes to the ER (Simmen *et al*, 2005). This implicates PACS-2 as a regulator of the ER localization of polycystin-2. PACS-2 interacted with transiently overexpressed polycystin-2 in HEK 293T cells and with endogenous protein (Figure 5A and B). The interaction between polycystin-2 and PACS-2 is direct (data not shown), and is mediated by the identical acidic motif that

binds to PACS-1 (Figure 5C). To characterize PACS-2 more, we examined vesicular coat preferences for PACS-1 and PACS-2 (Figure 5D). Co-immunoprecipitation analyses showed that PACS-1 and PACS-2 associate with different vesicular coat complexes. While PACS-1 associated with both the AP-1 and AP-3 clathrin adaptors (Figure 5D; Crump *et al*, 2001), PACS-2 selectively associated with COPI; this association could be disrupted in the PACS-2admut construct, a mutant analogous to PACS-1admut. To identify which region of PACS-2 is required for the interaction with COPI, the 889 residues of



PACS-2 were divided into four segments. Glutathione-S-transferase (GST) fusion proteins corresponding to each of the four regions of PACS-2 were purified, and incubated with cell lysates (Figure 5E). Protein complexes were captured with glutathione-agarose and bound COPI was identified by Western blot. This analysis showed that the region of PACS corresponding to the PACS-1 cargo/adaptor-binding domain, GST-PACS<sup>37-179</sup>, bound directly to COPI. To test whether PACS-2 connects TRPP2 to COPI by forming a ternary complex, GST-PKD2<sup>S812D</sup> was incubated with purified COPI in the presence or absence of PACS<sup>37-179</sup>. GST capture showed an approximately three-fold greater amount of COPI associated with GST-PKD2<sup>S812D</sup> in the presence of PACS-2 than in its absence (Figure 5F). These data show that a ternary complex can form *in vitro*, and identify PACS-2 as a connector protein that can directly link acidic cluster motifs with COPI. They also demonstrate that PACS-2admut functions as a dn mutation, because of its abrogated COPI binding. We next used PACS-2admut to examine polycystin-2 localization after interference with PACS-2 and found that this mutant shifted polycystin-2 from the ER to the Golgi compartment (Figure 5G). Fluorescence polarization revealed that the furin-binding domain of PACS-1 and the homologous region of PACS-2 bound the acidic cluster of polycystin-2 with similar affinities, whereas the acidic cluster di-leucine-binding domain of GGA3 (Kato *et al*, 2002b) failed to interact with the phosphorylated polycystin-2 peptide (Figure 5H). However, neither dn PACS-1 nor dn PACS-2 alone resulted in the surface expression of a Tac-polycystin-2 fusion protein. Only the coexpression of both dn PACS proteins dramatically increased polycystin-2 surface expression (Figure 5I). Therefore, the trafficking and surface expression of polycystin-2 appears to be a two-step process directed by PACS-2 at the ER, and by PACS-1 at Golgi/TGN compartment of the secretory pathway. An interdependent role involving both PACS proteins was confirmed by RNA interference of PACS-1 and PACS-2 expression in *Xenopus* oocytes. siRNA directed against either xPACS-1 or xPACS-2 did not alter whole cell conductance (Figure 5J); only microinjection of both siRNAs

generated a significant increase in whole cell currents in oocytes expressing WT polycystin-2 (Figure 5K).

