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PIGEA-14, a Novel Coiled-coil Protein Affecting the Intracellular Distribution of Polycystin-2*

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Employing a yeast two-hybrid screen with the COOH terminus of polycystin-2, one of the proteins mutated in patients with polycystic kidney disease, we were able to isolate a novel protein that we call PIGEA-14 (polycystin-2 interactor, Golgi- and endoplasmic reticulum-associated protein with a molecular mass of 14 kDa). Molecular modeling only predicts a coiled-coil motif, but no other functional domains, in PIGEA-14. In a subsequent two-hybrid screen using PIGEA-14 as a bait, we found GM130, a component of the cis-compartment of the Golgi apparatus. Co-expression of the PIGEA-14 and PKD2 cDNAs in LLC-PK$_1$ and HeLa cells resulted in a redistribution of PIGEA-14 and polycystin-2 to the trans-Golgi network, which suggests that PIGEA-14 plays an important role in regulating the intracellular location of polycystin-2 and possibly other intracellular proteins. Our results also indicate that the intracellular trafficking of polycystin-2 is regulated both at the level of the endoplasmic reticulum and that of the trans-Golgi network.

PKD2 was identified as one of the two genes mutated in patients with autosomal-dominant polycystic kidney disease (1). It encodes polycystin-2, a 968-amino acid protein with six putative transmembrane domains, whose NH_2 and COOH termini are predicted to extend into the cytoplasm. Subsequent to the identification of polycystin-2, three related proteins have been found, which are called polycystin-2L, polycystin-2L2, and mucolipin. These four proteins apparently constitute a branch of the large family of Trp (transient receptor potential) proteins, a novel class of cation channels (2). Although for polycystin-2 (3–6), polycystin-2L (7), and mucolipin (8) cation channel activity indeed has been demonstrated, no reports have been published yet whether polycystin-2L2 can also conduct cations.

So far the physiological function of polycystin-2 and the mechanism of cyst formation have remained a mystery (9). A lot of evidence indicates that the majority of polycystin-2 resides in the endoplasmic reticulum both in cell lines (10–12) and in the kidney (6, 10, 12), although there are contradicting results (3, 13). Furthermore a retention signal for the endoplasmic reticulum has been identified in the COOH terminus of polycystin-2 (10). Because many mutations in the *PKD2* gene are predicted to lead to the synthesis of a truncated protein (14), a possible scenario could be that a mutant protein escapes from the endoplasmic reticulum and reaches the plasma membrane, where it results in the uncontrolled influx of Ca^{2+} ions.

One entry point to a better functional understanding of polycystin-2 lies in the identification of interacting proteins. Polycystin-2 and polycystin-1 have been shown to associate through their respective COOH termini (15, 16), which indicates that they participate in the same regulatory network. The interaction of polycystin-2 with TRPC1 (17) emphasizes the cation channel properties of polycystin-2. Our own results (11) as well as those of another group (18) suggest that polycystin-2 is connected to the actin cytoskeleton. In this report we identify a novel protein with a coiled-coil domain, which is connected both to polycystin-2 and to GM130, a constituent of the Golgi matrix.

MATERIALS AND METHODS

Yeast Two-hybrid Screen—The fragment coding for the COOH terminus of human polycystin-2 (amino acids 680–968) was subcloned into the bait plasmid pPC97 (19). MaV103 yeast cells were first transformed with the bait construct and then with a human kidney cDNA library in the prey plasmid pPC86 (Invitrogen). Approximately 4×10^5 transformants were first screened for histidine prototrophy in the presence of 50 mM 3-aminotriazole (Sigma) and subsequently assayed for activation of the *URA3* and *LacZ* genes. A second yeast two-hybrid screen was carried out with PIGEA-14 as a bait and the same human kidney cDNA library as a prey.

Generation of Deletion Mutants of PIGEA-14 and Polycystin-2—To define which regions within polycystin-2 and PIGEA-14 were necessary for the respective interactions, a number of deletion mutants were constructed by PCR using *Pfu* polymerase. All of the constructs were sequenced prior to use to confirm the absence of mutations.

Expression Plasmids—The synthesis of a full-length human polycystin-2 protein with an epitope tag from the influenza virus hemagglutinin protein at its COOH terminus was driven by the eukaryotic expression vectors pcDNA3 (Invitrogen) and pUHD 10–3 (kind gift from Hermann Bujard). PIGEA-14 was cloned into the eukaryotic expression vector pEBG, which encodes fusion proteins with glutathione *S*-transferase (kind gift from Tom Force).

To generate a construct, in which the COOH terminus of polycystin-2 is located on the extracellular side, the CD8 cDNA was digested with SgrAI (the recognition site for SgrAI site is located in the region encoding the extracellular domain of CD8). The fragment encoding the region beyond the sixth membrane-spanning domain of polycystin-2 was PCR-amplified with oligonucleotides containing recognition sites for AvaI at their 5′-end, cut with AvaI, and inserted into the SgrAI site of CD8. The correct orientation of the insert was confirmed by DNA sequencing.

Cell Culture and Transfection Protocols—COS-7 cells were transiently transfected using the DEAE-dextran method (20). HtTA-1 (kind gift from Hermann Bujard) and LtTA-2,22 cells (HeLa and LLC-PK$_1$ cells producing a tetracycline-controlled transactivator, respectively) were stably transfected using poly-L-ornithine (21) and selected with puromycin at a concentration of 1 μg/ml (Calbiochem). To co-express the PIGEA-14 and PKD2 cDNAs, HtTA-1 and LtTA-2,22 cells inducibly producing the HA[1] epitope-tagged polycystin-2 protein were stably transfected with pEBG/PIGEA-14 using hygromycin at a concentration of 300 μg/ml (Calbiochem).

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[1] The abbreviations used are: HA, hemagglutinin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; Endo H, endoglycosidase H.

