Rab25 regulates integrin expression in polarized colonic epithelial cells

Moorthy Krishnan¹, Lynne A. Lapierre¹,4, Byron C. Knowles¹,2 and James R. Goldenring¹,2,3,4

¹Section of Surgical Sciences and the Epithelial Biology Center,  
²Department of Cell and Developmental Biology,  
³Vanderbilt-Ingram Cancer Center,  
Vanderbilt University Medical Center, Nashville, TN 37232, USA  
⁴Nashville VAMC, Nashville, TN

Running Title: Rab25 regulates colonic cell polarity

Abstract

Rab25 is a tumor suppressor for colon cancer in humans and mice. To identify elements of intestinal polarity regulated by Rab25, we have developed Caco2-BBE cell lines stably expressing shRNA for Rab25 and lines rescuing Rab25 knockdown with re-expression of rabbit Rab25. Rab25 knockdown decreased α2, α5 and β1-integrin expression. We observed co-localization and direct association of Rab25 with α5β1-integrins. Rab25 knockdown also up-regulated Claudin-1 expression, increased transepithelial resistance and increased invasive behavior. Rab25 knockdown cells showed disorganized brush border microvilli with decreases in villin expression. All of these changes were reversed by reintroduction of rabbit Rab25. Rab25 knockdown altered the expression of 29 gene transcripts including the loss of α5-integrin transcripts. Rab25 loss decreased expression of one transcription factor, ETV4, and over-expression of ETV4 in Rab25 knockdown cells reversed losses of α5β1-integrin. The results suggest that Rab25 controls intestinal cell polarity through the regulation of gene expression.
Key words: Rab25/α5-integrin/β1-integrin/ETV4/Caco2 cells

Introduction

Epithelial cells maintain a polarized barrier between the external and internal milieu by assembling discrete apical and basolateral domains separated by tight and adherens junctions (Marrs et al., 1995). The polarized domains in the epithelial cells allow trafficking of critical cell adhesion molecules to apical or basolateral domains and also define regions for elaboration of apical specializations, including brush border microvilli (Mellman and Nelson, 2008). Perturbations of these polarized functions are thought to induce some of the earliest changes that predispose to transformation and epithelial to mesenchymal transformation (Royer and Lu, 2011). This process of increasing losses in polarity appears to be incremental, such that loss of individual components, by themselves, are not able to induce dysplastic changes, but multiple coincident aberrations can lead to the loss of polarized function and disorganization of the epithelial monolayer (Jones et al., 2006). These results all suggest that alterations in membrane protein trafficking pathways have the potential to lead to deleterious changes in cell polarity.

The successful establishment of polarized epithelial domains is facilitated by discrete intracellular vesicle trafficking pathways that deliver cargoes to appropriate membrane pathways. These pathways have been delineated in detail in renal MDCK cells, where a number of investigations have demonstrated the separation of membrane recycling pathways for basolateral recycling, apical recycling and transcytotic pathways between the apical and basolateral membranes (Wang et al., 2000; Lapierre et al., 2001; Tzaban et al., 2009; Lapierre et al., 2012). The Rab small GTPases and their effectors are critical regulators of these polarized membrane trafficking pathways (Mellman and Nelson, 2008). In addition to mediation of dynamic intracellular trafficking pathways for recycling, Rab proteins are also key mediators of the initiation of MDCK cells polarity and the establishment of apical specializations such as
primary cilia (Boehlke et al., 2010; Bryant et al., 2010; Roland et al., 2011). Much less is known about trafficking pathways in polarized intestinal epithelial cells. The junctions of polarized intestinal epithelial cells are clearly undergoing continuous remodeling mediated by vesicle trafficking (Shen et al., 2008). In addition, correct trafficking of integrins to appropriate plasma membrane surfaces is required for polarized intestinal epithelial function (Kuwada and Li, 2000). Furthermore, the recent identification of mutations in Myosin Vb as the cause of the loss of microvilli in neonates with Microvillus Inclusion Disease has highlighted the importance of membrane trafficking pathways in establishing the components of the apical intestinal brush border (Erickson et al., 2008; Muller et al., 2008).

We have previously demonstrated that the epithelial-specific small GTPase, Rab25 (Goldenring et al., 1993) is a candidate tumor suppressor in the colon (Nam et al., 2010). Other studies have also implicated Rab25 in carcinogenesis in other epithelial cancers (Cheng et al., 2004; Cheng et al., 2006; Cheng et al., 2010; Vuoriluoto et al., 2011; Dozynkiewicz et al., 2012). Human colon cancers demonstrate a stage-independent reduction in Rab25 expression (Nam et al., 2010). Moreover, while Rab25-deficient mice do not manifest a significant intestinal phenotype, the combination of Rab25 knockout with either expression of mutant APC\textsuperscript{Min} or heterozygous loss of Smad3 induces accelerated intestinal neoplasia (Nam et al., 2010). In particular, Rab25\textsuperscript{−/−};Smad3\textsuperscript{+/−} mice develop large, regionally invasive colon cancers. While these studies established Rab25 as a tumor suppressor in the colon, they could not address the range of cell polarity changes that might account for the induction of neoplasia.

We have now sought to investigate the molecular mechanisms responsible for the aberrant effects of Rab25 loss in enterocytes through the examination of the knockdown of Rab25 expression in polarized Caco2-BBE cells. Caco2-BBE cells are a critical model of
intestinal epithelial cells because they polarize and elaborate a mature and well-organized microvillar brush border during 15 days of culture on permeable Transwell filters (Peterson and Mooseker, 1992). We have found that shRNA-mediated knockdown of Rab25 expression in Caco2-BBE cells leads to a loss of α5β1-integrins at the plasma membrane and these changes are reversed by reintroduction of rabbit Rab25. Moreover loss of Rab25 expression also elicited alterations in claudins, increases in transepithelial resistance, increases in soft agar colony formation and increases in cell invasion. Rab25 loss also caused alterations in a discrete set of genes including down-regulation of transcripts for α5-integrin and up-regulation of sucrase isomaltase transcripts. Importantly, Rab25 loss also down-regulated expression of the transcriptional regulator ETV4, and reintroduction of ETV4 into Rab25 knockdown cells led to normalization of α5-integrin and sucrase isomaltase expression. These findings demonstrate that Rab25 regulates intestinal cell polarity through regulation of gene transcription.

