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Polycystin-2 takes different routes to the somatic and ciliary plasma membrane

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Polycystin-2 (also called TRPP2), an integral membrane protein mutated in patients with cystic kidney disease, is located in the primary cilium where it is thought to transmit mechanical stimuli into the cell interior. After studying a series of polycystin-2 deletion mutants we identified two amino acids in loop 4 that were essential for the trafficking of polycystin-2 to the somatic (nonciliary) plasma membrane. However, polycystin-2 mutant proteins in which these two residues were replaced by alanine were still sorted into the cilium, thus indicating that the trafficking routes to the somatic and ciliary plasma membrane compartments are distinct. We also observed that the introduction of dominant-negative Sar1 mutant proteins and treatment of cells with brefeldin A prevented the transport into the ciliary plasma membrane compartment, whereas metabolic labeling experiments, light microscopical imaging, and high-resolution electron microscopy revealed that full-length polycystin-2 did not traverse the Golgi apparatus on its way to the cilium. These data argue that the transport of polycystin-2 to the ciliary and to the somatic plasma membrane compartments originates in a COPII-dependent fashion at the endoplasmic reticulum, that polycystin-2 reaches the cis side of the Golgi apparatus in either case, but that the trafficking to the somatic plasma membrane goes through the Golgi apparatus whereas transport vesicles to the cilium leave the Golgi apparatus at the cis compartment. Such an interpretation is supported by the finding that mycophenolic acid treatment resulted in the colocalization of polycystin-2 with GM130, a marker of the cis-Golgi apparatus. Remarkably, we also observed that wild-type Smoothened, an integral membrane protein involved in hedgehog signaling that under resting conditions resides in the somatic plasma membrane, passed through the Golgi apparatus, but the M2 mutant of Smoothened, which is constitutively located in the ciliary but not in the somatic plasma membrane, does not. Finally, a dominant-negative form of Rab8a, a BBSome-associated monomeric GTPase, prevented the delivery of polycystin-2 to the primary cilium whereas a dominant-negative form of Rab23 showed no inhibitory effect, which is consistent with the view that the ciliary trafficking of polycystin-2 is regulated by the BBSome.

Introduction

Once considered an evolutionary relic, primary cilia (also called monocilia) are now believed to play essential roles in signaling pathways and organ development. They are singular extensions of the plasma membrane and contain an axoneme of nine peripheral microtubule doublets but no central pair of microtubules, and therefore are believed to be immotile in most circumstances. Stimulated not least by the identification of most of the genes mutated in cystic kidney diseases, the primary cilium has become an intensely investigated organelle (Satir and Christensen, 2007). Mutations in the *PKD1* (The European Polycystic Kidney Disease Consortium, 1994) and *PKD2* (Mochizuki et al., 1996) genes result in autosomal-dominant

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polycystic kidney disease. Orthologues of polycystin-1 and polycystin-2, the proteins encoded by the *PKD1* and *PKD2* genes, were first localized to the ciliated endings of sensory neurons in *Caenorhabditis elegans* (Barr and Sternberg, 1999) before they were detected in primary cilia of mammalian cells (Pazour et al., 2002; Yoder et al., 2002). Meanwhile, a plethora of cyst-associated proteins has been detected in primary cilia, among them IFT88/Polaris (the protein mutated in a murine model of polycystic kidney disease), fibrocystin/polyductin (mutated in patients suffering from autosomal-recessive polycystic kidney disease), most of the nephrocystins (mutated in patients suffering from nephronophthisis), the BBS proteins (mutated in patients suffering from Bardet-Biedl syndrome), OFD1 (a protein mutated in patients with oral-facial-digital syndrome, which is also associated with polycystic kidney disease), and cystin (mutated in a spontaneous model of polycystic kidney disease in the mouse; Yoder, 2007). No consensus has yet emerged for the role of primary cilia in mammalian cells (Witzgall, 2005a). Not only have they been attributed a chemo- and mechanosensory role, but they are also felt to be essential for signal transduction in the hedgehog, Wnt, and planar cell polarity pathways (Bisgrove and Yost, 2006; Singla and Reiter, 2006; Wang and Nathans, 2007).

The intracellular location of polycystin-2 has been discussed controversially. Whereas some groups favor a retention of polycystin-2 in the ER, others have argued for its trafficking to the plasma membrane (Witzgall, 2005b). The initial characterization has resulted in the identification of a retention signal for the ER in the C terminus of polycystin-2, and indeed mutant proteins lacking most of the C terminus have been shown to escape from the ER (Cai et al., 1999; Hanaoka et al., 2000). Further analysis of the C terminus has yielded more evidence for the importance of this portion of polycystin-2. Using a two-hybrid screen with the C terminus as a bait, we were able to isolate PIGEA-14 (also called Chibby), a novel protein which in addition interacts with the Golgi matrix component GM130 (Hidaka et al., 2004) and which may also be involved in β-catenin signaling (Takemaru et al., 2003; Li et al., 2007). A stretch of acidic amino acids in the C terminus of polycystin-2 mediates the interaction with PACS-1 and PACS-2 (Köttgen et al., 2005). The association between polycystin-2 and the PACS proteins is regulated through the phosphorylation of a serine residue at position 812 of polycystin-2 (Köttgen et al., 2005), but there is conflicting evidence whether the phosphorylation status is important for the retention of polycystin-2 in the ER (Cai et al., 2004; Köttgen et al., 2005).

It is generally agreed upon that polycystin-2 reaches the primary cilium (Pazour et al., 2002; Yoder et al., 2002), but how it gets there is unknown. There are essentially three alternative routes by which integral membrane proteins may become integrated into the ciliary plasma membrane (Witzgall, 2005b). At the current state of knowledge the least likely route is the transport of vesicles into the primary cilium via intraflagellar transport and their subsequent fusion with the ciliary plasma membrane anywhere along the length of the cilium. Although contrary reports have been published (Jensen et al., 2004), it is believed that the cilium does not contain vesicles (Kozminski et al., 1993); furthermore, it is not clear whether a membrane fusion machinery is present in the cilium. A second hypothesis states that integral membrane proteins are first incorporated into the nonciliary plasma membrane and then move into the ciliary plasma membrane. In a third model, integral membrane proteins reach a docking station at the base of the cilium where they are integrated into the ciliary plasma membrane and then move up and down the cilium. We now present evidence that polycystin-2 uses distinct pathways to reach the nonciliary and ciliary plasma membrane compartments, thus indicating that the third model is correct. To emphasize the distinction between the two plasma membrane compartments in ciliated cells, we suggest calling the plasma membrane compartment lining the cell body the somatic plasma membrane.

Results

A motif adjoining the cytoplasmic side of the fourth membrane-spanning segment is critical for the insertion of polycystin-2 into the somatic plasma membrane

It has been demonstrated that polycystin-2 mutant proteins terminating after amino acids 703 (Cai et al., 1999) and 741 (Hanaoka et al., 2000) efficiently travel to the somatic plasma membrane, whereas the full-length polycystin-2 protein does not. In the course of another set of experiments we discovered that C-terminal truncation mutants of polycystin-2 with 582 amino acids in length or longer were still incorporated into the plasma membrane, whereas truncation mutants with 547 amino acids and shorter were again retained in intracellular membrane compartments (Fig. 1, a and b). This finding indicated that polycystin-2 not only contained a retention signal for the ER in its C terminus but also an export signal embedded in the middle of the protein. When we went on to narrow down the amino acids responsible for the integration of polycystin-2 into the plasma membrane by making additional truncation mutants we arrived at the result that polycystin-2 (1–576) reached the plasma membrane but polycystin-2 (1–573) is unable to do so (Fig. 1, c and d).

A comparison of the human, murine, rat, canine, bovine, and chicken polycystin-2 sequences showed that the region from amino acids 572 to 577 was highly conserved between the different species (Fig. 2 a). This encouraged us to exchange single amino acids in the context of the polycystin-2 (1–703) mutant. The polycystin-2 (1–703) mutant has been well characterized in several publications. It escapes from the ER and reaches both the somatic and the ciliary plasma membrane compartments (Cai et al., 1999; Gallagher et al., 2006) where it can still act as a cation channel, although with characteristics different from the full-length protein (Koulen et al., 2002). Because truncations in themselves may disturb the local protein structure, we felt that amino acid substitutions were more informative. Both the substitution of amino acids KLF (572–574) and of amino acids KFI (575–577) by alanines maintained the sensitivity of the mutant proteins to endoglycosidase H, thus indicating that the two substitution mutants were retained in the ER (Fig. 2 b). This finding was corroborated by a cell surface biotinylation assay that showed a markedly reduced biotinylation

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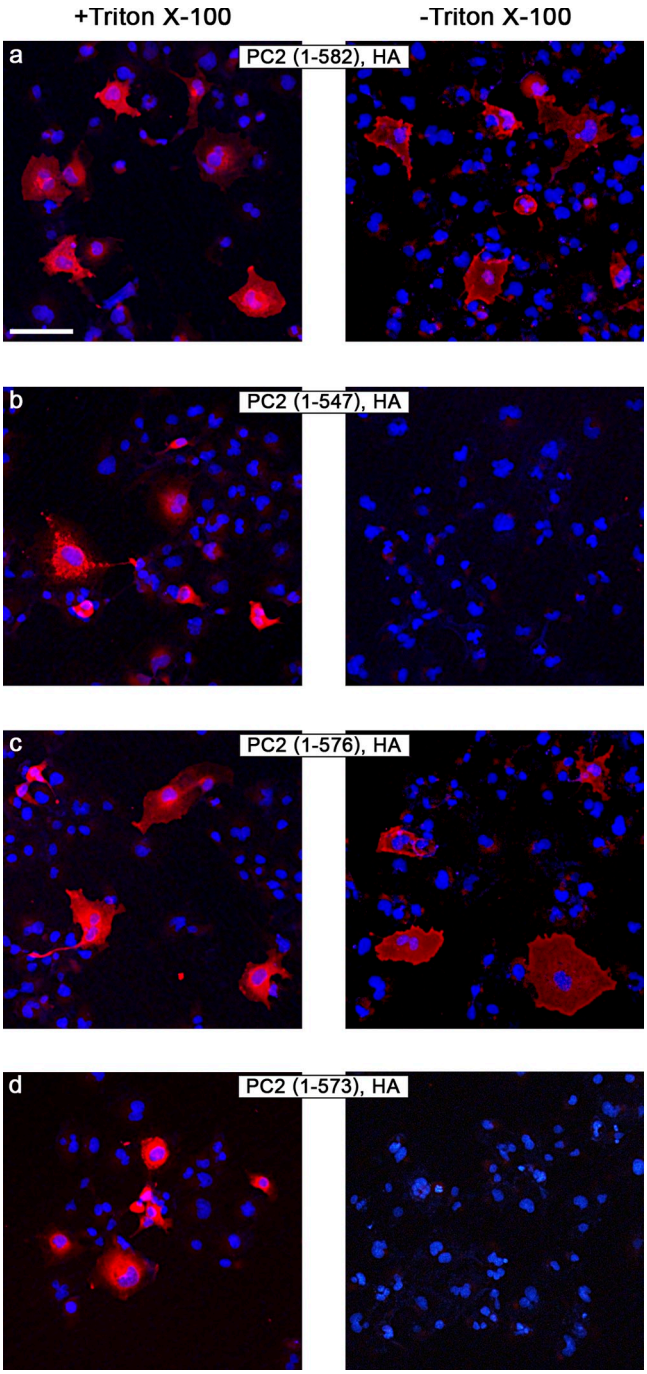

Figure 1. **Evidence for an export motif in polycystin-2.** COS-7 cells were transiently transfected with expression plasmids for the polycystin-2 mutant proteins indicated. 2 d later the cells were stained with an antibody directed against an extracellular domain (loop 1) of polycystin-2 (red) in the presence and absence of the detergent Triton X-100. Only when the mutant proteins reached the plasma membrane could a signal be detected in the absence of Triton X-100. Nuclei were counterstained with Hoechst 33258 (blue) to demonstrate the presence of cells. Bar, 100 µm.

of the two polycystin-2 substitution mutants in COS-7 cells (a nonciliated cell line) and in LLC-PK_1 cells (a ciliated cell line; Fig. 2, c and d). Finally, single amino acid substitutions were assayed in COS-7 cells, which demonstrated that the K572A and F576A mutants reached the somatic plasma membrane as inefficiently as the triple-alanine mutants KLF572-574AAA and KFI575-577AAA, whereas the other mutants were transported to the same extent to the somatic plasma membrane as the non-mutated polycystin-2 (1–703) construct and the plasma membrane protein Na^+/K^+-ATPase, which we used as a control. No full-length polycystin-2 was detected at the cell surface (Fig. 2 e).

