Rab10 GTPase regulates ER dynamics and morphology

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We have identified Rab10 as an ER-specific Rab GTPase that regulates ER structure and dynamics. We show that Rab10 localizes to the ER and to dynamic ER-associated structures that track along microtubules and mark the position of new ER tubule growth. Rab10 depletion or expression of a Rab10 GDP-locked mutant alters ER morphology, resulting in fewer ER tubules. We demonstrate that this defect is due to a reduced ability of dynamic ER tubules to grow out and successfully fuse with adjacent ER. Consistent with this function, Rab10 partitions to dynamic ER-associated domains found at the leading edge of almost half of all dynamic ER tubules. Interestingly, this Rab10 domain is highly enriched with at least two ER enzymes that regulate phospholipid synthesis, phosphatidylinositol synthase (PI-4P) and CEPT1. Both the formation and function of this Rab10/PI-4P/CEPT1 dynamic domain are inhibited by expression of a GDP-locked Rab10 mutant. Together, these data demonstrate that Rab10 regulates ER dynamics and further suggest that these dynamics could be coupled to phospholipid synthesis.

The endoplasmic reticulum (ER) is a large membrane-bound compartment composed of multiple structurally distinct domains spread throughout the cytoplasm of eukaryotic cells. A particularly striking feature of the ER is its extremely dynamic nature and ability to maintain its continuity during rearrangements of its complex morphology. In recent years, some of the factors that regulate ER dynamics have been identified. In animal cells, ER tubules are generated when they are pulled along microtubules and contact an adjacent ER region where they subsequently undergo a homotypic fusion reaction. This homotypic fusion reaction results in the formation of a three-way junction, which produces the reticular peripheral ER morphology. Homotypic ER fusion has been shown to be regulated by the atlastin family of dynamin-like GTPases. It is not known what guides the growth and tethering between ER tubules and molecular motors on microtubules during dynamics and fusion.

Numerous membrane compartments derive functional specificity from the precise combination of Rab proteins and SNAREs that guide fusion between donor and acceptor compartments. These GTP-binding proteins regulate the fusion of donor and target membranes in a process regulated by GTP hydrolysis. In vitro systems for ER formation suggest that a Rab GTPase could also regulate ER fusion. Here we identify Rab10 as an ER-specific Rab GTPase that regulates ER tubule extension and fusion at the leading edge of dynamic ER tubules.

RESULTS

A Rab GTPase is required for ER assembly in vitro

On the basis of previous data implicating a Rab GTPase in ER assembly, we aimed to isolate Rab proteins localized to ER membranes. We started with ER vesicles isolated from unfertilized Xenopus laevis egg extracts. These ER vesicles fuse to form a tubular ER network in the presence of hydrolysable GTP, as previously described. We investigated whether pre-incubation of ER vesicles with recombinant Rab GDI would be inhibitory. Indeed, Rab GDI pre-incubation inhibits in vitro ER fusion and tubule formation. Rab GDI also inhibits ER formation when assayed quantitatively using a previously described Ca²⁺ release assay. These results demonstrate that our in vitro ER network formation assay is sensitive to Rab GDI addition and is likely to require a Rab protein.

To identify a Rab protein tightly associated with Xenopus ER membranes, we optimized a purification scheme using the GTP-binding ability of Rab proteins. We investigated whether pre-incubation of ER vesicles with non-inhibitory buffer containing 500 mM KCl to remove cytosolic or loosely associated proteins. Washed vesicles were solubilized with 1% digitonin and applied to a GTP-agarose column. A control sample was alternatively pre-incubated with 1 mM GTP-γS to pre-block GTP-agarose binding sites before GTP-agarose binding. The GTP-binding proteins were eluted from the column with GTP, and analysed by SDS–PAGE. The unique bands with relative molecular masses of approximately 25,000 and 45,000 were excised and analysed by mass spectrometry. The M₉ ~ 45 K band was identified as tubulin and the M₉ ~ 25 K band included several Rab proteins represented to different degrees of sequence coverage: Rab11 (40%), Rab8/10 (29%), Rab7 (20%), Rab2 (18%) and Rab1 (17%).