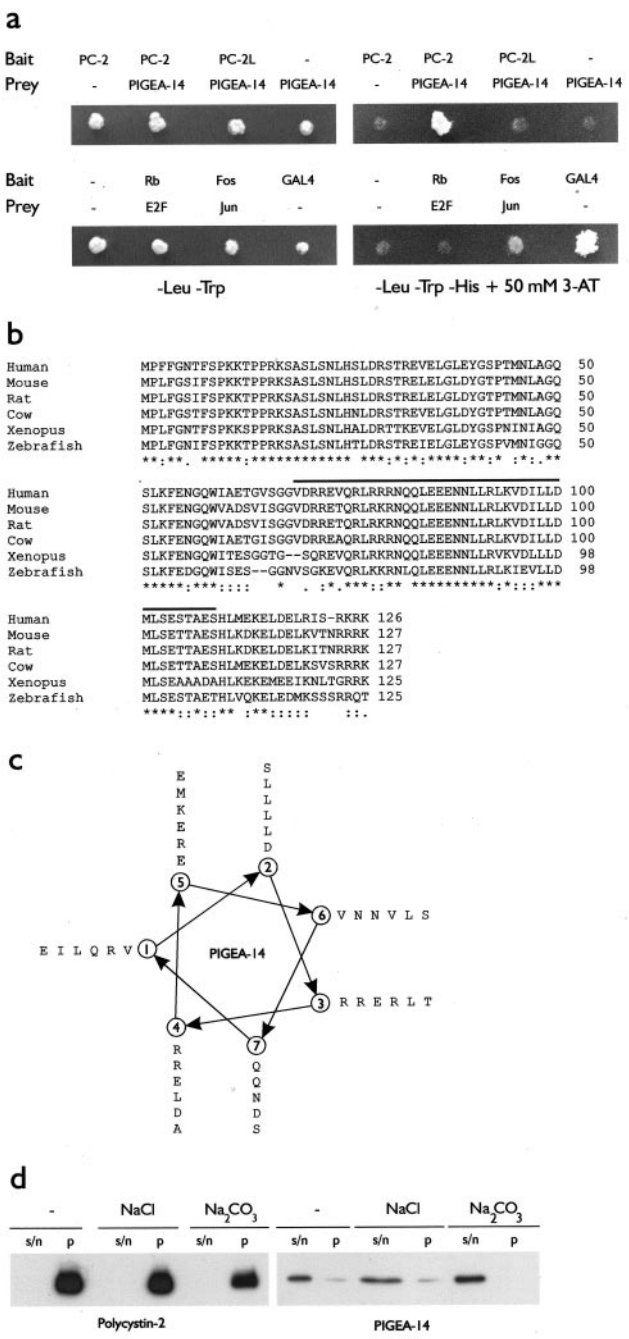

FIG. 1. **PIGEA-14, a soluble coiled-coil protein interacting with polycystin-2.** *a*, two-hybrid assay for the interaction between PIGEA-14 and polycystin-2. Yeast containing various combinations of bait and prey plasmids were first grown in the absence of leucine and tryptophan before being replica-plated onto selective agarose lacking histidine and containing 50 mM of 3-aminotriazole (*3-AT*). Only a bait-prey combination of the COOH terminus of polycystin-2 (*PC-2*) and PIGEA-14 was able to confer prototrophy for histidine; the COOH terminus of polycystin-2L (*PC-2L*) was not. The *two bottom panels* show the negative control lacking bait and prey proteins and positive controls of increasing strength. *b*, sequence comparison between PIGEA-14 proteins from different species using the CLUSTAL algorithm (the respective accession numbers are as follows: human, AL021707; mouse, AK003719; rat, AF393211; cow, AF393210; *Xenopus*, AF393212; and zebrafish, AF393213). The coiled-coil domain is indicated by the *bar* above the sequence. *c*, helical wheel presentation of the coiled-coil domain of PIGEA-14 demonstrates the leucine zipper motif at position 2 and the abundance of charged amino acids at positions 3–5. *d*, PIGEA-14 is not an integral membrane protein. After LLC-PK_1 cells were homogenized in a Dounce homogenizer, the cell lysate was centrifuged for 1 h at 100,000 × *g*, and equivalent aliquots of the supernatant and pellet fraction were analyzed by Western blot. It can be seen that the large majority of PIGEA-14 resides in the supernatant and that subjecting the cell homogenate to an alkaline pH (*Na_2CO_3*) before centrifugation results in the transfer of even more PIGEA-14 into the supernatant, whereas a treatment with 1.5 M NaCl (*NaCl*) had little effect. The integral membrane protein polycystin-2 is exclusively present in the pellet fraction.

Co-precipitation and Western Blot Experiments—The cells were lysed in 1× PBS, 1% Triton X-100. Swollen glutathione-agarose, protein A-Sepharose, or protein G-agarose beads were incubated with lysate containing 2–3 mg of protein for at least 4 h at 4 °C (for immunoprecipitations, 50 μl of 12CA5, 50 μl of 9E10, 50 μl of OKT8, and 2 μl of the anti-GM130 antiserum were included as well). The beads were washed with cell lysis buffer, resuspended in SDS sample buffer, and analyzed by Western blot. The mouse monoclonal antibody 12CA5, which is directed against an epitope of the hemagglutinin protein (hybridoma supernatant diluted 1:30), a mouse monoclonal anti-GST antibody (diluted 1:3,000; Sigma), and a sheep polyclonal anti-GM130 antibody (diluted 1:2,000; kind gift from Francis Barr) were used as primary antibodies; horseradish peroxidase-conjugated goat anti-mouse and donkey anti-sheep antibodies (diluted 1:10,000; Sigma) served as secondary antibodies.

Immunocytochemistry—Transfected cells were cultured on coverslips for at least 2 days. After fixation with Bouin's solution (0.1 M sodium phosphate, pH 7.4, 15% saturated picric acid, 2% formaldehyde) for 20 min, the cells were permeabilized in 1× PBS, 2% bovine serum albumin, 0.1% Triton X-100 for 45 min and then incubated with the primary antibody (diluted in 1× PBS, 2% bovine serum albumin) for 2 h at room temperature. The following primary antibodies were used: the mouse monoclonal anti-HA epitope antibody 12CA5 (diluted 1:30), a mouse monoclonal anti-GST antibody (diluted 1:1,000; Sigma), a rabbit polyclonal anti-GST antibody (diluted 1:200; Santa Cruz Biotechnology), a rabbit polyclonal antibody against Sec61β, a marker of the endoplasmic reticulum (diluted 1:100; kind gift from Martin Pool and Bernhard Dobberstein), the mouse monoclonal anti-CD8 antibody OKT8 (diluted 1:30; kind gift from Walter Nickel), a sheep polyclonal antibody against GM130 (diluted 1:250; kind gift from Francis Barr), a mouse monoclonal antibody against acetylated tubulin (diluted 1:500; Sigma), and a rabbit polyclonal antibody against TGN46 (diluted 1:250; kind gift from George Banting). After three washes with 1× PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (diluted 1:150; Cappel), Cy3-conjugated anti-mouse IgG (diluted 1:300; Dianova), fluorescein isothiocyanate-conjugated anti-rabbit IgG (diluted 1:200; Cappel), Cy3-conjugated anti-rabbit IgG (diluted 1:300; Dianova) or fluorescein isothiocyanate-conjugated anti-sheep IgG (diluted 1:40; Sigma) for 1 h at room temperature. Following the incubation with the secondary antibody, the cells were washed with 1× PBS and mounted in bicarbonate-buffered glycerol, pH 8.6. Pictures were taken with a Zeiss laser confocal scanning microscope and then processed with Adobe Photoshop.

