Trafficking of Crumbs3 during Cytokinesis Is Crucial for Lumen Formation

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Although lumen generation has been extensively studied through so-called cyst-formation assays in Madin-Darby canine kidney (MDCK) cells, an underlying mechanism that leads to the initial appearance of a solitary lumen remains elusive. Lumen formation is thought to take place at early stages in aggregates containing only a few cells. Evolutionarily conserved polarity protein complexes, namely the Crumbs, Par, and Scribble complexes, establish apicobasal polarity in epithelial cells, and interference with their function impairs the regulated formation of solitary epithelial lumina. Here, we demonstrate that MDCK cells form solitary lumina during their first cell division. Before mitosis, Crumbs3a becomes internalized and concentrated in Rab11-positive recycling endosomes. These compartments become partitioned in both daughter cells and are delivered to the site of cytokinesis, thus forming the first apical membrane, which will eventually form a lumen. Endosome trafficking in this context appears to depend on the mitotic spindle apparatus and midzone microtubules. Furthermore, we show that this early lumen formation is regulated by the apical polarity complexes because Crumbs3 assists in the recruitment of aPKC to the forming apical membrane and interference with their function can lead to the formation of a no-lumen or multiple-lumen phenotype at the two-cell stage.

INTRODUCTION

During kidney development, cells of the metanephric mesenchyme undergo massive morphological changes to form the renal vesicle, an epithelial structure surrounding a central lumen (Saxen, 1987). This process is termed mesenchymal-to-epithelial transformation and is tightly controlled at the transcriptional level (Boutet et al., 2006; Thiery and Sleeman, 2006). The induction of the epithelial lumina is felt to depend on the induction of several polarity genes (Ikenouchi et al., 2003; Martinez-Estrada et al., 2006; Whiteman et al., 2008), but exactly how the products of these polarity genes lead to initial lumen formation is still unclear. Several tissue culture models have been used to identify mechanisms underlying lumen formation (O’Brien et al., 2002). Among these, cyst-formation and tube-formation assays allow the study of epithelial lumen formation de novo. It is generally believed that lumen formation is related to the formation and localized positioning of apical membrane, although additional mechanisms like apoptosis have been demonstrated to play a role in several contexts (Lubarsky and Krasnow, 2003). In Madin-Darby canine kidney (MDCK) cells, it has been shown that single epithelial cells (ECs) embedded in extracellular matrix (ECM) express apical proteins on their plasma membrane. These are thought to be endocytosed during the subsequent cell divisions, to form vesicular apical compartments (VACs) and to be exocytosed at the position of the forming lumen (Martin-Belmonte and Mostov, 2008). In this model, lumen formation has been shown to be completed at the four-cell stage. Others have reported lumen formation already in two- to three-cell aggregates, although underlying mechanisms remain elusive (Ferrari et al., 2008; Jaffe et al., 2008).

To successfully generate an apical lumen, ECs must acquire apicobasal polarity (Martin-Belmonte and Mostov, 2008). During the initiation of polarization, it is generally believed that phosphorylation-dependent mutual exclusion of apical and basolateral proteins leads to the separation of distinct apical and basolateral domains (Shin et al., 2006). At the boundary between both domains mammalian ECs form a tight junction (TJ), serving not only as a tight intercellular seal, but also as a reinforcement of membrane polarity (Shin et al., 2006). Apicobasal polarity is regulated by three evolutionarily conserved protein complexes, namely the apical Crumbs and Par complexes and the basolateral Scribble complex. In fact, in cyst-formation assays, interference with the proper regulation of polarity proteins leads to the generation of either multiple lumina or no lumen at all, as demonstrated for Crumbs3 (Crb3; Straight et al., 2004; Shin et al., 2005; Martin-Belmonte et al., 2007; Torkko et al., 2008; Schluter and Margolis, 2009). Crumbs (Crb) proteins have been demonstrated to be important determinants of apical membrane identity (Wodarz et al., 1995; Lemmers et al., 2004a; Fogg et al., 2005; Omori and Malicki, 2006; Fan et al., 2007). Thus it is tempting to speculate that polarity proteins are the master regulators for the formation of a lumen with a central role for Crb in the definition of the first apical membrane and subsequent lumen.

Although the knowledge of proteins involved in apicobasal polarity and lumen formation is growing, the mecha-
nisms underlying the initial formation of a solitary lumen are not yet well characterized. Here we report that a single MDCK cell forms a cyst with a Crb3a-positive lumen during the first cell division. We demonstrate that the initial apical membrane gets established during and after cytokinesis through delivery of a Crb3-positive membrane from apical recycling endosomes that are trafficked along the mitotic spindle apparatus. The definition of the apical membrane eventually gives rise to the first lumen, because Crb3 knockdown reduces the size of the formed lumina or abolishes lumen formation altogether. Finally, Crb3 assists in the recruitment of Par–αPKC complex proteins to the forming apical membrane, where they serve to reinforce apical identity.

MATERIALS AND METHODS

Antibodies and Chemicals

Rabbit polyclonal Crb3a, rabbit polyclonal PATJ, and rabbit polyclonal PALS1 have been previously described (Roh et al., 2002; Makarova et al., 2003). Rabbit polyclonal canine Prominin-1 antibody was raised against the peptide C3KAKYRMSD6EDWYD (100% identical to human), coupled to KLH. Rabbits were immunized at Protein&tech Group (Chicago, IL). Mouse monoclonal GP135 antibody was a gift from George Oprian (State University of New York). Mouse monoclonal α-tubulin, mouse monoclonal polyglutamylated-tubulin, mouse anti-Flag M2, mouse monoclonal γ-tubulin antibodies, nocodazole, and cytochalasin B were purchased from Sigma (St. Louis, MO). Mouse antibodies against Rab11 were purchased from BD Biosciences (San Jose, CA). Rat polyclonal E-cadherin, mouse monoclonal ZO-1, and Alexa Fluor conjugated secondary antibodies, DAPI, and phalloidin-rhodamine were purchased from Invitrogen (Carlsbad, CA). Atypical protein kinase C (αPKC) myristoylated pseudosubstrate was purchased from Calbiochem (San Diego, CA).

