Published in final edited form as:

Dev Dyn. 2012 September ; 241(9): 1385–1395. doi:10.1002/dvdy.23826.

Rab11 regulates planar polarity and migratory behavior of multiciliated cells in *Xenopus* embryonic epidermis

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Abstract

Background—*Xenopus* embryonic skin is composed of the superficial layer with defined apicobasal polarity and the inner layer lacking the apical domain. Multiciliated cells (MCCs) originate in the inner layer of the epidermal ectoderm and subsequently migrate to the surface. How MCCs acquire the apicobasal polarity and intercalate into the superficial layer during neurulation is largely unknown. As Rab11-dependent vesicle trafficking has been implicated in ciliary membrane assembly and in apical domain formation in epithelial cells, we assessed the involvement of Rab11 in MCC development.

Results—Here we report that Rab11 is specifically enriched and becomes apically polarized in skin MCCs. Interference with Rab11 function by overexpression of a dominant negative mutant or injection of a specific morpholino oligonucleotide inhibited MCC intercalation into the superficial layer. Dominant negative Rab11-expressing MCC precursors revealed intrinsic apicobasal polarity, characterized by the apical domain that is not normally observed in inner layer cells. Despite the presence of the apical domain, the cells with inhibited Rab11 function were randomly oriented relative to the plane of the tissue, thereby demonstrating a defect in planar polarity.

Conclusion—These results establish a requirement for Rab11 in MCC development and support a two-step model, in which the initial polarization of MCC precursors is critical for their integration into the superficial cell layer.

Keywords

epidermis; cell polarity; Rab11; intercalation; multiciliated cells; *Xenopus*

INTRODUCTION

Cell polarity, a key property of eukaryotic cells, is essential for cell behavior during embryonic development and adult tissue homeostasis. All epithelial tissues are characterized by the apicobasal polarity, which is regulated by the apical protein complexes, including atypical PKC/Par3/Cdc42 and the Crumbs complex, and by the basolateral complexes, such as Lethal giant larvae/Discs large/Scribble (reviewed in Bryant and Mostov, 2008; St

Johnston and Sanson, 2011). These proteins function as intrinsic regulators of cell polarity and also control the formation of intercellular junctions, located at the boundary of the apical and the basolateral domains (Shin et al., 2006; Etienne-Manneville, 2011). The acquisition of epithelial characteristics, or epithelialization, is a common phenomenon that accompanies neural tube formation, somitogenesis, and multiple sites of organogenesis and branching morphogenesis (Lowery and Sive, 2004; Andrew and Ewald, 2010; Watanabe and Takahashi, 2010; Datta et al., 2011). This process involves both cell-intrinsic mechanisms and non-cell autonomous mechanisms that depend on the polarity of surrounding cells (Baas et al., 2004; Umeda et al., 2006; Bryant et al., 2010; Campbell et al., 2010). The relative contribution of these mechanisms to the establishment and maintenance of epithelial polarity remains largely unknown.

Xenopus embryonic skin is composed of the inner and the outer (superficial), cell layers and contains cells with distinct functions, such as multiciliated cells (MCCs), secretory goblet cells and ion transport cells or ionocytes (Hayes et al., 2007; Dubaissi and Papalopulu, 2011; Quigley et al., 2011). The assembly of this complex tissue depends on the intercalation of some inner cells into the superficial cell layer (Stubbs et al., 2006; Dubaissi and Papalopulu, 2011; Quigley et al., 2011; Werner and Mitchell, 2012). One type of intercalating cells are MCCs, which are specified in the inner layer of epidermal ectoderm (Drysdale and Elinson, 1992; Deblandre et al., 1999). The ciliated cell precursors intercalate into the superficial layer which has defined epithelial properties, such as tight junctions and the apical surface (Fesenko et al., 2000; Chalmers et al., 2003; Stubbs et al., 2006). When the cells reach the superficial layer by late neurula stages, they develop the apical surface, differentiate into MCCs, and start ciliogenesis (Stubbs et al., 2006). Thus, the development of MCCs represents an *in vivo* embryological and cell-biological model for studies of epithelial polarization.