Immunogold Electron Microscopy—The cells were grown on Petri dishes and then treated as described above for immunocytochemistry. The primary antibodies used were the mouse monoclonal anti-HA epitope antibody 12CA5 and the mouse monoclonal anti-GST antibody; the secondary antibody (diluted 1:40) was conjugated to 6-nm gold particles (Dianova). Following the incubation with the secondary antibody, the cells were washed with 1× PBS and fixed with 1× PBS, 2% glutaraldehyde. Subsequent to a treatment with osmium, the cells were dehydrated and embedded in Durcupan ACM. Sections of 90 nm thickness were prepared, stained with uranyl acetate, and analyzed by electron microscopy.

Cellular Fractionation and Deglycosylation Experiments—Cells grown on Petri dishes were washed twice in 1× PBS and scraped into a microcentrifuge tube. After centrifuging for 5 min at 4,000 rpm, the cell pellet was resuspended in 1 ml of ice-cold homogenization buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin) and homogenized with a motor-driven pestle. Post-nuclear supernatants were prepared by centrifugation at 1,000 × *g*, and then the supernatant was divided into three tubes. One tube was left on ice for 30 min without any treatment, one tube was treated for 30 min with 1.5 M NaCl (final concentration), and one tube was treated with 0.2 M Na_2CO_3 (final concentration) pH 11.0. These homogenates were centrifuged at 100,000 × *g* for 1 h at 4 °C, the supernatants were recovered, and the pellets were washed with the respective buffers before being resuspended in SDS sample buffer. Equal amounts of supernatant and pellet fractions were analyzed by Western blot. When a deglycosylation experiment was to be performed, the membrane pellet was resuspended in denaturation buffer and incubated with Endo H

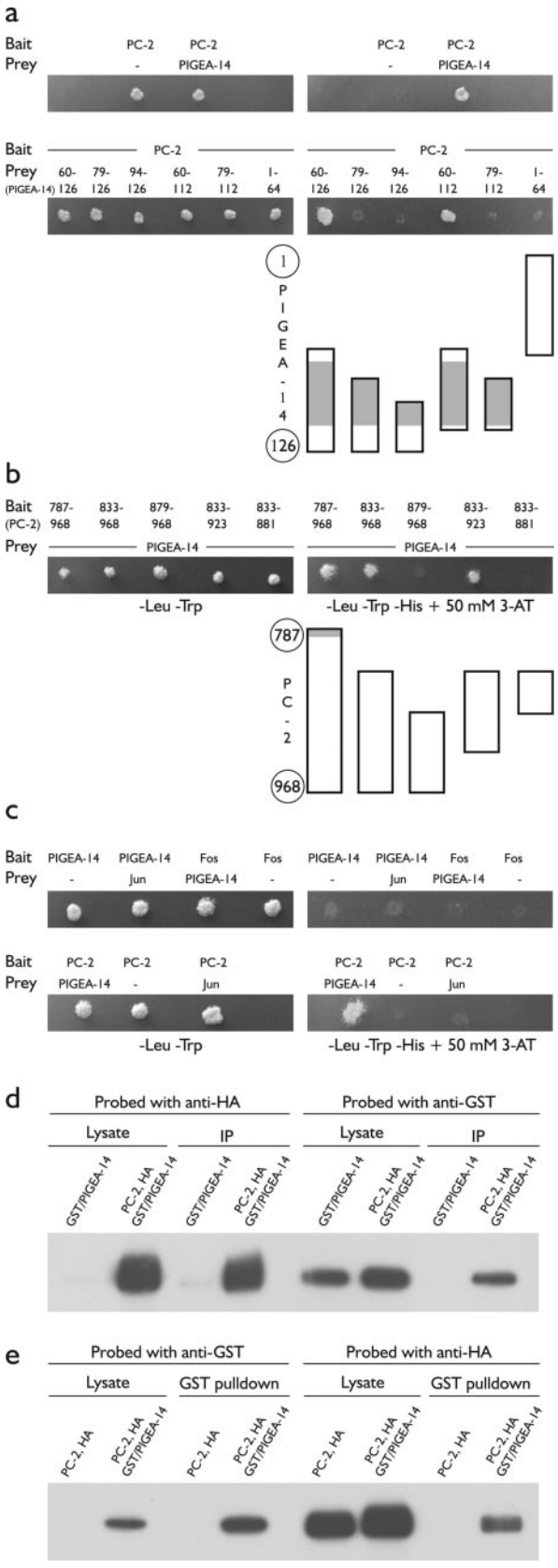

FIG. 2. **Corroboration of the interaction between PIGEA-14 and polycystin-2.** *a*, various deletion constructs of PIGEA-14 were tested for their interaction with the COOH terminus of polycystin-2 (*PC-2*). Only those constructs containing the entire coiled-coil domain (*shaded*) of PIGEA-14 still interacted with polycystin-2. *b*, in the case of polycystin-2, a region beyond its coiled-coil domain (*shaded*) was necessary for its interaction with PIGEA-14. *c*, a yeast two-hybrid assay demonstrates that the leucine zipper domains of c-Fos and c-Jun do not interact with PIGEA-14 and polycystin-2. *d* and *e*, stably transfected LLC-PK$_1$ cells either inducibly producing a HA epitope-tagged polycystin-2 protein or inducibly producing a HA epitope-tagged polycystin-2 protein and constitutively producing a GST/PIGEA-14 fusion protein were used for immunoprecipitation (*IP*) with the anti-HA epitope antibody (*d*) and for a GST pull-down experiment (*e*). The GST/PIGEA-14 protein was co-immunoprecipitated only when polycystin-2 was present (*lane PC-2, HA + GST/PIGEA-14* in *d*) but not in its absence (*lane GST/PIGEA-14* in *d*), and polycystin-2 was pulled down only when GST/PIGEA-14 was present (*lane PC-2, HA + GST/PIGEA-14* in *e*) but not in its absence (*lane PC-2, HA* in *e*).

and peptide *N*-glycosidase F according to the manufacturer's instructions (New England Biolabs).

RESULTS

Isolation of PIGEA-14—We used the COOH terminus of human polycystin-2 to perform a two-hybrid screen with a human adult kidney cDNA library. Of ~400,000 transformants, 28 clones were isolated that grew in the absence of histidine and uracil and also turned blue in a β-galactosidase assay. Eight of those clones turned out to code for a novel protein that we call PIGEA-14 (pronounced "piggy 14"), a 126-amino acid protein with a calculated molecular mass of 14.2 kDa (Fig. 1*a*). It is highly conserved in various vertebrate species (Fig. 1*b*) but was not detected in *Drosophila melanogaster* and *Caenorhabditis elegans*. According to information from the human genome project, PIGEA-14 is located on human chromosome 22q12-13. It is organized in five exons, which are delineated by consensus splice donor and acceptor sites (GenBank^{TM} accession number BK005534). The only predicted structural motif is a coiled-coil domain extending from amino acid Val^{69} to amino acid Ser^{109} in the human protein (Fig. 1, *b* and *c*). No putative membrane-spanning segments were revealed *in silico*, and indeed only a minor portion of PIGEA-14 was detected in the pellet fraction after subcellular fractionation and subsequent centrifugation at 100,000 × *g* (Fig. 1*d*).