Evidence for only partially overlapping sorting pathways of polycystin-2 into the somatic and ciliary plasma membrane

To obtain a dynamic picture of the intracellular movements of polycystin-2, pulse-chase experiments were performed in HeLa cells (another nonciliated cell line) and in LLC-PK_1 cells. By radioactively labeling the cells for a short period of 5 min we were able to follow the fate of newly synthesized polycystin-2 for up to 24 h. Except for the appearance of a slower migrating band after ~4 h, no post-translational modifications were observed for the full-length polycystin-2 protein over this period in both cell lines; furthermore, distinct bands were present even after a chase of 24 h (Fig. 3 a). Because the top band disappeared after a digest with calf intestinal phosphatase (not depicted), this shows that full-length polycystin-2 becomes phosphorylated soon after it is synthesized. Immunoprecipitated polycystin-2 was also subjected to treatment with PNGase F and endoglycosidase H. Whereas PNGase F removes any *N*-linked sugar residues regardless of their modification and therefore can be used to determine whether a protein is *N*-glycosylated, endoglycosidase H will only remove the sugar residues if a glycoprotein has not reached the mid-Golgi compartment. The digestion with both enzymes yielded faster migrating protein species (Fig. 3, b and c) and confirms that full-length polycystin-2 does not reach the mid-Golgi compartment (Cai et al., 1999; Hidaka et al., 2004). The pulse-chase results therefore strongly argue against the possibility that full-length polycystin-2 moves rapidly through the Golgi apparatus and is then quickly degraded before reaching the plasma membrane. More complex glycosylation patterns were apparent in the case of the polycystin-2 (1–703) protein, where a smear of a higher molecular weight was seen after ~2 h in addition to two lower bands (Fig. 3 d). The smear was transformed to a lower molecular weight after a digest with PNGase F but was still present after a digest with endoglycosidase H (although its mobility increased a little), and it therefore represents a form that has moved to the mid-Golgi apparatus and beyond (Fig. 3, e and f). The two lower bands, however, were reduced to a single faster-migrating species both by PNGase F and endoglycosidase H. They therefore represent a pool of protein that has not reached the mid-Golgi compartment even over a period of 24 h.

Because the ciliary plasma membrane only constitutes a small portion of the total plasma membrane area, biochemical assays such as biotinylation of intact cells and pulse-chase experiments may not indicate whether an integral membrane protein is indeed absent from the ciliary plasma membrane. We therefore transiently transfected LLC-PK_1 cells with expression constructs for the HA epitope–tagged mutant polycystin-2 proteins and stained them with antibodies against the HA epitope and acetylated tubulin. Somewhat unexpectedly, the cilia contained even those mutant proteins that no longer reached the somatic

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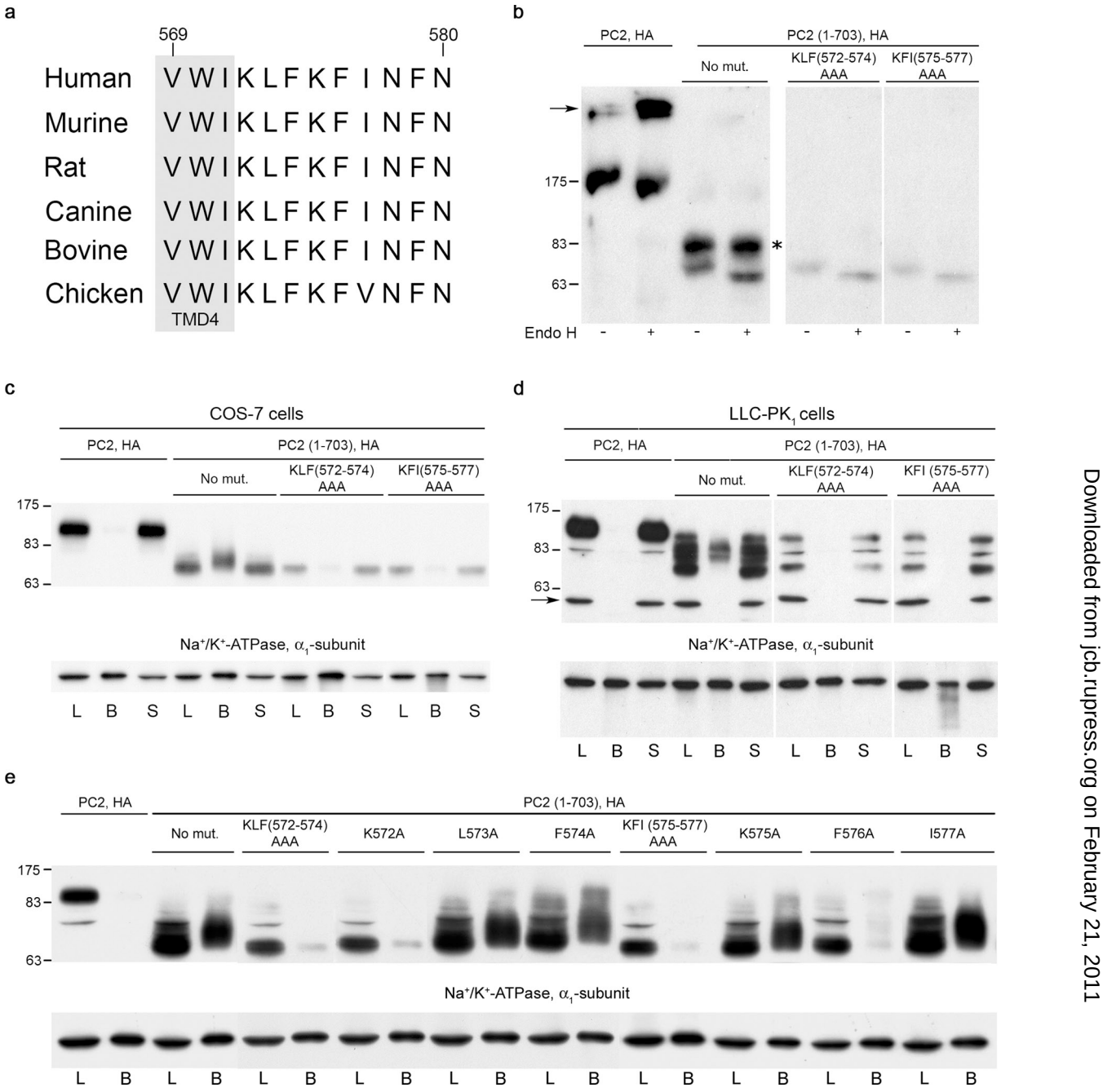

Figure 2. **Endoglycosidase H sensitivity and cell surface biotinylation of mutated polycystin-2 proteins.** (a) Sequence comparison of polycystin-2 from various species. Shown are amino acids 569–580 of the human protein; the last three amino acids of the presumptive fourth membrane-spanning segment (TMD4) are highlighted in gray. (b) Endoglycosidase H_f (Endo H) assay of HA epitope–tagged full-length polycystin-2 (PC2, HA) and of polycystin-2 (1–703) without additional mutation (No mut.) and with alanine substitutions at positions 572–574 and 575–577. The full-length polycystin-2 protein and the triple-alanine substitution mutants are fully sensitive to treatment with Endo H, but the polycystin-2 (1–703) protein without additional mutations is not (asterisk indicates the resistant band). The band indicated by the arrow probably represents full-length polycystin-2 that did not enter the gel. (c–e) Surface biotinylation of COS-7 (c and e) and LLC-PK_1 cells (d) transiently transfected with expression plasmids for the indicated proteins. The α_1 subunit of the Na^+/K^+-ATPase served as a control for efficient biotinylation of plasma membrane proteins. When proteins were (mostly) retained in intracellular membrane compartments, no signal or only a weak signal was detected after precipitation with NeutrAvidin beads. The arrow in d indicates a nonspecific band. Molecular masses (in kD) are indicated on the left of panels b–e. L, whole-cell lysate; B, proteins precipitated with NeutrAvidin beads; S, proteins left in the supernatant after precipitation with NeutrAvidin beads.

plasma membrane (Fig. 4). This indicated that the trafficking pathways of polycystin-2 into the somatic and ciliary plasma membrane were different. However, it left open the question of where the two routes diverged. The fact that the full-length polycystin-2 protein was always completely sensitive to Endo H in the pulse-chase experiments suggested an early branching of the two pathways, i.e., at the level of the ER, the ER-Golgi intermediate compartment, or at the cis-Golgi apparatus.

Therefore, we transiently transfected an expression plasmid for polycystin-2 (1–703) together with expression plasmids

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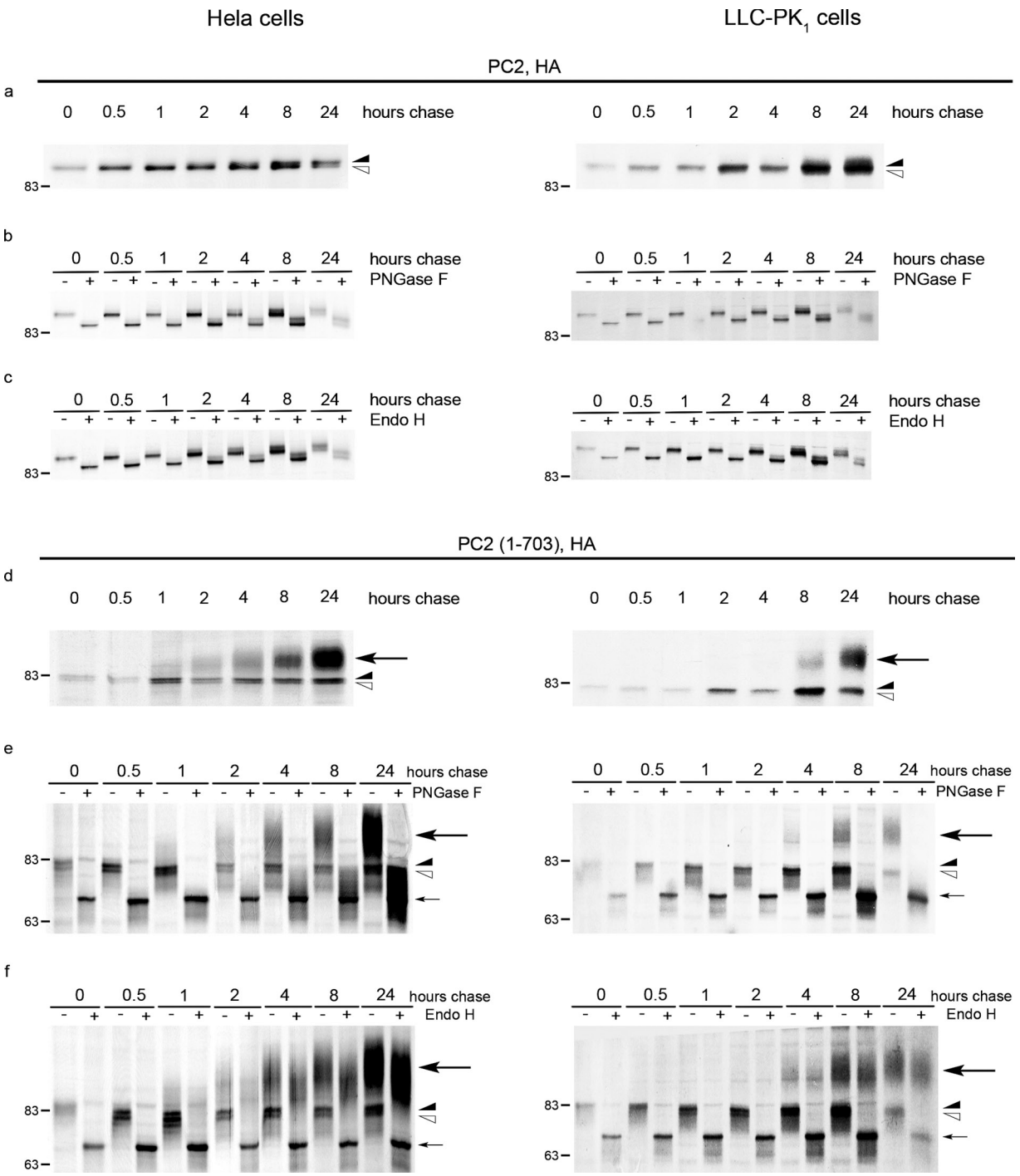

