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A Rab8 Guanine Nucleotide Exchange Factor-Effector Interaction Network Regulates Primary Ciliogenesis*[S]

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Background: Exocytosis at the plasma membrane mediated by Rabin8 is essential for primary ciliogenesis.
Results: Rabin8 activation involves the relief of its autoinhibition. Rabin8 interacts with the exocyst component Sec15 upon its activation.
Conclusion: The Rab8 guanine nucleotide exchange factor-effector interaction is important for exocytosis and primary ciliogenesis.
Significance: The study sheds light on our understanding of the regulation of exocytosis and ciliogenesis.

Primary cilia are microtubule-based solitary membrane projections on the cell surface that play important roles in signaling and development. Recent studies have demonstrated that polarized vesicular trafficking involving the small GTPase Rab8 and its guanine nucleotide exchange factor Rabin8 is essential for primary ciliogenesis. In this study, we show that a highly conserved region of Rabin8 is pivotal for its activation as a guanine nucleotide exchange factor for Rab8. In addition, in its activated conformation, Rabin8 interacts with Sec15, a subunit of the exocyst and downstream effector of Rab8. Expression of constitutively activated Rab8 promotes the association of Sec15 with Rabin8. Using immunofluorescence microscopy, we found that Sec15 co-localized with Rab8 along the primary cilium. Inhibition of Sec15 function in cells led to defects in primary ciliogenesis. The Rabin8-Rab8-Sec15 interaction may couple the activation of Rab8 to the recruitment of the Rab8 effector and is involved in the regulation of vesicular trafficking for primary cilium formation.

Primary cilia contain signaling proteins on their surface and serve as "antennas" in many eukaryotic cells to sense the external signals (1–3). Defects in primary cilium formation have been implicated in many disorders such as polycystic kidney disease and Bardet-Biedl syndrome (4). The formation of primary cilia ("primary ciliogenesis") involves the assembly of microtubule substructures (axonemes) and directional transport of proteins to the cilium surface (5, 6). Recently, a number of studies using genetic and biochemical approaches demonstrated the importance of vesicular trafficking in primary ciliogenesis. The small GTPase Rab8 and its guanine nucleotide exchange factor (GEF)[2] Rabin8 play critical roles in cilium formation, presumably by regulating the delivery and docking of secretory vesicles to the cilium membrane (7–9). Furthermore, Rabin8 associates with the BBSome, a protein complex containing proteins linked to Bardet-Biedl syndrome (7). Rab11, which regulates vesicular trafficking from the *trans*-Golgi network and recycling endosomes to the plasma membrane, is localized at the base of the primary cilia (10, 11). The GTP-bound form of Rab11 binds directly to Rabin8 and stimulates the GEF activity of Rabin8 toward Rab8 (10) and recruitment of Rabin8 to the transport carriers *en route* to the cilia (11). Earlier works in the budding yeast *Saccharomyces cerevisiae* demonstrated that Sec2p (a Rabin8 homolog) interacts with GTP-Ypt32p (a Rab11 homolog), which, together with phosphatidylinositol 4-phosphate, recruits Sec2p to the *trans*-Golgi network for vesicle budding and subsequent delivery to the plasma membrane (12, 13). Therefore, a cascade of Rab function involving Rab11 and Rab8 is evolutionarily conserved in eukaryotic cells (14). The cascade model not only applies to exocytosis but also to other stages of membrane trafficking such as endosomal docking and fusion (15–19).

The targeting of secretory vesicles to specific domains of the plasma membrane is mediated by the exocyst, an octameric protein complex consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (20–23). Members of the exocyst are evolutionarily conserved from yeast to human (21, 24). In budding yeast, the exocyst component Sec15p associates with secretory vesicles and interacts with the Rab GTPase Sec4p in its GTP-bound form (25). This interaction appears to be evolutionarily conserved in higher eukaryotes, as Rab8, the homolog of Sec4p, was shown to interact directly with Sec15 (26), and the exocyst has been implicated in epithelial cystogenesis under the control of Rab8 (27). Thus, the exocyst functions together with Rab proteins to mediate vesicle docking and fusion at the plasma membrane.

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[2] The abbreviations used are: GEF, guanine nucleotide exchange factor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate).

Recent studies have implicated the exocyst in primary cilium formation. It was shown that the exocyst component Sec8 localizes at the base of the primary cilium in Madin-Darby canine kidney cells (28). When overexpressed, another exocyst component, Sec10, led to longer primary cilia (29). The exocyst was also implicated in the deciliation-associated process (30). However, how the exocyst and the Rab proteins are coordinated during ciliogenesis remains unclear.

Here, we investigated the molecular mechanisms that regulate Rabin8 activity and interactions. We demonstrate that Rabin8 activation involves the relief of its autoinhibitory conformation. We further show that the exocyst component Sec15 directly interacts with Rabin8 in its activated conformation. A molecular network containing both the GEF (Rabin8) and effector (Sec15) of Rab8 plays important roles during primary ciliogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and RNA Interference—hTERT-RPE1 cells were grown in DMEM/nutrient mixture F-12 with 10% FBS. For analysis of ciliogenesis, hTERT-RPE1 cells were grown overnight in medium containing 10% FBS and then starved for 48 h in Opti-MEM to induce cilium formation. Cilium length was measured and statistically analyzed using Student's *t* test. For transfection, the genes of interest (*SEC15*, *SEC10NT*, or *RAB8A*) were cloned in-frame into the pEGFP-C1 or pJ3EGFP vector.[3] Plasmids were transfected into cells using FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen). The sequences used for the knockdown of *SEC15* isoforms are 5′-AACAAGTGACGGATACTAATA-3′ and 5′-AAAGATATCATTCGATGTAGA-3′. The control luciferase siRNA target sequence is 5′-AACGTACGCG-GAATACTTCGA-3′. For siRNA treatment, cells were grown in DMEM and 10% FBS on coverslips. siRNA transfection was carried out in Opti-MEM with Oligofectamine (Invitrogen). The cells on coverslips were fixed 48–72 h after transfection. The remaining cells on plates were lysed for Western blot analysis.

