Rab11a regulates syntaxin 3 localization and microvillus assembly in enterocytes

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ABSTRACT

Rab11a is a key component of the apical recycling endosome that aids in the trafficking of proteins to the luminal surface in polarized epithelial cells. Utilizing conditional Rab11a-knockout specific to intestinal epithelial cells, and human colonic epithelial CaCo2-BBE cells with stable Rab11a knockdown, we examined the molecular and pathological impact of Rab11a deficiency on the establishment of apical cell polarity and microvillus morphogenesis. We demonstrate that loss of Rab11a induced alterations in enterocyte polarity, shortened microvillar length and affected the formation of microvilli along the lateral membranes. Rab11a deficiency in enterocytes altered the apical localization of syntaxin 3. These data affirm the role of Rab11a in apical membrane trafficking and the maintenance of apical microvilli in enterocytes.

KEY WORDS: Rab11, Rab8, Enterocyte, Intestinal polarity, Microvilli, Syntaxin 3

INTRODUCTION

The maintenance of polarity in epithelial cells requires the establishment of specialized apical structures including apical microvilli and primary cilia (Rodriguez-Boulan and Powell, 1992). In intestinal epithelia, which do not possess primary cilia, the apical microvilli are organized into a tightly packed brush border structure that provides for an amplified absorptive surface. Investigations over the past several years have established a role for elements of the apical recycling system in the regulation of apical brush border assembly and maintenance. As in the case of primary cilia formation (Nachury et al., 2007; Westlake et al., 2011; Westlake et al., 2007), the small GTPases Rab11a and Rab8a both participate in trafficking required for the assembly of the apical intestinal microvilli (Dhekne et al., 2014; Knowles et al., 2014). Rab8a and Rab11a regulate plasma membrane recycling by nucleating multi-protein complexes that participate in the processes required for targeted delivery of recycling cargoes back to membrane surfaces (Baetz and Goldenring, 2013; Bryant et al., 2010; Roland et al., 2011). Previous investigations have suggested that knockout of Rab8a in mice leads to a deficit in apical microvillus structures (Sato et al., 2014; Sato et al., 2007). Similarly, inactivating mutations in MYO5B, which occur in microvillus inclusion disease, elicit profound losses in apical microvilli and alterations in polarity (Knowles et al., 2014; Ruemmele et al., 2010; Thoener et al., 2014). Our recent investigations have demonstrated that associations of Rab11a and Rab8a with MYO5B account for specific aspects of the enterocyte phenotypes observed in microvillus inclusion disease (Knowles et al., 2014). All of these studies place the endosomal recycling system at the center of regulation of the apical brush border.

Studies over the past decade have established the particular association of Rab11a with the apical recycling system in polarized epithelial cells. Whereas the plasma membrane recycling system in non-polarized cells utilizes Rab11a as a general regulator, in polarized systems, Rab11a appears to localize specifically within the apical recycling system and does not participate in basolateral recycling of cargoes such as transferrin receptor. Thus, Rab11a is functionally linked to the trafficking of apical cargos in polarized epithelial cells. Previous studies have noted the crucial role for Rab11a in the apical recycling of the H+/K⁺-ATPase in gastric parietal cells. In human parietal cells, immuno-isolation of H⁺/K⁺-ATPase-containing tubulovesicles has demonstrated the presence of Rab11a on parietal cell recycling vesicles as well as SNARE proteins required for membrane fusion events. The SNARE core complex components must be delivered to the membranes by trafficking machinery, and failure to target these proteins disrupts vesicular trafficking in polarized cells (Reales et al., 2011). In parietal cells, fusion of recycling tubulovesicles to the apical surface is mediated by the syntaxin 3 (STX3), VAMP2 and SNAP25 core SNARE complex (Calhoun et al., 1998; Karvar et al., 2002). STX3 appears to play a general role in targeting of vesicle fusion events at the apical membranes in epithelial cells. Notably, expression of mutant STX3, without targeting sequence, disrupts the apical delivery of proteins in MDCK cells (Sharma et al., 2006). Recently, mutations in STX3 and STXB2 (also known as Munc18-2) have been identified in neonates with severe neonatal diarrheal disease and alterations in enterocyte polarity associated with apical microvilli loss (Stepensky et al., 2013; Wiegnerink et al., 2014). These studies all point to regulation of apical membrane delivery through the recycling endosome system as being crucial to the establishment of apical membrane specializations.