### Phosphorylatable acidic clusters: a general mechanism of ion channel trafficking?

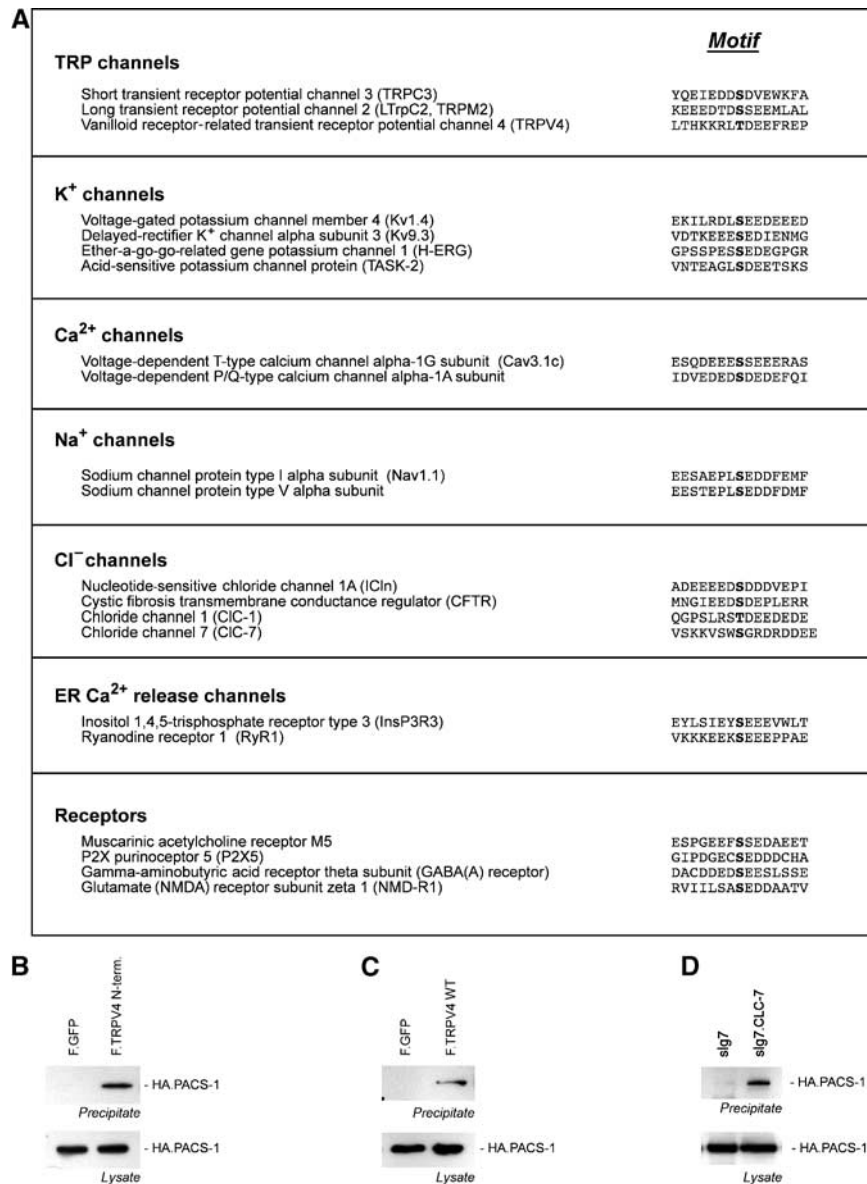
Our findings suggest that PACS family members participate in a novel mechanism for ER release and forward transport of ion channels with a phosphorylatable acidic cluster. A search of the NCBI database for proteins with phosphorylatable acidic clusters (D/ED/ED/ESD/ED/ED/E) revealed that many ion channels and membrane receptors contain potential PACS-binding motifs (Figure 6A). We tested several candidates for interaction with PACS molecules. The calcium-permeable, nonselective cation channel TRPV4 is a member of the TRP subfamily of temperature-sensitive ion channels (reviewed in Nilius *et al*, 2004). Although TRPV4 appears at the plasma membrane, a substantial fraction is retained in subcellular compartments (data not shown). TRPV4 interacts with PACS-1 (Figure 6B and C), as does the intracellular Cl<sup>-</sup> channel CLC-7 (reviewed in Jentsch *et al*, 2002). CLC-7<sup>1-123</sup> fused to an immunoglobulin tag (sIg7.CLC-7) co-precipitates HA-tagged PACS-1 (Figure 6D). Thus, PACS proteins may direct the localization of TRPV4, CLC-7 and other ion channels to distinct subcellular compartments.