Antibodies—Mouse anti-His_6 antibody was purchased from Covance (Berkeley, CA). Rabbit anti-Rab11 antibody was from US Biological (Swampscott, MA). Mouse anti-c-Myc antibody 9E10 and rabbit anti-transferrin receptor antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-acetylated α-tubulin monoclonal antibody and rabbit anti-γ-tubulin polyclonal antibody were purchased from Sigma. Rabbit anti-Glu-tubulin antibody was purchased from Millipore (Billerica, MA). Secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 594 for immunofluorescence were purchased from Invitrogen.

Recombinant Protein Expression and in Vitro Binding Assays—The NusA-His_6-Rab8a fusion protein was expressed in the pET43 vector as described previously (31, 32). The NusA-His_6-Rab8a fusion protein was purified from bacteria with TALON metal affinity resin (Clontech). The generation of Rabin8 fusion proteins was as described previously (10). GST fusion proteins were expressed in pGEX vectors in bacteria and purified using glutathione-Sepharose 4B. The beads were then washed four times with binding buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, and 1% Triton X-100) and used directly for *in vitro* binding assays. Sec15 was expressed in the presence of [^{35}S]methionine using a TNT Quick kit (Promega). The translation products were then incubated with protein-coupled glutathione-Sepharose beads in binding buffer for 1.5 h at room temperature. After washing four times with binding buffer, the bound proteins were separated by 10% SDS-PAGE. The gels were dried, and the protein bands were visualized by phosphorimaging. To examine the interaction between Rabin8 and Rab11, His_6-Rab11a was expressed in pET32a. The purified proteins were dialyzed overnight with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM DTT. Purified Rab11a was incubated with GST-Rabin8 immobilized on glutathione-Sepharose in binding buffer containing GTPγS for 2.5 h at 4 °C. After four washes with binding buffer, bound Rab11 was analyzed by SDS-PAGE and Western blotting using antibodies against the His_6 epitope. The binding of GST-Rab8 fusion proteins to *in vitro* translated Rabin8 or Rabin8Δ(300–305) was carried out under the same conditions as described above except that Rab8 was preloaded with 50 μM GDPβS.

GEF Activity Assays—Rab8 was cleaved from the NusA-His_6-Rab8a fusion protein using thrombin. GST-Rabin8 and GST-Rabin8Δ(300–305) were expressed and purified as described above, and GST was removed using thrombin. The GEF activity of Rabin8 or Rabin8Δ(300–305) toward Rab8 was determined as previously described (10). Data were statistically analyzed using Student's *t* test ($n = 3$).

Immunofluorescence Microscopy—For Sec15, Rab11, and transferrin receptor staining, cells were fixed with 4% paraformaldehyde in PBS for 5 min at 37 °C and then permeabilized with 0.2% Triton X-100 in PBS. For cilium staining, cells were fixed with 4% paraformaldehyde in PBS for 5 min, followed by fixation with ice-cold methanol for 3 min. The cells were then permeabilized with 1% Triton X-100 in PBS for 10 min, blocked in PBS with 3% BSA, and incubated sequentially with primary and secondary antibodies.

RESULTS

Rabin8 Interacts with Sec15—In yeast, Sec15p was shown to interact with Sec2p, the GEF for the Rab protein Sec4p involved in exocytosis (33). Here, we examined the interaction between human Sec15 and Rabin8. GST-Rabin8 fusions with serial deletions at the C terminus were constructed (Fig. 1*A*). These Rabin8 fusion proteins were conjugated to glutathione-Sepharose 4B and used to perform binding assays with *in vitro* translated [^{35}S]-methionine-labeled Sec15 or NusA-His_6-tagged Rab11a. Sec15 interacted with a Rabin8 fragment lacking the C-terminal 130 amino acids; full-length Rabin8 (amino acids 1–460) and Rabin8 (amino acids 1–400) had much weaker interactions with Sec15 (Fig. 1*B*). In addition, the region of Rabin8 containing amino acids 262–331 was sufficient to bind Sec15 (supplemental Fig. S1). This region is adjacent to the coiled-coil domain (amino acids 84–255) of Rabin8, which mediates guanine nucleotide exchange of Rab8 (the GEF domain) (34, 35).

[3] Details of the constructs are available upon request.