Constructs and Cell Lines

**GFP-Crb3a constructs.** The Crb3a cDNA was described previously (Makarova et al., 2003). Green fluorescent protein (GFP) was cloned into human Crb3a extra-cellular domain according to standard procedures. Briefly, an ectopic EcROV site was inserted into Crb3 via site-directed mutagenesis (primer sequence: GCACGGTGTTCCTCAGACGGAATCGC; and reverse: CTCACTGCGTCCA). GFP was amplified via PCR and ligated in-frame into the EcROV site in Crb3a. The whole construct was subcloned via PCR into the BamHI and Clal sites of pRevTRE vector (Clontech, Mountain View, CA). Crb3 ΔΔEI was cloned through PCR from pRevTRE GFP-Crb3a and cloned BamHI/Clal into pRevTRE.

**mCherry-E-cadherin construct.** mCherry was PCR-amplified from pmCherry-N1 (Clontech; primer forward: TTTGGATATCCCTGACGGCGCATGGTGAACGAGCGGAGGAGG, primer reverse: TTTAATTACCTTACTGGGACAGCCTGCAG). PCR product was digested with EcoRI and ligated with the PCR product. E-cadherin was PCR-amplified (primer forward: TTTTGGATATCCCTGACGGCGCATGGTGAACGAGCGGAGGAGG, primer reverse: TTTAATTACCTTACTGGGACAGCCTGCAG). Digestion with EcoRI and digestion (Clontech) and digested with Apol pQXIP (Clontech) was digested with EcoRI and ligated with the PCR product. PCR was performed with primers (Clontech) vector system and transduced into MDCK cell lines. The cells were selected with 5 μg/ml puromycin for 2 wk. Knockdowns were confirmed by immunofluorescence and Western blotting.

**CRISPR/Cas9 knockdown cell lines** were previously described (Whitman et al., 2008). The second Crb3 target sequence used for Supplemental Figure S7 is GCACATGCAGCTGAT. Briefly, hairpin constructs against canine Crb3 mRNA were cloned into the pBiren-RetroQ (Clontech) vector system and transduced into MDCK cell lines. The cells were selected with 5 μg/ml puromycin for 2 wk. Knockdowns were confirmed by immunofluorescence and Western blotting.

**PCR of Rab11a.** Rab11a was done from a complete Hs. Rab11a EST (Invitrogen Clone ID: 5792753). Human Flag-Rab11a was cloned using the following PCR primers: Forward primer: TTTAATTACCTTACTGGGACAGCCTGCAG; Reverse primer: TTTTGGATATCCCTGACGGCGCATGGTGAACGAGCGGAGGAGG. PCR products were engineered to insert an Hs. Rab11a restriction site following by a Flag-epitope sequence at the 5′end and an EcoRI restriction site at the 3′end. Reverse primer: CGAGAGACTGATCTTGCTGG; and forward primer: GCTGGAGGACATGGTGAACGAGCGGAGGAGG. PCR products were digested with XbaI (Clontech) and digested with Apol pQXIP (Clontech) was digested with EcoRI and ligated with the PCR product. PCR was performed with primers (Clontech) vector system and transduced into MDCK cell lines. The cells were selected with 5 μg/ml puromycin for 2 wk. Knockdowns were confirmed by immunofluorescence and Western blotting.

**Western Blotting**

Western blots were performed according to standard procedures. Briefly, one 10-cm dish of cells was lysed in 500 μl RIPA buffer (20 mM Tris, 150 mM sodium chloride, 5 mM EDTA, 10 mM sodium phosphate, 1% [vol/vol] glycerol, 1% [vol/vol] NP40, 1% [v/v] sodium deoxycholate, and 0.1% [v/v] SDS). Protein lysate, 50 μg, was separated on a 10% SDS-PAGE gel. Western blot transfers were performed according to standard procedures. The membranes were blocked with 5% BSA in TBS at 4°C overnight, and stained with primary antibody overnight. After three 10-min washes on the shaker, the membranes were probed with secondary antibody plus DAPI for 2 h. Filters were transferred onto glass slides, and after addition of Prolong Gold antifade reagent (Invitrogen) covered under glass coverslips, and analyzed by epifluorescence ( Eclipse T2000-U, Nikon Instruments, Melville, NY) or confocal (FV500; Olympus) microscopy.

**RESULTS**

Crb3 exists in two splice isoforms: Crb3a and Crb3b. The carboxy-terminus of Crb3a ends in the sequence ERLI and this sequence binds to Pals1 as well as Par6 and is an evolutionarily conserved determinant of apical identity (Wodarz et al., 1995; Makarova et al., 2003; Lemmers et al., 2004a). To further investigate the role of Crb3a during MDCK cyst formation, we cloned GFP-Crb3a into a Tet-inducible plasmid where we could control expression, because overexpression of Crb3a can disrupt apical basal polarity (Figure 1A; Roh et al., 2003). Using this plasmid in the absence of Tet induction, the basal expression level of the vector was sufficient to drive GFP-Crb3a expression at low levels. We obtained MDCKII clones with varying expression levels of GFP-Crb3a (Figure 1B).

**Tissue Culture and Cyst Formation Assay**

MDCKII cells were cultivated in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/1 penicillin, and 100 U/1 streptomycin (Invitrogen). For Geltrex cyst assays, 80–90% confluent cells were detached with enzyme-free PBS-based (no immunofugand) buffer (for 30 min), resuspended in DMEM with 2% Geltrex (Invitrogen), and seeded on a 100% Geltrex base in eight-well chamber coverslips (Labtek, Thermo Fisher Scientific, Rochester, NY) using 250 μl of 2 × 10⁶ cells/ml for each well. Cells were fixed with 4% paraformaldehyde after periods of 12 h to 6 d. Tissue culture medium was replaced every 3 d.

Immunofluorescent staining of cysts in Geltrex was carried out in chamber coverslips. After fixation, samples were permeabilized with 0.1–0.5% SDS in PBS or 0.1% Triton X-100 in PBS. Samples were blocked in PBS plus 2% goat serum (PBSG). Primary antibody staining was performed in PBSG for 3 h. After four 30-min washes in PBSG, secondary antibody with DAPI and/or phalloidin-rhodamine in PBSG was added overnight. After four 30-min washes in PBSG, cells were analyzed by confocal laser microscopy (FV500, Olympus; Center Valley, PA). Three-dimensional rotational imaging was performed with Velocity software (Perkin Elmer-Cetus, Waltham, MA). Live-cell reconstitution was performed on a Delavitation RT microscope (Olympus) and reconstructed with Imaris software (Bitplane Scientific Solutions, Saint Paul, MN). Cyst-assays in collagen were performed as previously described (Shin et al., 2005).