Based on these previous findings, we have hypothesized that Rab11a plays a crucial role in apical trafficking and membrane...
recycling required for maintenance of microvilli in polarized enterocytes. In this study, we report that Rab11a regulates apical intestinal enterocyte polarity. Utilizing mice with a Rab11a-knockout specific to intestinal epithelial cells (Rab11a^{fl/fl};villin-cre') and stable Rab11a knockdown in CaCo2-BBE cells, we demonstrate that Rab11a loss results in a decrease in microvilli length, presentation of microvilli along the lateral membranes, mislocalization of apically targeted proteins, and mislocalization of both Rab8a and Rab11b. Most notably, Rab11a^{fl/fl};villin-cre‘ mouse duodenum and CaCo2-BBE Rab11a-KD cells displayed mislocalization of STX3 and the presentation of microvilli in the basolateral compartment. These results indicate that Rab11a is a crucial regulator of apical microvillar maintenance in polarized enterocytes.

RESULTS
Disruption of apical polarity is observed in Rab11a^{fl/fl};villin-cre‘ mouse duodenum

To examine the role of Rab11a in the establishment of brush borders in enterocytes, we developed Rab11a^{fl/fl};villin-cre‘ (hereafter denoted Rab11a^{ΔIEC}) mice, which display loss of Rab11a expression specifically in intestinal epithelial cells (Yu et al., 2014a). Previous work published by our laboratory has demonstrated that MYO5B-coupled Rab11a-dependent trafficking is required for proper apical trafficking and brush border maintenance in enterocytes (Knowles et al., 2014). In wild-type duodenal sections, MYO5B immunostaining was concentrated sub-apically in enterocytes below the brush border, and microvilli stained positively for ezrin (Fig. 1A). In contrast, the Rab11a^{ΔIEC} duodenum displayed redistribution of MYO5B away from the apical membrane with increased staining in the cytosol and nucleus (Fig. 1A). Ezrin staining in both wild-type and Rab11a^{ΔIEC} duodenum showed discrete apical localization in the enterocytes (Fig. 1A). Interestingly, increased ezrin was also observed along the basolateral membrane in the Rab11a^{ΔIEC} duodenum. Similar to ezrin, villin-1 was observed both in the apical membrane and along the basolateral membrane in Rab11a^{ΔIEC} enterocytes (Fig. 1B). Interestingly, both alkaline phosphatase and mucin-like protocadherin (MLPCDH) showed diffusely subapical staining in the Rab11a^{ΔIEC} enterocytes compared with tight apical staining in wild-type enterocytes (Fig. 1B). In contrast, in Rab11a^{ΔIEC} duodenum enterocytes, apical NHE3
staining was decreased whereas cytoplasmic NHE3 was increased (Fig. 1B).

Given the apparent alterations in brush border markers in Rab11a^{AIEC} duodenum, we next examined enterocyte ultrastructure. Examination using scanning electron microscopy (SEM) demonstrated that microvilli in Rab11a^{AIEC} duodenum were uniformly short compared with wild type enterocytes, although they displayed a normal packing distribution (Fig. 2A). Transmission electron microscopy (TEM) confirmed the shortened length of apical microvilli in Rab11a^{AIEC} duodenum, and also identified microvilli projecting from the lateral membranes in some enterocytes (Fig. 2A). Given that both Rab8a and Rab11a have been implicated in the growth of apical microvilli, we also compared the ultrastructure of both Rab8a-knockout (KO) mice and the Rab11a^{AIEC} mouse duodenum by TEM and SEM. As previously reported, microvillar growth and density were markedly perturbed in Rab8a-KO mice. In contrast with the pattern in the Rab11a^{AIEC} duodenum, Rab8a-KO mice showed a markedly dispersed pattern of microvilli (Fig. 2B). In areas with microvilli, the microvilli length in Rab8a-KO mouse enterocytes was markedly reduced compared to the microvilli of the Rab11a^{AIEC} mice or wild-type mice (Fig. 2B) (Sato et al., 2007). Quantification of microvillar length from transmission electron microscopy images showed a significant 50% decrease in the length of microvilli in both Rab11a^{AIEC} and Rab8a-KO mouse duodenum (Fig. 2C) as compared with control duodenal

![Fig. 2. SEM and TEM of Rab11a- and Rab8a-knockout mice display aberrant intestinal microvilli.](image)
enterocytes. Interestingly, we also observed a significant increase in the width of microvilli in Rab11a<sup>IEC</sup> duodenum (Fig. 2C).

