A rapid, membrane-dependent pathway directs furrow formation through RalA in the early *Drosophila* embryo

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**ABSTRACT**

Plasma membrane furrow formation is crucial in cell division and cytokinesis. Furrow formation in early syncytial *Drosophila* embryos is exceptionally rapid, with furrows forming in as little as 3.75 min. Here, we use 4D imaging to identify furrow formation, stabilization, and regression periods, and identify a rapid, membrane-dependent pathway that is essential for plasma membrane furrow formation in vivo. Myosin II function is thought to provide the ingression force for cytokinetic furrows, but the role of membrane trafficking pathways in guiding furrow formation is less clear. We demonstrate that a membrane trafficking pathway centered on Ras-like protein A (RalA) is required for fast furrow ingression in the early fly embryo. RalA function is absolutely required for furrow formation and initiation. In the absence of RalA and furrow function, chromosomal segregation is aberrant and polyplloid nuclei are observed. RalA localizes to syncytial furrows, and mediates the movement of exocytic vesicles to the plasma membrane. Sec5, which is an exocyst complex subunit and localizes to ingressing furrows in wild-type embryos, becomes punctate and loses its cortical association in the absence of RalA function. Rab8 also fails to traffic to the plasma membrane and accumulates aberrantly in the cytoplasm in *RalA* disrupted embryos. RalA localization precedes F-actin recruitment to the furrow tip, suggesting that membrane trafficking might function upstream of cytoskeletal remodeling. These studies identify a pathway, which stretches from Rab8 to RalA and the exocyst complex, that mediates rapid furrow formation in early *Drosophila* embryos.

**KEY WORDS:** Syncytial divisions, RalA, Rab8, Exocytosis

**INTRODUCTION**

The ability to form a plasma membrane furrow is essential to most cellular and tissue-level developmental processes. Plasma membrane furrow formation is required for cytokinesis and the generation of multicellular tissues. In the *Drosophila* embryo, the first nine rounds of nuclear mitoses occur deep within the syncytial yolk. However, at cycle 10, nuclei migrate out to the embryonic periphery and sequential transient rounds of plasma membrane furrow formation occur in rapid succession during mitotic cycles 10-13. These furrow processes then culminate in a final, permanent furrowing event that encapsulates individual nuclei in a contiguous plasma membrane forming the embryonic epithelium at cycle 14 (reviewed by Schejter and Wieschaus, 1993; Sullivan and Theurkauf, 1995). The early syncytial fly embryo is therefore a furrow-making machine, rapidly making and disassembling thousands of interconnected furrows in the time-scale of a few minutes. Understanding how cells are able to coordinate changes in cytoskeletal and membrane trafficking networks to produce these dynamic ingestions of the plasma membrane should inform our understanding of the general processes that are available to cells to drive furrow formation in animal cells.

Ras-like protein A (RalA) is a small GTPase that was originally identified as a key downstream target of the Ras oncoprotein (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994; White et al., 1996). Subsequent studies have demonstrated that RalA can function through the exocyst complex to control directed membrane addition (Moskalenko et al., 2002). The exocyst complex is an octameric protein complex that directs the targeting and tethering of vesicles to the plasma membrane. The Sec5 and Exo84 exocyst subunits directly bind to active GTP-bound RalA, and this interaction drives exocyst complex assembly and function (Moskalenko et al., 2002, 2003; Fukai et al., 2003). In multicellular organisms, exocyst components are required for many cellular processes involving directed membrane trafficking, including epithelial polarity (Grindstaff et al., 1998; Yeaman et al., 2001; Langevin et al., 2005; Blankenship et al., 2007), photoreceptor morphogenesis (Beronja et al., 2005), synapse formation (Mehta et al., 2005) and cell abscission (Fielding et al., 2005; Gromley et al., 2005). The exocyst complex has additionally been found to bind to a different class of small GTPases, the Rab proteins, which are key mediators of membrane trafficking pathways. During lumen formation and ciliogenesis in mammalian cells, Rab8 binds to the Sec5 subunit of the exocyst complex (Bryant et al., 2010; Knödler et al., 2010; Feng et al., 2012), and the Rab11 recycling endosome protein can directly associate with the exocyst subunits Sec5 and Sec15 (Zhang et al., 2004; Beronja et al., 2005; Jafar-Nejad et al., 2005; Langevin et al., 2005; Wu et al., 2005).

The formation of a plasma membrane cleavage furrow is an obligate step in successful cytokinesis. It is well established that both an actomyosin contractile ring as well as membrane trafficking are required for animal cell cytokinesis (reviewed by Pollard, 2010; Neto et al., 2011; Schiel and Prekeris, 2013). However, the relative contributions of these two pathways in directing the progression of the cytokinetic furrow are unclear. One advantage of studying the syncytial furrows in early *Drosophila* embryos is that furrow formation occurs *in vivo*, at a predictable time, and their occurrence at the surface of the embryo facilitates imaging with modern confocal microscopy approaches. Additionally, working in *Drosophila* permits the use of defined genetic alleles, whereas studies on tissue culture cells rely on partial disruption through RNAi. Finally, syncytial cell cycles and furrow formation are exceptionally rapid, enabling the visualization of several rounds of furrow formation in a short time period (Zalokar and Erk, 1976; Foe and Alberts, 1983; Foe et al., 2000).

