COMMENTARY

Regulation of podocalyxin trafficking by Rab small GTPases in epithelial cells

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ABSTRACT

The characteristic feature of polarity establishment in MDCK II cells is transcytosis of apical glycoprotein podocalyxin (PCX) from the outer plasma membrane to the newly formed apical domain. This transcytotic event consists of multiple steps, including internalization from the plasma membrane, transport through early endosomes and Rab11-positive recycling endosomes, and delivery to the apical membrane. These steps are known to be tightly coordinated by Rab small GTPases, which act as molecular switches cycling between active GTP-bound and inactive GDP-bound states. However, our knowledge regarding which sets of Rabs regulate particular steps of PCX trafficking was rather limited. Recently, we have performed a comprehensive analysis of Rab GTPase engagement in the transcytotic pathway of PCX during polarity establishment in 2-dimensional (2D) and 3-dimensional (3D) MDCK II cell cultures. In this Commentary we summarize our findings and set them in the context of previous reports.

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Introduction

Epithelia form monolayers that line most of the major cavities of the body. Epithelial cells act as sensory receptors, provide mechanical barrier, and facilitate transport across epithelial sheet. These functions can be performed thanks to asymmetrical division of their plasma membrane into 2 functionally and morphologically distinct domains, namely, the apical domain facing the organ lumen, and the basolateral domain contacting the underlying extracellular matrix. The most comprehensively characterized and most widely used epithelial cell line is Mardin-Darby canine kidney (MDCK) II.¹ In addition to forming flat monolayers on ordinary culture dishes, MDCK II cells form spheroid-like structures called “cysts” with a hollow lumen inside when they are cultured in the presence of extracellular matrix. The formation of this lumen provides an instructive *in vitro* model for studying mechanisms underlying the establishment of epithelial polarity and so far its usage allowed researchers to identify many important polarity regulators.²

One of the proteins localized exclusively to the apical domain, and hence often used as its marker, is podocalyxin (PCX), also known as gp135.³ PCX is a type I transmembrane protein with a short cytoplasmic tail and a large highly sialylated and glycosylated mucin domain

(Fig. 1). During the growth of MDCK II cells into cysts, PCX is transported from the outer plasma membrane to the newly formed apical domain and its transcytosis is a characteristic feature of the polarity establishment (transcytotic pathway is graphically summarized in Fig. 2). Moreover, delivery of PCX to the apical domain has been shown to be required for lumen opening during polarity development in MDCK II cells,^{4–7} which is likely a result of its high negative charge causing the repulsion between 2 adjacent membrane layers. Therefore, PCX is not only a good model cargo for studying apical transport but is also important for proper cyst morphogenesis.

Important coordinators of intracellular membrane trafficking in eukaryotes are Rab small GTPases—molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states and regulate formation, transport, tethering, and fusion of transport vesicles. In their active state, Rabs can interact with their specific downstream effectors, which carry out various functions.^{8–10} We have recently performed comprehensive colocalization and knockdown (KD) screenings of all 60 Rab GTPases in order to identify the candidate Rabs, which regulate PCX trafficking in MDCK II cells during polarity establishment, and we discovered interesting dissimilarities in cells growing under 2-dimensional (2D)

Figure 1. A schematic model of podocalyxin structure. The extracellular part of PCX consists of an extensively sialylated and glycosylated mucin domain, a cysteine-rich globular domain, and a juxtamembrane stalk. This is followed by a single-pass transmembrane domain, and a cytoplasmic tail, which can interact with various proteins as indicated. Adapted from Nielsen and McNaghy, 2009.⁵²

and 3-dimensional (3D) culture conditions (Fig. 3).⁷ Here, we describe our recent findings and observations in the context of previous reports.