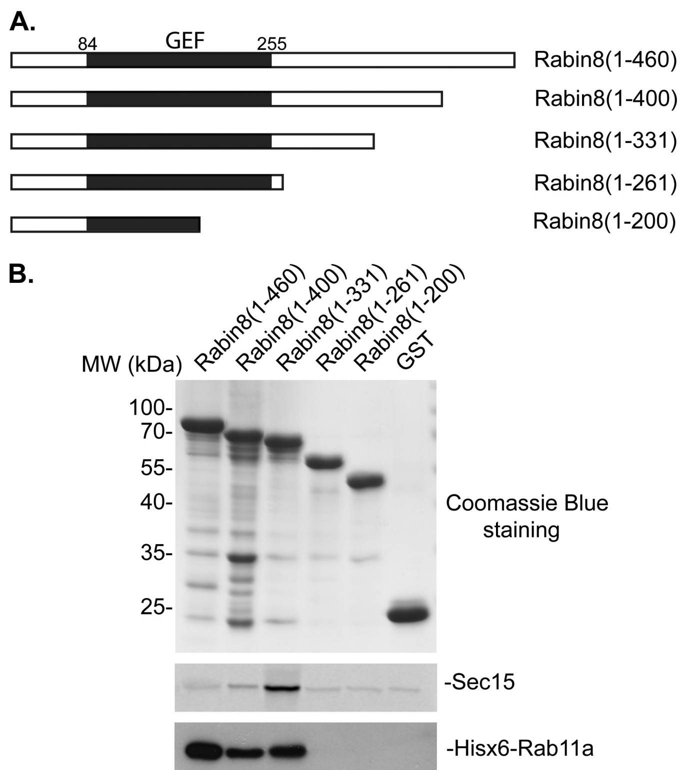

FIGURE 1. **Rabin8 interacts with Sec15.** *A*, diagram of GST fusion constructs of Rabin8 with serial C-terminal deletions. *Numbers* indicate the amino acid sequences. *Black boxes* indicate the GEF domain (amino acid 84–255). *B*, interaction between Rabin8 and Sec15. The Coomassie Blue-stained gel shows the different Rabin8 fusion proteins coupled to glutathione-Sepharose (*upper panel*). GST alone was used as a control. Sec15 was translated *in vitro* in the presence of [^{35}S]methionine (*middle panel*). The binding of Sec15 to the individual Rabin8 fusion proteins was detected with a PhosphorImager (*middle panel*). For comparison, the interaction of Rab11a (His$_6$-Rab11a) with the same set of Rabin8 proteins is also included (*lower panel*).

The same region of Rabin8 that mediates Sec15 binding was also critical for the interaction with the GTP-bound form of Rab11 (Fig. 1*B*) (10). However, Rab11a bound to full-length Rabin8, whereas Sec15 interacted better with the C-terminally truncated form of Rabin8. Therefore, the C terminus of Rabin8 probably blocks its interaction with Sec15.

Rab11 Binding-deficient Rabin8 Mutant Has Enhanced Binding to Rab8 and Sec15—To understand the mechanisms of Rabin8 activation, we first sought to identify the key residues in Rabin8 that are needed for its interaction with Rab11. Because Rabin8 and yeast Sec2p both interact with the upstream Rab proteins (*i.e.* Rab11 and Ypt32p) through their C-terminal halves, we compared the sequences of Rabin8 and Sec2p located C-terminal to their GEF domains (Fig. 2*A*). The overall sequence conservation is very low, and secondary structure prediction suggested that this region is mostly unstructured. However, a small region of six amino acids (SLYNEF) is identical between Rabin8 and Sec2p. Moreover, this stretch is predicted to form part of an α-helix. In many cases, α-helix structures have been found to constitute part of the Rab interaction site for Rab effector proteins. Thus, we made a deletion mutant of Rabin8, Rabin8Δ(300–305), and tested its protein interactions. As shown in Fig. 2*B*, Rabin8Δ(300–305) indeed had much reduced binding to Rab11. However, it had a stronger interaction with Sec15 compared with wild-type Rabin8 (Fig. 2*B*). The dose-dependent binding of Sec15 to Rabin8Δ(300–305) is shown in Fig. 2*C*. Quantification of wild-type Rabin8 binding to activated Rab11 (Rab11(Q70L)) is shown in Fig. 2*D*. The K_d for binding was ~54 μM.

Because Rabin8 is the GEF protein that interacts with the GDP-bound form of Rab8, we examined the interaction of Rabin8Δ(300–305) with GDP-loaded Rab8 or the Rab8(T22N) mutant. Compared with wild-type Rabin8, the Rabin8Δ(300–305) mutant had enhanced binding to both forms of Rab8 (Fig. 2*E*). Next, we examined whether the deletion mutant affects the guanine nucleotide exchange activity of Rab8. Purified Rab8a was preloaded with [^{3}H]GDP, and the nucleotide dissociation was monitored over time (see "Experimental Procedures"). As shown in Fig. 2*F*, Rabin8 promoted the dissociation of [^{3}H]GDP from Rab8a, consistent with previous reports (10, 31, 32). The addition of Rabin8Δ(300–305) led to a even faster dissociation of [^{3}H]GDP from Rab8a compared with wild-type Rabin8. This is consistent with the observation that Rabin8Δ(300–305) bound better to Rab8 than the wild-type protein (Fig. 2*E*). The deletion of amino acids 300–305 may cause conformational changes that relieve the self-inhibition of Rabin8, allowing its subsequent interaction with Rab8 and Sec15.