We examined whether Rab proteins are displaced from ER vesicles by concentrations of Rab GDI that inhibit ER formation. ER vesicles were...
To further characterize the identified Rab candidates, we generated fluorescently tagged human homologue expression constructs of a subset of these candidates. These Rab constructs were transiently co-transfected into COS-7 cells with a luminal ER marker (KDEL-targeted) to determine localization of each Rab relative to the ER by live-cell confocal fluorescence microscopy. Several Rab proteins previously shown to localize to endocytic compartments gave the expected localization and did not localize to the ER membrane (including Rab5, Rab7 and Rab11; Supplementary Fig. S1a). X. laevis Rab8/10 has two human paralogues (Rab8 and Rab10; Fig. 2a and Supplementary Fig. S2a). Despite their high sequence similarity (Supplementary Fig. S2a), Rab8 localized to vesicles (Fig. 2a top panel), consistent with its previously defined localization. In contrast, Rab10 co-localized with multiple domains of the ER, including the nuclear envelope and peripheral ER cisternae and tubules (Fig. 2a and Supplementary Video S1), even with low transfection levels (Supplementary Fig. 2b). Rab10 also localized to the Golgi (labelled with GPP130-eGFP; Supplementary Fig. S2c), consistent with previous reports. Rab10 does not localize to the mitochondria, early endosomes or recycling endosomes (Supplementary Fig. S2c). Although previous studies have not assayed whether Rab10 co-localizes with ER markers, images of Rab10 in Caenorhabditis elegans reveal a reticular structure reminiscent of tubular ER (ref 22,23).

Rab10 regulates ER morphology

The mechanisms that regulate the Rab family of GTPases are well known and GTP-binding state mutations can be engineered to alter the activity of Rab proteins. We generated two mutants of Rab10: a GTP-locked Q68L mutation and a GDP-locked T23N mutation.

Figure 1 Purification of GTP-binding proteins from a X. laevis in vitro ER assembly assay. (a) ER vesicles from fractionated X. laevis eggs were analysed directly (t0) or incubated for 60 min (t60) at 25°C with GTP, no GTP or GTP-γS. Alternatively, vesicles were pre-incubated for 20 min at 25°C with Rab GDI or washed with 0.5 M KCl buffer and then incubated with GTP. The resulting vesicles or tubules were visualized by fluorescence microscopy with octadecyl rhodamine. Scale bars, 10 μm. (b) Ca²⁺ efflux from ER vesicles was measured with aequorin in a luminometer during the course of the ER tubule formation assay. The reactions were performed in the presence of GTP, GTP-γS or GTP following pre-incubation with 5 μM Rab GDI, 20 μM Rab GDI, 40 μM Rab GDI or 40 μM boiled Rab GDI. (c) Strategy used to purify GTP-binding proteins from ER vesicles. (d) Bound proteins from the purification shown in c were eluted with GTP and analysed by SDS–PAGE and silver staining. Control samples were pre-incubated with GTP-γS before application to the GTP-agarose column (second lane). Arrowheads mark the band that was used to identify Rab11, Rab8/10, Rab7, Rab2 and Rab1. Figure shows two lanes spliced from the same gel. (e) Reactions in b were alternatively spun down and separated into soluble (S) and membrane (M) fractions. Soluble and membrane proteins were analysed by immunoblotting (IB) with antibodies recognizing Xenopus Rab8/10. Note that Rab 8/10 is displaced from the ER vesicles by concentrations of Rab GDI that inhibit tubule formation (as assayed in a and b). Uncropped images of blots are shown in Supplementary Fig. S9.
Figure 2 Rab10 localizes to the ER and regulates tubular ER morphology. (a) COS-7 cells co-expressing mCh-tagged human Rab8 (mCh–Rab8) or Rab10 (mCh–Rab10) and a luminal ER protein (KDEL–venus) were localized by confocal fluorescence microscopy (top and bottom panels, respectively). The third and fourth panels show a merged image and a zoom of the outlined region, respectively (Rab in green; ER in red). Note that Rab10 localizes throughout the ER, and Rab8 does not. (b) As in a for cells expressing KDEL–venus with mCh–Rab10 T23N or mCh–Rab10 Q68L, as indicated. Note the expansive cisternae in Rab10-T23N-expressing cells. (c) Method for quantitative analysis of ER shape. Three identical 28-pixel-wide line segments were drawn on the images beginning at the nuclear envelope away from the microtubule organizing centre (yellow rectangles); the Renyi entropy threshold setting was used to select only ER cisternae (middle panel image) or the total ER (third panel, green + blue). Dividing the number of ER cisternae pixels (green) by the number of total ER pixels (green + blue) gives the percentage of ER cisternae in each line segment. The remaining ER was defined as ER tubules. Scale bars, 10 µm (a–c). (d) Percentage of the ER comprising cisternae (using analysis described in c) for cells expressing KDEL–venus alone or together with mCh–Rab10 WT, mCh–Rab10 Q68L or mCh–Rab10 T23N. Means ± standard errors were calculated from each condition in d, n = 50 cells for each condition, 3 regions per cell. ***P < 0.001.

