TBC1D9B functions as a GTPase-activating protein for Rab11a in polarized MDCK cells

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INTRODUCTION

Collectively Rab GTPases form a large family of evolutionarily conserved proteins (42 subfamilies in humans) that coordinate vesicle fission, transport, tethering, and fusion (Stenmark, 2009). Their function is regulated by specific guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors. Whereas GEFs activate their cognate Rabs by promoting the exchange of GDP for GTP, GAPs facilitate the hydrolysis of GTP to GDP, returning Rabs to their inactive, GDP-bound state. Studies in the past few years have identified a family of Tre2-Bub2-Cdc16 (TBC) domain–containing proteins, several members of which have Rab GAP activity. This function depends on two conserved motifs in their TBC domain: an arginine or R finger within an IxxDxxR motif, and a glutamine or Q finger within an YxQ motif. Whereas the conserved Y residue in the Q finger interacts with the conserved switch II glutamate residue of the Rab GTPase, the R and Q residues of Rab proteins coordinate the Rab-bound GTP, promoting its hydrolysis (Pan et al., 2006; Gavriljuk et al., 2012). The Rab specificity and GAP activity have been defined for some TBC-domain proteins (Fukuda, 2011; Frasa et al., 2012), but there is limited information for the other family members, including TBC1D9B.

Structurally, TBC1D9B is composed of two N-terminal GRAM domains, a central TBC domain–containing proteins, and a C-terminal EF hand motif (Doerks et al., 2000; Frasa et al., 2012). The closest nonvertebrate orthologue to TBC1D9B is the
yeast protein Gyp2p, which has a similar domain architecture (but has only one GRAM domain) and apparently acts as a GAP for Ypt31p, a homologue of Rab11a (Sciorra et al., 2005). No studies have defined whether vertebrate TBC1D9B has GAP activity or have determined its Rab specificity.

Rab11a is a well-studied Rab GTPase that regulates exocytosis and is critical for a variety of cellular processes, including membrane protein recycling (Ulrich et al., 1996; Wang et al., 2000b), transcytosis (Wang et al., 2000b; Su et al., 2010; Xu et al., 2011), regulated exocytosis (Khandelwal et al., 2008, 2013), cytokinesis (Fielding et al., 2005; Wilson et al., 2005; Yu et al., 2007), ciliogenesis (Knodler et al., 2010; Westlake et al., 2011), lumenogenesis (Descoleaux et al., 2008; Bryant et al., 2010), and autophagosome formation (Fader et al., 2008; Longatti et al., 2012). Whereas many studies have focused on identifying Rab11a effectors (e.g., FIP1-5 and Sec15A; Hales et al., 2001; Lindsay et al., 2002; Wallace et al., 2002; Zhang et al., 2004; Wu et al., 2005; Oztan et al., 2007; Horgan and McCaffrey, 2009; Baetz and Goldenring, 2013), much less is known about the specific GEFS and GAPS that regulate vertebrate Rab11a activity, and the majority of insights have come from studies in model organisms. In yeast, the multisubunit TRAPII complex is reported to act as a GAP for Ypt31p and Ypt32p (Jones et al., 2000; Morozova et al., 2006), which is an additional orthologue of Rab11a. However, the purified TRAPII complexes may lack GEF activity in mammalian cells (Yamasaki et al., 2009). In addition, the Drosophila protein Crag acts as a GEF for Rab11 in photoreceptor cells (Xiong et al., 2012) and for Rab10 in vitro studies (Yoshimura et al., 2010). In the case of GAPs, the TBC-domain-containing protein Evi5 regulates border cell migration during Drosophila oocyte development by acting as a Rab11a GAP (Laflamme et al., 2012). However, whether Evi5 acts as a GAP for vertebrate Rab11a is controversial. Some reports indicate that it lacks Rab11a GAP activity, whereas others point to the opposite conclusion (Dabbeekeh et al., 2007; Fuchs et al., 2007; Westlake et al., 2007; Hehnly et al., 2012). Another TBC-containing protein, TBC1D14, appears to bind to Rab11a and Rab11b and regulate autophagosome formation but apparently does not function as a GAP but does function as a Rab11a/b effector (Longatti et al., 2012). Thus, the regulatory proteins that regulate the vertebrate Rab11a GTPase cycle remain to be defined and characterized.

Using a polarized Madin–Darby canine kidney (MDCK) cell culture model, coupled with in vitro assays, we observed the following. Although able to interact with Rab11a, Rab11b, and Rab4a in a nucleotide-dependent manner, TBC1D9B only stimulated Rab11a GTP hydrolysis in vitro. Furthermore, endogenous TBC1D9B colocalized with Rab11a-dependent endosomes, as well as with cargoes that transit these endosomes, including immunoglobulin A (IgA) bound to the polymeric immunoglobulin receptor (pIgR), the archetypal basolateral-to-apical transcytotic protein. We further observed that overexpression of TBC1D9B, but not an inactive mutant, slowed the rate of basolateral-to-apical IgA transcytosis, where short hairpin RNA (shRNA)–mediated depletion of TBC1D9B increased the rate of this process. Furthermore, expression of TBC1D9B decreased the amount of active Rab11a in the cell and concomitantly disrupted the interaction between Rab11a and its effector, Sec15A. We discuss the likelihood that TBC1D9B acts as a Rab11a GAP in polarized MDCK cells, where it regulates the Rab11a-dependent IgA-pIgR transcytotic pathway. We also discuss the possibility that TBC1D9B may act as an effector of Rab11b and/or Rab4a.

RESULTS

TBC1D9B interacts with specific Rab GTPases

TBC1D9B is an ~140-kDa TBC domain–containing protein with a comparable domain architecture to Gyp2p (Figure 1A). Furthermore, the overall similarity of these proteins is reasonably high at 49% and increases to 64% when the TBC domains are assessed individually. The TBC domain of TBC1D9B contains conserved R, Y, and Q residues, which form the “R” and “Q” fingers that are critical for GTP hydrolysis in other TBC proteins (Figure 1B; Pan et al., 2006; Gavriljuk et al., 2012).

To identify possible substrates for TBC1D9B, we first tested whether TBC1D9B could bind to Rab GTPases—Rab11a in particular—using a yeast two-hybrid assay with TBC1D9B as bait and different Rabs as prey (Supplemental Figure S1). A positive interaction was found between TBC1D9B and Rab11a-Q70L (abbreviated Rab11a-QL), a mutant of Rab11a that was proposed to be defective in GTP hydrolysis (Adari et al., 1988; Sternmark et al., 1994; Ulrich et al., 1996). Notwithstanding a previous report that this mutant is able to hydrolyze GTP and therefore may not be constitutively

FIGURE 1: Domain structure of TBC1D9B and sequence comparisons of its TBC domain with other TBC domain–containing proteins. (A) Schematic of human TBC1D9B and Saccharomyces cerevisiae Gyp2p domain structure. Predicted GRAM domains are colored pink, TBC domains are colored green, and EF hands are colored light blue. (B) Alignment of the indicated region of the TBC domains using MAFFT software (Larkin et al., 2007; Katoh and Standley, 2013). Sequences including the arginine (R) and glutamine (Q) fingers are shaded gray; key amino acids in the R and Q fingers are shaded red. Identical residues are indicated with an asterisk, conserved amino acid substitutions by a colon, and semiconserved substitutions by a period. The percentage of identity (Id) or similarity (Sim) between the TBC domains using MAFFT software (Larkin et al., 2007; Katoh and Standley, 2013).

| TBC1D9B | TBC1D9A | TBC1D8A | TBC1D8B | Gyp2p | TBC1D10A | TBC1D10B | TBC1D11 | Evi5 | dmaEvi5 | TBC1D4 | TBC1D1 | TBC1D30 | Gyp1p |
|---------|---------|---------|---------|-------|----------|----------|---------|------|--------|-------|--------|---------|-------|
| (546) YSLATEEIDRLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (552) YNLATEEIDRLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (553) CCLATEEIDRLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (552) CCLATEEIDRLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (272) PQQARMDEIEERLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (154) DPKWLOVIERLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (396) DPQWLOVIERLoKSMPEHPAPFOKGE-LGIAERKRLVTLATAFRPFIFVAFIAGO | (559) ESSPDQASICTRIEIPFHAFDFK摇了另一方面6QGQQLYXKIAKAYSVD1E.POAVG | (195) T5Y30C50L30X50E50P50FX50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 | (147) TSACEK50V50R50K50E50A50T50P50FX50P50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 | (159) LTQWQ150L30X50E50P50FX50P50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 | (841) LTRQWW150L30X50E50P50FX50P50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 | (127) DDRAQ150L30X50E50P50FX50P50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 | (404) LTRQWW150L30X50E50P50FX50P50K50D50Q50L50W50F50M50Y50L50S50D50V50F50G50 |
| 87% | 87% | 87% | 87% | 87% | 35% | 35% | 30% | 46% | 29% | 29% | 30% | 30% | 30% |
| 93% | 84% | 87% | 87% | 64% | 52% | 53% | 48% | 49% | 49% | 47% | 48% | 57% | 39% |
TBC1D9B is a Rab11a GAP