## Discussion

### PACS molecules direct the subcellular localization of polycystin-2

PACS-1 is a cellular sorting protein that directs the endosome-to-Golgi trafficking of furin by connecting the acidic cluster-containing domain of furin to the AP-1 (reviewed in Thomas, 2002). Dominant negative mutations of PACS-1 that selectively disrupt the interaction with AP-1 cause mislocalization of furin from the TGN to endosomal compartments (Crump *et al*, 2001). Complementing the PACS-1-mediated endosome-to-Golgi retrieval of cargo proteins is the role of PACS-2 in Golgi-to-ER retrieval of cargo proteins. PACS-2 localizes to the ER (Simmen *et al*, 2005) and binds COPI, an adaptor that

**Figure 5** Trafficking and surface expression of polycystin-2 is controlled by PACS-1 and PACS-2. (A) The carboxy-terminal domain of polycystin-2, fused to the sIg7 tag, precipitates HA-PACS-2 (full length) from transiently transfected HEK 293T cells. (B) Immunoprecipitation of endogenous polycystin-2 from COS M6 immobilizes native PACS-2. (C) Mutation of serine 812 to alanine (PKD2 S812A) or mutation of the acidic cluster (PKD2 D815-817A), but not the serine 812 to aspartic acid mutation abrogates the interaction between polycystin-2 and PACS-2. The interactions were tested using the carboxy-terminal domain of WT or mutant polycystin-2 fused to the sIg7 tag and the HA-tagged PACS-2 in transiently transfected HEK 293T cells. (D) Adaptor co-immunoprecipitations. HA-tagged PACS-1, PACS-2, PACS-1admut or PACS-2admut were immunoprecipitated with anti-HA. Co-immunoprecipitating AP-1, AP-3 and COPI were detected by Western blot analysis. (E) Domain mapping for the interaction of PACS-2 with COPI. COPI was pulled down from cellular lysates using GST-tagged fragments of PACS-2 (amino-acid residues are indicated) (control = GST, input = 1% of initial lysate). (F) Ternary complex formed between recombinant COPI, PACS-2 and polycystin-2. Immobilized GST-PKD2<sup>741-871</sup> S812D immobilized  $\beta$ -COP in the presence of PACS-2<sup>37-179</sup>. (G) The Tac-polycystin-2 localizes to the ER. Interference with PACS-1 function by expression of a dn PACS-1 protein does not alter the subcellular distribution of polycystin-2, whereas expression of a dn PACS-2 protein allows polycystin-2 to exit the ER and translocate to the Golgi apparatus. (H) Affinities between polycystin-2 and PACS-1, PACS-2 or GGA3 were compared using fluorescent polarization. A fluorescein-tagged polycystin-2 phosphopeptide RSFPRSLDDpSEEDDDEDSGHSSRRR, derived from the putative PACS-binding site of polycystin-2, was incubated with different GST fusion proteins as indicated. The upper panel depicts the time-dependent interaction between the polycystin-2 peptide and the respective fusion proteins. In the lower panel, fluorescein-tagged polycystin-2 peptide (66 nM final concentration) was added to serially diluted GST fusion proteins to determine the dissociation constants (kD). (I) Surface labelling of nonpermeabilized cells expressing Tac-tagged polycystin-2. Neither dn PACS-1 nor dn PACS-2 alone increases the surface expression of polycystin-2 compared to control. In six randomly picked areas, only 1  $\pm$  1 cells were positive expressing polycystin-2 alone ( $n = 4$ ), 2  $\pm$  1 cells were positive coexpressing dn PACS-1 ( $n = 2$ ) and 4  $\pm$  2 cells were positive coexpressing dn PACS-2 ( $n = 2$ ). Coexpression of both dn PACS proteins dramatically increased the surface expression of polycystin-2 to 26  $\pm$  5 positive cells ( $n = 4$ ). (J) Injection of either xPACS-1 or xPACS-2 siRNA had no effect on the whole cell conductance of either water-injected oocytes (gray bars) or oocytes expressing polycystin-2 (black bars) ( $n = 10$ ). (K) Combined injection of xPACS-1 and xPACS-2 siRNA significantly increased the whole cell conductance of polycystin-2-expressing oocytes, whereas it had no effect on water-injected oocytes (gray bars: control siRNA; black bars: xPACS-1 and xPACS-2 siRNA) ( $n = 10$ ).

