Synaptotagmin-like proteins control the formation of a single apical membrane domain in epithelial cells

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The formation of epithelial tissues requires both the generation of apical–basal polarity and the coordination of this polarity between neighbouring cells to form a central lumen. During de novo lumen formation, vectorial membrane transport contributes to the formation of a singular apical membrane, resulting in the contribution of each cell to only a single lumen. Here, from a functional screen for genes required for three-dimensional epithelial architecture, we identify key roles for synaptotagmin-like proteins 2-a and 4-a (Slp2-a/4-a) in the generation of a single apical surface per cell. Slp2-a localizes to the luminal membrane in a PtdIns(4,5)P2-dependent manner, where it targets Rab27-loaded vesicles to initiate a single lumen. Vesicle tethering and fusion is controlled by Slp4-a, in conjunction with Rab27/Rab3/Rab8 and the SNARE syntaxin-3. Together, Slp2-a/4-a coordinate the spatiotemporal organization of vectorial apical transport to ensure that only a single apical surface, and thus the formation of a single lumen, occurs per cell.

Epithelia represent the most fundamental tissue in metazoa, forming complex layers of cells such as the skin or kidney tubules. The epithelial plasma membrane is divided into two domains: apical and basolateral, separated by cellular junctions, dependent on the asymmetric delivery and segregation of membrane proteins and lipids1,2. Such plasma membrane asymmetry allows the formation of a central lumen, and hence the evolution of specialized functions for different metazoan tissues3. Epithelial cells create lumens through an array of morphogenetic mechanisms. Despite this diversity, a series of common molecular events creates biological tubes: vectorial transport to a nascent apical domain, de novo apical plasma membrane biogenesis, and secretion and expansion of the luminal space4,5. Transport of apical proteins to the initial site for apical-membrane formation, at which the Par3–aPKC–Cdc42 polarity complex is established, is controlled by a Rab11a/8a GTPase cascade and its effectors, the exocyst and Myo5B (refs 6,7). At the lumen, phosphoinositide asymmetry is concomitantly established with PtdIns(4,5)P2 and PtdIns(3,4,5)P3, localizing to, and specifying, the apical and basolateral domains, respectively8,9. How vectorial exocytic transport is coordinated and directed so that each cell has a single apical initiation site, and thus the tube has a single lumen, however, is largely unclear. Similarly, how such machineries are controlled at the transcriptional level during morphogenesis of epithelial tissues is poorly understood1.

Here, we report a functional screen for regulators of three-dimensional (3D) epithelial polarity using MDCK cyst cultures, based on transcriptional, RNA-mediated interference (RNAi) and morphogenetic analysis.

Synaptotagmin-like proteins (Slps) 1-5 are a family of Rab effectors involved in regulated exocytosis10. Slps harbour an amino-terminal Rab-binding domain (also called the Slp homology domain SHD) and tandem carboxy-terminal C2 domains involved in Ca2+ and phospholipid binding, and function in tethering secretory vesicles to the plasma membrane11. In Drosophila melanogaster, a single divergent Slp paralogue, bitesize (Btsz), functions in epithelial polarization12, although whether mammalian Slps function in polarity generation is unknown.

We demonstrate that Slp2-a and related family member Slp4-a function in distinct, but complementary, steps of apical transport.
A screen for regulators of 3D epithelial polarization.

(a) Experimental design for the function screen of regulators of 3D epithelial polarity. MDCK cells were cultured for 36 h in 2D or 3D (n = 3), and control (2D) and experimental (3D) RNA samples were analysed using the Affymetrix Canine Genome 2.0 platform. The significance of the data was determined by LIMMA analysis (false discovery rate (FDR) < 0.05). A set of significantly upregulated (>2-fold) genes was pooled with other genes of interest and then gene overexpression was validated by RT-qPCR. Bioinformatic pathway analyses revealed that some genes were connected in common functional pathways. A final set of 47 candidates was selected for stealth siRNA design. MDCK cells were transfected with siRNAs individually or in pools and cultured to grow cysts. The silencing efficiency of the siRNA was determined by RTqPCR. Then, cells were fixed and stained for Podxl, β-catenin and nuclei to quantify normal lumen formation. The RNAi screening finally resulted in 16 positive hits (see Methods). (b) RNAi screening for polarization regulation. Lumen formation efficiency was quantified for each of the listed 47 siRNA treatments. Green dotted line, normal levels as found in control; red dotted line, the threshold considered for the definition of a positive hit (lumen formation < 75% of control; P < 0.05); green stars, positive hits; red dots, knockdowns where the efficiency was below 60%. *P < 0.05; n = 3; error bars represent s.d. (c) Examples of phenotypes induced by RNAi in the screen. Seventy-two hour MDCK cysts transfected with siRNA from four positive candidates (SMTNL2, CLDN2, RHOU and FUZ) and stained with the apical marker Podxl (red), the basolateral marker β-catenin (β-cat; green), the tight junction marker ZO-1 (white) and nuclei (blue). Scale bars, 5 μm. (d) Epithelial cancers with a downregulated 3D polarity gene set. The expression levels of the candidate gene set in all cancer versus normal expression data sets were analysed using Oncomine (www.oncomine.org). The graph shows the number of downregulated genes per type of indicated epithelial cancers (P < 0.05; n varies in each tissue). (e) Frequency of gene downregulation in breast and kidney cancer versus normal tissue microarray data sets (Oncomine, P < 0.05). The yellow arrowheads denote genes downregulated in both breast and kidney cancer.
to form a lumen de novo. Slp2-a controls the positioning of Slp4-a/Rab27-positive vesicles to target exocytosis to a single PtdIns(4,5)P₂-enriched lumen. Slp4-a regulates the tethering of these vesicles, through the association with the apical SNARE syntaxin-3 (Stx3), to mediate vesicle delivery to the lumen. Thus, through a functional, multi-step screen, we have identified a previously uncharacterized mechanism for coordinated vectorial transport, crucial to form a single apical domain.

RESULTS
Identification of 14 previously uncharacterized regulators of epithelial morphogenesis

We performed a multi-step, functional screen for regulators specifically of 3D epithelial architecture and morphogenesis. We first conducted a microarray-based differential expression analysis comparing the transcriptome of MDCK cells undergoing apical–basolateral polarization either in the traditional monolayer culture (2D), or as 3D cysts grown in basement membrane extract (3D), wherein MDCK cells self-assemble to form a 3D monolayer (Fig. 1a). Notable transcriptional differences were observed during 3D morphogenesis with 1,597 upregulated, and 1,304 downregulated probes detected (Supplementary Fig. S1). To prioritize functional analyses, upregulated genes were subjected to bioinformatic analysis to reconstruct potential molecular pathways and known components of epithelial polarization. Using this approach, a set of 99 upregulated genes was selected for secondary validation by quantitative PCR (qPCR; Supplementary Fig. S2). Next, we characterized Slp2-a localization in MDCK cysts. On plating MDCK into 3D, the apical podocalyxin (Podxl) localized into vesicles and delivered to the contact between two cells, where lumen is formed de novo⁷⁻⁸,¹⁸. In early aggregates, Slp2-a localized to the plasma membrane, enriched at cellular junctions (Fig. 2b and Table 1). A pool of Slp2-a became apparent on internalized Podxl-positive transcytosing vesicles near the cell–cell contact (Fig. 2b, 16–20 h). On lumen formation (24–48 h), Slp2-a localized to the apical membrane (Fig. 2b, 48 h).

To analyse a possible relevance of these genes in vivo, we analysed whether this gene set was downregulated in human cancers, suggesting an importance in the maintenance of a differentiated epithelial phenotype in vivo (Fig. 1d). Notably, renal, breast and skin cancers presented with the strongest downregulation of this gene set. We selected one of these genes, SYTL2 (encoding the protein Slp2-a), which was significantly downregulated in several epithelial cancer data sets (Fig. 1e), to characterize its role in lumen formation.

Slp2-a associates with, and regulates the formation of, the luminal membrane

The mammalian Slp family has been shown to regulate primarily Rab27-dependent membrane trafficking and secretion¹⁵⁻¹⁷, but the function of these proteins in mammalian epithelial morphogenesis is unknown. We confirmed that Slp2-a protein levels were upregulated in 3D when compared with 2D cultures by western blot (14-fold enrichment at 72 h; Fig. 2a), validating the qPCR data (Supplementary Fig. S2). Next, we characterized Slp2-a localization in MDCK cysts. To analyse a possible relevance of these genes in vivo, we analysed whether this gene set was downregulated in human cancers, suggesting an importance in the maintenance of a differentiated epithelial phenotype in vivo (Fig. 1d). Notably, renal, breast and skin cancers presented with the strongest downregulation of this gene set. We selected one of these genes, SYTL2 (encoding the protein Slp2-a), which was significantly downregulated in several epithelial cancer data sets (Fig. 1e), to characterize its role in lumen formation.