In both COS-7 (Fig. 2b) and HeLa cells (Supplementary Fig. S3a), the fluorescently tagged wild-type (WT) and mutant Rab10 proteins are localized to the ER (although mCh–Rab10 T23N is also cytosolic). Immunoblot analysis shows that transfected cells express 2–3 fold-more fusion protein relative to endogenous Rab10 (Supplementary Fig. S3b). Confocal fluorescence microscopy reveals that the expression of the mCh–Rab10 T23N mutant alters peripheral ER shape, resulting in more cisternal regions and fewer tubules (compared with mCh–Rab10 WT or controls; Fig. 2a,b). We developed a quantitative assay to measure the effect of Rab10 T23N expression...
on ER morphology by determining the ratio of cisternae to tubules (as described in the Methods, Fig. 2c). The peripheral ER was 23% cisternal in control cells expressing KDEL–venus alone (Fig. 2d). Cells co-transfected with KDEL–venus and mCh–Rab10 WT or mCh–Rab10 Q68L showed similar results (19 and 21% cisterna, respectively, Fig. 2d). In contrast, cells expressing KDEL–venus and mCh–Rab10 T23N had a statistically significant increase in the percentage of cisternae (52%; Fig. 2d). The effect of mCh–Rab10 T23N expression on ER shape is intermediate in magnitude to overexpressing Climp63 (80% cisternal; Supplementary Fig. S4c,d), an ER protein known to proliferate cisternal ER (ref. 25).

We next investigated the effect of Rab10 depletion on peripheral ER shape. Cells were co-transfected with control or Rab10 short interfering RNA (siRNA) together with an ER marker (KDEL–venus) to visualize shape (Fig. 3a). Rab10 siRNA treatment led to a noticeable expansion of cisternal ER and fewer ER tubules (Fig. 3a). Immunoblot analysis confirmed Rab10 depletion (Fig. 3b). We quantified the effect of Rab10 depletion on peripheral ER shape as before. Rab10-siRNA-treated cells had a significant increase in cisternal ER relative to the control (68% versus 25%, respectively; Fig. 3a,c). We did not observe an effect of Rab10 depletion on Golgi morphology, even though Rab10 also localizes to Golgi (Supplementary Fig. S4a).

Rab10 alters the efficiency of ER tubule extension and fusion

Rab10 deletion or Rab10 T23N expression led to an increase in ER cisternae; this phenotype is similar to when ER tubule-shaping proteins, reticulons, are depleted16,26 or microtubules are depolymerized2. Thus, Rab10 may play a role in maintaining and/or generating the structure of ER tubules. We next examined whether Rab10 regulates ER tubule extension and fusion. Using live-cell, time-lapse fluorescence microscopy images of COS-7 cells transfected with KDEL–venus alone, and mCh–Rab10 WT or mCh–Rab10 T23N, we measured the number of ER tubule extension events occurring within a 10 μm² square over a 5 min time period and determined whether the extended tubules could successfully fuse. Fusion was considered successful if an ER tubule grew out, contacted the adjacent ER and generated a three-way junction maintained for more than 30 s (Fig. 4a, top panel). An unsuccessful event was when an ER tubule extended into another section of the ER but did not successfully fuse (Fig. 4a, bottom panel). Cells expressing KDEL–venus alone or co-transfected with mCh–Rab10 WT had similar successful fusion rates of 74% (mean of 9.5 out of 12.9 events) and 79% (mean of 10.2 out of 13.0 events), respectively (Fig. 4b,c). In contrast, cells expressing KDEL–venus with mCh–Rab10 T23N had a reduced rate of both successful fusion and extension (51%, 5.5 out of 10.7 events; Fig. 4b,c). We observed a similar change when we instead normalize the events relative to the total area of ER in the 10 μm² square (Supplementary Fig. S5b). Thus, Rab10 depletion or Rab10 T23N expression reduces the efficiency of ER tubule extension and fusion, explaining how the loss of functional Rab10 results in depleted tubular ER morphology.

A Rab10/PIS dynamic domain leads ER tubule extension and fusion

Live-cell imaging reveals Rab10 structures localized not only to the ER, but also to dynamic ER-associated domains (Fig. 2a and Supplementary Video S1). Time-lapse images of these Rab10 dynamic domains relative to a general ER marker show that they precede the path of new ER tubule growth (Fig. 5a). In animal cells, most ER dynamics are dependent on the microtubule network2,3. We examined whether these Rab10 dynamic domains track along microtubules by imaging cells co-transfected with BFP–Rab10, mCh–ct-tubulin and an ER marker (Supplementary Fig. S6a). BFP–Rab10 localized to the ER network and to dynamic domains tracking along microtubules (Supplementary Fig. S6a)27. To depolymerize microtubules, we treated cells with nocodazole (5 μM) for 60 min (refs 3,27), and measured whether Rab10-mediated dynamics were reduced. We performed overlays of ER images taken 1 min apart at three time points: before, 20 min post and 60 min post nocodazole treatment (Supplementary Fig. S6b). These overlays reflect the extent to which the ER network and Rab10 dynamic domains move during a 1 min time period. Rab10 dynamic domains stop moving by 60 min post nocodazole treatment, when virtually all microtubules are depolymerized. These data demonstrate that Rab10 dynamics depend on microtubules.