In addition to affecting the GEF activity, the interaction of GTP-bound Rab11a with Rabin8 also mediates the recruitment of Rabin8 to the recycling endosomes (11). Here, we examined the localization of Rabin8Δ(300–305) in hTERT-RPE1 cells. Wild-type Rabin8 co-localized with endogenous Rab11a in the perinuclear region. However, Rabin8Δ(300–305) was mostly diffused in the cytoplasm (Fig. 3). The same pattern of localization was observed with the constitutively activated form of Rab11a expressed in the cell (supplemental Fig. S2). This observation suggests that the loss of interaction of Rabin8 with GTP-bound Rab11 results in its dissociation from the membranes, where it is thought to carry out its function in activating Rab8 during exocytosis. We also noticed a slight difference in the distribution of Rab11a in cells expressing Rabin8Δ(300–305), suggesting that this mutant somewhat affected the morphology of the recycling endosomes. Finally, hTERT-RPE1 cells expressing the Rabin8Δ(300–305) mutant had slightly shorter primary cilia compared with cells expressing wild-type Rabin8 (supplemental Fig. S3). It is possible that, although the Rabin8Δ(300–305) protein is more active *in vitro*, the lack of its localization in the recycling endosomes compromises its function in the cell.

Sec15 Is Localized to Primary Cilia—Recent studies have demonstrated that both Rab8 and Rabin8 are important regulators of primary ciliogenesis (7, 8). Because Sec15 interacts with both Rab8 and Rabin8, we examined whether Sec15 is localized to primary cilia. Using a monoclonal antibody against Sec15, we performed immunofluorescence microscopy in quiescent hTERT-RPE1 cells. As shown in Fig. 4, similar to Rab8, Sec15 was localized along the primary cilia. In addition, comparison of Sec15 localization with respect to Glu-tubulin (detyrosinated α-tubulin, a marker of primary cilia) and γ-tubulin (a marker of basal bodies) further confirmed its ciliary localization.

Inhibition of Sec15 Function Blocks Primary Ciliogenesis—To test the possible role of the exocyst in ciliogenesis, we generated