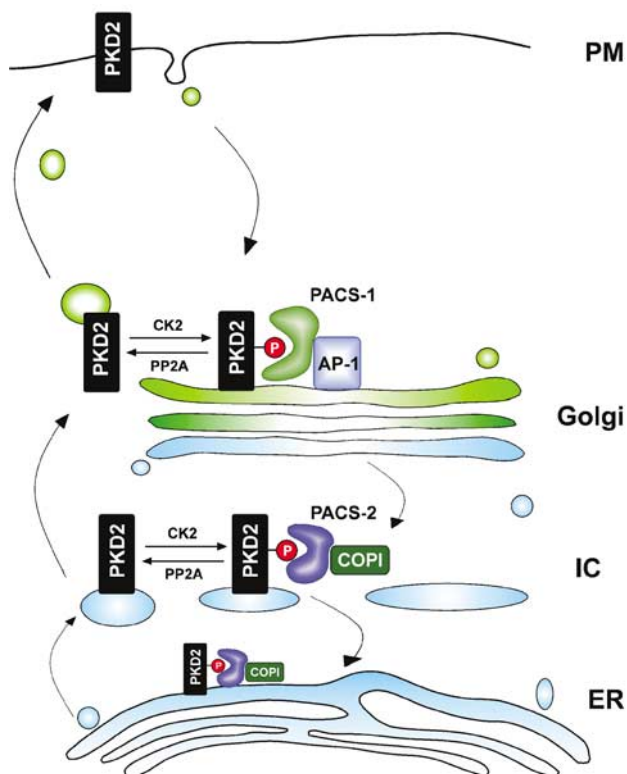


**Figure 6** PACS interacts with acidic clusters of other ion channels. **(A)** Database search for ion channels with acidic clusters. **(B)** Flag-tagged TRPV4<sup>1-486</sup> and TRPV4 WT **(C)** interact with HA-tagged PACS-1. **(D)** CLC-7<sup>1-123</sup> fused to an immunoglobulin tag (slg7:CLC-7) co-precipitates HA-tagged PACS-1.

directs the retrieval of cargo protein from the Golgi to ER (reviewed in Murshid and Presley, 2004). The interaction between COPI and PACS-2 suggests that this protein complex regulates the retrieval of polycystin-2 from the Golgi to the ER (Figure 7). Newly synthesized polycystin-2 may immediately undergo phosphorylation to mark it for PACS-2-mediated retrieval to the ER compartment, as CK2 is conveniently associated with the ER (Faust *et al*, 2001), and most of the cellular polycystin-2 is phosphorylated on S812 *in vivo* (Cai *et al*, 2004). Following dephosphorylation of S812, polycystin-2 is routed to the TGN, where it is sorted for its final destination. Polycystin-2 traffics to the Golgi apparatus in the absence of PACS-2, but fails to accumulate at the plasma membrane (Figure 5), suggesting that PACS-1 directs the retrieval of polycystin-2 from endosomal compartments back to the TGN. Polycystin-2 escapes the PACS-mediated

retrieval mechanism and accumulates at the plasma membrane only when both PACS molecules are absent or upon inhibition of CK2 activity. Thus, polycystin-2 trafficking is sequentially controlled at two different levels by PACS-2 and PACS-1, which direct Golgi-to-ER and endosome-to-TGN retrieval, respectively. These findings clearly resolve the hitherto puzzling opposition of polycystin-2 localizations: the ER retention of transiently expressed polycystin-2 (Cai *et al*, 1999; Koulen *et al*, 2002; Grimm *et al*, 2003) versus the presence of endogenous polycystin-2 at the plasma membrane of renal tubular cells (Foggensteiner *et al*, 2000; Newby *et al*, 2002; Scheffers *et al*, 2002; Luo *et al*, 2003). By regulating its interaction with PACS proteins, we could direct polycystin-2 to localize to ER or plasma membrane, a major advance in the understanding of polycystin-2 trafficking.





**Figure 7** Model of polycystin-2 trafficking. Polycystin-2 (PKD2), phosphorylated on serine 812 by CK2, binds to PACS-2/COPI and is retrieved from the intermediate compartment (IC) back to the endoplasmic compartment (ER). Dephosphorylation of polycystin-2 by PP2A releases polycystin-2 from this interaction, and facilitates its translocation to the plasma membrane. PACS-1/AP-1 mediates the retrieval of polycystin-2 to the Golgi/TGN compartment.