TBC1D9B stimulates Rab11a GTP hydrolysis

Next, we sought to determine whether TBC1D9B had GAP activity against any of its binding partners. We first performed comparative protein structure modeling using the known three-dimensional (3D) structure of the TBC1D4 TBC domain as a template (Protein Data Bank [PDB] file 3QYB; Park et al., 2011). Figure 3A shows the predicted structure of the TBC domain of TBC1D9B superimposed on the known structure of the TBC1D4 TBC domain. The structural overlap parameter obtained was 64%, confirming that the two structures were structurally related (Geourjon et al., 2001). In addition, the putative catalytic residues (R359, Y392, and Q594) of TBC1D9B were exposed similarly to those of TBC1D4 and other TBC proteins such as Gyp1p (unpublished data). Thus, the sequence homologies and predicted structure are consistent with the possibility that TBC1D9B was a functional GAP.

Next, we evaluated whether TBC1D9B had measurable GAP activity. Because we were unable to express sufficient quantities of the full-length protein in Escherichia coli (TBC1D9B has a molecular weight of ~140 kDa), we synthesized fragments of TBC1D9B and tested these for GAP activity (Supplemental Figure S2A). The fragment 301–810, which we refer to as TBC1D9B-(301–810), retained maximal in vitro GAP activity against Rab11a and was used in our subsequent studies (Supplemental Figure S2B). Of the 14 Rabs tested, a significant increase in mean GAP activity was observed only in the case of Rab8a and Rab11a (Figure 3B). However, no GAP activity was detected for the other Rabs, including Rab4a, or the other Rab11 family members Rab11b and Rab25. Further analysis revealed a linear dependence between the TBC1D9B-(301–810) concentration and the initial rate of Rab11a-GTP hydrolysis (Figure 3, C and D). At 4 μM, the extent of GTP hydrolysis was 9.7 times greater than GTP hydrolysis in the absence of TBC1D9B-(301–810) (Figure 3C), and the calculated rate of GTP hydrolysis was 4.8 times that of the intrinsic rate of Rab11a GAPase activity in the absence of TBC1D9B-(301–810) (Figure 3D). The catalytic efficiency of wild-type TBC domain was calculated as 158.3 M−1 s−1.

As a control for these studies, we generated variants of TBC1D9B that contained mutations in key amino acids exposed in the active site of the protein (Figures 1B and 3A) and were therefore likely to lack activity. To screen for inactive TBC1D9B mutants, we used non-polarized HEK cells. In these cells, we observed a significant redistribution of Rab11a from recycling endosomes (which appear as a juxtanuclear cluster in these images) to the cytosol when TBC1D9B was overexpressed (Supplemental Figure S3A). However, this redistribution was not observed in cells expressing TBC1D9B-(301–810) (Figure 3B). The catalytic efficiency of wild-type TBC domain was calculated as 158.3 M−1 s−1.

As an additional method to screen for TBC1D9B interactions, we determined whether flag-TBC1D9B could coimmunoprecipitate a broader array of GFP-tagged, GTP-locked mutants of Rab proteins previously implicated in endocytic and exocytic membrane traffic. Of the Rabs tested, we observed that Rab11a-QL, Rab11b-QL, and Rab4a-QL formed significant interactions with flag-TBC1D9B (Figure 2). In contrast, little interaction was observed with nominally GTP-locked mutants of Rab3a, Rab5a, Rab6a, Rab7, Rab8a, Rab10, Rab13, Rab25, Rab27a, or Rab33a (Figure 2). Taken together, these results indicate that TBC1D9B forms interactions with a selective number of Rab GTPases, including Rab11a, Rab11b, and Rab4a.
FIGURE 3: TBC1D9B in vitro GAP activity. (A) TBC1D4 (PDB: 3QYB) was used as a template (gray) to model the 3D structure of the TBC1D9B TBC domain (green). (B) GTP hydrolysis by the indicated wild-type, GST-tagged Rab loaded with $[$γ-32P]GTP and incubated with 2 μM of either wild-type or mutant TBC1D9B-(301-810) for 60 min at 30°C. Data are corrected for reactions lacking the TBC1D9B fragment. Those values significantly different from the group means, assessed by ANOVA, are indicated (*$p$ < 0.05). (C) Kinetics of Rab11a GTP hydrolysis loaded with $[$γ-32P]GTP and incubated with 0, 0.5, 1, 2, or 4 μM recombinant TBC1D9B-(301-810). (D) Initial rates of Rab11a GTP hydrolysis plotted against the concentration of wild-type or mutant TBC1D9B-(301-810). (E) In vitro GAP assays performed in the presence of Rab11a or Rab8a. In these reactions, Mg$^{2+}$ mixed at a 1:1 M ratio with GTP, was added at a final concentration of 0.5 mM. Alternatively, the reaction was supplemented with 5 mM MgCl$_2$. Reactions were incubated for 30 min at 30°C. Data were normalized to control reactions in which no TBC1D9B-(301-810) was added. Values for TBC1D9B-(301-810) that were significantly different ($p$ < 0.05) from matched incubations performed in the presence of TBC1D9B-RYQ/AAA (301-810) or between the indicated reactions are identified with an asterisk. (F) Top, full-length flag-TBC1D9B-WT or
inactive mutant, we showed that it lacked significant GAP activity in the in vitro assays described earlier (Figure 3, B–D).

We were initially surprised that Rab8a-QL served as a substrate for TBC1D9B in the GAP assays (Figure 3B) but did not coimmunoprecipitate with flag-TBC1D9B (Figure 2). Additional experimentation revealed that these differences were likely related to the amount of Mg2+ used in these assays. Whereas the in vitro GAP assays were initially performed in minimal concentrations of Mg2+ (GTP bound with Mg2+ was used in the assays at a final concentration of 0.5 mM), similar to previous reports (Krügmann et al., 2004; Haas et al., 2005; Fuchs et al., 2007; Yoshimura et al., 2008), the coimmunoprecipitation studies in Figure 2 were performed in the presence of an additional 2.5 mM Mg2+ (Oztan et al., 2007; Tsun et al., 2013). Of interest, when we removed Mg2+ from the coimmunoprecipitation studies, we observed interactions between GFP-Rab8a-QL and flag-TBC1D9B (see later discussion of Figure 5C). In contrast, supplementing the concentration of Mg2+ in the GAP assays by 5 mM MgCl2 (Du et al., 1998; Albert et al., 1999; Casanova et al., 1999) evoked a significant increase in Rab11a GTPase activity (Figure 3E) but not for Rab8a (Figure 3E) or any of the other Rab GTPases tested (Supplemental Figure S2C). Finally, we expressed full-length flag-TBC1D9B in HeLa cells and then recovered the protein by immunoprecipitation. Like TBC1D9B-(301-810), the full-length protein showed GTPase activity only against Rab11a and not Rab8a (Figure 3F).

In summary, these results demonstrate that when the assay reactions are supplemented with 5.0 mM Mg2+, TBC1D9B shows greatest activity toward Rab11a.

