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Trafficking through Rab11 Endosomes Is Required for Cellularization during *Drosophila* Embryogenesis

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Summary

Background: Embryonic cleavage leads to the formation of an epithelial layer during development. In *Drosophila*, the process is specialized and called cellularization. The trafficking pathways that underlie this process and that are responsible for the mobilization of membrane pools, however, remain poorly understood.
Results: We provide functional evidence for the role of endocytic trafficking through Rab11 endosomes in remobilizing vesicular membrane pools to ensure lateral membrane growth. Part of the membrane stems from endocytosed apical material. Mutants in the endocytic regulators *rab5* and *shibire/dynamin* inhibit basal-lateral membrane growth, and apical endocytosis is blocked in *shibire* mutants. In addition, *shibire* controls vesicular trafficking through Rab11-positive endosomes. In *shibire* mutants, the transmembrane protein Neurotactin follows the secretory pathway normally but is not properly inserted in the plasma membrane and accumulates instead in Rab11 subapical endosomes. Consistent with a direct role of *shibire* in vesicular trafficking through Rab11 endosomes, Shibire is enriched in this compartment. Moreover, we show by electron microscopy the large accumulation of intracellular coated pits on subapical endocytic structures in *shibire* mutants. Finally, we show that Rab11 is essential for membrane growth and invagination during cellularization.
Conclusion: Together, the data show that endocytic trafficking is required for basal-lateral membrane growth during cellularization. We identify Rab11 endosomes as key trafficking intermediates that control vesicle exocytosis and membrane growth during cellularization. This pathway may be required in other morphogenetic processes characterized by the growth of a membrane domain.

Introduction

Epithelia separate different environments within an organism. This property relies on the existence of different membrane domains, the apical surface that forms the lumen of most organs, and the basal-lateral surface. Epithelia can form via two cellular pathways: the mesenchyme-to-epithelium transition as studied in *M*adin *D*arby *c*anine *k*idney (MDCK) cells is the most common pathway, and the other is embryonic cleavage, which leads to the formation of primary epithelial tissues in a variety of organisms [1, 2]. Although the processes are different, the columnerization of flat squamous epithelial cells and embryonic cleavage are both accompanied by the growth of a basal lateral membrane domain. The extent of membrane growth varies between tissues. The cellular mechanisms underlying polarized membrane growth remain poorly understood.

In MDCK cells, the process depends mostly on vesicular sorting along the secretory pathway from the trans Golgi network (TGN) [3, 4] and on vesicular insertion at adherens junctions via the recruitment of the multiprotein complex called exocyst [5–7]. In other cases, such as cultured hepatocytes, however, the process relies on vesicular sorting through endocytic trafficking pathways [3]. In this case, called transcytosis, all proteins are first targeted to the future apical surface and endocytic targeting from the apical domain to another membrane domain ensures the formation of the basal-lateral membrane surface. The contribution of membrane trafficking pathways to the growth and polarization of the cell surface during embryonic cleavage has not been thoroughly addressed yet.

Drosophila cellularization, a specialized form of embryonic cleavage, is a very good system for addressing this problem [8–10], although the process may in part rely on specific mechanisms that may not apply to other cases of epithelial morphogenesis. In the early syncytial embryo, the nuclei undergo 13 synchronous division cycles in a common cytoplasm. Upon entry into cycle 14, the cell surface increases, and the plasma membrane invaginates between the nuclei. This invagination results in a columnar epithelium. Cellularization proceeds in two distinct phases, slow phase and fast phase. The process is characterized by the polarized growth of the cell surface as revealed by surface-labeling experiments in living embryos [8]. Distinct membrane behaviors are observed in slow phase and fast phase. When the plasma membrane is labeled at the onset of cellularization, the surface marker gradually disappears during slow phase from the apical surface rich in microvilli and accumulates instead along the growing basal-lateral surface. If the surface is labeled instead at the onset of fast phase, a patch of unlabeled membrane appears in fast phase in the apical-lateral membrane. Two different mechanisms could underlie the disappearance of apical surface marker specific to the slow phase and its basal-lateral accumulation [8]. The process could depend on the direct apical insertion of intracellular unlabeled membrane and a sorting between the newly inserted and recipient membranes within the plane of the epithelium. Conversely, the process could stem from an endocytic-based membrane transfer from the apical to the basal-lateral surface in a manner akin to transcytosis.

The transport of vesicles along the endocytic pathway proceeds via a series of organelles defined in part by

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the transport of cargo molecules (e.g., the transferrin receptor) and in part by the specific localization of regulatory molecules. Internalized vesicles at the plasma membrane are transported to early endosomes, also called sorting endosomes. This step is dependent on the activity of the small GTPase Rab5 [11, 12] as well as on Dynamin [13]. Dynamin is encoded by *shibire* in *Drosophila* [14, 15] and controls vesicle budding and internalization in a large number of tissues. Vesicles are then transported back to the plasma membrane, destined for the late endosome and lysosome degradation pathway, or transported to the pericentriolar recycling endosome. The recycling endosome is involved in the recycling of vesicles back to the plasma membrane. In polarized epithelial cells, the apical recycling endosome is required for cell polarization as well as for transcytosis [3, 16]. Trafficking through recycling endosomes is dependent on the small GTPase Rab11 in mammals as well as in *Drosophila* [17, 18]. Rab11 localizes to this organelle in both vertebrates and invertebrates [18, 19]. Dynamin was also shown to control vesicle budding from recycling endosomes in mammalian cells [20].

In order to address the implication of endocytic trafficking in plasma membrane growth during cellularization, we tested the role of key effectors of these pathways. We more specifically probed the requirement for Dynamin, Rab5, and Rab11.