Several models can explain the mechanism of MCC precursor integration into the superficial cell layer. One potential model is that the MCC precursors migrate (or intercalate) into the apical surface in a directed fashion, and establish junctional communication with the surrounding superficial epithelial cells to acquire the apical-basal polarity. Alternatively, these cells may develop an intrinsic apical-basal polarity, which then helps them to intercalate into the superficial layer. To distinguish between these possibilities, we examined a role for the Rab11 GTPase, which regulates the establishment of the apicobasal polarity and cell junctions in many systems (Desclozeaux et al., 2008; Roeth et al., 2009; Bryant et al., 2010). Rab11-dependent vesicle trafficking is required for directional protein targeting to the plasma membrane and serves to regulate cell and tissue morphology, cell migration and ciliogenesis (Ullrich et al., 1996; Jones et al., 2006; Jing and Prekeris, 2009; Das and Guo, 2011; Kawauchi, 2011). Importantly, Rab11 initiates lumen formation in MDCK cells by targeting active Cdc42 to the presumptive apical domain (Bryant et al., 2010). Additionally, Rab11-dependent recycling of E-cadherin is important for the establishment of cell polarity during epithelial cell morphogenesis and the maintenance of adherens junctions in *Drosophila* embryonic ectoderm (Desclozeaux et al., 2008; Roeth et al., 2009). In the developing mouse brain, trafficking of N-cadherin by Rab5 and Rab11 is required for neuronal migration relative to radial glia fibers (Kawauchi et al., 2010). Recent studies

showed that Rab11 is located at the base of cilia and initiates ciliary membrane formation by targeting Rabin 8, another GTPase, to the centrosome (Knödler et al., 2010; Westlake et al., 2011). Together, these findings make Rab11 a possible candidate for the regulation of MCC precursor polarity, migration and ciliogenesis.

Based on these known functions of Rab11, we hypothesized that Rab11 is involved in MCC development and might regulate their apico-basal polarity and ciliogenesis. When Rab11 function was downregulated by a dominant-negative mutant and a specific morpholino oligonucleotide, MCC precursors failed to intercalate into the superficial layer. Nevertheless, they established a clear apical-basal polarity, and developed the apical domain within the inner cell layer, despite being misoriented relative to the apical surface. These results indicate a role of Rab11 in polarizing MCCs in the plane of the epithelial tissue, a process that is commonly referred to as planar polarity. Our observations support a model, in which MCCs acquire the apicobasal polarity in a Rab11- and cell junction-independent manner, but require Rab11 for planar polarity and for their radial intercalation into the superficial epithelial layer.

RESULTS

Rab11 is expressed in MCCs and their precursors in *Xenopus* epidermis

We first examined Rab11 localization in *Xenopus* skin by immunostaining of embryos sectioned at neurula and tailbud stages. In contrast to the previous study, in which Rab11 was detected in secretory goblet cells (Hayes et al., 2007) at the tailbud stage, Rab11 was enriched in MCCs, visualized by an antibody to acetylated tubulin (Fig. 1A, St. 28). All cells that were stained for Rab11 in these experiments were also positive for acetylated tubulin, indicating that neither goblet cells nor ionocytes were stained. Since at earlier stages, MCC precursors also contain acetylated tubulin (Chu and Klymkowsky, 1989), we used the same antibody to assess the distribution of Rab11 at neurula stages. At stage 16, we observed that MCC precursors contain high level of acetylated tubulin, although the signal was weaker than in differentiated MCCs, and express Rab11 (Fig. 1A, St. 16). Next, we examined whether the reported Rab11 distribution in pericentriolar vesicles (Ullrich et al., 1996; Knödler et al., 2010) is maintained in these cells. Consistent with this view, Rab11 was concentrated at the apical surface of differentiated MCCs, where γ -tubulin-positive basal bodies are located (Fig. 1B; Park et al., 2008). Apical view of MCCs showed that Rab11 is localized adjacent to basal bodies (Fig. 1C). Some Rab11 staining was detectable at acetylated tubulin-positive cilia (Fig. 1A, insets). These observations imply that Rab11-dependent vesicle trafficking is involved in MCC development.