Results

**Rab25 is up regulated during polarization in Caco2-BBE cells.**

While recent studies have suggested that Rab25 is a tumor suppressor in mice and humans for colon cancer (Nam et al., 2010), no previous investigations have examined the association of Rab25 with polarity and vesicle trafficking in colonic cell lines. We investigated the expression of Rab25 in eight different colorectal cancer cell lines. Figure 1A demonstrates that all of the cell lines with some capacity to polarize and differentiate expressed Rab25 protein. In contrast, the poorly differentiated SW480 and SW620 lines did not express Rab25. We therefore sought to examine whether Rab25 contributes to intestinal epithelial cell polarity using the human colonic Caco2-BBE and HCA-7 cell lines that develop highly polarized monolayers when grown on Transwell filters. In particular, Caco2-BBE cells are a unique model for intestinal cell polarity...
because they develop full polarization expression of resident microvillar structural proteins as well as brush border enzymes (Peterson and Mooseker, 1992). The Caco2-BBE cells were grown on Transwell filters and the protein expression was analyzed at 3, 8 and 15 days of culture on filters. As previously noted (Peterson and Mooseker, 1992), expression of the brush border proteins villin-1 and sucrase-isomaltase (SI) increased with polarization of Caco2-BBE cells (Figure 1B,D). Caco2-BBE cells expressed low level of Rab25 on day 3 with increasing levels of expression during polarization over 15 days on Transwells (Figure 1C,D). No significant changes were observed in the expression of other Rab proteins including Rab11a, Rab5, Rab8a and Rab21 (Figure S1A). Since our earlier studies had implicated Rab25 in the expression of integrins (Nam et al., 2010), we evaluated integrin expression during polarization. Interestingly, as previously noted (Levy et al., 1998), expression of both α2-integrin and β1-integrin was highest at 3 days in culture and decreased during polarization (Figure 1B,D). In contrast, expression of α5-integrin increased during polarization similar to the pattern for Rab25 (Figure 1B,D). We observed a similar increase in Rab25 and α5-integrin expression during polarization in HCA-7 cells, however levels of α2-integrin and β1-integrin remained stable throughout 15 days of culture on Transwells (Supplemental Figure 1B). These results suggested that increases in Rab25 and α5-integrin expression might be characteristic of colonic cell polarization.

We next sought to evaluate whether changes in Rab25 protein expression were a reflection of changes in gene expression during polarization. The RT-PCR and quantitative PCR analysis (Figure 1E,F) showed that the expression of Rab25 mRNA increased significantly on both 8 and 15 days in culture for Caco-2-BBE cells. Thus, Rab25 gene and protein expression were both increased during the process of Caco2-BBE cell polarization.
Knockdown of Rab25 alters integrin expression and localization in polarized Caco2-BBE cells.

The integrins are heterodimeric transmembrane proteins composed of α and β subunits, which heterodimerize in different combinations. In recent years, increasing evidence indicates that alterations in Rab25 regulate integrin expression, especially α5- and β1-integrins, both in vivo and in vitro (Cheng et al., 2004; Caswell et al., 2007; Nam et al., 2010; Lapierre et al., 2011). Therefore we examined the role of Rab25 in the regulation of multiple isoforms of α and β integrins at different stages of polarity. We established Caco2-BBE cell lines stably expressing 1) scrambled shRNA (Control), 2) shRNA directed against human Rab25 (Rab25KD) and 3) Rab25KD cells with reintroduction of rabbit Rab25 (Rescue). In Rab25KD cells, Rab25 mRNA (Figure 2A) and protein expression (Figures 2B and S2A) were greatly reduced throughout culture for up to 15 days. Rescue with rabbit Rab25 expression did not alter the knockdown of endogenous human Rab25 mRNA in the Caco2-BBE cells (Figure 2A). The expression of Rabbit Rab25 in Rescue cells remained stable throughout 15 days of polarized cell culture (data not shown). Of note, Rab25 knockdown did not elicit any significant changes in the expression of Rab5, Rab8a, Rab11a or Rab21 (Figure S1C).

We next examined the effects of Rab25 knockdown and rescue on the expression of integrins. As seen in Figure 1B, α5–integrin expression increased with polarity, but Rab25KD cells showed a prominent loss of α5–integrin expression throughout 15 days in culture (Figures 2B and S2A). Nevertheless, reintroduction of untagged-rabbit Rab25 in Rescue cells restored α5-integrin expression to control levels (Figure 2B). It is important to note that we prepared the rescued stable line with untagged-Rab25, because we found that expression of mCherry-tagged rabbit Rab25 did not rescue the changes induced by Rab25 knockdown (data not shown). While
α2-integrin expression decreased with polarity in Control cells, Rab25 knockdown elicited further decreases in expression. However, in the Rab25 Rescue line, α2-integrin levels were maintained at the levels seen in Control cells (Figures 2B and S2B). The maintenance of α2-integrin expression may reflect the stable expression levels for Rab25 throughout the culture period consistent with expression from pCB6-Rab25. While β1-integrin levels also declined in Control cells during 15 days of polarization on filters, Rab25KD cells showed significant decreases in β1-integrin expression (Figure 2B). The decreases in β1-integrin expression were reversed by reintroduction of rabbit Rab25 in Rescue cells, but only to the levels of Control cells at 3, 8 or 15 days in culture (Figures 2B and S2B)). We also observed a decrease in Fibronectin expression during culture of cells on filters, but this was not significantly altered by either knockdown of Rab25 expression or Rescue (Figures 2B and S2C).

Since these studies all suggested that loss of Rab25 altered integrin protein expression, we evaluated the distribution of integrins by immunofluorescence microscopy. Unfortunately, no specific antibodies are available to assess α2-integrin expression with immunofluorescence, so we examined the distribution of α5-integrin and β1-integrin. Rab25KD cells showed a decrease in overall α5-integrin expression and a marked decrease in plasma membrane α5-integrin at 3, 8 and 15 days in culture (Figure 3A). These decreases were abrogated in the Rab25 Rescue cells. Rescue cells (Figure 3A). No changes were observed for lateral membrane expression of E-cadherin in Rab25KD or Rab25 Rescue cells throughout 15 days in culture (Figure 3B). Similar to the findings for α5-integrin, in Rab25KD cells we also observed losses of β1-integrin expression, especially at the plasma membrane, that were again rescued with reintroduction of rabbit Rab25 (Figure 3C).
Given the changes in integrin expression, we also sought to evaluate the expression of mRNA transcripts for α5-integrin and β1-integrin. Figure S3A demonstrates that knockdown of Rab25 elicited decreases in α5-integrin mRNA expression that were significant at 8 and 15 days of polarized culture. Nevertheless, Rab25 Rescue cells showed normal levels of α5-integrin mRNA expression. In contrast we did not see any decreases in β1-integrin mRNA in the Rab25KD-Caco2-BBE cell lines during 15 days of culture (Figure S3B). These studies indicated that Rab25 might influence gene expression in Caco2-BBE cells.