**Disruption of apical polarity in Rab11a-KD CaCo2-BBE cells**

We next produced CaCo2-BBE cells with stable knockdown of either Rab8a or Rab11a to examine whether loss of these Rab proteins produced similar phenotypes in an enterocyte-like cellular model. The CaCo2-BBE subclone cell line from the CaCo2 parental cell line was used, as these cells form extensive microvilli when grown on permeable Transwell filters (Peterson and Mooseker, 1992). For this study, lentiviral short hairpin RNA (shRNA) vectors targeting Rab8a and Rab11a (KD cells), and a control shRNA were used for transduction of CaCo2-BBE cells. Both Rab8a and Rab11a mRNA and protein were reduced by greater than 50% when compared to the control cells, and we were able to rescue these cell lines with lentiviral vectors encoding mCherry fusions of mouse Rab8a or Rab11a (supplementary material Fig. S1). We examined staining for ezrin and F-actin (phalloidin) in CaCo2-BBE control, Rab11a-KD and Rab8a-KD cells by confocal fluorescence microscopy. In control cells, both ezrin and phalloidin-stained F-actin were located at the apical surface (Fig. 3). In Rab11a-KD cells, as in the Rab11a<sup>IEC</sup> mice, both ezrin and phalloidin staining were present at the apical membrane, but in addition, prominent F-actin and ezrin staining was also observed along the lateral membranes, often producing expanded lateral lumens (Fig. 3). In Rab8a-KD cells, ezrin and phalloidin staining was distributed on both the apical and basolateral surfaces of cells, and these cells appeared to lose contact inhibition and pile on top of each other (Fig. 3).

To assess changes by ultrastructure, we also examined the CaCo2-BBE cell lines by SEM and TEM (Fig. 4). Control cells demonstrated a normal arrangement of mature brush border microvilli (Fig. 4A). As assessed by both SEM and TEM, Rab11a loss in CaCo2-BBE cells caused the formation of sparse immature apical microvilli (Knowles et al., 2014) and lateral membrane microvilli were evident in TEM images (Fig. 4A,B). Re-expression of mCherry–Rab11a re-established a normal pattern of brush border microvilli (supplementary material Fig. S1B). Utilizing both SEM and TEM in Rab8a-KD cells, we observed that Rab8a-KD caused the formation of sparse immature apical microvilli (Fig. 4A). Re-expression of mCherry–Rab8a re-established the normal apical brush border microvilli (supplementary material Fig. S1B). Thus, both Rab8a and Rab11a appear to be essential for normal apical polarity and microvilli growth in enterocytes, and loss of Rab11a produces lateral lumens on the basolateral surface of enterocytes.

The integrity of the basolateral compartment is maintained in Rab11a<sup>IEC</sup> enterocytes and Rab11a-KD CaCo2-BBE cells

We next examined the effects of reducing Rab11a on components of the basolateral compartment. To assess the integrity of the basolateral compartment, we immunostained Rab11a<sup>IEC</sup> mice duodenum tissue samples for E-cadherin. Rab11a loss did not appear to affect E-cadherin staining in the duodenum as it was distributed along the basolateral surface as observed in control tissues (Fig. 5A). We also immunostained Rab11a<sup>IEC</sup> duodenum tissue samples for Na<sup>+</sup>/K<sup>-</sup>- and p120 catenin (also known as CTNND1), and they were both distributed along the basolateral compartment as observed in the control (supplementary material Fig. S2). To further assess the integrity of the basolateral surface in the absence of Rab11a, we immunostained CaCo2-BBE cells for claudin-1, β-catenin and E-cadherin. In control cells, claudin-1 and β-catenin were distributed along the lateral surface, and E-cadherin was positioned at the junctions (Fig. 5B). In the Rab8a-KD cells, claudin-1 was maintained at its lateral position, but β-catenin was redistributed to the cytoplasm. Interestingly, E-cadherin accumulated throughout the apical and lateral membranes in Rab8a-KD cells, and also accumulated sub-apically (Fig. 5B). In Rab11a-KD cells, claudin-1 and β-catenin were distributed along the lateral surface, but E-cadherin was redistributed along both the apical and lateral surfaces, as observed previously in CaCo2-BBE
MYO5B-KD cells (Fig. 5B) (Knowles et al., 2014). Thus, in CaCo2-BBE Rab11a-KD cells the basolateral integrity was maintained, but E-cadherin appears to be partially mislocalized to the apical surface. The discrepancy between the CaCo2-BBE Rab11a-KD cells and the Rab11a⁰°iec duodenum tissue samples might be due to compensation in vivo in the tissues.

Fig. 4. SEM and TEM examination of Rab11a KD and Rab8a KD cells demonstrates deficits in apical microvilli. Control, Rab11a-KD and Rab8a-KD cells were examined by SEM and TEM. (A) Rab8a KD cells demonstrated more immature and sparser microvilli than Rab11a KD cells. Scale bars are as indicated. (B) TEM of enlarged lateral spaces containing microvilli in CaCo2-BBE Rab11a-KD cell. Scale bars: 2 μm (main panel); 500 nm (inset). All results are representative of four separate experiments.