Results

rab5 and *shibire* Are Required for Membrane Invagination in Slow Phase

The overexpression of Rab5S43N, a dominant-negative variant of Rab5, was shown to block endocytosis in a number of *Drosophila* tissues [21, 22]. Because cellularization coincides with the zygotic induction of gene expression, we cannot express high enough levels of the UAS-Rab5S43N transgene. To circumvent this problem, we prepared GST+Rab5S43N in vitro and injected the proteins in embryos during cycle 13, 10–15 min before cellularization. Injection of GST+Rab5S43N (Figure 1A) reduces the speed of membrane invagination near and opposite to the site of injection during slow phase (Figure 1B, 55%, n = 33, movie S1); this is in contrast to the injection of GST alone (Figure 1B, 0%, n = 53) and of GST+Rab5WT (Figure 1B, 5%, n = 59). Successive stages of membrane invagination in an embryo injected with GST+Rab5S43N are shown in Figure 1C. The delay in membrane invagination can be seen via a comparison of the relative positions of the membrane front (Figure 1C, white arrows) and of the nuclei (Figure 1C, black arrowheads) at the end of slow phase (+26'), and in fast phase (+29' and +32'). Confocal images (Figure 1D) show the different positions of the membrane front (marked with an antibody to Slam [23, 24], red) and of the plasma membrane (marked with an antibody to the transmembrane protein Neurotactin, Nrt, green) within the injected (Figure 1D, right) and the control area (Figure 1D, left). Similar results are obtained with another dominant-negative variant of Rab5, Rab5N142I (data not shown). However, no invagination defect was observed after injection of a dominant-negative form of Rab1, Rab1N124I, which is involved in ER-to-Golgi transport (Figure 1B, 0%, n = 73). These observations support the fact that the injection of Rab5S43N has a specific effect during cellularization.

We could further detail the role of endocytosis from the plasma membrane by using the temperature-sensitive allele of *shibire/dynamin*. We can precisely control the timing of gene inactivation by shifting embryos to the restrictive temperature at 32°C. In *shibire-ts* mutant embryos this causes the total inhibition of membrane invagination in slow phase (Figures 2E and 2F), unlike in control embryos, where cellularization proceeds normally (Figures 2A and 2B). In contrast, both control and *shibire-ts* mutant embryos cellularize when the embryos are shifted to the restrictive temperature at the beginning of fast phase (Figures 2C, 2D, 2G, and 2H), albeit at a slightly slower rate in *shibire-ts* mutants. The specific requirement for *shibire* during slow phase is remarkable in light of the fact that, in wild-type embryos, apical membrane-labeled material is cleared from the plasma membrane specifically in slow phase [8]. In agreement with the known role of *shibire/dynamin* in the internalization of plasma membrane vesicles, we show that apically labeled membrane is no longer endocytosed in a *shibire* mutant (Figures 2I and 2J), as opposed to wild-type control embryos (Figures 2K and 2L).

Together, the data so far suggest that endocytosis from the apical plasma membrane is required throughout slow phase in order for the basal-lateral surface to grow.

Inhibition of Plasma Membrane Insertion of Neurotactin in a *shibire* Mutant

Although our results are consistent with the known role of *shibire* in plasma membrane endocytosis, Dynamin has also been implicated in vesicle budding and trafficking from the TGN and from recycling endosomes [20]. We therefore checked the possibility that other trafficking defects might account for the role of *shibire* during cellularization. Neurotactin is a transmembrane protein synthesized de novo during slow phase. In control embryos, the protein traffics through the secretory pathway and, at steady state, Nrt is predominantly localized at the plasma membrane (Figures 3A and 3C, arrows). Small vesicular staining is also detected in the Golgi (ref [8] and Figures 3A and 3C, arrowheads). We shifted *shibire* mutant embryos at the restrictive temperature at the onset of cellularization (e.g., when Nrt is not synthesized yet), incubated them for 20 min during slow phase, and fixed the embryos to monitor the localization of Nrt. The striking result is that Nrt is not properly inserted in the plasma membrane (compare Figures 3C and 3D; see also Figure 3G) and accumulates instead in a large subapical compartment (Figures 3B and 3D, yellow arrows, and Figure 3G). This compartment is not the Golgi apparatus, which can be detected with the middle-trans Golgi marker p120 [25]. In control and *shibire* mutant embryos, p120 is indeed mostly concentrated in the basal cytoplasm in the form of small ($<2\mu$) vesicular structures (Figures 3E and 3F). The very different distribution of these two proteins suggests that Nrt follows the secretory pathway normally but fails to be inserted in the