**Rab25 regulates integrin localization in Caco2-BBE cells.**

Caswell et al. (Caswell et al., 2007) have previously demonstrated that over-expressed Rab25 in ovarian cancer cells directly interacts with α5β1-integrin. We therefore sought to examine whether Rab25 associated directly with α5β1-integrin in Caco2-BBE cells. We first compared the distribution of endogenous Rab25 with endogenous Rab11a. We observed only a small overlap of endogenous Rab25 staining vesicles with Rab11a (Figure 4A). The Mander’s coefficients for colocalization and Pearson’s coefficient were higher than those seen for colocalization between the Golgi marker GM130 and Rab25, but still showed only a minor overlap (Figure 4E). A recent investigation in ovarian cells has suggested that Rab25 may associate with elements of the late endosomal system (Dozynkiewicz et al., 2012). We therefore also examined whether Rab25 containing vesicles were late endosomes. Figure 4B demonstrates that there was little overlap of endogenous Rab25 staining with Rab7 staining and both Mander’s coefficients and Pearson’s coefficient were generally lower than those seen with Rab11a (Figure 4E), suggesting that the Rab25 vesicles were distinct from Rab7-staining late endosomes. We next examined whether integrins localized with Rab25 on trafficking vesicles. Figure 4C and 4D show that both α5-integrin and β1-integrin staining was present in Rab25-containing vesicles.
High Mander’s coefficients were seen with both integrins, with greater than 20% of each associated with Rab25-containing vesicles. Finally, we examined whether Rab25 directly interacted with the integrins in Caco2-BBE cells. For these studies, we constructed a second Rescue line stably expressing myc-tagged rabbit Rab25 in Rab25KD cells. Unlike the fluorescent protein chimeras, the smaller myc tag allowed rescue. By immunoisolating myc-Rab25 from rescued cells and evaluating integrin co-isolation in western blots (Figure 4E), we found that both α5-integrin and β1-integrin were co-isolated with myc-Rab25. We also determined that immunoprecipitating antibodies against endogenous α5-integrin could co-isolate myc-Rab25 (Figure 4F). All of these studies suggest that Rab25-containing vesicles can traffic α5β1-integrins.

**Rab25 alters the development of the brush border.**

To examine the effects of Rab25 loss on the formation of the apical brush border, we examined Control, Rab25KD and Rab25 Rescue cell lines cultured for 15 days on Transwell filters by Scanning Electron Microscopy (SEM). Figure 5A demonstrates that, compared to the well organized brush border observed in Control cells, the Rab25KD cells showed a sparser and more disorganized brush border with clustering of microvillar groups rather than a tightly packed brush border. Reintroduction of rabbit Rab25 elicited a return of the more organized brush border configuration. Given these findings, we next examined the expression and distribution of the brush border components villin-1 and sucrase isomaltase. By western blots, villin protein levels were only slightly decreased, by Rab25 knockdown at all three days studied (Figures 5B and S4A). By immunofluorescence, we did observed a decrease in villin staining in apical region of the cells (Figure 5C). This loss in apical villin was restored with reintroduction of Rab25 in Rescue cells.
In contrast with villin, in Rab25KD cells we observed a surprising up-regulation of sucrase isomaltase expression, especially at 8 and 15 days of culture (Figures 5B and S4B). In Rescue cells, sucrase isomaltase levels returned to those observed in Control cells. By immunofluorescence, 15 day cultured cells showed elevated SI staining which was distributed throughout the cytoplasm. However, there was an apparent absence of SI in the apical membranes (Figure 5D). Re-expression of Rab25 in Rescue cells re-established a more normal pattern of SI distribution in the apical membranes. As with the integrins, we also evaluated the effects of Rab25 knockdown and Rescue on the expression of SI mRNA transcripts (Figure 5E). Interestingly, Rab25 knockdown elicited a marked elevation of SI transcript expression that returned to control levels in cells rescued with rabbit Rab25 (Figure 5E). The results indicate that Rab25 regulates both assembly of the brush border and gene transcription of brush border components.

**Rab25 depletion alters claudin expression.**

Tight junctions (TJ) provide a continuous intercellular seal, mediate paracellular transport, act as fence between apical and basolateral membranes and regulate cell polarity (Shen et al., 2008). Since depletion of Rab25 caused perturbation of the apical membrane specializations, we evaluated whether loss of Rab25 affected tight junction proteins and their function. We first measured the transepithelial resistance (TER) in all three cell lines from day 3 to 15 (Figure 6A). The TER values increased from day 3 to 15 in all three cell lines. However, compared to Control cells, we observed a prominent increase in TER in Rab25 KD cells after 8 days in culture. Rescue cells with rabbit Rab25 expression showed lower TER than Controls throughout the 15 days in culture (Figure 6A).
The alterations in TER suggested that Rab25 might regulate tight junction components. We therefore analyzed tight junction proteins by western blots (Figures 6B and S4C,D). Similar to previous findings (Buzza et al., 2010), the Control Caco2-BBE cells expressed low levels of Claudin-1 in less polarized cells (day 3) with increasing amounts observed at 8 and 15 days in culture (Figures 6B and S4C). In contrast, we observed a decrease in the expression of Claudin-2 in Control cells during the 15 days in culture. In the Rab25KD cells, we observed a prominent increase in Claudin-1 expression. Overall, levels of Claudin-2 expression during polarizing cell culture showed little significant change except at 15 days (Figure S4C). In Rab25 Rescue cells, we found a decrease of Claudin-1 expression towards control levels (Figures 6B and S4C). In addition, we also examined the expression of Claudins 3, 4 and 7. Expression of Claudin-3 and Claudin-4 was stable throughout the 15 days in culture in Control cells. Rab25KD cells showed small consistent decreases in the expression of Claudin-3 and Claudin-4 that were reversed in the Rab25 Rescue cells (Figures 6B and S4C). Claudin 7 expression increased during the 15 days in culture for all three cell lines, but expression was not affected by loss of Rab25 expression (Figure S4D). We also did not observe any changes in the expression of either ZO-1 or occludin in any of the cell lines throughout the 15 days in polarizing culture (Figure S4D).

We next evaluated the distribution of claudins using immunostaining (Figures S5-S8). These studies confirmed that Rab25KD caused an increase in Claudin-1 staining with more prominent extension of Claudin-1 along the lateral membrane compared to Control or Rescue cells (Figure S5). In contrast, in Rab25KD cells, we observed prominent decreases in the staining of tight junctions for Claudin-2, Claudin-3 and Claudin-4 (Figures S6-8). Overall, these studies suggest that alterations in the ratios between Claudin-1 and Claudin-2 expression could account for the differences in TER observed in Rab25KD cells.
Rab25KD cells show increased colony formation and cell invasion.

Since alterations in integrins and tight junction proteins can lead to changes in cancer cell invasion, we examined these properties in the Control, Rab25KD and Rescue cell lines. In soft agar colony formation assays, we observed a two-fold increase in the number of colonies formed and a significant increase in colony size in Rab25KD cells compared with Control cells (Figure 7A). Reintroduction of rabbit Rab25 in Rescue cells reversed the effects of Rab25 knockdown. In general, Caco2-BBE cells show slow migration in cell invasion assays. We studied cell invasive capacity using an xCELLigence system (Roche) for continuous monitoring of the cell migration (Figure 7B). We did not observe any migration until approximately 25 hours in culture after which we observed an acceleration in migration by Rab25KD cells that was not observed in either Control or Rescue cell lines. These findings indicate that loss of Rab25 can promote invasive characteristics in Caco2-BBE cells.

Modulation of Rab25 alters the gene expression.