Fig. 5. Rab11a loss does not cause loss of basolateral integrity in enterocytes. (A) Sections of wild-type and Rab11a⁰°iec mouse duodenum were stained for Rab11a (red) and E-cadherin (green), with the merged image at the right including DAPI nuclear stain (blue). In Rab11a⁰°iec mouse duodenum, E-cadherin was maintained in the basolateral compartment of enterocytes, but the cells did appear to lose some contact inhibition. Scale bars: 20 μm. (B) Left panels: control CaCo2-BBE, Rab8a KD and Rab11a KD cells were stained for claudin-1 (red) and β-catenin (green) with the merged image shown at right including DAPI nuclear stain (blue). In control cells, claudin-1 and β-catenin were distributed along the basolateral surface. In Rab8a-KD cells, claudin-1 was maintained at its basolateral position, but β-catenin was shifted to a cytoplasmic localization. In Rab11a-KD cells, claudin-1 and β-catenin were distributed along the basolateral surface. Right panels: cells were stained for E-cadherin. In control cells, E-cadherin was positioned in a junctional localization. In Rab8a KD cells, E-cadherin was accumulated in the cytosol, but was still present on the lateral membranes. In Rab11a-KD cells, E-cadherin was redistributed to both the apical and basolateral surfaces. Arrowheads at the right in X-Y images indicate the position of the corresponding X-Z image. Scale bars: 10 μm. All results are representative of three separate experiments.
Loss of Rab11a causes mislocalization of Rab8a and Rab11b

Previous work performed in MDCK cells has demonstrated that loss of Rab11a causes a concomitant increase in Rab8a to compensate for Rab11a loss, and Rab11a, through Rab8a, activates Rab8a (Bryant et al., 2010). Because we observed that E-cadherin basolateral localization was unaffected in Rab11a-expressing enterocytes, we analyzed whether other Rab proteins could compensate for Rab11a loss by immunostaining Rab11a mouse duodenum sections for Rab8a and Rab11b. Both Rab8a and Rab11b were distributed sub-apically in control samples (supplementary material Fig. S3A). In the Rab11a mouse samples, Rab8a was dispersed throughout the cytoplasm (supplementary material Fig. S3A). Moreover, in these samples, Rab11b was dispersed throughout the cytoplasm away from its normal distribution and accumulated with increased fluorescence intensity throughout the enterocytes (supplementary material Fig. S3A). We next immunostained the CaCo2-BBE cell lines for Rab8a and Rab11b. Differences in the lateral marker p120 in sections from wild-type and IEC samples (Fig. 5A). Interestingly, the apical localization of Rab8a and Rab11b was unaffected. In Rab11a-KD cells, Rab8a staining was increased and both Rab8a and Rab11b were dispersed throughout the cytoplasm away from their normal distribution (supplementary material Fig. S3B). In Rab8a-KD cells, Rab8a was lost from the cells, and the localization of Rab11b was unaffected. In Rab11a-KD cells, Rab8a staining was increased and both Rab8a and Rab11b were dispersed throughout the cytoplasm away from their normal distribution (supplementary material Fig. S3B). These findings demonstrate that loss of Rab11a leads to an altered distribution of Rab8a and Rab11b both in vitro and in Rab11a mouse samples. These alterations in other Rab proteins might reflect an attempt by enterocytes to compensate for Rab11a loss.

Rab11a loss causes redistribution of STX3

Rab11a has recently been implicated, through atypical protein kinase C (aPKC) and mammalian STE20-like protein kinase 4 (Mst4, also known as STK26), in promoting the phosphorylation of ezrin, which is required for proper microvilli formation (Dhekne et al., 2014). To examine the status of phosphorylated ezrin and known ezrin kinases, we immunostained Rab11a mouse duodenum for Mst4, aPKC and phosphorylated ezrin, radixin and moesin (ERM) proteins. In control samples, Mst4 was distributed throughout the cytoplasm of enterocytes with a distinct sub-apical pool (Fig. 6A). In Rab11a samples, the Mst4 sub-apical pool was diminished (Fig. 6A). aPKC was distributed along the apical surface in both the control and Rab11a samples (Fig. 5A). Interestingly, the apical distribution of phosphorylated ERM proteins (P-ERM) was the same in both the control and Rab11a samples (Fig. 6A). To analyze the distribution of ezrin and P-ERM in the lateral membranes, we compared the distribution of ezrin and P-ERM to the lateral marker p120 in sections from wild-type and Rab11a duodenum (Fig. 6B). In wild-type enterocytes, we observed no enrichment of ezrin at the lateral membranes and there was negligible signal for P-ERM. In Rab11a enterocytes, as noted above, ezrin was observed at the lateral membranes, however the signal for P-ERM remained at the minimal detectable level. These results suggest that much of the lateral ezrin is not phosphorylated. We next immunostained the CaCo2-BBE cell lines for phosphorylated ERM proteins in comparison with the basolateral marker Na+/K+-ATPase (Fig. 6C). In control cells, P-ERM staining was sharply localized to the apical microvillar surface (Fig. 6C). In both Rab8a-KD and Rab11a-KD cells, P-ERM proteins were localized to the apical surface (Fig. 6C). Thus, alterations in microvillar structure in Rab11a samples did not correlate directly with changes in ezrin phosphorylation.