Rab10 dynamic domains are reminiscent of a recently described dynamic ER domain enriched in the ER enzyme PIS (ref. 28). Photo-activation experiments demonstrated that this dynamic PIS domain is ER-derived29. PIS catalyses the conversion of diacylglycerol precursors and inositol to PI at the ER. To determine whether Rab10 and PIS co-localize, we transfected COS-7 cells with mCh–PIS, BFP–Rab10 and KDEL–venus. We find Rab10 and PIS co-localized at the ER, Golgi and at dynamic domains (Fig. 5b and Supplementary Video S2). These dynamic domains are tightly associated with the ER membrane over time, but lack ER luminal marker staining (Fig. 5b and Supplementary Video S2). Out of 444 puncta (from 10 cells), 68% were positive for both Rab10 and PIS, 24% were positive only for Rab10 and 8% were positive only for PIS (Fig. 5c). Thus, Rab10 and PIS co-localize at dynamic ER-associated domains.

Rab10 regulates ER extension and fusion, and partitions with PIS to dynamic ER-associated domains; we therefore examined whether these dynamic domains are found at the leading edge of ER extension and fusion events. We acquired live-cell, time-lapse confocal images of COS-7 cells co-transfected with BFP–Rab10 WT, mCh–PIS and KDEL–venus (to visualize all ER tubule dynamics) and measured the percentage of total dynamics led by a Rab10/PIS punctum (within a 10 μm² square over a 5 min period). This analysis revealed that Rab10/PIS puncta are localized to the leading edge of 42.3% of dynamic events (mean = 4.3 out of 10.2 events; Fig. 5d). Therefore, nearly half, but not all, ER tubule dynamics are led by Rab10/PIS.

To determine whether the GTP-binding state of Rab10 would affect the ability of Rab10 and PIS to co-localize, we expressed the Rab10 T23N mutant. Rab10 T23N expression reduced the percentage of puncta labelled with both markers (to 54%; Fig. 5c). We measured the ability of these Rab10 T23N/PIS puncta to mediate ER extension and fusion and found that the percentage of dynamic
Figure 3 Depletion of endogenous Rab10 reduces tubular ER morphology. (a) COS-7 cells were co-transfected with a luminal ER marker (KDEL–venus) and either control or Rab10 siRNA, and were then visualized by confocal fluorescence microscopy to detect changes in ER morphology (note the expansive cisternae in the Rab10-siRNA-depleted sample). Scale bars, 10 µm. (b) Immunoblot analysis with antibodies against Rab10 reveals efficient depletion of Rab10 by siRNA. GAPDH protein levels were measured as a loading control. (c) Percentage of the ER comprising cisternae for cells transfected as in (a) with control or Rab10 siRNA (analysis performed as described in Fig. 2c,d). Means ± standard errors were calculated from each condition, n = 50 cells, 3 regions per cell. *** P < 0.001. Uncropped images of blots are shown in Supplementary Fig. S9.

Figure 4 Rab10 regulates ER tubule dynamics and fusion. (a) COS-7 cells expressing KDEL–venus and mCh–Rab10 WT or mCh–Rab10 T23N (top and bottom panel, respectively) were imaged live to visualize ER tube extension and fusion events at the time points indicated (in seconds). Arrowheads mark the sites of successful (top panels) or unsuccessful fusion (bottom panels). (b) Cells described in (a) were imaged live to measure the number of tubular ER extension events that occurred in a 10 µm² square over a 5 min time course. These tubular extensions were scored as either resulting in a successful or unsuccessful fusion event. (c) The successful and unsuccessful fusion events measured in (b) were alternatively graphed as a percentage of events leading to fusion. (d) COS-7 cells expressing KDEL–venus to visualize ER extension and fusion were co-transfected with control siRNA (top panel) or Rab10 siRNA (bottom panel) and were imaged at the indicated time points (in seconds). Arrowheads mark sites of successful fusion (top panel) or unsuccessful fusion (bottom panel). Scale bars, 2 µm (a, d). (e) As in b for d. (f) As in c for d. Means ± standard errors were calculated for b, c, e, f, n = 30 cells for each condition, two 10 µm² squares for each cell. *** P < 0.001.

