Depletion of apical transport proteins perturbs epithelial cyst formation and ciliogenesis

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Summary

Epithelial cells are vital for maintaining the complex architecture and functions of organs in the body. Directed by cues from the extracellular matrix, cells polarize their surface into apical and basolateral domains, and connect by extensive cell-cell junctions to form tightly woven epithelial layers. In fully polarized cells, primary cilia project from the apical surface. Madin-Darby canine kidney (MDCK) cells provide a model to study organization of cells as monolayers and also in 3D in cysts. In this study retrovirus-mediated RNA interference (RNAi) was used to generate a series of knockdowns (KD) for proteins implicated in apical transport: annexin-13, caveolin-1, galectin-3, syntaxin-3, syntaxin-2 and VIP17 and/or MAL. Cyst cultures were then employed to study the effects of these KDs on epithelial morphogenesis. Depletion of these proteins by RNAi stalled the development of the apical lumen in cysts and resulted in impaired ciliogenesis. The most severe ciliary defects were observed in annexin-13 and syntaxin-3 KD cysts. Although the phenotypes demonstrate the robustness of the formation of the polarized membrane domains, they indicate the important role of apical membrane biogenesis in epithelial organization.

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Key words: Cilia, Cyst, Epithelial lumen, Morphogenesis, Polarization

Introduction

Epithelial cells form layers that constitute boundaries between the interior of vertebrates and invertebrates and the outside environment (Gibson and Perrimon, 2003; Nelson, 2003). These epithelial layers can form cysts such as acini in the pancreas or follicles in the thyroid or, by branching, build tubes similar to in the intestine or in nephrons (Gumbiner, 1992; Montesano et al., 1991b; Vainio and Muller, 1997). Each epithelial cell is polarized into an apical and a basolateral plasma membrane domain, separated by junctional complexes (Bacallao et al., 1989; Margolis and Borg, 2005; Umeda et al., 2006). The apical surface is the unique membrane domain characteristic of epithelia that defines the lumen in cysts and tubes (Lubarsky and Krasnow, 2003; O’Brien et al., 2002). How apical membrane domains are generated remains a key issue in development.

One prime model to study epithelial cell biology is the Madin-Darby canine kidney (MDCK) cell system. When these cells are plated on solid (glass or plastic) or porous (filter) supports, they proliferate and move to form islets of cells, eventually fusing to a continuous monolayer of confluent cells that are connected by lateral and tight junctions (TJs) (Bacallao et al., 1989; Drubin and Nelson, 1996; Misfeldt et al., 1976; Tsukita et al., 1999). The apico-basal orientation in these 2D cultures is defined by a strong spatial cue from the artificial support. MDCK cells secrete laminin and assemble extracellular matrix (ECM) at their basal surface facing the support. Assembly of the basement-membrane-like matrix defines the opposite surface, which faces the culture medium, to become the apical side of the cell layer (Drubin and Nelson, 1996; Ecay and Valentich, 1992; O’Brien et al., 2001). Polarization of MDCK cells in 2D cultures is a robust process, where apical components become sorted to the free surface, but this culture system is not very well suited to study the biogenesis of the apical lumen in cysts and tubes. Cells in 2D cultures do not form an enclosed lumen.

However, one can also study epithelial polarization in MDCK cells in 3D cultures (Elsdale and Bard, 1974; O’Brien et al., 2002; Wang et al., 1994; Zegers et al., 2003). If the MDCK cells are grown in suspension culture (no matrix cue for polarity), they form hollow spherical cysts, wherein the apical surface and the cortical actin meshwork face the outside, whereas the basal surface lines the cyst lumen – a situation not encountered in vivo. When these cells are later placed in a collagen gel (a primary cue for epithelial polarity), polarity is ‘reversed’ without cell-layer disruption (Ojakian and Schwimmer, 1994; Wang et al., 1990a; Wang et al., 1990b). The apical proteins become internalized and degraded during the inversion process, followed by the generation of a new apical surface, established by biosynthetic delivery of apical components (Wang et al., 1994; Wang et al., 2000).