**Rab11a binds to the TBC domain of TBC1D9B**

Next, we explored the nature of the interaction between TBC1D9B and Rab11a. In general, GAPs are believed to interact most robustly to Rab in their GTP-bound state and less so with Rab’s in their GDP-bound state (Will and Gallwitz, 2001; Haas et al., 2005; Fuchs et al., 2007; Hannemann et al., 2012). Consistent with this finding, we observed that immunoisolated flag-tagged TBC1D9B coimmunoprecipitated approximately five times more avidly with Rab11a-QL than with Rab11a-WT or Rab11a-SN (Figure 4A). Mutations of the R and/or Q fingers in the TBC domain are reported to alter TBC–protein interactions in vitro, either enhancing it (Will and Gallwitz, 2001; Haas et al., 2005; Itoh et al., 2006) or impairing it (Itoh et al., 2006). In the case of TBC1D9B, we observed that full-length flag-TBC1D9B-RYQ/AAA coimmunoprecipitated Rab11a-WT in a manner that was greater than for the wild-type flag-TCB1D9B protein (Figure 4B). Thus, our results were similar to those reported for RUTBC-3/RabGAP-5, USP6NL/RN-Tre, and Gyp6p, all of which show increased binding to variants of their cognate TBC GAPs that contain mutations in their active-site residues (Will and Gallwitz, 2001; Haas et al., 2005).

The foregoing results indicate that R559 and Q562, and Q564 are unlikely to mediate the binding between TBC1D9B and Rab11a. To assess which domain(s) of TBC1D9B were responsible for binding Rab11a, we generated glutathione S-transferase (GST)-tagged fragments of TBC1D9B containing or lacking the TBC domain (Figure 4C). These were used to pull down GFP-Rab11a-WT produced in HEK cells (Figure 4D). Fragments lacking the TBC domain did not interact with GFP-Rab11a (Figure 4D, left), whereas all fragments containing the TBC domain, including those with flanking N- and C-terminal amino acids, interacted with Rab11a (Figure 4D, right). As expected, the TBC1D9B-(301-810)-RYQ/AAA mutant retained the ability to interact with Rab11a (Figure 4D, lane #2[Sup]). Finally, we observed that, like the full-length protein, flag-TBC1D9B-(301-810) showed the greatest interaction with Rab11a-QL and less with Rab11a-WT or Rab11a-SN (Figure 4, E–G).

The foregoing results demonstrate that Rab11a interacts with TBC1D9B via its TBC domain and that this interaction is dependent on the nucleotide state of Rab11a. We further observe that mutants that alter the TBC active site show increased binding to Rab11a.

**TBC1D9B interacts with Rab11b and Rab4a in a nucleotide-dependent manner**

Next, we further explored the interaction of TBC1D9B with Rab11b and Rab4a. Although not substrates for TBC1D9B (Figure 3B), both Rabs were found to coimmunoprecipitate with flag-TBC1D9B expressed in HEK cells (Figure 2). Of interest, when wild-type (WT), GTP-locked (QL), and GDP-locked (SN) mutants of Rab11b were compared in an immunoprecipitation assay (Figure 5A), we observed that flag-TBC1D9B interacted most efficiently with Rab11b-QL. In a somewhat similar manner, both the WT and QL forms, but not the SN mutant, of Rab4a also interacted with TBC1D9B (Figure 5B). However, we did note that TBC1D9B interacted more strongly with Rab4a-WT than Rab4a-QL. Whereas full-length flag-TBC1D9B showed the rank efficiency for interactions with its cognate Rabs as Rab11b > Rab11a > Rab4a (Figure 2), the efficiency of interaction for the GST-TBC1D9B-(301-810) fusion protein was Rab11b > Rab4a > Rab11a (Figure 5, E, F, and H). Thus, there were some differences in the interactions between the full-length protein and the 301–810 protein fragment. We also studied the interaction between TBC1D9B and Rab8a, which was observed when the Mg2+ in the buffer was removed. Under these conditions, there was little difference in the binding efficiency between flag-TBC1D9B and Rab8a-QL and Rab11a-QL (Figure 5C). However, when the assay was supplemented with 2.5 mM Mg2+, neither Rab8a-WT nor Rab8a-QL interacted with flag-TBC1D9B (Figure 5D). Oddly, in the presence of Mg2+, Rab8a-SN showed a strong interaction with flag-TBC1D9B.

These results demonstrate that in the presence of 2.5 mM Mg2+, TBC1D9B selectively interacts with GTP-locked mutants of Rab4a and Rab11b but not with GDP-locked variants or with Rab8a-WT/-QL.

**TBC1D9B localizes to Rab11a-positive endosomes**

To better understand the site of TBC1D9B function in the cell, we studied its subcellular localization in polarized epithelial MDCK cells. In these cells, endogenous TBC1D9B was vesicular in appearance and accumulated in the apical cytoplasm of the cell up to the level of the apical membrane (defined by GP-135 or actin staining in Figure 6A). TBC1D9B did not localize to EEA1-positive early endosomes or to LAMP2-labeled late endosomes/lysosomes (Figure 6, B–D; Manders coefficient of colocalization of 0.031 ± 0.005 and 0.035 ± 0.005, respectively). TBC1D9B also partially localized with the giantin-labeled Golgi (coefficient of colocalization of 0.005, respectively).
FIGURE 4: Rab11a binds to the TBC domain of TBC1D9B. (A) Left, coimmunoprecipitation of flag-tagged TBC1D9B with GFP-Rab11a-wild-type (WT), GTP-locked GFP-Rab11aQ70L (QL), or GDP-locked GFP-Rab11aS25N (SN) coexpressed in HEK cells. Anti-flag antibody was used to recover flag-TBC1D9B, and coimmunoprecipitated GFP-Rab11a was detected using an anti-GFP antibody. Controls included use of IgG instead of the flag-tag antibody and expression of GFP-Rab11a alone (first lane). Right, 2% of each lysate was resolved by SDS–PAGE and the indicated proteins detected by Western blot. Bottom, quantification of coimmunoprecipitations. Values were normalized to the total expression of each protein first and then to the values obtained in experiments using the Rab11a-WT lysate. (B) HEK cells were cotransfected with GFP-Rab11a and either flag-TBC1D9B-wild-type (WT) or the flag-TBC1D9B-RYQ/AAA mutant (RYQ). Flag-tagged TBC1D9B was recovered by immunoprecipitation, and the amount of GFP-Rab11a was quantified. Control reactions were performed for cells that only expressed GFP-Rab11a, or in some cases IgG was substituted for the anti-flag antibody. Right, 2% of each lysate was resolved by SDS–PAGE and the indicated proteins detected by Western blot. Bottom, amount of coimmunoprecipitated GFP-Rab11a normalized to the amount recovered from the TBC1D9B-WT lysate. (C) Fragments of TBC1D9B used in pull-down studies. (D) Top, GST-TBC1D9B fragments, labeled according to C, were used to affinity capture GFP-Rab11a in HEK cell lysates. Here #2RYQ denotes the use of fragment #2 with the RYQ/AAA mutations. GST alone was used as a control. Bottom, GST constructs were resolved by SDS–PAGE and proteins blotted with anti-GST antibody. (E, F) Top left, GST-TBC1D9B-#2 fragment was used to affinity capture Rab11a. (G) Relative binding to Rab11a-WT (normalized to the values obtained in experiments using the Rab11a-WT lysate).
There was a significant redistribution of Rab11a from recycling endosomes to the cytosol when TBC1D9B-WT, but not when the inactive mutant TBC1D9B-RYQ/AAA, was expressed (Supplemental Figure S2, A and B). This is the expected result for HEK cells if the GDP-bound form of Rab11a increased as a result of expressing TBC1D9B-WT. Finally, when Rab11a expression was depleted using a specific shRNA, endogenous TBC1D9B's normal accumulation in the apical half of the cell was altered, and its distribution became more dispersed throughout the cell (Supplemental Figure S5D).

Taken together, these results indicate that TBC1D9B is associated with endocytic vesicles, including those likely to contain active Rab11a. Moreover, the loss of Rab11a expression results in an altered distribution of TBC1D9B, further pointing to an interaction between these proteins.

TBC1D9B regulates basolateral-to-apical transcytosis but not transferrin recycling or epidermal growth factor degradation

If TBC1D9B acts as a Rab11a-GAP, we reasoned that increasing its expression should down-regulate Rab11a activity and in doing so alter Rab11a-dependent trafficking events. We first tested the effects of TBC1D9B expression on the efficiency of basolateral-to-apical IgA transcytosis, a known Rab11a-dependent trafficking event (Casanova et al., 1999; Wang et al., 2000b). As expected, expression of TBC1D9B-WT, but not TBC1D9B-RYQ/AAA, significantly slowed the rate of IgA transcytosis, particularly at early time points (Figure 8A). We also tested the effects of TBC1D9B-WT expression on the fraction of IgA that recycles apically, a trafficking event that shows partial dependence on Rab11a (Wang et al., 2000b). Although the effect was not pronounced, there was a small but significant inhibition in the amount of IgA that recycled apically (Figure 8B). We also tested two pathways known to be Rab11a independent, including basolateral recycling of transferrin (Tf) and degradation of basolaterally internalized epidermal growth factor (EGF; Barbieri et al., 2000; Wang et al., 2000b). In both cases, expression of TBC1D9B-WT (or TBC1D9B-RYQ/AAA) had no significant effect (Figure 8, C and D).