A deletion mutagenesis analysis showed that the minimal domain necessary for the interaction of PIGEA-14 with polycystin-2 comprised amino acids Ile^{60}–Met^{112}, and therefore the coiled-coil domain of PIGEA-14 (Fig. 2*a*). A coiled-coil domain has also been found in the COOH terminus of polycystin-2 (amino acids 769–796) and therefore served as an obvious candidate for the interacting counterpart in polycystin-2. The deletion mutant extending from Gly^{833} to Ser^{923} represented the minimal domain in polycystin-2 that was still able to interact with PIGEA-14, therefore showing that the coiled-coil domain in polycystin-2 is not necessary for the interaction with PIGEA-14 (Fig. 2*b*). Because we were worried that the interaction of PIGEA-14 with polycystin-2 was due to the stickiness of its coiled-coil domain, we assayed PIGEA-14 and polycystin-2 against coiled-coil domains from other proteins. When PIGEA-14 was tested against the leucine zipper domains of c-Fos and c-Jun and polycystin-2 was tested against that of c-Jun, no interaction was demonstrated in yeast, thus indicating that the interaction was specific (Fig. 2*c*). We also assayed PIGEA-14 against the COOH terminus of polycystin-2L, and again no interaction was observed (Fig. 1*a*). To confirm the interaction in mammalian cell lines, stably transfected LLC-PK$_1$ and HeLa cells were established that inducibly produce a HA epitope-tagged human polycystin-2 protein and constitutively produce a GST/PIGEA-14 fusion protein. Both by immunoprecipitation and in a GST pull-down assay the interaction between polycystin-2 and PIGEA-14 was confirmed (Fig. 2, *d* and *e*).

PIGEA-14 Affects the Intracellular Distribution of Polycystin-2—Because its primary sequence did not provide any hints on the possible function of PIGEA-14, we performed another

two-hybrid screen using PIGEA-14 as a bait. Of 720,000 transformants, 24 clones grew in the absence of histidine and uracil and turned blue in a β-galactosidase assay. Three of those clones represented GM130, a protein associated with the Golgi matrix and two of those clones represented PIGEA-14. Polycystin-2 was not isolated, which may not be too surprising because it is predicted to contain six transmembrane segments and therefore either does not even reach the nucleus or if it does, it is probably unstable or does not fold properly. As in the case of the interaction between PIGEA-14 and polycystin-2, the interaction between PIGEA-14 and GM130 and the self-interaction of PIGEA-14 depended on the presence of the coiled-coil domain of PIGEA-14, although the responsible interaction domains seemed to vary somewhat (Fig. 3*a*). Again the association between PIGEA-14 and GM130 was confirmed by co-precipitation in mammalian cells (Fig. 3*b*).

The finding that PIGEA-14 interacts with GM130 prompted us to take a closer look at the intracellular distribution of PIGEA-14. Stably transfected HeLa cells were established that inducibly produced polycystin-2 either in the absence or presence of PIGEA-14. In the absence of exogenous PIGEA-14, polycystin-2 was distributed in a reticular pattern in the cytoplasm of most cells as shown before (11), consistent with its location in the endoplasmic reticulum (Fig. 4*a*), but in a minority of cells we also found a patchy distribution of polycystin-2 (Fig. 4*b*). Similarly, in the absence of polycystin-2, PIGEA-14 was found in a fine reticular pattern in the cytoplasm (Fig. 4*c*). After the induction of polycystin-2, however, the distribution of PIGEA-14 and of polycystin-2 changed dramatically to an identical patchy pattern, and upon higher magnification a lumen could be noticed in those "patches" (Fig. 4*d*), which indicated that they did not represent aggregated protein but rather vesicular organelles.

Because we were worried that the redistribution of both proteins was due to the overexpression of the PIGEA-14 and PKD2 cDNAs and not to a specific interaction, we performed several control experiments. In HeLa cells stably producing PIGEA-14 and a truncated polycystin-2 protein lacking the region beyond amino acid 703, PIGEA-14 was still found in a reticular pattern in the cytoplasm, whereas the truncated polycystin-2 protein reached the plasma membrane (Fig. 5*a*). This strongly argues that the interaction between PIGEA-14 and polycystin-2 and therefore also the redistribution of PIGEA-14 upon the induction of full-length polycystin-2 depended on the COOH terminus of polycystin-2 (because both the full-length and truncated polycystin-2 protein were epitope-tagged, we were able to make sure that they were synthesized at comparable levels). A second set of control experiments was based on the generally accepted assumption that the COOH terminus of polycystin-2 extends into the cytoplasm. Therefore it would be predicted that polycystin-2 would not be able to interact with PIGEA-14 if its COOH terminus would be located on the opposite side of the membrane. If a redistribution was still observed with such a construct, then it probably represented a nonspecific effect from overexpressing two cDNAs. We tested this hypothesis by generating fusion proteins between CD8, an integral membrane protein normally located in the plasma membrane, and the COOH terminus of polycystin-2. In one construct the COOH terminus of polycystin-2 was fused to the intracellular portion of CD8, whereas in the second construct it was inserted into the extracellular portion of CD8. The transient transfection of COS-7 cells with expression plasmids encoding the GST/PIGEA-14 and the CD8/polycystin-2 fusion proteins yielded the following distribution patterns. Similar to what we observed in HeLa cells, the CD8 protein with the COOH terminus of polycystin-2 attached to its intracellular