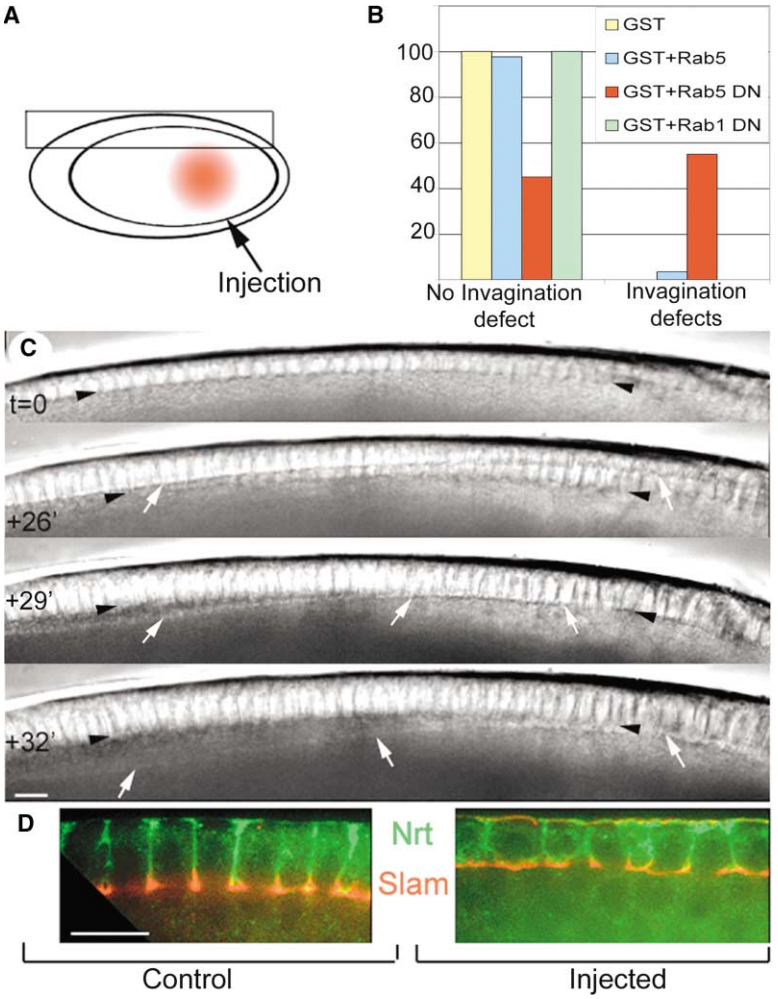

Figure 1. Role of Rab5 during Membrane Invagination

(A) Injection of GST, GST+Rab5WT, GST+Rab5S43N, or GST+Rab1N124I in one half of living embryos during cycle 12–13, about 15 min prior to cellularization. The boxed area represents the portion of the embryo shown in (C).

(B) Results obtained for GST alone (yellow, n = 53), GST+Rab5WT (blue, n = 59), GST+Rab5S43N (red, n = 33), and GST+Rab1N124I (green, n = 73).

(C) Images of a representative embryo injected with GST+Rab5S43N with a cellularization phenotype visible at the end of slow phase (+26'). The delay in membrane invagination is visible by the relative positions of the membrane front (white arrows) and of the basal extent of the nuclei (black arrowheads). This delay persists during fast phase (+29' and +32'). The left part shows the control half and the injected half is to the right.

(D) Confocal images of an embryo injected similarly with GST+Rab5S43N and fixed toward the end of slow phase. Neurotactin (Nrt, green) shows the plasma membrane, and Slam (red) marks the membrane front in the control (left) and injected (right) halves of the embryo. The scale bar represents 10 μm.

plasma membrane apically. Note that Toll, a maternally provided transmembrane protein that is already present at the plasma membrane at the onset of cellularization, remains in the plasma membrane, unlike Nrt in a *shibire* mutant (Figure 3G). The trafficking defects observed with Nrt, a newly synthesized protein en route to the plasma membrane, suggest that *shibire* controls another step in vesicular trafficking.

Trafficking Defects through Rab11 Recycling Endosomes in *shibire* Mutants

In the search for markers of intracellular compartments in which Nrt accumulates, we found Rab11. In *Drosophila*, during slow phase, Rab11 accumulates mostly in the subapical cytoplasm in two large pericentriolar endocytic compartments (Figures 4A and 4C, red arrows, and 4E). An additional staining is seen in small puncta (<1 μ) concentrated mostly in the basal cytoplasm and, in some cases, in the apical cytoplasm. These small puncta colocalize with Golgi markers, unlike the large apical staining (data not shown). At steady state, Nrt is virtually absent from the large apical Rab11 endosomes (Figures 4A and 4C), although some colocalization is occasionally detected in the small Rab11-positive Golgi puncta (Figure 4C, arrowheads). However, in a *shibire* mutant, the subapical accumulation of Nrt predominantly colocalizes with Rab11 in the large subapical pericentriolar endosomes (Figures 4B and 4D, yellow).

The sequestration of Nrt in Rab11 apical endosomes is unlikely to be the indirect effect of perturbing globally endocytic traffic in a *shibire* mutant. Indeed, we used an antibody to Shibire to detect the protein and found a clear enrichment of Shibire in Rab11 endosomes (Figure 5A), although the colocalization is not total. Shibire may mark a subdomain of recycling endosomes, as reported for other endosomal proteins [19]. Moreover, the localization of Rab11 and of Shibire follows a similar developmental regulation. The proteins colocalize in subapical endosomes during slow phase but not in fast phase (Figures 5B–5E), when *shibire* is in fact no longer required for cellularization (Figure 2).

These results argue that *shibire* is also required for vesicular budding from Rab11 recycling endosomes during cellularization. In support of this conclusion, we find, by using transmission electron microscopy, very large amounts of intracellular subapical coated pits in *shibire* mutant embryos (Figure 6). We observe large subapical endocytic structures, all of which contain one or more dark uncleaved vesicle (Figure 6, black arrows). In wild-type embryos, however, we have not been able