Here, we use 4D time-lapse analysis to characterize some of the very first morphological events that occur at the embryonic plasma membrane. Our results define the temporal and spatial dynamics of...
furrow formation during mitotic cycles 10-13, and show that furrows possess three distinct phases encompassing ingression, stabilization, and disassembly. We additionally demonstrate a requirement for RalA protein function in the formation of plasma membrane furrows. In the absence of RalA function, furrow formation does not initiate, Rab8 fails to traffic to the cell surface, and the exocyst complex subunit Sec5 loses its cortical localization. These results are consistent with a fundamental requirement for directed membrane addition in the initiation and ingestion of plasma membrane furrows.

RESULTS
Rapid furrow formation and regression in the early syncytial embryo
The early Drosophila embryo undergoes 13 rounds of division in the absence of cytokinesis before the process of cellularization packages individual nuclei into an epithelial array. These rounds of division are rapid, with the 13 cycles occurring in a 2-h time span at 25°C. The last four divisions occur after nuclei have migrated out to the embryonic periphery. With the nuclei arranged into a common cortical plane, cytokinetic-like plasma membrane furrows rapidly ingress to separate the nuclei, prevent chromosomal missegregation, and provide attachment points for spindle assembly and positioning (Foe and Alberts, 1983; Sullivan et al., 1993). We have used high-resolution temporal and spatial imaging to examine the timing and dynamics of furrow formation and regression during these divisions (Fig. 1; supplementary material Movie 1).

We first characterized the furrows that form during cycles 10-13 by analyzing embryos expressing a plasma membrane marker, Gap43:mCherry (Gap43:mCh). We observed that furrow dynamics can be separated into three distinct phases: (1) furrow formation; (2) furrow stabilization; and (3) furrow regression. The events of furrow formation, stabilization and regression occur continuously over the total cycle time. As successive cycles proceed, the nuclei crowd closer together, and furrows reach deeper into the embryo (Fig. 1I). Furrow ingression occurs on average in 3.8, 5.7, 6.1 and 11.3 min during cycles 10, 11, 12 and 13, respectively (Table 1). Furrows are more irregular in the earliest cortical cycle (cycle 10) but become

![Fig. 1. Spatial and temporal dynamics of syncytial furrow formation.](image)

(A-D) Still-frame views at a single z-plane of a wild-type (WT) Drosophila embryo expressing the membrane marker Gap43:mCh at t=0, 3, 7.5 and 15 min during cycle 12. (A) At the onset of cycle 12, Gap43 remnant furrows from cycle 11 persist near the apical surface. (B,C) As the cycle proceeds, the broader furrow tip ingresses into the viewing plane (B), and then moves deeper into the embryo as thinner, more medial portions of the furrow come into the viewing plane (C). (D) The furrow retracts apically and is lost from the viewing plane. (E-H) Schematic depicting the corresponding apical-basal views for orientation. (I) Quantification of mean furrow lengths for syncytial cycles 10-13 (∑n=10). Mean furrow lengths are 2.3 µm, 4.2 µm, 6.1 µm and 8.2 µm at cycle 10, 11, 12 and 13, respectively. (J) Quantification of calculated mean ingression speeds and retraction speeds for cycles 10-13 (∑n=10) (see Table 1). Error bars indicate s.e. Scale bar: 10 µm.
successively more regular and hexagonally packed with each proceeding cycle. After furrow ingression has occurred, furrows are relatively stable in depth for a period of 2-3 min (Table 1). After this short stable period, the syncytial furrows begin to deform as mitosis proceeds, and furrow regression rapidly occurs with different temporal dynamics than furrow formation. Furrow regression occurs on average in 2.5, 3.3, 3.9 and 4.8 min during cycles 10, 11, 12 and 13, respectively (Table 1). Furrow regression is often not complete, with small regions of remnant furrows (0.5-1.5 µm in depth) apparent as a new cycle of furrow formation initiates (Fig. 1A). Interestingly, ingression speeds for each cycle remain fairly consistent (Fig. 1J), at ~0.7 µm/min. However, furrow retraction rates progressively increase with development (Fig. 1J), suggesting that ingression and regression are controlled by different mechanisms.

As furrow formation is one of the first morphological events that occurs at the plasma membrane in the *Drosophila* embryo, and as the embryo makes hundreds of furrows in a matter of a few minutes,
we chose to focus further on the mechanisms that drive rapid furrow formation in the early embryo.