For immunofluorescent staining of cells grown in monolayers, cells were grown on six-well Transwell filters (pore size 0.4 μm, Corning Costar, Lowell, MA). The filters were cut, fixed in 4% paraformaldehyde for 1 h, permeabilized in 0.1% SDS or 0.1% Triton X-100, blocked for 1 h in PBSG, and stained with primary antibody overnight. After three 10-min washes on the shaker, the cells were probed with secondary antibody plus DAPI for 2 h. Filters were transferred onto glass slides, and after addition of Prolong Gold antifade reagent (Invitrogen) covered under glass coverslips, and analyzed by epifluorescence (Eclipse TE2000-U, Nikon Instruments, Melville, NY) or confocal (FV500; Olympus) microscopy.

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Crumbs3 and Early Lumen Formation

Crumbs3 exists in two splice isoforms: Crb3a and Crb3b. The carboxy-terminus of Crb3a ends in the sequence ERLI and this sequence binds to Pals1 as well as Par6 and is an evolutionarily conserved determinant of apical identity (Wodarz et al., 1995; Makarova et al., 2003; Lemmers et al., 2004a; Mori and Malicki, 2006). The carboxy-terminus of Crb3a ends in the sequence CLPI, and this isoform binds to importins but its role in epithelial cells is not as well understood as Crb3a (Fan et al., 2007). Knockdown of Crb3, in MDCK cells leads to a “no lumen” phenotype when the cells are grown in three-dimensional culture (Torkko et al., 2008).

To further investigate the role of Crb3a during MDCK cyst formation, we cloned GFP-Crb3a into a Tet-inducible plasmid where we could control expression, because overexpression of Crb3a can disrupt apical basal polarity (Figure 1A; Roh et al., 2003). Using this plasmid in the absence of Tet induction, the basal expression level of the vector was sufficient to drive GFP-Crb3a expression at low levels. We obtained MDCKII clones with varying expression levels of GFP-Crb3a (Figure 1B).
**GFP-Crb3a Localizes to the TJ and Apical Domain as well as Cilia and Promotes the Expansion of the Apical Domain**

When grown on Transwell filters, MDCK cells form monolayers. As already reported GFP-Crb3a localizes to the apical membrane domain (Roh et al., 2003), indicated by colocalization with the apical marker GP135, to the TJ, shown by colocalization with zonula occludens protein 1 (ZO-1), and to cilia, colocalizing with the cilia marker polyglutamylated tubulin (Figure 1C). This localization pattern was independent of the GFP-Crb3a expression level (data not shown).

When grown in collagen for 8 d, the cells form cysts with a single lumen (Figure 1D). Interestingly and in accordance with previous reports (Wodarz et al., 1995; Lemmers et al., 2004b; Omori and Malicki, 2006), increasing amounts of exogenous Crb3a induces apical membrane expansion, leading to apical membrane folding (Figure 1D, arrowheads). In comparison, MDCK wild-type cells present a flat apical membrane domain. GFP-Crb3a–expressing clones were isolated for 2 wk to generate a stable cell line. Clones were isolated, grown for 3 d, lysed in RIPA buffer, and analyzed by Western blot. The basal GFP-Crb3a expression in the absence of induction was sufficient to yield acceptable protein levels. (C) GFP-Crb3a localizes to the apical membrane, TJ, and cilia. MDCKII cells were grown on transwell filters for 4 or 7 d to obtain cilia, fixed, and costained with GP135, ZO-1, E-cadherin, or polyglutamylated tubulin. (D) Overexpression of GFP-Crb3a increases the size of the apical membrane domain. GFP-Crb3a–expressing MDCKII clones were embedded in collagen as previously described (Straight et al., 2004), grown for 7 d, fixed, and stained for the indicated markers. Clone 6 is a low-level expresser and only shows a moderate expansion of the apical membrane (arrowhead), whereas clone 7 expresses GFP-Crb3a at a higher level and displays folds of surplus apical membrane on the luminal side (arrowheads). (E) MDCKII wild-type cells grown in collagen for 7 d possess a flat apical membrane positive for endogenous Crb3 and GP135.

**The First MDCK Cell Division Creates a Cyst with a Crb3a-positive Apical Membrane**

Because low-level expression of GFP-Crb3a allows cyst formation with solitary lumina, we set out to use this system for examining early stages of cyst formation. It was previously reported that the presence of laminin in the extracellular matrix had an influence on the speed of polarization and thus on the mechanism of lumen formation (Martin-Belmonte et al., 2008). Thus we used a Geltrex (Invitrogen)-based cyst-formation assay, which has the advantage of growth-factor reduction, increasing the reproducibility of the experimental results. We embedded the cells into the ECM and fixed and stained them after 12–24 h of incubation (Figure 2). Through nonenzymatic cell detachment before embedding, we protected the GFP tag, which was located in the extracellular region of Crb3a, but still obtained a predominant single cell suspension (Supplemental Figure S1, A and B). MDCK cells were not cell cycle synchronized, so that all stages of the cell cycle can be visualized at a single time point at 12 h, whereas at 24 h cells had formed predominantly two-cell aggregates. The total amount of observed structures at 24 h was only insignificantly reduced compared with directly after embedding, suggesting that the observed two-cell stages were derived from cell divisions and not migrational and adhesion events (Supplemental Figure S1, A and B). Cells were stained for α-tubulin to determine the mitotic stage and for E-cadherin to visualize cell adhesions (Figure 2A–C, Supplemental Figure S1, C–E).