Rab11 is polarized in MCC precursors

To study early steps of MCC development, we examined their precursors, which originate in the inner cell layer of *Xenopus* epidermal ectoderm (Drysdale and Elinson, 1992; Deblandre et al., 1999). As Rab11 localizes to the pericentriolar region, we labeled the cells using a basal body protein rather than acetylated tubulin (Fig. 2). Centrin-2 is a centriolar/basal body marker of MCC differentiation in vitro (Laoukili et al., 2000; Vladar and Stearns, 2007). We observed that after gastrulation centrin-2 expression is gradually upregulated in MCC

precursors that are positioned just below superficial cell junctions (Figs. 2A and B). Interestingly, the centrin-2-positive material was polarized in the cytoplasm, even at earlier stages (Fig. 2A). At later stages, acetylated tubulin was similarly concentrated towards the apical embryonic surface (Fig. 2B, St 16), possibly reflecting the polarization of the centrioles/basal bodies with respect to the future direction of migration.

Using centrin-2 as a marker for MCC precursors, we examined Rab11 distribution in these cells. Since the Rab11 signal was undetectable at stage 14, we assessed the localization of exogenous Rab11. Injection of a low dose of Rab11 RNA (0.1 ng) revealed Rab11 near the polarized centriolar material in 74.3 % of MCC precursors (Figs. 2C and D). By contrast, the GDP-bound form of Rab11 (Rab11S25N), when expressed at comparable levels (Fig. 2D), did not concentrate in the pericentriolar area, as observed in other system (Fig. 2C; Ullrich et al., 1996; Ren et al., 1998). These observations suggest that active Rab11 may regulate polarized vesicle trafficking to the presumptive apical surface of MCC precursors.

Rab11 is required for the intercalation of ciliated cell precursors

MCC precursors intercalate into the superficial layer and start to differentiate during neurula stages (Stubbs et al., 2006). To test whether Rab11 is involved in the intercalation process, we interfered with its function by microinjecting early embryos with 1 ng (high dose) of mRNAs encoding wild type Rab11 or Rab11S25N, a dominant negative mutant (Ullrich et al., 1996; Knödler et al., 2010). In stage 23 embryos, in which MCC have completed their migration and all localize in the superficial layer (Deblandre et al., 1999; Stubbs et al., 2006), Rab11S25N RNA interfered with the ability of MCC precursors to intercalate, since 20 % of centrin-2-positive cells (n=1198) have remained in the inner layer (Figs. 3A and B). The phenotype was not restored until stage 28, indicating MCC precursor migration is blocked by Rab11S25N expression (Fig. 3C). When the embryos were injected with the same amount of RNAs for wild-type Rab11 or the GTP-bound form (Rab11Q70L; Ullrich et al., 1996), the migration of MCC precursors was not affected (Figs. 3A and B). The migration defect was observed in 5.8 % of examined MCC precursors (n=1047) in embryos injected with GFP-CAAX RNA as compared to 6.9 % (n=1181) in embryos coinjected with GFP-CAAX and wild-type Rab11 RNAs. In uninjected embryos, the migration defect was in 1.5% of MCC precursors (n=1163). These observations indicated that wild-type Rab11 did not have a gain-of-function effect on MCC migration. Similar levels of Rab11 proteins were expressed in these experiments (Fig. 3D). These results suggest that Rab11 regulates the intercalation of MCC precursors into the superficial cell layer.

Next, we tested whether Rab11 is required for the intercalation of ionocytes (Mir et al., 2007; Dubaissi and Papalopulu, 2011; Quigley et al., 2011). At stage 25, the intercalation of Foxi1e-positive ionocytes into the superficial layer was complete in both control and Rab11S25N-injected embryos (Fig. 3E; Dubaissi and Papalopulu, 2011; Quigley et al., 2011), indicating that Rab11 function is specific for MCC intercalation.