**RalA localizes to the plasma membrane and furrows during syncytial cell cycles**

The RalA small GTPase is an essential regulator of targeted membrane addition and directs vesicular tethering activity at the plasma membrane (Moskalenko et al., 2002, 2003; Fukai et al., 2003). RalA is therefore a good candidate protein to probe the role of directed membrane addition during furrow formation in the early embryo. To examine whether RalA is involved in furrow formation, we first imaged RalA localization in vivo. During cycles 1-9 of the syncytial nuclear divisions, UAS-mCherry:RalA (mCh:RalA) is localized in puncta basal to the plasma membrane (supplementary material Fig. S1A-C). At the onset of cycle 10, these puncta disappear, and RalA localizes to the plasma membrane (supplementary material Fig. S1B,C). Throughout cycles 10-13, RalA localizes to the plasma membrane as furrows ingress around the nuclei (Fig. 2A-H). To ensure that the localization of the mCh:RalA construct represented that of endogenous RalA populations, we fixed wild-type (WT) embryos and stained with a RalA antibody (Teodoro et al., 2013). Planar and apical-basal images of fixed embryos show that endogenous RalA is present during syncytial stages and localizes in a manner similar to mCh:RalA (Fig. 2I-J‴).

We also examined mCh:RalA; Histone:GFP-expressing embryos in order to study the relationship between RalA localization and the cell cycle as indicated by chromosomal morphologies (Fig. 2K-O; supplementary material Movie 2). RalA-marked furrows are apparent from the earliest stages of furrow formation (Fig. 2B), but are not present at the level of the nuclei until prophase (Fig. 2K,L). At the end of prophase, furrow ingression halts and the furrows enter a stabilization phase that persists through prometaphase and metaphase (Fig. 2M). During the initiation of anaphase, the extended furrow begins to regress, and regression continues through the end of telophase (Fig. 2N,O). Furrow behaviors appear to occur continuously at these stages, as new furrows form immediately after regression of the furrows of the previous cycle (supplementary material Movies 1 and 2).

**Defective early development and furrow formation in RalA mutant embryos**

Given the localization of RalA to furrows and the plasma membrane, we suspected that it might be required for furrow formation in the early Drosophila embryo. To address this, we examined the effect of disrupting RalA (Ralα – FlyBase) function during early development. Embryos maternally deficient for RalA function (RalAPL56 germline clone embryos; Ghiglione et al., 2008) were scored under oil for gross tissue morphologies to determine if RalA is required for early morphogenesis. RalAPL56 embryos were severely disrupted in development: 86.5% of embryos showed defects during the syncytial nuclear divisions, 7.7% first displayed defects during cellularization, and 5.8% became defective during germband extension (Fig. 3A). These results suggest an essential maternal requirement for RalA function in the early embryo.

We further performed RNAi analysis of RalA function in the embryo. OreR (WT) embryos injected with RalA-specific dsRNA also demonstrated early morphological defects. In the early embryo, dsRNA injection often results in a partial knockdown of RalA, destabilizing maternally and zygotically expressed mRNAs while maternal protein stores may remain unaffected. RalA dsRNA-injected embryos were unable to complete germband extension, with 11.5% of embryos showing defects during germband extension, 50% first demonstrating defects during cellularization, and 38.5% becoming defective during the syncytial nuclear divisions (Fig. 3B). These results confirm the RalAPL56 mutant analysis, and suggest the possibility that all furrow-forming events (i.e. those that generate the transient syncytial and permanent cellularization furrows) require RalA function.

To examine the effects of disrupting RalA function on furrow formation, we examined GFP:MoeABD (an F-actin marker) in WT and RalA mutant backgrounds. In WT embryos, GFP:MoeABD localizes to furrows (Fig. 4A-D), illustrating the canonical model that the F-actin cytoskeleton supports furrow formation (Foe et al., 2000; Albertson et al., 2008; Cao et al., 2008). We then examined GFP:MoeABD in RalAPL56 germline clone embryos to address whether furrows and their associated F-actin networks could form in the absence of RalA function. RalAPL56 embryos were unable to produce regular F-actin-coated furrow canals, with only the borders of the apically associated F-actin nuclear caps remaining (Fig. 4E-H). We then examined F-actin behaviors in fixed embryos in which the mitotic stage could be determined. In WT embryos, F-actin-marked furrows are present in embryos undergoing DNA condensation and chromosome alignment (Fig. 4I,K). In RalAPL56 embryos, F-actin is present in puncta basal to the plasma membrane during prophase (Fig. 4J,L), but is never found on furrows. This suggests either that RalA may direct F-actin formation at the furrow, or that
Disruption of syncytial nuclear divisions in RalA mutant embryos

If furrow formation is defective during the syncytial divisions, then RalA mutant embryos would be expected to demonstrate defects in the ability to properly segregate chromosomes (Zalokar and Erk, 1976; Foe and Alberts, 1983; Sullivan et al., 1993). To better understand the mechanisms by which furrows support chromosomal segregation, we imaged the chromosomal marker Histone:RFP in RalAPL56 embryos. We observed that widespread nuclear fusions occur during cycles 10-13, affecting nearly 90% of nuclei. Early syncytial cycles, in which nuclei are spaced further apart, are not as dependent on RalA function, with ~12% of mitotic divisions failing and producing polyploid nuclei (Table 2). However, by cycle 13, nearly all nuclei have suffered a chromosomal missegregation event (Table 2). We further observed that there are two major types of nuclear fusion event in RalA mutant embryos. The first type we classified as ‘mitotic collapse’ nuclear fusion events, which are a result of the failure of sister chromosomes to remain separated at the end of anaphase (Fig. 5E-E‴; supplementary material Movie 3). The second type of nuclear fusion event observed was a fusion of chromosomes originating from adjacent dividing nuclei, which we classified as ‘adjacent nuclear fusion’ (Fig. 5F-F‴; supplementary material Movie 4). Both types of fusion resulted in 4n nuclei; however, adjacent nuclear fusions can drive larger, highly polyploid nuclei if more than two nuclei fuse.