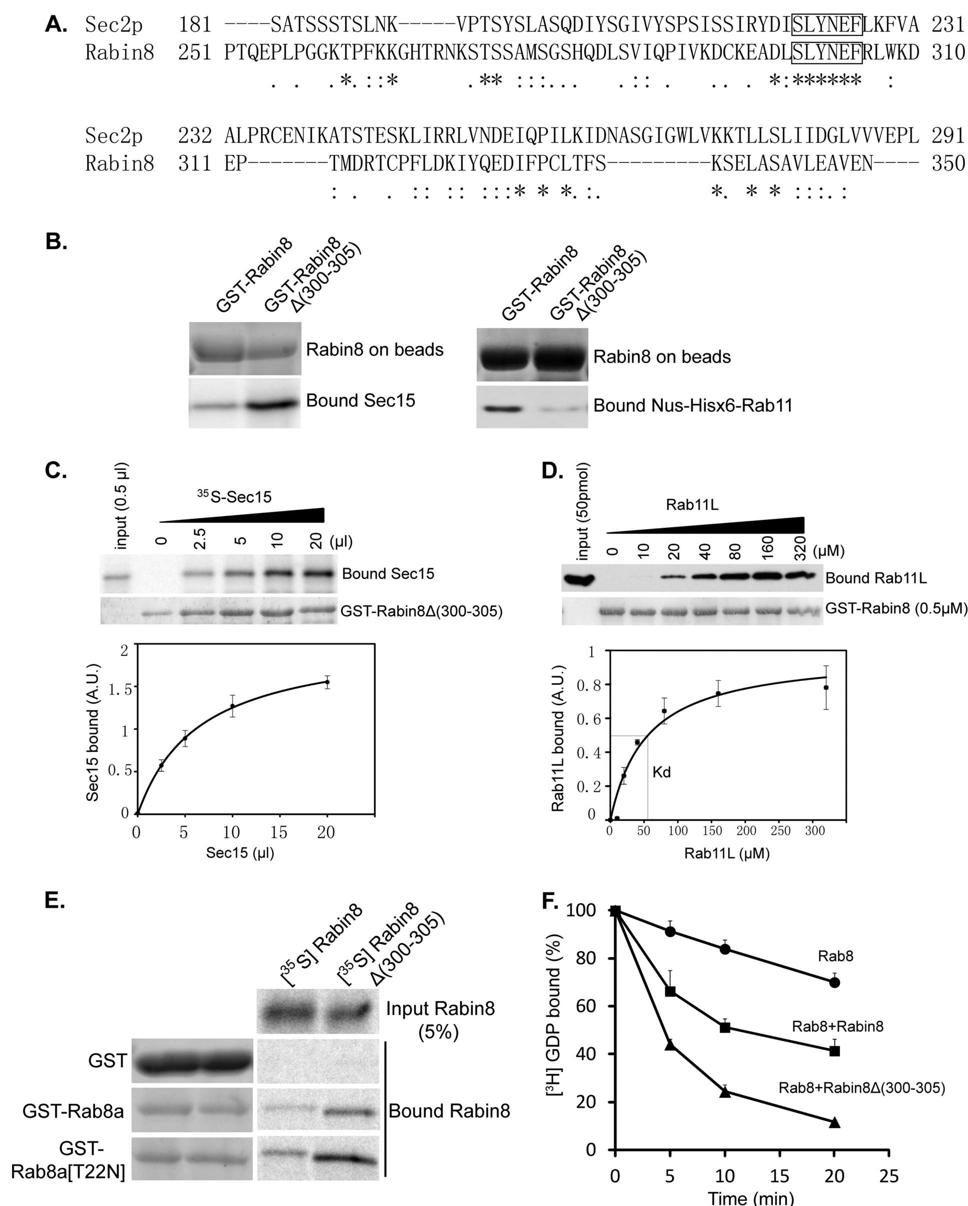

FIGURE 2. *A*, sequence comparison between Sec2p and Rabin8. *Asterisks* indicate identical amino acids. *Double dots* indicate amino acids of high similarity, and *single dots* indicate amino acids of low similarity. Amino acids 300–305 (SLYNEF) are identical in Sec2p and Rabin8. *B*, comparison of the binding of Rab11 and Sec15 to wild-type Rabin8 and the Rabin8Δ(300–305) mutant. The Coomassie Blue-stained gel shows the Rabin8 and Rabin8Δ(300–305) fusion proteins coupled to glutathione-Sepharose (*upper panels*). Rab11 expressed as a NusA-His$_6$-tagged fusion protein (*lower right panel*) had had weaker binding to the Rabin8 mutant. *In vitro* translated Sec15 bound more strongly to the Rabin8 mutant (*lower left panel*). *C*, dose-dependent interaction of Sec15 with Rabin8Δ(300–305). Various amounts of *in vitro* translated [^{35}S]methionine-labeled Sec15 were incubated with 0.5 μM GST-Rabin8Δ(300–305). Sec15 binding was standardized to 1 μl of ^{35}S-labeled Sec15 input (defined as 1 arbitrary unit (*A.U.*)). Bound Sec15 is plotted against input amounts. *Error bars* indicate S.D. (n = 3). *D*, affinity of Rabin8-Rab11(Q70L) interaction. GST-Rabin8 (0.5 μM) coupled to glutathione-Sepharose was incubated with various concentrations of NusA-His$_6$-Rab11(Q70L) in the binding assay. Bound NusA-His$_6$-Rab11(Q70L) was detected by Western blotting using anti-His$_6$ antibody. The amount of bound Rab11(Q70L) was standardized to 50 pmol of purified Rab11(Q70L) (defined as 1 arbitrary unit). The dissociation constant (K_d) was calculated by fitting the data using SigmaPlot software with a single rectangular hyperbola equation: $B = B_{max}X/(K_d + X)$, where B is bound and X is free Rab11. The K_d for the Rabin8-Rab11(Q70L) interaction was ~54 μM. *E*, the Rabin8Δ(300–305) mutant binds to Rab8 more strongly than Rabin8. Wild-type Rab8a and Rab8a(T22N) were expressed as GST fusion proteins and conjugated to glutathione-Sepharose. Rabin8 and Rabin8Δ(300–305) were *in vitro* translated in the presence of [^{35}S]methionine. The binding of Rabin8 to Rab8a and Rab8a(T22N) in the presence of GDPβS was examined. *Left panels*, Coomassie Blue-stained gel showing GST fusion proteins used in the binding reaction; *right panels*, input (5%) and bound Rabin8 and Rabin8Δ(300–305). Compared with wild-type Rabin8, the Rabin8Δ(300–305) mutant bound better to Rab8 and Rab8(T22N). *F*, analysis of the release of [^{3}H]GDP from Rab8 catalyzed by Rabin8 or Rabin8Δ(300–305). The *circles* indicate Rab8 only; the *squares* indicate Rab8 in the present of wild-type Rabin8; and the *triangles* indicate Rab8 in the present of Rabin8Δ(300–305). Rabin8Δ(300–305) was more potent than wild-type Rabin8 in promoting GDP release from Rab8 ($p < 0.01$, $n = 3$).

siRNA oligonucleotides targeting human *SEC15*. Human *SEC15* has three isoforms, so we used mixed siRNA oligonucleotides targeting all of them. These oligonucleotides knocked down *SEC15* in hTERT-RPE1 cells by 55% as determined by Western blotting (Fig. 5*A*). We then examined the formation of primary cilia in these cells by immunostaining with an antibody against acetylated α-tubulin, a marker for primary cilia. The average length of primary cilia in the luciferase siRNA-treated cells was 3.4 ± 0.4 μm ($n = 60$). The cilia in cells transfected with siRNA oligonucleotides against *SEC15* were significantly

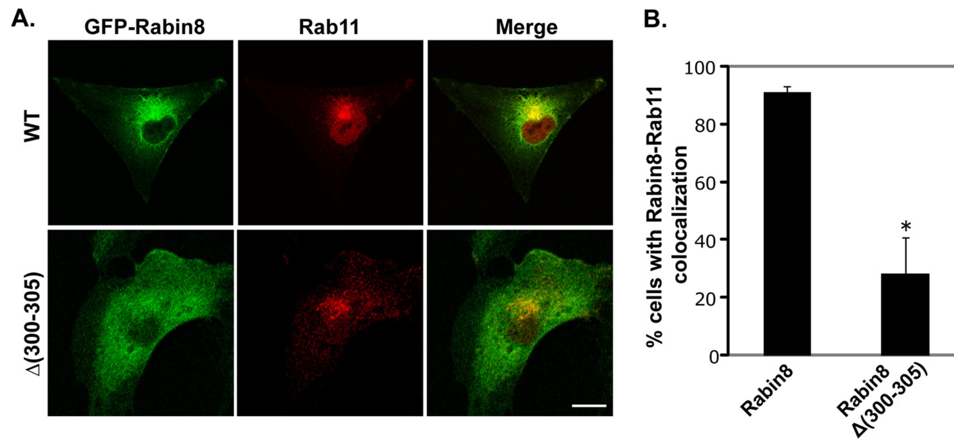

FIGURE 3. *A*, GFP-Rabin8 mostly co-localizes with endogenous Rab11 in the cells, whereas GFP-Rabin8Δ(300–305) is diffused in the cytoplasm. *Scale bar* = 10 μm. *B*, percentages of cells that have co-localization of endogenous Rab11 with GFP-Rabin8 or GFP-Rabin8Δ(300–305). Three groups of experiments were performed; >100 cells were counted in each group. *Error bars* indicate S.D.($p < 0.01$).