Table 1 Localization and function of synaptotagmin-like proteins, and their mutants, in MDCK cyst formation.

| Protein | Construct | Early aggregate | Cell localization | Open lumen | Mutant effect | Phenotype rescue |
|---------|-----------|-----------------|-------------------|-----------|--------------|-----------------|
| Slp2-a  | Full length | Unpolarized PM | Vesicles/junctions PM | Subapical/junctions PM | - | No Rab binding |
|         | SHD       | Vesicle/cytosol | Vesicle/gyotysol | Vesicle/cytosol | - | - |
|         | ΔC2AB     | Vesicle/endosome | Vesicle/endosome | Subapical | - | - |
|         | Linker    | Cytosol         | Cytosol          | Cytosol | - | - |
|         | ΔSHD      | Unpolarized PM  | Unpolarized PM   | Unpolarized PM | - | - |
|         | C2AB      | Unpolarized PM  | Unpolarized PM   | Unpolarized PM | - | - |
|         | mut E11A/R32A | Unpolarized PM | Unpolarized PM | No Rab binding | No | No |
|         | mut V18A  | Unpolarized PM  | Unpolarized PM   | No Rab binding | No | No |
| Slp4-a  | Full-length | Apical PM | Vesicles | Apical PM | Yes | Yes |
|         | SHD       | nuc/cytosol     | nuc/cytosol     | nuc/cytosol | - | - |
|         | ΔC2AB     | Cytosol         | Cytosol         | Cytosol | - | - |
|         | Linker    | Cytosol         | Cytosol         | Cytosol | - | - |
|         | ΔSHD      | Unpolarized PM  | Unpolarized PM   | Unpolarized PM | - | - |
|         | C2AB      | Unpolarized PM  | Unpolarized PM   | Unpolarized PM | - | - |
|         | mut V21A  | Vesicle         | Vesicles        | Subapical/junctions PM | No Rab3/8 binding | No |
|         | mut W118S | Vesicle         | Vesicles        | Subapical | No Rab binding | No |
|         | mut K - Q | Vesicles       | Vesicles        | Subapical/junctions PM | No Rab3/8 binding | No |
| Slp454a | Unpolarized PM | Subapical/junctions PM | No SNARE binding | No | - |
| Δ305–354 | Unpolarized PM | Subapical/junctions PM | No SNARE binding | No | - |

This table represents a qualitative summary of results regarding WT or mutant Slp protein localization (at different time points) and its ability to rescue the silencing of endogenous protein expression. PM, plasma membrane; nuc, nucleus; mut, mutant.
Figure 2 Slp2-a is required for epithelial morphogenesis. (a) Top, western blot (WB) showing the induction of Slp2-a in MDCK cells growing in 2D and 3D at different time points (24–72 h). Bottom, expression quantified by densitometry (n = 4). (b) Localisation of GFP-Slp2-a during lumen formation. MDCK cells stably expressing GFP-Slp2-a were grown in 3D and fixed at different time points. Cysts were stained with Podxl (red) and β-catenin (β-cat; blue). The arrows indicate localization to apical plasma membrane; the arrowheads indicate localization to cell–cell junctions; scale bar, 5 μm. (c) Downregulation of Slp2-a by siRNA. MDCK cells were transfected with a pool of siRNA to knockdown canine Slp2-a, and siRNA efficiency was analysed by western blotting. Cells were transfected with a pool of siRNA to knockdown Slp2-a or control siRNA and plated to form cysts for 72 h. Markers are Podxl (red), β-catenin (green) and nuclei (blue). The arrows indicate apical membrane localization; the arrowheads indicate localization to intracellular apical vesicles. Scale bars, 5 μm. (d) Quantification of cysts with normal lumens in cells transfected with control siRNA or Slp2-a siRNA. Values are mean ± s.d. (n = 5; ≥100 cysts per experiments). (e) Knockdown of Slp2-a by siRNA in cells stably expressing GFP-Slp2-a. MDCK cells stably expressing GFP-Slp2-a were transfected with Slp2-a or control siRNAs. Total lysates were blotted for Slp2-a using α-tubulin as a loading control. (f) Rescue effect of GFP-Slp2-a in cells silenced for Slp2-a on lumen formation. Cells were stained for Podxl (red) and β-catenin (blue). Scale bars, 10 μm. (h) Quantification of cysts with normal lumens in cells expressing GFP-Slp2-a and transfected with control or Slp2-a siRNAs, n = 3. (i) Slp2-a localization during lumen initiation. In early aggregates (12 h), Slp2-a localizes to cell–cell junctions at sites of apical vesicle fusion. After the lumen is initiated (24 h), Slp2-a remains polarized at the apical membrane. Green lines, Slp2-a; blue ovals, nuclei; black lines, basolateral membrane. In all panels error bars represent s.d.; *P < 0.05; **P < 0.005; L, lumen; areas outlined in micrographs are magnified in the associated images. Uncropped images of blots are shown in Supplementary Fig. S8. 

The SHD and C2 domains play non-redundant roles in targeting Slp2-a to membranes

Slp-family proteins share an N-terminal Rab27-binding domain (the SHD), a linker region and two C-terminal tandem C2 domains (phospholipid and/or protein interaction sites)20,21. Slp2-a could potentially therefore connect Rab GTPases and phosphoinositides during lumen formation7–9. To elucidate the control of Slp2-a localization, we analysed Slp2-a domains during cyst formation. In early aggregates, the C2 domains localized to the plasma membrane and to cell–cell junctions, but not to Podxl vesicles (Fig. 3a and Supplementary Fig. S4b, 12–20 h). Once lumens formed, the C2A/B fragment localized exclusively to the apical membrane (Fig. 3a and Supplementary Fig. S4b, 24–48 h). In contrast, the SHD fragment was predominantly cytoplasmic, and partially localized to Podxl vesicles in early aggregates and subapically in mature cysts (Fig. 3a, bottom panels). Deletion of the C2 domains (GFP-Slp2-aΔC2A/B) resulted in a similar localization to the SHD, whereas the linker region...
Figure 3 Slp2-a requires SHD and C2A/B domains for correct localization. (a) Localization of GFP–Slp2-a C2A/B, and SHD during lumen morphogenesis. MDCK cells stably expressing different GFP–Slp2-a constructs were grown in 3D to form cysts. Cysts were fixed at different time points (12, 20, 24 and 48 h) and co-stained to detect Podxl (red) and β-catenin (β-cat; blue). The arrowheads indicate apical membrane; the arrows indicate localization to cell-cell junctions. Scale bars, 5 μm. (b) Scheme of the Slp2-a constructs used. Different domains and truncated forms of Slp2-a were cloned for characterizing Slp2-a function. (c) PIP-binding ability of Slp2-a. GST-tagged full-length Slp2-a (GST–FL–Slp2-a) and ΔC2A/B (GST–Slp2-aΔC2A/B), which should be unable to bind phospholipids, were expressed and purified in bacteria. PIP-stripe membranes were incubated with 1 μg ml⁻¹ of GST (control), GST–FL–Slp2-a or GST–Slp2-aΔC2A/B and then membranes were blotted with anti-GST. A scheme of the PIP-stripe membrane is shown. The arrowheads indicate specific PIP₂ binding. The red lines highlight the PIP₂ species. LPA, lysophosphatidic acid. LPC, lysophosphatidylcholine. PI₁, phosphatidylinositol. PE, phosphatidylethanolamine. PC, phosphatidylcholine. S1P, sphingosine-1-phosphate. PA, phosphatidic acid. PS, phosphatidylserine. (d) Co-localization of Cherry–Slp2-a and PtdIns(4,5)P₂ during early cyst formation. MDCK cells stably expressing Cherry–Slp2-a were transfected with the PtdIns(4,5)P₂ probe (PHD–GFP) and grown in cysts. Cysts were fixed at different time points (12, 20, 36 h) and co-stained to detect Podxl (blue). The arrowheads indicate apical membranes; the arrows indicate cell-cell junction membrane localization. Scale bars, 5 μm. (e) Apical Slp2-a localization depends on PtdIns(4,5)P₂. Cysts expressing PHD–GFP (top panels) and Cherry–Slp2-a (bottom panels), were treated with ionomycin, which stimulates endogenous PLC activity to deplete membrane PtdIns(4,5)P₂, and were analysed by video-microscopy (0.1 s exposure every 1 s). Still images at different time points after ionomycin addition are presented. The arrowheads indicate apical membrane localization. Scale bars, 10 μm. (f) Schematic of Slp2-a association with the apical plasma membrane. Slp2-a C2A/B domains bind PIP₂ and localize Slp2-a to the lumen initiation site and the apical membrane. The SHD domain binds apical vesicles. For all panels, areas outlined in micrographs are magnified in the associated images. Uncropped images of blots are shown in Supplementary Fig. S8.

was cytoplasmic (Supplementary Fig. S4). These results suggest that whereas the SHD binds to apical vesicles, the C2 domains target Slp2-a to membranes.