Figure 3. **Pulse-chase labeling of full-length polycystin-2 and polycystin-2 (1–703).** Stably transfected HeLa and LLC-PK$_1$ cells inducibly producing HA epitope–tagged full-length polycystin-2 (a–c) and polycystin-2 (1–703; d–f) proteins were metabolically labeled with ^{35}S-methionine/cysteine. The HA-tagged proteins were immunoprecipitated with an anti-HA epitope antibody, digested with PNGase F and endoglycosidase H (Endo H) when indicated, run on a polyacrylamide gel, and visualized by autoradiography. (a–c) The full-length polycystin-2 can be digested with both glycosidases at any time point after the pulse. Because the top band (filled arrowhead) disappeared after a digest with calf intestinal phosphatase (not depicted), it represents a phosphorylated form of polycystin-2. (d–f) The truncated polycystin-2 protein migrated as a smear (large arrow) ~2 h after the pulse. The smear was resistant to endoglycosidase H and therefore probably represents protein that has progressed beyond the cis-Golgi apparatus. Because both bottom bands (filled and open arrowhead) collapsed into one faster-migrating band (small arrow) after endoglycosidase H treatment, they represent a pool of truncated polycystin-2 that did not reach the mid-Golgi apparatus. Molecular masses (in kD) are indicated on the left of each panel.

for the Sar1 mutant proteins T39N and H79G, which disrupt the COPII-dependent budding of vesicles from the ER (Aridor et al., 1995), into LLC-PK$_1$ cells. Both the GDP-locked T39N and the GTP-locked H79G Sar1 mutant resulted in a strongly decreased presence of the polycystin-2 (1–703) protein in the cilium (Fig. 5, a–h). Polycystin-2 (1–703) was absent from the cilium in all of the cells synthesizing the H79G mutant (positive cilia: 0/11) and in 10 out of 14 cells synthesizing the T39N

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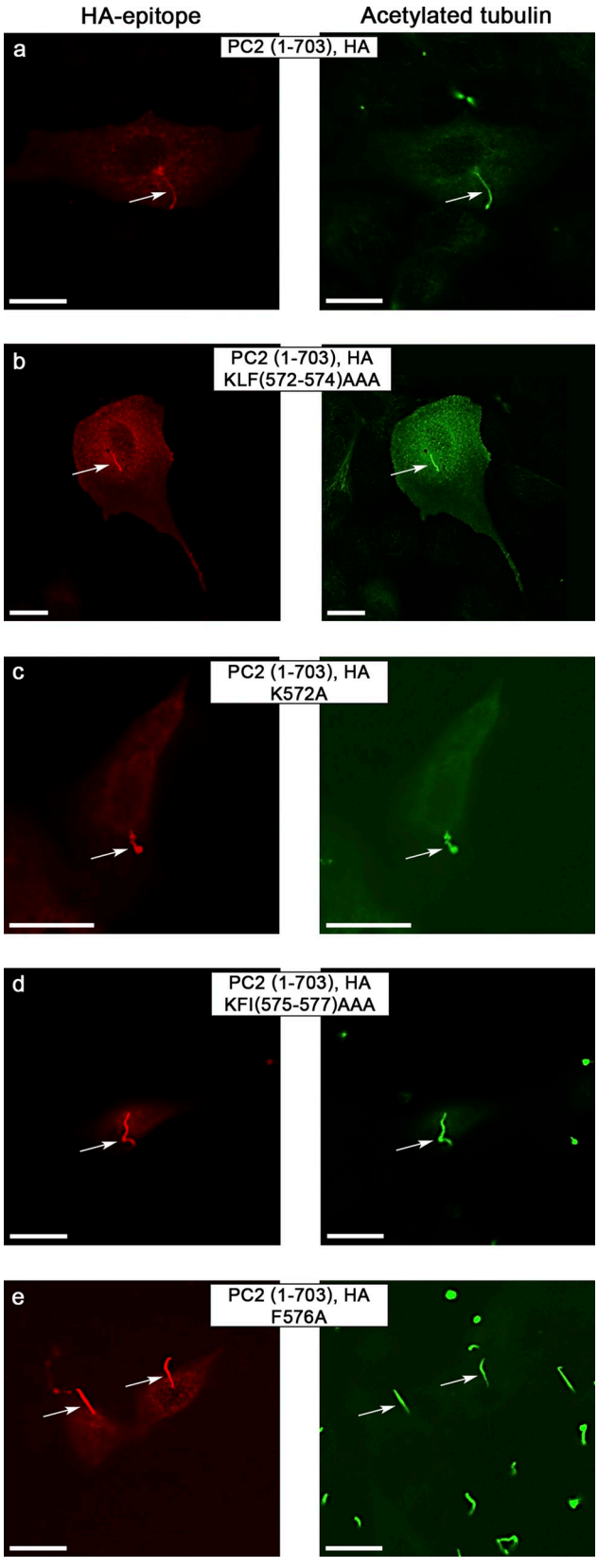

Figure 4. **Ciliary trafficking of polycystin-2 mutant proteins.** LLC-PK_1 cells were transiently transfected with expression plasmids for HA epitope–tagged polycystin-2 (1–703) proteins without and with the indicated alanine substitutions. 3–4 d after transfection, cells were double stained for the HA epitope and for the primary cilia marker acetylated tubulin. Primary cilia are indicated by arrows. Bar, 15 µm.

mutant (positive cilia: 4/14). This argued that trafficking into the cilium originated with a COPII-dependent transport from the ER. Treatment of LLC-PK_1 cells with the Golgi-disrupting agent brefeldin A also prevented both the incorporation of the full-length polycystin-2 and the polycystin-2 (1–703) mutant protein into primary cilia (Fig. 5, i–p; Fig. S1), which demonstrates that polycystin-2 has to reach the Golgi apparatus before being routed into the cilium (although it does not move through it as indicated by the pulse-chase experiments and the experiments described in the following paragraphs).

In another set of experiments we resorted to light microscopic imaging to provide a dynamic picture of the intracellular trafficking of polycystin-2. LLC-PK_1 cells were stably transfected with expression plasmids for fusion proteins between ECFP and *N*-acetylgalactosaminyltransferase-2, a marker for all compartments of the Golgi apparatus (Röttger et al., 1998; White et al., 2001), and TGN38, a marker of the trans-Golgi network (McNamara et al., 2004), respectively. Subsequent transient transfections were performed to synthesize EGFP fusion proteins with full-length polycystin-2 and polycystin-2 (1–703), respectively, in those cells. Insertion of EGFP into the N terminus of full-length polycystin-2 (after amino acid 157), and fusion of EGFP to the C terminus of polycystin-2 (1–703) still permitted the transport of either polycystin-2 construct into the cilium (Fig. 6, a and j). 3–4 d after transfection, the cells were incubated for 5 h at 15°C to arrest newly synthesized protein between the ER and the Golgi apparatus. Upon returning the cells to 37°C the further transport of the polycystin-2 proteins was observed. Whereas polycystin-2 (1–703) rapidly reached the Golgi apparatus (Fig. 6, k and l) and the trans-Golgi network (Fig. 6, m and n), we did not detect full-length polycystin-2 in these compartments (Fig. 6, b–e; Video 1). Identical experiments were performed with the (KFI575-577AAA) mutants of full-length and truncated polycystin-2 in order to investigate the intracellular route of polycystin-2 mutant proteins that no longer reached the somatic plasma membrane but only the primary cilium. In neither case did we observe trafficking through the Golgi apparatus and the trans-Golgi network (Fig. 6, f–i and o–r).

Further evidence for different trafficking pathways of polycystin-2 into the somatic and the ciliary plasma membrane was sought by electron microscopy. We used the same EGFP fusion proteins described in the previous paragraph but inserted an additional 12–amino acid tetracysteine motif. The tetracysteine motif contains four cysteine residues that are able to bind the biarsenical fluorophore ReAsH. ReAsH in turn is used to photoconvert diaminobenzidine into an electron-dense product that can be visualized in the electron microscope. In contrast to immunogold labeling where the gold particles are often distributed in a somewhat scattered fashion around the target and the ultrastructural details are poorly preserved, the ReAsH technique has the big advantage of a well-maintained ultrastructure paired with an extremely high resolution because the diaminobenzidine precipitates form right at the ReAsH-labeled protein (Gaietta et al., 2002). Transiently transfected cells expressing the respective expression constructs were identified through the EGFP portion of the fusion proteins. After ReAsH labeling the

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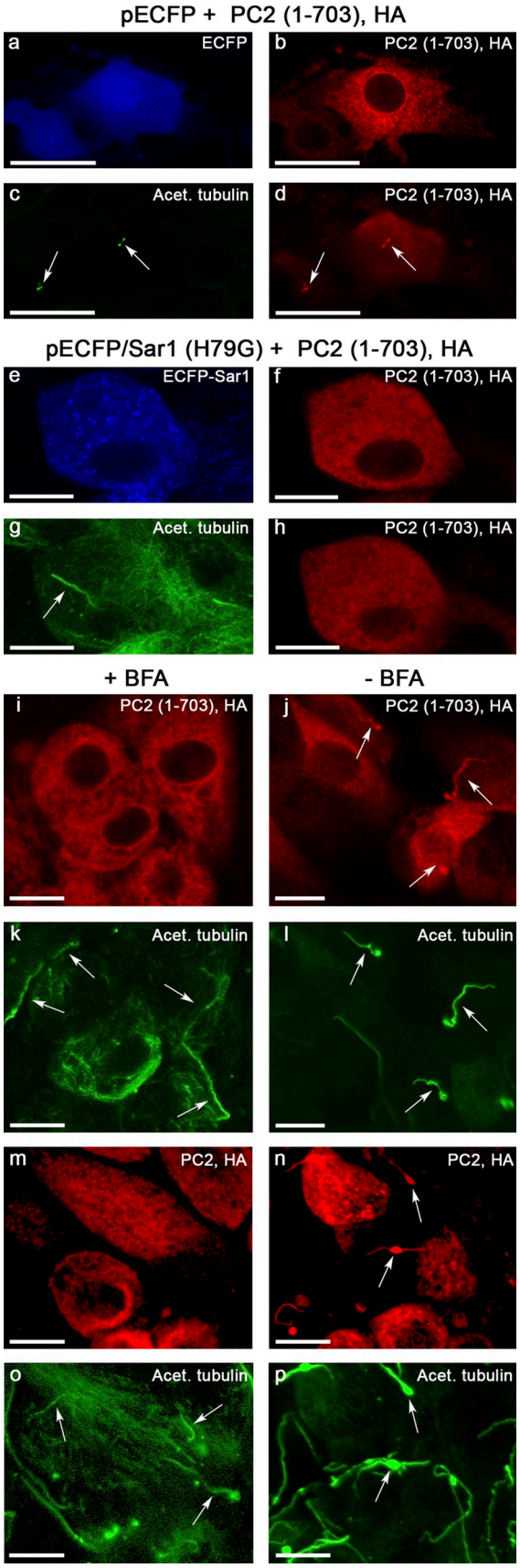