Finally, we rationalized that if TBC1D9B negatively regulates IgA transcytosis by its action on Rab11a, then depletion of TBC1D9B should increase the rate or extent of IgA transcytosis. TBC1D9B expression was knocked down in MDCK cells using specific shRNAs (shRNA-2), which decreased TBC1D9B expression by 88% (Figure 6E). We observed a modest but significant increase in the kinetics of IgA transcytosis (Figure 8F). However, no significant effect was observed if an inefficient shRNA (shRNA-1) was used, acting as a second negative control. As expected, shRNA-mediated depletion of TBC1D9B had no effect on the Rab11a-independent pathways, basolateral recycling of Tf, or degradation of basolaterally internalized EGF (Supplemental Figure S6, A and B). These results confirm a role of TBC1D9B in basolateral-to-apical transcytosis of IgA, a Rab11a-dependent pathway.

Expression of TBC1D9B-WT reduces the amount of active Rab11a

The foregoing results are consistent with the possibility that TBC1D9B acts as a Rab11a GAP but do not directly address whether TBC1D9B affects the amount of active Rab11a in the cell. To examine this possibility more directly, we determined whether expression

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capture GFP-Rab11aQ70L (QL) or GFP-Rab11aS25N (SN) from HEK cell lysates. Right, 2% of each lysate was resolved by SDS–PAGE and GFP-Rab11a detected by Western blot using anti-GFP antibody. Bottom left, GST constructs were resolved by SDS–PAGE and proteins detected using an anti-GST antibody. (G) Quantification of data from E and F. Values were normalized to total expression of the protein of interest and then to the values obtained for the GFP-Rab11a-WT pull down. For A, B, and G, data are from at least three independent experiments, and the mean ± SEM is shown. Values significantly different from the group means, as assessed by ANOVA, are indicated (*p < 0.05).
FIGURE 5: TBC1D9B interacts with Rab4a and Rab11b. (A) Left, coimmunoprecipitation of flag-tagged TBC1D9B with GFP-Rab11b-WT (11b-WT), GFP-Rab11b-Q70L (11b-QL), or GDP-locked GFP-Rab11bS25N (11b-SN) coexpressed in HEK cells. Anti-flag antibody was used to immunoprecipitate flag-TBC1D9B, and anti-GFP antibody was used to detect the coimmunoprecipitated GFP-tagged Rab proteins. IgG was used as a nonspecific control. Right, 2% of each lysate was resolved by SDS–PAGE and overexpressed proteins detected by Western blot. Values were normalized to the total expression of each protein and then normalized to the amount of Rab11b-WT lysate. (B) Same as in A, but HEK cells coexpressed flag-TBC1D9B and GFP-Rab4a-WT (4a-WT), GFP-Rab4aQ72L (4a-QL), or GFP-Rab4aS27N (4a-SN). (C) Same as in A, but the cells expressed Rab8aQ67L (8a-QL) or Rab11aQ70L (11a-QL), and after lysis, the incubations were performed in the absence of MgCl2. (D) Same as in A, but HEK cells coexpressed flag-TBC1D9B with GFP-Rab8a-WT (8a-WT), GFP-Rab8aQ67L (8a-QL), GFP-Rab8a-T22N (8a-TN). (E–G) Top left, GST alone or GST-TBC1D9B (301-810) (#2 fragment) was used to affinity capture GFP-Rab11a-QL and GFP-Rab11b-QL (E), GFP-Rab4a-QL (F). or GFP-Rab8a-QL (G). Right, 2% of each lysate was resolved by SDS–PAGE and GFP-Rab-QL detected by Western blot using anti-GFP antibody. Bottom left, GST constructs were resolved by SDS–PAGE and proteins detected on Western blots using an anti-GST antibody. (H) Quantification of data from E–G. Values are normalized to the total expression of the protein first and then to the values obtained for the GFP-Rab11a-WT pull down. Data were obtained from at least three independent experiments, and the mean ± SEM is shown. Values significantly different from the group means, as assessed by ANOVA, are indicated (*p<0.05).
of TBC1D9B-WT altered the amount of active Rab11a in the cell and thereby affected its association with its effector Sec15A. As predicted, expression of TBC1D9B-WT decreased the amount of colocalization between Rab11a and Sec15A, whereas expression of TBC1D9B-RYQ/AAA was without effect (Figure 9, A and B). We also observed that the normally apically distributed Sec15A became more random in distribution when cells expressed TBC1D9B-WT but not when TBC1D9B-RYQ/AAA was expressed (Figure 9A, middle). The total amount of Sec15A protein expressed in the cell was not affected by expression of TBC1D9B-WT (Supplemental Figure S7). However, a twofold increase in the expression of Sec15A was observed in cells expressing TBC1D9B-RYQ/AAA (Supplemental Figure S7B).

We also sought to determine whether shRNA-mediated depletion of TBC1D9B would increase the amount of colocalization between Rab11a and its effector, Sec15A, or whether it would affect the amount of Rab11a-GTP in the cell. However, depletion of TBC1D9B has no significant effect on the amount of colocalization between Rab11a and Sec15A (Figure 9C). To measure the amount of Rab11-GTP in the cells, we used an effector-based pull-down assay, using purified GST-tagged Sec15A to recover active Rab11a-GTP from cell extracts. We found no effect of decreasing TBC1D9B expression on the amount of Rab11a-GTP (Figure 9, D and E). However, when TBC1D9B-RYQ/AAA was expressed, we observed a 2.5-fold increase in the nominal amount of Rab11a-GTP, as indicated by increased interaction between Rab11a and Sec15A (Figure 9E). In contrast, expression of TBC1D9B-WT reduced the amount of interaction between Rab11a and Sec15, likely by decreasing the amount of Rab11a-GTP in the cell (Figure 9, G and H).

In summary, overexpression of TBC1D9B decreases the amount of Rab11a-GTP in the cell and, in doing so, alters the interaction of the common recycling endosome and basolateral early endosomes; and (G) TBC1D9B, actin, and Rab11a (which is associated with the apical recycling endosomes in these cells). In A and C–G, single optical sections from the region just under the apical membrane (subapical), just above the nucleus (supranuclear), or at the level of the nucleus along the lateral membranes (medial) are shown. Boxed regions are magnified in the images below the word ZOOM. Scale bar, 5 μm. In B, data are mean ± SEM from three experiments (n > 100 cells).

FIGURE 6: Subcellular localization of TBC1D9B in MDCK cells. Filter-grown MDCK cells were immunolabeled to show the distribution of the following endogenous proteins. (A) TBC1D9B and the tight junction–associated protein ZO-1 (xy-section, left), the apical membrane protein gp-135 (xz-section, top right), or actin (xz-section, bottom right). (B) Coefficient of colocalizations for endogenous TBC1D9B and the following markers: (C) TBC1D9B, actin, and EEA1 (an early endosome marker); (D) TBC1D9B, actin, and Lamp2 (a late endosome/lysosome marker); (E) TBC1D9B, actin, and giantin (a Golgi marker); (F) TBC1D9B, ZO-1, and TfR (a marker of the common recycling endosome and basolateral early endosomes); and (G) TBC1D9B, actin, and Rab11a (which is associated with the apical recycling endosomes in these cells). In A and C–G, single optical sections from the region just under the apical membrane (subapical), just above the nucleus (supranuclear), or at the level of the nucleus along the lateral membranes (medial) are shown. Boxed regions are magnified in the images below the word ZOOM. Scale bar, 5 μm. In B, data are mean ± SEM from three experiments (n > 100 cells).
FIGURE 7: Localization of TBC1D9B at sites of active Rab11a function. (A) Filter-grown MDCK cells were pulse labeled with 200 μg/ml IgA at the basolateral surface for 20 min at 18°C and chased for 20 min at 37°C in the presence of apical Cy5-labeled anti-IgA antibodies. The cells were fixed and then immunolabeled to detect endogenous TBC1D9B (green), endogenous Rab11a (red), and transcytosed IgA (blue). In the merged panel, the boxed region is magnified in the bottom right inset. Magnified views of the individual channels are found in the insets under ZOOM. (B, C) MDCK cells were stained for endogenous TBC1D9B and either the (B) pIgR or (C) endogenous Sec15A. (D) Coefficient of colocalization for endogenous TBC1D9B and the indicated proteins. (E, F) MDCK cells were transfected with cDNA encoding GFP-Rab11a-WT (E) or GFP-Rab11a-QL (F) and after 72 h fixed and then stained for endogenous TBC1D9B. (G) Coefficient of colocalization for TBC1D9B and the indicated Rab11a construct. In (B, C, E, and F), the boxed region is magnified in the panels under the label ZOOM. Scale bar, 5 μm. In D and G, data were obtained from at least three independent experiments, and the means ± SEM are shown (n ≥ 150 cells). In G, a Student’s t test was used to show that difference between these two sample groups was significantly different (*p < 0.05).
TBC1D9B is a Rab11a GAP