Notably, the distribution of the C2 domains resembles PtdIns(4,5)P₂ localization during cyst formation. Furthermore, Slp2-a, and paralogues, bind selectively to PtdIns(4,5)P₂ (refs 12,17), although it could bind also to phosphatidylycerine. We found that C2 domains bind specifically to PIP₂ species, but not to phosphatidylycerine (Fig. 3c). Lact-C2–GFP, a probe for phosphatidylycerine, presents non-polarized membrane localization in cysts (Supplementary Fig. S4e). Given the established role of PtdIns(4,5)P₂ in apical membrane specification, and higher cellular abundance, we reasoned that PtdIns(4,5)P₂ may target Slp2-a to plasma membranes. During cyst formation, Slp2-a and PtdIns(4,5)P₂ co-localized at the plasma membrane, becoming progressively enriched to the lumen in morphogenesis (Fig. 3d). Both PtdIns(4,5)P₂ and Cherry–Slp2-a disappeared rapidly from the apical membranes on ionomycin treatment, which causes PIP depletion at the membrane (Fig. 3e and Supplementary Video S1). Taken together, these results confirm that Slp2-a requires the C2 domains for PtdIns(4,5)P₂ binding and apical membrane localization, whereas the SHD region targets Slp2-a to apically destined vesicles (Fig. 3f).

Slp2-a targets Rab27 vesicles to the lumen initiation site to form the lumen

Nearly all described functions of mammalian Slps required the SHD domain. In contrast, Btsz, the sole Slp parologue in Drosophila, does not require a Rab-binding domain for epithelial
Figure 4 Slp2-a binds Rab27 to form the apical membrane. (a) Knockdown of Slp2-a in cells stably expressing GFP–Slp2-aΔSHD or Btsz2–GFP (Btsz2) at 72 h after siRNA transfection. C, control; KD, knockdown; endog, endogenous; WB, western blot. (b) Quantification of cysts with normal lumens in cells expressing GFP–Slp2-aΔSHD or Btsz2–GFP and transfected with siRNA to Slp2-a or control (n = 3). (c) Rescue effect of GFP–Slp2-aΔSHD and Btsz2–GFP in cells knocked down for Slp2-a on lumen formation at 72 h post siRNA transfection. Podxl, red; β-catenin, blue. Note, Btsz2–GFP localization is not polarized on the plasma membrane of cysts. (d) Knockdown of Rab27a/b and Rab3b by siRNA. MDCK cells were transfected with different siRNA duplexes targeting canine Rab27a, Rab27b or Rab3b. After 72 h RNA extracts were quantified by RT-qPCR; n = 3. (e) Effect of Rab27a/b or Rab3b knockdown on cyst formation. Cysts were fixed 48 h after transfection. Silencing of Rab27a/b or Rab3b was sufficient to disrupt cyst formation and accumulate Podxl in vesicles (arrowheads). Podxl, red; β-catenin, green; nuclei, blue. (f) Quantification of cysts with normal lumens in cells transfected with siRNA targeting Rab27a, Rab27b, Rab27ab or Rab3b (n = 3). (g) Rab–GT-Pase interaction with Slp2-a mutants V18A and E11A/R32A. GST (control) or GST–Slp2-a (WT, V18A and E11A/R32A) beads were used to pulldown fluorescent protein-tagged Rab3b, Rab8a or Rab27a from total cell lysates. Bottom lane, Coomassie staining of an independent polyacrylamide gel loaded with GST–Slp2-a constructs and a representative input. (h) Quantification of normal cysts in cells expressing GFP–Slp2-a WT, V18A and E11A/R32A mutants transfected with siRNA against Slp2-a or control (n = 3). (i) Images of GFP–Slp2-a V18A and E11A/R32A cysts after Slp2-a knockdown. Cysts were fixed 48 h after transfection. Podxl, red; β-catenin, blue. The arrows indicate apical membrane. (j) Co-localization of Rab27 and Slp2-a during cyst morphogenesis. MDCK cells stably expressing Cherry (Ch)–Slp2-a and GFP–Rab27a were grown as cysts and fixed after 16, 20, or 24 h. Podxl, blue; β-catenin, white. (k) Effect of the downregulation of Slp2-a on Rab27a subapical localization; the arrows indicate co-localization of Podxl and Rab27a. In all panels values are means ± s.d. of n independent experiments; ∗P < 0.05; ∗∗P < 0.005; L, lumen; scale bars, 10 μm; areas outlined in micrographs are magnified in the associated images. Uncropped images of blots are shown in Supplementary Figs S8 and S9.

and an SHD-deleted Slp2-a mutant (GFP–Slp2-aΔSHD). Importantly, neither GFP–Slp2-aΔSHD nor Btsz2–GFP was able to rescue the defects caused by endogenous Slp2-a knockdown (Fig. 4a–c and morphogenesis33 (Supplementary Fig. S5b), suggesting Rab–Slp interactions may be dispensable for epithelial polarity. To address this possibility, we expressed the epithelial Btsz protein (Btsz2–GFP)
Supplementary Fig. S4b). These results reveal that the SHD region is required for epithelial morphogenesis in MDCK cysts.

To determine which Rab GTPase interactions are required for Slp2-a function, we analysed Rab that interact with other Slop (ref. 10). Slp2-a bound to Rab3b, Rab8a, Rab27a and to a lower extent Rab3a (Supplementary Fig. S5A). Although Rab8a/b are required for cyst formation7,18, only knockdown of both Rab27a/b strongly perturbed lumen formation (Fig. 4d–g), confirming partial isoform redundancy noted from knockout mice24. In contrast, although Rab3a-d isoforms are expressed in MDCK (data not shown), silencing of Rab3b was sufficient to disrupt cyst formation (Fig. 4d–g), suggesting Rab3b may have subtle non-redundant roles in apical transport25,26, and epithelial polarity. These results suggested that Slp2-a could mediate the targeting of apical vesicles loaded with Rab27a/b, Rab3b and/or Rab8a/b.

Next, we generated Slp2-a SHD mutants to disrupt the interaction with specific Rab based on the structure of the Slp2-a/Rab27 interaction20. The introduction of a V18A mutation in Slp2-a completely abolished the interaction with Rab3b and Rab8a, while preserving Rab27a binding; E11A/R32A mutations also disrupted the binding to Rab27 (Fig. 4g). Although both mutants retain apical localization (Fig. 4i and Table 1), GFP–Slp2-aV18A, but not GFP–Slp2-aE11A/R32A, completely rescued the Slp2-a knockdown phenotype (Fig. 4h and Supplementary Fig. S5f). Together, these data indicate that although Slp2-a can bind multiple Rabs, Rab27a/b is necessary and sufficient for Slp2-a function in lumen morphogenesis.

Next, we analysed Rab27b localization. Before lumen formation, GFP–Rab27a co-localized with Podxl and Slp2-a in vesicles transcytosing to the lumen (Fig. 4j, top and middle panels and Supplementary Fig. S5c). Once lumen initiation was completed, Rab27a localized to a subapical compartment, whereas Slp2-a localized apically (Fig. 4j, bottom panels and Supplementary Fig. S5c). Finally, Slp2-a knockdown caused the scattering of Rab27a vesicles close to the plasma membrane (Fig. 4k, bottom panels). Thus, Slp2-a is required to localize Rab27a. Taken together, these results indicate that Slp2-a binds to Rab27a-loaded apical vesicles and targets them to initiate the lumen.