A different way to assess the efficiency of MCC precursor intercalation into the superficial ectodermal layer is to measure their apical surface. We, therefore, examined the ZO-1-positive apical domain in the epidermis of stage 28 embryos that were injected with Rab11 and Rab11S25N RNAs, along with GFP-centrin-2 RNA to label MCCs (Haigo et al., 2003;

Yasunaga et al., 2011). Rab11S25N, but not wild-type Rab11, inhibited apical area expansion in MCCs after their integration into the superficial layer (Figs. 3F). This result is consistent with the requirement of Rab11 for the migratory behavior of MCC precursors. Of note, we consistently find that the apical area of goblet cells surrounding MCCs is increased in DN-Rab11-injected embryos (data not shown), although whether this is a direct effect of Rab11S25N on goblet cells or an indirect result of inhibited MCC intercalation is currently unknown.

We next used Rab11-specific morpholino (MO) injection to independently evaluate Rab11 function in MCC development (Fig. 4). This MO efficiently blocked Rab11 translation *in vivo* from the Rab11 mRNA containing MO target site at its 5' UTR, but had no effect on translation of mRNA lacking the target site (Fig. 4A). Moreover, the Rab11 MO significantly reduced the level of endogenous Rab11 (Fig. 4B). Injection of Rab11 MO caused MCC precursor migration defects that were similar to the effect of Rab11S25N RNA (Fig. 4C). At stage 23, these cells revealed the patterns of centrin-2 and acetylated tubulin that resembled those of MCC precursors at stage 14-16 (Fig. 2 and Fig. 4D), indicating MCC development might be delayed. After intercalation, the apical area of MCCs in Rab11 morphants was smaller than that of CO MO-injected embryos (Fig. 4E), further suggesting that Rab11 is required for MCC precursor intercalation.

Requirement of Rab11 for the orientation of the apical domain in MCC precursors

Rab11 is known to function in epithelial morphogenesis by trafficking apical and junctional proteins (Desclozeaux et al., 2008; Roeth et al., 2009; Bryant et al., 2010). To test whether the inhibition of intercalation is due to apical-basal polarity defects in MCC precursors, we assessed the localization of the tight junction marker, ZO1 and atypical PKC, an apical marker. In Rab11S25N RNA-injected embryos, MCC precursors that failed to move into the superficial layer by stage 23 developed ectopic ZO-1-positive domain (59.9 % cells, n= 167) (Fig. 5A). The stained structures were also positive for atypical PKC, indicating that they have apical properties (Fig. 5B). In these cases, the orientation of ectopic apical structures and basal bodies was randomized relative to the apical surface of the superficial cells (Fig. 5C). These findings indicate that Rab11 functions to regulate the planar polarity (i. e., proper orientation) of MCC precursors, which might be required for their integration into the superficial cell layer.

DISCUSSION

In this study, we examined a role for Rab11 in the development of MCCs in *Xenopus* embryonic epidermis. We found that Rab11 is enriched in MCCs and becomes polarized during early stages of MCC development. Interference with the Rab11 function by a dominant-negative mutant and with a specific morpholino antisense oligonucleotide resulted in the failure of MCC precursors to intercalate into the superficial ectodermal layer. Although the apical domain formed in MCC precursors even in the presence of the dominant negative Rab11 mutant, it was randomly oriented with respect to the apical surface of the epidermis. Based on these observations, we conclude that Rab11 functions to regulate the planar polarity of MCC precursors and their intercalation into the superficial cell layer.

Whereas the molecular mechanism of the intercalatory behavior of epidermal MCCs is largely unknown, the polarization of these cells appears to be a critical step in this process. Consistent with this possibility, intercalating MCC precursors have been reported to form protrusions that are first integrated into the apical surface of the superficial cell layer, followed by the expansion of the apical surface (Stubbs et al., 2006). We found that both centrin-2 and Rab11 are apically polarized in MCC precursors, similarly to differentiated MCCs. In polarized epithelial cells, the microtubule network from subapically localized centrosomes regulates apical targeting of recycling endosomes (reviewed in Müsch, 2004). Therefore, polarized centrosomes in MCC precursors may contribute to the regulation of spatially restricted vesicular trafficking to mediate further changes in cell polarization (Fig. 6A). This polarization is predicted to take place in the intercalating inner cells, such as MCC and ion transport cells (Hayes et al., 2007; Dubaissi and Papalopulu, 2011; Quigley et al., 2011). Our observations indicate that the localized expression of Rab11 in MCC precursors controls MCC-specific intercalation in *Xenopus* ectoderm, whereas the mechanism mediating the intercalation of ion transport cells remains currently unclear.