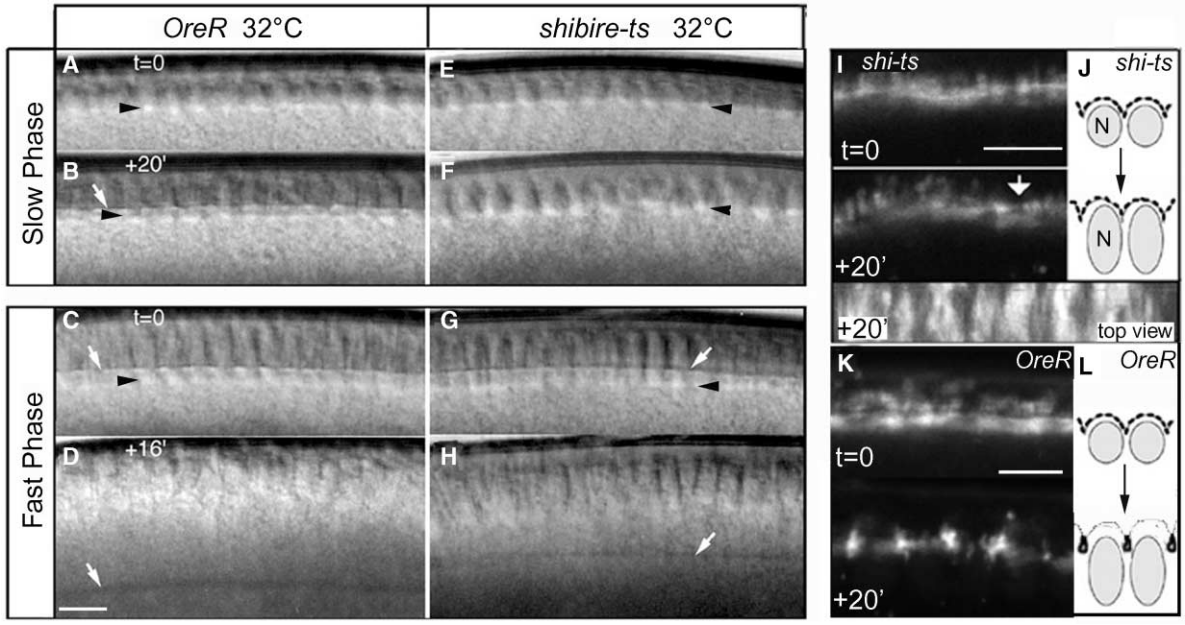

Figure 2. *shibire* Controls the Invagination of the Plasma Membrane and Apical Endocytosis

OreR (A–D) and *shibire-ts* (E–H) embryos at successive stages of cellularization at the restrictive temperature 32°C. The white arrows mark the front of the membrane, and the black arrowheads indicate the basal extent of the nuclei. Note the absence of membrane invagination during slow phase in *shibire-ts* embryos (E and F) compared to the wild-type embryos (A and B). However, cellularization progresses normally in control (C and D) and *shibire-ts* embryos ([G and H] albeit at a slightly slow speed) if the embryos are placed at 32°C during fast phase. (I–L) Membrane-labeling experiments with WGA at the onset of cellularization in a *shibire-ts* mutant embryo placed at 32°C (I and J) and in a wild-type embryo (K and L). At 20 min after labeling, the fluorescent WGA is still at the surface and not internalized in a *shibire* mutant (I, arrow). The irregular fluorescence of the plasma membrane is due to the presence of numerous villous projections at the surface. (J and L) Schematized representation of the experiment showing the nuclei (N) and the labeled surface (dotted line). the scale bar represents 5 μm.

to detect any such vesicular structure in the subapical cytoplasm (data not shown), suggesting that they may form very rapidly and transiently in the wild-type. Given the role of Dynamin/Shibire in severing vesicles, these observations support the role of *shibire* in vesicular traffic from subapical endosomes in addition to its role in plasma membrane endocytosis.

Rab11 Is Required for Membrane Invagination

We further tested the role of trafficking through recycling endosomes by using a dominant-negative form of Rab11, Rab11S25N, which is an effector of this process [17, 18]. Injection of GST+Rab11S25N during cycle 12–13 causes a striking inhibition of membrane invagination during slow phase (Figure 7 and Movies S2 and S3). Such an effect is not detected when either GST or GST+Rab11WT are injected in embryos at the same stages (Figure 7B).

In vertebrates, Rab11 interacts with members of the Arfophilin family of proteins [26]. The *Drosophila nuclear-fallout* (*nuf*) mutation [27, 28] encodes for a centrosomal protein that is a member of the Arfophilin family, suggesting that, in *Drosophila, rab11* and *nuf* may function in a similar functional pathway. In support of this proposal, *nuf* is required for the formation of membrane furrows prior to cellularization as well as during slow phase [27, 28]. In addition, the apical Rab11 endosomes are assembled at the centrosomes, as revealed by the centrosomal protein γ-Tubulin (Figure 4E). Interestingly, we found that, on top of the cellularization defects, Rab11S25N-injected embryos show a distinct "nuclear fallout" phenotype (Figures 7D, 7E, and 7F) reminiscent of *nuf* mutant embryos. Instead of being regularly aligned at the cortex, the nuclei are disorganized and occasionally "fall" inside the embryo.

We describe two classes of phenotypes, distinguished by their severity. In both cases, the phenotype is concentrated in the area of the embryo that was injected, at the injection site, and opposite to it. Class 1 (weak) represents 50% of the injected embryos ($n = 56$) and shows weak nuclear defects and membrane invagination defects (Figures 7C and 7E and Movie 2). Of the injected embryos, 37.5% fall in class 2 (strong) and have strong nuclear and membrane invagination defects in slow phase (Figures 7D and 7F and Movie 3). A typical example of the class 1 phenotype is shown at successive time points in Figure 7C.

These data further support the role of trafficking through Rab11 endosomes to ensure membrane growth during cellularization.

Discussion

The formation of an epithelium during cellularization is associated with the polarized growth of the plasma membrane. Probing the dynamics of the cell surface shows that the plasma membrane is remodeled in a stage-specific manner [8]. We here identify several ve-