Podocalyxin trafficking in 2D culture system

A single MDCK II cell seeded on an uncoated culture dish divide until forming a confluent 2D monolayer. During this process its membrane segregates into apical and basolateral domains, which are separated by tight junctions. Previous studies have suggested that in 2D system PCX was segregated into the apical domain through lateral diffusion rather than transcytotic recycling, although the authors noted that some PCX staining was visible in Rab11-positive endosomes, indicating that transcytosis occurs to some extent.⁴ Our recent observations further showed that, upon cell contact with the dish surface, PCX was indeed rapidly internalized, passed through early endosomes, and then was directed to Rab11-positive recycling endosomes, before being delivered to the apical domain (Fig. 2, upper).⁷ Thus, transcytosis rather than simply lateral diffusion is likely to be the mechanism of PCX segregation into the apical domain during polarity establishment.

The detailed mechanism of PCX endocytosis is largely unknown. Meder and colleagues have reported in their

study that PCX showed different localization at the early steps of polarity establishment in MDCK II cells from other apical proteins like placenta alkaline phosphatase (PLAP) or gp114, which, in contrast to PCX, remained localized all over the plasma membrane even after cells attached to the support.⁴ PLAP contains a clathrin recruitment motif¹¹ and in HEp2 epithelial cells is abundantly present in clathrin-coated vesicles,¹² suggesting its endocytosis to be clathrin-dependent. PCX in MDCK II cells is internalized much earlier than PLAP, which implies that the mechanism of its endocytosis differs from that of PLAP. Formation of plasma membrane-derived clathrin-coated vesicles is known to be tightly regulated on several endocytic stages by Rab5,¹³⁻¹⁵ yet the Rab5 knockdown (KD) did not induce accumulation of PCX at the basal membrane in freshly attached MDCK II cells. Instead, Rab5-depleted cells developed into 2D monolayers with PCX present at “lateral lumens” between adjacent cells. This phenotype was observed only when Rab5A or Rab5B, but not Rab5C, was knocked down.⁷ Interestingly, Stoops and colleagues have reported that a subset of PCX molecules, before their delivery to the apical domain, briefly traversed the basolateral membrane.¹⁶ It is thus tempting to speculate that, even though the primary endocytic event induced by the cell attachment is Rab5-independent, PCX traversing the basolateral membrane en route to the apical domain is endocytosed from the lateral site in a Rab5A/B-dependent manner. Consequently, blocking this secondary endocytic event by Rab5A or Rab5B depletion may cause retention of PCX at the lateral membrane and ectopic lumen formation. A similar lateral lumen phenotype was observed in Rab32-depleted cells,⁷ suggesting that Rab32 may also be involved in PCX internalization from the lateral membrane. Although there have been no reports showing direct involvement of Rab32 in clathrin-mediated endocytosis, Rab32 was observed at clathrin-coated vesicles in human melanoma cells.¹⁷ In our recent study, the only Rab whose knockdown impaired the primary endocytic event and caused actual retention of PCX near the basal membrane in freshly attached cells was Rab14.⁷ Actually, Rab14 has been reported to regulate apical targeting in MDCK cells and to be involved in early endocytic events,^{18,19} although the exact mechanism of its action is yet to be elucidated.

After internalization, PCX was directed to Rab11-positive perinuclear recycling endosomes (Fig. 2, upper), entering the so-called “slow” recycling pathway.²⁰ Rab11A itself has been reported to be involved in polarity establishment in 2D MDCK cells through interaction with its effectors Rab11-FIP2 and myosin Vb. Myosin Vb depletion or overexpression of its tail domain prevented the delivery of apical cargoes to the apical membrane²¹⁻²³ and also caused