Polarized localization of Rab11 in MCC precursors implies a function in cell polarization through the control of apical and junctional proteins. During MDCK cyst formation, Rab11 regulates the targeting of active Cdc42 to promote apical membrane and lumen formation (Bryant et al., 2010). Disruption of Rab11 function results in multi-lumen formation, implying that some apical proteins are mislocalized (Bryant et al., 2010). In newly polarized MDCK monolayers, Rab11 contributes to epithelial polarity by regulating the recycling of E-cadherin to the basolateral surface (Lock and Stow, 2005; Desclozeaux et al., 2008). In this system, Rab11S25N mutant induces abnormal accumulation of E-cadherin at the apical surface, which inhibits normal polarization (Desclozeaux et al., 2008). In MCC precursors, Rab11S25N did not interfere with the formation of the apical domain, suggesting that the targeting of apical and junctional proteins to the plasma membrane occurs normally. However, these apical domains were randomly oriented relative to the apical surface, as compared to MCCs that successfully integrated into the superficial layer. This observation suggests that Rab11 is not necessary for the establishment of the intrinsic apical-basal polarity in these cells, but functions to orient MCC precursors in the tissue plan (i. e., establish planar polarity) (Fig. 6B). The misorientation of the apical domain in these cells may prevent their integration into the superficial layer. Our results are reminiscent of the reported function of Rab11 for cell intercalation during *Drosophila* tracheal morphogenesis, for which the balance between internalization and recycling of the junctional proteins appears critical (Shaye et al., 2008). Thus, the analysis of endocytosis may help assess whether the Rab11-dependent recycling of E-cadherin or other adhesion receptors is necessary for MCC intercalation.

Acquisition of the apical-basal polarity by differentiating MCCs might play a causal role in MCC precursor intercalation into the superficial layer (Fig. 6). It appears that the Rab11-independent apical domain formation and the Rab11-dependent planar polarity of MCC precursors are both necessary for their migration into the superficial cell layer. To further study the relationship between the orientation of the apical domain and MCC precursor intercalation, one would need to understand the roles of cell polarity proteins that are

responsible for the intrinsic apical-basal polarity. For example, in *Drosophila* renal tubules, overexpression of atypical PKC mutant, which disrupts apicobasal polarity in intercalating satellite cells, also inhibits their migration into the epithelium (Campbell et al., 2010). In addition, both polarity and migratory behavior of these cells depend on the polarity of neighboring epithelial cells (Campbell et al., 2010). Therefore, we currently cannot exclude an alternative possibility that the intercalation of MCC precursors helps establish their planar polarity, due to junctional communication with superficial layer cells. In summary, Rab11 is involved in both planar polarity of MCC precursors and their intercalatory behavior, however, the causal relationship between these two processes still remains to be clarified.

The cellular targets of Rab11 are largely unknown. Rabin8, a guanine nucleotide exchange factor for Rab8, is one of the downstream effectors of Rab11 during ciliogenesis (Bryant et al., 2010; Knödler et al., 2010; Westlake et al., 2011). The Rab11-Rabin8-Rab8 cascade is involved in two independent processes, de novo lumen formation from unpolarized cells and ciliary membrane formation (Das and Guo, 2011). Consistent with the reported requirement of Rab11 in ciliogenesis, we observed fewer and shorter cilia in Rab11S25N-expressing MCCs as compared to uninjected MCCs (Suppl. Fig. 1). At present, we cannot separate a role of Rab11 in cilia formation from its function in cell polarity and intercalation, which precedes MCC differentiation and ciliogenesis. Future studies are needed to examine the relationship between the polarization of MCC precursors and ciliogenesis.