**Slp4-a also functions in lumens biogenesis**

In addition to Slp2-a, mammalian cells express four other Slp-family proteins (Slp1-5), and four closely related Slac2s (Slp homologue lacking C2 domains)10. To determine whether other Slop proteins function in epithelial polarization, we analysed their expression during lumen formation. Notably, whereas Slp2-a was the sole Slp upregulated in 3D at early times (Fig. 5a, 3D-14 h), Slp1 and Slp4-a were upregulated at later times (Fig. 5a, 3D-36 h).

In contrast to Slp1, Slp4-a silenced cysts presented acute defects with the formation of multiple lumens and internal vesicles (Fig. 5b,c). In addition, Slp4-a knockdown did not affect polarity or ciliogenesis in monolayers (Supplementary Fig. S3a,b). Moreover, we observed that Slp2-a is specifically induced before Slp4-a in 3D cultures (Fig. 5d), suggesting that Slp2-a is required earlier than Slp4-a in lumenogenesis.

We next examined the localization of Slp4-a. Endogenous Slp4a/b and GFP–Slp4-a associated with apical membranes at all stages of polarization (Fig. 5e and Supplementary Fig. S6d), thus presenting a different localization pattern from Slp2-a (Fig. 3 and Table 1). Next, we examined Slp4-a domains in cysts. The C2 domains (C2A/B), either alone or in tandem, localized to apical and basolateral plasma membranes (Fig. 5f, left panels, Supplementary Fig. S6e and Table 1), suggesting that they confer nonspecific plasma membrane localization.

In support of this, GST–Slp4-a C2 domains bound promiscuously to PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (Fig. 5g,h and Supplementary Fig. S6c). In contrast, the C2A domain of Slp2-a bound specifically to PtdIns(4,5)P2, and localized apically (Supplementary Fig. S4d), suggesting that only Slp2-a is able to tether Rab27 vesicles to apical PtdIns(4,5)P2-enriched plasma membrane. Together, these data highlight a requisite of the SHD-linker region for apical targeting of Slp4-a, representing a major difference from Slp2-a, and suggesting non-redundant roles for Slp2-a and Slp4-a in lumen formation.

**Slp4-a apical localization and function depend on Rab and syntaxin interaction**

Slp4-a functions in docking of secretory granules with the plasma membrane, a function modulated by the SHD region, which interacts with Rab3, Rab8 and Rab27 family members10. Therefore, we examined the contribution of Rab binding to Slp4-a function.

To elucidate the role of Rab binding, we examined the ability of SHD mutations to bind to Rab27/3/8 and to rescue the phenotype of endogenous Slp4-a knockdown (Fig. 6a,b)27. In contrast to apical wild-type (WT)-Slp4-a, removal of the Rab-interacting region of Slp4-aSHD resulted in both cytoplasmic and non-polarized membrane localization of Slp4-a (Fig. 6c, Supplementary Fig. S6e and Table 1), as for Slp4-aW118R (no Rab binding) expression (Fig. 6a–c, Supplementary Fig. S6f and Table 1). Uncoupling of Slp4-a from Rab3b (V21A) resulted in subapical localization (Fig. 6c and Supplementary Fig. S6f). In contrast, co-uncoupling of Rab8 and Rab3b (I18A) resulted in the targeting of a pool of Slp4-a to the basolateral membrane, in addition to subapical vesicles (Fig. 6a–c, Supplementary Fig. S6f and Table 1). Whereas WT-Slp4-a was able to rescue endogenous Slp4-a knockdown, none of the Rab-binding mutants was able to restore lumen formation (Fig. 6b,c and Table 1). These results indicate that Rab27, Rab8 and Rab3 binding are required for Slp4-a localization and function. The different localization of the mutants also suggests that Rab27 is required for Slp4-a targeting to vesicles, Rab8 would be necessary to exclude Slp4-a from the basolateral membrane, and Rab3 may be important for subsequent Slp4-a transport to the lumen initiation membrane.

Next we analysed the role of phospholipid binding for Slp4-a, using a phospholipid-uncoupled mutant (K > Q; ref. 16). Slp4-aK0-Q localized to subapical puncta of apical membranes (Supplementary Fig. S6g and Table 1), and conferred a partial rescue to morphogenesis (Fig. 6b), suggesting that although the C2 domains can confer membrane localization, interaction of Slp4-a with other factors (Rabs and SNARE complexes) may partially compensate for the lack of C2 domain function.

Slp4, in contrast to other Slops, binds to SNARE proteins by its linker domain28,29. To analyse SNARE binding, we generated a chimera construct of Slp4-a bearing a Slp5 linker domain (Slp4-S54) that was unable to bind to Stx3 (Fig. 6d). Slp4-S54 showed a subapical and basolateral localization, and failed to rescue lumen formation on endogenous Slp4-a knockdown (Fig. 6e,f and Supplementary Fig. S6f and Table 1), suggesting an important role for Stx3 binding to Slp4-a. Next, we mapped the Stx3-binding domain of Slp4-a, and
Figure 5 Slp4-a is required for epithelial morphogenesis. (a) Analysis of Slps and Slac2s expression in 2D versus 3D. Slp1, Slp2-a, Slp4-a, Slp5, Slac2A and Slac2B expression was evaluated at different time points (14 and 36 h) by RT-qPCR in MDCK cells grown in 2D and 3D. Slp3, Slac2C and the related Rab27 effectors Noc2 and rabphilin3 were not expressed in MDCK (n = 4). (b) Effect of Slp4-a silencing on lumen formation. Cells stably expressing Slp4, Slp1 or scramble shRNA were plated to form cysts for 72 h. Cells were stained to detect Podxl (red), β-catenin (β-cat; green) and nuclei (blue). The arrowheads indicate intracellular Podxl vesicles. (c) Quantification of cysts with normal lumens in cells expressing scramble, Slp1 or Slp4-a shRNA (n = 3). (d) Quantification of Slp2-a and Slp4-a mRNA in cells grown on filters or in Matrigel. MDCK cells were grown on filters to confluence (2D) or in Matrigel (3D). mRNA expression was evaluated at different times by RT-qPCR. Data were normalized to 2D levels at 14 h post-plating. Left panel, Slp2-a (blue lines) and Slp4-a (green lines) mRNA expression patterns in 2D (solid lines) or 3D (dashed lines). Right panel, mRNA expression as the 3D/2D coefficient at different time points (n = 3). (e) Localization of GFP–Slp4-a in stably expressing cells during lumen formation. Cysts were stained for Podxl (red) and β-catenin (blue). The arrowheads indicate Slp4-a co-localization with Podxl (red) and β-catenin (blue). The arrows indicate the basolateral plasma membrane. (f) Phospholipid-binding ability of Slp2-a and Slp4-a C2 domains. Purified GST-tagged Slp2-a and Slp4-a C2A and C2B domains were incubated with beads covered with phosphoinositides or phosphatidylserine (PS). In all panels values are means ± s.d. of n independent experiments; *P < 0.05; **P < 0.005; L, lumen; scale bars, 10 μm; areas outlined in micrographs are magnified in the associated images. Uncropped images of blots are shown in Supplementary Fig. S9.
Figure 6 Slp4-a binding to the plasma membrane, Rabs and Stx3 is required for apical membrane formation. (a) Rab-GTPase binding to Slp4-a mutants. Purified GST-tagged Slp4-a (WT, 118A, V21A, W118S and ΔSHD) or GST (control) proteins were used to pulldown fluorescent-protein-tagged Rab3b, Rab8a or Rab27a from total cell lysates. Membranes were blotted with anti-GFP or anti-Rab8a. Bottom lane, Coomassie staining of an independent polycrylamide gel loaded with different GST–Slp4-a constructs and inputs. WB, western blot. (b) Quantification of cysts with normal lumens in GFP–Slp4-a (WT, ΔSHD, 118A, V21A, W118S and K > Q) cells expressing scramble or Slp4-a shRNA (n = 3). (c) Rescue effect of GFP–Slp4-a WT, Rab-binding defective mutants or membrane-binding defective Slp4-a shRNA. Total lysates were blotted for Stx3 and GAPDH (loading control). (d) Co-immunoprecipitation assay of Slp4-a binding to Stx3, T7–Slp4 or T7–Slp454 beads were incubated with FLAG–Munc18-2 and FLAG–Stx3 lysates. FLAG-tagged proteins were detected with HRP-conjugated anti-FLAG. Input 1:10 of immunoprecipitate (IP) volume. (e) Quantification of cysts with normal lumens in the absence of the Stx3–Slp4 interaction using cells expressing GFP–Slp454 or GFP–Slp4-aΔ305–354 and knocked down for Slp4-a (n = 3). (f) Localization of GFP–Slp454 in Slp4-a silenced cells. Podxl, red; β-catenin, blue. Arrowheads show vesicular localization; arrows show basolateral membrane. (g) Localization of Cherry–Stx3 and GFP–Slp4-a during cyst development. Cherry–Stx3 co-localized with GFP–Slp4-a at the periphery of early aggregates (arrowheads), intracellular vesicles and cell–cell contacts. As lumens formed, Cherry–Stx3 concentrated at the nascent luminal membrane with GFP–Slp4-a (arrowheads). Nuclei (blue). (h) Intracellular localization of GFP–Stx3 (arrows) in Slp4-a knockdown cysts (72 h). β-catenin, red; nuclei, blue. (i) GFP–Slp4-a localization in 48 h cysts knocked down for Stx3. Podxl (red), β catenin (green) and nuclei (blue). Note the Slp4-a basolateral mis-localization in the Stx3 knockdown cysts (yellow arrows). The arrowheads indicate Podxl vesicles. (j) Downregulation of Slx3 in MDCK cells stably expressing Slp4-a shRNA. Total lysates were blotted for Stx3 and GAPDH (loading control). (k) Quantification of the effect of Stx3 silencing in cyst formation (n = 3). In all panels values are means ± s.d of n independent experiments; *P < 0.05; **P < 0.005; scale bars, 5 μm; areas outlined in micrographs are magnified in the associated images. Uncropped images of blots are shown in Supplementary Fig. S10.