During early stages (prepro- to metaphase) of the first cell division, GFP-Crb3a localized primarily intracellularly to the area of the α-tubulin–positive spindles (Figure 2A, Supplemental Figure S1C). At later stages of the first cell division, GFP-Crb3a localized to both cell poles (Figure 2B, Supplemental Figure S1D). With further progression of the telophase stage (as visualized by the developing midzone microtubule bundle), increasing amounts were visible on the side of the newly developing nuclei facing the opposite daughter cell (Figure 2B, outlined arrows) and in some cases became apparent at the tip of the midzone microtubule bundle (Figure 2B, bold arrows, Supplemental Figure S1D). In late telophase and two-cell stages, GFP-Crb3a plasma membrane staining became visible at the membrane between the two daughter cells (Figure 2, B and C). Apparent intracellular Crb3a-positive structures disappeared with further develop-
Figure 2. Lumen formation occurs during the first cell division of ECM-embedded MDCKII cells through positioning of apical membrane in the plane of division. (A–C) GFP-Crb3a–expressing (green) MDCKII cells were grown as single-cell suspensions in Geltrex-containing medium and were fixed at 12 h (for mitotic stages) or 24 h (for two-cell stages) after embedding. Cells were stained for E-cadherin (blue), α-tubulin (red), and DAPI (gray) and analyzed by confocal laser microscopy. (A, collapsed Z-stacks) During early stages of cell division GFP-Crb3a localizes to cytosolic compartments located around the mitotic spindle apparatus and partitions to both sides of the condensing nucleus during metaphase. (B, collapsed Z-stacks) At later mitotic stages, it relocates from a position at the lateral side of the nucleus (subpanel a) to the medial side of the two new daughter nuclei (outlined arrows) and finally forms a nascent lumen in the plane of the cell adhesion at the two-cell stage (C, collapsed Z-stacks). Note that GFP-Crb3a is evident at the tips of the midzone microtubule bundles (B, subpanels a and b, white arrows). (D) Single Z-section of a GFP-Crb3a–expressing two-cell stage, costained against ZO-1 (red) and DAPI (gray). (E, single Z-section) GFP-Crb3a only locates to spindle poles during early but not late cell divisions. GFP-Crb3a–expressing MDCKII cells in Geltrex-containing medium grown from single-cell suspensions were fixed after 8 d and stained for α-tubulin (red) and DAPI (blue). (F) Live-cell imaging of lumen formation during MDCKII cell division. MDCKII cells were sequentially virally transduced with GFP-Crb3a (in pRevTRE) and mCherry-E-cadherin (in pQCXIP). Images were three-dimensionally reconstructed at individual time points. The adhering membranes between two-daughter cells are displayed in side view, showing an emerging Crb3a-positive apical lumen.

ment of the apical membrane. This led us to speculate that Crb3a-positive vesicles traffic along microtubular routes to their subapical destination, where they arrive during telophase and are subsequently exocytosed to reinforce the initially aggregated apical membrane.

During the subsequent interphase, the developed two-cell structure possesses an emerging lumen with apical membrane identity, as shown by GFP-Crb3a localization (Figure 2C, Supplemental Figure S1E). This emerging lumen is surrounded by a ZO-1–positive TJ (Figure 2D). Both cells adhere through an E-cadherin–positive adherens junction surrounding the TJ (Figure 2, C and D, and Supplemental Movie 1). Staining performed with Crb3a-specific antibody confirmed these results (Supplemental Figure S2A). These data clearly demonstrate that dividing MDCK cells form a solitary lumen already at a two-cell stage. Interestingly, no more Crb3a trafficking to the spindle poles occurs at later cell divisions, promoting the idea that this trafficking mechanism is unique to the early cell divisions of MDCK cells (Figure 2E). Also, the functionality of this mechanism does not depend on the type of extracellular matrix, because MDCK cell divisions produce lumina as well, when grown in collagen type I (data not shown). This is in accordance with a previous report demonstrating lumina in MDCK two-cell aggregates when suspended in collagen, although cell divisions had not been observed in this context (Ferrari et al., 2003).

To further delineate the sequence of events leading to early lumen formation, we sequentially coinjected the GFP-Crb3a MDCK cells with mCherry-tagged E-cadherin. In addition to the typical GFP-Crb3a staining, these cells display lateral mCherry-E-cadherin staining and maintain their ability to form hollow cysts (Supplemental Figure S, 3A and B). We embedded these double-fluorescent cell lines into ECM, performed live-cell imaging for 24 h, and reconstructed the dataset three-dimensionally (Figure 2F, Supplemental Figure S3C). The figures show the time after embedding into the ECM with live-cell imaging beginning 6 h after embedding. Single cells were chosen, as judged by size, GFP-Crb3a and mCherry-E-cadherin staining (Supplemental Figure S3C).

At early time points, accumulated GFP-Crb3a is seen in intracellular organelles. Images in Figure 2F focus on the plane between the two daughter cells initially formed by E-cadherin accumulation. At time 17:45 (h:min), regions were detectable where Crumbs3a was concentrated in patches that excluded E-cadherin. Subsequently, GFP-Crb3a intensity increased at the forming apical domain. Additionally, GFP-Crb3a–positive structures can be seen in proximity of the eventual lumen (Figure 2F). At time 19:00, a fully developed hollow GFP-Crb3a–positive emerging lumen is visible, excluding mCherry-E-cadherin, and indicating the completion of the first mitosis (Supplemental Figure S3C). In this system, however, recycling endosomes proved difficult to observe because of high cell motility at the one- to two-cell stage, causing structures to often leave the focal plane.

In mature cysts, Crb3a colocalizes with other apical proteins like GP135 (Figure 1E). To examine whether Crb3a colocalizes with GP135 in the previously characterized Crb3a-positive endogenous compartments, we performed costainings during the first MDCK cell division. Indeed, GFP-Crb3a as well as endogenous Crb3a and GP135 show a strong colocalization in these intracellular structures as well as at the emerging lumen (Figure 3A and Supplemental Figure S2A).

The process of Crb3a-positive membrane trafficking from the plasma membrane of single cells to internal compartments and back to the forming apical membrane is reminiscent of recycling endosomal trafficking, as published for Rab11-positive endosomes (Hobdy-Henderson et al., 2003). Rab11-positive endosomes and Rab11 family–interacting proteins have been implicated in the biosynthetic trafficking
and recycling of apically targeted proteins (Wang et al., 2000; Cresawn et al., 2007). Indeed, GFP-Crb3a–positive intracellular compartments show a strong staining for Rab11 during this first cell division (Figure 3B and Supplemental Figure S2B). At the two-cell stage, when Crb3a becomes localized to the newly formed apical membrane, it loses colocalization with Rab11, which remains in a subapical area, indicating complete delivery of Crb3a to the newly formed apical surface.

**Crb3a Trafficking Depends on the Microtubular Cytoskeleton during First Cell Division**

Because Crb3a is a transmembrane protein and redistributes along the mitotic spindle during this first cell division (Figure 2, A–C), we hypothesized that the tubulin cytoskeleton directs Crb3a vesicles during initial partitioning and formation of apical membrane between the two daughter cells. Interestingly, and in contrast to a previous report (Ferrari et al., 2008), GFP-Crb3a does not colocalize with actin during the initial cell division before the two-cell stage (Figure 4A).