Different syncytial cycles in RalA mutant embryos were characterized by different ratios of mitotic collapse nuclear fusions and adjacent nuclear fusions. In WT embryos, nuclear fusion events are rare throughout the syncytial divisions (1.2% of nuclear divisions, supplementary material Table S1). During cycle 10, RalAPL56; Histone:RFP germline clone embryos display mitotic collapse-driven fusions in 11.7% of mitoses (Table 2). No adjacent nuclear fusions are observed during cycle 10 (Table 2). Cycle 11 also shows predominately mitotic collapse nuclear fusions in RalA mutant embryos (Fig. 5B, Table 2), whereas cycle 12 demonstrates a mix of mitotic collapse and adjacent nuclear fusions (Table 2), and cycle 13 has mostly adjacent nuclear fusions (Fig. 5D, Table 2).

Since RalAPL56 embryos display increasingly defective phenotypes as nuclei become more numerous and more crowded, we hypothesized that RalA might function by regulating the formation of furrows, which in turn provide potential anchor points for the spindle machinery and maintain the physical separation of chromosomes during mitosis.

RalA directs membrane addition required during furrow formation and ingression

As our data suggest that RalA function may drive a furrow ingression pathway, and published data demonstrate that RalA can physically bind to members of the exocyst complex and direct exocyst assembly (Moskalenko et al., 2002, 2003; Fukai et al.,
Table 1. Quantification of syncytial furrow periods and speeds

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Ingression time</th>
<th>Stabilization time</th>
<th>Retraction time</th>
<th>Ingression speed</th>
<th>Retraction speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.75±0.31</td>
<td>2.06±0.09</td>
<td>2.53±0.15</td>
<td>0.66±0.02</td>
<td>0.68±0.05</td>
</tr>
<tr>
<td>11</td>
<td>5.67±0.89</td>
<td>2.58±0.18</td>
<td>3.25±0.41</td>
<td>0.70±0.02</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>12</td>
<td>6.05±0.67</td>
<td>2.90±0.29</td>
<td>3.93±1.53</td>
<td>0.82±0.01</td>
<td>0.96±0.10</td>
</tr>
<tr>
<td>13</td>
<td>11.25±4.91</td>
<td>3.05±0.62</td>
<td>4.80±1.06</td>
<td>0.70±0.01</td>
<td>1.14±0.06</td>
</tr>
</tbody>
</table>

Mean (±s.e.) furrow ingression, stabilization and retraction times (min) and ingression and retraction speeds (μm/min) during cycles 10-13 of syncytial furrow behaviors, as determined from Gap43:mCherry-expressing embryos (n=10).

We hypothesized that RalA might guide a membrane delivery mechanism required to form furrows. To test this, we examined the in vivo delivery of a cell surface-associated protein (Gap43:mCh) that is trafficked to the plasma membrane (Martin et al., 2010). In WT embryos, Gap43:mCh is present in punctate, cytoplasmic populations prior to cycle 10. During cycles 10 and 11 these large, punctate pools of Gap43:mCh disappear as the protein begins to localize to the plasma membrane (supplementary material Fig. S1D-F). In a RalAPL56 mutant background, Gap43:mCh is present in vesicular pools prior to cycle 10, similar to the Gap43:mCh population in WT embryos (data not shown). However, with each syncytial cycle these Gap43:mCh pools remain in their punctate localization and delivery to the plasma membrane is severely disrupted (Fig. 6D-F). Additionally, no evidence of furrow formation, as marked by Gap43:mCh, is observed (Fig. 6D-F). This strongly suggests that RalA is required for exocytic trafficking to the plasma membrane during these early cycles, and that RalA-dependent trafficking is necessary for the earliest events of furrow formation.

To examine a potential molecular mechanism of RalA function, we determined the localization of a component of the exocyst complex, Sec5, which has been shown to physically interact with RalA in MDCK cells (Moskalenko et al., 2002, 2003). Anti-Sec5 staining in WT embryos revealed that the localization of Sec5 was strikingly similar to that of RalA during cycles 10-13. In RalA mutant embryos, Sec5 localization to the cortex is lost, and it accumulates within the cytoplasm (Fig. 6H-J). Prior to cycle 10 and the onset of furrow formation, Sec5 does not localize to RalA puncta in WT embryos, suggesting that these puncta might represent inactive RalA protein populations (supplementary material Fig. S1G). These results are consistent with a model in which active RalA directs the localization of exocytic machinery to the incipient furrows for the delivery of cytoplasmic membrane stores.