### Regulation of PACS binding

The interaction of polycystin-2 with adaptor proteins PACS-1 and PACS-2 is regulated by the CK2-dependent phosphorylation of serine 812. Polycystin-1 binds polycystin-2 and reportedly facilitates the translocation of polycystin-2 to the plasma membrane (Hanaoka *et al*, 2000). Accordingly, we speculated that polycystin-1 shields the PACS-binding site of polycystin-2. However, neither full-length polycystin-1 nor the carboxy-terminal domain of polycystin-1 affected the interaction between polycystin-2 and PACS proteins (data not shown). Interaction between polycystin-2 and PACS proteins is curtailed by phosphorylation of serine 812 by CK2. The active CK2 holoenzyme is composed of two catalytic subunits ( $\alpha$  and  $\alpha'$ ) and two regulatory subunits ( $\beta$  subunits). Although CK2 phosphorylates a large number of cellular proteins, the regulation of CK2 activity remains rather mysterious (reviewed in Litchfield, 2003). Whereas expression of the catalytic CK2  $\alpha$  subunit is inhibited by TGF- $\beta$ 1 (Cavin *et al*, 2003), the activity of CK2 is stimulated by INF- $\gamma$  or src-mediated tyrosine phosphorylation (Donella-Deana *et al*, 2003; Mead *et al*, 2003). Interestingly, adenomatous polyposis coli (APC) protein, upregulated by some Wnt molecules (Papkoff *et al*, 1996), inhibits CK2 activity (Homma *et al*, 2002). During renal tubulogenesis, requisite Wnt signalling could modulate CK2 activity and polycystin-2 localization (Dressler, 2002). Alternatively, activation of PP2A could release polycystin-2 from its interaction with PACS molecules

(Molloy *et al*, 1998) and promote localization of polycystin-2 to the plasma membrane. Coexpression of polycystin-1 did not affect the binding of PACS-1 to polycystin-2 at the plasma membrane; however, modulation of CK2 or PP2A may require ligand-mediated activation of polycystin-1.

### PACS molecules interact with phosphorylatable acidic clusters of other ion channels

Important cellular functions are defined by the activity of ion channels, mandating complex mechanisms to control their trafficking to the cell surface. Many channels reside in the ER until homo- or heterodimeric interactions with other pore-forming or auxiliary subunits permit their exit from the ER. These interactions shield retention signals, such as the RKR, RSRR, RXT motifs in KIR, GABA $_B$  receptor and CFTR, or they mask assembly motifs, such as the PL(Y/F)(F/Y)xxN motif in the nicotinic acetylcholine receptor channel (reviewed in Deutsch, 2003). Other signals promote anterograde trafficking of ion channels such as the diacidic motifs present in KIR 1.1 and 2.1 channels (Ma *et al*, 2001). Recently, 14-3-3 was shown to release ion channels in a phosphorylation-dependent manner from the ER, providing insight into the regulation of forward trafficking of channels with a dibasic N-terminal  $\beta$ -COP-binding site (O'Kelly *et al*, 2002). Our database search for ion channels with acidic clusters detected this motif in many ion channels (Figure 6A). We demonstrated that even less perfect matches such as the partial acidic cluster of TRPV4, containing a threonine rather than a serine, mediate binding of PACS-1. The presence of candidate PACS-binding motifs within many ion channels suggests a broad and important role for PACS proteins in the sorting of ion channels, and thereby an important mechanism for controlling the activity of ion channels.

### Conclusions

Our results demonstrate that PACS-1 and PACS-2 direct the localization of polycystin-2 and alter the permeability of the plasma membrane to cations such as calcium. The interaction between polycystin-2 and PACS proteins is mediated by a phosphorylatable acidic cluster within the carboxy-terminus of polycystin-2. Our findings clarify the molecular mechanisms that retain polycystin-2 in the ER and sort it to the plasma membrane. The presence of acidic cluster sorting motifs within the cytoplasmic domains of a large number of ion channels suggests that PACS proteins may play an important role in the trafficking of ion channels between secretory pathway compartments.