Since our investigations suggested that loss of Rab25 was associated with decreases in α5-integrin mRNA expression, we performed gene microarray analysis of mRNA transcripts in Control, Rab25KD and Rescue Caco2-BBE cell lines. We sought to identify transcripts whose expression was either significantly increased or decreased in Rab25KD cells and then restored to control levels in Rescued cells. We identified only 29 genes with greater than four-fold changes between Control and Rab25KD that were normalized in the Rescue cell line (Table 1). 22 transcripts were up-regulated with loss of Rab25 and 7 transcripts were down-regulated. Among these transcripts, as seen by qPCR, α5-integrin was down-regulated and SI was up-regulated. Pathway analyses suggested that most of the genes altered by Rab25 knockdown were related to either glucose metabolism or retinoic acid pathways.
While these findings suggested that Rab25 loss could effect significant changes in gene transcription, we have found no evidence, by either isolation of nuclei or by immunofluorescence staining, that Rab25 traverses into the nucleus in Caco2-BBE cells (data not shown). Among the 29 genes, we identified only one transcription factor: ETS translocation variant 4 (ETV4, also known as E1AF or PEA3). We examined the expression of ETV4 during polarization of Caco2-BBE cells. No antibodies are presently available against ETV4 for immunoblot analysis, so we evaluated ETV4 expression by IF. Interestingly, the expression of ETV4 immunostaining (Figure S9) decreased with polarity. By immunostaining, Rab25 KD cells had lower expression of ETV4 in the nucleus. Rescue of Rab25 elicited an increase in nuclear ETV4 staining (Figure S9).

We therefore examined whether ETV4 could be the downstream target of the loss of Rab25 by overexpressing mCherry-ETV4 in Rab25 KD cells (Figure 8) and evaluating rescue of the Rab25KD phenotype. Over-expression of ETV4 caused a reduction of SI protein and an increase in α5 and β1-integrins expression similar to that observed in cells rescued with either untagged or Myc-tagged rabbit Rab25 (Figure 8). These results suggest that Rab25 controls gene transcription of these critical polarity proteins through the regulation of ETV4 expression.

**Discussion**

A number of investigations over the past decade have led to the concept that a loss of polarity is a central driver in the early stages of carcinogenesis. These changes at cellular junctions can involve the mis-trafficking of adhesion molecules such as integrins, junctional components such as claudins, or alterations in the assembly of proteins such as catenins. All of these scenarios lead to losses in polarized function that individually do not induce phenotypic transformation. Rather it appears that multiple deficits must be present to induce frank carcinogenesis. Previous studies have demonstrated that Rab25 loss, in combination with other alterations in cell behavior,
can lead to accelerated tumor formation in the intestine and colon (Nam et al., 2010). The results
detailed in the present studies indicate that Rab25 loss can alter not only trafficking pathways for
integrins and claudins, but it can also influence gene expression of a discrete set of transcripts
relevant to the maintenance of polarity.

The loss of aspects of cellular polarity is a major determinant of cell behavior. Previous
studies by Bretscher (Bretscher, 1992), Roberts (Roberts et al., 2001) and Pellinen et al.
(Pellinen et al., 2006) showed that most of the integrins are internalized from the membrane into
recycling compartments and recycled back to cell surface using small GTPases. Norman and
Mills (Cheng et al., 2004; Caswell et al., 2007) have previously implicated Rab25 as a tumor
promoter in ovarian cancer cells, where increases in Rab25 expression cause changes in α5β1-
integrins. In contrast, we have observed that membrane localization of β1-integrin in enterocytes
is disrupted in Rab25 knockout mice (Nam et al., 2010). Previous studies have also shown that
loss of β1-integrin in the intestinal enterocytes promotes intestinal tumor formation in the mouse
and knockdown of β1-integrin expression in Caco-2 cells leads to increased invasive properties
(Kuwada and Li, 2000). The over-expression of α5β1-integrin in HT29 colon carcinoma cells
dramatically reduced tumorigenicity (Varner et al., 1995). While the different tumor suppressor
and tumor promoter activities of Rab25 are not clear, Norman and colleagues have suggested
that when CLIC3 is expressed, Rab25 is a tumor promoter, while it acts as a tumor suppressor in
cells lacking CLIC3 expression (Dozynkiewicz et al., 2012). Indeed, we have found only low
levels of CLIC3 expressed in Caco2-BBE cells (data not shown). Nevertheless, in both ovarian
and intestinal systems, there appears to be consistent data that Rab25 can associate directly with
α5β1-integrin. Caswell, et al. demonstrated evidence for a direct association of over-expressed
Rab25 in ovarian cells (Caswell et al., 2007). In our present investigations, we have also
observed an association of Rab25 with α5β1-integrins in Caco2-BBE cells. These results suggest that Rab25 mediates context dependent trafficking decisions that manifest in cell-specific decisions in cargo presentation at epithelial cell surfaces.

Importantly, while both Rab11a and Rab11-FIP1C/RCP have been implicated in the regulation of α5β1-integrin trafficking (Caswell and Norman, 2006; Caswell et al., 2007; Lober et al., 2010), Rab25 loss did not influence the expression or distribution of either Rab11a or Rab11-FIP1C/RCP (data not shown). Previous studies in breast cancer cells have demonstrated that α2β1-recycling is mediated by Rab21 (Pellinen et al., 2006; Pellinen et al., 2008). We did not observed any change in Rab21 in the Rab25 KD cell line. We also observed reductions in the expression of α2-integrin in Rab25KD Caco2-BBE cells. All of these findings support a complex and dynamic relationship of targeted trafficking events in polarized epithelial cells.

Recent investigations have demonstrated that elements of the recycling system regulate the protein composition of the adherens and tight junctions. In MDCK cells, expression of a mutant of Rab11-FIP2 with a serine to glutamate mutation that mimics phosphorylation by MARK2/Parlb causes a loss of both E-cadherin from adherens junctions and occludin from tight junctions (Lapierre et al 2012). Interestingly the cells expressing the S227E mutation do not display any changes in transepithelial resistance, in part because they show an increase in Claudin-1 and a decrease in Claudin-2 expression. In the present studies, we have not observed significant alterations in adherens junction proteins with loss of Rab25. But we did find an increase in the expression of Claudin-1 compared to a prominent decrease in the expression of Claudin-2 and to a lesser extent decreases in Claudins 3 and 4 in Rab25 knockdown cells. While we have not observed localization of claudins in Rab25-containing vesicles, so it is presently not clear how vesicle trafficking pathways are involved in these changes in junctional properties.
The increased expression of Claudin-1 as well as the decreased expression of Claudin-2 are expected to lead to a decrease in monolayer permeability through the formation of cation selective ion channels at the tight junctions with particular affinity for the sodium ions and a resultant increase in transepithelial resistance (Hou et al., 2006; Van Itallie and Anderson, 2006). Thus, Rab25KD Caco2-BBE cells also demonstrated increased transepithelial resistance. In addition, Rab25KD Caco2-BBE cells also displayed increased soft agar colony formation and invasive cell behavior. While these properties may seem counterintuitive, previous investigations have noted that, as was observed for Rab25 loss (Nam et al., 2010), increases in Claudin-1 expression were noted previously in human colon cancers independent of tumor stage (Shiou et al., 2007; Krishnan et al., 2010). Thus alterations in barrier junctions, combined with changes in integrins may lead to invasive properties due to changes in the distribution and function of junctional components. In this case, tighter junctional associations of cells during invasion may lead to a more efficient cell invasion front.