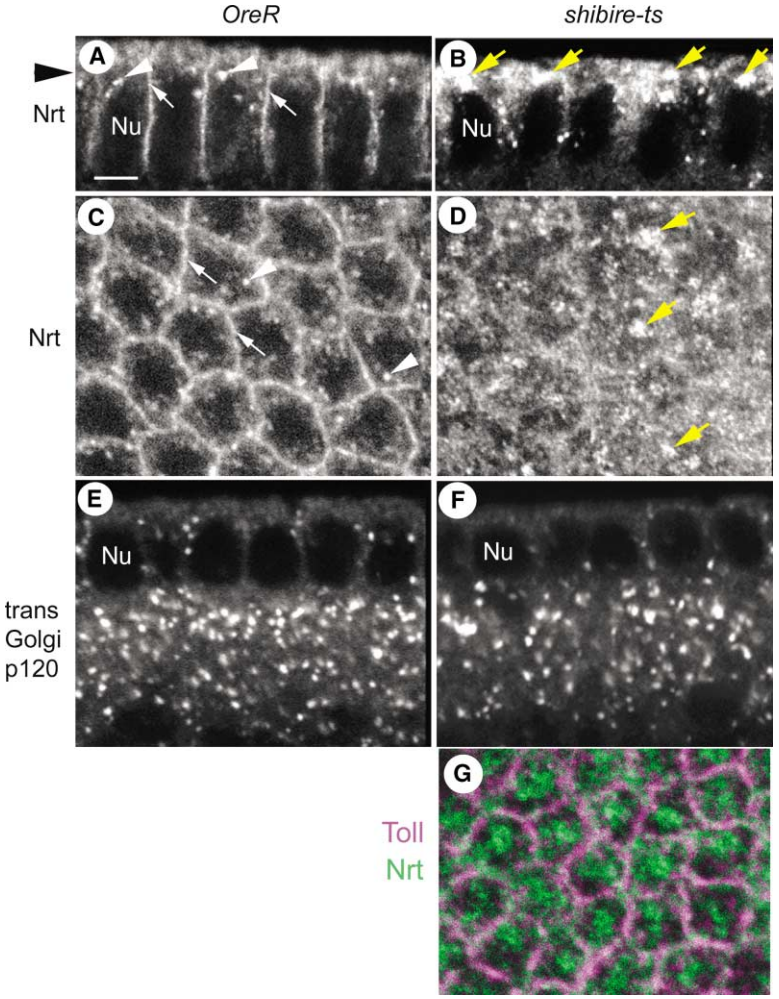

Figure 3. Plasma Membrane Insertion Defects in *shibire* Mutants

Confocal images of wild-type *OreR* embryos and *shibire-ts* embryos placed at 32°C at the onset of cellularization, fixed 20 min later, and stained with an antibody to Neurotactin (Nrt) ([A–D] in white and [G] in green), the Golgi p120 protein (E, F), or Toll (G, purple). Panels (C) and (D) are subapical confocal sections at a position indicated by the black arrowhead in (A) and (B).

(A and C) In control embryos, the zygotically induced transmembrane protein Nrt mostly accumulates at the plasma membrane (white arrows) and is also detected in small (<1 μm) vesicular structures (arrowheads) that colocalize with Golgi markers (see [8]).

(B and D) In contrast, in *shibire-ts* embryos, Nrt is mostly concentrated in large (around 3 μm) subapical aggregates (yellow arrows) just above the nuclei (Nu) and cannot be detected at the plasma membrane (compare C) and (D).

(E and F) In both wild-type and *shibire* embryos, the Golgi, marked with p120, has a distinct distribution, mostly concentrated in small (<2 μm) punctate structures in the basal cytoplasm. Although double Nrt/p120 antibody stainings could not be done because both antibodies are raised in the mouse, note that Nrt does not accumulate in the Golgi in *shibire* mutants. (G) Toll, a transmembrane protein already present at the plasma membrane before the shift at 32°C, remains at the cell surface (purple), unlike de novo synthesized Nrt (green). The scale bar represents 5 μm.

sicular-trafficking regulators that control distinct steps of endocytic trafficking and are required for the growth of the basal-lateral surface (Figure 8).

In the temperature-sensitive mutant *shibire/dynamin* we show that apical endocytosis is blocked and that lateral membrane growth is inhibited, suggesting that apical membrane material contributes in part to the basal-lateral surface. This is supported by the fact that the injection of a dominant-negative Rab5 protein also affects membrane invagination during slow phase. In addition, *shibire* is required for trafficking through Rab11 recycling endosomes during cellularization, as reported in mammalian cells [20]. First, in a *shibire* mutant, the transmembrane protein Nrt is not normally inserted in the plasma membrane and accumulates in part in subapical Rab11 recycling endosomes. Second, Shibire is enriched in Rab11 recycling endosomes. This compartment is organized in a subapical pericentriolar fashion and subsequently disperses in smaller vesicular structures at a stage when *shibire* is no longer required for cellularization. Finally, in *shibire* mutants, apical endocytic structures accumulate large amounts of coated pits, suggesting a defect in the vesicular budding from this compartment. Together, the data suggest that vesicle exocytosis from Rab11 endosomes is required for the growth of the lateral surface. Consistent with this model, we find that injection of dominant-negative Rab11 also leads to a strong reduction in the invagination of the plasma membrane.

The injection of dominant-negative Rab11 also affects the organization of nuclei at the cortex of the embryo in a manner that strongly resembles the maternal effect *nuclear-fallout* (*nuf*) mutation. Interestingly, *nuf* is also required for the formation of membrane furrows and for cellularization. In *nuf* mutant embryos, the membrane-associated Dah protein accumulates in vesicles in the cytoplasm instead of accumulating at the tip of the pseudo-cleavage furrows. Finally, both Nuf and Rab11 localize at centrosomes. These data suggest that the centrosomal protein Nuf contributes indirectly to the formation of membrane furrows via the perinuclear assembly of Rab11 endosomes at the centrosomes.

The identification of Rab11 endosomes as a key intermediate in the trafficking of vesicles necessary for lateral membrane growth raises two questions. Where does the membrane come from? Where is it eventually inserted? The facts that *shibire* and *rab5* are both required for basal-lateral membrane growth and that in a *shibire* mutant apically labeled membrane is not internalized suggests that the plasma membrane present in the vil-