Because nocodazole treatment prevents cell division altogether, it is impossible to test the microtubular role for Crb3a trafficking during cell division, as seen at 36 h after seeding (Figure 4B; phenotype occurred in all observed cells). Cells treated with nocodazole fail to divide, whereas many cysts in the DMSO-treated control have already reached the four-cell stage. Nevertheless, the nocodazole-treated cells often display vacuolar GFP-Crb3a–positive structures along the periphery, and these structures also possess the apical marker GP135, but not the basolateral protein E-cadherin, which often localizes in proximity to Crb3a-positive areas (Figure 4, B and C). Interestingly, most of the observed GFP-Crb3a–positive vesicles are negative for Rab11 (Figure 4C). We speculate that these vesicles might represent mature apical membrane, which have failed to be delivered to its proper destination.

To test the role of the actin cytoskeleton in Crb3a trafficking, we inhibited actin polymerization through cytochalasin B treatment. On cytochalasin B–mediated depolymerization of the actin cytoskeleton, cells are still able to produce mitotic spindles and undergo nuclear mitosis, but they lose the ability to undergo cytokinesis because of inhibition of contractile actin ring assembly (Figure 4D). Strikingly, GFP-Crb3a is still able to partition to the spindle poles under actin-depolymerizing conditions. Furthermore it retains its ability to traffic back to the medial side of the two daughter nuclei during anaphase. Because there is no efficient bundling of the midzone microtubules (Figure 4Dc, bold arrow) and no intercellular membrane where the endocytosed membrane could traffic to, GFP-Crb3a–positive vesicles accumulate in a broader area between the two daughter nuclei (Figure 4Dd). It is important to note that a majority of these vesicles do not colocalize with Rab11, but rather with GP135 (Supplemental Figure S4), pointing to a mature apical identity and underlining the notion that disruption of tubulin polymerization causes a defect of membrane-delivery, but not membrane maturation.

To further test the importance of Rab11-containing endosomes in cytokinetic membrane trafficking, we expressed wild-type, dominant active (Q70L), and dominant negative (S25N) Rab11a in GFP-Crb3a cells and then studied early apical membrane formation (Supplemental Figure S5). We found that dominant negative but not wild-type or dominant active Rab11a perturbed early apical membrane formation. At the two-cell stage, GFP-Crb3a could be seen localized to emerging lumens in the cells expressing control, wild-type, and dominant active Rab11a, whereas GFP-Crb3a was diffusely localized in the cells expressing dominant negative Rab11a. Taken together, our data suggest that during MDCK cell divisions, Crb3a gets internalized into Rab11-positive vesicles and is transported along microtubular routes to the newly forming apical membrane.

**Members of the Crb3 Complex, But Not the Par3/Par6/aPKC Complex Colocalize with Recycling Endosomes during the First Cell Division**

At the TJ, Crb3a forms an apical protein complex with PALS1 and PATJ (Wang and Margolis, 2007). The Par complex consists of Partitioning defective 3 (Par3), Par6, and aPKC, and both protein complexes are involved in the establishment and maintenance of apicobasal epithelial polarity (Wang and Margolis, 2007). Thus we were interested in investigating whether apical polarity proteins would traffic along the same route as Crb3a during the first MDCK cell division.

Indeed, PALS1 and PATJ colocalize with Crb3a in the cytoplasmic recycling endosomes, as seen for the GFP-tagged proteins and endogenous PALS1 (Figure 5, A and B, and Supplemental Figure S6). Interestingly, endogenous Par3 and aPKC cannot be found in these structures and only appear at the newly formed tight junction or lumen, respectively (Figure 5, C and D).
Knockdown of Crb3 Abolishes Lumen Formation at the Two-Cell Stage

Crumbs proteins have been implicated in the definition of apical membrane identity and in lumen formation (Wodarz et al., 1995; Omori and Malicki, 2006; Torkko et al., 2008). In mature MDCK cysts, Crb3a localizes to the apical membrane (Figures 1, D and E). Thus we were interested in examining whether and how Crb3a is involved in the initial lumen formation of dividing MDCK cells. To address this question, we established Crb3 short hairpin RNA (shRNA) knockdown MDCKII cell lines. After infection, we isolated clones, lysed them after 3 d, and analyzed protein expression by Western blotting (Supplemental Figure S7, A and B). Crb3a expression was significantly suppressed, whereas expression of PATJ, PALS1, aPKCζ, and the apical marker Prominin1 was unchanged. The expression of GP135 was reduced to some extent in some, but not of the cell clones compared with their controls (Supplemental Figure S7, A and B).

To assess the impact of Crb3 knockdown on the initial lumen formation, we embedded the cells into Geltrex as previously described and performed cyst-formation assays. Different stages of cell divisions were visualized by confocal laser microscopy (Figure 6A). At mature two-cell stages, we determined the average size of an early apical membrane or lumen—if present—at the attaching membranes between the two daughter cells in the control cell line. Lumen size could be judged by the size of GP135 enrichment with simultaneous E-cadherin exclusion. Early apical structures measured between 6 and 11 μm, as visualized by GP135 staining. Thus we grouped the acquired two-cell datasets into “no lumen,” “small lumen” (≤5 μm) or “regular lumen” (>5 μm) phenotypes (quantification in Figure 6B and Supplemental Figure S7C). Although the control cells showed an average amount of 34% of regular-sized lumina and 13% of small lumina (Figure 6, Aa and B), Crb3a knockdown cell lines seemed to have lost their ability to form these early lumina almost completely. Clone 3 displayed a residual amount of 1.4% regular-sized lumina and 13% of small lumina (Figure 6, Ac and B). The residual two-cell stages had no GP135-positive membrane or lumen at all at the attaching membranes of the daughter cells, because E-cadherin staining was continuous (Figure 6Aa and B). The residual early lumen formation could not be observed in clone 22 (Figure 6B) and with a second hairpin (Supplemental Figure S7C).
though lumen formation was strongly impaired by Crb3 knockdown, the cellular uptake of GP135 and its partitioning to the spindle poles seems to be unaffected at early stages of cell divisions (Figure 6Ad), similar to wild-type MDCK cells (Figure 3A).