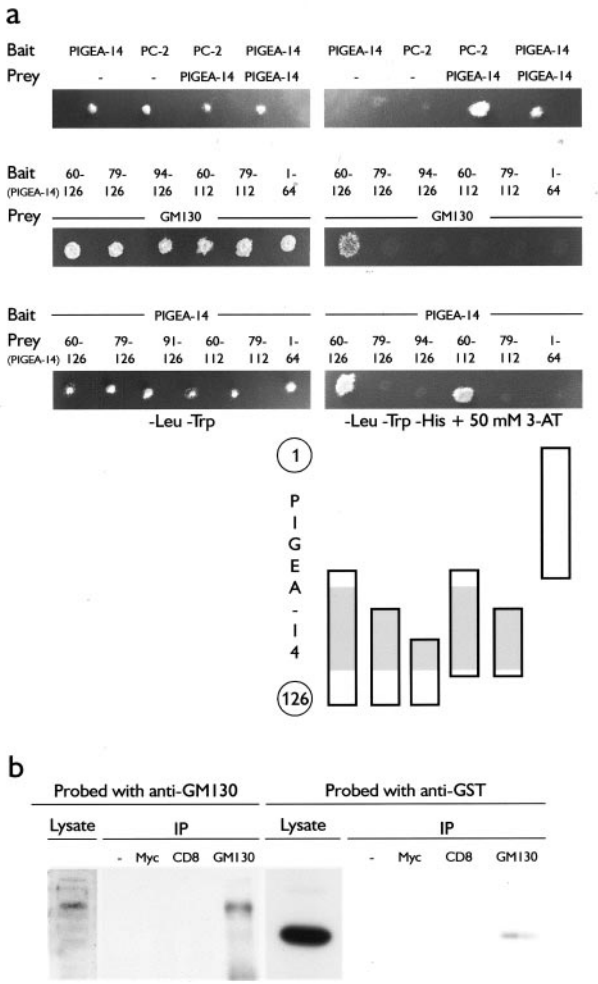

FIG. 3. **Interaction of PIGEA-14 with GM130 and with itself.** *a*, using a yeast two-hybrid assay it can be appreciated that the interaction between PIGEA-14 and GM130 and the self-interaction of PIGEA-14 is mediated by the coiled-coil domain of PIGEA-14. Apparently, however, a more extended interface is required for the interaction between PIGEA-14 and GM130, because a COOH-terminal truncation of PIGEA-14 ending shortly before the coiled-coil motif already abrogates the association of the two proteins. *b*, interaction between PIGEA-14 and GM130 in LLC-PK_1 cells. A cell lysate was prepared from stably transfected LLC-PK_1 cells constitutively synthesizing a GST/PIGEA-14 fusion protein. Whereas the immunoprecipitation performed with an anti-GM130 antiserum resulted in the clear co-precipitation of the GST/PIGEA-14 protein, an immunoprecipitation with monoclonal antibodies against Myc (monoclonal antibody 9E10) and CD8 (monoclonal antibody OKT8) did not; an incubation with protein G beads but without any antibody served as a negative control (−).

portion was detected in a reticular and patchy distribution (Fig. 5, *b* and *c*). However, when the COOH terminus of polycystin-2 was inserted into the extracellular portion of CD8, the fusion protein was clearly detected in the plasma membrane (Fig. 5*d*). Again very comparable with the situation in HeLa cells, the GST/PIGEA-14 fusion protein was distributed in a reticular fashion in the cytoplasm (Fig. 5*e*). The co-transfection of the respective expression plasmids only then led to a co-localization of polycystin-2 and PIGEA-14 when the COOH terminus of polycystin-2 was located in the cytoplasm (Fig. 5, *f* and *g*). These experiments support the notion that the particular redistribution of both proteins depended on their physical interaction and was not simply a result of overexpressing two cDNAs.

To learn what intracellular compartment PIGEA-14 and polycystin-2 were redirected to upon their simultaneous production, we performed double immunocytochemistry experi-

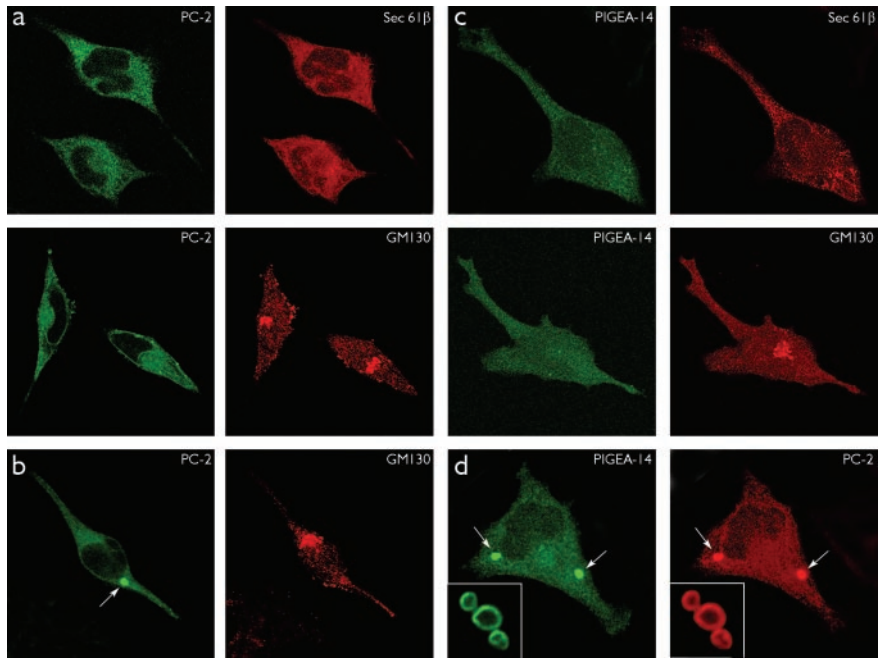

FIG. 4. **Intracellular distribution of polycystin-2 and PIGEA-14.** Stably transfected HeLa cells inducibly producing a HA epitope-tagged polycystin-2 protein with or without the constitutive synthesis of a GST/PIGEA-14 fusion protein were generated. In the absence of PIGEA-14, polycystin-2 was mostly seen in a reticular distribution in the cytoplasm, where it co-localized with Sec 61β, a marker of the endoplasmic reticulum (*a*), but in some cells a patchy distribution of polycystin-2 was noticed (*arrow* in *b*). GM130 was used as a marker for the Golgi apparatus. When the expression of the PKD2 cDNA was kept turned off, PIGEA-14 was exclusively distributed in a reticular pattern in the cytoplasm (*c*), but upon the induction of polycystin-2 a dramatic redistribution of both PIGEA-14 and polycystin-2 was observed (*arrows* in *d*). Both proteins now co-localized in vesicular structures (*insets* in *d*).

ments with markers for various cytoplasmic organelles. No co-localization was detected with ERGIC-53, a marker for the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus, nor with various markers of the Golgi apparatus such as Golgi 58K protein, mannosidase II, Gal T1, Giantin, and also (somewhat surprisingly) not with GM130. TGN46, however, a marker of the trans-Golgi network, was found to co-localize with PIGEA-14 and polycystin-2 in HeLa cells (Fig. 6, *a* and *b*). In HeLa cells not producing exogenous PIGEA-14 and polycystin-2, however, TGN46 was seen in close neighborhood to the nucleus (Fig. 6*c*), indicating that in cells producing PIGEA-14 and polycystin-2, TGN46 also changed its distribution.