Figure 2. Subcellular localization of PCX in MDCK II cells. Schematic models presenting PCX localization during cell polarization into 2D monolayers (xz cross-section view; upper) and 3D cysts (xy cross-section view; lower). PCX is shown in green, actin in red, and nucleus in blue. Transcytosis of PCX in both culture conditions occurs in the following orders: internalization of PCX from the outer plasma membrane (endocytosis), traffic through early and Rab11-positive recycling endosomes, and then export to the newly formed apical (or luminal) membrane (exocytosis). Candidate Rabs involved in each step of PCX trafficking in 2D monolayers and 3D cysts discussed in this Commentary are shown at the top and bottom, respectively. The Rabs with putatively different functions in PCX trafficking in 2D and 3D cells are shown in boldface. Numbers shown as superscript indicate reference numbers and asterisks indicate the Rabs that were identified only based on phenotypes of KD cells with no mechanical insight. © Mrozowska and Fukuda, 2016. Originally published in *J. Cell Biol.* doi:10.1083/jcb.201512024.⁷

the subapical retention of PCX in MDCK II 3D cysts.²⁴ We have confirmed the involvement of Rab11A in PCX trafficking in 2D MDCK II cells, where Rab11A KD caused retention of PCX near apical membrane and in scattered vesicles. However, this defect was observed only at early stages of cell growth and after extended culturing cells formed a normally polarized 2D monolayer with PCX being present at the apical domain.⁷ Casanova and colleagues have reported that Rab25, a close homolog of Rab11 (sometimes called Rab11C) that can also interact with Rab11-FIP2 and myosin Vb,^{25,26} was involved in apical recycling similarly to Rab11A, and that overexpression of Rab25 inhibited the apical delivery of polymeric immunoglobulin receptor.²⁷ In our observations, however, Rab25 depletion by specific siRNA treatment did not cause any defect in apical recycling or localization of PCX.⁷ It is thus possible that in 2D MDCK II cells Rab25 is dispensable for PCX trafficking, but in prolonged culture it can partially compensate for lack of Rab11A in Rab11A-depleted cells. In addition to Rab11-FIP2 and myosin Vb, the active form of Rab11A can interact with Rabin8, a guanine nucleotide exchange factor (GEF) for Rab8.^{28,29} A Rab11A–Rabin8–Rab8A cascade governs PCX delivery to the apical domain in MDCK II cysts and its disruption caused intracellular accumulation of PCX.³⁰ In Rab8A-KD

Figure 3. Rab GTPases affecting the localization of PCX in 2D and 3D MDCK II cells. The graph groups all Rabs knocked down in our screening (yellow circle) and Rabs whose knockdown (KD) influenced PCX trafficking in 2D monolayers (green circle) and 3D cysts (red circle). Rabs in the green and red overlap region influenced PCX trafficking under both conditions. © Mrozowska and Fukuda, 2016. Originally published in *J. Cell Biol.* doi:10.1083/jcb.201512024.⁷

2D MDCK II cells we also observed subapical accumulation of PCX-containing vesicles, which persisted even after the cells became fully polarized, in contrast to Rab11A-KD cells. A similar phenotype occurred in MDCK II cells with knocked down Rab13,⁷ which belongs to the same Rab subfamily as Rab8 and shares more than 60% amino acid identity with Rab8A.³¹ Rab13 has been shown to regulate apical delivery of cargoes transported through recycling endosomes.³² It is also responsible for maintaining the integrity of tight junctions and for the delivery of junctional proteins.^{33,34} In this last process Rab13 cooperates with Rab8A through the common effector JRAB/MICAL-L2 and together they coordinate the assembly of tight and adherent junctions.³⁵ These observations may suggest that PCX delivery to the apical membrane requires functional cell junctions. Stoops and colleagues have recently shown that the periciliary ring serves as the “hot spot” for PCX delivery to the apical membrane, but the spot is only used for newly synthesized PCX proteins and not for proteins in the recycled pool.¹⁶ On the other hand, it has previously been shown that in MDCK cells another apical protein, aminopeptidase, was delivered to the apical domain in close proximity to cell-cell contact sites and this process was not affected by cycloheximide treatment, indicating that observed exocytotic events should occur for the transcytosed pool rather than the freshly synthesized protein pool.³⁶ It is thus highly probable that the KD of Rab13 and/or Rab8A impairs PCX exocytosis by disrupting the delivery site for the recycled pool of PCX.