### Materials and methods

#### Materials

Flag-tagged versions of polycystin-2 and point mutations were generated by PCR and standard cloning techniques. The PACS-1 constructs (Molloy *et al*, 1998; Crump *et al*, 2001) and polycystin-2 antiserum (Foggensteiner *et al*, 2000) have recently been described. The immunoglobulin tag contains the leader sequence of CD5 followed by the CH2 and CH3 domain of human immunoglobulin and the transmembrane region of CD7. The CD16.7 tag contains the extracellular domain of CD16, followed by the transmembrane region of CD7. Both tags have been previously described (Tsiokas *et al*, 1997). Recombinant proteins, fused to the MBP, GST or HIS tag, were generated as previously described (Kim *et al*, 1999). Tac-polycystin-2 fusion cDNAs were constructed as follows: a *Sal*I site was inserted by PCR after the transmembrane sequence of human

Tac/CD25 in pGEMT-Easy (Promega) with the oligos GCT CAC CTG GCA CGC TCG ACA GAG GAA GAG TAG and CTA CTC TTC CTC TGT CGA CGC TGC CAG GTG AGC. Another in-frame *SalI* site was generated by PCR at the beginning of the cytosolic tail sequence of human polycystin-2 with the oligo ATA TCG TCG ACA GGG CTA CCA TAA AGC TTT G and the fused cDNA was inserted into pcDNA3 (Invitrogen). For the construction of dn PACS-2 with the oligos CCA GTG GAC AAG TGG CTG CAG CTG CGG CCG CTG CAG CTT CCT TGC AGT ATC CTC and GAG GAT ACT GCA AGG AAG CTG CAG CGG CCG CAG CTG CAG CCA CTT GTC CAC TGG, the amino acids 89–96 were PCR-mutated to alanines as previously described for dn PACS-1. Thioredoxin (TRX)-PACS-2 FBR sequence (residues 37–179) of human PACS-2 was expressed using pET32 (Novagen). GST fusion proteins containing the PACS-2 fragments corresponding to residues 1–36 (amino-terminal region), 37–179 (cargo/adaptor-binding region (FBR)), 37–179<sub>8A</sub> (FBR containing an E<sub>89</sub>TDLALTF→Ala<sub>8</sub> substitution, 180–467) and 468–889 were generated by subcloning the respective DNA fragments into pGEX3X (Amersham). DRB was purchased from Alexis (Germany). TBB was kindly provided by LA Pinna (University of Padua), and subsequently synthesized. Briefly, a suspension of benzotriazole (1.00 g, 8.39 mmol) and sodium acetate (2.81 g, 34.2 mmol) in acetic acid (7 ml) was added to bromine (2.0 ml, 39 mmol). The reaction mixture was heated to 120°C in a sealed tube for 60 h, and poured into water (50 ml); the resulting precipitate was recrystallized from ethanol:water (1:1). The raw product was purified by repeated recrystallization from acetic acid to give pure TBB (158 mg) with a melting point of 261–263°C (Wiley and Hussung, 1957).

### Co-immunoprecipitations

*In vitro* binding assays were performed as previously described (Crump *et al*, 2001). For the PACS-2-COPI binding assays,  $8 \times 10^6$  A7 cells were lysed in mRIPA (1% NP-40, 1% deoxycholate, 150 mM NaCl, 50 mM Tris, pH 8.0, supplemented with protease inhibitors (COMPLETE, Roche)) and incubated with 25 µg of PACS-2-GST fragments followed by streptavidin-agarose pull-down and Western blot with anti-β-COP. To detect PKD2–PACS-2–COPI ternary complex formation, 10 µg of GST or GST-calnexin fusion proteins was preincubated with glutathione-agarose, and then mixed with 30 µg of Trx-PACS-2-FBR or 2 µg of purified COPI, or both in 25 mM HEPES (pH 7.2), 25 mM KCl, 2.5 mM MgOAc, 0.1 mM DTT, protease inhibitors and 5 mg/ml BSA. The bound material was analyzed by Western blot with anti β-COP. Western blot analysis and co-immunoprecipitations were performed as recently described (Kim *et al*, 1999; Benzing *et al*, 2001), and representative results of at least three independent experiments are shown.