EXPERIMENTAL PROCEDURES

Plasmid constructs and antibodies

Xenopus Rab11 cDNA clone was purchased from Open Biosystems (accession number: BC081187) and its coding sequence was subcloned into pXT7 (untagged Rab11) or pCS2+-EGFP (GFP-Rab11). S25N and Q70L mutants were made by single-primer based mutagenesis as described (Itoh et al., 2005) and the mutations were confirmed by sequencing. Rab11 mRNA which is sensitive to Rab11 MO has an additional 5' UTR sequence which is a target site of Rab11 MO. Constructs encoding GFP-CAAX (Ossipova et al., 2007) and Centrin-2 GFP (Yasunaga et al., 2011) were previously described. Antibodies against Rab11 (Zymed, rabbit and BD biosciences, mouse), γ -tubulin (GTU-88, Sigma, mouse), acetylated-tubulin (6-11 B-1, Sigma, mouse), α -tubulin (B-5-1-2, Sigma, mouse), GFP (B-2, Santa Cruz, mouse), ZO-1 (Invitrogen, mouse; Zymed, rabbit), PKC ζ (C-20, Santa Cruz, rabbit) were used. Rabbit polyclonal antibodies for *Xenopus* centrin-2 were raised against the complete coding sequence of *Xenopus* centrin-2 fused to GST and affinity-purified with the membrane-blotted antigen using standard techniques (Itoh et al., 2005). Anti-Foxi1 antibody was affinity purified from sera of rabbits immunized against the peptides STNNTLYNREGTEV (aa 360-373) and NSSPGTDDSPKRS (aa 253-266) of the nonconserved carboxy-terminal region of the protein (Suri et al., 2005).

mRNAs and morpholinos

Capped mRNA was made by in vitro transcription with T7 or SP6 promoter using mMessage mMachine kit (Ambion). For morpholino (MO) injection, control MO (5'-CTC

CGC ACC CGG TGA TGC GAT TTG G-3') and Rab11-specific MO (5'-TAC CCA TCG TCG CGG CAC TTC TGA C -3') were purchased from Gene Tools.

Manipulation of *Xenopus* embryos and injection

Xenopus laevis eggs were fertilized in vitro and dejellied with 3 % cysteine (pH 7.5-7.8) (Yasunaga et al., 2011). The embryos were cultured in 0.1× Marc's modified Ringer's solution (MMR) (Newport and Kirschner, 1982) until indicated embryonic stages (Nieuwkoop and Faber, 1994). For microinjection of mRNA or MO, four-cell embryos were transferred into 3 % Ficoll/0.5× MMR and 10 nl solution per injection was injected into 2-4 cells, according to the purpose of experiments. Indicated amounts in figure legends are the amount of injected mRNA or MO per blastomere. To test the phenotypes, animal pole injection in 1 dorsal and 1 ventral side was performed. After injection, the medium was changed to 0.1 × MMR before gastrulation.

Embryo lysate preparation and immunoblotting

Xenopus embryos were lysed in the buffer, containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1mM EDTA, 1 % Triton X-100, 10 mM NAF, 1 mM Na₃VO₄ and 1 mM PMSF). The supernatant was subjected to immunoblot assay after centrifugation at 12,000 g for 10 min at 4°C. For immunoblotting of MO-injected lysates, animal caps were dissected in 0.6× MMR at stage 9 and cultured until intact embryos developed to stage 15/16. Immunoblot analysis was carried out according to the standard protocol (Itoh et al., 1998). Image was captured by LAS-3000 luminescent analyzer system (Fujifilm).

Immunohistochemistry

The embryos were fixed with Dent's fixative (80% Methanol: 20% DMSO) for 2hr at RT or overnight at 4°C and rehydrated with PBS washing. For cryosectioning, the embryos were embedded in the solution containing 15% cold fish gelatin and 15% sucrose. To observe ciliated cells in apico-basal axis, the embryos were cryosectioned in the transverse plane. Cryosectioned samples were incubated with blocking solution for 30 min, primary antibodies for 2 hr and secondary antibodies for 30 min. For whole mount immunostaining, dehydrated embryos with methanol were incubated with blocking solution for 1 hr and followed by overnight incubation for primary and secondary antibodies (Lee et al., 2008). To see the apical surface of ciliated cells, skin was peeled off from tadpole stage embryos and mounted on a slide glass, after whole mount immunostaining.