between Rab11a and Sec15A. In contrast, expression of TBC1D9B-RYQ/AAA has the opposite effects.

**DISCUSSION**

Rab11a is a critical regulator of exocytosis, but there is a limited understanding of the proteins that regulate its GTPase cycle. Our studies focused on TBC1D9B, a protein that has been included in several biological screens (Itoh et al., 2006; Fuchs et al., 2007; Yoshimura et al., 2007; Ishibashi et al., 2009; Popovic et al., 2012), but there are no reports on whether it has GAP activity, nor is there information about its Rab substrates. In this work, we identified TBC1D9B as Rab GAP for vertebrate Rab11a. This conclusion derives from the following observations: 1) TBC1D9B is the closest homologue of the yeast protein Gyp2p, which is reported to be a GAP for the Rab11a homologue Ypt31p (Sciorra et al., 2005); 2) the TBC domain of TBC1D9B interacts with Rab11a and accelerates Rab11a GTPase activity in vitro, whereas the TBC1D9B-RYQ/AAA mutant is without effect; 3) endogenous TBC1D9B is found as associated with Rab11a-positive compartments; 4) in HEK cells, overexpression of TBC1D9B causes Rab11a to dissociate from recycling endosomes, apparently resulting in its redistribution to the cytosol; 5) in MDCK cells, overexpression of TBC1D9B impairs IgA transcytosis, a biological activity dependent on Rab11a, whereas down-regulation of TBC1D9B causes a modest but significant increase in IgA transcytosis; and 6) overexpression of TBC1D9B decreases the apparent levels of the GTP-bound form of Rab11a in MDCK cells.

We calculated the catalytic efficiency of TBC1D9B toward Rab11a to be $158 \text{ M}^{-1} \text{s}^{-1}$, which is modest compared with the catalytic efficiencies reported for TBC1D1 ($5.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$), TBC1D4 ($2.8 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$), and TBC1D20 ($2.7 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$; Sklan et al., 2007; Park et al., 2011) but comparable to the activity of other TBC-domain containing proteins such as the Caenorhabditis elegans homologue of TBC1D2, TBC-2 ($\sim 50 \text{ M}^{-1} \text{s}^{-1}$; Chotard et al., 2010), and full-length TBC1D20 ($360 \text{ M}^{-1} \text{s}^{-1}$; Sklan et al., 2007). However, the value we calculated for TBC1D9B is likely to be an underestimate, as we did not determine the catalytic efficiency of the full-length protein or under different concentrations of Mg$^{2+}$, which we showed affected TBC1D9B activity. Although some GAPs are reported to have multiple Rab substrates (Du et al., 1998; Nottingham et al., 2011; Frasa et al., 2012; Lachmann et al., 2012), our experiments indicate that TBC1D9B may be selective for Rab11a. Indeed, we observed that TBC1D9B has only low levels of activity against a number of Rab GTPases, including Rab11b and Rab4a, both of which interacted with TBC1D9B in their GTP-locked states. We also observed that the TBC domain of TBC1D9B has activity against Rab8a; however, this was noted only when low concentrations of Mg$^{2+}$ were present in the assays. Thus, it is possible that under Mg$^{2+}$-limiting conditions, Rab8a may serve as an additional substrate for TBC1D9B. We also observed that TBC1D9B was
FIGURE 9: TBC1D9B has GAP activity in cells. (A) Localization of endogenous Rab11a and endogenous Sec15A in cells transduced with adenovirus encoding CFP-TBC1D9B-WT or the inactive mutant (-RYQ/AAA). Top, from the subapical region of the cell; bottom, xz-sections. (B, C) Coefficient of colocalization for the fraction of Rab11a that colocalizes with Sec15A (or vice versa) in MDCK cells transduced with adenovirus encoding CFP-TBC1D9B-WT or -RYQ/AAA (B) or in cells expressing shRNA-1 or shRNA-2 against canine TBC1D9B (C). In B, GFP expression was used as control, and in C, scrambled shRNA was used as control. (D) GST-Sec15A–mediated pull down of activated Rab11a in MDCK cells expressing scrambled shRNA (Control) or shRNA-2 specific for canine TBC1D9B. Left, immunoprecipitate from the pull down was analyzed by Western blot, using anti-GST and anti-Rab11a antibodies. Right, 2% of each lysate was analyzed by Western blotting, using anti-TBC1D9B and anti-Rab11a antibodies. (E) Data from D and F are quantified and
localized to Rab11a-containing endosomes and much less to EEA1-positive (and Rab5-positive) early endosomes or LAMP2-positive late endosomes/lysosomes. Moreover, only Rab11a-dependent endocytic pathways—for example, basolateral-to-apical transcytosis—were affected when TBC1D9B was overexpressed or when its endogenous protein levels were decreased by shRNA. Our results indicate that TBC1D9B is Rab11a specific at concentrations of Mg²⁺ in the 2.5–5.0 mM range. However, additional experiments may reveal that this protein targets other Rab proteins in other pathways, other cell types, or other physiological and developmental contexts.

In addition to TBC1D9B, there may be additional Rab11a GAPs, as is the case for other Rab GTPases (Fukuda, 2011; Frasa et al., 2012; Chaineau et al., 2013). This could explain why depleting endogenous TBC1D9B had only a modest effect on the rate of IgA transcytosis. In addition, the transcytotic pathway is also regulated by other RabS (including Rab3b/d, Rab17, and Rab25; Hunziker and Peters, 1998; Zacchi et al., 1998; Larkin et al., 2000; Wang et al., 2000b; van IJzendoorn et al., 2002), whose activities are unlikely to be affected by the loss of TBC1D9B expression. The presence of additional Rab11a GAPs could also explain why TBC1D9B depletion had no effect on the nominal levels of GTP-Rab11a in the cell (as assessed by binding to its Sec15A effector). Examples of potential Rab11a GAPs include Evi5, which has been described as a Rab11GAP in Drosophila melanogaster (Laffamme et al., 2012). However, whether Evi5 acts as a Rab11a GAP in mammalian cells is still controversial. For example, mammalian Evi5 interacts with Rab11a and can regulate Rab11a interaction with its effectors but apparently in a manner that is independent of its GAP activity (Westlake et al., 2007; Hehnly et al., 2012). Of interest, TBC1D14 lacks GAP activity toward Rab11a but does interact with Rab11a in its GTP-bound state, indicating that it acts as an effector rather than a GAP for this GTPase (Longatti et al., 2012). Furthermore, TBC1D11/GAPCen, a Rab4 and Rab6 GAP, and TBC1D15, a Rab7 GAP, are reported to act as GAPs for Rab11a in vitro (Zhang et al., 2005; Fuchs et al., 2007). However, their activity has not been assessed in cells. Finally, we note that TBC1D9B is a member of a subgroup named TBC-A (Gabernet-Castello et al., 2013), which includes TBC1D8, TBC1D8B, TBC1D9, TBC1D9B, and Gyp2p. With the exception of TBC1D9B, which was studied here, the other mammalian TBC proteins in this subgroup are poorly characterized, and no substrates have been reported. However, it is possible that because of their high similarity, they could share similar substrates. In a similar manner, TBC1D10A and TBC1D10B were both identified as GAPs for Rab27A and, in a different study, as GAPs for Rab35 (Itoh and Fukuda, 2006; Hsu et al., 2010).