In addition, to direct interactions of Rab25 with α5β1-integrins that would be expected to influence trafficking, we also observed a prominent influence of Rab25 on the expression of α5-integrin mRNA transcripts. Rab25 did not influence the mRNA expression of β1-integrin, but did lead to a decrease in both total β1-integrin protein expression as well as a loss of β1-integrin protein expression at plasma membrane surfaces, similar to that observed in Rab25-deficient mice (Nam et al., 2010). We also found that β1-integrin protein expression decreased with the assumption of mature polarity (Schreider et al., 2002), while α5-integrin and Rab25 protein expression increased during maturation of the polarized monolayer. These results suggest that alterations in β1-integrin may be secondary to the major regulation of α5-integrin gene expression as the primary focus for the influence of Rab25 in intestinal cells. No such
mechanism has previously been observed in other systems such as ovarian cancer, which manifest non-polarized epithelial phenotypes.

Previously, Rab proteins have not been associated strongly with changes in gene regulation. A recent report has noted an increase in a number of metabolic genes in association with over-expression of Rab25 in ovarian cancer cells (Cheng et al., 2012). Little if any evidence exists for translocation of Rab small GTPases to the nucleus. Indeed, we have not been able to find any evidence for Rab25 relocation to the nucleus in Caco2-BBE cells (data not shown). Nevertheless, our findings do indicate that loss of Rab25 does lead to discrete changes in gene expression. Loss of Rab25 leads to both up-regulation and down-regulation of genes involved in polarity. The most prominent of these changes are the loss of transcripts for α5-integrin and the marked up-regulation of transcripts for SI. Loss of Rab25 causes the down-regulation of the expression of the transcriptional regulator ETV4. Re-expression of ETV4 re-establishes the normal expression of both α5-integrin and sucrase isomaltase. It therefore appears that a regulatory cascade exists for control of ETV4 expression that is influenced by Rab25 and transcription of ETV4 mRNA is an intermediary regulator of polarity in enterocytes. While the results with ETV4 expression in Caco2-BBE cells suggest a tumor suppressor role for ETV4, previous studies have suggested that ETV4 is up-regulated in invasive colon cancer (Jung et al., 2011). Nevertheless, other studies have identified ETV4 as a key regulator of epithelial cell organization during development (Kuure et al., 2010). Thus, as is the case with other critical regulators such as the EGF receptor, expression of ETV4 in the context of the differentiated intestinal phenotype may promote polarity, while expression in the context of a dedifferentiated neoplastic phenotype may promote invasive properties.
In summary, the present investigations demonstrate that Rab25 regulates intestinal epithelial cell polarity through alterations in integrin gene expression. At the present time, it is not possible to pinpoint the connection between Rab25 and the regulation of ETV4 transcription. Further studies will be required to identify transcriptional regulators that may associate with Rab25-containing trafficking membranes. One would assume that sequestration of these factors in the cytoplasm with Rab25-containing vesicles might account for ETV4 transcriptional modulation in a manner similar to that observed for regulators such as β-catenin. Nevertheless, these studies do demonstrate that vesicular trafficking proteins can both directly influence the localization of critical regulators of polarity as well as affect polarity through the modulation of the cellular transcriptional profile.

**Experimental Procedures**

**Cell Culture and Reagents:**

Caco2-BBE cells were a gift from Dr. Matthew Tyska (*Vanderbilt University, Nashville, TN, USA*). Cell culture dishes and Transwells were purchased from Costar (Cambridge, MA, USA). The human colon cancer Caco2-BBE and HCA-7 cell lines and HEK293T cells were grown in D-MEM media with 10% heat-inactivated fetal bovine serum (FBS) and 1% Glutamine and penicillin/streptomycin. The cells were cultured on Transwell polycarbonate filters for all the experiments and media was changed every day throughout the culture duration. The following antibodies are used in this study: Rab25: for western blot (WB) 1:1000 Sigma-Aldrich Catalog # R4405 or affinity purified rabbit anti-Rab25 1:100 (Nam et al., 2010) and murine monoclonal anti-Rab25 12C3 for immunofluorescence (IF) 1:50 (Goldenring et al., 1993); α5-Integrin: for WB 1:2000 BD Biosciences Catalog # 610633, and for IF 1:100 BD Biosciences Catalog # 555651; α2-Integrin: for WB 1:500 BD Biosciences Catalog # 611016; β1-Integrin: for WB
1:1000 BD Biosciences Catalog # 610467 and for IF 1:50 Millipore Catalog # MAB1959; Fibronectin: for WB, 1:1000 BD Biosciences Catalog # 610077; β–Actin: for WB 1:5000 Sigma-Aldrich Catalog # A5316; SI: for WB, 1:500 Sigma-Aldrich HPA011897 and for IF 1:100 rabbit anti-SI (a gift from Dr. Matthew J. Tyska, Vanderbilt University, Nashville TN); Villin-1 for both WB and IF 1:1000/1:50 Cell Signaling Catalog # 2369; Claudin-1: for both WB and IF 1:2000/1:200 Invitrogen Catalog # 51-9000; Claudin-2: for both WB and IF 1:1000/1:100 Invitrogen Catalog # 51-600; Claudin-3: for both WB and IF 1:1000/1:100 Invitrogen Catalog # 34-1700; Claudin-4: for both WB and IF 1:2000/1:200 Invitrogen Catalog # 32-9400; Claudin-7: for WB 1:500 Invitrogen Catalog # 37-4800; ZO-1: for WB 1:500 Invitrogen Catalog # 61-7300), Occludin (WB 1:1000 Invitrogen Catalog # 71-1500), DsRed (WB 1:250 Clontech Catalog # 632496; Rab11a: rabbit anti-Rab11a for both WB and IF 1:2000/1:200 (Ducharme et al., 2006); Rab5: for WB 1:2500 Cell signaling Catalog # 3547; Rab21: for WB 1:2000 Sigma-Aldrich Catalog # R4405; Rab7: for IF 1:200 Abcam Catalog # Ab50533; Myc-Tag: for WB/ and immunoprecipitation (IP) 1:3000/1:50 Vanderbilt Antibody and Protein Resource (VAPR) Catalog # VAPR9E10; Rab8a: affinity-purified rabbit anti-Rab8a for WB 1:3000 (Roland et al., 2007). The secondary antibodies (Cy3, Cy5 and HRP conjugated) were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Agar was from Lonsa (Rockland, USA) and High Capacity cDNA Reverse Transcription Kits were purchased from Applied Biosystems (Foster City, CA USA)

**Rab25 knockdown and DNA constructs:**

The lentiviral vectors for shRNA targeting human Rab25 and scrambled control shRNA were purchased from Open Biosystems (Lafayette, CO). The shRNA against Rab25 and control shRNA were transfected in HEK239T cells along with packaging vector (pR8.2) and ENV
plasmid (pMDG.2) using Effectine reagent (Qiagen, Valencia CA). The next day the HEK cells were washed and refed with the same media used for the Caco-2 BBE cells. After another 48hrs the cell supernatant was collected and filtered through a 0.45 μM filter. Caco2-BBE cells were infected with 50% lentiviral containing media and 50% fresh media with polybrine (5 mg/ml). The cells were stably selected (after 72-96 hrs of infection) using puromycin (2 μg/ml) and stable cell lines were maintained with 1 μg/ml puromycin. The Rab25 knockdown was confirmed by immunoblotting using anti human-Rab25 antibodies. The cells stably expressing rabbit Rab25 were established by transfection of pCB6-rabbit Rab25 (Casanova et al., 1999) into Rab25 knockdown cells and clones were selected with 200 μg/ml of G418 and 1 μg/ml puromycin. The rabbit Rab25 sequenced was recloned into pMYC-C2 vector between EcoRI and SalI. This myc-tagged rabbit Rab25 expression vector was then used to establish stable rescue lines by transfection into Rab25 knockdown cells and G418 selection as above.