Single MDCK cells can also be directly embedded into the collagen gel, where the cells divide and form aggregates that differentiate into hollow spheres with an organization closely mimicking cyst architecture in vivo (Montesano et al., 1991b; O’Brien et al., 2002; Wang et al., 1990a; Wang et al., 1990b). These are formed by a single layer of cells with their apical surface lining the inside lumen and the basal domain facing the outside collagen gel. Cystogenesis proceeds by the formation of cell-cell contacts and apico-basal polarization (O’Brien et al., 2002; Wang et al., 1994). Cells that are trapped inside the aggregate and have lost...
The cellular mechanisms responsible for polarizing epithelial cells have been studied intensively. Three protein complexes (Par3-Par6-aPKC; Crumbs–Stardust–Discs-lost; Scribble–Discslarge–Lethal-giant-larvae) have been identified that act together to direct cell-cell interactions and surface polarization (Hurd et al., 2003; Knust and Bossinger, 2002; Roh et al., 2003; Tenentzapf and Tepass, 2003; Yamanaka et al., 2003). Recent studies have also implicated a key role for phosphoinositides in the generation of separate apical and basolateral plasma membrane domains (Martin-Belmonte et al., 2007). How these protein complexes interact with the membrane trafficking machinery to generate routes for newly synthesized proteins and lipids that form the polarized cell surface is not yet known. Sorting in these routes occurs both in the trans-Golgi network and in the endosome-and-recycling-circuits that connect the apical and the basolateral plasma membrane domains (Ang et al., 2004; Bonifacino and Rojas, 2006; Gravotta et al., 2007; Wang et al., 2000). The basolateral targeting machinery might be connected to cell junctions in MDCK cells. A network of acentrosomal cortical microtubules has been implicated in delivering the basolateral cargo to the membrane, where the exocyst protein complex might be involved in docking and exocytosis of the carrier vesicles (Grindstaff et al., 1998; Hua et al., 2006; Lipschutz and Mostov, 2002; Reilein et al., 2005; Yeaman et al., 2004). Less is known about the generation of the apical targeting capability (Schuck and Simons, 2004). Several candidate proteins potentially involved in sorting and targeting have been identified, mostly by work in 2D filter cultures of MDCK cells.

In this paper, we have selected some of these candidate proteins for a study in 3D cyst cultures to investigate their role in MDCK cell cystogenesis. Previously the effects of overexpression of Crumbs-3 (Roh et al., 2003) and PATJ (Shin et al., 2005), and the KD of the genes encoding FAPP2, Lethal giant larvae proteins and PALS1 (Straight et al., 2004; Vicent et al., 2006; Yamanaka et al., 2006) were analyzed in MDCK cyst cultures. Depletion of these proteins perturbed the 3D organization of the cells in the cysts. We have silenced the expression of annexin-13, caveolin-1, galectin-3, syntaxin-3 and VIP17 and/or MAL by retrovirus-mediated RNA interference (RNAi) (Schuck et al., 2004), and performed a systematic analysis of the resulting effects on polarization and cyst morphogenesis. All of these proteins have been implicated in apical transport by previous work (Cheong et al., 1999; Delacour et al., 2006; Lafont et al., 1998; Low et al., 1998; Puertollano et al., 1999; Sharma et al., 2006; Sotgia et al., 2002). Here, we found that all KDs caused perturbations of cyst morphogenesis and often of surface polarization as well. Several KDs also affected ciliogenesis. We conclude that interfering with the expression of factors implicated in apical transport in all cases resulted in perturbed cystogenesis.

Results

RNA silencing

Cyst formation in collagen I culture takes several days, therefore precluding the use of transiently transfected siRNAs for gene silencing. To achieve stable KDs for the examination of the long-term effects of the depletion of the proteins on polarization and epithelial morphogenesis, we employed retrovirus-mediated RNAi previously developed in our lab (Schuck et al., 2004). The target gene mRNA levels were efficiently reduced for the genes under study, and the average reductions of gene expression ± s.d. (n=6-12) were: annexin-13 (86±2.5%), caveolin-1 (91±2.7%), syntaxin-3 (77±2.4%) and VIP17/MAL (91±2.4%). The protein levels were reduced accordingly (Friedrichs et al., 2007; Schuck et al., 2004). Two additional KDs for galectin-3 (90±1.8%) (Friedrichs et al., 2007) and crumbs-3 (92±1.5%), which have also been previously implicated in apical transport and the polarized membrane formation (Delacour et al., 2006; Roh et al., 2003), were generated for this study. For the caveolin-1 KD and the galectin-3 KD efficient protein depletion was also demonstrated by immunostaining and immunoblotting (supplementary material Fig. S1). All KDs were

![Fig. 1. Morphology of the cysts and polarization of different markers in the control and crumbs-3 knockdown (Crb-3 KD) cells. (A–H) Control and Crb-3 KD cysts (A–D and E–H, respectively) were immunostained with antibodies against the apical markers podocalyxin (Pcx) or carcinoembryonic antigen (CEA), the basolateral membrane marker E-cadherin (E-cad), or ZO-1 for cell junctions as indicated. AlexaFluor®488-conjugated secondary antibodies were applied to visualize the different markers in green. All cysts were also stained for actin (TRITC-phalloidin; red) indicating outlines of lumina and individual cells. DAPI staining (blue) shown nuclei. Arrowheads in G indicate aberrant localization of E-cadherin at the luminal surface in a Crb-3 KD cyst. Scale bars, 30 μm.](Image)
also tested for the reduction of mRNA levels in the cyst cultures at day 14. Quantitative PCR analysis from RNA in cysts (supplementary material Fig. S1) confirmed the persistency of KD efficiencies in continuous long-term culture, in agreement with earlier findings (Schuck et al., 2004).