An interesting but perplexing finding is that TBC1D9B-RYQ/AAA, like RA mutants in other TBC-containing proteins (Will and Gallwitz, 2001; Haas et al., 2005), shows increased interaction with its cognate Rab. Moreover, we observed that expression of TBC1D9B-RYQ/AAA increases the nominal amount of Rab11a-GTP in the cell (measured by showing increased association between Rab11a and one of its effector proteins, Sec15A). These results are consistent with the possibility that this mutant acts in a dominant manner. However, this does not square with the lack of an obvious dominant effect on IgA transcytosis. One possibility is that TBC1D9B-RYQ/AAA stabilizes the Rab11a/Sec15A interaction, but any gains achieved in forming this interaction (likely at the level of tethering transcytotic vesicles) are offset by slowing the dissociation of Rab11a from Sec15A before vesicle fusion. Thus, it is possible that this mutant does have a dominant effect, but it was not revealed by our experiments. One difference between TBC1D9B and other TBC GAPs studied is that a single R-to-A mutation commonly used in other studies (Will and Gallwitz, 2001; Haas et al., 2005; Itoh et al., 2006; Hannemann et al., 2012) is not enough to produce an inactive protein. This may indicate that other residues in or near the catalytic pocket of TBC1D9B can substitute or that the interaction between TBC1D9B and Rab11a is not adequately represented by the current structural models.

Although we identified TBC1D9B as a Rab11a GAP, there remains much to understand about its physiological functions in the cell. Of great interest are previous reports that the pIgR is transcytosed more efficiently when it binds IgA (Song et al., 1994), which stimulates activation of protein kinase C, release of inositol 1,4,5-trisphosphate, and increase in intracellular calcium (Cardone et al., 1996). Of importance, TBC1D9B contains an EF hand, a well-described Ca²⁺-binding motif in other proteins. Thus, one possibility is that Ca²⁺ binding to the EF hand may allosterically impair the function of TBC1D9B and in doing so increase the amount of active Rab11a and therefore extent of pIgR-IgA transcytosis. One possible way that Ca²⁺ may exert this influence would be by altering the function of the N-terminal GRAM domains, which by analogy to other proteins are likely to regulate the association of TBC1D9B with phospholipids (Doerks et al., 2000; Tsujita et al., 2004).

Finally, it has been proposed in the yeast secretory system that Gyp2p may be recruited by a Rab that acts downstream of Ypt31/32, possibly as an effector of Sec4p (or a similar GTPase), to regulate secretory vesicle maturation. By engaging Gyp2p, Sec4p would ensure compartmental identity by promoting the inactivation and dissociation of Ypt31/32 from maturing vesicles (Sciorra et al., 2005). By analogy, we hypothesize that Rab4a and/or Rab11b could recruit TBC1D9B and in doing so generate endosomal microdomains/ compartments that are depleted of active Rab11a. In the case of Rab4, this would be a mechanism to sequester Rab4a cargoes, which undergo rapid recycling (Sonnichsen et al., 2000; Grant and Donaldson, 2009), from the relatively slow recycling pathways usually associated with Rab11a. Similarly, it could explain how the cell generates Rab11b-positive microdomains, which are critical for the recycling of cargoes distinct from those regulated by Rab11a (Lapierre et al., 2003; Silvis et al., 2009; Butterworth et al., 2012).
MATERIALS AND METHODS

Antibodies and reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Rhodamine-phalloidin and TO-PRO-3 were obtained from Molecular Probes-Invitrogen/Life Sciences (Grand Island, NY). Antibodies used were as follows: mouse anti-flag M2 and rabbit anti-GFP (Abcam, Cambridge, MA), mouse anti-Rab11a (8H10) and rabbit anti-Rab11a (Goldening et al., 1996; Lapierre et al., 2003), goat anti-GST (GE Healthcare, Pittsburgh, PA), rat anti-ZO1 hybridoma R40.7; culture supernatant (D. A. Goodenough, Harvard University, Cambridge, MA), mouse anti-gp135 hybridoma 3F2/D8 (G. Ojakian, State University of New York, New York, NY), mouse anti-EEA1 (BD Biosciences, San Jose, CA), mouse anti-LAMP-2 antibody AC17 (E. Rodriguez-Boulan, Cornell University, New York, NY), mouse anti-giantin (Adam Linstead, Carnegie Mellon University, Pittsburgh, PA), mouse anti-Tf receptor antibody H68.4 (Invitrogen, Carlsbad, CA), goat anti-Rab4a (Santa Cruz Biotechnology, Dallas, TX), mouse anti-Rab8a (BD Biosciences), human polymeric IgA (purchased from J. P. Vaerman, Catholic University of Leuven, Leuven, Belgium), mouse anti-pigR antibody SC166 (Solari et al., 1985), goat anti-Sec15A (Santa Cruz Biotechnology), and goat anti–glyceraldehyde-3-phosphate dehydrogenase (Proteintech, Chicago). Horseradish peroxidase–, Cys–, and Dylight 488–, 549–, and 649– conjugated, affinity-purified, and minimal cross-reacting secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cloning of TBC1D9B and sequence analysis

For mammalian expression, full-length human TBC1D9B (Accession Number NP_942568.2) was obtained from Open Biosystems (Thermo Fisher Scientific, Waltham, MA). The cDNA was amplified by PCR and cloned into pEGFP-C1 vector for GFP tagging or pCDN3.1+ for flag tagging. The inactive mutant was produced by mutagenesis using the QuickChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), and the following residues were mutated: R559 to A, Y592 to A, and Q594 to A. Sequences were verified using ImageJ software (National Institutes of Health, Bethesda, MD). Values were normalized to total expression of each protein.

Cell culture and transfection

HeLa and HEK 293FT (HEK; Invitrogen) cells were grown in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (pen/strep) in a 37°C incubator gassed with 5% CO2. HeLa and HEK 293FT (HEK; Invitrogen) cells were grown in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (pen/strep) in a 37°C incubator gassed with 5% CO2. Cells were plated on coverslips or on 10-cm tissue culture plates according to the experiment requirements, and after 24 h they were transfected with DNA using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

MDCK type II cells expressing the wild-type rabbit plgR (pWe) have been described (Breitfeld et al., 1989). Cells were grown in MEM (Cellgro, Herndon, VA) containing 10% (vol/vol) FBS (Hyclone) and 1% penicillin/streptomycin (pen/strep) in a 37°C incubator gassed with 5% CO2. Cells were cultured on 0.4-μm and 12-mm Transwells (Costar, Cambridge, MA) and used 3–4 days after culture as described (Breitfeld et al., 1989). Cells were transfected in suspension as described previously (Mo et al., 2010), using the appropriate vector and Lipofectamine 2000 reagent.

Immunoprecipitation

Immunoprecipitations were performed as described previously with minor modifications (Ozkan et al., 2007). Briefly, HEK cells were cotransfected, as described, with flag-tagged TBC1D9B and GFP-tagged Rab constructs. The GFP-tagged Rab constructs with Q-to-L mutation in their switch II active region, used for the Rab screen (Figure 2A), were described previously (Azouz et al., 2012). After 24–48 h transfection, HEK cells were washed with ice-cold phosphate-buffered saline (PBS), gently scraped into cold PBS using a flexible cell scraper, and then recovered by centrifugation at 1500 × g. The cells were resuspended in IP buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 2.5 mM MgCl2, 5 mM EDTA, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 5 μg/ml each of peptatin, leupeptin, and antipain), and after incubation for 30 min on ice, the cells were passed 20 times through a 25-gauge syringe and the lysate centrifuged 10 min at maximum speed in a 5424R microcentrifuge at 4°C (Eppendorf, Westbury, NY). The supernatants were transferred into a new tube and then incubated overnight at 4°C on a rotator with 1 μg of anti-flag M2 antibody or 1 μg of nonspecific mouse IgG as a control. Next, 30 μl of a 50% (vol/vol) slurry of Protein G Sepharose 4FastFlow beads (GE Healthcare) was added to each tube, and the reaction was incubated at 4°C for 1 h on a rotator. Tubes were centrifuged for 1 min at 2000 × g, and the beads were washed five times with IP buffer and then resuspended in 25 μl of 2× Laemmli sample buffer. When the Rab screen was performed (Figure 2A), the washes were made using the IP buffer supplemented with 300 mM NaCl. When stated in the text, MgCl2 was omitted from the IP buffer. The samples were heated at 70°C for 10 min, the cell lysate was resolved by SDS–PAGE, and Western blots, performed as described previously (Ozkan et al., 2007), were probed with the indicated primary antibodies. To quantify the binding efficiency of the communoprecipitating proteins, the bands detected in the immunoblots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Values were normalized to total expression of each protein.