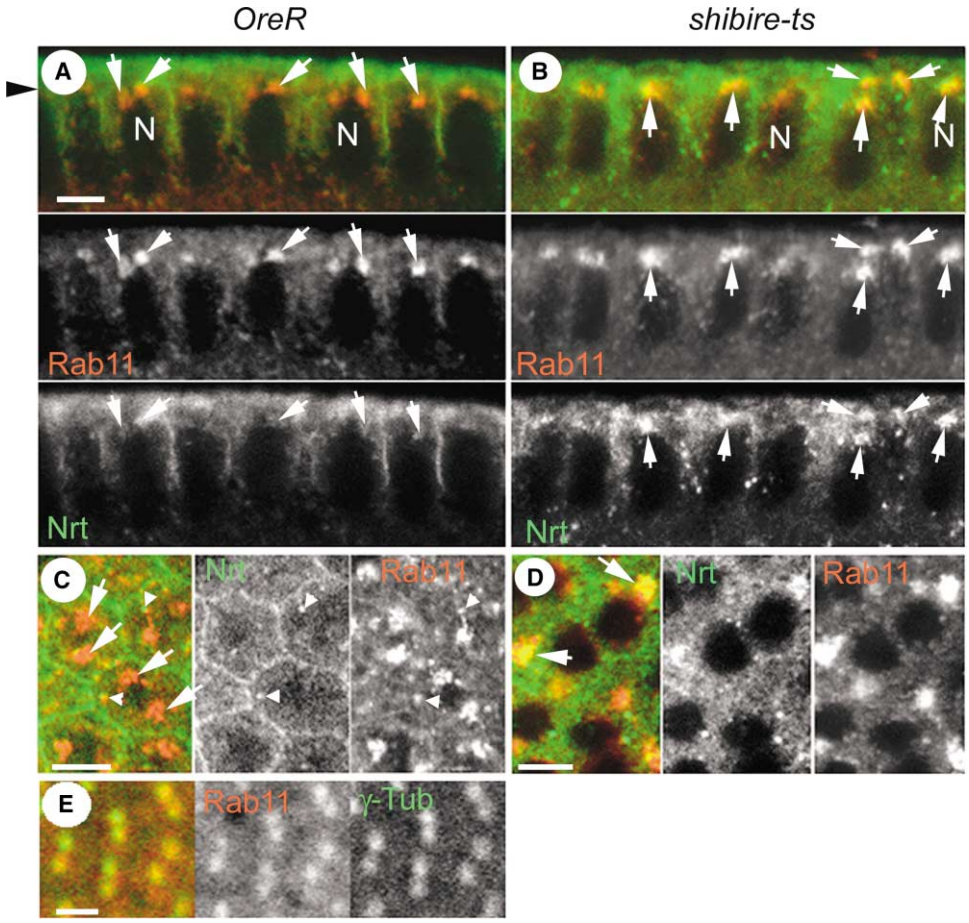

Figure 4. Trafficking Defects through Rab11 Endosomes in *shibire* Mutants

Confocal images of control *OreR* (A and C) and *shibire-ts* (B and D) mutant embryos fixed and stained 20 min after they were shifted to the restrictive temperature of 32°C at the onset of cellularization. Nrt is shown in green and Rab11 in red. Panels (C) and (D) are subapical sections at the position indicated by the black arrowhead in (A) and (B). (A and C) At steady state, Nrt is not visible in the large subapical Rab11 endosomes in the wild-type (red, arrows). Rab11 endosomes form large subapical perinuclear aggregates (arrows) as well as small vesicular structures in which Nrt is occasionally present (arrowheads). These small punctate structures colocalize with Golgi markers such as p120. (B and D) In contrast, in *shibire* mutants, Nrt is significantly present in the large apical Rab11 endosomes (arrows). (E) Rab11 is enriched in the centrosomes marked with γ-Tubulin. N denotes the nuclei. The scale bar represents 5 μm.

lous projections accounts in part for the growth of the lateral surface. The plasma membrane is indeed a huge reservoir whose capacity largely exceeds the membrane needed for lateral membrane growth during slow phase. Trafficking through Rab11 endosomes appears to also be essential for cellularization, as assayed by the injection of a dominant-negative form of Rab11 and as revealed by the role of *shibire* in vesicular trafficking through this compartment. In a *shibire* mutant, the transmembrane protein Nrt, which is de novo synthesized and traffics normally through the Golgi, accumulates in part in Rab11 endosomes. This suggests that secretory material might also contribute to the growth of the basal-lateral surface. This is supported by the fact that the injection of the drug Brefeldin A, an inhibitor of Endoplasmic Reticulum (ER) to Golgi transport, blocks cellularization and that the Golgi structural protein Lava-Lamp is required for cellularization [9]. Note that our failure to detect any invagination defect after injection of dominant-negative Rab1 could stem from the fact that Rab2 may compensate for ER-to-Golgi vesicular transport. Together, our data suggest that Rab11 endosomes constitute a point of integration of vesicles from the secretory and endocytic pathways necessary for the rapid exocytosis of vesicles required for the growth of the lateral membrane (Figure 8). Note that the ER was suggested to be a membrane reservoir required in part for the formation of pseudopods, another example of rapid membrane growth during phagocytosis [29]. In this process, an early step called "focal exocytosis" is controlled in part by Dynamin-2 [30].

After trafficking through Rab11 endosomes, where might the vesicles be finally inserted? The sites of membrane insertion are likely to dictate how polarity arises [1]. In light of the published data, the most likely proposal would state that vesicles are inserted at the basal junctions, a transient adherens junction that forms between the membrane front called "furrow canal" and the lateral surface [31]. The adherens junction is indirectly required for lateral membrane growth and basal-lateral targeting

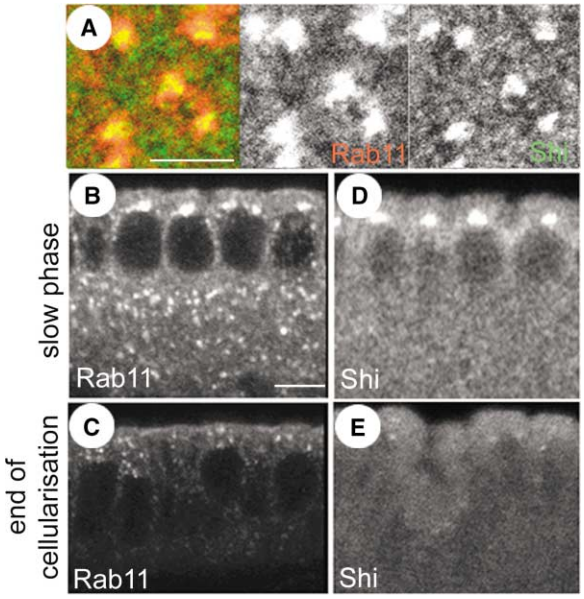

Figure 5. Shibire and Rab11 Colocalization

(A) Subapical confocal section of an embryo double stained with an antibody against Rab11 (red) and Shibire/Dynamin (green) during slow phase. Shi is enriched in Rab11 endosomes. Rab11 is present in subapical aggregates during slow phase (B) but not in fast phase (C). A similar developmental pattern is observed with Shi (compare [D] and [E]). The scale bar represents 5 μm.

in polarizing MDCK cells through the recruitment of Sec6 and Sec8, key components of the exocyst that localize in part at adherens junctions and control vesicle insertion, presumably from the Golgi apparatus [5, 6]. Consistent with a role of junctions in regulating vesicular insertion, when basal adherens junctions fail to form, as in *slam* mutant embryos, membrane invagination is blocked and Nrt is less efficiently inserted in the plasma membrane [23].