Fluorescence microscopy and quantification

Immunofluorescence images were captured using a fluorescence microscope (AxioImager, Zeiss) and Axiovision imaging software (Zeiss). Centrin-2-positive cells in the inner layer were counted in the injected side. The quantification results were pooled from two independent experiments. Results are shown as means ± standard errors (s. e.), statistical analysis was carried out with SigmaPlot (Systat Software, Inc.). P-values were determined using Student's t-test. Image processing and measurements of apical area, orientation and protein band intensity were performed using Image J software (NIH). Scale bars in figures are 10 µm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Carlo Iomini for comments on the manuscript and members of the Sokol laboratory for helpful discussions. We also thank Takayuki Yasunaga and Keiji Itoh for their help in the preparation of the antibody to centrin 2. This work has been supported by NIH grants to S. S. and D. W.

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- Rab11 is enriched and polarized in multiciliated skin cells and their precursors.
- Interference with Rab11 function in ciliated cell precursors causes migration defects and formation of randomly oriented apical domains.
- Rab11 is required for the initial polarization of ciliated cell precursors that is necessary for their intercalation into the superficial ectodermal layer.

Fig. 1.

Rab11 localizes to the pericentriolar region in multiciliated cells (MCCs) A: Transverse sections of tailbud (stage 28) or neurula (stage 16) embryos were co-immunostained with anti-Rab11 and anti-acetylated tubulin (Ac-tub) antibodies. Nuclei are stained with DAPI. Arrows indicate Ac-tub-positive MCC precursors. Insets show the presence of Rab11 at cilia as revealed by costaining with acetylated tubulin antibody (arrowheads). Dotted lines mark the apical surface of embryonic epidermis. B: The embryos were immunostained for Rab11 and γ -tubulin (γ -tub). Arrow indicates the apicobasal polarity of MCC and dotted lines mark cell boundaries. C: Apical view of a MCC at stage 28, marked with dotted lines. Scale bar, 10 μ m.

Fig. 2.

Rab11 has a polarized distribution during MCC precursor migration A: Four-cell embryos were injected with GFP-CAAX RNA (0.1 ng) to label cell boundaries and fixed at indicated stages. The embryos were sectioned and stained with anti-GFP and anti-centrin-2 (Cen2) antibodies. B: MCC precursors are labeled in neurula stage embryos by immunostaining for acetylated tubulin (Ac-tub) and centrin-2 (Cen2). C: Four-cell embryos were injected with RNAs encoding GFP-Rab11WT (0.1 ng) or GFP-Rab11S25N (0.1 ng). The embryos were fixed at stage 14 and immunostained with anti-GFP and anti-centrin-2 (Cen2) antibodies. Dotted lines mark the boundaries of MCC precursors. Arrow indicates polarized Rab11. D: Immunoblotting reveals equal expression levels of GFP-Rab11 constructs in lysates of injected embryos (stage 11). Uninjected embryo lysate is a negative control, α-tubulin (α-tub) is a control for loading. Scale bar, 10 μm.

Fig. 3.