Yeast two-hybrid assay

For yeast two-hybrid experiments the MATCHMAKER GAL4 Two-Hybrid System (Clontech Laboratories, Mountain View, CA) was used. TBC1D9B-WT or TBC1D9B-RA were cloned into pLexA vectors. Human Rab4, Rab5, Rab6, Rab7, Rab11a, and Rab33 were cloned into pB42AD vectors. Experiments were performed according to the directions supplied by the manufacturer.

Purification of GST-fusion proteins

cDNAs encoding full-length mammalian Rab GTPases fused to GST were described previously (Pan et al., 2006) and kindly provided by D. G. Lambright (University of Massachusetts, Worcester, MA). GST-Rab11b was subcloned from GFP-hRab11b (kindly provided by Robert Edinger, University of Pittsburgh, Pittsburgh, PA) into the pGEX4T1 vector. TBC1D9B fragments (residues 450–950, 450–810, and 810–1250) were subcloned into the pGEX4T1 vector. TBC1D9B fragments (residues 450–950, 450–810, and 810–1250) were subcloned into the pGEX4T1 vector. Plasmids encoding the Rab or TCB1D9B fragments were introduced into BL21-CodonPlus (DE3)-RIPL. E. coli cells (Agilent Technologies, Santa Clara, CA) by transformation and expression induced with 0.5 mM isopropyl-β-D-thiogalactoside for 16 h at 30°C. Cells were collected by centrifugation at 8000 × g and the cell pellet dried in a lyso buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 8, 500 mM NaCl, 0.5% [vol/vol] Triton X-100, 5 mM MgCl2, 1 mM dithiothreitol [DTT], 1 mM PMSF, and 5 μg/ml each of peptatin, leupeptin, and antipain). When purifying the Rabs, 200 μM GDP was included in the lysis buffer. DNA

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was sheared by treating the lysates with 10 sonic pulses (15-s duration each) using a Model 100 Sonic Dismembrator (Fisher Scientific). After centrifugation for 30 min at 4°C at 15,000 × g, the supernatants were recovered and subsequently incubated with reduced glutathione (GSH) Sepharose-4B beads (GE Healthcare) for 3 h at 4°C with rotation. The proteins bound to the beads were then recovered by centrifugation at 5000 × g, washed four times with lysis buffer, and GST-fusion proteins released from the beads by incubating them with elution buffer (10 mM GSH in 100 mM Tris, pH 8, containing 1 mM DTT). Proteins were concentrated fourfold and equilibrated in dialysis buffer (10 mM Tris, pH 7.5, 0.1 mM DTT, 2 mM MgCl₂, 150 mM NaCl) using Amicon-Ultra-4 centrifugal filters (EMD Millipore, Billerica, MA). Quantification of proteins was performed using the bicinchoninic acid assay (BCA) Kit (Promega, Madison, WI). Glycerol was added to a final concentration of 10% (vol/vol) and the purified proteins were divided into aliquots, snap-frozen using liquid nitrogen, and then stored at −80°C.

In vitro GAP assay

The efficiency of GTP binding to purified Rabs and GAP assays were performed as described previously (Haas et al., 2005; Fuchs et al., 2007; Yoshimura et al., 2008). To estimate the former, 100 pmol of GST-Rab protein was mixed on ice with 100 μl of assay buffer (50 mM HEPES-NaOH, pH 6.8, 1 mM DTT, 0.2 mg/ml bovine serum albumin, 1 mM EDTA, pH 8.0, and 0.5 mM of an equal mixture of GTP and MgCl₂) containing 2 μl of [γ-32P]GTP (10 mCi/ml; 4500 Ci/mmol; MP Biomedicals, Solon, OH), and the reaction was then incubated for 15 min at 30°C. The efficiency of binding was evaluated by nitrocellulose-filter assay (Kabcenell et al., 1990). For GAP assays, the indicated concentration and fragment of TBC1D9B was added to this reaction mixture and then incubated at 30°C for up to 60 min. In some experiments, 5 mM MgCl₂ was added in the GAP reaction as described previously (Du et al., 1998; Albert et al., 1999; Nottingham et al., 2011; Lachmann et al., 2012). A 2-μl aliquot of the reaction was counted directly to measure the specific activity in cpn/picomole GTP. The 32Pi release was quantified by taking duplicate 2-μl samples from the GAP reaction and mixing them with 795 ml of ice-cold activated charcoal (5% vol/vol in 50 mM NaH₂PO₄), which specifically binds to Pi. The charcoal was recovered by centrifugation at 10,000 × g, and the radioactivity within a 400-μl aliquot of the cleared supernatant was measured by Cerenkov counting in a Wallac 1409 liquid scintillation counter (PerkinElmer, Waltham, MA). The amount of hydrolyzed GTP (32Pi release) was calculated from the specific activity of the reaction mixture (Yoshimura et al., 2008) and normalized to the fraction of GTP-bound Rab, which was measured as described using the nitrocellulose-binding assay (Kabcenell et al., 1990). Where indicated, data were fitted to a pseudo-first-order Michaelis–Menten model in which y(t) = Amax[1 − exp(−Kobs t)], where Amax corresponds to the maximum amount of hydrolyzed GTP and Kobs is the observed rate constant for the GTP hydrolysis. For each tested concentration of TBC1D9B-(301-810), a Kobs value was obtained, and the catalytic efficiency (Kcat/Km) relative to the intrinsic rate constant of GAP hydrolysis (Kcat) was calculated from the linear dependence between Kobs and the evaluated GAP concentrations, Kobs = Kcat + (Kcat/Km) [GAP] (Pan et al., 2006). Initial velocities (V) were calculated as V = AmaxKobs, where Amax and Kobs were obtained from the described fitted pseudo-first-order curves.

A previously described procedure was used to measure the GAP activity of full-length TBC1D9B (Nottingham et al., 2011). Briefly, HeLa cells were transfected with either flag-TBC1D9B-WT or flag-TBC1D9B-RYQ/AAA, and 24 h later, the cells were solubilized in lysis buffer as described and the total amount of protein was quantified using the BCA Kit (Promega, Madison, WI). One milligram of total protein was incubated with 0.5 μg of anti-flag antibody prebound to Protein G Sepharose 4 FastFlow beads for 2 h at 4°C with end-to-end rotation. The flag-TBC1D9B-beads were washed three times with lysis buffer, four times with PBS, and two times with assay buffer. An aliquot was kept to confirm the efficiency of the immunoprecipitation, and the remaining beads containing bound GAP were directly added to the described GAP assay. Untransfected HeLa cells were lysed, treated as described, and used as a control in the GAP assay.

Detection of interactions between TBC1D9B fusion proteins and Rab GTPases

HEK cells were transfected with cDNA encoding GFP-Rab constructs (either wild-type or mutant forms) and lysed 24–48 h post-transduction in IP buffer containing 1 mM GTP. The cell lysate was centrifuged and the supernatant “precleared” by incubating the lysate with GSH Sepharose-4B beads (GE Healthcare) for 30 min at 4°C. After a brief centrifugation at 2000 × g, an aliquot of lysate containing 250 μg of total protein was mixed with 1 μg of purified TBC1D9B fusion protein and interacting complexes recovered by the addition of 25 μl of GSH beads and incubation for 3 h at 4°C on a rotator. The reactions were centrifuged for 1 min at 2000 × g, and the beads were washed five times with IP lysis buffer. The beads were resuspended in 25 μl of 2× Laemmli sample buffer, heated at 70°C for 10 min, the proteins resolved by SDS–PAGE, and Western blots probed with the indicated primary antibodies. When stated in the text, no MgCl₂ was added to the IP buffer. To quantify the binding efficiency in the pull-down experiments, we quantified the bands detected in the immunoblots using ImageJ software. Values were normalized to total expression of each protein in the lysate and the corresponding amount of GST protein used in each experiment.