Cyst morphology, and polarization of control and crumbs-3 depleted cells

To generate MDCK cysts, the control cells – transduced with control retrovirus (Schuck et al., 2004) – and the different KD cells were trypsinized to yield single-cell suspensions, mixed with the collagen I gel and plated. Cells were generally cultured for 14 days in standard medium containing 5% FCS. To study the morphology and polarization of the resulting cysts we used immunofluorescence confocal microscopy. MDCK cell cysts were stained with antibodies against the apical proteins podocalyxin (Podxl, gp135, hereafter referred to as Pcx; Fig. 1A) or carcinoemryonic antigen (CEA, gp114; Fig. 1B), basolateral E-cadherin (Fig. 1C) or ZO-1 at the TJs (Fig. 1D). Primary antibodies were labeled with AlexaFluor®488-conjugated secondary antibodies to visualize the localization of the different markers in green. TRITC-phalloidin was used to mark actin filaments and DAPI to stain the nuclei (shown in red and blue, respectively; Fig. 1A-D).

After 14 days, control cells had proliferated and differentiated into cysts that had a single lumen (Fig. 1A-D) in most cases. Phenotypes derived from control and different KD cells were readily distinguishable already at 10 days. The final classification of the phenotypes was done after 14 days (Fig. 3). We determined this time as the endpoint. The cells in the control cysts developed polarized surfaces, in which Pcx and CEA were found at the apical membrane (Fig. 1A,B, respectively), contrasting with E-cadherin at the basolateral membrane (Fig. 1C). ZO-1 staining marked the TJs appearing in a chicken-wire-like pattern, also defining the borderline between the apical and the basolateral membrane (Fig. 1D). 3D rendering (using Velocity® software by Improvision) of the confocal stacks was applied to better visualize the 3D organization of the cysts by showing the distribution of the TJs (ZO-1 staining) in the different KDs (Fig. 2). Actin staining, by using TRITC-phalloidin, was used to reveal the outlines of the individual cells and to outline the dense actin meshwork beneath the apical surface, a landmark for luminal surfaces in polarized MDCK cysts (Pollack et al., 1998).

To analyze the various KDs, we used the following set of characteristic features to decide whether or not cyst formation was disturbed: (1) the formation of a single spherical lumen, (2) the polarized distribution of apical and basolateral markers, (3) the polarized distribution of the TJs and, (4) the concentration of actin beneath the apical surface. With the exception of lumen formation, none of these features readily lend themselves to quantification. We therefore mostly had to rely on qualitative descriptions of the various phenotypes. However, these descriptions are based on several independent experiments. For the classification of the morphological phenotypes 100-200 cysts were examined in each case (Fig. 3). Notably, the overall classification based on the comparison of the lumen formation is here used to give a general idea of the severity of polarity perturbation.

Crumbs-3 has previously been shown to have an essential role in the formation of the junction-associated complexes required for apical-membrane biogenesis (Hurd et al., 2003; Knust et al., 1993; Roh et al., 2003). We observed that crumbs-3-depleted cells formed mostly aggregates with multiple irregular-shaped small lumina or with no defined lumen formation (Fig. 1E-H indicates the immunostaining with different markers; see also Fig. 3). The overall size of the Crb-3 KD cysts was often smaller than those derived from the control cells. Although, apical markers (Pcx and CEA; Fig. 1E,F, respectively) and basolateral markers (E-cadherin and ZO-1; Fig. 1G,H, respectively) mostly polarized properly, these also sometimes colocalized on the luminal surface (e.g. E-cadherin, as indicated by arrowhead in Fig. 1G). Moreover, in ~15% of the crumbs-3 KD cysts (which are included in the ‘no lumen formed’ class; Fig. 3) a dense actin meshwork was also seen on the basal side facing the collagen gel instead of being concentrated underneath nascent luminal surfaces in the cell aggregates (supplementary material Fig. S2). Thus, as expected, the crumbs-3 KD led to a severe impairment of the polarization process. Although the crumbs-3 KD cells proliferated (Fig. 1E-H), they could not establish the architecture characteristic of normal cysts (Fig. 1A-D).

Cyst morphology and polarization of annexin-13, caveolin-1, galectin-3, syntaxin-3 and VIP17 and/or MAL KD cells