### Immunofluorescence

To determine the subcellular localization of CD16.7-PKD2 chimeras, transiently transfected COS M6 cells were fixed with acetone/methanol and incubated with the primary antibody as indicated, followed by the appropriate fluorescein-conjugated secondary antibody. Images were obtained using a confocal microscope (LSM 510; Zeiss, Jena, Germany). To determine the surface expression of proteins, transiently transfected COS M6 cells were detached in 0.5 mM EDTA/PBS and labelled with primary and secondary antibodies at 4°C. Cells were fixed in 1% formaldehyde/PBS and analyzed by FACS. To determine the subcellular localization of Tac-PKD2 chimeras, BSC40 cells were plated at 100 000 in 35-mm dishes, and transfected the subsequent day with pcDNA3-Tac-PKD2 chimera using GENEporter (GTS) according to the manufacturer's recommendations. After 24 h, the cells were infected with adenovirus expressing either the *trans* activator alone or in combination with PACS-1admut, PACS-2admut or both (m.o.i.: 20). At 24 h after infection, cells were processed for immunofluorescence. For intracellular staining, cells were fixed in formaldehyde/PBS and permeabilized in blocking buffer (0.5 Triton X-100,

0.2% goat serum in PBS). Staining was performed with anti-mannosidase II from K Moremen (1:1000) and anti-2A3A1HTac antibody (1:400). Antibodies were visualized using Alexa antibodies (goat anti-IgG1 594 for 2A3AH1 and anti-rabbit 488 for mannosidase). Images were captured using  $\times 63$  oil immersion objective on a Zeiss Axioplan 2 microscope. For external binding, cells were incubated for another hour at 4°C in growth medium containing anti-2A3A1H (1:400) and then fixed. Bound antibody was revealed using Alexa goat anti-IgG1 594 antibody. There was no permeabilization step; Triton X-100 was omitted from the blocking buffer.

### Electrophysiology

Oocytes were isolated by partial ovariectomy from *X. laevis*. cRNAs were synthesized *in vitro* using the mMessage mMachine kit (Ambion). At 12–24 h after isolation, stage V–VI oocytes were injected with 30 nl of water containing 10 ng cRNA of WT polycystin-2 or various mutations of polycystin-2. Voltage clamp experiments were performed at 3–6 days after injection of cRNAs. Whole cell currents of oocytes were recorded using the Turbo TEC 03X voltage/current clamp amplifier (npi electronic, Tamm, Germany) and 2 M KCl microelectrodes with resistances of 0.5–2 MΩ. All whole cell voltage clamp experiments were performed at 20°C in ND96 solution (composition (in mM): 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES). Equimolar substitution of Cl<sup>-</sup> by gluconate and Na<sup>+</sup> by N-methyl-D-glucamine (NMDG) was carried out as indicated. Oocytes were clamped at 0 mV, and voltage ramps from -100 to +50 mV were applied every 1.2 s. Conductance was determined in the negative voltage range (-100 to 0 mV). Data are presented as original recordings or as mean ± s.e.m. (*n* = number of experiments). Unpaired and paired Student's *t*-test was used for statistical analysis. A *P*-value of <0.05 was accepted to indicate statistical significance. The experiments were performed with five different batches of oocytes.

### Fluorescence polarization

To determine the affinities between polycystin-2 and PACS-1, PACS-2 and GGA3, we performed fluorescence polarization assays as described previously (Benzing *et al*, 2000). Briefly, GST fusion proteins were expressed in bacteria and purified on glutathione-Sepharose beads (GST.PACS-1<sup>85–280</sup>, GST.PACS-2<sup>117–266</sup> and GST.GGA3<sup>1–146</sup>). The fluorescent polycystin-2 phosphopeptide was linked with FITC to the peptide amino-terminus via a β-alanine linker (Jerini AG, Berlin, Germany). Fluorescence polarization anisotropy was measured using a Panvera Beacon 2000. Fluorescein-tagged peptide was mixed in (66 nM final concentration) and fluorescence polarization measured at 22°C after a 120-s delay. GST fusion proteins were serially diluted to measure the binding curves.

### RNA interference

To silence gene expression of xPACS-1 or xPACS-2, synthetic siRNA was injected into *Xenopus* oocytes 3–4 days prior to voltage clamp experiments. The siRNA target sequences were AACTTCAGGGCT CAAAACGT and AATAACCGGCAACAGAATT for xPACS-1 and xPACS-2, respectively (based on *Xenopus* sequences BI444377 and BG360195).

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