**Immunoblot**

The Caco2-BBE-Control, Rab25KD and Rescue (both untagged rabbit Rab25 and myc-rabbit Rab25) cells were plated on 6 well Transwells for 3, 8 and 15 days then washed with PBS and lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 10 minutes at 4°C. The lysate was sonicated for 25 seconds and centrifuged at 16000g for 10 min. The supernatant was transferred to a new tube and protein concentration was determined by BCA method using the Pierce BCA protein assay reagent (Pierce, Rockford, CA). 20 μg of total protein was suspended in 1X SDS Sample buffer, heated for 10 min. at 70°C, resolved on a 6-12% SDS-PAGE gel (Laemmli, 1970) and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The blots were blocked in 5% DMP/TBS-T (5 % dry milk powder in Tris-buffered saline, 0.01% Tween-20). The blots were incubated for 2
hrs at RT or overnight at 4°C with primary antibody diluted in 1% DMP/TBS-T. The blots were then washed 5 times for 5 minutes at RT with TBS-T (0.01% Tween-20) and incubated for 1 hour at RT with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch), washed 5 times for 5 minutes at RT with TBS-T (0.01% Tween-20) followed by 1 wash with just TBS. Specific labeling was detected by chemiluminescence reagent (Pierce, Rockford, CA) with detection using Kodak BioMax ML film. The film was scanned and the amount of proteins was evaluated by densitometry using Image J software. The relative band intensity for each protein was obtained through normalization to the intensity of immunoreactive β-Actin bands. The statistical significance was calculated using an unpaired students t-test with Graphpad software. The final graph was made using Prism software.

**Immunofluorescence**

The Caco2-BBE-Control, Rab25KD and Rescue cells were plated on 12 well Transwells for 3, 8 and 15 days, and then washed with PBS and fixed. For claudin staining, Transwells were fixed with -20°C methanol for 5 minutes at -20°C. For all other staining, the cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at RT. Cells were washed 3 times with PBS then blocked/extracted for 30 minutes at RT in 10% normal goat serum (Jackson ImmunoResearch), 0.3% TritonX-100 in PBS. Transwells were incubated for 2 hours at RT or overnight at 4°C with primary antibodies diluted in 1% normal donkey serum, 0.005% Tween-20 in PBS. Transwells were washed 3 times for 15 minutes at RT with 0.005% Tween-20 in PBS (PBS-T), then incubated for 1 hour at RT with secondary antibodies diluted as above, washed 3 times in PBS-T, once in PBS, then rinsed in water and mounted with ProLong Gold plus DAPI (Invitrogen). All images were captured with an Olympus FV1000 confocal microscope or a Zeiss 510 LSM confocal microscope (Vanderbilt Cell Imaging Shared Resource) using a 60X oil immersion
objective with an NA of 1.42 and a 5X optical zoom using the microscope’s software. The individual images were converted to tif files with the FV1000 or Zeiss LSM software and then Adobe Photoshop was used to produce the final figures.

For dual staining of Rab25 with α5-integrin, β1-integrin and Rab7 using TSA kit (PerkinElmer, Waltham MA), The Caco2-BBE cells were plated on 12 well Transwells for 8 days, washed with PBS and fixed. in 4% paraformaldehyde for 20 minutes at RT. Cells were washed 3 times with PBS then block/extracted for 30 minutes at RT in 10% normal goat serum (Jackson ImmunoResearch), 0.3% TritonX-100 in PBS. Transwells were incubated for 2 hours at RT or over night at 4°C with primary antibodies diluted in 1% normal donkey serum, 0.005% Tween-20 in PBS. Transwells were washed 3 times for 15 minutes at RT with 0.005% Tween-20 in PBS-T, then incubated for 1 hour at RT with secondary antibodies diluted as above, washed 3 times in PBS-T, once in PBS. The cells were blocked as above and incubated with Rab25 antibody (12C3) overnight at 4°C. The cells were washed 5 times for 5 minutes at RT with PBS-T and then cells were incubated with specific secondary antibody conjugated with HRP. The cells washed 5 times for 5 minutes. The secondary antibody signal was amplified using Cyanine-3 fluorophore-labeled tyramide amplification reagent for 7 minutes. The transwell were washed 3 times for 5 minutes and rinsed in water and mounted with ProLong Gold plus DAPI (Invitrogen).

All images were captured with an Olympus FV1000 confocal microscope or a Zeiss 510 LSM confocal microscope as above. The colocalization of Rab25 with Rab11a, Rab7, α5-integrin, β1-integrin was quantitated and analyzed using Imaris Software (version 7.3.1, Bitplane AG, Switzerland). For each colocalization analysis, 5-10 entire X-Y confocal images were analyzed. The thresholds value and single time points were selected from 2D scatter plot and
histogram mode for the both channels. The colocalization matrices were built and Pearson correlation coefficient and Mander’s coefficients were calculated.

**Polymerase chain reaction quantitation:**

The Control, Rab25KD and rescue cell lines were plated on 6 well Transwell filters and total RNA was extracted using Trizol reagent according to the manufacturer’s instructions. One microgram of RNA was treated with RQ1 RNase-free DNase (Promega) and used for cDNA synthesis with random hexamers. For RT-PCR, cDNA was made using Advantage 2 RT-PCR kit (Clontech, Mountain View, CA, USA) according to the manufacturer’s instructions. 2 μl of 5 times diluted cDNA was amplified using gene specific primers. β–Actin amplification was used as control to normalize the transcript expression. For quantitative PCR, the cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with a mix of random and oligo-dT primers according to the manufacturer’s instructions. Real-time PCR was performed using an Applied Biosystems StepOnePlus real-time PCR system with Express SYBR Green ER Supermix (Applied Biosystems) and performed in triplicate for each transcript. The real-time PCR data were analyzed by the comparative C_T method as described by Schmittgen and Livak (Schmittgen and Livak, 2008). The primers were purchased from Sigma Genesys (St Louis, MO, USA) and validated for melting temperature and efficiency. The primers for the qPCR analysis used were as follows:

Rab25-sense primer: 5’-GACCTCAGCCCTGGGACTCTAC-3’
Rab25 antisense primer: 5’-TGATGCAACAGGCCCTCTTCTC-3’
α5-Integrin sense primer: 5’-TGGAGTCTCCTGACTGTCCAGC-3’
α5-Integrin antisense primer: 5’-TCGCTCAGTGGCTCCTTCTC-3’
β1-Integrin sense primer: 5’-ATTGGCCCTTGCATTACTGCTG-3’
β1-Integrin antisense primer: 5’-AGCTACCTAACTGTGACTATGG-3’
SI sense primer: 5’-TGGCAAGAAAGAATTTAGTGGA-3’
SI antisense primer: 5’-TTATTTCTACATTGACAGGATC-3’
Villin-1 sense primer: 5’-AAGAAAGCCAATGAGCAGGAGAAG-3’
Villin-1 antisense primer: 5’-TTCTCAATGCGCCACACCTG-3’
GAPDH sense primer: 5’-AGATCCCTCCAAAATCAAGTG-3’
GAPDH antisense primer: 5’-GGCAGAGATGATGACCCTTTT-3’

**Scanning electron microscopy**

A total of 100,000 cells were plated and grown on 12 well Transwells. After 15 days in culture, cells were washed 3 times with PBS. The cells were fixed with 3% glutaraldehyde in SEM buffer (0.1 M sodium phosphate buffer, pH 7.4, 0.1 M sucrose) at 4°C overnight. Cells were washed with SEM buffer twice, treated with 1% osmium tetroxide (in SEM buffer) on ice for 1 hour, and washed 3 times with SEM buffer. The cells were dehydrated with serial dilutions of ethanol (35%, 50%, 70%, 95% and 100%) for 15 minutes each and then incubated with hexamethyldisilazane (HMDS). After the HMDS treatment, samples were mounted on stubs and coated with gold in a sputter coater. The cells were analyzed using an FEI Quanta™250 SEM (Vanderbilt Cell Imaging Shared Resource).

**Transepithelial resistance (TER) measurements**

The cell lines were plated on 24 well Transwells and media was changed every day. The TER was measured using a Millicell-ERS (Millipore) at 3 different places on each Transwell. The mean was recorded and the results were expressed in Ωcm².

**Soft agar colony growth.**

A soft agar proliferation assay was performed as described previously (Kuwada and Li, 2000). After 3 weeks, the colonies were treated with Thiazolyl Blue Tetrazolium Bromide (0.5mg/ml)
for 2 hours at $37^\circ$ C. The colonies were counted using a GelCount™ colony-counter (Vanderbilt Epithelial Biology Center Shared Resource). The results represent the mean of three individual experiments.

**Microarray Analysis**

The Control, Rab25KD and Rescue cell lines were plated on Transwells for 8 days. On day 8, total RNA was isolated and treated with RNase-Free DNase as mentioned above. The RNA from replicate samples was submitted to Genomics Shared Resource microarray facility at Vanderbilt University. The RNA samples were hybridized to Human Gene 1.0 ST Affymetrix arrays.

**Immunoprecipitation**

Caco2-BBE Rab25 KD cells transfected with myc-Rab25, were grown for 5 days on Transwells. The cells were washed twice with PBS and lysed with lysis buffer (30mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM Mg-Acetate and 1% CHAPS). After 30 minutes incubation rotating end-over-end at $4^\circ$C, the lysates were centrifuged at 15,000 g for 15 minutes at $4^\circ$C to remove the cell debris. 100 µg of lysate protein were pre-cleared with 50 µl of Dynabeads for 2 hrs at $4^\circ$C. For immunoprecipitation, the anti-myc antibody, anti-α5-Integrin antibody or non-specific IgG were incubated with Dynabeads for 1 hour at room temperature. The beads were washed 2 times with PBS and re-suspended in 200 mM triethanolamine (pH 8.2) and incubated for 10 min at room temperature. The beads were crosslinked with 20 mM dimethyl pimelimidate dihydrochloride for 30 minutes at room temperature. The Dynabeads were washed with 50 mM Tris-HCl (pH 7.5) for 15 minutes and then 3 times with TBS. The washed Dynabeads were incubated with pre-cleared lysates overnight. Beads were washed with lysis buffer 3 times and eluted with 1.5%
SDS-PAGE sample buffer and incubation for 10 minutes at 70°C. The supernatant was analyzed by SDS-PAGE as above.

**Cell invasion assay**
To assess cell invasion capacity, we utilized an XCELLigence system (Roche Applied Science, IN, USA). In a CIM Plate 15, 150 µl of serum-containing media was added to the bottom chamber. The background value was measured in the RTCA DP analyzer. All the cell lines were trypsinized and plated in the top chamber with serum free media. The CIM plate was left in the culture hood for 10 minutes and then it was moved to a 37°C incubator and assembled in the RTCA DP analyzer. The membrane in the CIM plate equilibrated for 1 hr. After 1 hour, the cell index number was measured every 15 minutes for 55 hours.

**Statistical analysis**
Descriptive statistics including mean values and SD/SEM were calculated using Microsoft Excel and P values were calculated using an unpaired students t-test with Graphpad software. All data represent at least three independent experiments and are expressed as the mean±SD/SEM unless otherwise indicated.

**Acknowledgments**
Confocal fluorescence imaging was performed through the use of the VUMC Cell Imaging Shared Resource supported by National Institute of Health (NIH) Grants CA68485, DK20593, DK58404 and HD15052. This work was supported by NIH National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants RO1 DK048370 and RO1 DK070856 (to J.R.G.). We thank James Higginbotham for assistance with Caco2 cell sorting.

**Author Contributions:**
Krishnan performed experiments, prepared figures and wrote manuscript.
Lapierre performed experiments and reviewed manuscript.
Knowles performed experiments and wrote manuscript.

Goldenring designed experiments, prepared figures and wrote manuscript.

**Conflict of Interest:**

None of the authors have any conflicts of interest.

**REFERENCES**


pathways for IgG transport by FcRn are distinct and display an inherent polarity. J Cell Biol 185, 673-684.


Table 1: Rab25-regulated gene transcripts. Control, Rab25KD and Rescue Caco2-BBE cell lines were grown on 6-well Transwells for 8 days. Total RNA was isolated using Trizol and treated with RNase-free DNAse. The replicate total RNA samples from separate experiments were submitted for gene expression analysis by Affymetrix microarrays. Gene transcripts were identified with greater than four-fold changes between Control and Rab25KD cells that were reversed in the rabbit Rab25 Rescue cells.