identified amino acids 305–354 to be essential for Stx3 binding (Supplementary Fig. S6h). Consistently, expression of a construct lacking amino acids 305–354 of Slp4-a (GFP–Slp4-aΔ305–354) also failed to localize to the apical plasma membrane and to rescue lumen formation on endogenous Slp4-a knockdown (Fig. 6e and Supplementary Fig. S6j and Table 1).

Stx3 functions as a critical apical SNARE. In cysts, GFP–Stx3 co-localized with Slp4-a at the nascent luminal membrane (Fig. 6g). Notably, Stx3 knockdown resulted in disruption of lumen formation and the redistribution of a pool of GFP–Slp4-a to the basolateral membrane (Supplementary Fig. S6i–k), and a similar localization observed on the removal of the Stx3-binding region of Slp4-a (Supplementary Fig. S6j).
Our data thus far indicate that Slp2-a and Slp4-a function in surface per cell accumulation and initiation of de novo vesicle formation. Cells knocked down for Slp2-a/Slp4-a and GFPRab27 in 24 h cysts. Slp4-a or Rab27a co-localized with Slp2-a membrane. (arrowheads indicate Slp2-a/Slp4-a co-localization at the nascent luminal junctions is unaffected. Note the accumulation of Podxl (red) in vesicles (arrows). The arrowheads indicate scattered Podxl vesicles. (c) Effect of Slp4-a knockdown on Slp2-a localization. After Slp4-a knockdown, Slp2-a localization at cellular junctions is unaffected. Note the accumulation of Podxl (red) in vesicles (arrowheads). (d) Effect of Slp2-a overexpression on GFP-Slp4-a and GFP-Rab27 in 24 h cysts. Slp4-a or Rab27a co-localized with Slp2-a and β-catenin (blue) at cellular junctions (arrows). (e) Effect of double Slp2-a/Slp4-a knockdown on lumen formation. Cells knocked down for Slp4-a, Slp2-a or Slp4-a/Slp2-a for 48 h were fixed and stained for nuclei (blue), Podxl (red) and β-catenin (green). The arrowheads indicate apical plasma membranes. (f) Quantification of cysts with normal lumens in control, Slp2-a knockdown, Slp4-a knockdown or Slp2-a/Slp4-a double knockdown (n = 3). (g) Quantification of cysts presenting two or more apical surfaces per cell in control, Slp2-a knockdown, Slp4-a knockdown or Slp2-a/Slp4-a double knockdowns (n = 3). In all panels values are mean ± s.d. from n independent experiments; NS, not significant; **P < 0.005; scale bars, 5 μm; areas outlined in micrographs are magnified in the associated images. (h) Model of Slp2-a/Slp4-a function in epithelial polarization. Top panel, Slp2-a targets Rab27-positive endosomes to the PIP2-enriched membrane. Slp4-a binds to Rab3 and Stx3 to be delivered to the lumen initiation site in Rab27-positive vesicles. As Slp4-a is delivered in Rab27-positive vesicles, its targeting depends on Slp2-a function. Therefore, Slp2-a directs localization of the Slp4-a/Stx3-influenced vesicle tethering activity to the single PIP2-enriched membrane, resulting in multiple apical domains in the same cell.

In contrast, Slp4-a knockdown did not disrupt the apical localization of Stx3 (Fig. 6h). These data suggest that Stx3 association with Slp4-a, by interaction with the linker domain, directs recruitment of Slp4-a to apically destined vesicles, and initiation of de novo lumen formation.

**Slp2-a regulates Slp4-a function to produce a single apical surface per cell**

Our data thus far indicate that Slp2-a and Slp4-a function in distinct, non-redundant steps in Rab-dependent transport to form the lumen. We thus examined their localization during lumen formation. In early aggregates, Slp2-a localized mainly to cell–cell junctions, whereas Slp4-a co-localized with Podxl at the cell–extracellular matrix interface (Fig. 7a, 16 h, top panels and Supplementary Fig. S7c). As internalized Podxl transcytosed to the cell–cell contact, Slp4-a co-localized to these vesicles, whereas Slp2-a was mainly at the cell–cell junctions (Fig. 7a, 20 h and Supplementary Fig. S7c). Finally, Slp2-a/Slp4-a co-localization was evident once lumens formed (Fig. 7a, 24 h and Supplementary Fig. S7c).

Figure 7 Slp2-a regulates Slp4-a targeting to determine single apical membrane formation. (a) Slp2-a and Slp4-a localization during lumen initiation. Cysts stably expressing GFP–Slp4-a and Cherry–Slp2-a were fixed after 16, 20 and 24 h. Podxl (blue, bottom panels) and β-catenin (β-cat; blue, top panels). The arrows indicate vesicular Slp4-a (black) and Slp2-a (blue, top panels). The arrowheads indicate Slp2-a/Slp4-a co-localization at the nascent luminal membrane. (b) Effect of Slp2-a knockdown (KD) on Slp4-a localization. Slp4-a localization becomes basolateral after Slp2-a knockdown and co-localizes partially with Podxl (red) in vesicles (arrows). The arrowheads indicate scattered Podxl vesicles. (c) Effect of Slp4-a knockdown on Slp2-a localization. After Slp4-a knockdown, Slp2-a localization at cellular junctions is unaffected. Note the accumulation of Podxl (red) in vesicles (arrowheads). (d) Effect of Slp2-a overexpression on GFP–Slp4-a and GFP–Rab27 in 24 h cysts. Slp4-a or Rab27a co-localized with Slp2-a and β-catenin (blue) at cellular junctions (arrows). (e) Effect of double Slp2-a/Slp4-a knockdown on lumen formation. Cells knocked down for Slp4-a, Slp2-a or Slp4-a/Slp2-a for 48 h were fixed and stained for nuclei (blue), Podxl (red) and β-catenin (green). The arrowheads indicate apical plasma membranes. (f) Quantification of cysts with normal lumens in control, Slp2-a knockdown, Slp4-a knockdown or Slp2-a/Slp4-a double knockdown (n = 3). (g) Quantification of cysts presenting two or more apical surfaces per cell in control, Slp2-a knockdown, Slp4-a knockdown or Slp2-a/Slp4-a double knockdowns (n = 3). In all panels values are mean ± s.d. from n independent experiments; NS, not significant; **P < 0.005; scale bars, 5 μm; areas outlined in micrographs are magnified in the associated images. (h) Model of Slp2-a/Slp4-a function in epithelial polarization. Top panel, Slp2-a targets Rab27-positive endosomes to the PIP2-enriched membrane. Slp4-a binds to Rab3 and Stx3 to be delivered to the lumen initiation site in Rab27-positive vesicles. As Slp4-a is delivered in Rab27-positive vesicles, its targeting depends on Slp2-a function. Therefore, Slp2-a directs localization of the Slp4-a/Stx3-influenced vesicle tethering activity to the single PIP2-enriched membrane, resulting in multiple apical domains in the same cell.