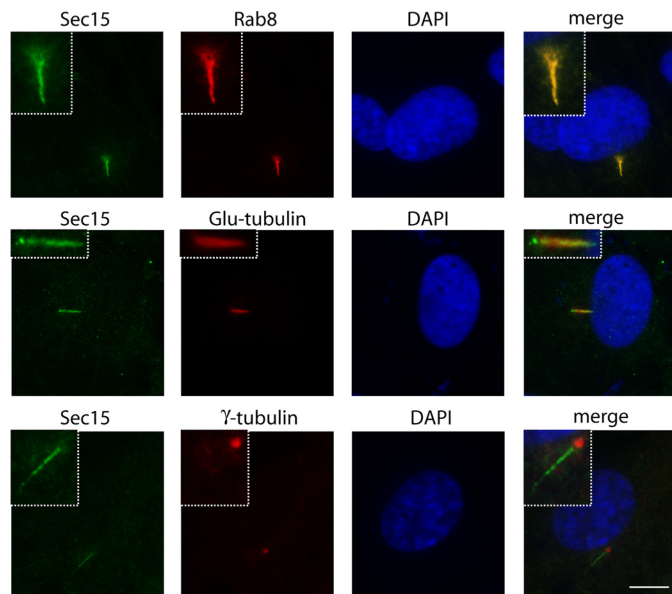

FIGURE 4. **Sec15 co-localizes with Rab8 in primary cilia.** hTERT-RPE1 cells were immunostained with a mouse monoclonal antibody against Sec15 (*green*) together with a rabbit polyclonal antibody against Rab8 (*red*, *upper panel*), Glu-tubulin (*red*, *middle panel*), or γ-tubulin (*red*, *lower panel*). Sec15 co-localized with Rab8 and Glu-tubulin at primary cilia. Nuclei were stained with DAPI (*blue*), and the merged images are shown (*right panels*). *Scale bar* = 10 μm. *Insets* show cilium staining at a higher magnification.

shorter (2.3 ± 0.5 μm, $n = 60$; $p < 0.01$). We were not able to perform RNAi rescue experiments because expression of Sec15 in cells had a strong dominant-negative effect as discussed below. To complement the RNAi experiment, we also used a dominant-negative approach to inhibit the function of Sec15. It was shown previously that overexpression of an N-terminal fragment (amino acids 1–370) of Sec10 (Sec10NT) blocked the proper assembly of Sec15 to the exocyst complex (37, 38). GFP-Sec10NT was expressed in hTERT-RPE1 cells. We found that cells transfected with GFP-Sec10NT had much shorter primary cilia (0.9 ± 0.4 μm, $n = 45$) than the untransfected cells (3.5 ± 0.6 μm, $n = 60$; $p < 0.01$) (Fig. 5, *B* and *C*). In some cases, the primary cilia were not even detectable in the Sec10NT-overexpressing cells.

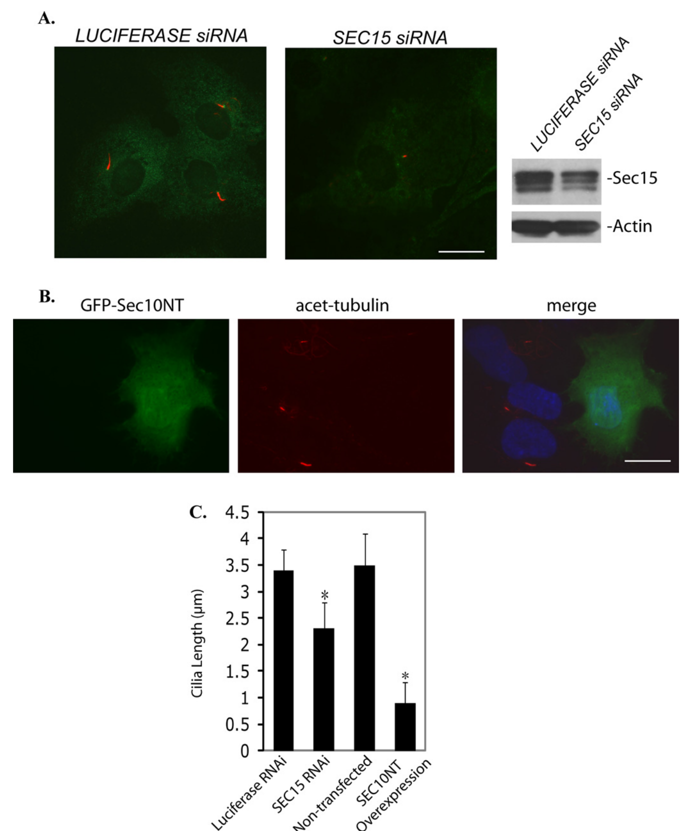

FIGURE 5. **RNAi knockdown of *SEC15* or overexpression of Sec10NT blocks primary ciliogenesis.** *A*, hTERT-RPE1 cells were treated with siRNA oligonucleotides against luciferase (used as a control) or human *SEC15* isoforms. The cells were immunostained with a mouse monoclonal antibody against acetylated α-tubulin (*red*) to detect primary cilia and with a rabbit polyclonal antibody against γ-tubulin (*green*) to label the basal bodies. The levels of *SEC15* knockdown and actin (used as a control) were examined by Western blotting (shown to the right). *B*, cells expressing a GFP-tagged N-terminal fragment of Sec10 (GFP-Sec10NT) were fixed and immunostained with an antibody against acetylated α-tubulin (*red*) to detect primary cilia. Primary cilia appeared to be shorter in cells expressing GFP-Sec10NT compared with untransfected cells in the same field. The nuclei were stained with DAPI (*blue*), and the merged images are shown (*right panel*). *Scale bar* = 10 μm. *C*, statistical analysis of the effect of *SEC15* knockdown and Sec10NT overexpression on cilium length ($n \geq 45$). *, $p < 0.01$. *Error bars* indicate S.D.