Figure 5. **The ciliary trafficking of polycystin-2 requires COPII and an intact Golgi apparatus.** (a–h) LLC-PK$_1$ cells were transiently transfected with 8 µg of an expression plasmid for ECFP (a–d) or a fusion protein between ECFP and the dominant-negative Sar1 (H79G) protein (e–h) and with 6 µg of an expression plasmid for HA epitope–tagged polycystin-2 (1–703). 3 d after transfection, the cells were either stained for the HA epitope or double-stained for the HA epitope and acetylated tubulin. Whereas ECFP had no effect on the ciliary location of the truncated polycystin-2 protein, the ECFP-Sar1 (H79G) fusion protein prevented the trafficking of the truncated polycystin-2 into primary cilia. a/b, c/d, e/f, and g/h represent corresponding panels. (i–p) LtA-2,22 cells were stably transfected with expression plasmids for HA epitope–tagged full-length and polycystin-2 (1–703) proteins, and treated with brefeldin A (+BFA; i, k, m, and o) or with the solvent methanol (−BFA; j, l, n, and p). Double staining for the HA epitope and acetylated tubulin revealed that in the presence of brefeldin A the truncated and the full-length polycystin-2 protein no longer reached primary cilia. i/k, j/l, m/o, and n/p represent corresponding panels. Primary cilia are indicated by arrows. Bars: (a–h) 25 µm, (i–p) 10 µm.

cells were fixed, photoconverted, embedded in plastic, and serially sectioned. When we studied the Golgi region of the cells that synthesized polycystin-2 (1–703) it was obvious that the Golgi apparatus was stained by the diaminobenzidine precipitate (Fig. 7 b). In cells synthesizing full-length polycystin-2 (Fig. 7 a), however, the Golgi apparatus was not labeled, which again supported the interpretation that the full-length protein did not move through the Golgi apparatus on its way to the primary cilium. EGFP-negative control cells also showed no diaminobenzidine precipitate at the Golgi apparatus, which emphasized the specificity of the technique (Fig. 7 c). One possible prediction of the experiments with brefeldin A would have been that polycystin-2 is detected in the cis compartment of the Golgi apparatus. However, if most of the polycystin-2 protein is rapidly transported back to the ER, and if that portion of polycystin-2, which moves further on to the primary cilium, either quickly moves through the cis-Golgi apparatus or only briefly touches the cis-Golgi apparatus and is then quickly diverted, polycystin-2 will not reach high enough levels to be detected in this compartment. In an attempt to increase the levels of polycystin-2 in the cis-Golgi apparatus we treated cells with mycophenolic acid, a strong inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH) (Franklin and Cook, 1969). Because this enzyme catalyzes the rate-limiting step in the synthesis of GTP, and because G proteins play essential roles in various vesicular trafficking pathways, we reasoned that mycophenolic acid treatment may lead to higher polycystin-2 levels in the cis-Golgi apparatus. Indeed, we were able to observe vesicular structures in which polycystin-2 colocalized with GM130, a marker of the cis-Golgi apparatus (Nakamura et al., 1995), upon treatment with mycophenolic acid (Fig. S2).

We wanted to find out whether the trafficking route of polycystin-2 to the primary cilium was exceptional or whether similar observations could also be made for other proteins. Smoothened, an integral membrane protein involved in hedgehog signaling, is located at the somatic plasma membrane in the absence of hedgehog and moves into the cilium in its presence (Corbit et al., 2005; Milenkovic et al., 2009). The constitutively active M2 mutant of Smoothened, which is mutated in patients with basal cell carcinoma (Xie et al., 1998), however, is constantly located in the primary cilium (Corbit et al., 2005). Fusion proteins between EYFP and wild-type and the M2 mutant of Smoothened, respectively, were subjected to the same

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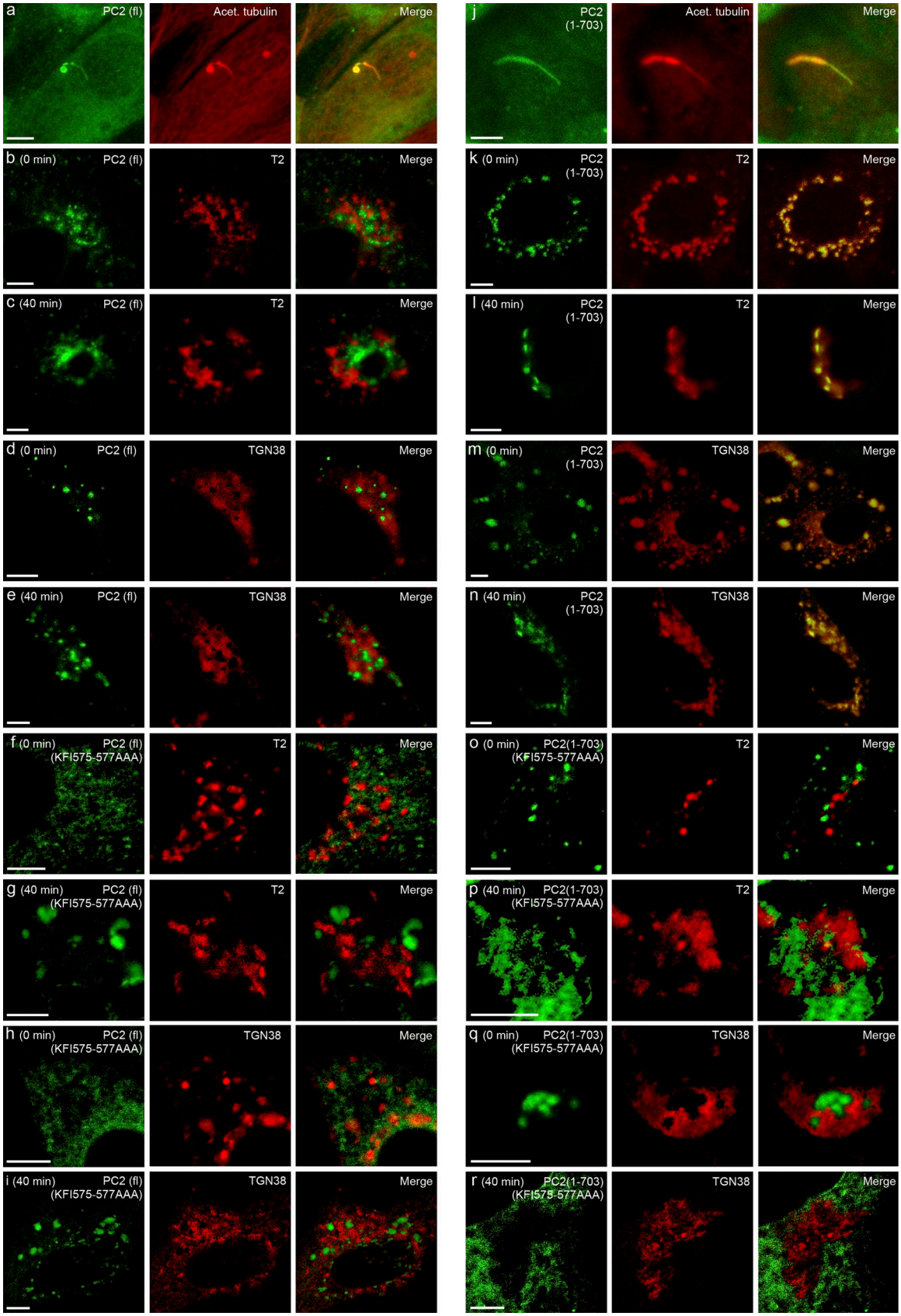

Figure 6. **Trafficking of full-length polycystin-2 and polycystin-2 (1–703) with respect to the Golgi apparatus and the trans-Golgi network.** (a and j) Immunostaining for acetylated tubulin (red) demonstrates that EGFP fusion proteins with full-length polycystin-2 (PC (fl)) and truncated polycystin-2 (PC2 (1–703)) still enter the primary cilium. (b–e and k–n) LLC-PK_1 cells stably synthesizing ECFP fusion proteins with *N*-acetylgalactosaminyltransferase-2 (T2) and TGN38 (shown in red for better visibility) were transiently transfected with expression plasmids coding for an EGFP fusion protein with the respective polycystin-2

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temperature shift protocol as full-length and truncated polycystin-2. Wild-type Smoothened moved through the Golgi apparatus and the trans-Golgi network on its way to the somatic plasma membrane upon release from the 15°C block (Fig. 8, a–d), but we found no evidence that the M2 mutant used this pathway on its way to the primary cilium (Fig. 8, e–h).

Rab8a is essential for the trafficking of polycystin-2 into the primary cilium

We have previously identified PIGEA-14 (also called Chibby) as a protein interacting with the C terminus of polycystin-2 (Hidaka et al., 2004). PIGEA-14 also interacts with GM130, and the synthesis of (high levels of) PIGEA-14 and polycystin-2 leads to a striking redistribution of both proteins into the trans-Golgi network (Hidaka et al., 2004). Furthermore, the germline inactivation of the gene encoding PIGEA-14 causes morphological and functional defects of kinocilia in the airways (Voronina et al., 2009). This encouraged us to investigate the role of PIGEA-14 in the trafficking of polycystin-2 to the primary cilium. The human PIGEA-14 protein is 126 amino acids long and contains a putative coiled-coil domain between amino acids 69 and 109, which is necessary for the interaction with both polycystin-2 and GM130 (Hidaka et al., 2004). We therefore speculated that PIGEA-14 deletion mutants would interfere with the function of the endogenous PIGEA-14 protein. Expression constructs encoding fusion proteins between ECFP and two different portions of PIGEA-14 (amino acids 1–64 and amino acids 65–126, respectively) were transiently transfected and microinjected into stably transfected LLC-PK_1 cells inducibly producing HA epitope–tagged full-length polycystin-2 and polycystin-2 (1–703). A statistically significant effect, however, was only found for the trafficking of polycystin-2 (1–703) upon the transient transfection of PIGEA-14 (65–126) (Fig. S3 a), and not for the other conditions tested (Fig. S3, a and b). This negative result could not be explained by the ineffectiveness of the PIGEA-14 constructs because both constructs led to a decrease in the number of cells elaborating cilia (Fig. S3 c).