events led by a Rab10/PIS punctum is reduced to 25.9% in the presence of BFP–Rab10 T23N (mean = 1.9 out of 7.3 events; Fig. 5d). Strikingly, when ER dynamic events are led by a Rab10 T23N/PIS punctum, only 11.5% result in a successful fusion event, compared with 90.7% for Rab10 WT/PIS puncta (Fig. 5e). These data demonstrate that the GTP-binding state of Rab10 regulates the ability of Rab10/PIS dynamic domains to lead and direct ER extension and efficient fusion.
**Figure 5** Rab10 and PIS co-localize to the leading edge of dynamic ER tubules. (a) A COS-7 cell co-expressing luminal ER marker (KDEL-venus) and mCh–Rab10 imaged by live-cell confocal fluorescence microscopy to visualize ER dynamics; the top panel shows overlay, and the bottom panel shows KDEL-venus alone to illustrate the absence of the ER from the Rab10 domain. Arrowheads mark the position of a Rab10 dynamic domain (in red; white arrowheads) not initially labelled with the luminal ER marker (in green; yellow arrowheads follow the movement of the KDEL marker behind the Rab10 domain). (b) As in a for cells expressing KDEL-venus, mCh-tagged human PIS (mCh-PIS) and either BFP–Rab10 WT (top panel) or BFP–Rab10 T23N (bottom panel) (PIS in red; Rab10 in blue; KDEL in green). Note that Rab10 and PIS co-localize at dynamic domains not initially labelled with KDEL. Arrowheads mark positions of successful and unsuccessful fusion events (top and bottom panel, respectively) that follow the BFP–Rab10/mCh–PIS dynamic domain structures. Scale bars, 2 μm.

**Rab10 regulates the formation of a dynamic domain enriched in PIS and CEPT1**

The ER is the main site of synthesis for numerous phospholipids. We therefore examined whether other lipid-synthesizing proteins, besides PIS, also localize to Rab10 dynamic domains. At the ER, CEPT1 converts diacylglycerol precursors to phosphatidylethanolamine or phosphatidylcholine. CEPT1 (mCh-tagged) localizes throughout the ER and accumulates at dynamic domains in a manner similar to PIS.
We also examined the effect of Rab10 T23N expression on PIS punctum movement over 60 sec (Fig. 7c). Using the Pearson–Spearman co-localization coefficient to quantify these data, we found that Rab10 puncta are less mobile in the presence of Rab10 T23N when compared with Rab10 WT (33.7% versus 56.2%, respectively, Fig. 7d). Together, these data demonstrate the requirement of functional Rab10 for the formation and dynamics of the Rab10/PIS/CEPT1 domain.

Atlastin and Rab10 do not overlap in function or location

Rab10 dynamic domains do not lead all ER dynamics; however, the atlastin machinery6–7 could be responsible for the remaining events. Atlastin is ubiquitously expressed, partitions specifically to ER tubules and accumulates at three-way junctions5,6,26,27. We localized GFP–Atl3 to ER tubules and to punctate structures at three-way junctions (Fig. 8a). These GFP–Atl3 puncta do not co-localize with the Rab10 dynamic puncta (Fig. 8b). Expression of GFP–Atl3 is unable to rescue the expansion of ER cisternae caused by BFP–Rab10 T23N expression or Rab10-siRNA treatment (Fig. 8b,c). In fact, lack of functional Rab10 alters the localization of GFP–Atl3. Atlastin puncta also do not co-localize with dynamic domains labelled by Rab10 and PIS (Fig. 8d and Supplementary Video S5). Indeed, depletion of atlastin and depletion of Rab10 give nearly opposite ER phenotypes: atlastin depletion from COS-7 cells results in elongated ER tubules5, whereas Rab10 depletion leads to fewer ER tubules (Fig. 3). Thus, Rab10 and atlastin machineries probably regulate separate and non-redundant aspects of ER dynamics (see model in Supplementary Fig. S8a).

DISCUSSION

We have demonstrated that Rab10 localizes to the ER and regulates ER tubule dynamics. Rab10 depletion or expression of a GDP-locked mutant Rab10 mutant reduces both the frequency of ER tubule extension and the efficiency of ER tubule fusion (see model in Supplementary Fig. S8b). Consequently, the absence of functional Rab10 leads to a more cisternal and less tubular ER morphology; a phenotype similar to depletion of reticulon proteins16,26 or depolymerization of microtubules27. Rab10 has a unique localization complementing its function by accumulating in a dynamic domain positioned at the leading edge of nearly half of all dynamic ER tubules. These Rab10 dynamic domains do not initially label with ER luminal or membrane proteins, but predict the path of where the growing ER tubule will travel.

A particularly compelling feature of the studied Rab10 dynamic domains is that these domains also accumulate at least two proteins involved in phospholipid biogenesis (see model in Supplementary Fig. S8a). These proteins, CEPT1 and PIS, are each integral membrane proteins of the ER, yet, they partition with Rab10 at the leading edge of dynamic ER tubules. Even though the Rab10 dynamic domain does not label with luminal or general ER membrane proteins, the localization of the ER proteins PIS and CEPT1 at this domain suggest that it is continuous with the ER membrane. Previous data support the model that dynamic domains containing PIS are either continuous with or derived from the ER (ref. 28). It has been shown that photo-activatable GFP-tagged PIS when activated eventually localizes to structures at the dynamic ends of the ER (ref. 28). Here we show