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Next we examined whether their activities were mutually dependent. Knockdown of Slp2-a caused the scattered distribution of small Podxl vesicles and the redistribution of GFP–Slp4-a to the lateral plasma membrane, partially co-localizing with Podxl vesicles (Fig. 7b, arrows). In contrast, Slp4-a knockdown cysts presented clusters of Podxl vesicles (Fig. 7c), but GFP–Slp2-a localized normally to cell–cell contacts, suggesting that Slp2-a localization and function are independent, or upstream, of Slp4-a. Furthermore, overexpression of GFP–Slp2-a forced endogenous Rab27a and Slp4-a mis-recruitment to cell–cell contacts at early time points (Fig. 7d), whereas the converse effect of GFP–Rab27a on Slp2-a was not observed (not shown). These results suggest that Slp2-a functions to regulate the positioning of Rab27 vesicles upstream of Slp4-a-mediated vesicle docking, thus controlling the position of the apical membrane and subsequent lumen.

To test this hypothesis, we silenced Slp2-a and Slp4-a alone or together (Fig. 7e–g). Strikingly, we observed that whereas Slp4-a knockdown induced accumulation of Podxl vesicles close to the membrane (Fig. 7e, middle panels), Slp2-a knockdown cysts possessed some cells simultaneously developing more than one apical membrane (Fig. 7e, top panels, quantification Fig. 7g). Moreover, although dual knockdown cysts presented a mixture of both phenotypes (Fig. 7e, bottom panels), they perturbed single lumen formation to a level resembling that of Podxl knockdown induced accumulation of Podxl vesicles close to the membrane (Fig. 7e, top panels, quantification Fig. 7g). These data support our model that Slp2-a and Slp4-a function in a spatiotemporal cascade to control vectorial apical transport (Fig. 7h), a fact supported by their sequential transcriptional upregulation during cyst formation.

An interesting question is how Slp2-a/4-a may coordinate this vectorial transport to form a single lumen. In non-polarized cells, both Slp2-a and Slp4-a are considered as negative regulators of secretion, on the basis of the fact that their overexpression attenuates secretory granule release\(^ {15-17,27,32}\). Indeed, Slp4-a can interact with the closed (non-fusion-forming) conformation of SNARE complexes\(^ {29}\). To this end, transient overexpression of Slp2-a or Slp4-a in the presence of endogenous protein consistently reduced single-lumen-formation rates (Supplementary Fig. S7A,B), a trend that could be strongly reversed by expression of SHD-deleted Slp2-a/4-a, suggesting that they may act as negative regulators of vesicle trafficking. However, Slp2-a/4-a are also clearly required for single lumen formation, thus suggesting a scenario where, rather than being considered as negative or positive regulators of exocytosis, Slp2-a/4-a act as molecular traffic wardens\(^ {32}\), controlling vectorial exocytosis through ensuring vesicles dock and fuse only at singular membrane domains to form a single, coordinated luminal space between neighbouring cells.

We identified, for the first time, a transcriptionally regulated molecular pathway that controls the formation of a single apical surface per cell, addressing a major, long-term unanswered question in cell biology. The study of the transcriptional machinery responsible for lumenogenesis in vivo presents a major future challenge to both cell and developmental biology.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

M.G.-S., A.E.R.-F., D.M.B., S.V. and F.M-B. designed the experiments. M.G.-S., A.E.R.-F., D.M.B., S.V., T.S., I.B.R., I.B.D., N.S., K.Y. and C.I.S. did the experimental work. M.G.-S., A.E.R.-F., D.M.B., K.E.M. and F.M-B. analysed the experiments. P.R.B. and M.F. provided reagents. F.M-B., D.M.B. M.G.-S. and A.E.R.-F. wrote the manuscript.
COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Two-step functional screening (microarrays, RT-qPCR, RNAi, Oncomine).

A microarray-based differential expression analysis was conducted using the Affymetrix Canine Genome 2.0 platform. MDCK type II cells were grown in 1% growth media and a total RNA was isolated at day 36 and purified using Trizol and 3 μg of RNA was used for microarray analysis. To disrupt C2 domain (J. Hammer, NIH, USA). For bacterial expression of GST-tagged full-length and mutant proteins, Slp2-a and Slp4-a were cloned into pGEX-4T1 vector (Promega) and used bioinformatic/bibliographic searches to analyse all 1,597 upregulated genes. The resulting list was used to select a maximum of 100 upregulated genes for RT-qPCR validation. To further examine this list, we used bioinformatic/bibliographic searches to analyse all 1,597 upregulated genes for previously published references into function, and Gene Ontology terms from human or mouse orthologues in NCBI databases. We selected genes with Gene Ontology terms, or references into function related to processes or mechanisms involved in changes in cell signalling, cell architecture and organ morphogenesis.

The list included a comprehensive list of Gene Ontology terms related to cell polarity, membrane trafficking, cell-to-cell junction assembly and remodelling, cell cycle regulation, cytoskeleton regulation and cell division, among others (complete list on demand). The second selection approach used STRING software (http://string-db.org/) to select genes interacting with pathways previously known to have a role in epithelial architecture or morphogenesis. From the resulting list, we selected 99 genes on the basis of bibliographic research and designed specific primers to perform qPCR analysis validation of their overexpression pattern in 3D cell cyst formation.

After qPCR validation, a stealth siRNA library was custom designed to target 47 validated candidate genes (Invitrogen). To perform the siRNA screening, MDCK cells were transfected with siRNA using Nucleofector-II (Lonza). Transfected cells were cultured for two days in 3D conditions and RNA extracts were analysed by RT-qPCR to check the silencing efficiency (Supplementary Table S2). Gene expression silencing was verified by RT-qPCR procedures (SYBR RT-qPCR premix, Applied Biosystems), and normalizing to GAPDH or HPRT expression. For functional analyses, transfected MDCK cells were grown for three days in Matrigel to form cysts, and lumen formation efficiency was quantified by confocal microscopy using the following markers to assess lumen formation: localization of the apical protein Podxl, integrity of the actin cytoskeleton (F-actin: phalloidin), adherens junctions (β-catenin), tight junctions (ZO-1), nuclei (DNA; DAPI).

Antibodies. Antibodies against α-tubulin (1:5,000; T9026, Sigma-Aldrich), Rab27a (1:200; R6655, Sigma-Aldrich), GFP (1:500; a5455, Invitrogen), Rab8a (1:1,000, 610845, BD Biosciences), mEPF/Cherry (1:230; PM005, MBL), Stx3 (1:200; Ab4113, Abcam), GST (1:5,000; sc138), β-catenin (1:1,000; sc7199) and Slp4-a (1:100; 34448) from Santa Cruz Biotechnology were commercial primary antibodies. The Slp-2a antibody was raised as a polyclonal serum against the Slp-2a C-terminal region and used as previously described64. Podxl antibody was a gift from the Okajian laboratory (State University of New York Downstate Medical Center, USA). ZO-1 (1:500; R4076) was from DSHB. Peroxidase-conjugated donkey anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies for western blots (Jackson Immunoresearch Laboratories). Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 405, 488, 555 or 647; Invitrogen) and TOPRO-3 or DAPI (for nuclear/DNA staining) were used in the microscopy protocols.

Plasmids. Slp2-a and Slp4-a (full length and mutants) were cloned into either pEGFP-C1/C2 or pmCherry-C1 vector (Takara Bio). The human Slp-4a complementary DNA template was from Open Biosystems (Thermo Fisher). Plasmids provided were: pEGFP-Slp1 (f. Peranen, University of Helsinki, Finland), pEGFP-STX3a (M. ter Beest, University of Chicago, Illinois, USA), pENTR-Rab3a/b/c/d (B. Goud, Institut Curie, Paris, France). GFP-Rab8a (1:1,000, sc7199) and Slp4-a (1:100; 34448) were used in Stx3 binding experiments as previously described21. GFP-Rab8a, Slp4-a and Slp4a54a constructs22 into a GFP–Slp4-a28 plasmid. T7-tagged Slp4-a, Slp454a and Slp4-a linkers were used for in vitro StxB binding experiments as previously reported22. The Slp4-a construct lacking the StxB-3’-interacting amino acids of the linker domain (A305–354) was cloned into pEGFP-C1. All constructs were verified by sequencing.