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**Figure 1: Rab25 expression increases with polarity in polarized epithelial cell lines.** (A) Endogenous expression of Rab25 in colorectal cancer cell lines. Blots were probed with a polyclonal antibody that was specific for human Rab25. (B) Protein expression for enterocyte differentiation markers Villin-1 and Sucrase Isomaltase (SI) and Integrins β1, α2 and α5 were analyzed in Caco2-BBE cells grown on Transwells for 3, 8 and 15 days. (C) Rab25 protein expression was assessed in protein lysates from Caco2-BBE cells grown on Transwells for 3, 8 and 15 days by immunoblot. (D) Densitometric analysis of protein expression in Caco2-BBE cells. *p<0.0001, #p<0.0005 and $p<0.01 compared to protein expression at 3 days. (E) Rab25 mRNA levels were analyzed by RT-PCR in Caco2-BBE cells plated as above. (F) The Rab25 mRNA expression was analyzed by quantitative real time PCR (normalized to GAPDH) in Caco2-BBE cells in different stages of polarization. Values (fold-change) are mean ±standard deviation from three separate experiments. **p<0.0005 compared to mRNA expression at 3 days.
**Figure 2: Alteration of Rab25 expression alters expression of integrins.** Caco2-BBE cells lines were prepared stably expressing scrambled shRNA (Control, C), shRNA targeting human Rab25 (KD, K) and Rab25KD cells stably re-expressing rabbit Rab25 (Rescue, R). Cells were grown on Transwell filters for 3, 8 and 15 days. (A) Quantitative RealTime PCR was utilized to analyze expression of endogenous human Rab25. *p<0.001 compared with days matched Control, #p≤0.0005 compared with expression on day 3. Data are representative of three separate experiments. (B) Cell lysates were analyzed for human Rab25 (Hu Rab25), Integrins β1, α2, α5 and Fibronectin by immunoblotting. The membrane was reprobed for β–actin as a loading control.
Figure 3. Rab25 depletion causes a loss of integrins from Caco2-BBE cells. (A) All three Caco2-BBE cell lines at different stages of polarity were fixed with methanol and stained for α5-integrin. X-Z confocal fluorescence images are shown and are representative of at least 3 experiments. (B) Cells grown as in (A) were stained for E-cadherin. X-Z confocal fluorescence images are shown and are representative of at least 3 experiments. (C) To test the localization for β1-integrin Caco2-BBE cells were fixed with paraformaldehyde and stained for human β1-integrin. X-Z confocal fluorescence images are shown above single X-Y sections. The experiments were repeated at least 3 times. Bar = 10 μm.
Figure 4. Rab25 associates with α5β1-integrin in Caco2-BBE cells. (A) Caco2-BBE cells were grown on transwell and stained for endogenous expression of Rab25 and Rab11a. For the colocalization of Rab25 with (B) Rab7, (C) α5-integrin and (D) β1-integrin, the BBE cells were grown on transwell for 8 days, fixed with 4% paraformaldehyde and stained using a TSA kit. Bars = 10 um. (E) Colocalization between Rab25 and other immunostains was determined and Mander’s comparison coefficients and Pearson coefficient were calculated. (F) Rab25KD cells stably transfected with myc-rabbit Rab25 were grown on Transwells filters for 5 days in culture, lysed with 1% CHAPs buffer and immunoprecipitations were performed with anti-myc or non-
specific IgG and immunoisolates were probed on western blots for $\alpha_5$-integrin, $\beta_1$-integrin and myc. Input staining at right was for 20% of the lysate used in the immunoprecipitations. (G)

Cells were lysed as in (F) and immunoprecipitations were performed with anti-human $\alpha_5$-integrin or non-specific IgG and immunoisolations were analyzed in western blots for myc and $\alpha_5$-integrin. Input staining at right was for 20% of the lysate used in the immunoprecipitations. Results are representative of 3 separate experiments.
Figure 5: Rab25 loss alters the Caco2-BBE apical brush border. (A) Scanning electron microscopy was used to visualize the apical brush border in Control, Rab25KD and Rab25 Rescue cells. Cells were grown on Transwells filter for 15 days and fixed with 3% glutaraldehyde in SEM buffer. Bar = 10 µm (left and middle panels); 1 µm (right panels) (B) Protein expression of Villin-1 and the apical membrane-anchored hydrolase sucrase isomaltase (SI) were analyzed in total cell lysates by western blot at different stages of polarity in Control, Rab25KD and Rescue cells. (C) The three cell lines were fixed with 4% paraformaldehyde on the indicated days and stained for villin-1. X-Z confocal fluorescence images shown are representative of 3 separate experiments. Bar = 10 µm. (D) Control, Rab25KD and Rescue Caco2-BBE cells were grown for 15 days on Transwell filters, fixed with 4% PFA and stained for SI (red in merge) and F-actin (green in merge). X-Z confocal fluorescence images shown are
representative of 3 separate experiments. Bar = 10 μM. (E) RNA was isolated from all 3 cell lines and SI mRNA expression was analyzed by qRT-PCR. The data are from at least 3 separate experiments and are presented as the mean±standard deviation. #p<0.001 compared with 3 day control value, $p≤0.05$ compared to 8 day control value, *p<0.0001 compared to days matched control and +p<0.001 compared to Rab25KD.
Figure 6: Rab25 depletion alters the transepithelial resistance and claudin expression. (A) Transepithelial resistance (TER) was measured in all cell lines from day 3 to 15 on Transwell filters. During this period, the media was changed every day and TER was measured in 24-well Transwell inserts. Results are expressed in Ωcm² and are representative of three separate experiments. (B) Equal amounts of total protein lysates (20 μg) from Control, Rab25KD and Rescue cells were analyzed by western blots for tight junction proteins. The blots were subsequently stripped and reprobed for β-actin as a loading control. The results are representative of 3 separate experiments.
Figure 7: Rab25 loss promotes soft agar colony formation and increases the cell migration in Caco2-BBE cells. (A) Control, Rab25KD and Rescue cell lines were mixed with 0.6% low
melting agarose in DMEM media. Cells were allowed to grow for 21 days. After 21 days, colonies were counted using a Gelcount™ colony counter. Rab25KD cells showed significant increases in the number as well as the diameter of colonies compared to Control and Rescue cells. \*p≤0.005 compared to number of colony formed in control and \#p≤0.0001 compared to size of the colony formed in control. (B) The three Caco2-BBE cell lines were plated in the top chambers of xCELLigence CIM Plates with serum free media and serum-containing media was loaded into lower chamber. The cells were incubated in the RTCA DP Analyzer inside the incubator and measurements were taken every 15 minutes for 55 hrs. The representative traces from one experiment show that Rab25KD cells showed increased migration after 24 hrs, whereas Control and rescue cells showed more indolent migration throughout. The experiments were performed in triplicate and repeated three times.
Figure 8: Rab25 regulates protein expression through expression of the transcription factor ETV4. (A) Control, Rab25KD, and Rab25KD cells rescued with either untagged rabbit Rab25 (R) or myc-tagged rabbit Rab25 (M) were plated on 6-well Transwells. For ETV4 expression, the Rab25KD cells were transiently transfected with mCherry-ETV4 (E). After 8 days in culture, the cells were lysed with RIPA and analyzed by western blot for endogenous human Rab25, β1-integrin, α5-integrin and SI. The membrane was reprobed for myc and mCherry to demonstrate expression of the myc-Rab25 and mCherry-ETV4, respectively. (B) Quantitative analysis of 3 separate experiments. *p<0.001, #p<0.05 compared with control value.