Immunofluorescence and colocalization analysis

For immunofluorescence studies, MDCK cells were washed in PIPES-KOH buffer (80 mM 1,4-piperazinediethanesulfonic acid [PIPES]–KOH, pH 6.8, containing 2 mM MgCl₂ and 5 mM ethylene glycol tetraacetic acid) and then fixed for 5 min with 4% (vol/wt) formaldehyde (Electron Microscopy Sciences, Hatfield, PA) dissolved in PIPES-KOH buffer (adjusted to pH 6.5 by the addition of HCl), followed by 10 min in 4% (vol/wt) paraformaldehyde dissolved in 100 mM sodium borate buffer (pH 11.0; Bacallao and Stelzer, 1989; Apodaca et al., 1994). After fixation, unreacted paraformaldehyde was washed for 10 min at room temperature with PBS containing 20 mM glycine, pH 8.0, and 75 mM NH₄Cl, with the addition of 0.1% Triton X-100. Fixed cells were incubated with block buffer (0.025% [vol/vol] saponin and 8.5 mg/ml fish-skin gelatin dissolved in PBS) for 60 min at room temperature. Cells were incubated with primary antibody, diluted in block buffer for 1 h at room temperature or 16 h at 4°C, washed three times with block buffer for 5 min, and then incubated with fluorescence-labeled secondary antibodies (diluted in block buffer) for 1 h at room temperature. After three additional 5-min washes with block buffer, the cells were rinsed with PBS, fixed with 4% (vol/vol) paraformaldehyde in 100 mM sodium cacodylate, pH 7.4, for 10 min at room temperature, and then mounted as described previously (Apodaca et al., 1994).

To label transcytosing IgA, filter-grown MDCK cells were washed with MEM/bovine serum albumin (BSA; MEM containing 0.45 g/ml NaHCO₃, 0.6% [vol/vol] BSA, 10 mM HEPES, pH 7.4, and pen/strep/Fungizone) and incubated at 18°C for 15 min. IgA, 200 μg/ml, was internalized from the basolateral surface of the cells for 20 min at
18°C. The cells were then incubated for 20 min at 37°C, during which 25 μg/ml Cy5-labeled anti-human IgA was added to the apical media. The cells were then washed with ice-cold MEM/BSA and then PBS and finally fixed with 4% (wt/vol) paraformaldehyde in 100 mM sodium cacodylate, pH 7.4, for 10 min at room temperature (Oztan et al., 2007). Unreacted paraformaldehyde was quenched, and the cells were labeled as described.

Images were captured using an HCX PL APO 100x/numerical aperture (NA) 1.4 oil objective (Leica, Wetzlar, Germany) and the appropriate laser lines of a Leica TCS SPS CW-STED confocal microscope (in normal confocal mode). The photomultipliers were set at 800–1000 V and zoom at 4×, and 8-bit images were collected using four to eight line averages combined with four to eight frame averages. Serial 0.25-μm z-sections were acquired. The images (512 × 512 pixels) were imported into Velocity 3D software (PerkinElmer, Waltham, MA) and, after image reconstruction, exported as TIFF files. The contrast of the latter was corrected in Photoshop CS5 (Adobe, San Jose, CA), and the composite images were prepared in Adobe Illustrator CS5.

Measures of colocalization were performed as described previously (Khandelwal et al., 2008; Khandelwal et al., 2013). Briefly, stacks of dual-labeled confocal sections were imported into Velocity software, background noise was removed using the fine (3 × 3) median noise reduction filter, and a scatter plot of voxel intensities for each of the markers was generated using the colocalization function. The images were thresholded using a fixed value of 40, and Mx, the Manders colocalization coefficient, was calculated for the entire 3D volume. An Mx value of 1.0 indicates that the ratio for all x intensity values that have a corresponding y intensity (i.e., are colocalized) divided by the sum of all x intensity values is 100%. In contrast, a value of 0.0 indicates that there is no colocalization.

**Antibody production for anti-TBC1D9B in rabbits**

The anti-TBC1D9B antibody is a rabbit polyclonal antibody made by Cocalico Biologicals (Reamstown, PA), using a KLH-conjugated peptide EEDEPAPHELQDAARELQ (amino acids 1082–1100) from the human TBC1D9B isoform (NP_942568.2). The antibody production was approved by the University of Pittsburgh Institutional Animal Care and Use Committee, Protocol 0809099-1. This antibody was used in immunoblots and immunofluorescence studies.

**Adenovirus production and transduction**

Full-length TBC1D9B-WT and -RYQ-AAA were cloned into the pECPF vector (Clontech Laboratories) and subcloned into pAdLX vector (Hardy et al., 1997). These constructs were transfected into Cre8 cells, and the adenovirus was produced and amplified as previously described (Henkel et al., 1998; Khandelwal et al., 2008). Other constructs used were previously described: GFP/HA-Rab11a-WT, GFP/HA-Rab11a-S25N, GFP-Rab25 (Khandelwal et al., 2008), and GFP-Rab8a-T22N (Khandelwal et al., 2013). Adenovirus was transduced into MDCK cells as described previously (Henkel et al., 1998; Oztan et al., 2007). Adenoviral-mediated expression of CFP-TBC1D9B was detected by immunofluorescence using anti-GFP staining.

**Lentivirus shRNA production and transduction**

To decrease the expression of canine TBC1D9B, shRNA sequences were generated using an (AA)N19 algorithm and iRNAi software (www.mekentosj.com). The target sequences were (5′−3′): shRNA-1, GACGACATGTCCATGTCCT; shRNA-2, GGTTGAGAGACAGTCAGC; and scrambled, CGCGAGTGATGCACGGCT. The sequences were cloned into the pLKO.1 vector (Addgene) and lentiviruses produced in 293FT cells using the ViralPower packaging kit (Invitrogen) according to the manufacturer’s instructions. Infection of the MDCK cells was performed as described previously (Bryant et al., 2010), and cells expressing the shRNA were enriched by incubating the transduced cells in medium containing 5 μg/ml puromycin. The expression of endogenous TBC1D9B was detected by reverse transcription (RT)-PCR using the RETROscript First Strand Synthesis Kit (Life Technologies), followed by PCR using Taq polymerase (Life Technologies). PCR primers, designed using the predicted sequences of canine TBC1D9B (XM_538581.4), were as follows (5′–3′): forward primer, GCAGGAGCAGTGTCCGGGT, and reverse primer, CACCGTGCGGATGCGATGTT. Actin was used as control. Sequencing was used to confirm the identity of the PCR products. To quantify the effectiveness of the shRNAs, PCR reaction products were separated on agarose gels, photographed using a Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA), and then quantified using ImageJ software. Values were normalized to actin expression. To deplete cells of Rab11a, shRNA specific for canine Rab11a was described previously (Bryant et al., 2010) and kindly provided by D. M. Bryant (University of California, San Francisco, San Francisco, CA).

**125I-IgA transcytosis, 125I-IgA apical recycling, 125I-Tf recycling, and 125I-EGFR degradation**

The postendocytic fates of 125I-IgA, 125I-Tf and 125I-EGF were determined as described previously (Apodaca et al., 1994; Maples et al., 1997; Leung et al., 1999; Oztan et al., 2007).

**Effector-based, pull-down assay to measure activated Rab11a**

For this assay, a GST-ratSec15A full-length construct was used to produce recombinant protein as described previously (Oztan et al., 2007). MDCK cells were transduced with CFP-TBC1D9B-WT or -RYQ/AAA adenovirus as described. After 48 h, the cells were solubilized in IP buffer as described, and the lysate was incubated with 1 μg GST-Sec15A for 2 h at room temperature with rotation. GSH Sepharose-4B beads were used to pull down GST-Sec15A and the endogenous GTP-bound Rab11a associated with Sec15A. The beads were washed four times with lysis buffer and resuspended in 2x Laemmlr sample buffer. The samples were heated at 95°C for 2 min and resolved by SDS–PAGE, and Rab11a was detected by Western blot. A similar protocol was used with MDCK cells transduced with lentivirus expressing scrambled shRNA or shRNA-2 targeting canine TBC1D9B.

**Statistical analysis**

Data are reported as mean ± SEM. Statistically significant differences between means were determined using a two-tailed Student’s t test; p < 0.05 was considered statistically significant. One-way analysis of variance (ANOVA), with Bonferroni’s correction, was used when making multiple comparisons.

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