Recent publications have provided evidence that monomeric GTPases of the Rab family, in particular Rab8a, Rab17, and Rab23 (Nachury et al., 2007; Yoshimura et al., 2007), and a set of proteins mutated in patients with Bardet-Biedl syndrome (the BBSome; Nachury et al., 2007), regulate the growth of cilia. Because Rab proteins are involved in intracellular vesicle trafficking and because the extension of cilia requires the incorporation of new membrane material, we considered them promising candidates to mediate the entry of transport vesicles containing polycystin-2 into the ciliary membrane. Stably transfected LLC-PK_1 cells synthesizing a fusion protein between EGFP and polycystin-2 (1–703) were grown to confluency in order to make sure that most cells would form primary cilia. Then they were microinjected with expression plasmids for dominant-negative Rab8a and Rab23 mutant proteins. Whereas the truncated polycystin-2 protein was only very rarely detected in primary cilia after microinjection of the Rab8a construct (Fig. 9; positive cilia: 3/32, i.e., 9.4%), microinjection of the expression plasmid for the dominant-negative Rab23 protein (positive cilia: 26/34, i.e., 76.5%) and for CFP (positive cilia: 52/60, i.e., 86.7%) had no effect in most cells (not depicted). Therefore, the trafficking of polycystin-2 into primary cilia depends on Rab8a but obviously not on Rab23.

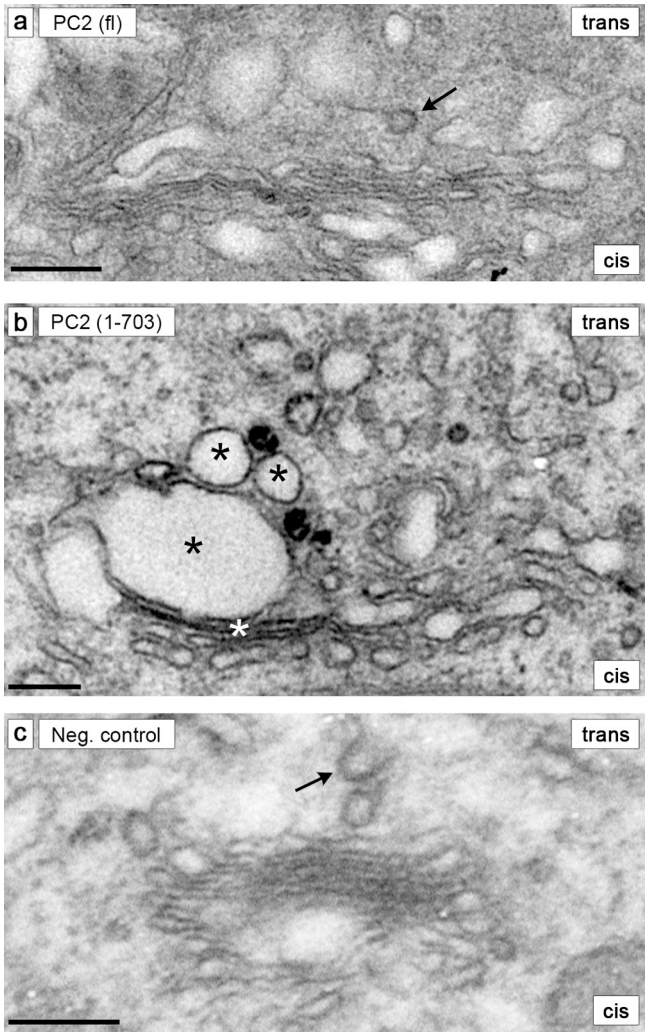

Figure 7. **Ultrastructural localization of full-length polycystin-2 and polycystin-2 (1–703).** COS-7 cells were transiently transfected with expression plasmids for full-length polycystin-2 (a) and polycystin-2 (1–703) (b and c) fused with EGFP and the tetracysteine motif. (a and b) Cells with EGFP fluorescence. Asterisks in b indicate Golgi cisternae with an osmiophilic diaminobenzidine precipitate in a cell producing the truncated polycystin-2 protein. No such precipitate can be seen in a cell producing full-length polycystin-2. (c) A cell without EGFP fluorescence served to demonstrate background staining. The arrows in a and c point to clathrin-coated budding vesicles marking the trans-Golgi network. Bar, 200 nm.

proteins. 3–4 d later, the cells were incubated for 5 h at 15°C before being incubated at 37°C. Immediately before (0 min) and 40 min after (40 min) shifting the cells from 15°C to 37°C, the cells were fixed and visualized in a confocal laser scanning microscope. No overlapping signal can be seen between full-length polycystin-2 and the two marker proteins (b–e), whereas a good overlap was observed for the truncated polycystin-2 protein (k–n). (f–l and o–r) Analogous experiments were performed for the (KFI575-577AAA) mutants of full-length polycystin-2 and polycystin-2 (1–703). For neither mutant protein were we able to detect a colocalization with *N*-acetylgalactosaminyltransferase-2 and TGN38. Bar, 5 µm.

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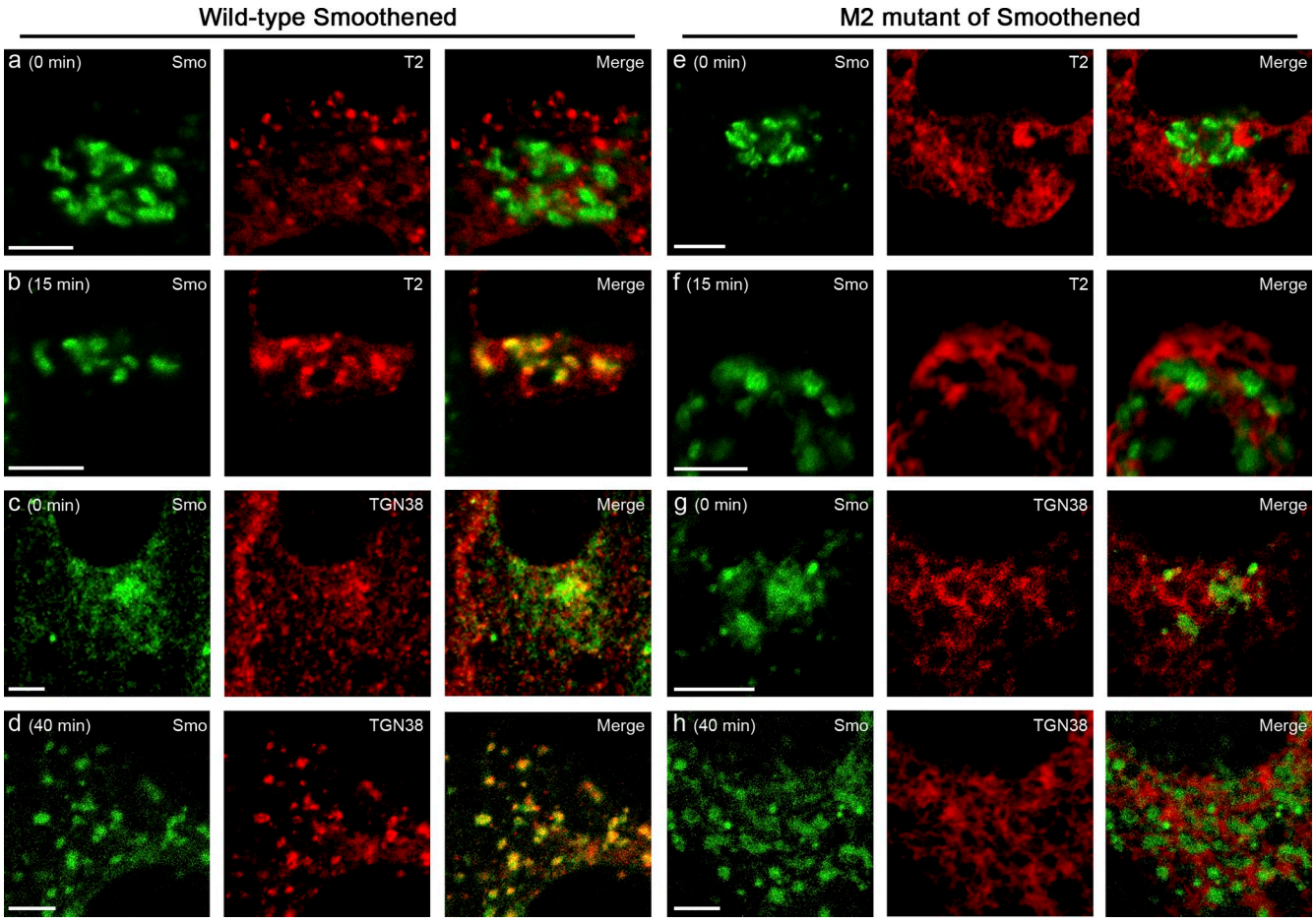

Figure 8. **Trafficking of wild-type Smoothened and the M2 mutant of Smoothened.** LLC-PK_1 cells stably synthesizing ECFP fusion proteins with *N*-acetylgalactosaminyltransferase-2 (T2) and TGN38 (shown in red for better visibility) were transiently transfected with expression plasmids coding for an EYFP fusion protein with the respective Smoothened proteins (shown in green for better visibility). 3–4 d later, the cells were incubated for 5 h at 15°C before being incubated at 37°C. Immediately before (0 min) as well as 15 min (15 min) and 40 min (40 min) after shifting the cells from 15°C to 37°C, the cells were fixed and visualized in a confocal laser scanning microscope. Overlapping signals were seen between wild-type Smoothened and the two marker proteins (a–d), whereas no overlap was observed for the M2 mutant of Smoothened (e–h). Bar, 5 µm.

Discussion

Different scenarios have been suggested regarding the subcellular location of polycystin-2 (Witzgall, 2005b). One says that polycystin-2 resides in the ER and in the ciliary plasma membrane, another argues for the constitutive location of polycystin-2 in the somatic and ciliary plasma membrane compartments, and a third poses that the exit of polycystin-2 from the ER to the somatic plasma membrane depends on the presence of other proteins such as polycystin-1 (Witzgall, 2005b). Whichever of these models will turn out to be correct, one crucial question to be answered concerns the mechanism by which polycystin-2 and other integral membrane proteins traffic to the primary cilium.

At least in the cellular systems used by us, the full-length polycystin-2 protein reaches the ciliary, but not the somatic, plasma membrane, whereas the truncated polycystin-2 protein lacking virtually the complete C terminus is integrated into both plasma membrane compartments. Several conclusions can be drawn from these observations. First, a barrier has to exist that separates the ciliary from the somatic plasma membrane because not only the full-length polycystin-2 protein but also other proteins such as the type 6 serotonin receptor (Brailov et al., 2000) and the type 3 somatostatin receptor (Händel et al., 1999) are restricted to the ciliary plasma membrane at least in some cell types. Second, the C terminus is not necessary for polycystin-2 to be sorted into the primary cilium. Third, the finding that the truncated polycystin-2 protein reaches both plasma membrane compartments could be explained by the promiscuous trafficking of the mutant protein, but it also leaves open the possibility that the trafficking routes to the somatic and ciliary plasma membranes are distinct. Our current study supports the latter model because the substitution of K572 and of F576 by alanines prevented the insertion of the truncated polycystin-2 protein into the somatic but not into the ciliary plasma membrane. There is precedent for the importance of a lysine residue in the intracellular linker region between the fourth and fifth membrane-spanning domain as an export motif of cation channels. The K^+ channels IK1 and SK3, which are usually integrated into the somatic plasma membrane, are both retained in intracellular membrane compartments upon the mutation of the respective lysine residues to alanine (Jones et al., 2005). We are not aware, however, of any publications that have reported on

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the significance of a single phenylalanine residue for the export of an integral membrane protein out of the ER. Di-phenylalanine and phenylalanine-tyrosine motifs such as that present in the C terminus of ERGIC-53 have been demonstrated to interact with COPII (Kappeler et al., 1997), but the single phenylalanine residue and its position in the middle of the protein make that unlikely for polycystin-2. Preliminary experiments in which the KLFKFI motif of polycystin-2 was inserted into the potassium channel Kir 2.1, which like polycystin-2 contains six membrane-spanning domains, indicate that it was not able to substitute for the ER and Golgi export signals of Kir 2.1 (unpublished data). We also tested whether the KLFKFI domain was functional in the asialoglycoprotein receptor subunit H1, a protein with a single membrane-spanning domain, but again found no evidence that it was able to substitute for the endogenous Golgi export signal (unpublished data). This argues that the KLFKFI domain exerts its effect in a context-specific fashion.