Members of the Crb3 complex have been shown to directly interact with members of the Par3/Par6/aPKC complex (Hurd et al., 2003b; Lemmers et al., 2004b). We previously reported that Crb3a was able to recruit Par6 to the membrane in unpolarized cells (Hurd et al., 2003b). Thus we hypothesized that the delivery of Crb3a complex to the forming apical membrane might serve as an anchor recruiting Par3/Par6/aPKC to this location. Immunostaining of two-cell stages for aPKC/H9256 in Crb3 knockdown cell lines reveal that little or no aPKC/H9256 is present at the membrane between the two daughter cells, where lumen formation is absent (Figure 6Ab). At two-cell stages with residual lumen formation we found aPKCζ staining to be limited to the site of the small lumina, where it accumulated with high intensity (Figure 6Ac). Because apical membrane areas are strongly reduced in size and number, aPKCζ-positive areas are reduced to the same extent. This indicates that Crb3a-positive membranes might recruit aPKCζ possibly through interaction with Par6, to the forming apical membrane.

**GFP-Crb3a Rescue of Lumen Formation**

The canine Crb3 short hairpin target sequences are directed against both canine Crb3a and -b isoforms. Because Crb3a shows the higher degree of conservation and has a well-established role in cell polarity, we generated GFP-Crb3a rescue cell lines of Crb3 shRNA interference (shRNAi) clone 3. After 2 wk of selection, the expression of the rescue constructs was determined by Western blotting (Figure 7A). Both full-length GFP-Crb3a (FL) and the truncated protein missing its PDZ-binding motif (ΔERLI) were expressed at similar levels. The knockdown of endogenous Crb3a was maintained in the empty vector control and in both rescue cell lines, compared with MDCK wild-type cells.

The generated cell lines were tested in the lumen-formation assay as described in Materials and Methods, fixed after 24 h, and stained for aPKCζ and E-cadherin. Two-cell stages were visualized through confocal microscopy, and lumen diameters were measured and statistically analyzed (Figure 7, B–D). Cell lines transduced with GFP-Crb3a FL showed a significant rescue with strong aPKCζ recruitment (Figure 7Bb), Par3 at tight junctions (Supplemental Figure S8), and lumen diameters predominantly >5 μm (Figure 7C), whereas control vector-transduced cells only showed a residual amount of lumen formation and aPKCζ staining similar to the results of the original knockdown clone (Figures 6, A and B, and 7, Ba and C). Interestingly, GFP-Crb3a ΔERLI-transduced cells showed a partial rescue of lumen formation, although the amount of lumina >5 μm was decreased (Figure 7, Bc and C). On average, full-length rescued cells possessed lumen diameters about 5.2 μm, whereas GFP-Crb3a ΔERLI presented lumen diameters averaging 3.2 μm.

Figure 5. Members of the Crb3 complex, but not Par3–aPKC complex, are recruited to Crb3a-positive endosomes. MDCKII cells were embedded in Geltrex as previously described, fixed at different times, and stained with the indicated antibodies. Single Z-sections are displayed. (A and B) Exogenously expressed GFP-PALS1 and GFP-PATJ co-localized with Crb3a. (C and D) Par3 and aPKCζ do not initially co-localize with GFP-Crb3a, but get enriched at the newly forming TJ and/or apical membrane between the two daughter cells and at the emerging lumen.
apical membrane domain, promoting apical identity (Plant
ylation and thus excluding basolateral proteins from the
has been shown to maintain epithelial polarity by phosphor-

We wanted to further test the role of aPKC in apical mem-
nulation of aPKC
Some cells with impaired lumen formation showed minimal accu-
GP135 (subpanel b), with a simultaneous accumulation of aPKC
strongly decreased lumen size (subpanel c). Knocked-down cells
formed intracytoplasmic accumulations of the apical marker

Inhibition of aPKCΔζ Impairs Apical Membrane Definition
We wanted to further test the role of aPKC in apical mem-
brane determination. aPKC is a member of the Par polarity
complex and is indispensable for the establishment of epi-
thelial polarity in numerous systems (Shin et al., 2006). aPKC
has been shown to maintain epithelial polarity by phosphory-
ating and thus excluding basolateral proteins from the
apical membrane domain, promoting apical identity (Plant

DISCUSSION
MDCK cyst formation has been used as a model for the study of
epithelial cell polarity for several years but the underlying
mechanisms regulating the emergence of the very first lumen
remain unclear (O’Brien et al., 2002). Labarsky and Krasnow
(2003) summarized data on lumen formation and concluded
that lumen formation was related to the delivery of apical
membrane. We demonstrate that MDCK cells can create
apicobasal polarity and a primary lumen from a single sym-
metric cell division. A similar mechanism has been demon-
strated before in zebrafish neurulation, where neuroepithe-
lial cells divide during neural keel and rod formation,
resulting in two separate cells with symmetric Par3 position-
ning surrounding a newly formed lumen (Tawk et al., 2007).
We propose that this might be a general mechanism repre-
sented in various types of epithelial cells.

**Internalization, Partitioning, and Delivery of Crb3a-positive Membrane Through Rab11-positive Endosomes Lead to Lumen Formation from MDCK Mitosis**

During the first cell division, MDCK cells are able to inter-
nalize apical membrane components from their plasma
membrane, partition them to the spindle poles of the newly
forming nuclei, and finally deliver them to a membrane area
between the two daughter cells, thus constituting a first
lumen (Figures 2 and 3). We have found that the internalized
fraction of Crb3a colocalizes with Rab11-positive endosomes
during cell division (Figure 3B, Supplemental Figure S2B).

Rab GTPase family–positive recycling endosomes have been
identified as important players of apical trafficking
(van Ijzendoorn, 2006; Sato et al., 2007; Nokes et al., 2008).
Especially the Rab11-family seems to play a central role in
apical trafficking and consists of Rab11a, -11b, and -25 (Bhar-
tur et al., 2000). Rab11-positive recycling endosomes together
with Rab11-FIP3 (Rab11 family–interacting protein 3) have
also been implicated in membrane delivery that is required
during cytokinesis and abscission. In interphase, proteins of
the apical recycling endosomes localize to areas around
centrosomes (Horgan et al., 2004). During mitosis, Rab11a

![Image](image_url)
aggregates near the spindle poles, and these aggregates are symmetrically inherited by both daughter cells (Hobdy-Henderson et al., 2003). Thus it seems likely that the targeting of apical proteins such as Crb3a into Rab11-positive vesicles plays a role in the initial formation of the apical membrane and ongoing apical membrane trafficking. Indeed we were able to demonstrate that dominant negative Rab11a blocked GFP-Crb3a trafficking and formation of early apical membrane (Supplemental Figure S5).