Cells and 3D culture. T23-MDCKII and MDCKII cells were grown as previously described25. MDCK cells stably expressing GFP-Slp-2a (full length and mutants), Cherry–Slp2-a, GFP–Rab27a, GFP–Rab8a and PLCδ3–PH–GFP (PHD–GFP) were made by co-transfection with the blasticidin-resistant gene and selected for ten days with 0.3 μg/ml blasticidin. MDCK cells stably expressing GFP–Slp1, GFP–Stx3 or GFP–Slp4-a (full length and mutants) were selected for ten days using G418 (0.5 mg/ml). Cells and Transwell cultures were prepared as described before33.

Microscopy. Immunofluorescence microscopy of cysts was performed as previously described34. Per condition, >100 cysts per experiment were analysed. For early time points, cysts were grown up to 24 h and two/three-cell stage cysts were classified on the basis of Podxl and β-catenin localization either as formed preapical-patch or presence of internal vesicles.

RNAi. The RNAi sequences and qPCR primers are listed in Supplementary Table S3. Briefly, 25 nucleotide stealth siRNA duplexes targeting messenger RNA sequences of canine Slp-2a were purchased from Invitrogen. Sequences were submitted to BLAST search to ensure targeting specificity. For siRNA transfection, MDCK cells were trypsinised and then nucleofected (Lonza) with siRNA duplexes or scrambled siRNA. After 24-h incubation, cells were resuspended and plated in 12-well plates and in coverglass chambers coated with Matrigel to grow cysts. Total cell lysates from 3D cultures were analysed by western blotting or RT-qPCR to confirm the siRNA efficiency.

Stable RNAi was achieved by viral short hairpin RNA (shRNA), essentially as previously described26. In all instances, knockdown was verified by western blot or RT-qPCR procedures (Brilliant-II SYBR Green Kit, Agilent), and normalizing to GAPDH expression. RNAi and RT-qPCR primers are presented in Supplementary Table S3. shRNA lentiviruses were constructed in pLKO.1-puro according to the Addgene pLKO.1 protocol (www.addgene.org) using iRNAi (www.mekentosj.com), and target sequences were based on an (AA)19 algorithm. RNAi sequences were submitted to BLAST (NCBI) to verify target specificity, with SYTL4 sequences targeting common regions for Slp4-a and Slp4-b transcripts. GFP-tagged human Slp4-a, which is not targeted by anti-canine shRNA, was used for Slp4-a knockdown and rescue experiments.

Virus production and transduction. Lentivirus production was performed essentially as previously described27. For lentivirus transduction, subconfluent MDCK cultures, 1–4 h after plating, were infected with virus-containing supernatants for 12–16 h at 37°C. Viral supernatants were then diluted 1:1 with growth medium, and cultured for a further 48 h. Transduced cells were selected by passage into appropriate antibiotic-containing medium. Puromycin (3 μg/ml) and blasticidin (12.5 μg/ml) were used.

Statistics. Single lumen formation was quantified as previously described28. The percentage of cysts with a single lumen was determined, and normalized to control cysts as 100%. Values are mean ± s.d. from ≥3 replicate experiments, with n ≥ 100 cysts per replicate. For RT-qPCR experiments, the percentage of remaining mRNA in each knockdown condition was normalized to the HPRT level, and represented as a percentage of the control (scramble shRNA) mRNA levels. The significance was calculated using a paired, two-tailed Student’s t-test. *p < 0.05, **p < 0.01.

Rab–GTPase pulldown. Rab GTPases–Slp protein pulldowns were performed using HEK293T cells overexpressing GFP-tagged Rab proteins, and GST-tagged Slp proteins. HEK293T cells expressing GFP-tagged Rab proteins were lysed in 0.1% SDS, 1% Triton X-100, 0.5 mM dihydroethanol and 1× TBS buffer with a protease inhibitor cocktail and sodium orthovanadate. Cell debris and nuclei were removed by centrifugation at 14,000g for 2 min at 4°C, and lysates were precleared and incubated in rotation with 100 ng of the relevant GST protein–loaded beads (GE Amersham) for 30 min, using GST alone as the control, in the presence of a non-hydrolysable GTP analogue (Sigma). Beads were centrifuged and washed five times, dried using aspiration, and resuspended in 10 μl Laemmli buffer before western blot analysis.

Co-immunoprecipitation assays. Co-immunoprecipitation assays in COS-7 cells were performed essentially as described previously29,30. In brief, pEF-FLAG-Stx3, pEF-FLAG-Munc18-2, pEF-T7-Stx4 and pEF-T7-Stx4p45a (a linker domain-swapping construct between Shp2 and Stx4p45a) or pEF-T7-Stx4p45a linker deletion constructs (that is, linker, amino acids 144–354; F1, amino acids 144–240; F2, amino acids 215–304; and F3, amino acids 272–354) were transfected into COS-7 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.
Cells were collected 48 h after transfection and homogenized in a homogenization buffer\textsuperscript{28,29}. After removal of insoluble materials by centrifugation, cell lysates were obtained. The associations between T7-tagged proteins and FLAG-tagged Stx3/Munc18-2 in the cell lysates were evaluated by immunoprecipitation using anti-T7 tag antibody-conjugated agarose beads (Merck Biosciences) as described previously\textsuperscript{36}. Immunoreactive bands were visualized with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (1:10,000 dilution; Novagen, 69522-4) and HRP-conjugated anti-FLAG tag M2 antibody (1:10,000 dilution; Sigma-Aldrich, A8592) and detected by enhanced chemiluminescence (GE Healthcare). The Rab-GTPase co-immunoprecipitation assay was performed as previously described\textsuperscript{7}.

**PIP-strip and lipid bead protein binding assays.** A solution of 1 μg ml\textsuperscript{-1} of purified protein (GST, GST-Slp2-a, GST-Slp4-a) was incubated with PIP-strip membranes according to the instructions of the manufacturer (Echelon Bioscience). Lipid beads (Echelon) prepared with different phosphoinositides or phosphatidylserine were incubated with 2 μg of purified GST–C2A or GST–C2B from Slp2-a or Slp4-a, washed five times, dried and resuspended in 100 μl sample buffer before western blot analysis. Enhanced chemiluminescence blotting of membranes was developed by immunostaining with an anti-GST antibody (Sigma-Aldrich) and HRP-conjugated donkey anti-mouse IgG (Jackson Immunoresearch).