Our data argue strongly that the route of integral membrane proteins such as polycystin-2 to the primary cilium does not occur via the somatic plasma membrane but rather directly to the cilium. Previous (more indirect) evidence supports such a conclusion. A RVxP motif in the N terminus of polycystin-2 is necessary to target the truncated polycystin-2 protein to the cilium. This motif was also sufficient to sort the transferrin receptor, a somatic plasma membrane protein, into the cilium (Geng et al., 2006). Because no components of the vesicle fusion machinery such as SNARE proteins have been described in the cilium, it is difficult to imagine how integral membrane proteins can be delivered to the ciliary plasma membrane even if transport vesicles entered the cilium. Indeed, ultrastructural investigations have demonstrated the appearance of ciliary components at the base of the cilium but not at the somatic plasma membrane (Bouck, 1971; Deretic and Papermaster, 1991). Potential regulatory proteins such as the monomeric G protein Rab8a and various Bardet-Biedl proteins (the "BBSome") are located at the basal body and are essential for ciliogenesis (Nachury et al., 2007; Yoshimura et al., 2007). Our results provide evidence that Rab8a regulates the trafficking of polycystin-2 to the cilium. In addition to Rab8a, Rab17 and Rab23 have been found necessary for the elaboration of primary cilia (Yoshimura et al., 2007), but only Rab8a and not Rab23 seems to be necessary for polycystin-2 to reach the ciliary membrane compartment, so obviously different monomeric G proteins regulate distinct intracellular trafficking routes. Consistent with the importance of Rab8a is the observation that the synthesis of dominant-negative Rab8 in *Xenopus laevis* leads to retinal degeneration (Moritz et al., 2001). Retinal photoreceptor cells contain a structure called the connecting cilium, which is related to the primary cilium and which mediates the transport of proteins (such as rhodopsin) from the cell body of the photoreceptor cells to their outer segments. Our attempts to localize transport vesicles with polycystin-2 at the base of the cilium using γ-tubulin as a marker were not successful (Fig. S4), but that could be due to the fact that γ-tubulin does not mark the docking station for those vesicles.

Neither in our current study nor in previous publications (Cai et al., 1999; Hidaka et al., 2004) was any evidence for the trafficking of full-length polycystin-2 through the Golgi

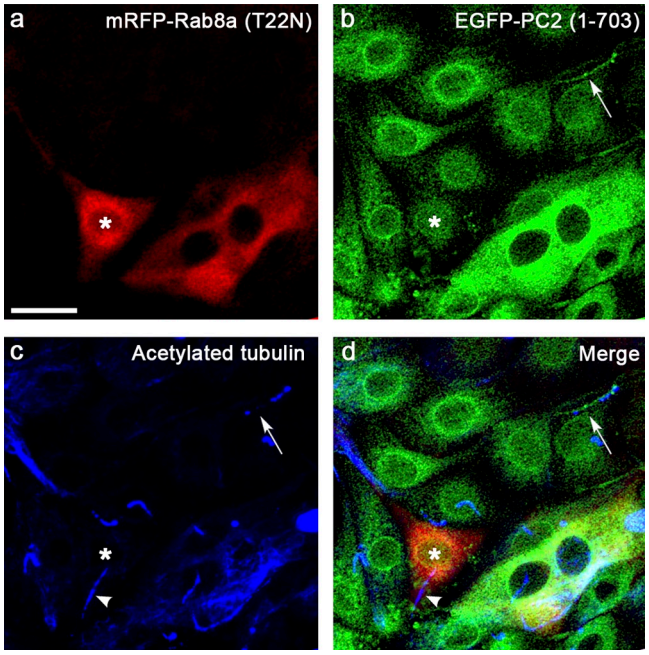

Figure 9. **Rab8a is necessary for the trafficking of polycystin-2 into the primary cilium.** Stably transfected LLC-PK$_1$ cells inducibly synthesizing a fusion protein between EGFP and polycystin-2 (1–703) were microinjected with an expression plasmid encoding a fusion protein between RFP and the dominant-negative Rab8a T22N mutant protein. The cell marked by an asterisk synthesizes both Rab8a T22N (a) and polycystin-2 (1–703) (b), but its primary cilium (c and d, arrowhead) does not contain polycystin-2 (1–703). In other cells not synthesizing Rab8a T22N, the primary cilium is positive for polycystin-2 (1–703) (b–d, arrow). Bar, 20 μm.

apparatus found. However, our pharmacological experiments with brefeldin A indicate that an intact Golgi apparatus is necessary for polycystin-2 to reach the cilium. Because except for very few examples (Saunders et al., 1985) proteins only become resistant to Endo H once they reach the mid-Golgi compartment, we argue that full-length polycystin-2 reaches the cis compartment of the Golgi apparatus in order to move to the ciliary plasma membrane but that the majority of it is returned to the ER. We admit, however, that the ciliary plasma membrane represents only a small portion of the total plasma membrane and that Endo H assays of proteins obtained from whole-cell extracts (just as the biotinylation of intact cells) may not be sensitive enough to detect ciliary membrane proteins. Although it is formally possible that the full-length polycystin-2 protein is first transported through the Golgi apparatus and only a small portion of it moves on to the primary cilium whereas the majority of it is degraded, the stability of polycystin-2 as shown in the pulse-chase experiments does not support this interpretation. Our light and electron microscopic data also argue against the idea that full-length polycystin-2 moves through the Golgi apparatus in order to reach the cilium. Therefore, the interaction of polycystin-2 with the cis compartment of the Golgi apparatus, which appears as a necessary intermediate step on the way to the cilium as indicated by our Brefeldin experiments, is probably only transient. The inhibition experiments with mycophenolic acid in which we were able to colocalize polycystin-2 with GM130 at the cis-Golgi compartment favor such a model. We think that the lower intracellular GTP levels upon

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mycophenolic acid incubation probably prevented the fast dissociation of polycystin-2 from the cis-Golgi apparatus and resulted in its accumulation to high enough levels for it to be detectable by immunofluorescence.

We propose the following model as an attempt to combine the data available so far (Fig. 10). Polycystin-2 is constantly released in a COPII-dependent fashion from the ER to the Golgi apparatus but is immediately returned through the action of the retrieval signal in its C terminus (we favor this name over the term retention signal). Only a small portion of polycystin-2 will be able to escape from the retrograde transport to the ER and is transported further from the cis-Golgi apparatus to the primary cilium. The route of an integral membrane protein via the cis-Golgi compartment, i.e., without traversing the Golgi apparatus, to the primary cilium probably is not restricted to polycystin-2 because we also found no evidence that the M2 mutant of Smoothened traffics through the Golgi apparatus. In cells in which the retrieval signal of polycystin-2 is masked, as may be the case in the presence of polycystin-1 (Hanaoka et al., 2000), or lost as in C-terminally truncated polycystin-2 proteins, polycystin-2 moves further through the Golgi apparatus and reaches the somatic plasma membrane. For this step the presence of a positive signal, such as the newly identified export motif represented by K572 and F576, is necessary. We also want to point out that mutations in the *PKD2* gene have been described that lead to a frame shift after codon 566 (Viribay et al., 1997) and a truncation after codon 584 (Torra et al., 1999). Our results predict different intracellular locations for the two mutant proteins but it is not known whether these mutations result in different phenotypes. The presence of the full-length polycystin-2 protein in the plasma membrane is still discussed controversially. We propose that under various (patho)physiological conditions the KLFKFI motif modulates the transport of polycystin-2 to the somatic plasma membrane. In the case that the physiological location of full-length polycystin-2 is the ER and the primary cilium (and not the somatic plasma membrane), then the KLFKFI motif could be a potential therapeutic target in order to impede the misdirected trafficking of mutant polycystin-2 proteins to the somatic plasma membrane where they may lead to poorly controlled Ca^{2+} currents.

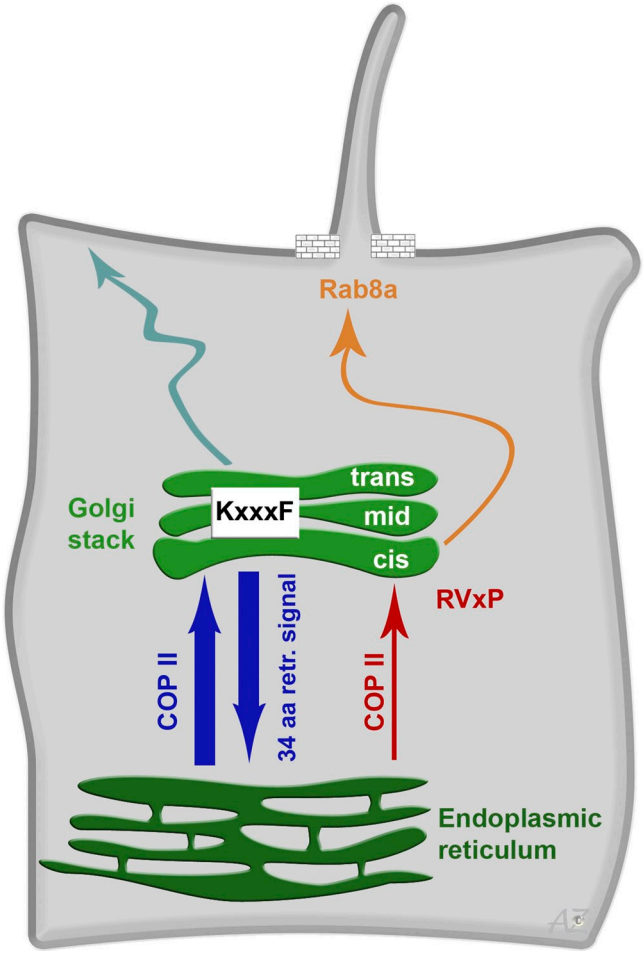

Figure 10. **Model for the trafficking of polycystin-2 to the primary cilium.** Most of the wild-type polycystin-2 protein leaves the ER in a COPII-dependent fashion and is rapidly returned from the Golgi apparatus due to the presence of a 34–amino acid retrieval signal in its C terminus (dark blue arrows). A small portion of wild-type polycystin-2 will reach the cis-Golgi apparatus (red arrow) and is diverted to the primary cilium (orange arrow), where the delivery is controlled by Rab8a. Whether the ciliary targeting signal "RVxP" in the N terminus of polycystin-2 acts at the cis-Golgi compartment or at a more distal location is not known. Under some circumstances, e.g., due to the interaction between polycystin-2 and polycystin-1 or upon the loss of the retrieval signal in the C terminus, polycystin-2 will also reach the somatic plasma membrane through the Golgi apparatus (light blue arrow). Passage through the Golgi apparatus depends on the "KxxxF" motif in loop 4.