Given that altered ezrin phosphorylation could not account for the shortened microvilli in Rab11a samples, we investigated other possible causes of this phenotype. We assessed whether Rab1a loss might affect apical vesicle fusion machinery in Rab11a samples. To investigate this possibility, we immunostained the Rab1a mouse duodenum samples for STX3 (an apical SNARE) and STX4 (a basolateral SNARE protein). In control samples, STX3 was present along the apical surface of enterocytes (Fig. 7A). In contrast, STX3 was localized to both the apical surface and to small vesicular puncta below the apical surface in Rab11a mouse samples (Fig. 7A). Of note, at the base of villi in Rab11a enterocytes STX3 localized normally to the apical membrane only (Fig. 7A2), and at the villus tips, punctate intracellular sub-apical staining was also observed (Fig. 7A1). In control samples, STX4 was distributed throughout the cytoplasm in small punctate vesicles, as well as in distinct lateral membrane staining (Fig. 7B). Similar STX4 staining was observed in Rab11a mouse duodenum samples (Fig. 7B). We also immunostained the CaCo2-BBE cell lines for STX3. In controls cells, STX3 staining was confined to the apical membranes. In Rab8a-KD cells, STX3 accumulated more sub-apically than in control samples (Fig. 8A). In addition, STX3 staining was more intensely apical after re-expression of mCherry–Rab8a in Rab8a-KD cells (Fig. 8B). In Rab11a-KD cells, STX3 was redistributed from the apical surface to cytoplasmic vesicles and to lateral membrane spaces (Fig. 8A). To elucidate the origin of the intracellular cytoplasmic vesicles observed with STX3 staining, we immunostained both Rab11a mouse samples and Rab11a-KD cells for STX3 and Lamp2a (supplementary material Fig. S4). In Rab11a mouse samples, STX3 and Lamp2a-containing vesicles were both observed in the apical regions of enterocytes, but there was no overlap between the staining for the two proteins (supplementary material Fig. S4A). In Rab11a-KD cells, STX3 also did not localize with Lamp2a-positive vesicles (supplementary material Fig. S4B). As expected, re-expression of mCherry–Rab11a in Rab11a-KD cells re-established the apical localization of STX3 (Fig. 8B). Thus, loss of Rab11a might impair apical localization of key trafficking proteins and cause deficits in proteins that are important for microvilli elongation.

DISCUSSION

The plasma membrane recycling system is crucial for both the establishment and the maintenance of apical polarity in epithelial cells. Knocking down Rab11a in MDCK cells disrupts the initial formation of the apical surface (Bryant et al., 2010). Similarly, knockdown of MYO5B alters the establishment of apical lumen MDCK cells (Roland et al., 2011). In this study, we demonstrate that Rab11a contributes to the maintenance of normal apical polarity in enterocytes. In Rab11a samples and CaCo2-BBE Rab11a-KD cells, apically trafficked proteins were mislocalized, microvilli length was reduced and microvilli were aberrantly present on the basolateral membrane. While this work was in revision, another group also published information on a similar intestine-targeted Rab11a-knockout mouse model (Sobajima et al., 2015). Many of the aspects of the effects of Rab11a loss in this mouse were similar to those that we have observed including short microvilli and expansion of the intracellular lysosome system. However, in contrast with our
studies, Sobajima, et al. did not report the lateral microvilli that we observed in both mouse and cell line studies with loss of Rab11a (Sobajima et al., 2015). Nevertheless, they did notice mislocalization of the apical brush border protein DPPIV to the basolateral membranes. In contrast they did report the presence of scattered microvillus inclusions, which we did not observe. It might be that differences in precise phenotype are due to strain and/or construct differences or microbiome-specific issues. In any case, the results indicate that loss of Rab11a in the intestinal enterocytes leads to aberrant trafficking of apical membrane components. In enterocytes, towards the villus tips, we also observed expansion of sub-apical vesicular compartments that did not correspond with lysosomes. These compartments appeared to also contain STX3. Loss of Rab11a also elicited redistribution and upregulation of Rab8a and Rab11b. Interestingly, the enterocytes of patients with STX3 mutations show many of the same characteristics as those in the Rab11a<sup>DIEC</sup> duodenum, including the presence of lateral microvilli (Stepensky et al., 2013; Wiegerinck et al., 2014). Previous investigations have noted the presence of STX3 on Rab11a-containing recycling vesicles (Lapierre et al., 2007). Inhibition of trafficking through the apical recycling system in MDCK cells causes accumulation of STX3 with Rab11a in internal collapsed membrane cisternae (Lapierre et al., 2007). These findings suggest that Rab11a facilitates the proper localization of STX3 to the apical surface.