Annexin-13

There are two isoforms of annexin-13 in MDCK cells, annexin-13a and annexin-13b (Lecat et al., 2000). Both regulate apical transport, but annexin-13a is localized basolaterally and annexin-
Addition of recombinant annexin-13a and annexin-13b to the permeabilized MDCK cells has previously been shown to enhance apical transport, whereas annexin-13a also inhibited transport of vesicular stomatitis virus (VSV) G-protein to the basolateral surface (Lafont et al., 1998; Lecat et al., 2000). Our data demonstrated that annexin-13 KD cells, depleted of both annexin-13a and annexin-13b, had severe problems in generating a polarized cyst structure. Fig. 4 shows representative single stacks of confocal images of annexin-13 KD, caveolin-1 KD, galectin-3 KD, syntaxin-3 KD and VIP17 KD cysts (panels 1, 2, 3, 4 and 5, respectively) stained for actin (TRITC-phalloidin; in red) and for different polarity markers, Pcx, CEA, E-cadherin or ZO-1 (in green; panels A, B, C or D, respectively). Nuclei are shown in blue. Annexin-13 KD cells proliferated, forming large aggregates that rarely had a well-defined single lumen (Figs 3, 4). As judged by the localization of the different markers Pcx, CEA, E-cadherin and ZO-1 (Fig. 4, panels A1, B1, C1 and D1, respectively), the formation of the polarized membrane domains appeared robust in annexin-13-depleted cells. Of the apical markers, Pcx was generally targeted to the surfaces of the nascent lumina or to surface domains marked by an intense actin meshwork (Fig. 4A1). CEA was distributed in a more punctate manner, yet also to the nascent lumina or surface domains (Fig. 4B1). E-cadherin (Fig. 4C1) was sometimes mislocalized to the luminal surfaces (arrowheads). Assembly of E-cadherin and ZO-1 at the cell junctions (Fig. 4C1,D1) was often impaired and, although continuous and merging between the individual cells, the TJs of the annexin-13-depleted cells often extended close to the basal side (Fig. 4D1, see also Fig. 2B). Thereby, the length of the lateral membrane was reduced owing to the extension of the apical surface domain towards the basal membrane. Somewhat similar phenotypes have previously been observed in Rab10 KD and in the Crumbs-overexpressing MDCK cysts (Roh et al., 2003; Schuck et al., 2007). Finally, the distribution of the actin meshwork was often aberrant (Fig. 4A1,B1,C1,D1), because this was not only abundant at the luminal but also at the basal surface (facing the collagen matrix) in the annexin-13 KD cysts.

Caveolin-1
This protein had been previously implicated in apical delivery of GPI-anchored proteins (Sotgia et al., 2002). However, careful studies in different caveolin-deficient cell types have demonstrated no defects in apical delivery (Le Lay and Kurzchalia, 2005; Manninen et al., 2005; Zurzolo et al., 1994). Unlike in control cysts, the majority of caveolin-1 KD cysts had multiple lumina (Figs 3, 4). These lumina were separated by only a monolayer of cells (Fig. 4). Often, these seemingly separate lumina were found to be connected with each other. Such assemblies of continuous lumina were also found in the galectin-3 KD and VIP17 and/or MAL KD cysts (see below). Actin staining was generally intense at the luminal surfaces and even more enhanced in cell-cell...
Fig. 4. Morphology of the cysts and polarization of different marker proteins in knockdown cells. Columns 1. to 5. shown the following KD cells. 1., annexin-13 (Anx-13 KD); 2., caveolin-1 (Cav-1 KD); 3., galectin-3 (Gal-3 KD); 4., syntaxin-3 (Stx-3 KD); and 5., VIP17 KD. Panel groups A-D show immunostaining of the different KD cysts. Shown in green (AlexaFluor®488) are: A, apical membrane marker podocalyxin (Pcx); B, carcinoembryonic antigen (CEA); C, basolateral membrane marker E-cadherin (E-cad); D, tight-junction marker ZO-1. DAPI was used to stain nuclei (blue) and TRITC-phalloidin to show the actin meshwork (red). Arrowheads indicate (C) luminal and (D) aberrant localization of E-cadherin and ZO-1, respectively, observed sometimes in the different KDs (see text). Scale bars, 30 μm.
contacts (Fig. 4, panels A2, B2, C2, D2), where the filaments coalesced with intense E-cadherin expression (panel C2). Sometimes only a thin actin belt was observed between the adjacent lumina in the multiple lumen cysts. However, the apical and basolateral markers (Fig. 4, panels A2, B2, C2) were normally polarized, and the TJVs (Fig. 4, panel D2) showed no manifest defects in the caveolin-1 KD cysts (see also Fig. 2D).

Galec-tin-3
Recent studies have implicated this protein in the apical transport in a route that delivers lactase-phlorizin hydrolase and the neurotrophin receptor (p75) to the apical surface in the MDCK cells (Delacour et al., 2006). Dynamic changes in the expression and targeting of galec-tin-3 have been reported in polarized cells and during cystogenesis (Bao and Hughes, 1995). Galec-tin-3 has also been linked to nephrogenesis and pathogenesis of the polycystic kidney disease (Chiu et al., 2006; Winyard et al., 1997).

The galec-tin-3 KD cysts had a variable morphology (Figs 2-4). Depletion of galec-tin-3 seemingly interfered with the cellular orientation required to drive the polarized formation of a single lumen and of an apical surface in a defined direction. Instead, the galec-tin-3 KD cells (Fig. 4, panels A3, B3, C3, D3) formed convoluted luminal surfaces that did not enclose well-defined lumina. Pcx (Fig. 4A3) usually coaligned with the actin meshwork underlining these surfaces, whereas CEA (Fig. 4B3) was found not only at these convoluted 'pre-luminal' surfaces but also to be accumulating intracellularly. The generation of the TJs (Fig. 4D3) appeared normal in ZO-1 staining. Although, the intensity of the ZO-1 staining at the TJs varied, these structures still appeared continuous (Fig. 2C), similar to those in multilumina Cav-1 KD cysts (Fig. 2D). Localization of E-cadherin (Fig. 4C3) was more variable, in that it was abundant at the cell-cell contacts and also sometimes abnormally localized at the nascent luminal surfaces (Fig. 4C, arrowheads). Similarly, the dense actin meshwork was lining the convoluted surfaces, with the most intense expression at the contact sites of the surfaces (Fig. 4C).