**RalA directs membrane addition through the Rab8 GTPase**

Recent work in our laboratory suggests that Rab8 is a key mediator of membrane addition during furrow formation in the early *Drosophila* embryo (L.M.M., Z.Z., J.T.B., unpublished). To determine Rab8 localization relative to RalA, embryos co-expressing mCh:RalA and YFP:Rab8 were examined. Rab8 compartments are present in the cytoplasm as furrow formation initiates (Fig. 7B). As furrow ingression proceeds, cytoplasmic Rab8 compartments become depleted and low levels of Rab8 accumulate at the furrow in a cortical array (Fig. 7C). At these stages, mCh:RalA is also present in a cortical localization at the furrow and demonstrates colocalization with YFP:Rab8 (Fig. 7C).

As RalA and Rab8 colocalize at the furrow cortex, and both RalA and Rab8 have been shown to be essential for furrow formation (Fig. 4I-L; L.M.M., Z.Z., J.T.B., unpublished), we tested their functional interdependence. To determine if Rab8 localization is dependent on RalA function, *RalA* dsRNA was injected into a YFP:Rab8 background. Indeed, RalA knockdown resulted in the mislocalization of YFP:Rab8 rather than cycling from a punctate to cortical location, Rab8 remained in cytoplasmic puncta (Fig. 7F-F″) and these puncta also appeared larger in size. These results are suggestive of a failure of Rab8 trafficking to the cell surface and might explain the underlying failure of furrow formation in RalA mutant embryos. YFP:Rab8 does not localize to the apical surface in water-injected or *RalA* dsRNA-injected embryos (Fig. 7G,H).

Next, we performed the converse experiment of injecting *Rab8* dsRNA into a mCh:RalA background. RalA localization at the apical surface of the plasma membrane was unaffected (Fig. 7K-K″). However, furrows were unable to extend in embryos with compromised Rab8 function (Fig. 7L-L″). Rab8 puncta, which normally clear from the cytoplasm by the end of cycle 10, were still present at cycle 12 (Fig. 7L-L″). As Rab8 localization is severely affected by disrupting RalA function, and RalA still localizes appropriately to the cell cortex, it is likely that RalA functions upstream of Rab8 in a membrane delivery pathway. RalA, in turn, might require a Rab8-dependent exocytic trafficking pathway to reinforce cortical RalA populations.

Finally, we examined the effect of disrupting Sec5 function on RalA localization (Fig. 7M,N). Similar to Rab8 RNAi, apical RalA localization is unaffected in Sec5 dsRNA-injected embryos, while furrow formation is disrupted, suggesting that RalA might function upstream of exocyst complex assembly and Sec5 function.

**RalA is enriched at the furrow tip relative to F-actin during furrow formation**

As furrows fail to form from their earliest stages in *RalA* mutant embryos, we further examined the relationship between the F-actin cytoskeleton and the RalA/Sec5/Rab8 membrane trafficking pathway. Embryos co-expressing mCh:RalA and GFP:MoeABD were imaged at a single focal plane basal to a forming furrow. We then asked whether either protein appears first in the focal plane on

Table 2. Chromosomal segregation defects in RalA mutant embryos

<table>
<thead>
<tr>
<th>Cycle</th>
<th>WT divisions completed</th>
<th>Mitotic collapse nuclear fusions</th>
<th>Adjacent nuclear fusions</th>
<th>WT divisions started</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>91 (88.3%)</td>
<td>12 (11.7%)</td>
<td>0 (0%)</td>
<td>103</td>
</tr>
<tr>
<td>11</td>
<td>135 (88.8%)</td>
<td>15 (9.8%)</td>
<td>1 (0.7%)</td>
<td>152</td>
</tr>
<tr>
<td>12</td>
<td>114 (64.4%)</td>
<td>36 (20.3%)</td>
<td>14 (7.9%)</td>
<td>177</td>
</tr>
<tr>
<td>13</td>
<td>18 (12.7%)</td>
<td>33 (23.4%)</td>
<td>65 (46.1%)</td>
<td>141</td>
</tr>
</tbody>
</table>

Quantification (number and percentage) of mitotic nuclear fusions and adjacent nuclear fusions observed in RalAPL56 embryos. As mitotic cycles progress, the number of adjacent nuclear fusions increases exponentially (n=9).
the ingressing furrow. We observed that RalA is strongly detected at the furrow from the earliest moments that the ingressing furrow can be imaged (Fig. 8B). RalA is present in the broader furrow tip at levels comparable to the rest of the furrow (Fig. 8B,C). F-actin, however, appears to have an asymmetric enrichment on syncytial furrows: it is present at low levels at the furrow tip, but then is more strongly enriched on the more apical portions of the furrow (Fig. 8B,C). These observations, in combination with the finding that F-actin is localized in puncta in the absence of RalA function (Fig. 4J,L and Fig. 6H,J), suggest that membrane trafficking, as indicated by RalA, might precede F-actin cytoskeletal remodeling during furrow formation.

We also examined furrow retraction, and asked whether RalA or F-actin disassembly occurs first. Here, again, we observed a differential behavior of RalA and F-actin. During retraction, F-actin depolymerization occurs first and the furrow loses the F-actin cortical assembly (Fig. 8E). Interestingly, this F-actin disassembly occurs during anaphase, and further deformation of the regularity of the furrow correlates with this period (Fig. 8E).