Why does cellularization involve such a pathway to ensure the growth and invagination of the plasma membrane? The comparison with other examples of cells in which the plasma membrane is rapidly remodeled provides insight into this question. Endocytic trafficking is usually much more efficient than secretory trafficking at remobilizing membrane pools. Antigen-presenting cells and migrating *Dictyostelium* ameobae [32] can, for instance, recycle their whole surface within a few minutes. In a situation where the membrane grows rapidly, remobilizing preexisting membrane pools at the plasma membrane and in the secretory pathway through recycling endosomes might be a very efficient way to transfer membrane "en masse" toward a defined site of the plasma membrane. Note that endocytic recycling is indeed involved in the formation of membrane protrusions [33, 34], ruffles, and lamellipodes [35–37] but also in other cases in which invaginations form, for example during cytokinesis (reviewed in [10]). *rab11* is required for late stages of cytokinesis in the *C. elegans* embryo [38]. Endobrevin/VAMP8, a regulator of endosome trafficking, is also required for cytokinesis in mammalian cells, together with Syntaxin 2 [39]. Trafficking through recycling endosomes may also provide an efficient way to couple surface polarization to membrane growth. Our findings are consistent with the similarities between cellularization and cytokinesis. Through the identification of a trafficking pathway involved in the growth of the lateral surface during cellularization, our work may also

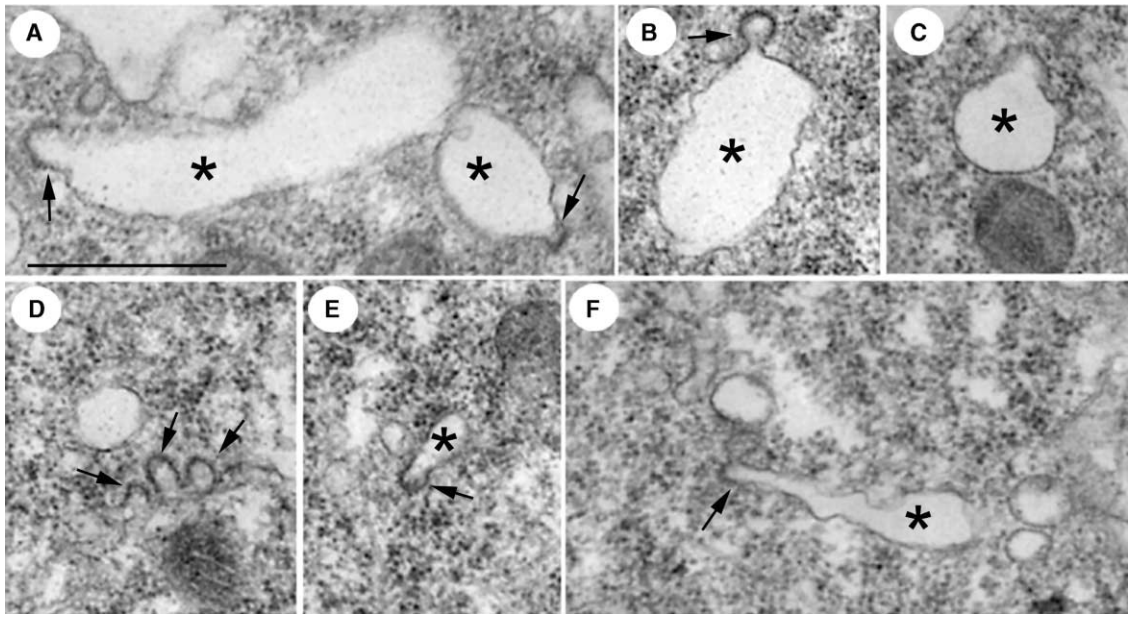

Figure 6. Accumulation of Intracellular Coated Pits on Endocytic Structures in *shibire* Mutants

Transmission electron microscographs of *shibire-ts* embryos after a 20 min shift at the restrictive temperature, 32°C, during slow phase. The subapical cytoplasm contains endocytic structures (asterisks) decorated with dark coated pits (arrows). The scale bar represents 500 nm.