Syntaxin-3
This SNARE protein has been shown to localize apically in polarized epithelial cells (Low et al., 1996). Several studies have demonstrated involvement of syntaxin-3 in apical transport and recycling (Low et al., 1998; Low et al., 2006; Sharma et al., 2006). Cysts formed from syntaxin-3 KD MDCK cells (Fig. 4, panels A4, B4, C4, D4) were significantly smaller than those from the control cells (P<0.001) or from other KDs, also investigated with this regard (see below). Unlike the Crb-3 KD (Fig. 1), the majority of the syntaxin-3 KD cysts had a single lumen (Figs 3, 4). Some of the syntaxin-3 KDs generated only small cell aggregates without defined lumina. Notably, unlike in the other KDs, those cysts with multiple lumina were rarely formed (Fig. 3). A substantial fraction (~10%) of the syntaxin-3 KD cell cysts formed cell aggregates that had an intense actin meshwork underneath the basal instead of the apical surface (supplementary material Fig. S2). Such an inverse organization of the cell cytoskeleton was similar to that observed in some of the crumbs-3 KD cysts (supplementary material Fig. S2). However, we did not observe inverse organization of actin at the periphery of the control cysts. The apical markers Pcx and CEA (Fig. 4, panels A4 and B4, respectively) polarized mostly normally, although CEA was also sometimes found at the lateral surfaces (Fig. 4B4). The basolateral (Fig. 4C4) and junctional (Fig. 4D4; Fig. 2E) markers appeared normal. Since knockdown >75% could not be achieved, the observed phenotypic variation could be owing to varying amounts of syntaxin-3 that were still present in the cells.

Syntaxin-2, another member of the syntaxin family, is localized to both basolateral and apical domains in MDCK cells, and has been implicated in cell surface transport (Kauppi et al., 2002; Li et al., 2002; Low et al., 1996; Quinones et al., 1999). Although, analysis of syntaxin-2 KD cysts revealed no significant perturbation of single-lumen formation (Fig. 3, supplementary material Fig. S3), we discovered that, similar to syntaxin-3 KD, syntaxin-2 KD cells also formed often small lumina and cysts that were significantly smaller than control cysts (P<0.001).

VIP17 and/or MAL
This protein has been described in several studies to be implicated in apical transport and the regulation of apical endocytosis (Cheong et al., 1999; Frank, 2000; Puertollano et al., 1999; Zacchetti et al., 1995) (SwissProt no.: Q28296). The VIP17 KD cells formed large cysts with usually multiple and large lumina (Figs 3, 4). The lumina of VIP17 KD cysts often formed an uneven basal margin with characteristic bulging towards the collagen matrix (e.g. Fig. 4A5).

Some of the VIP17 KD cysts had abnormal localization (lateral, intracellular) of apical markers Pcx and CEA. In some cases the expression levels of Pcx and CEA were reduced (Fig. 4, panels A5 and B5, respectively). E-cadherin was not only localized basolaterally but was also found on the luminal surfaces (Fig. 4C5, arrowhead). ZO-1 staining in the VIP17 KD cells (Fig. 4D5) revealed seemingly uniform (Fig. 2F) TJ structures around the apical surfaces. However, unlike in other KDs, a pronounced punctate ZO-1 staining was observed, indicating an accumulation of intracellular ZO-1 in the vicinity of the membrane in the VIP17 KD cysts (Fig. 4D5, arrowheads; see also Fig. 2F). The actin meshwork in the VIP17 KD cells was concentrated underneath the luminal surfaces (Fig. 4, panels A5, B5, C5, D5).

Lumen size
Notably, not only the overall luminal morphology, but also the size of the cysts derived from the different KDs varied. The cysts formed from VIP17 KD and annexin-13 KD cells appeared generally larger than the control cysts (Figs 1, 4). However, syntaxin-3 KD cysts (Figs 1, 4) were significantly smaller than control (P<0.001) and also VIP17 KD cysts (P<0.001), even compared with Stx-3 KD cysts. This suggests a crucial role for syntaxin-3 in determining lumen size in cysts. Similarly, the smaller – compared with control cysts – single-lumen cysts (P<0.001) often observed in syntaxin-2 KD cells suggest a role for syntaxin-2 in determining lumen size. In addition to its function as a SNARE protein in vesicular fusion at the cell surface, syntaxin-2 has been implicated as a tissue morphogen (also known as epimorphin) (Hirai et al., 2007). The observed small size of cysts in the collagen culture might also reflect general inhibition of growth, because syntaxin-2 function has also been linked to cell division, specifically to cytokinesis (Low et al., 2003). However, although the depletion of galec-tin-3 has recently been shown to result in slowed down proliferation of the MDCK cells (Friedrichs et al., 2007), this did not limit the generation of normal sized lumens per se (Friedrichs et al., 2007) (this study), which we observed to be the consequence of suppressed expression of cell-surface and apical syntaxins in cysts.