We finally extended our findings to LLC-PK_1 cells, a highly differentiated porcine kidney epithelial cell line. In LLC-PK_1 cells producing either PIGEA-14 or polycystin-2, a reticular distribution was detected for both proteins (data not shown). When PIGEA-14 and polycystin-2 were synthesized together, their distribution changed markedly to a TGN46-positive compartment (Fig. 7, *a* and *b*), and again the distribution of TGN46 was altered drastically (Fig. 7, compare *b* and *c*). Immunogold electron microscopy demonstrated the intense labeling of vesicular structures near the nucleus (Fig. 7*d*); sometimes these vesicular structures even appeared to be assembled in a honeycomb-like fashion (Fig. 7*e*). Because our data suggested that polycystin-2 is increasingly able to escape to the trans-Golgi network in the presence of PIGEA-14, we predicted that its *N*-linked sugar residues should have been processed and become resistant to Endo H as we have observed for a truncated polycystin-2 protein lacking the COOH terminus (10). As a matter of fact, both in the absence and in the presence of PIGEA-14 polycystin-2 was still completely sensitive to Endo H, indicating that its *N*-linked sugar residues were not processed in the Golgi apparatus (Fig. 7*f*). Recently, increasing evidence has been accumulated for the role of primary cilia in the pathogenesis of cyst formation, and we therefore asked whether PIGEA-14 is also located in this organelle. We could easily detect primary cilia on the apical surface of LLC-PK_1 cells, but neither in the absence nor in the presence of polycystin-2 was PIGEA-14 found there (Fig. 8, *a* and *b*).

DISCUSSION

There is conflicting evidence on the subcellular location of polycystin-2, one of the two proteins mutated in patients suffering from autosomal-dominant polycystic kidney disease. One set of experiments argues that full-length polycystin-2 is located exclusively or at least predominantly in the endoplasmic reticulum (6, 10–12), whereas other groups have provided data demonstrating the presence of polycystin-2 in the plasma membrane (3, 13). Immunocytochemical staining, biotinylation studies, glycosidase sensitivity, and density gradient centrifugations have been used in favor of one or the other argument without leading to a consensus. One reason for such a discrepancy may lie in the fact that the subcellular location of polycystin-2 may very well depend on the biological context, *i.e.* the cell line and tissue used for a study. An argument for this hypothesis comes from the finding that in the absence of polycystin-1, polycystin-2 was located in the endoplasmic reticulum of Chinese hamster ovary cells, but in its presence polycystin-2 was able to reach the plasma membrane (3). In other words, the protein composition of a given cell may determine whether polycystin-2 is retained in the endoplasmic reticulum or is able to reach the plasma membrane.

A better understanding of the basic mechanisms regulating the subcellular location of polycystin-2 should therefore be very useful to decide whether the endoplasmic reticulum or the plasma membrane is the physiological site of action of polycystin-2 in a given organ or cell. It is generally acknowledged that the COOH terminus of polycystin-2 harbors a retention signal for the endoplasmic reticulum (10), and because many mutations in the *PKD2* gene are predicted to lead to the synthesis of a truncated protein (14), the mislocalization of a mutant polycystin-2 protein and the resulting aberrant calcium currents could be one explanation for the formation of cysts. Our two-hybrid screen using the COOH terminus of polycystin-2 as a bait has led to the identification of PIGEA-14, a novel coiled-coil protein not only interacting with polycystin-2 but also with itself and with GM130. GM130 is a peripheral membrane protein that is associated with the cis-compartment of the Golgi apparatus through its interaction with GRASP65, a myristoylated protein. It prob-

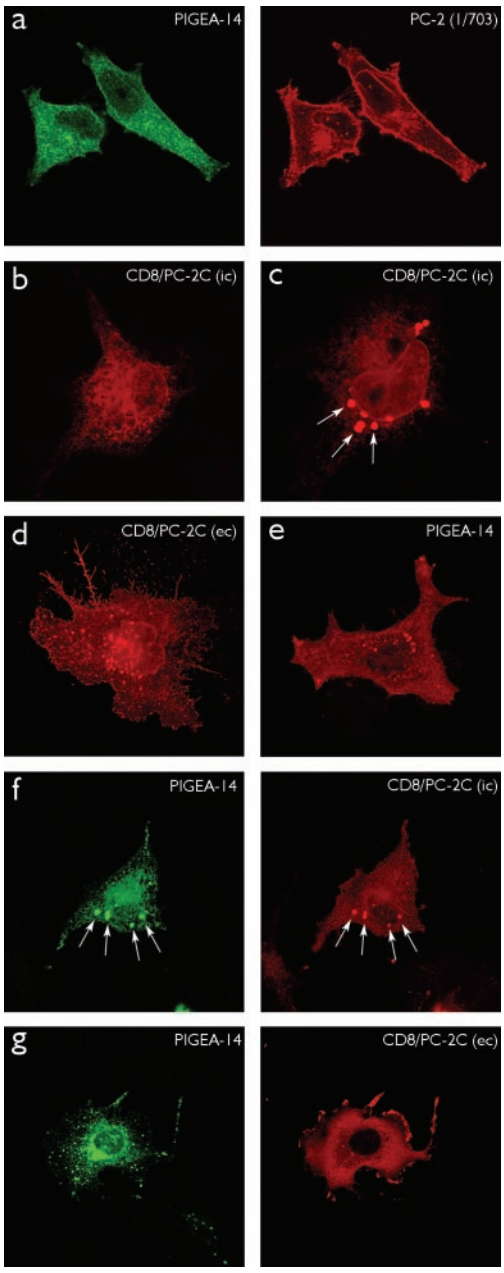

FIG. 5. **An interaction between PIGEA-14 and the COOH terminus of polycystin-2 is necessary for the co-localization of both proteins.** *a*, stably transfected HeLa cells constitutively producing a GST/PIGEA-14 protein and inducibly producing a HA epitope-tagged polycystin-2 protein, which still contained all six membrane-spanning segments but was truncated after amino acid 703 (*PC-2 (1/703)*), were generated. In these cells the truncated polycystin-2 protein reached the plasma membrane, and PIGEA-14 always assumed a reticular distribution in the cytoplasm. *b–g*, expression plasmids encoding fusion proteins between CD8 and the COOH terminus of polycystin-2 were constructed. One fusion protein consisted of the complete extracellular domain, the membrane-spanning segment, and a short cytoplasmic tail of CD8, to which the COOH terminus of polycystin-2 was attached (*CD8/PC-2C (ic)*). The second fusion protein consisted of the same CD8 fragment, but in this case the COOH terminus of polycystin-2 was inserted into the extracellular domain of CD8 (*CD8/PC-2C (ec)*). Whereas the CD8/PC-2C (ic) protein was detected in a reticular (*b*) and a patchy (*arrows* in *c*) distribution in singly transfected COS-7 cells, the CD8/PC-2C (ec) protein reached the plasma membrane (*d*). The GST/PIGEA-14 fusion protein was typically found in a reticular pattern in the cytoplasm (*e*). When COS-7 cells were co-transfected with the expression plasmid for the GST/PIGEA-14 protein and one of the aforementioned CD8/PC-2C expression plasmids, a co-localization of PIGEA-14 and the respective CD8/PC-2C fusion protein was only observed when the COOH terminus of polycystin-2 extended into the cytoplasm (*arrows* in *f*). The fusion protein, in which the COOH terminus of polycystin-2 was inserted into the extracellular domain of CD8, still reached the plasma membrane and did not alter the reticular cytoplasmic distribution of PIGEA-14 (*g*).