Recent investigations have suggested that localization of MST4 and aPKC to the apical surface of enterocytes is dependent on MYO5B-coupled Rab11a-dependent trafficking, both of which facilitate ezrin phosphorylation, which stimulates microvilli growth (Dhekne et al., 2014). Unexpectedly, in Rab11a<sup>DIEC</sup> duodenum apical trafficking of aPKC is not affected and Mst4 trafficking to the apical surface is only moderately influenced. Consequently, phosphorylation of ezrin (P-ERM staining) appears to remain restricted to the apical membrane in Rab11a<sup>DIEC</sup> duodenum, despite the presence of ezrin at the lateral membranes. The reason for the discrepancies between our results and previous studies is not clear. The previous investigations utilized the LS174R-W4 colon cancer cell line, which displays unusual presentation of patches of apical like membrane in non-polarized cells (Dhekne et al., 2014). Thus, the strong effects of Rab11a knockdown observed in that report might be a result of the partially polarized system. It is notable that although transferrin receptor recycling is strongly controlled by Rab11a in non-polarized cells (Green et al., 1997; Ulrich et al., 1996), transferrin is excluded from the Rab11a-containing apical recycling system in polarized cells (Lapiere et al., 2001; Wang et al., 2000). Alternatively, the mild phenotype that we have observed in both mouse and cell line studies with loss of Rab11a (Sobajima et al., 2015). Nevertheless, they did notice mislocalization of the apical brush border protein DPPIV to the basolateral membranes. In contrast they did report the presence of scattered microvillus inclusions, which we did not observe. It
observed might have accrued from compensation by other Rab proteins. Thus, we did observe that Rab8a and Rab11b were reorganized in Rab11a-deficient tissue. Indeed, E-cadherin localization appeared normal in Rab11a<sub>IEC</sub> duodenum, whereas in Rab11a-KD CaCo2-BBE cells E-cadherin was misdirected to the apical surface. Thus, there might be different levels of compensation possible in <i>in vitro</i> and <i>in vivo</i> systems.

Rab11a in enterocytes appears to be integral to the formation of the normal apical surface. Previously, we demonstrated that MYO5B works in concert with both Rab8a and Rab11a to maintain both apical and basolateral polarity in enterocytes. In particular, we observed that the interaction between Rab11a and MYO5B was necessary for proper recycling of membranes macropinocytosed from the apical surface. Independently, Rab11a appears to maintain normal apical polarization, but parts of normal Rab11a function might be compensated for in Rab11a<sub>IEC</sub> duodenum samples. Similarly, some aspects of normal Rab8a function can be compensated for by Rab8b, and only with dual knockout of both Rab8a and Rab8b genes can the complete phenotype be appreciated (Sato et al., 2014). Still it is notable that Rab8b does not interact with MYO5B (Roland et al., 2009).

Our previous studies and those presented here also demonstrate an important dynamic along the intestinal villi.

Fig. 8. Both MYO5B and Rab11a are required for proper localization of STX3. (A) CaCo2-BBE cell lines were stained for STX3. In control cells, STX3 was localized to the apical surface. In Rab8a-KD cells, STX3 accumulated in vesicles below the apical surface. In Rab11a-KD cells, STX3 was redistributed away from the apical surface into the cytoplasm and to lateral membranes. Scale bars: 10 μm. (B) STX3 immunostaining (green) was evaluated in Rab8a KD cells with mCherry–Rab8a rescue (red) or Rab11a KD cells with mCherry–Rab11a rescue (red). Rab8a re-expression in Rab8a-KD re-established discrete apical localization of STX3. Rab11a re-expression in Rab11a-KD cells, re-established the normal apical localization of STX3. Arrowheads at the right in X-Y images indicate the position of the corresponding X-Z image. Scale bars: 10 μm. All results are representative of three separate experiments.
investigations have noted differences in protein expression in enterocytes as they migrate out of the crypt towards the villus (Barnard et al., 1989; Jakab et al., 2011; Levy et al., 2009; Suzuki et al., 2009). We have previously observed in duodenal samples from microvillus inclusion disease patients that the brush borders in enterocytes of the proximal villus are intact (Knowles et al., 2014); loss of microvilli and development of microvillus inclusions only occur in the upper half of the villus. It is unclear from published findings whether patients with STX3 or Munc-18-2 mutations also show similar patterns of differential loss of brush border integrity during migration towards the villus tips (Stepensky et al., 2013; Wiegnerck et al., 2014). Nevertheless, it appears that loss of Rab11a and loss of functional MYO5B do not affect initial formation of the enterocyte brush border, but rather disrupt maintenance of polarity and brush border integrity in the enterocytes of the upper villus tip. These findings suggest alteration of enterocyte final differentiation at the villus tips might provide an effective strategy for ameliorating neonatal diarrhea in patients with mutations that affect apical trafficking in enterocytes.