Ciliogenesis
The final event in the polarization process of MDCK cells is the outgrowth of a primary cilium. This structure can be visualized by
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staining for acetylated tubulin, a component of the ciliary axoneme. In control cysts, primary cilia extended from the ciliary centrioles located in the center of the apical membrane and projected to the cyst lumen. The cilia were ≈50 μm in length (Fig. 5B).

We analyzed each KD phenotype with respect to cilia formation by comparing the cysts stained for acetylated α-tubulin through the z-stacks in the different KDs. Single sections of z-stacks show the ciliogenesis phenotypes in the different KDs (Fig. 5C1-6). The long cilia extending into the lumenal space in control cells (Fig. 5B) were used as a comparative criterion to judge the level of ciliogenesis in the different KDs (Fig. 5D). The defects in ciliogenesis in the different KD cysts were also visualized by 3D rendering of the confugal stacks by using the Velocity® software (Fig. 5A). It has previously been shown that depletion of crumbs-3 in epithelial cells results in defective ciliogenesis (Fan et al., 2004). We also observed that ciliogenesis was frequently perturbed in crumbs-3 KD cysts. The cilia were truncated and, instead, a subapical acetylated tubulin-positive network was often observed in these cells (Fig. 5C). More severe defects were found in the annexin-13 KD and syntaxin-3 KD cells (Fig. 5A,C), in which most
cysts showed no or severely truncated cilia. Defects in ciliogenesis (Fig. 5D) were also observed in galectin-3 and VIP17 KD cysts (Fig. 5A,C), whereas most of the caveolin-1 KD cysts developed elongated cilia (Fig. 5A,C).

Discussion
This and previous studies demonstrate that the 3D cultures of MDCK cells in collagen gels are useful models to study the formation of apical lumina. We have shown here that proteins implicated in apical transport and apical-membrane formation (annexin-13, crumbs-3, galectin-3, syntaxin-3, syntaxin-2 and VIP17 and/or MAL) gave rise to different characteristic phenotypes when the proteins are depleted using RNAi. We have shown previously that depletion of FAPP2, a phosphatidylinositol (4)-monophosphate-binding Golgi protein involved in apical transport led to the formation of cysts with a collapsed lumen (Vieira et al., 2006).

Depletion of most of the proteins selected for this study perturbed normal morphogenesis and lumen formation in collagen cysts. One of the main phenotypes was the formation of cysts with multiple lumina. This phenotype was observed in caveolin-1 KD, galectin-3 KD and VIP17 KD cells (Fig. 3). It should be noted that an intermediate stage containing multiple lumina was observed during normal cyst formation and at day 14 (endpoint of our assay), a fraction of control cysts <30% remained multifiluminal. However, since there was no indication that the phenotype was alleviated upon prolonged culture of KD cysts, it is unlikely that these phenotypes merely represented delayed cyst maturation. O'Brien et al. have suggested that such excessive lumen formation could be owing to a block in how the cells identify the free surface, which will become the luminal surface (O'Brien et al., 2001; O'Brien et al., 2002). This phenotype could be because of partial mislocalization of basal components, such as integrins and laminin, to the apical-luminal side of the polarizing cells, therefore introducing a basal cue that drives assembly of ECM to the interior side of the cysts (Ecay and Valentich, 1992; Ojakian and Schwimmer, 1994; Saelman et al., 1995; Yu et al., 2005). Caveolin-1 is known to regulate focal adhesions, and the trafficking and assembly of integrins, the main collagen receptors in MDCK cells (Cavalcanti-Adam et al., 2007; Echarri and Del Pozo, 2006; Gaus et al., 2006). Disrupted caveolin function might perturb the basolateral cue required for initiation of normal cystogenesis. Alternatively, caveolin-deficient cells might fail to internalize and thereby inactivate apically mislocalized integrin complexes leading to disrupted free surface cue. Also, galectin-3 has been implicated in the regulation of cell-ECM interactions. It has been proposed to regulate integrin function as well as the secretion and assembly of ECM components, such as laminin (Ochieng et al., 1998). Disruption of these functions could explain the abnormal polarization and morphogenesis seen in the galectin-3 KD cysts. Recent results employing quantitative single-cell force microscopy have shown that galectin-3 KD cells have altered adhesion to the laminin substratum (Friedrichs et al., 2007). It is important to note that, unlike collagen I employed in this study, the commonly used alternative matrix Matrigel (Martin-Belmonte et al., 2007; O'Brien et al., 2001; Roh et al., 2003; Yamanaka et al., 2006), provides a ready-made ECM including laminin (Kleinman and Martin, 2005; O'Brien et al., 2001). In collagen gels, this ECM has to be produced by the MDCK cells. These differences might influence the variety of phenotypes observed in 3D culture systems. In fact, the Gal-3 KD phenotype was not observed in Matrigel (our unpublished observations).