In addition to colocalizing with Rab11, Crb3a accumulates at the end of the midzone microtubule bundle and colocalizes with it during telophase of the first MDCK cell division (Figure 2B). Live-cell imaging shows a strong increase in the initial GFP-Crb3a signal at the membrane between the two daughter cells at the end of cell division (Figure 2F, Supplemental Figure S3C). Together, these data lead us to speculate that Rab11-positive endosomes deliver apical membrane to the site of abscission, leading to the formation of the first lumen. Because the exact partitioning mechanism for apical recycling endosomes is still elusive, we performed costainings between GFP-Crb3a–positive aggregates and cytoskeletal proteins tubulin or actin, respectively. We found that at all stages of mitosis, GFP-Crb3a–positive compartments localize along or in proximity of the tubulin cytoskeleton (Figure 2, A–C). Conversely, we found that colocalization with actin only occurred at very early or late stages of mitosis (Figure 4A). Nocodazole-dependent inhibition of tubulin polymerization led to an impairment of nuclear division with vesicles of apical identity localized to random submembranous spots (Figures 4, B and C). In contrast inhibition of actin polymerization with cytochalasin B impaired cytokinesis but not nuclear division (Figure 4D). Crb3a partitioning to the opposite spindle poles was still intact despite actin inhibition, leading to the notion that the actin cytoskeleton is expendable for this process. This indicates that trafficking of Crb3a in early cell division is predominantly microtubule based.

**Crb3 and Apical Membrane Identity**

Although trafficking along distinct routes, both members of the Crb3– and Par–aPKC-complexes become enriched at the attaching membranes of the two daughter cells, in proximity of the midzone microtubules. All Crb3-complex members traffic in Rab11-positive endosomes along microtubular routes, whereas Par–aPKC complex members that distribute evenly throughout the cytoplasm in single MDCK cells get concentrated at the forming apical membrane without association with Rab11-positive compartments.

Crb3a has been shown to recruit Par6 to the membrane of unpolarized cells through a direct interaction; furthermore; a direct interaction between Pals1 and Par6 has been demonstrated (Hurd et al., 2003b; Lemmers et al., 2004b). At the

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**Figure 7.** Expression of GFP-Crb3a rescues the knockdown of Crb3. Crb3 shRNA clone 3 was transduced with empty pRevTRE vector, pRevTRE GFP-Crb3a full-length (FL), or missing its PDZ-binding motif (ΔERLI). (A) Western blot of MDCK wild-type cells versus empty vector–infected cells shows a persisting knockdown of Crb3a. Probing with anti-GFP antibody reveals similar expression levels for FL and ΔERLI constructs. (B, collapsed Z-stacks) Empty vector–transduced cells presented primarily a no-lumen phenotype with minimal recruitment of aPKCζ to the site of cell adhesion similar to the original knockdown (subpanel a). Expression of GFP-Crb3a FL rescues lumen formation and aPKCζ recruitment (subpanel b). GFP-Crb3a ΔERLI expression partially rescues MDCK lumen formation with a smaller average lumen diameter, but not aPKCζ recruitment, which was minimal in all observed two-cell stages (subpanel c). (C) Quantification of phenotypes. Cells with a lumen size $>$5 μm were categorized as large lumen, and smaller sizes were counted as small lumen. GFP-Crb3a FL expression rescues lumen formation with predominantly large lumina. GFP-Crb3a ΔERLI-expression Crb3a shRNAi cells display a partial rescue with a greater number of small lumina. (D) The average lumen diameter is significantly smaller in ΔERLI-rescued cells (3.2 μm) compared with FL-rescued cells (5.2 μm).
end of MDCK division in our experimental setup, Crb3a assists in recruiting aPKC\(_{\text{z}}\) to the site of the nascent apical lumen, because aPKC\(_{\text{z}}\) is limited to residual areas of lumen formation in Crb3 knockdown conditions (Figure 7). Furthermore, although both constructs could rescue lumen formation partially, GFP-Crb3a FL more efficiently recruited aPKC\(_{\text{z}}\) than GFP-Crb3a \(\Delta\text{ERLI}\), and lumens were smaller in the GFP-Crb3a \(\Delta\text{ERLI}\) cells. GFP-Crb3a \(\Delta\text{ERLI}\) was able to partially rescue lumen formation despite its impaired ability to recruit aPKC\(_{\text{z}}\) (Figure 7). The partial rescue might be due to overexpression of GFP-Crb3a \(\Delta\text{ERLI}\) at levels above the endogenous Crb3a level. Residual endogenous Crb3a might also be responsible for small amounts of aPKC\(_{\text{z}}\) being recruited to the emerging lumen even without its PDZ-binding motif. If this was true, a residual amount of endogenous Crb3a being able to interact with Pals1 and aPKC might be sufficient to generate a functional tight junction and recruit small amounts of aPKC. The fact that the average lumen diameter in GFP-Crb3a \(\Delta\text{ERLI}\) rescued cells is reduced may reflect the impaired ability of GFP-Crb3a \(\Delta\text{ERLI}\) to contribute to TJ formation, limiting the size of the apical domain (Figure 7D). Finally, we cannot exclude the possibility of overlapping mechanisms of aPKC recruitment and TJ formation, which might result in a partial rescue even when overexpressing C-terminally truncated GFP-Crb3a.