The above results are consistent with a model whereby RalA-dependent membrane trafficking is the core machinery responsible for furrow formation, with F-actin coating of the furrow acting as a structural reinforcement.

**DISCUSSION**

We have demonstrated the furrow dynamics of one of the earliest membrane-shaping morphological events in the *Drosophila* embryo. We identified formation, stabilization and retraction phases in the plasma membrane furrows of the early syncytial embryo, and measured furrow lengths and ingestion and retraction rates. We show that furrow formation can be exceptionally rapid, occurring in as little as 3.75 min. Interestingly, the rate of furrow formation appears relatively consistent regardless of the cycle or the number of furrows that are forming in the embryo, in contrast to...
syncytial furrow retraction rates. The rate of furrow formation is similar to those reported for furrow ingression during the fast, but not slow, phase of cellularization (0.8 µm/min; Lecuit et al., 2002). The fast phase of cellularization is a process that, like syncytial furrow formation, is heavily dependent on targeted membrane addition (Lecuit and Wieschaus, 2000; Pelissier et al., 2003; Murthy et al., 2010; L.M.M., Z.Z., J.T.B., unpublished). Furrow retraction rates, by contrast, are not consistent between different mitotic cycles, and retraction speeds increase with syncytial mitotic cycles. This suggests that a simple reversal of the trafficking pathways might not drive furrow retractions. It is also worth noting that small regions of the furrows often never fully retract at the end of a cycle, and short ridges (0.5-1.5 µm) are present as a new cycle of furrow formation begins. This demonstrates that furrow formation and regression are continuously occurring throughout mitotic cycles 10-13, which would be consistent with a model in which cell cycle length drives furrow depth.

We have also shown that RalA function is essential from the earliest stages of furrow initiation. In the absence of RalA function, furrow failures lead to two distinct classes of failed nuclear divisions. These classes suggest that furrows are required for both spindle anchoring (the failure of which leads to the mitotic collapse phenotype) and for the separation of adjacent genomes (the failure of which leads to the fusion of adjacent nuclei). In the earliest

Fig. 6. RalA is required for membrane addition and syncytial furrow formation. (A–C) Control embryo expressing the plasma membrane marker Gap43:mCh at t=0, 5 and 13 min of cycle 12. (D–F) RalAPL56 embryo expressing Gap43:mCh at t=0, 4 and 10.5 min during cycle 12. Cytoplasmic membrane pools are unable to traffic to the plasma membrane to allow for furrow ingression. (G–G‴) Fixed WT embryo stained with anti-Sec5 (green) and for F-actin (red, phalloidin) and DNA (blue, Hoechst). Sec5 localizes to furrows during cycle 12 furrow ingression. (H–H‴) Fixed RalAPL56 embryos during mitotic cycle 12. Sec5 becomes delocalized and is distributed broadly within the cytoplasm. (I–I‴) Apical-basal views of WT fixed embryos stained for Sec5, F-actin and DNA. Sec5 localizes to the cortex and at furrows. (J–J‴) A RalAPL56 embryo demonstrates loss of Sec5 localization to the cortex and an absence of syncytial furrows. Scale bars: 10 µm.
cycles of furrow formation (cycles 10, 11), furrow function is most important for proper spindle function and the successful completion of individual mitoses. However, as the cycles progress and nuclei become more numerous (cycles 12, 13), furrows become increasingly important to each division through their maintenance of a physical barrier between chromosomes. In the absence of these furrows, adjacent mitotic figures are not properly fenced in, and mitotic fusions occur.

We further demonstrate the existence of a membrane trafficking pathway that directs rapid furrow formation. This pathway involves the combined function of RalA, Sec5 and Rab8. Sec5 and Rab8 become aberrantly localized in RalA mutant embryos, suggesting that RalA functions through the exocyst complex and Rab8 to direct furrow formation. Indeed, active GTP-bound RalA has been shown to directly bind to the Sec5 and Exo84 exocyst subunits, and this interaction drives exocyst complex assembly and function (Moskalenko et al., 2002, 2003; Fukai et al., 2003). Interestingly, Sec5 does not colocalize with RalA prior to cycle 10, suggesting that RalA is inactive prior to furrow formation. Activation of RalA might therefore initiate furrow formation through the recruitment of a vesicular targeting and tethering complex, i.e. the exocyst, to the plasma membrane (Fig. 9A). The exocyst, in turn, may bind to and recruit exocytic vesicles through direct subunit interactions with Rab8 (Fig. 9A) (Bryant et al., 2010; Knödler et al., 2010; Feng et al., 2012). It is interesting to note that both Rab8 and RalA are present as cytoplasmic puncta prior to cycle 10, and that RalA partially