Overexpression of Sec15 Arrests Trafficking from Recycling Endosomes and Blocks Primary Ciliogenesis—In yeast cells, overexpression of the exocyst component Sec15p leads to intracellular accumulation and clustering of secretory vesicles and a block of exocytosis (39). Similarly, in mammalian cells, overexpression of Sec15 leads to accumulation and clustering of Rab11-positive recycling endosomes, and membrane trafficking from the recycling endosomes to the plasma membrane is blocked (40). Although the mechanism for Sec15-induced vesicle clustering remains elusive, it was speculated that the vesicle docking property of Sec15 led to the clustering of vesicles upon overexpression conditions (39). Taking advantage of this observation, we overexpressed GFP-Sec15 in hTERT-RPE1 cells and examined whether ciliogenesis is affected. As shown in Fig. 6*A* (*upper panels*), the recycling endosomes, as detected by the staining of endogenous Rab11, were abnormally concentrated in the cytoplasm in Sec15-transfected cells. The labeling of endogenous Rab11 was much brighter compared with untransfected cells in the same field. In addition, GFP-Sec15 co-localized with the transferrin receptor in the cell (Fig. 6*A*, *lower panels*), and recycling of the endocytosed transferrin receptor to the plasma membrane was blocked in hTERT-RPE1 cells (supplemental Fig. S4), similar to the observation made by Zhang *et al.* (40) in HeLa cells. The cilia in GFP-Sec15-overexpressing cells were much shorter (1.0 ± 0.7 μm, $n = 60$) than those in the untransfected cells (3.4 ± 0.5 μm, $n = 60$; $p < 0.01$) in the same field or in cells transfected with GFP-Sec8 used as a control (3.3 ± 0.8 μm, $n = 60$, $p < 0.01$) (Fig. 6, *B* and *C*). These results suggest that Sec15 overexpression affects vesicular trafficking from the recycling endosomes and blocks the formation of primary cilia.