Apart from the “slow” recycling route through Rab11-positive pericentriolar endosomes, proteins can be delivered directly from early endosomes back to the plasma membrane (so-called “fast” recycling route).²⁰ The principal regulator of this pathway seems to be Rab35.^{37,38} It is important to note that Rab35 is the only known Rab that in its GTP-bound state can directly interact with PCX,⁶ although there are no studies showing that PCX is also transported to the apical domain through the fast recycling pathway. Cauvin and colleagues have recently shown that Rab35 was present at freshly generated endosomes, where it recruited its effector, lipid phosphatase OCRL, and facilitated clearing of PtdIns(4,5)P₂ (phosphatidylinositol 4,5-bisphosphate) from the endosomal membrane. Depletion of Rab35 or OCRL resulted in retention of internalized proteins in early endosomes abnormally rich in PtdIns(4,5)P₂, which subsequently induced excessive actin polymerization.^{38,39} Interestingly, Rab35 depletion in 2D MDCK II cells induced a similar phenotype – internalized PCX was retained in endosomes abnormally rich in actin. This phenotype was largely mimicked by OCRL KD, indicating that PCX exit from early endosomes was regulated through orchestrating Rab35 and OCRL.⁷ However, we cannot rule out the

possibility that direct interaction between Rab35 and PCX also contributes to this process.

Docking of PCX-containing vesicles to the apical membrane is mediated by Rab27A/B, which links the vesicles to the target membrane through its effector Slp2-a.⁴⁰ Tandem C-terminal C2 domains of Slp2-a recognize PtdIns(4,5)P₂-enriched apical membrane, while the N-terminal SHD domain binds to Rab27A/B localized on the vesicle surface.^{41,42} Consistent with this mechanism, KD of Rab27A/B or Slp2-a impaired vesicle docking and caused their retention under the apical membrane.^{7,41}

Podocalyxin trafficking in 3D culture system

When a single MDCK II cell is embedded in extracellular matrix analogs, such as matrigel or collagen, it spontaneously forms a 3D cyst with PCX localized at the luminal membrane. The trafficking pattern of PCX during cyst development is similar to the one in 2D cells, although the endocytosis is substantially delayed in comparison to 2D conditions (Fig. 2).⁷ Internalized PCX traffics to early endosomes and Rab11-positive recycling endosomes, and subsequently is delivered to the apical membrane initiation site (AMIS), where it assists in lumen opening.^{5,7,30}

At the initial stage of cyst formation (before lumen initiation), PCX is localized at the cortical membrane, where it forms a complex with ezrin and NHERF1. β 1-integrin activation by extracellular matrix components leads to PKC β II-dependent phosphorylation of PCX and NHERF1, disassembly of the PCX–ezrin–NHERF1 complex, and PCX internalization.⁵ Even though the signaling cascade, starting from β 1-integrin activation and leading to PCX internalization, has been well characterized, the engagement of Rab GTPases in this process is poorly understood. KD of endocytic Rab5B induced formation of large actin-rich vacuoles where PCX was retained,⁷ but the exact mechanism behind this phenotype is unclear. Rab35 depletion also affected PCX internalization in 3D cell culture, causing its retention at the outer plasma membrane. However, as we describe in detail further in this Commentary, the mechanism of Rab35 action in 2D and 3D cell cultures was remarkably different.

Like in 2D culture, in 3D cysts PCX is directed to Rab11-positive recycling endosomes and subsequently delivered to the cell-cell contact site, where the lumen is initiated. Rab11A recruits Rab8A/B to recycling endosomes through binding to Rabin8³⁰ and together they associate with myosin Vb and deliver PCX from recycling endosomes to the AMIS. Rab10, which can also associate with myosin Vb, was not required for proper

PCX targeting.²⁴ Indeed, in agreement with this report, our knockdown screening showed that both Rab11A and Rab8A depletion induced subapical accumulation of PCX, while Rab10 depletion had no effect.⁷ KD of Rab25 on the other hand, which did not influence PCX trafficking in 2D monolayers, in matrigel culture induced a phenotype similar to that of Rab11A-KD.^{7,30} Rab11A additionally recruits the exocyst complex through binding to Sec15A.⁴³ KD of Sec15A or Sec10, an exocyst subunit directly interacting with Sec15A, resulted in a great decrease in single lumen formation and an accumulation of PCX in Rab11A-positive vesicles.³⁰