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Figure S1. Transcriptional profiling of 3D vs. 2D MDCK cultures. (A) MDCK cells were grown in 2D and 3D conditions and purified RNA extracts were submitted for Canine Genome 2.0 Affymetrix cDNA chip microarray analysis. Mean results of overexpressed (green) and downregulated (red) genes are plotted. Significance of upregulated and downregulated genes was calculated using FDR-LJMA analysis (FIESTA software). (B) Signal-ratio (3D/2D) M-A plot of microarray results presented in A.
Figure S2 RT-qPCR validation of selected genes. Relative mRNA expression of a selected group of genes upregulated during cyst formation. MDCK cells were plated either in 2D, or 3D to grow cysts and lysates were collected for 2D at 36h, and at 14 and 36h in 3D. After mRNA extraction and cDNA polymerization, samples were analyzed by qPCR using gene-specific exon-spanning reactions and quantitative data was analyzed using SDS software (Applied Biosystems), and normalized to 2D expression levels. Different charts are used to distribute results in different scales using relative arbitrary units (top chart, ≤2-fold; bottom-left, ≤10-fold; bottom-right >10-fold). N=4.
**Figure S3** Slp-family proteins are not essential for 2D cell polarity or ciliogenesis. (A) Confocal microscopy images of 6d culture of MDCK cells in transwells. Cells transfected with Slp2-a siRNA or Slp4-a shRNA or controls were grown in a 2D-monolayer in transwells for 6d, fixed and stained to detect Podxl (red), β-catenin (green) and DNA (blue). Three confocal sections are shown for each treatment. Top panels show x-y apical section. Middle panels show x-y mid-height section. Bottom panels show x-z section. Bars, 20 μm. (B) Quantification of cell packing in monolayers shown in A. Cell packing measurements are cell height-width ratios of control (black bars) or Slp-KD cells (white bars). Values represent the average of three different experiments ± S.D. N=3 (>150 cells/experiment, *P<0.05). (C) Confocal microscopy images of 6d culture of MDCK cells in transwells. Cells treated as in A were fixed and stained to detect primary cilia using acetyl-tubulin as a marker (green) and DNA (blue). Bars, 10 μm. (D) Quantification of ciliated cells and cilia length in monolayers shown in C. Values represent the average of three different experiments ± S.D. N=3 (>100 cells/experiment). (E) Localization of apical and basolateral proteins in control cysts or silenced for Slp2-a. 14 hours after plating, cysts were treated with α-GFP for 30' at 4°C, after washing, they were grown for 24 hours more, allowing lumen development to occur. Then, they were fixed and staining for β-catenin (blue) and a secondary antibody to detect the uptake α-GFP. Immediately after binding (14h), control and Slp2-a silenced cysts showed peripheral GFP-PCX (green) and α-GFP (red). 24 hours after treatment, control cysts presented podocalyxin, mainly in the apical plasma membrane (arrows), revealing PCX-GFP is transcytosed from peripheral membranes to the new developed apical membrane. However, Slp2-a knock-down cysts showed accumulations of vesicles containing GFP-PCX (green) and α-GFP (red), suggesting a defect in trafficking of transcytosed PCX. Yellow, colocalization of podocalyxin and bound antibodies, blue indicates β-catenin. Bar, 10 μm. (F) Quantification of antibody localization after uptake in control or Slp2-a KD cysts. Cysts in Figure A were analyzed by confocal microscopy. Cysts were classified as “vesicles”, “apical plasma membrane” (APM) or “not-internalized” depending on the localization presented by the α-GFP (Red) antibody was found in internal vesicles, apical membrane or peripherally, respectively. Values are mean ± SD from three different experiments. N=3 (>100 cysts/ experiment; **, P<0.005; *, P<0.05).
Figure S4  Slp2-a-ΔC2AB, Slp2-a-ΔSHD, Slp2-a-linker and PS-probe localization. (A) Confocal microscopy images of cysts stably expressing GFP-Slp2-a linker domain. Cells were plated to form cysts for 20-24-48h, fixed and stained to detect Podxl (red) and β-catenin (blue). Bar, 10 μm. (B) Confocal microscopy images of cysts stably expressing GFP- Slp2-a-ΔSHD. Cells were plated to form cysts for 20-24-48h, fixed and stained to detect Podxl (red) and β-catenin (blue). Bar, 10 μm. (C) Confocal microscopy images of cysts stably expressing GFP- Slp2-a-ΔC2AB. Cells were plated to form cysts for 12-24h, fixed and stained to detect Podxl (red) and β-catenin (blue). Bar, 10 μm. (D) Confocal microscopy images of cysts stably expressing GFP- Slp2-a C2A (left panels) and C2B (right panels). Cells were plated to form cysts for 12-24h, fixed and stained to detect Podxl (red) and β-catenin (blue). Bar, 10 μm. (E) Confocal microscopy images of cysts stably expressing a phosphatidylserine (PS) fluorescent probe (Lact-C2-GFP). Cells were plated to form cysts for 72h, fixed and stained to detect Podxl (red) and F-actin (blue). Note the lack of asymmetric polarization of PS at the PM. Bar, 10 μm.
**Figure S5** Slp2-a controls apical membrane trafficking through interaction with Rab27a. (A) Slp2-a-interacting Rab GTPases. GST-Slp2-a or GST (control) beads were used to pull-down fluorescent protein-tagged Rab3a/b/c/d, Rab8a, Rab11a or Rab27a from total cell lysates. The table indicates relative binding results from 3 different experiments. (B) Immunoprecipitation of Rab27-GTP in GFP-Slp2-a or GFP-Btsz2 MDCK cysts. GFP-Slp2-a or GFP-Btsz2 were immunoprecipitated using anti-GFP (or control) beads and analyzed to detect binding of endogenous Rab27. (C) Localization of GFP-Rab27a during cyst morphogenesis. MDCK cells stably expressing GFP-Rab27a were grown to form cysts and fixed after 16, 20, 24, 48 and 72h. Samples were stained for Podxl (red), and β-catenin (blue). Confocal microscopy images correspond to five different stages of lumen initiation (AP vesicle aggregation [16h], vesicle fusion [20h], preapical-patch formation [24h], lumen expansion [48h], and mature cyst [72h]). Arrowheads, Podxl-positive vesicles; arrows, lumen. Bar, 5 μm (left panels). (D) Confocal microscopy images of endogenous Rab27arb. MDCK cells were plated to form cysts for 72h and stained to detect Rab27b (green), Podxl (red) and F-actin (blue). Bar, 10 μm. (E) Confocal microscopy images of Slp2-a and Rab27 in cysts at 72h. MDCK cells stably expressing Cherry-Slp2-a and GFP-Rab27a were grown as cysts and fixed at 4d and stained to detect Podxl (blue). Bar, 10 μm. (F) Western-blot of Slp2-a downregulation by siRNA in WT, V18A or E11A/R32A-Slp2-a-GFP stably expressing MDCK cells. Tubulin was used as loading control.
Figure S6 Localization of other mutants and constructs of Slp4-a. (A) Quantification of the silencing of Slp4-a by RT-qPCR. Values represent the average of ≥ three different experiments ± S.D., normalized to control levels (**, P < 0.005). (B) Scheme of Slp4-a, and Slp4-a domain fragments utilized. (C) PIP-binding assay. GST-tagged Slp4-a WT and C2A, C2B or C2AB were expressed and purified in bacteria. PIP-strip membranes were incubated with 1 μg/ml concentration of either GST-Slp4-a WT, C2A, C2B or C2AB fusion proteins and then membranes were blotted with an anti-GST antibody. A scheme of the PIP-strip membrane is shown (left panel). (D) Confocal microscopy images of 72h cysts stained with anti-Slp4-a antibody (green) and nuclei (blue). Note apical localization of Slp4-a. Bar, 20 μm. (E) Confocal microscopy images of cells stably transfected with GFP-Slp4-a fragments. Cells stably expressing GFP-Slp4-a linker, C2A, C2B or ΔSHD fragments were plated to form cysts for 72 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (F) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (G) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (H) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (I) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (J) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm. (K) Confocal microscopy images of cells stably transfected with GFP-Slp4-a K>Q mutant which uncouples phospholipid binding. Cells were plated to form cysts for 48 h, fixed and stained to detect Podxl (red) and nuclei (blue). Arrowheads, subapical vesicular localization of Slp4-a. Bar, 10 μm.
Figure S7 Overexpression and colocalization analysis of Slp2-a and Slp4-a during cyst morphogenesis. (A) Quantification of cysts with normal lumens in cells transiently overexpressing GFP (control), or GFP-Slp2-a constructs (WT, ΔSHD, or SHD). Values are mean ± SD from three different experiments. N=3 (>100 cysts/experiment; *, P < 0.05; **, P < 0.005). (B) Quantification of cysts with normal lumens in cells stably overexpressing GFP (control), or GFP-Slp4-a constructs (WT, ΔSHD, SHD, Linker, C2A, C2B and C2AB-GFP). Values are mean ± SD from three different experiments. N=3 (>100 cysts/experiment; *, P < 0.05; **, P < 0.005). (C) Correlation analysis for Slp2-a and Slp4-a quantitative-colocalization using Podxl (black bars, apical membrane marker) and β-catenin (white bars, basolateral marker) in three different stages during cyst morphogenesis. GFP-Slp2-a or GFP-Slp4-a expressing cells were grown to form cysts for 12 (early), 24 (preapical) or 72h (lumen), fixed and stained to analyze Podxl or β-catenin localization. Quantitative colocalization analysis between GFP signal and each marker was performed using ImageJ. Values are mean ± SD from three independent cyst cultures. N=3 (>10 cysts/experiment).
Figure S8 Scans of uncropped blots.
Figure 6A

Figure 6D

Figure 6J

Figure S8 continued
Figure S8 continued
**Supplementary Information**

**Supplementary Table 1** RTqPCR validation of gene overexpression (2D, 14h, 36h)

**Supplementary Table 2** RNAi and RNA expression analysis in silencing experiments

**Supplementary Table 3** Quantification of lumen morphogenesis (RNAi screening)

**Supplementary Video 1** Slp2-a apical localization depends on PIPs. MDCK cells stably expressing PHD-GFP and Slp2-Cherry were grown in cysts and set up for videomicroscopy. Cysts were treated with ionomycin for 5 minutes during recording.