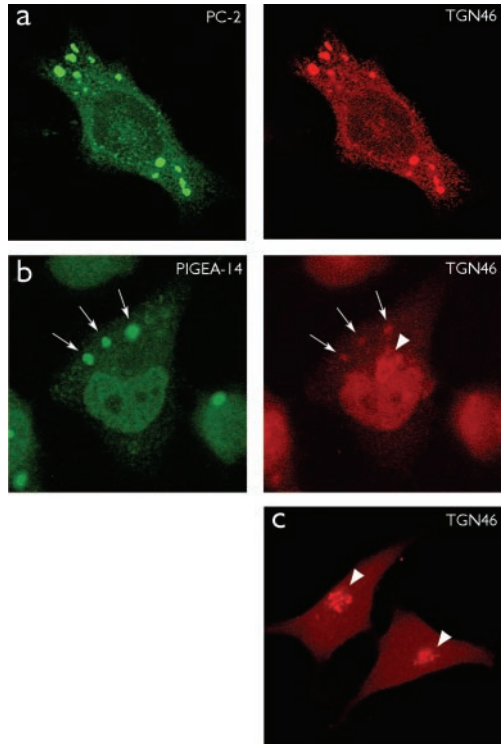

FIG. 6. **PIGEA-14 and polycystin-2 co-localize in the trans-Golgi network.** When a GST/PIGEA-14 fusion protein and a HA epitope-tagged polycystin-2 protein were produced in stably transfected HeLa cells, both proteins co-localized with TGN46, a marker for the trans-Golgi network (*a* and *b*). It should be noted that in HeLa cells not producing either protein, TGN46 was typically concentrated near the nucleus (*c*). Such a perinuclear location of TGN46 could still be seen in some cells producing both PIGEA-14 and polycystin-2 (*arrowhead* in *b*), although a portion of TGN46 was already redistributed (*arrows* in *b*).

ably acts as a docking station for p115, another peripheral membrane protein, which is attached to COPII vesicles through its interaction with Rab1 and to COPI vesicles through its interaction with the integral membrane protein giantin (22, 23). Because many proteins involved in endoplasmic reticulum-to-Golgi trafficking contain coiled-coil motifs (among them p115 and GM130) and because of its particular location and its redistribution upon the induction of polycystin-2, a role for PIGEA-14 in intracellular vesicle trafficking can be easily envisioned. It is also remarkable that polycystin-2L, which does not interact with PIGEA-14, contains no retention signal for the endoplasmic reticulum in its COOH terminus (own observations). Polycystin-2 and polycystin-2L are well conserved in their COOH termini, but the homology breaks down in the region of polycystin-2, which contains the retention signal and which is responsible for the interaction with PIGEA-14.

The easiest explanation for the co-localization of PIGEA-14 and polycystin-2 with TGN46 would be that PIGEA-14 and polycystin-2 reach the trans-Golgi network. It is remarkable, however, that the shape of the TGN46-positive compartment changes. This may indicate that none of the three proteins is present in the trans-Golgi network and that TGN46, PIGEA-14, and polycystin-2 have all three been redirected to an altogether different compartment. We were also surprised by the fact that polycystin-2 in the TGN46-positive compartment was still sensitive to a treatment with the glycosidase

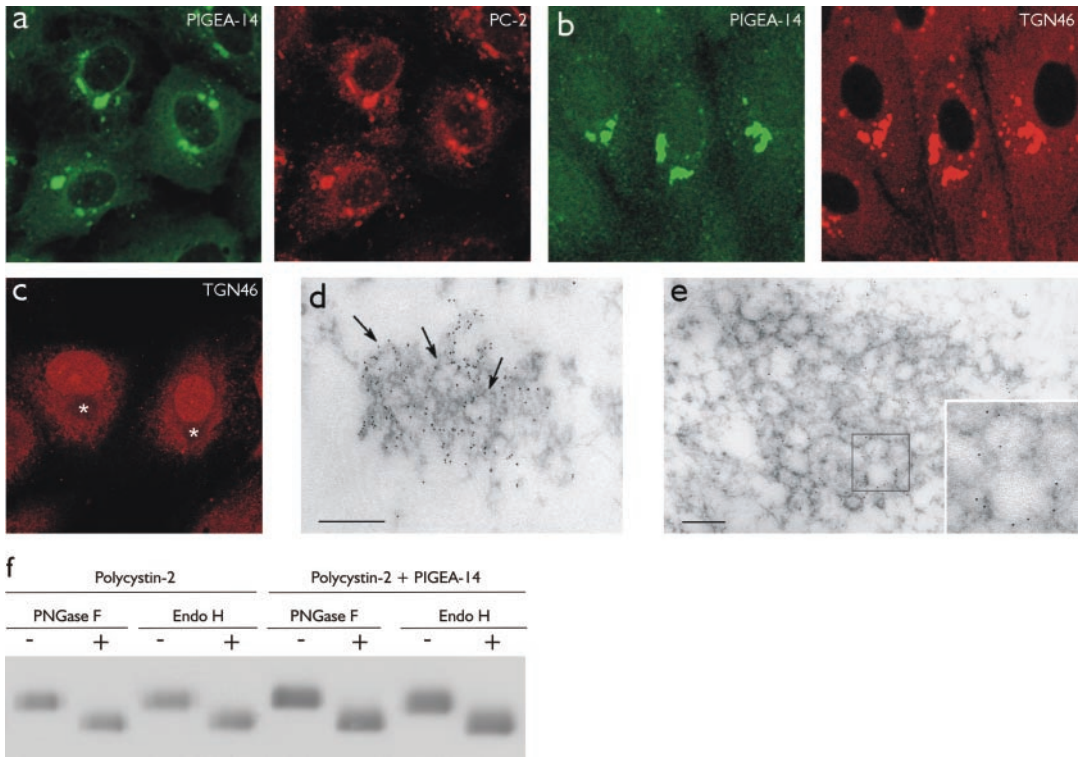

FIG. 7. **Distribution of PIGEA-14 and polycystin-2 in LLC-PK$_1$ cells.** *a* and *b*, the synthesis of a GST/PIGEA-14 fusion protein and a HA epitope-tagged polycystin-2 protein in the porcine kidney epithelial cell line LLC-PK$_1$ leads to the accumulation of both proteins in a TGN46-positive compartment. *c*, in LLC-PK$_1$ cells not producing either protein, TGN46 was typically distributed around the nucleus; the nonstained region (*asterisks*) probably represents the Golgi apparatus. *d* and *e*, immunogold electron microcopy with the anti-GST antibody (*d*) and the anti-HA epitope antibody (*e*) demonstrates the presence of the GST/PIGEA-14 fusion protein and of the HA-tagged polycystin-2 protein in the periphery of vesicular structures (*arrows* in *d*). In some cells a honeycomb-like assembly of vesicles was noticed as shown in *e* (*inset* shows gold particles at a higher magnification). *f*, no difference was detected with respect to the Endo H sensitivity of polycystin-2 in the absence and presence of PIGEA-14, in either case the sugar residues were still completely removed. *Bars* in *d* and *e*, 250 nm.