Fig. 7. RalA directs membrane growth through Rab8. (A-D″) Planar views of embryo expressing mCh:RalA and YFP:Rab8 at t=0, 3, 11 and 18.5 min during cycle 13. Rab8 is present in cytoplasmic compartments, but also colocalizes cortically with RalA on the ingressing furrow (C). (E-E″) Transverse section through furrows of water-injected YFP-Rab8-expressing embryo at t=0, 3, 6 and 15 min during cycle 13. Rab8 displays the normal transition from compartmental to cortical localization at the furrow. (F-F″) RalA dsRNA-injected embryos expressing YFP:Rab8 at t=0, 3, 6 and 15 min during cycle 13; view is 1.5 μm below the apical surface. Rab8 fails to transition into a cortical array, and cytoplasmic puncta increase and grow with time. (G-H″) YFP:Rab8 is not present at the apical surface of the embryo in water-injected (G-G″) nor RalA dsRNA-injected (H-H″) embryos. (I-J″) Water-injected mCh:RalA-expressing embryos at t=0, 2.5, 7.5 and 11.5 min during cycle 11, shown at the apical surface (I-I″) and 1 μm basal to the apical surface (J-J″). (K-L″) Rab8 dsRNA-injected embryos expressing mCh:RalA at t=0, 2.5, 7.5 and 11.5 min during cycle 11, shown at the apical surface (K-K″) and 1 μm basal to the apical surface (L-L″). RalA localization is not disrupted at the apical surface; however, furrows are unable to ingress and RalA puncta remain basal to the plasma membrane. (M-N″) Sec5 dsRNA-injected embryos at t=0, 2.5, 7.5 and 11.5 min during cycle 11. Sec5 compromised embryos localize RalA at the apical surface (M-M″), but lack furrow-associated RalA and furrow formation fails as observed in a basal plane 1 μm down (N-N″). Scale bars: 10 μm.
remains in a punctate distribution when Rab8 function is knocked down. Previous work has illustrated a reinforcing loop, in which exocyst complex subunits traffic to the cell surface on vesicular intermediates (Yeaman et al., 2001, 2004; Langevin et al., 2005). It is interesting to note that membrane trafficking is required for the local enrichment of F-actin and RalA protein. Rgl has been identified as a potential RalA guanine nucleotide exchange factor (GEF) in the adult fly and neuroblasts (Mirey et al., 2003; Carmena et al., 2011), and the characterization of Rgl in the early embryo might reveal the sites of active RalA protein. Alternatively, membrane might be recycled back to cytoplasmic Rab8 stores through endocytic pathways, a mechanism that has also been shown to operate during early furrow formation through Dynamin-dependent endocytosis (Lecuit and Wieschaus, 2000; Pelissier et al., 2003; Rikhy et al., 2015).

It is interesting to contrast these results with the mechanisms known to regulate cleavage furrow formation during animal cell cytokinesis. The major force driving cleavage furrow ingression is believed to originate through the action of a contractile actomyosin ring (reviewed by Fededa and Gerlich, 2012). However, it is clear that membrane trafficking pathways are required for cytokinesis as well [reviewed by Albertson et al. (2005); Prekeris and Gould (2008)]. Teasing apart the relative contributions of these pathways to cytokinesis has been difficult. Syncytial furrow formation in the early Drosophila embryo is believed to be a myosin-independent process (Royou et al., 2004), and the geometric constraints of the embryo make assempling and orienting myosin ring contraction into the interior of the embryo challenging. Our results demonstrate that exocytic membrane addition is required for syncytial furrow formation and ingression, and are consistent with a model in which targeted membrane addition directs furrow formation through the accretion of membrane material at the incipient furrow. As our antibody staining and fluorescent protein fusions reveal total RalA protein dynamics, we could not distinguish between inactive GDP-bound and active GTP-bound RalA. It therefore remains to be seen whether RalA is activated locally in cortical subdomains, or if it is generally activated at the plasma membrane and then further processes, such as regulated vesicular fusion or cytoskeletal function, guide the localization of furrow formation. However, Rab8 localization to the cortex occurs specifically in an apicolateral region of the furrow (Fig. 7E,G; L.M.M., Z.Z., J.T.B., unpublished), suggesting that RalA/exocyst function might be locally active at the furrow. Rab8 has been identified as a potential Rab8 guanine nucleotide exchange factor (GEF) in the adult fly and neuroblasts (Mirey et al., 2003; Carmena et al., 2011), and the characterization of RalA in the early embryo might reveal the sites of active Rab8 protein. Alternatively, membrane might be recycled back to cytoplasmic Rab8 stores through endocytic pathways, a mechanism that has also been shown to operate during early furrow formation through Dynamin-dependent endocytosis (Lecuit and Wieschaus, 2000; Pelissier et al., 2003; Rikhy et al., 2015).

The ease of imaging early embryos and the availability of the rich genetic and molecular tools that Drosophila can offer suggest that the transient syncytial furrows should provide a potent system for studying the interplay between membrane trafficking pathways and cytoskeletal networks in driving the formation of plasma membrane furrows.
MATERIALS AND METHODS

Fly stocks and genetics
Fly stocks were maintained at 25°C by standard procedures. All UAS transgenic flies were crossed with mwhTub-Gal4VP16 67C;15 (D. St Johnston, Gurdon Institute, Cambridge, UK) maternal driver females, and second generation embryos were analyzed. Fly stocks used: UAST-mCh:RalA, RalAPL56, FRT19A/FM7 (Bourbon et al., 2002; Ghiglione et al., 2008), Histone:GFP (Bloomington Stock Center), Histone:RFP (Bloomington Stock Center), Sqh-GFP:MoesinABD (Kiehart et al., 2000) and Sqh-Gap43:mCherry (Martin et al., 2010).