We next investigated whether co-localization of PIS and CEPT1 to dynamic puncta requires functional Rab10. Expression of the GDP-locked mutant Rab10 T23N results in a marked decrease in the accumulation of CEPT1 at dynamic puncta, as well as a reduction in the size of PIS-labelled puncta (Fig. 7b and Supplementary Video S4). We also examined the effect of Rab10 T23N expression on PIS puncta dynamics by overlaying time-lapse snapshots of GFP–PIS taken at t₀ and t₆₀ sec, and measuring the extent of PIS punctum movement over time (Fig. 7c). Using the Pearson–Spearman co-localization coefficient to quantify these data, we found that PIS puncta are less mobile in the presence of Rab10 T23N when compared with Rab10 WT (33.7% versus 56.2%, respectively, Fig. 7d). Together, these data demonstrate the requirement of functional Rab10 for the formation and dynamics of the Rab10/PIS/CEPT1 domain.
Figure 7 Rab10 regulates the formation of Rab10/PIS/CEPT1 dynamic puncta. (a) A COS-7 cell co-expressing mCh-CEPT, GFP-PIS and BFP-Rab10 WT (CEPT1 in red; PIS in green; Rab10 WT in blue). Note that CEPT1, PIS and Rab10 co-localize throughout the ER and at punctate structures. (b) A COS-7 cell co-expressing mCh-CEPT, GFP-PIS and BFP-Rab10 T23N (CEPT1 in red; PIS in green; Rab10 T23N in blue). Note that CEPT1 no longer forms punctate structures and PIS puncta are markedly smaller. For a and b, the third and fourth panels show a merged image and a zoom of the outlined region, respectively; scale bars, 10 μm (top panel) and 2 μm (bottom panel). (c) Cells as in a and b were imaged live, with two images acquired, taken 1 min apart. These images were superimposed to visualize the change in GFP-PIS over a 1 minute time period with BFP-Rab10 WT (left panel) or BFP-Rab10 T23N (right panel) expression. Each image shows GFP-PIS at $t=0$ (green) and $t=60$ s (red). Immobile puncta appear yellow. Note that the 60 s overlay for GFP-PIS expressed with BFP-Rab10 T23N shows very little change in PIS dynamics. Scale bars, 2 μm. (d) Percentage of co-localization for cells expressing GFP-PIS with BFP-Rab10 WT or BFP-Rab10 T23N, as measured by Spearman-Pearson’s co-localization coefficient. Note that the higher the percentage of co-localization, the less overall dynamics. Means ± standard errors were calculated from each condition, $n=10$ cells for each condition. ***$P<0.001$. 
Figure 8 Atlastin and Rab10 do not overlap in function or location. (a) Atl3 accumulates at three-way junctions. Confocal images of COS-7 cells co-expressing GFP-tagged human Atl3 (GFP–Atl3 in green) and an ER luminal marker (mCh–KDEL in red). (b) COS-7 cells expressing GFP–Atl3 (green) and mCh–KDEL (red) with BFP–Rab10 (blue) WT (top panel) or BFP–Rab10 T23N (bottom panel). Note the expansion of cisternal ER induced by Rab10 T23N expression, even in the presence of Atl3. (c) COS-7 cells were co-transfected with GFP–Atl3 (green), mCh–KDEL (red) and either control or Rab10 siRNA; note the expansive cisternae in the Rab10 siRNA-depleted sample, even in the presence of Atl3. (d) Live-cell images of COS-7 cells expressing GFP–Atl3 (green), mCh–PIS (red) and BFP–Rab10 WT (blue). Note that Atl3 puncta do not co-localize with Rab10/PIS puncta. For all images, the second to last images show merged images. The last panels show zoomed images of the outlined regions in the merge panels. Scale bars, 10 μm.

that functional Rab10 is required for CEPT1 and PIS to co-localize and partition to dynamic domains within the ER. Our studies have not yet addressed whether CEPT1 and PIS are similarly required for Rab10 dynamic domain formation.

A popular theory posits that Rab proteins and components of the phospholipid synthesis pathway confer not only membrane identity, but also recruit other necessary components to regulate different parts of the fusion process. On the basis of the co-localization...
between Rab10, PIS and CEPT1 at the leading edge of dynamic ER events and the requirement of functional Rab10 for the formation of this dynamic domain, we suggest that a Rab10 complex could mediate ER extension and fusion along the microtubule cytoskeleton, and PIS and CEPT1 could synthesize the phospholipids necessary for ER growth and/or fusion (Supplementary Fig. S8). Together, these connections introduce the intriguing model that new ER tubule growth, fusion and phospholipid synthesis could all be coupled. However, it remains to be determined whether active lipid synthesis occurs in Rab10/PIS/CEPT1 structures located at the leading edge of dynamic ER tubules and if so, what effect blocking phospholipid synthesis at these positions would have on ER tubule dynamics. An alternative possibility is that lipid synthesis does not drive ER tubule growth, but rather the synthesis of specific phospholipids at the tip of dynamic ER tubules facilitates transfer of these phospholipids to other membrane-bound compartments during ER dynamics. This theory was initially proposed in ref. 28, in the description of PIS dynamic domains. However, the PIS dynamic domains did not co-localize with membrane-bound compartments during ER dynamics. This theory was initially proposed in ref. 28, in the description of PIS dynamic domains. However, the PIS dynamic domains did not co-localize with any of the organelle markers investigated28, but it is possible that transfer could occur during transient contacts. Future studies will be required to determine whether there is a physical link between Rab10 and these lipid-synthesizing enzymes or with molecular motor proteins.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS
G.K.V. and A.R.E. designed the experiments and wrote the manuscript. A.R.E. performed the experiments and data analysis.