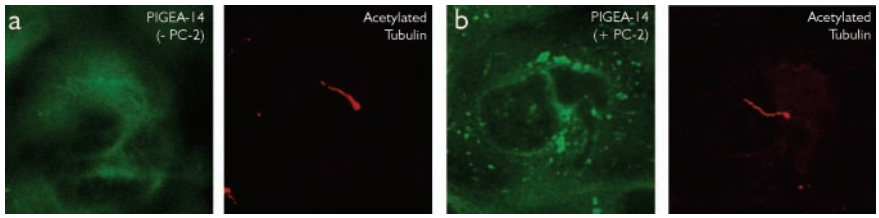

FIG. 8. **PIGEA-14 is not detected in primary cilia.** Double immunocytochemistry of LLC-PK$_1$ cells for PIGEA-14 and acetylated tubulin (a marker for primary cilia) shows that PIGEA-14 is not present in primary cilia either in the absence (*a*) or in the presence (*b*) of the HA epitope-tagged polycystin-2 protein.

Endo H, whereas a mutant polycystin-2 protein lacking the COOH terminus is resistant to treatment with Endo H (10). This would argue that the full-length polycystin-2 protein takes a different intracellular route than the mutant protein, which would be predicted to migrate through the Golgi apparatus. If this scenario is true, the interpretation of studies on the intracellular location of full-length polycystin-2 using Endo H sensitivity has to be reconsidered. There obviously remain a number of open questions such as the identity of the interaction motifs in the respective proteins and the identification of the various protein complexes. One also has to wonder why polycystin-2 gets stuck in the TGN46-positive compartment and does not move further to the plasma membrane. It is worth pointing out in this context that the R1394H mutation in the SUR1 subunit of the ATP-sensitive K^+ channel leads to the accumulation of the mutant SUR1 protein in the trans-Golgi network (24), so obviously there is another checkpoint for the assembly of functional ion channels at this level. We believe that the identification of PIGEA-14 could be an important first step to a better understanding of the intracellular trafficking of polycystin-2.

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REFERENCES

1. Mochizuki, T., Wu, G., Hayashi, T., Xenophontos, S. L., Veldhuisen, B., Saris, J. J., Reynolds, D. M., Cai, Y., Gabow, P. A., Pierides, A., Kimberling, W. J., Breuning, M. H., Deltas, C. C., Peters, D. J. M., and Somlo, S. (1996) *Science* **272,** 1339–1342
2. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) *Cell* **108,** 595–598
3. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) *Nature* **408,** 990–994
4. González-Perrett, S., Kim, K., Ibarra, C., Damiano, A. E., Zotta, E., Batelli, M., Harris, P. C., Reisin, I. L., Arnaout, M. A., and Cantiello, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1182–1187
5. Vassilev, P. M., Guo, L., Chen, X.-Z., Segal, Y., Peng, J.-B., Basora, N., Babakhanlou, H., Cruger, G., Kanazirska, M., Ye, C.-P., Brown, E. M., Hediger, M. A., and Zhou, J. (2001) *Biochem. Biophys. Res. Commun.* **282,** 341–350
6. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2002) *Nat. Cell Biol.* **4,** 191–197
7. Chen, X.-Z., Vassilev, P. M., Basora, N., Peng, J.-B., Nomura, H., Segal, Y., Brown, E. M., Reeders, S. T., Hediger, M. A., and Zhou, J. (1999) *Nature* **401,** 383–386
8. Raychowdhury, M. K., González-Perrett, S., Montalbetti, N., Timpanaro, G. A., Chasan, B., Goldmann, W. H., Stahl, S., Cooney, A., Goldin, E., and Cantiello, H. F. (2004) *Hum. Mol. Genet.* **13,** 617–627
9. Gallagher, A. R., Hidaka, S., Gretz, N., and Witzgall, R. (2002) *Cell. Mol. Life*

Sci. **59,** 682–693
10. Cai, Y., Maeda, Y., Cedzich, A., Torres, V. E., Wu, G., Hayashi, T., Mochizuki, T., Park, J. H., Witzgall, R., and Somlo, S. (1999) *J. Biol. Chem.* **274,** 28557–28565
11. Gallagher, A. R., Cedzich, A., Gretz, N., Somlo, S., and Witzgall, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4017–4022
12. Newby, L. J., Streets, A. J., Zhao, Y., Harris, P. C., Ward, C. J., and Ong, A. C. M. (2002) *J. Biol. Chem.* **277,** 20763–20773
13. Luo, Y., Vassilev, P. M., Li, X., Kawanabe, Y., and Zhou, J. (2003) *Mol. Cell. Biol.* **23,** 2600–2607
14. Deltas, C. C. (2001) *Hum. Mutat.* **18,** 13–24
15. Qian, F., Germino, F. J., Cai, Y., Zhang, X., Somlo, S., and Germino, G. G. (1997) *Nat. Genet.* **16,** 179–183
16. Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V. P., and Walz, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6965–6970
17. Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G., and Sukhatme, V. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3934–3939
18. Lehtonen, S., Ora, A., Olkkonen, V. M., Geng, L., Zerial, M., Somlo, S., and Lehtonen, E. (2000) *J. Biol. Chem.* **275,** 32888–32893
19. Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E., and Boeke, J. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10315–10320
20. Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1996) *Current Protocols in Molecular Biology*, pp. 9.2.1–9.2.10, John Wiley & Sons, New York
21. Dong, Y., Skoultchi, A. I., and Pollard, J. W. (1993) *Nucleic Acids Res.* **21,** 771–772
22. Barr, F. A., and Short, B. (2003) *Curr. Opin. Cell Biol.* **15,** 405–413
23. Gillingham, A. K., and Munro, S. (2003) *Biochim. Biophys. Acta* **1641,** 71–85
24. Partridge, C. J., Beech, D. J., and Sivaprasadarao, A. (2001) *J. Biol. Chem.* **276,** 35947–35952