Confocal microscopy and time-lapse imaging
Confocal images were acquired with an Olympus Fluoview FV1000 confocal laser scanning microscope with a 40×/1.35NA objective for fixed specimens. Time-lapse imaging was performed on a spinning-disk confocal microscope from Zeiss or Solamere Technologies Group with 40×/1.3NA or 63×/1.4NA objectives. Live imaging was performed with embryos that were dechorionated and placed on a gas-permeable membrane in Halocarbon 27 oil (Sigma). A coverslip was placed on embryos, which were then imaged over time. Fixed specimen confocal imaging was performed using 8 ms/pixel exposure settings, and live imaging was performed using 150-300 ms exposure times. Live imaging was acquired at fast (<1 image/s) or slow (1 image/30 s) rates. Furrow lengths and depths were determined by fine z-scale movie acquisitions (0.5 µm intervals). All movies were acquired at 25±1°C.

Embryo fixation and immunostaining
Embryos were dechorionated in 50% bleach solution and fixed for 1 h 15 min at the interface of heptane and 3.7% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) before being manually devitellinized and stained with Alexa 546-phalloidin (1:200; Molecular Probes), guinea pig anti-RalA (1:500; Teodoro et al., 2013), Hoechst (1:500; Sigma), mouse anti-Lamin (1:100; DSHB) or mouse anti-Sec5 (1:35; Murthy et al., 2010). Secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes) were used at 1:500. Embryos were mounted in Prolong Gold (Molecular Probes).

Fixed and time-lapse embryo analysis
Confocal and spinning disk images were edited using Adobe Photoshop. Channels for fixed and time-lapse images were leveled in Photoshop to show optimal protein populations.

Statistical analysis
Germline clone and siRNA scoring data were tested for statistical significance using a two-dimensional contingency table with a χ² test with α=0.005.

siRNA preparation
Primers for siRNA treatments were selected to represent independent regions of RalA through the use of the SNAPDRAGON RNAi design program, which bioinformatically selects against off-target effects (DSRC, Harvard). dsRNA was made using the Megascript T7 Transcription Kit (Ambion) and purified using QiaGen RNaseasy columns. Final concentration was determined with a NanoDrop ND1000 spectrophotometer (1550 ng/µl).

Scoring embryonic development
OreR and RalAPL56 embryos were dechorionated in 50% bleach, and then placed onto a transparent apple juice plate and observed over the course of 4 hours. RalA siRNA- and water-injected embryos were dechorionated in 50% bleach, then glued onto a coverslip. The embryos were dehydrated for 11 min, covered in Halocarbon 700 oil (Sigma), and then injected with RalA siRNA or water. The embryos were observed over the course of 4 hours.

Scoring nuclear fusion events
RalAPL56 embryos were live imaged, and individual nuclei were tracked throughout cycles 10-13. For each cycle, during prophase, all WT nuclei were counted. During anaphase, each nucleus was tracked over time to

Fig. 9. Model of RalA/Sec5/Rab8-mediated furrow formation. (A) Furrow formation initiates with the activation of RalA at the plasma membrane and the subsequent recruitment of exocyst complex assembly (Sec5) by RalA. Exocyst complex assembly functions to target and tether Rab8 vesicular compartments. (B) The fusion of Rab8 vesicles potentiates the ingestion of incipient furrows, and Rab8 begins to accumulate in a cortical array. Cortical exocyst complex function may be reinforced by trafficking of exocyst subunits on Rab8 vesicles. (C) Furrow ingression provides a scaffold for F-actin assembly, which supports furrow structural integrity. Cytoplasmic Rab8 compartments become depleted as trafficking to the cell surface progresses. (D) The timing of furrow formation, stabilization and retraction events relative to cell cycle stage.
identify if the nucleus underwent a WT division, a mitotic nuclear collapse, or adjacent nuclear fusion (s). Since two nuclei are involved in a single adjacent nuclear fusion, the total number of WT divisions started does not equal the total number of WT divisions completed when an adjacent nuclear division occurs. The fusion of one WT nucleus with an already polyploid nucleus was still counted as an adjacent nuclear fusion.

**siRNA injection and live imaging**

Embryos were prepared in the same manner as scored siRNA-injected embryos. After injection, embryos on a coverslip were immersed in Halocarbon 27 oil and placed onto a gas-permeable slide and imaged on the spinning disk confocal microscope.

**Germine clone preparation**

Heterozygous RalA 

mutant females were crossed with hemizygous ovoD, FRT72 males. Larvae were heat shocked twice for 2 hours over the course of 3 days to perform recombination events.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

R.M.H. performed the live imaging, immunostaining and knockdown experiments. R.M.H. performed initial characterization of RalA nuclear phenotypes. R.M.H. and J.T.B. designed research, analyzed data and wrote the article.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.120998/-/DC1

**References**