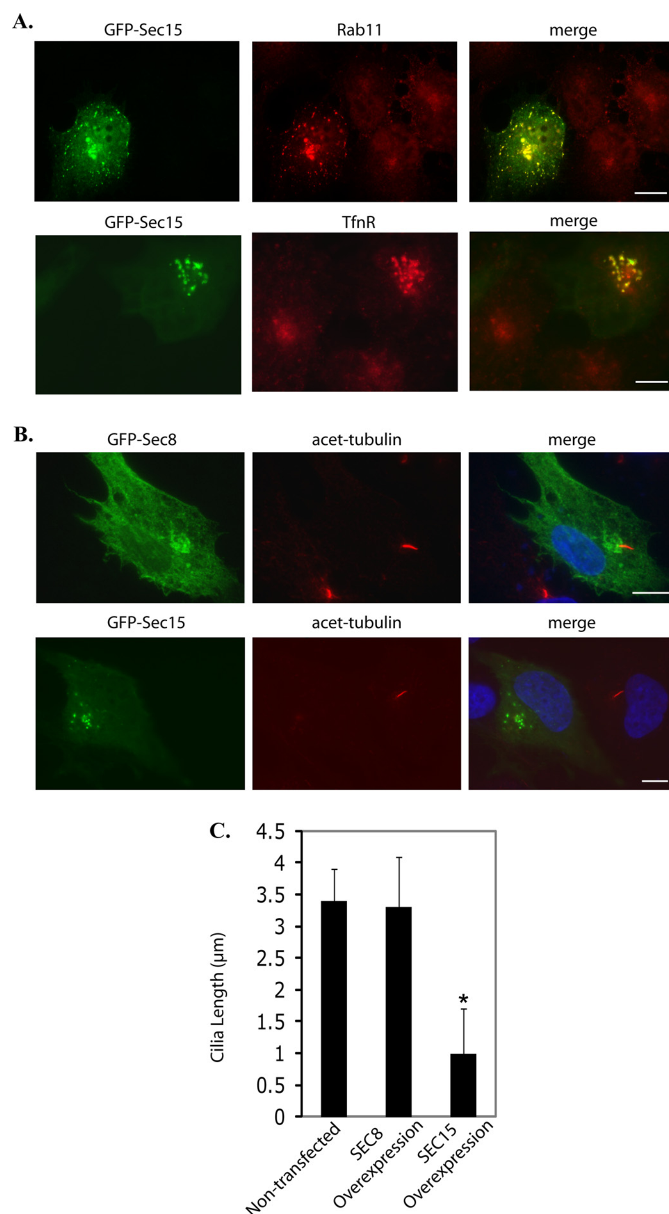

FIGURE 6. **Overexpression of Sec15 affects endosomal trafficking and blocks ciliogenesis.** *A*, cells overexpressing Sec15 have abnormal recycling endosome structures. Cells were immunostained with a polyclonal antibody against endogenous Rab11 (*red*). GFP-Sec15 (*green*) co-localized with Rab11. Rab11 staining in the Sec15-overexpressing cells was much brighter than in the untransfected cells, suggesting the clustering of recycling endosome structures (*upper panels*). The cells were also stained with antibodies against the transferrin receptor (*TfnR*; *red*). GFP-Sec15 co-localized with transferrin receptors (*lower panels*). *B*, GFP-Sec15 or GFP-Sec8 (used as a control) was transfected into hTERT-RPE1 cells and immunostained with an antibody against acetylated α-tubulin (*red*) to detect primary cilia. Primary cilia appeared to be shorter or undetectable in cells overexpressing GFP-Sec15 (*green*) compared with untransfected cells in the same field or in cells transfected with GFP-Sec8. The nuclei were stained with DAPI (*blue*), and the merged images are shown (*right panels*). *Scale bars* = 10 μm. *C*, statistical analysis of the effect of Sec15 overexpression on cilium length ($n = 60$). *, $p < 0.01$. *Error bars* indicate S.D.

DISCUSSION

Our work suggests a molecular mechanism for Rabin8 activation. Full-length Rabin8 likely has a self-inhibitory conformation. The conserved sequence SLYNEF functions as a "latch" that keeps Rabin8 in its closed conformation. We speculate that the GTP-bound Rab11 interacts with this region and may remove the latch and thus expose its GEF domain to catalyze the guanine nucleotide exchange of Rab8 and for its subsequent interactions with other proteins such as Sec15. It is unlikely that removal of the C terminus of Rabin8 or deletion of the SLYNEF sequence renders Rabin8 "sticky" because these mutants actually lost their binding to Rab11 but increased their interactions with Sec15 and Rab8. In cells, the Rabin8 mutant with the SLYNEF sequence deleted (Rabin8Δ(300–305), Rab11 binding-deficient) was diffused in the cytoplasm rather than associated with the vesicles, consistent with the role of Rab11 in the recruitment of Rabin8 to the vesicular carriers (11). On the other hand, the mislocalization of Rabin8Δ(300–305) in cells also makes functional studies of this "gain-of-function" mutant difficult.

A recent study demonstrated that Sec2p interacts with phosphatidylinositides on the membranes of secretory vesicles in addition to its interaction with GTP-Ypt32p; the interactions of Sec2p with both phosphatidylinositides and GTP-Ypt32p mediate the recruitment of Sec2p to the vesicles (13). It is possible that the phosphatidylinositide interaction also affects Ypt32p/Rab11 binding to Sec2p/Rabin8. Future crystallization of Rabin8 may provide important structural insights into the molecular details of Rabin8 activation.

Activated Rabin8 mediates the guanine nucleotide exchange of Rab8 and subsequent effector recruitment. Pioneering studies by Novick and colleagues delineated a molecular pathway

for polarized exocytosis in the budding yeast. In this pathway, the Rab protein Sec4p (Rab8 homolog) is activated by its GEF Sec2p (Rabin8 homolog) (33). Sec15p then binds to the GTP-bound form of Sec4p (25). Sec15p also binds to Sec2p; a Sec2p truncation mutant missing its C terminus binds more strongly to Sec15p (36). Our data described above indicate that the region of Rabin8 (amino acids 262–331) adjacent to the GEF domain is responsible for binding to both its upstream activator, Rab11, and Sec15. However, the modes of interaction of these two proteins with Rabin8 are different. Whereas GTP-Rab11 binds to full-length Rabin8, Sec15 binds more strongly to the C-terminally truncated version of Rabin8. It is likely that GTP-Rab11 first binds to and alleviates the autoinhibition of Rabin8, allowing Rabin8 to bind Sec15. Our data suggest that the GEF-Rab-effector interactions are conserved in the mammalian cells for exocytosis. This type of GEF-Rab-effector interaction has also been observed in other stages of membrane trafficking. A well studied example is the Rabaptin-5-Rabex-5 complex, which contains both the GEF and effector of Rab5; the concomitant Rab5 activation and effector function are required for early endosomal docking and fusion (41, 42). Combining mathematical modeling and kinetic analyses in cells, Zhu *et al.* (43) demonstrated that the Rab5 positive feedback loop may serve to convey sensitivity and precision for the activation of Rab5 on endosomes. Previous studies demonstrated that Sec15 also interacts with Rab11 on the recycling endosomes (26, 40, 44). Although future studied are needed to elucidate the implications of these interactions, we speculate that the binding of Sec15 to both Rab11 and Rab8 serves to coordinate vesicle budding from the recycling endosomes and vesicle tethering at the plasma membrane.

Whereas the GEF-Rab8-effector interaction network is important in yeast for polarized daughter cell growth, this network can be utilized in mammalian cells in processes such as primary ciliogenesis. Inhibition of Rab8 and Rabin8 function by RNAi or dominant-negative mutants was shown to block primary ciliogenesis (7, 8). Here, we have shown that disruption of the proper function of Sec15 leads to a block of endosomal recycling and impaired ciliogenesis.

In summary, our study suggests a mechanism of Rabin8 activation and demonstrates a molecular network containing both the GEF and effector of Rab8 that plays important roles in primary ciliogenesis. Future studies will provide more insights into the functions of the exocyst and the Rab proteins in primary ciliogenesis and shed light on the etiologies of ciliopathies.

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