Klinkert and colleagues have recently reported a novel role of Rab35 in docking of PCX-containing vesicles to the cleavage site of dividing MDCK II cells. They showed that during the first cell division activated Rab35 was localized at the cleavage furrow, where it tethered vesicles containing key apical proteins, e.g., aPKC, Cdc42, and Crumbs3, through direct interaction with a cytoplasmic tail of PCX. This interesting discovery shows a novel and unconventional mode of Rab-dependent vesicle targeting. Rab35 depletion disturbed docking of PCX-positive vesicles to the AMIS, leading to an inversion of apico-basal polarity of the cyst. Moreover, re-expression of a Rab35 binding-deficient mutant of PCX in PCX-KD cysts caused the polarity to invert.⁶ The authors explained this phenomenon through the following observations: PCX-containing vesicles also contained other key apical determinants, including aPKC, Cdc42, and Crumbs3, and when these vesicles failed to dock, these regulators relocated to the outer plasma membrane.⁶ However, KD of Rab27A or Rab11A, which also blocked the tethering of PCX-containing vesicles to the AMIS, did not induce polarity inversion, but rather caused subapical retention of these vesicles.^{30,41} These observations suggest that blocking of vesicle tethering to the AMIS is unlikely to be the sole reason for polarity inversion and an additional mechanism should be involved in Rab35-dependent polarity establishment. Because polarity inversion has previously been observed in cysts with perturbed pathways connected with Arf6 and β 1-integrin signaling^{5,44,45} and because Rab35 has already been shown to regulate β 1-integrin trafficking,⁴⁶ it is possible that a defect in Rab35-dependent β 1-integrin recycling is partially responsible for polarity inversion in Rab35-KD (or Rab35-knockout) cysts. It should be noted that this phenotype was mimicked by depletion of another Rab35 effector ACAP2, but not by OCRL that functions in PCX trafficking in 2D cell culture.⁷ Interestingly, ACAP2, also known as centaurin β 2, is a Rab35-associated GTPase-activating protein (GAP) for Arf6,⁴⁷ which together with Rab35 has been shown to regulate β 1-integrin trafficking.⁴⁶ We thus speculate that inverted polarity in Rab35-depleted cysts, in addition to being a result of a

defect in PCX-positive vesicle tethering, is also a consequence of dysregulation of interplay between Rab35, ACAP2, and Arf6, which leads to improper β 1-integrin recycling.

In 3D cysts, similarly as in 2D MDCK II cells, docking of PCX-containing vesicles to the apical domain is mediated by Rab27A and its effector Slp2-a, which binds to PtdIns(4,5)P₂ in the apical membrane and ensures single lumen formation. In addition, tethering and fusion of PCX-containing vesicles is controlled by Slp4-a in conjunction with Rab27/Rab3/Rab8. Consequently, KD of Slp2-a resulted in delayed fusion of PCX-containing vesicles with a site outside the AMIS (i.e., mislocalization of PCX), while KD of Slp4-a completely retained vesicles under the apical membrane.⁴¹ We have also confirmed that KD of Rab3 isoforms, Rab8A, and Rab27A resulted in accumulation of PCX under the apical membrane,⁷ endorsing the proposed mechanism of Rab3/Rab8/Rab27-coordinated function.

Discrepancies between 2D and 3D culture systems

When we compare Rab engagement in regulation of PCX trafficking in 2D and 3D culture described in the previous sections, we notice considerable differences between these 2 systems, despite the apparently similar trafficking pattern of PCX observed under 2D and 3D culture conditions (Fig. 2).