Materials and methods

Expression plasmids

The constructs encoding HA epitope–tagged human full-length polycystin-2 and polycystin-2 (1–703) proteins have been described previously (Gallagher et al., 2006). A fusion protein between EGFP and full-length polycystin-2 was created by inserting EGFP after amino acid 157 of polycystin-2. Further polycystin-2 mutant proteins were engineered in the context of the mammalian expression plasmids pcDNA3 (Invitrogen) and pEGFP (Takara Bio Inc.). For ReAsH labeling, expression plasmids were generated that encoded fusion proteins between the respective polycystin-2 proteins, the HA epitope, a tetracysteine motif, and EGFP. To produce fusion proteins between ECFP and PIGEA-14 mutant proteins, the respective sequences of the PIGEA-14 cDNA were amplified by PCR and subcloned into pECFP. The constructs coding for fusion proteins of ECFP with the Sar1 H79G and Sar1 T39N mutant proteins (Aridor et al., 1995) and the Rab23 S23N mutant protein (Guo et al., 2006) were generated by amplifying the inserts by PCR and cloning them into pECFP (Takara Bio Inc.). The Golgi apparatus and trans-Golgi network were visualized in live cells through fusion proteins between ECFP and *N*-acetylgalactosaminyltransferase-2 (White et al., 2001) and TGN38 (McNamara et al., 2004), respectively. All constructs were sequence verified to ensure the absence of any unwanted mutations.

Transfections and cell culture

Stably transfected LtTA-2,22 cells (LLC-PK_1 cells expressing the tetracycline-dependent transactivator) inducibly producing the HA epitope–tagged full-length and (1–703) mutant polycystin-2 proteins have been described previously (Gallagher et al., 2006). HtTA-1 cells (HeLa cells expressing the tetracycline-dependent transactivator; Gossen and Bujard, 1992) were transfected with 8 µg of the expression plasmids pUHD 10–3/PKD2, HA and pUHD 10–3/PKD2 (1–703), HA, respectively, together with 0.8 µg of the selection plasmid pBabe Puro (Morgenstern and Land, 1990) by using a calcium phosphate protocol (Chen and Okayama, 1987). COS-7 and LLC-PK_1 cells were transiently transfected using DEAE dextran/chloroquine (Ausubel et al., 1996), poly-L-ornithine (Dong et al., 1993), and Lipofectamine (Invitrogen), respectively. When cells were used for immunofluorescence, they were transferred onto coverslips 1 d after transfection.

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For the brefeldin A studies, the stably transfected LtTA-2,22 cells were plated onto coverslips. When the cells had reached 100% confluency, brefeldin A (Fluka) was added to the medium at a concentration of 0.5 µg/ml; the solvent methanol served as a negative control. In the case of the LtTA-2,22 cells producing full-length polycystin-2, brefeldin A was applied when the cells were induced; in the case of the LtTA-2,22 cells producing the polycystin-2 (1–703) protein, brefeldin A was added 24 h after the cells were induced. 48 h after treatment with brefeldin A the cells were subjected to immunofluorescence. For the mycophenolic acid experiments, stably transfected cells producing HA epitope–tagged full-length polycystin-2 were incubated for 6 h with 50 µM of mycophenolic acid. Mycophenolic acid was dissolved in ethanol and cells incubated with 0.2% of ethanol therefore served as a negative control. After the incubation, cells were fixed and stained for the HA epitope and the cis-Golgi marker GM130.

Live-cell imaging was performed in LtTA-2,22 cells stably transfected with expression plasmids for fusion proteins between ECFP and the Golgi marker enzyme *N*-acetylgalactosaminyltransferase-2 and the trans-Golgi marker protein TGN38, respectively. Individual clones were then transiently transfected with expression plasmids for fusion proteins between EGFP and polycystin-2 mutant proteins, and with expression plasmids for fusion proteins between EYFP and Smoothened. For imaging analysis, cells were plated onto µ-dishes (ibidi). To enhance the signal, the cells were incubated for 5 h at 15°C to block protein transport between the ER and the Golgi apparatus. After transferring the cells to Krebs-Henseleit buffer prewarmed to 37°C, pictures were taken every 2 min. Alternatively, cells were fixed immediately after the 15°C block as well as after 15 and 40 min of incubation at 37°C. The imaging setup consisted of an inverted microscope (Observer Z1; Carl Zeiss, Inc.) equipped with a c-Apochromat 63x/1.20w objective, a confocal laser scanning microscope (LSM 710; Carl Zeiss, Inc.) operated with the ZEN 2008 software, the polychromatic illumination system VisiChrome (Visitron), and the MetaMorph software package (Universal Imaging Corp.). Spectral unmixing was done on images taken with the built-in 34-channel detection unit of the laser scanning microscope and with an ImageJ (Abramoff et al., 2004) plug-in written by Joachim Walter. Further image processing was done using the Levels and Curves functions of Adobe Photoshop CS4.

To investigate the effect of PIGEA-14 mutant proteins, stably transfected LLC-PK_1 cells inducibly producing HA epitope–tagged full-length polycystin-2 and polycystin-2 (1–703) were transiently transfected with the pECFP/PIGEA-14 constructs. After 4 h of exposure to the lipofectamine/DNA mixture, the cells were washed and medium without doxycycline was added. 3 d later, the cells were fixed and stained with antibodies against acetylated tubulin and the HA epitope.

Immunofluorescence

Cells were fixed in 1x PBS (10 mM potassium phosphate, pH 7.5, and 140 mM NaCl), 4% paraformaldehyde for 1 h at room temperature, or in 100% methanol for 6 min at −20°C. After three washes with 1x PBS, they were either permeabilized for 15 min in 1x PBS, 0.25% Triton X-100, or left in 1x PBS. Then the cells were incubated for 2 h at room temperature with the primary antibody diluted in 1x PBS, 2% BSA, washed once in 1x PBS, 350 mM NaCl, twice with 1x PBS, and incubated with the secondary antibody for 1 h at room temperature. Next, the cells were washed once in 1x PBS, 350 mM NaCl, and twice with 1x PBS before they were stained for 1 min with 10 µg/ml of Hoechst 33258. Finally, the cells were washed three times with 1x PBS and mounted in 1x PBS, 40% glycerol. Images were taken with a confocal microscope (LSM 510; Carl Zeiss, Inc.), an Axioskop 20 and an Axiovert 200 fluorescence microscope (Carl Zeiss, Inc.). The following primary antibodies were used: a rabbit polyclonal anti-HA epitope antibody (cat. no. H 6908; diluted 1:100; Sigma-Aldrich), a mouse monoclonal anti-HA epitope antibody (cat. no. sc-7392; diluted 1:50; Santa Cruz Biotechnology, Inc.), a rabbit polyclonal anti–polycystin-2 antibody (directed against amino acids 331–343 of the human polycystin-2 protein; diluted 1:100), a mouse monoclonal anti-acetylated tubulin antibody (cat. no. T 6793; diluted 1:200; Sigma-Aldrich), a mouse monoclonal anti–γ-tubulin antibody (cat. no. sc-51715; diluted 1:50; Santa Cruz Biotechnology, Inc.), a mouse monoclonal anti-GM130 antibody (cat. no. 610822; diluted 1:40; BD), and a rabbit polyclonal anti-Arl13b antibody (diluted 1:100; Duldulao et al., 2009). Specific staining was visualized with FITC-conjugated goat anti–mouse IgG (cat. no. 55493; diluted 1:200; MP Biomedicals), Cy3-conjugated goat anti–mouse IgG (cat. no. 115–165-062; diluted 1:300; Jackson ImmunoResearch Laboratories), Cy3-conjugated goat anti–rabbit IgG (cat. no. 111–165-144; diluted 1:400; Jackson ImmunoResearch Laboratories), Cy5-coupled goat anti–mouse IgG (cat. no. 115–175-062; diluted 1:400; Jackson ImmunoResearch Laboratories), Alexa 488–conjugated goat anti–rabbit IgG (cat. no. A11034; diluted 1:600; Invitrogen), Alexa 488–conjugated goat anti–mouse IgG (cat. no. A11029; diluted 1:600; Invitrogen), Alexa 633–conjugated goat anti–rabbit IgG (cat. no. A21071; diluted 1:600; Invitrogen), and Alexa 633–conjugated goat anti–mouse IgG (cat. no. A21052; diluted 1:600; Invitrogen).

ReAsH labeling and electron microscopy

COS-7 cells were transiently transfected using DEAE dextran/chloroquine (Ausubel et al., 1996). 3 d later, the cells were washed three times with HBSS. After they were incubated for 1 h at 37°C with 450 nM ReAsH (resorufin arsenical hairpin binding reagent; Invitrogen), 12.5 µM EDT (1,2-ethanedithiol; Fluka), the cells were washed twice briefly with 500 nM BAL (2,3-dimercapto-1-propanol; Fluka) and then were incubated in 500 nM BAL for 20 min at 37°C, washed again three times with HBSS, and fixed with 2% glutaraldehyde overnight. Once the fixed cells were blocked for 30 min in 50 mM glycine, 10 mM KCN, 20 mM aminotriazole, 0.001% H_2O_2 and washed, a new buffer containing 1 mg/ml of diaminobenzidine was added. Photoconversion was performed by exposing the cells at 4°C to a 585-nm light source. Finally the cells were washed again and embedded in Durcupan (Fluka). 50-nm thin sections were analyzed in a transmission electron microscope (EM 902; Carl Zeiss, Inc.) equipped with a cooled CCD digital camera (TRS).

Metabolic labeling and immunoprecipitation

Cells were cultured in 100-mm Petri dishes and grown to ∼70% confluency. Then cells were washed twice with cysteine/methionine-free DME containing 2 mM L-glutamine and 5% dialyzed fetal calf serum before being preincubated in this medium for 30 min at 37°C. The labeling was performed in 4 ml fresh medium with 250 µCi Tran^{35}S-Label (MP Biomedicals) for 5 min at 37°C (pulse). To terminate the pulse, the cells were washed twice with chase medium (complete DME with 10% FCS), and chased for different times as indicated. After metabolic labeling the cells were washed twice with ice-cold 1x PBS and lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, pH 8.0, 50 mM NaF, 0.2 mM $NaVO_3$, 3 µg/ml of leupeptin, and 10 µg/ml of aprotinin). The lysate was transferred to a 1.5-ml microcentrifuge tube, passed multiple times through a needle to reduce its viscosity, and then centrifuged for 5 min at 14,000 rpm to remove insoluble material. For immunoprecipitation, 500 µl of radiolabeled cell lysate were incubated for 90 min at 4°C with 50 µl of supernatant from the 12CA5 hybridoma, which produces mouse monoclonal antibodies directed against the HA epitope. Then 25 µl of swollen protein A–Sepharose beads were added to precipitate the immune complexes, followed by further incubation for 90 min at 4°C. Subsequently, the beads were washed twice in the appropriate lysis buffer, resuspended in 50 µl of 1x SDS sample buffer (125 mM Tris-HCl, pH 6.7, 2.5% SDS, 10% glycerol, 2.5% β-mercaptoethanol, and 0.01% bromophenol blue), and incubated on ice for 30 min to elute the precipitated proteins from the beads.

SDS-PAGE and Western blotting

For immunodetection, proteins were separated on denaturing polyacrylamide gels without prior boiling and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). After transfer, the membrane was blocked overnight at room temperature in 1x PBS, 5% low-fat dry milk powder, 0.2% Tween 20, and then incubated for 1 h with the primary antibody. The following primary antibodies were used: the rat monoclonal anti-HA-epitope antibody 3F10 (cat. no. 11 867 423 001; diluted 1:4,000; Roche) and the mouse monoclonal anti-Na^+/K^+-ATPase α1-subunit (cat. no. A-277; diluted 1:3,000; Sigma-Aldrich). After washes with 1x PBS, 0.2% Tween 20, the membrane was incubated for 1 h with the HRP-conjugated goat anti–rat IgG secondary antibody (cat. no. 112–035-068; diluted 1:20,000; Jackson ImmunoResearch Laboratories) and HRP-conjugated goat anti–mouse IgG (cat. no. A3682; diluted 1:10,000; Sigma-Aldrich). To detect the secondary antibodies, a chemiluminescence detection kit (PerkinElmer) was used. For the detection of ^{35}S-labeled proteins, the gels were dried and exposed to RX NIF x-ray film (Fujifilm).