The most severe phenotypes with respect to lumen formation were observed in the annexin-13 KD and crumbs-3 KD cells (Figs 1, 4), whose 3D organization was impaired. Even in this case, as was found with the annexin-13 KD cells, Pcx still polarized where the actin meshwork was concentrated (Fig. 4A1). Pcx is a PDZ domain-containing scaffolding protein known to facilitate connection to the cortical cytoskeleton at the apical surface (Meder et al., 2005; Schneider et al., 2004). The sorting of Pcx to the apical surface being tightly linked to the actin cytoskeleton might stabilize its delivery and location at the luminal surface (Yu et al., 2007). Apparently, polarization and biogenesis of the apical membrane domain in the MDCK cells is so robustly hard-wired that parts of the program are executed despite serious disturbances in generating the correct epithelial architecture.

This robustness is even more obvious, when effects of the KDs on epithelial organization are assessed in 2D cultures on filter supports. After some delays in executing the programme, the KD cells appear to achieve mostly normal polarization – despite depletion of the proteins (our unpublished observations). Other investigators have reported similar results (Martin-Belmonte et al., 2007; Roh et al., 2003). One reason might be that the trafficking pathways to the polarized cell surface are redundant so that cargo can take another route to reach its destination when one route is blocked. In yeast this has been clearly demonstrated for the two pathways from the Golgi to the cell surface (Harsay and Schekman, 2002; Proszynski et al., 2005), and one can detect these blocks by the traffic jams that are caused by the rerouting of cargo from the blocked pathway to the intact route (Proszynski et al., 2005). There is evidence for more than one route both to the apical and the basolateral surface domains in MDCK cells (Jacob and Naim, 2001; Nelson and Rodriguez-Boulan, 2004). The genes encoding caveolin-1, galectin-3 and VIP17 and/or MAL have been knocked out in mice (Hsu et al., 2000; Le Lay and Kurzchalia, 2005; Schaeren-Wiemers et al., 2004). None of these knockouts show general epithelial polarization defects (Chiu et al., 2006; Colnot et al., 1998; Henderson et al., 2006; Iacobini et al., 2003; Winyard et al., 1997).

One additional interesting readout from our cyst experiments was the effect of the KDs on ciliogenesis. It has previously been shown that the depletion of the polarity protein crumbs-3 (Fan et al., 2004) leads to defective ciliogenesis. Here, we demonstrated dramatic inhibition of ciliary outgrowth in the annexin-13 KD and syntaxin-3 KD cells. Also, the other KDs affected ciliogenesis (Fig. 5). How these effects should be interpreted mechanistically is not clear. The ciliary membrane represents a domain of its own on the apical surface (Vieira et al., 2006). The cilia are formed during the last phase of polarization and cyst formation in the MDCK cells. Their biogenesis requires coordinated targeting, and the assembly of the ciliary base and centriole structures followed by axoneme and membrane outgrowth. Failures in this process perturb not only functions of single cells but also of the polarized tissue as a whole. Cilia are emerging as major mechanosensors and signaling centers and, therefore, have key roles in tissue function (Dawe et al., 2007; Reiter and Mostov, 2006; Simons and Walz, 2006; Wallingford, 2006).

Recent data suggest the existence of a barrier to the diffusion of membrane proteins into and out of the cilium at its base (Vieira et al., 2006). The protein composition of the ciliary plasma membrane is specialized for functions different from that of the adjacent apical membrane (Davis et al., 2006; Pazour, 2004). Some proteins are in both membrane domains, whereas others are unique to either one of them. This implies the existence of a transport route that delivers...
membrane carriers to the cilium with its own specific machinery. It appears unlikely that any of the proteins assessed here are be solely linked to the cilial transport machinery. Either the proteins have more than one function or their effects are indirect. Depletion of the proteins could inhibit earlier transport steps before the final membrane carrier destined for the cilial base is assembled. The addition of one more specific route to the cilium complicates the already complex traffic circuits connecting the apical, basolateral, and endosomal stations with the Golgi complex.

These studies could be readily extended into epithelial tubulogenesis (Montesano et al., 1991a; Pollack et al., 1998; Zegers et al., 2003). When MDCK cell cysts are treated with hepatocyte growth factor (HGF), they undergo transient epithelial-mesenchymal transition, whereby cords of cells outgrow from the cysts and develop into tubules. In the cord structures the cells need to re-polarize to form the tube lumen lined by the newly formed apical surface. The machinery for generating the apical surface that lines the tube will have need to be constructed such that it can be shut off and turned on again to function in normal organogenesis (Gumbiner, 1992; Montesano et al., 1991a; O’Brien et al., 2004). How this is achieved mechanistically remains a future challenge.