COMPETING FINANCIAL INTERESTS
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METHODS

In vitro ER formation and aequorin-based Ca2+ efflux assay. A light membrane fraction from Xenopus eggs was prepared as previously described. ER formation assays were performed by washing membranes once in membrane wash buffer (MWB; 50 mM HEPES/KOH at pH 7.5, 2.5 mM MgCl2, 250 mM sucrose and 150 mM KCl) followed by incubation in MWB with 1 mM ATP and 0.5 mM GTP at 25°C for 60 min according to methods described previously. Network staining was performed with octadecyl rhodamine and in vitro networks were visualized by fluorescence microscopy. Aequorin luminescence assays were performed as previously described, with measurements taken every 2.5 min.

GTP-binding protein precipitation and mass spectrometry. A light membrane fraction from Xenopus eggs was prepared as previously described. Membranes were incubated for 5 min at room temperature in high-salt MWB (MWB with 500 mM KCl). The high-salt-washed membranes were then solubilized in 1% digitonin and centrifuged, and the pelleted fraction was discarded. GTP-γS samples were incubated with 1 mM GTP-γS for 10 min at room temperature. Both sets of solubilized membranes were bound to GTP-γS-agarose beads in 150 mM MWB for 1 h at room temperature. Bound proteins were eluted with 10 mM GTP, subjected to SDS-PAGE and identified by mass spectrometry. All mass spectrometry analyses were performed by the Taplin MS facility at Harvard Medical School.

Constructs. KDEL–venus was a gift from E. Snapk (Albert Einstein College of Medicine, USA). GFP–PIS was a gift from T. Lee (Carnegie Mellon University, USA). mCherry–KDEL, GFP–mito, GFP–Rab7 and mCherry–tubulin were gifts from J. Friedman (Vezzolet laboratory). mCherry–Sec61β was described previously. GFP–Rab5 was described previously. GFP–AtIII was a gift from J. Friedman and generated by PCR-amplifying full-length AtIII (NCBI NM_015459) and cloning into the KpnI/XhoI sites of the pAcGFP–C1 vector (Clontech). mCherry–Clmp3β was a gift from J. Friedman and generated by PCR-amplifying full-length Clmp3β (NCBI NM_000625) and cloning into the NheI/BamHI sites of mCherry–tubulin. GFP–PIS was generated by PCR-amplifying full-length PIS (NCBI AF014807) and cloning into the BglII/KpnI sites of the pAcGFP–C1 vector. mCherry–PIS was generated by PCR-amplifying full-length PIS (NCBI AF014807) and cloning into the BglII/KpnI sites of pAcGFP–C1 vector, which had GFP excised and mCherry inserted into NheI/XhoI sites. mCherry–CEPT1 was generated by PCR-amplifying full-length CEPT1 (NCBI NM_006825) and cloning into the KpnI/XhoI sites of pAcGFP–C1, which had GFP excised and mCherry inserted into NheI/XhoI sites. mCherry–Rab8, mCherry–Rab10 and mCherry–Rab11 were generated by cloning mCherry into the NheI/BglII sites of pAcGFP–N1 (Takara Bio) and cloning human Rab8 (NCBI NM_016530), human Rab10 (NCBI NM_016530) or human Rab11 (NCBI NM_004663) with a stop codon into the BglII/EcoRI sites of that vector. mCherry–Rab7 was cloned into the BglII/EcoRI sites of pTagBFP-C. GFP–Rab10 T23N was generated by site-directed mutagenesis from GFP–Rab10. mCherry–Rab10 S34N was generated by site-directed mutagenesis from mCherry–Rab5.