First, regulation of PCX endocytosis from the outer plasma membrane appears markedly different. Rab14 KD, which significantly perturbed PCX endocytosis in 2D cells, had virtually no effect in cysts. Also Rab5B, which presumably affected secondary endocytic event from the lateral membrane in 2D cells, instead of causing PCX retention at the membranes, induced formation of large actin-rich vacuoles where PCX was retained. Interestingly, this vacuolar phenotype was observed only for Rab5B-depleted cysts, while in 2D cells lateral lumens were present both in Rab5A- and Rab5B-KD cells.⁷

Second, regulation of exocytosis of PCX-containing vesicles also varied. KD of Rab25, which did not influence PCX trafficking in 2D monolayers, in matrigel culture induced a phenotype similar to that of Rab11A-KD,^{7,30} indicating that, even though under 2D conditions Rab25 is dispensable for PCX trafficking, it is required in 3D cysts. Also, Rab8A, which regulates apical exocytosis of PCX both in 2D and 3D cell cultures, showed some dissimilarities in downstream events. Bryant and colleagues have shown that in 3D cysts Rab8A mediated activation of a principal regulator of cell polarity, Cdc42,⁴⁸ and that Cdc42 depletion resulted in the accumulation of PCX in Rab8A/Rab11A-positive vesicles,

suggesting that Cdc42 governed the exit from these vesicles.³⁰ Strikingly, in 2D MDCK cells functional deletion of Cdc42 affected only the basolateral domain and not the apical domain by selectively inhibiting basolateral trafficking.^{49–51} Another noteworthy difference lies in Rab13, which, while playing a major role in apical exocytosis of PCX under 2D culture conditions, was completely dispensable for its trafficking in 3D cysts.⁷ In 3D system, in addition to Rab27A and Rab8A, Rab3 (especially Rab3B) was also required for efficient vesicle delivery to the apical domain,⁴¹ yet in 2D MDCK II cells KD of either of Rab3 isoforms caused only temporary retention of PCX-containing vesicles near cell-cell contact sites, and this weak phenotype disappeared with culturing time.⁷

Another interesting difference was observed for Rab35 whose depletion caused different phenotypes in 2D and 3D culture systems and which exerted its function through interaction with different effectors (OCRL in 2D and ACAP2 in 3D cell cultures). We speculated that Rab35–ACAP2 in 3D cysts were involved in β 1-integrin recycling whose disruption induced polarity inversion. This process may not be required for polarity formation in 2D cell culture, since we did not observe any polarity reversal in 2D Rab35-depleted cells, which rather retained PCX in actin-rich clusters (= “OCRL-depletion phenotype”; see the section about PCX trafficking in 2D culture system).

Conclusions

The trafficking of PCX, a model apical cargo and a regulator of single lumen formation, in polarized epithelial cells is governed by a vast array of Rab GTPases. Here, we have summarized the mechanisms behind all known Rab-dependent trafficking steps of PCX, yet still much remains to be uncovered in this subject. For example, Rab engagement in PCX endocytosis from the outer plasma membrane is practically a terra incognita and its characterization can be greatly beneficial to further our understanding of the establishment of epithelial polarity. Unexpectedly, the set of Rabs responsible for PCX trafficking in 2D culture system differs from the one in 3D system (Fig. 3). The reason behind this is most probably different cues for endocytosis (binding of extracellular matrix components to β 1-integrin in 3D cell culture versus physical contact with the dish in 2D cell culture) that trigger different mechanisms of PCX uptake. Also, the final site of PCX delivery is fundamentally different – although in both systems PCX is delivered to the apical domain, in 3D cysts this domain is created at the cell-cell contact sites, while in 2D monolayers it is ultimately established at the free membrane facing the medium.

Which of these pathways is actually taken by PCX in living organism as well as which Rab GTPases regulate this pathway remains to be determined. Finally, different engagement of Rabs in PCX trafficking in 2D and 3D cell cultures raises another important question – is the trafficking of other apical (or basolateral) molecules also regulated by different sets of Rabs in these 2 culture conditions? To answer this question, investigations and detailed comparisons of polarized trafficking of other molecules in 2D and 3D cell cultures are anticipated in the future.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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