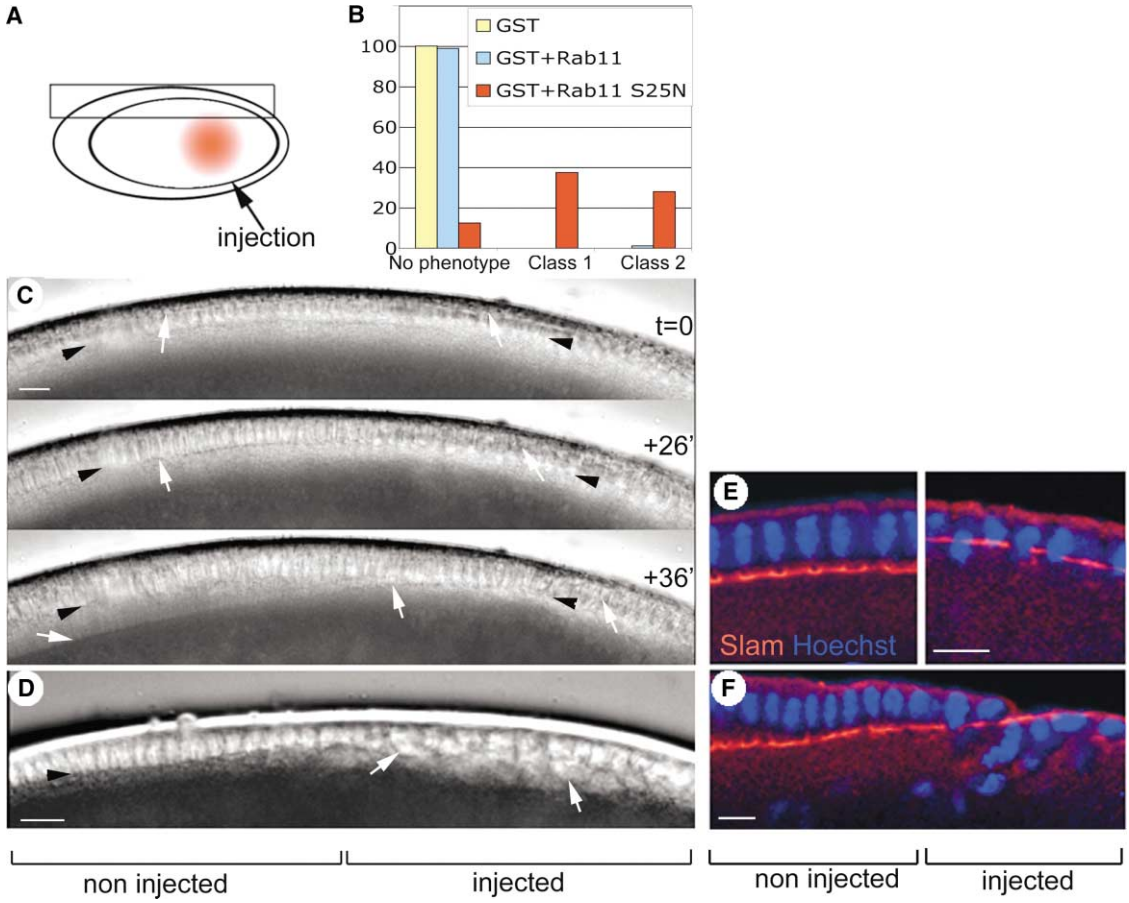

Figure 7. Role of Rab11 during Membrane Invagination

(A) Injection of GST, GST+Rab11WT, or GST+Rab11S25N in one half of living embryos during cycle 12–13, about 15 min prior to cellularization. The boxed area represents the portion of the embryo shown in (C) and (D).

(B) Results obtained for GST alone (yellow, n = 53), GST+Rab11WT (blue, n = 89), and GST+Rab11S25N (red, n = 56).

(C) Images of an embryo injected with GST+Rab11S25N with a cellularization phenotype visible at the end of slow phase (+26') and representative of the class 1. The defect in membrane invagination is visible by the relative positions of the membrane front (white arrow) and of the basal extent of the nuclei (black arrowhead). This delay persists during fast phase (+36'). The left part shows the control half, and the injected half is to the right.

(D) Nuclear defects in the injected half of an embryo shown during slow phase and representative of the class 2. Note the irregular arrangement of the nuclei in the injected area (right, white arrows) and compare this to their precise cortical alignment in the control area (left, black arrowhead). The position of the membrane is not yet visible in this image.

(E and F) Confocal images of a class 1 embryo (E) and a of class 2 embryo (F) stained with Hoechst to mark the nuclei and Slam to indicate the membrane front. The scale bar represents 10 μm.

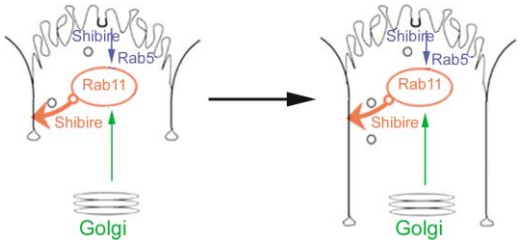

Figure 8. Model

Role of Rab11 subapical endosomes during lateral membrane growth. Rab11 is a central trafficking intermediate from the endocytic (blue) and secretory (green) pathways. Exocytosis from Rab11 endosomes (red) is required for vesicular insertion along the lateral surface and for membrane growth.

provide insight into the formation of an epithelial sheet during embryonic cleavage.

Experimental Procedures

Histology

Embryos are fixed with the heat/methanol protocol described in [8]. Mouse anti-Nrt antibody (BP106 clone, Hybridoma Bank) is used at 1/20; rabbit anti-Slam antibody (gift of R. Lehmann) is used at 1/1000; rat anti-Rab11 antibody (gift of R. Cohen) is used at 1/1000; rabbit anti-Toll antibody (gift of S. Wasserman) is used at 1/200; mouse anti-p120Golgi is used at 1/50 (Calbiochem, cat # 345865); and rabbit anti-Shibire is used at 1/200 (gift of M. Ramaswami). Membrane-labeling experiments with Alexa488 WGA (Molecular Probes) was performed on embryos as described in [8].

Imaging

Time lapse acquisition of living embryos during cellularization was performed on an inverted Zeiss Cell Observer system.

Transmission Electron Microscopy

shibire mutant embryos were incubated at 32°C for 20 min and immediately fixed with 25% glutaraldehyde and processed for TEM as described in [40].

Temperature Shift Experiments

shibire-ts or control embryos were prepared on a coverslip after a 30 min collection at 22°C. The embryos were aged 1 hr, and only synchronized embryos that entered cellularization were kept. These embryos were incubated at 32°C for 20 min and immediately fixed and stained.

Preperation and Injection of Recombinant Dominant-Negative Rabs

pGEX-2T-Rab1, pGEX-2T-Rab5, and pGEX-2T-Rab11 were cloned and mutagenized by PCR with QuickChange (Stratagene). All constructs were sequenced. The proteins were purified via standard protocols (information can be obtained upon request). Eluted GST+ Rabs were injected at a 200 μM concentration in embryos during cycle 12 or 13.

Supplemental Data

Supplemental movies are available with this article online at http://www.current-biology.com/cgi/content/full/13/21/1848/DC1.

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