Expression in mammalian tissue culture cells. COS-7 and HeLa cells (ATCC) were grown to 80% confluency in Dulbecco’s modified Eagle’s medium high-glucose (12430-062, Invitrogen) with 10% FBS and 1% penicillin/streptomycin in a 3.5 cm microscope dish (P35G-2-14-CGRD, MatTek). Plasmid transfections were performed in Opti-MEM media (31985-088, Invitrogen) with 5 μl Lipofectamine 2000 (31608-016, Invitrogen) and incubated in MWB with 1 μM ATP and 0.5 mM GTP at 25°C for 60 min according to methods described previously. Transfections were performed by the manufacturer’s directions, using DharmaFECT #1 transfection reagent (T-2001-02, Dharmacon). In brief, negative control (AM4635, Applied Bioscience) or Rab10 SMARTpool siRNA (L-010823-00-0005, Dharmacon, target sequences: 5'-GCAAGGGAGCAUGGUAUUA-3', 5'-CAGCUUAGCUAGUAUUC-3', 5'-GAUAGCUGUUUUCAUACUA-3', 5'-GAAAGUCAUUGAUAU-3') was mixed with the transfection reagent to a final concentration of 25 nM, and then applied to the cells for 48 h. In addition, various fluorescent plasmids were transfected, following the manufacturer’s directions, into each dish using Lipofectamine 2000 transfection reagent. The transfection was terminated by removing the transfection media and splitting the cells to a 3.5 cm microscope dish. Primary antibodies against Rab10 (1:500, #8127S, Cell Signaling Technologies) and GAPDH (1:30,000, #G9545, Sigma), and HRP-conjugated goat anti-rabbit (1:3,000, #A16154, Sigma) secondary antibodies were used. Signal was detected with SuperSignal West Pico Chemiluminescent solution (34080, Thermo Fisher Scientific). Relative protein levels were determined using the ImageJ (NIH) Gel Plotting Macro following the protocol outlined in the ImageJ instruction manual.

Immunoblot analysis and densitometry. Whole-cell lysates of COS-7 cells were resuspended in Laemmli sample buffer, boiled for 10 min, separated by SDS–PAGE and transferred to PVDF membrane. Primary antibodies against Rab10 (1:500, #8127S, Cell Signaling Technologies) and GAPDH (1:30,000, #G9545, Sigma), and HRP-conjugated goat anti-rabbit (1:3,000, #A16154, Sigma) secondary antibodies were used. Signal was detected with SuperSignal West Pico Chemiluminescent solution (34080, Thermo Fisher Scientific). Relative protein levels were determined using the ImageJ (NIH) Gel Plotting Macro following the protocol outlined in the ImageJ instruction manual.

Confocal microscopy. Confocal Z-stacks of the peripheral ER were collected using Metamorph Software (Molecular Devices) on a Nikon Eclipse TE2000-U with a ×100 objective, 1.40 NA (Nikon), fitted with a spinning-disc confocal system (Solamere Technology Group) and a Cascade II, a 16-bit electron-multiplying CCD (charge-coupled device) camera with a chip size of 512 × 512 pixels (Photometrics; pixel size at ×1,000: 0.09 μm per pixel, step size: 0.25 μm). Excitation of the fluorophores was performed using 405, 473 and 561 nm diode lasers.

ER cisternae analysis. The Z-stacks were imported into ImageJ (NIH) for analysis. Each Z-stack was first compressed to a maximum intensity projection. The displayed range of the projection was set to the minimum and maximum intensities of the image. The image was then converted from a bit depth of 16 to 8, where the minimum intensity of the 16-bit image was 0 in the 8-bit image, and the maximum intensity of the 16-bit image was 255. Using the Renyi entropy threshold setting, the 8-bit projection image was then converted to two separate binary thresholded images. The first image represents ER cisternae and the second image represents total ER. Three identical 28-pixel-wide line segments were drawn on the thresholded images, beginning at the nuclear envelope and ending at the cell periphery (and avoiding the microtubule organizing centre). To determine the area in pixels of ER covered by each line segment, we first measured the total area in pixels and the mean intensity of each line segment, and then divided the product of the mean intensity and the area by 255. Dividing the number of ER cisternae pixels by the number of total ER pixels gives the percentage of ER cisternae in each line segment. The remaining ER was determined to be ER tubes.

ER dynamics analysis. After imaging for 5 min, with exposures every 10 s, the time courses were imported to ImageJ (NIH) for analysis. Brightness and contrast were adjusted across the images using ImageJ. The time courses were analysed frame by frame for fusion events and for the presence of Rab10/PIS puncta, when applicable. Where indicated, nocodazole (Acros Organics) drug treatments were performed as previously described.

Pearson–Spearman co-localization coefficient. Acquired Z-stacks or one minute time courses were imported to ImageJ (NIH) for analysis. Each Z-stack was first compressed to a maximum intensity projection. The displayed range of the projection was set to the minimum and maximum intensities of the image. The image was then converted from a bit depth of 16 to 8, where the minimum intensity of the 16-bit image was 0 in the 8-bit image, and the maximum intensity of the 16-bit image was 255. The necessary images were overlaid and the Pearson–Spearman co-localization coefficient was determined using the PSC Colocalization plugin for ImageJ.

Statistical analysis. Statistical significance between two values was determined using a two-tailed, unpaired Student t-test (Graphpad Prism). Statistical analysis of three or more values was performed by one-way analysis of variance with Tukey’s post hoc test (Graphpad Prism). All data are presented as the mean ± standard error of the mean: ***P < 0.001, **P < 0.01.

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