Glycosidase treatment

Glycosidase treatment was essentially performed according to the manufacturer's instructions (New England Biolabs, Inc.). In the case of ^{35}S-labeled polycystin-2, the beads with the immunoprecipitated protein were resuspended in 50 µl buffer containing 2% β-mercaptoethanol, 1% SDS, 100 mM Tris, pH 7.5 before the samples were incubated for 1 h at 37°C with 1,000 U endoglycosidase H_f (Endo H) or 1,000 U peptide:*N*-glycosidase F (PNGase F). The reactions were terminated by the addition of 25 µl 3x SDS

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sample buffer. After a further incubation for 15 min at 37°C the samples were loaded on a protein gel and analyzed by autoradiography. When nonlabeled polycystin-2 protein was analyzed, whole-cell lysate containing 10–20 µg of protein was incubated for 90 min at 37°C with 1,000 U of endoglycosidase H_f.

Cell surface biotinylation

All steps were performed at 4°C. After cells were washed four times with 1x PBS-CM (10 mM potassium phosphate, pH 7.5, 140 mM NaCl, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$), they were incubated for 30 min in the same buffer containing 0.5 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific). The biotinylation reaction was quenched by incubating for 20 min with 1x PBS-CM containing 100 mM glycine. Then the cells were rinsed four times with 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0 and lysed in the same buffer supplemented with 1% Triton X-100. Whole-cell lysate corresponding to 130–170 µg of protein was immunoprecipitated overnight in a volume of 200 µl with 25 µl of NeutrAvidin beads (Thermo Fisher Scientific). After extensive washes with 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, and 50 mM Tris HCl, pH 7.5, 500 mM NaCl, 5 mM EDTA, pH 8.0, the NeutrAvidin beads were resuspended in SDS sample buffer and subjected to Western blot analysis.

Microinjection

LtTA-2,22 cells were stably transfected with a tetracycline-inducible expression plasmid coding for a fusion protein between the HA epitope–tagged polycystin-2 (1–703) protein and EGFP. When needed, the cells were cultured on coverslips until they reached confluency. Then expression was induced and plasmids (500 ng/µl) were injected with the InjectMan/FemtoJet System (Eppendorf) on a microscope (Axiovert 200; Carl Zeiss, Inc.). After microinjection, the cells were incubated at 37°C until the fluorescence-tagged protein was detected (typically up to 4 h). The injected plasmids coded for ECFP (Takara Bio Inc.), for a fusion protein between ECFP and amino acids 65–126 of PIGEA-14, for a fusion protein between mRFP and the dominant-negative Rab8a T22N mutant protein (Bravo-Cordero et al., 2007), and for a fusion protein between ECFP and the dominant-negative Rab23 S23N mutant protein (Guo et al., 2006).

Online supplemental material

Fig. S1 shows that the polycystin-2 (1–703) protein is not present in cilia after BFA treatment, as demonstrated by staining for Arl13b. Fig. S2 shows the colocalization of full-length polycystin-2 and GM130 upon treatment with mycophenolic acid. Fig. S3 shows the effect of PIGEA-14 mutant proteins on the ciliary trafficking of polycystin-2. Fig. S4 shows double staining for full-length polycystin-2 and γ-tubulin. Video 1 shows the imaging of full-length polycystin-2 and the Golgi marker *N*-acetylgalactosaminyltransferase-2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007050/DC1.

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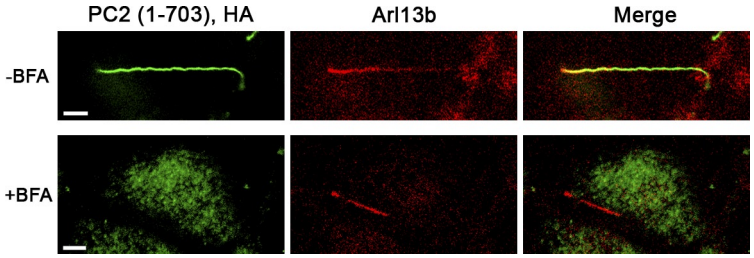

Figure S1. **Trafficking of polycystin-2 to the primary cilium depends on an intact Golgi apparatus.** LtA-2,22 cells were stably transfected with an expression plasmid for HA epitope–tagged polycystin-2 (1–703), and treated with brefeldin A (+BFA) or with the solvent methanol (−BFA). Double staining for the HA epitope and the monomeric G protein Arl13b, another ciliary marker, revealed that in the presence of brefeldin A the truncated polycystin-2 protein no longer reached primary cilia. Bar, 5 µm.

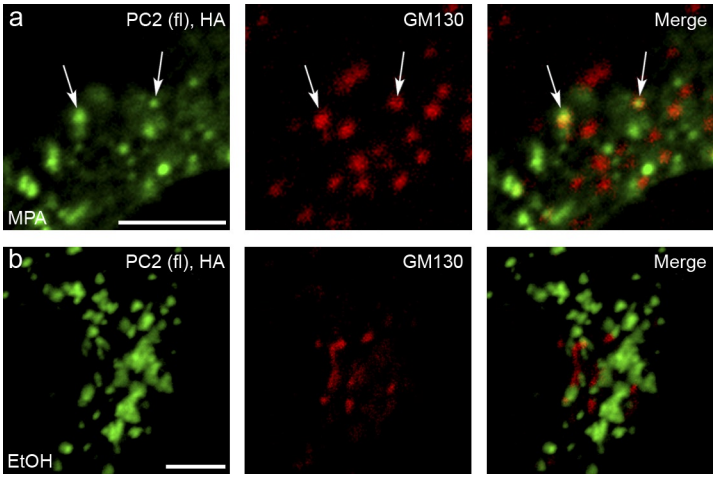

Figure S2. **Colocalization of full-length polycystin-2 and GM130.** Stably transfected LLC-PK_1 cells inducibly producing HA epitope–tagged full-length polycystin-2 were incubated for 6 h with 50 µM mycophenolic acid (MPA). Double staining for the cis-Golgi marker protein GM130 led to the identification of polycystin-2 vesicles colocalizing with GM130 (a, arrows). Cells incubated for 24 h with 0.2% of the solvent ethanol (EtOH) served as a negative control (b). Bar, 5 µm.

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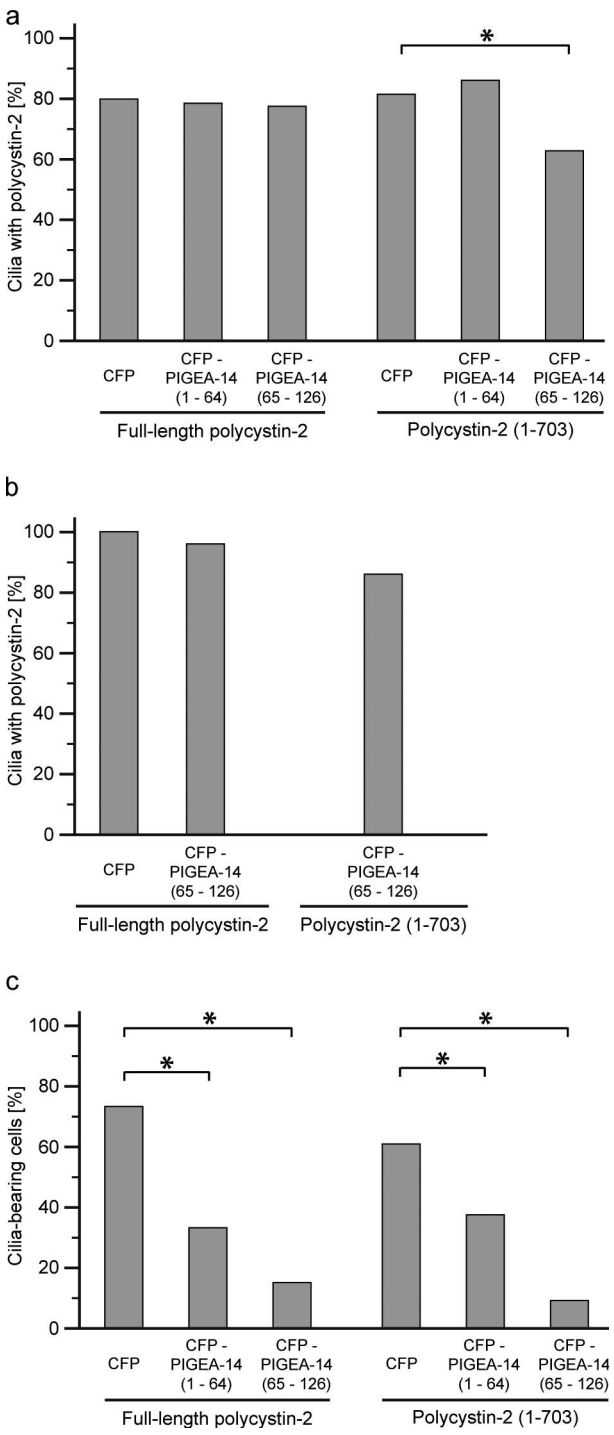

Figure S3. **Ciliary trafficking of polycystin-2 in the presence of PIGEA-14 mutant proteins.** (a) Stably transfected LLC-PK$_1$ cells inducibly producing HA epitope–tagged full-length polycystin-2 and polycystin-2 (1–703) were transiently transfected with expression constructs encoding ECFP fusion proteins with PIGEA-14 (1–64) and PIGEA-14 (65–126), respectively. Only under one experimental condition was a statistically significant effect observed. (b) Microinjection of the ECFP/PIGEA-14 (65–126) fusion protein had no effect on the trafficking of either polycystin-2 construct. (c) Both PIGEA-14 fusion proteins inhibited the formation of cilia, thus indicating that they are biologically active. In the transient transfection experiments, between 402 and 635 CFP-positive cells were counted, and at least three independent experiments were included. For the microinjection experiments, between 25 and 37 cells were counted. The asterisks indicate statistically significant differences at $P < 0.01$ as determined by a χ^2-test.

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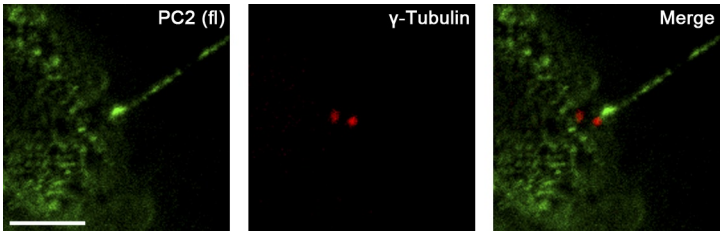
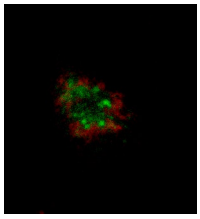


Figure S4. **Double staining for full-length polycystin-2 and γ-tubulin.** LtA-2,22 cells were stably transfected with an expression plasmid for HA epitope–tagged full-length polycystin-2 (PC2 (fl)). Double staining for polycystin-2 and γ-tubulin failed to reveal colocalization of the two proteins. Bar, 5 µm.



Video 1. **Imaging of full-length polycystin-2.** LLC-PK_1 cells stably synthesizing an ECFP fusion protein with *N*-acetylgalactosaminyltransferase-2 (shown in red for better visibility) were transiently transfected with an expression plasmid coding for an EGFP fusion protein with full-length polycystin-2. 3 d later the cells were incubated for 5 h at 15°C before being incubated at 37°C. Pictures were taken every 2 min over a period of 60 min, ~5 min after shifting the cells to 37°C. No overlapping signal can be seen between full-length polycystin-2 and *N*-acetylgalactosaminyltransferase-2.