A dominant negative form of Rab11 inhibits MCC precursor intercalation into the superficial cell layer. **A:** Four-cell embryos were injected with RNAs (1 ng) encoding WT (wild type), S25N or Q70L Rab11 constructs and GFP-CAAX RNA (0.2 ng) for lineage tracing. The embryos were fixed at stage 23 and immunostained for GFP and centrin-2 (Cen2). Arrow indicates a Cen2-positive cell in the inner layer. **B:** The percentage of Cen2-positive cells in the inner layer was calculated with respect to the total number of scored Cen2-positive cells (1243 for wild-type Rab11, 1198 for Rab11S25N and 560 for Rab11Q70L-expressing embryos). N is the number of examined embryos. **C:** The frequency of defective MCC precursors was compared in stage 23 (1181 cells for wild-type Rab11 and 1127 cells for Rab11S25N) and stage 28 (954 cells for wild-type Rab11 and 862 cells for Rab11S25N) embryos, as described in B. n.s.; not significant. **D:** Rab11 protein levels in stage 11 embryo lysates, revealed by anti-Rab11 antibody. α -tubulin is a loading control. **E:** Stage 25 embryos were immunostained for GFP and Foxi1e. The percentage of Foxi1e-positive cells in the inner layer was calculated with 10 embryos. The number of examined Foxi1e-positive cells is 951 for GFP-CAAX, 993 for wild-type Rab11 and 1062 for Rab11S25N. **F:** Four-cell embryos were injected with Rab11WT or S25N RNAs (1 ng each). GFP-Cen2 RNA (0.1 ng) was coinjected to mark RNA-injected MCCs. Whole mount staining for GFP and ZO-1 is shown for stage 28 embryos. The apical surfaces of MCCs were compared for 120 cells from 6 embryos in each group. A.U., arbitrary unit. All graphs show means $\pm$ standard errors.

Fig. 4.

Defect in MCC intercalation after Rab11 depletion. A: Two-cell embryos were injected with 25 ng of control (CO) or Rab11 morpholino (MO) per blastomere, along with Rab11 RNAs (1 ng per blastomere). Rab11r does not contain MO target sequence.

Stage 15/16 embryo lysates were subjected to immunoblot analysis with anti-Rab11 antibody. B: Four-cell embryos were injected with 50 ng of CO MO or Rab11 MO per each blastomere and levels of endogenous Rab11 were compared by immunoblotting as described in A. The number indicates the ratio of Rab11 and α-tubulin. C: The percentage of Cen2-positive cells in the inner layer was quantified in the indicated number of embryos. Total cell numbers examined are 1277 for CO MO and 1065 for Rab11 MO. D: The embryos injected with indicated MOs (25 ng) were fixed at stage 23 and immunostained for centrin-2 (Cen2) and acetylated tubulin (Ac-tub). Dotted lines mark the apical surface of superficial cells. E: Four-cell embryos were injected with indicated amount of CO MO or Rab11 MO, along with GFP-Cen2 RNA (0.1 ng). The apical areas of MCCs were compared as shown in Fig. 3F. All graphs show means ± standard errors.

Fig. 5.

Misoriented apical domains in the inner cells expressing dominant negative Rab11. **A:** Four-cell embryos were co-injected with Rab11WT or S25N mRNAs (1 ng), and GFP-CAAX RNA (0.2 ng) to label cell boundaries. Stage 23 embryos were immunostained for centrin-2 (Cen2) and ZO-1. Intense green signals are centrin-2-positive basal bodies. Arrows indicate apical structures in the inner cells. **B:** The embryos were immunostained for ZO-1 and aPKC. **C:** Apical domain orientation was scored by measuring the angle (α) between the surface plane and the tangent line to the apical surface of the MCC precursor. Cen2-positive cells in the superficial layer or inner layer were examined in Rab11WT or Rab11S25-injected embryos, respectively. Approximately 50 cells from 3 embryos were used for the quantification, and the experiment was repeated twice. Representative images for each group are shown in **A**.

Fig. 6.

A model for Rab11 role in MCC development. A: Rab11 orients MCC precursor intercalation. (a) MCC precursors are specified in the inner layer of embryonic epidermis and have polarized Rab11 distribution. (b) Directional trafficking of Rab11-positive vesicles orients the nascent apical domain in the direction of migration (grey arrow). (c) The cell intercalates into the superficial layer and expands its apical surface. B: Rab11 regulates the planar polarity of MCC precursors that is necessary for their intercalation. (a) During the intercalation process, MCC precursors develop apical and basolateral domains and become aligned according to the planar polarity of the ectoderm. (b) Rab11S25N disrupts planar polarization of MCC precursors, but does not +interfere with their intrinsic apicobasal polarity.