Materials and Methods

Generation of KDs using retrovirus-mediated RNAi

The methodology for the generation of stable knockdowns (KD’s) in polarized MDCK cells by retrovirus-mediated RNAi was performed as described in more detail previously (Schuck et al., 2004). In brief, MDCK cells were infected with retroviruses encoding short hairpin RNAs (shRNAs) designed for the depletion of the target genes (constructed in the vector RVH1-puro). The MDCK retroviral KD strains with stable genomic integration of the shRNA expression cassettes were selected in the presence of puromycin in the cell culture medium. The KD efficiency (the percentage reduction of the target gene mRNAs in comparison to the level in control cells) was determined by quantitative PCR. In addition to annexin-13, caveolin-1, syntaxin-3 and VIP17 and/or MAL KDs (Schuck et al., 2004), crumbs-3 and galectin-3 KDs were generated for this study. The design of the shRNAs used for the depletion of these genes in MDCK cells has been described elsewhere (Friedrichs et al., 2007; O’Brien et al., 2004). Target sequence 5′-GACATCATGAAGACGTAAG-3′ corresponding to the canine syntaxin-2 sequence was used for the syntaxin-2 KD enabling generation of the KD with an efficiency of 86±1.5%.

MDCK collagen-cyst cultures and immunoblotting of the total protein in cysts

Purified collagen-cyst cultures and KD MDCK cells were grown on plastic dishes and detached with trypsin when still subconfluent. Cells were resuspended in PBS and the cell number was determined using the Neubauer chamber. Collagen I solution was prepared by mixing 16 volumes collagen I (VitrogenTM), 2 volumes chilled 10× DMEM, 2 volumes 0.1 M NaOH, 1 volume 7.5% NaHCO3 and 1 volume 1 M HEPES pH 7.2 on ice. Fetal calf serum (FCS) was adjusted to 1% concentration. Cells were mixed with the collagen to obtain homogenous suspension of 2×10⁶ cells/ml and aliquots of the suspension was then pipetted onto 24-well plates. The plates were kept at 37°C to let the collagen solidify for 30 minutes, after which complete MEM culture medium containing 5% FCS was added into the wells. Cells were cultured at 37°C and the medium was replaced every other day. The differentiation into cysts was followed 10-14 days. Sometimes, to demonstrate the depletion of proteins by immunoblotting, cysts were at the end of the culture assay differentiation into cysts was followed 10-14 days. Sometimes, to demonstrate the presence of puromycin in the cell culture medium. The KD efficiency (the proportion of the total amount of a specific protein in a cell line) was determined by quantitative PCR. In addition to annexin-13, caveolin-1, syntaxin-3 and VIP17 and/or MAL KDs (Schuck et al., 2004), crumbs-3 and galectin-3 KDs were generated for this study. The design of the shRNAs used for the depletion of these genes in MDCK cells has been described elsewhere (Friedrichs et al., 2007; O’Brien et al., 2004). Target sequence 5′-GACATCATGAAGACGTAAG-3′ corresponding to the canine syntaxin-2 sequence was used for the syntaxin-2 KD enabling generation of the KD with an efficiency of 86±1.5%.

Antibodies, immunofluorescence analysis of cysts and statistical analysis

Mouse anti-podocalyxin (Pcx, gp135), carcinoembryonic antigen (CEA, gp114) and E-cadherin primary antibodies were developed in our lab or have been described elsewhere (Fullegk et al., 2006; Gumbiner and Simons, 1986; Meder et al., 2005). Rabbit caveolin-1 (N-20) and ZO-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology and Zymed, respectively. Mouse anti-acetylated β-tubulin antibody, TRITC-Phalloidin and DAPI were from Sigma and the rat galectin-3 (Mac-2) antibody from Cedarlane laboratories. At day 14, the cysts in the collagen I gel were rinsed 2×5 minutes in PBS, containing Mg²⁺ and Ca²⁺. Paraformaldehyde (4%) was added to fix the cells for 1 hour at room temperature (RT). Glycine (200 mM) in PBS was added for another hour to quench the excess aldehye. Cells in the cysts were permeabilized with 0.1% Triton X-100, and finally incubated for 1 hour in PBS-blocking solution supplemented with 0.2% fish skin gelatin and 0.2% BSA. The cysts were incubated overnight at RT with primary antibodies that were diluted 1:400 in the blocking solution. Excess amount of antibodies was washed off for 2 hours with PBS and by changing several times to fresh wash solution; thereafter, a 1:400 dilution of the AlexaFluor®488-conjugated secondary antibodies (Molecular Probes) in the blocking buffer was added to the cysts for incubation overnight. The next day, cysts were again washed with PBS. The blots of collagen gel containing the cysts were carefully removed from the culture wells with a spatula and mounted onto slides in Mowiol. Images with usually 0.2 mm z-stack sections (sometimes 0.4-mm or 0.6-mm sections were also acquired) were acquired using an Olympus Fluoview-1000 laser scanning confocal microscope and a 60× PLANPO OLSM oil objective (NA=1.10). The image sections shown of the apical and lumeneal surface(s) were from the middle of the cysts. For 3D rendering of the z-sets image stacks Velocity ⁸ (x64) software by Improvision was used. Cyst size was measured on-screen. The means and standard deviations of the largest diameter of cysts in the different samples were recorded from 50-100 images showing z-sections of cysts. Statistical significance for pairwise comparisons was determined by Student’s t-test similar to the analysis of statistical significance for cilogenesis and morphological phenotypes in the different cysts, whereas in the latter case in addition to the Student’s t-test, also ANOVA was used as an alternative method (cyst morphological classes).

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Epithelial cyst formation and ciliogenesis

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