**MATERIALS AND METHODS**

**Mice**

The conditional Rab11a floxed allele mice were derived from homologous recombination in embryonic stem cells as described previously by Yu, et al. (Yu et al., 2014a; Yu et al., 2014b). The Rab8a-knockout mice used for the study have also been described previously by Sakamori et al. (Sakamori et al., 2012; Sato et al., 2007). Data for mouse experiments were obtained from four individual mice for each genotype group. All experiments were performed using littermates unless stated otherwise. All animal experiments were performed according to approved guidelines.

**Cell lines**

Human colonic epithelial CaCo2-BBE and human embryonic kidney (HEK) 293 cell lines were grown in DMEM as detailed by Knowles et al. (Knowles et al., 2014). For CaCo2-BBE cells, 200,000 cells were plated and grown on 12-well Transwell filters for 15 days in culture unless stated otherwise, and medium was changed daily throughout the culture duration as detailed previously (Knowles et al., 2014).

Lentiviral shRNA vectors targeting Rab8a and Rab11a (Open Biosystems V3LHS_359728 and V3LHS_377860) and a control shRNA (Open Biosystems) were used for transduction of CaCo2-BBE cells. The CaCo2-BBE cells were transduced with lentiviral medium produced in HEK cells and selected using puromycin (Cellgro). Rescue of CaCo2-BBE Rab8a-KD and Rab11a-KD was performed using vectors that were synthesized from mouse Rab8a and Rab11a sequences resistant to silencing by the above shRNAs, cloned into pcDH lentiviral vectors, and selected using puromycin (Knowles et al., 2014).

RNA was isolated from CaCo2-BBE control, Rab8a-KD, and Rab11a-KD cell lines using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and treated using RQ1 RNase-free DNase (Promega). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with both oligo-dT and random hexamer primers as described previously by Lapiere, et al., 2012 (Lapiere et al., 2012). Real-time PCR was performed using a StepOnePlus real-time PCR system with Express SYBR Green ER Supermix (Applied Biosystems) and the following primer pairs: Rab8a-forward 5’-ACCGGGCTGTGCTCATATT-3’ and Rab8a-reverse 5’-GACGCTTCAACTCCACATT-3’; Rab11a-forward 5’-TTCGCGTCTGTCTGACATT-3’ and Rab11a-reverse 5’-TTGAAACTTCGGCCCT-3’; and GAPDH-forward 5’-AGATCCCTCCAAAATCAAG-TGG-3’ and GAPDH-reverse 5’-GGCAGAGATGATGACCCTTT-3’. The above primers were validated for efficiency and melting temperature, and the results were analyzed by the comparative CT method (Knowles et al., 2014).

**Tissue preparation**

All tissue samples were obtained through protocols approved by the Rutgers University IRB. All mouse duodenal sections from dissections were washed in PBS and fixed in 4% Paraformaldehyde or 2% Paraformaldehyde/2% Glutaraldehyde in 0.1 M sodium cacodylate pH 7.4. For immunofluorescence, samples were stored in 70% ethanol, embedded and sectioned for further staining with proper antibodies.

**Confocal immunofluorescence**

The CaCo2-BBE control, Rab8a-KD, and Rab11a-KD cell lines were plated on 12-well Transwell filters and cultured for 15 days. Cells were prepared for immunofluorescence as described by Knowles et al. (Knowles et al., 2014). All tissue images were captured with an Olympus FV-1000 confocal microscope (Olympus) using a 60× oil immersion objective with a numerical aperture of 1.42 and CaCo2-BBE cell images used the microscope’s software at a 3× optical zoom. The individual images were converted to tiff files with the FV-1000, and Photoshop (Adobe) was used to create the final figures.