Interestingly, we find Par3 staining restricted to the emerging TJ, but not at the forming luminal membrane, indicating that Par3 is expendable for apical aPKC\(_{\text{z}}\) recruitment. In contrast to this, Horikoshi et al. (2009) find that the interaction between Par3 and aPKC is required for the delivery of apical membrane in calcium-switch assays. The authors also demonstrate that Par3 is not always associated with Par6-aPKC. Under low calcium conditions Par6 and aPKC, in contrast to Par3, accumulate in cytoplasmically located vacuolar apical compartments (VACs). VACs were described more than 20 years ago as a compartment displaying apical features such as microvilli and apical proteins, appearing in epithelial cells under low-calcium or low-confluence conditions or when cells are embedded into ECM, such as collagen or agarose (Vega-Salas et al., 1987). In our lumen-formation assay, where cells get embedded into Geltrx containing laminin, we find only accumulation of Crumbs complex proteins, but not Par3 or aPKC\(_{\text{z}}\) in cyto-

**Figure 8.** Inhibition of aPKC\(_{\text{z}}\) impairs the regulated formation of a solitary lumen at the two-cell stage. GFP-Crb3a–expressing MDCKII cells were embedded in Geltrx as described. In some specimens, myristoylated aPKC\(_{\text{z}}\) pseudosubstrate (Calbiochem) was added to the liquid medium as indicated. Cells were fixed 24 h after embedding and stained with the indicated antibodies. (A, collapsed Z-stacks) In untreated cells, the majority of two-cell stages showed regular lumen formation, (subpanel a), whereas inhibitor-treated cells either showed ectopic localization of apical or tight junction components (subpanel c arrowhead) or occasionally had more than one lumen (subpanel b, arrows); (B) Quantification of two-cell stages with impaired lumen formation (ectopic localization of apical or tight junction components or multiple lumina) in aPKC\(_{\text{z}}\) pseudosubstrate–treated cells versus untreated cells.

![Diagram of MDCK lumen formation](image)

**Figure 9.** Model of MDCK lumen formation during cell division. Single cells embedded in extracellular matrix internalize Crb3-complex proteins into apical endosomal compartments. On formation of the spindle apparatus, the Crb3-complex members get transported to the spindle poles, whereas members of the Par-aPKC complex show a diffuse cytoplasmic localization. Crb3-complex proteins become apparent near newly nucleating microtubules that eventually form the midzone microtubules. The apical endosomes appear to get transported along the midzone bundle to the site of cytokinesis, where they are exocytosed. This lays the foundation for the formation of an apical membrane. Crb3a recruits aPKC\(_{\text{z}}\) through its PDZ-binding motif, most likely through interaction with Par6, to the forming apical membrane, where aPKC\(_{\text{z}}\) contributes to apical membrane identity through phosphorylation-dependent exclusion of basolateral proteins. Ultimately the separation of two polarized membrane domains gives rise to the formation of a tight junction. Pals1, Patj, and Par3 staining is restricted to the tight junction and excludes the apical membrane domain.
plasmatic accumulations, indicating a functional difference between VACs under low-calcium conditions and the recycling endosomes we found in these studies. More research will be required to compare the observed compartments.

Once recruited, αPKCζ might serve to further reinforce apical identity at the forming apical membrane domain. Previous work has shown that αPKC-dependent phosphorylation of the basolateral proteins Par1 and Lgl is sufficient to exclude them from αPKC-positive areas (Plant et al., 2003; Hurov et al., 2004; Suzuki et al., 2004), whereas Par1-kinase is able to phosphorylate Par3, leading to a disassembly of the Par-αPKC complex and thus its exclusion from Par1-positive areas (Benton and St. Johnston, 2003; Hurd et al., 2003a). This mutual exclusion of apical and basolateral proteins is thought to lead to the definition of two distinct apical and basolateral membrane domains. Figure 2D demonstrates this separation, as seen for GFP-Crb3a, representing the apical domain, and E-cadherin, representing the basolateral domain. On the border of the successfully separated membrane domains, a TJ is generated, fortifying the physical separation of both domains (Figure 2D, see ZO-1 staining; Shin et al., 2006). In accordance with this model, inhibition of αPKCζ with myristoylated pseudosubstrate leads to the ectopic localization of apical and tight junction proteins, in extreme cases even to the formation of multiple lumina already at a two-cell stage (Figure 8). This might be a result of incomplete exclusion of basolateral proteins from the forming apical domain with the result of several smaller apical areas which are separated by remaining domains of basolateral identity.

No-Lumen and Multiple-Lumen Phenotypes in Cyst Formation

In this article we present evidence that Crb3a is required for the definition of apical membrane identity. Knockdown of Crb3 leads to the absence of apical membrane and thus to the absence of emerging lumina at the two-cell stage (no-lumen phenotype, Figure 7). This result is supported by the previous observation that knockdown of Crb3 leads to the absence of lumina at later stages of cyst development (Torkko et al., 2008). Because one of our Crb3 hairpins showed a reduction of GP135 levels (Supplemental Figure S7A), we cannot rule out a role for GP135 in lumen formation. Meder et al. (2005) found a requirement of GP135 for lumen formation, although this result could not be reproduced by Cheng et al. (2005). Furthermore, the second hairpin we used did not cause a reduction of GP135, compared with the control cell line used, although reproducing the predominant no-lumen phenotype of the first hairpin (Supplemental Figure S7B). To resolve any doubt, we performed rescue experiments, clearly demonstrating that GFP-Crb3a could rescue lumen formation, thus proving its importance to the process (Figure 7).

The formation of the multiple-lumen phenotype may be more complex. A very recent article examined the delivery of αPKC in the division of single MDCK cells and presented a model in which down-regulation of Cdc42 resulted in a multiple-lumen phenotype (Jaife et al., 2008). In this model, apical membrane is delivered by the midzone microtubules, and Cdc42 knockdown-dependent deviation of the mitotic axis in later cell divisions would result in the ectopic delivery of apical membrane, resulting in additional ectopic lumina. We show that Crb3a-positive apical membrane gets endocytosed into apical recycling endosomes only during early cell divisions during MDCK cyst formation, whereas we failed to detect any internalized Crb3a at mature stages of cyst formation, where an apical surface is already present (Figure 2E). We conclude that Crb3a gets “trapped” at the apical surface by an as yet unidentified mechanism. Additionally, αPKCζ inhibition with myristoylated pseudosubstrate can result in a multiple-lumen phenotype already at the two-cell stage, without the possibility of ectopic membrane delivery caused by a disoriented mitotic axis in multiple cell divisions (Figure 8). Thus both mechanisms might work together in order to result in the observed multiple-lumen phenotype that has been reported in many publications (Straight et al., 2004; Shin et al., 2005; Jaife et al., 2008). Taken together, we have provided evidence that a cell division of a single MDCK cell embedded into ECM can create a lumen dependent on the delivery of Crb3a-positive apical membrane in Rab11-positive endosomes during the course of cytokinesis (Figure 9). Further work will clarify open questions, such as how the initial uptake of Crb3a at the one-cell stage takes place and what molecular mechanisms underlie the docking and fusion of Crb3a-positive vesicles during the formation of the very first lumen.

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