**Histology, SEM and TEM**

Mouse duodenal sections were de-paraffinized and were submitted to antigen retrieval in a pressure cooker using the target retrieval solution (Dako North America Inc.). Samples were then prepared for immunofluorescence as previously described and with the appropriate antibodies as detailed in supplementary material Table S1. All imaging was performed using the Olympus FV-1000. CaCo2-BBE cell lines and mouse tissue were prepared for SEM as described previously (Knowles et al., 2014). Samples were mounted on stubs and coated with gold in a sputter coater, and analyzed using an FEI Quanta 250 SEM (Vanderbilt Cell Imaging Shared Resource, Vanderbilt University). TEM samples from MVID patients were prepared and analyzed by the Vanderbilt Cell Imaging Shared Resource, Vanderbilt University. For quantification of microvillus length and width, TEM images were evaluated for 100 microvilli using an AMT Image Capture Engine to analyze images recorded with a DVC camera taking images at 1632×1632 pixels. Statistical differences between control and KO mouse microvilli were determined by a two-tailed Student’s t-test.

For analysis of ezrin and P-ERM staining, intestinal sections from wild type control and Rab11a<sup>floxed-villus-cre</sup> were dual immunostained for ezrin or P-ERM with p120 catenin to demark the lateral membrane. RGB images at equal exposures for each antibody were obtained using an Ariol SL-50 digitizing scanner in the VUMC Digital Histology Shared Resource. In regions of cells that were well-oriented along the villi, linear regions of interest (ROI) were drawn through the middle of cells to include the lateral membrane and cytoplasm (but exclude the apical membrane). Intensity profiles were generated with ImageJ Color Profiler plugin (NIH, http://rsbweb.nih.gov/ij/).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

B.C.K. designed and performed experiments, analyzed data and drafted the manuscript. V.G.W. designed and performed experiments, analyzed data and drafted the manuscript. S.Y. designed and performed experiments, analyzed data, and revised manuscript. J.T.R. analyzed data and revised manuscript. J.A.W. designed and performed experiments, analyzed data, and revised manuscript. L.A.L. performed
experiments, analyzed data and drafted the manuscript. M.D.S. designed experiments and revised manuscript. N.G. designed experiments, analyzed data and revised the manuscript. J.R.G. designed experiments, analyzed data and revised the manuscript.

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Supplementary material
Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.163303/-/DC1

References


Dube´ , N., Sinnett, D., Seidman, E. and Bendayan, M.


Supplemental Figures

Supplemental Figure 1. In CaCo2-BBE Rab8a-KD and Rab11a-KD cells expression of either Rab8a or Rab11a rescued the loss of apical microvilli. (A) Top: Western blot of CaCo2-BBE control and Rab8a-KD shRNA cell lines probed for Rab8a and β-actin, as a control, showing a reduction of Rab8a protein expression. Bottom: Western blot of CaCo2-BBE control and Rab11a-KD shRNA cell lines probed for Rab11a and β-actin, as a control, showing a reduction of Rab11a protein expression. (B) Top: Rescue of Rab8a-KD with silencing-resistant mCherry-Rab8a vector re-established mature microvilli. Bottom: Rescue of Rab11a-KD with silencing resistant mCherry-Rab11a vector re-established microvilli. Scale bars = 10 µm in the main panels and 1 µm in the insets.
Supplemental Figure 2. Basolateral compartment immunofluorescence staining in Rab11a$^{\DeltaIEC}$ mice duodenum showed no alteration in basolateral staining. Left: Na/K-ATPase staining in wild type and Rab11a$^{\DeltaIEC}$ mouse duodenum displayed a basolateral distribution. Right: p120 staining in wild type and Rab11a$^{\DeltaIEC}$ mouse duodenum displayed a basolateral distribution. Scale bars = 30 µm in the left panels and 50 µm in the right panels.
Supplemental Figure 3. Rab11a loss causes redistribution of Rab8a in enterocytes. (A) Rab11aΔIEC mouse duodenal enterocytes displayed a redistribution of both Rab8a and Rab11b from their normal subapical localization to cytoplasmic distributions. Scale bars = 50 μm. (B) Left: In CaCo2-BBE cells, Rab11a KD caused redistribution of Rab8a from its normal sub-apical and lateral localization to the cytoplasm. Right: In Rab11a-KD cells, Rab11b was redistributed from the sub-apical region to the cytoplasm. Scale bars = 10 μm.
Supplemental Figure 4. Rab11a loss caused mislocalization of STX3 to Lamp2a negative vesicles. (A) In normal duodenum, STX3 (magenta) was localized to the brush border and Lamp2a (green) localized in intracellular cytoplasmic vesicles. In Rab11aΔIEC mice duodenum, STX3 localized along the brush border with some STX3 localizing into the cytoplasm and showed little co-localization with Lamp2a. Scale bars =
50 µm. (B) In control and Rab8a-KD CaCo2-BBE cells, STX3 (green) localized to the brush border and Lamp2a (red) localized intracellularly without any overlap. In Rab11a-KD cells, STX3 was shifted intracellularly, but Lamp2a did not co-localize with STX3. Scale bars = 10 µm.
Supplemental Table 1. Antibodies used. ND=Not determined.

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