

University of Denver

Digital Commons @ DU

Electronic Theses and Dissertations

Graduate Studies

1-1-2015

Rab8 Directs Tubulation and Furrow Ingression During Epithelial Formation in *Drosophila Melanogaster*

Lauren MacKenzie Mavor
University of Denver

Follow this and additional works at: <https://digitalcommons.du.edu/etd>



Part of the [Cell Biology Commons](#), and the [Developmental Biology Commons](#)

Recommended Citation

Mavor, Lauren MacKenzie, "Rab8 Directs Tubulation and Furrow Ingression During Epithelial Formation in *Drosophila Melanogaster*" (2015). *Electronic Theses and Dissertations*. 1393.
<https://digitalcommons.du.edu/etd/1393>

This Dissertation is brought to you for free and open access by the Graduate Studies at Digital Commons @ DU. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ DU. For more information, please contact jennifer.cox@du.edu, dig-commons@du.edu.

Rab8 Directs Tubulation and Furrow Ingression During Epithelial Formation in *Drosophila Melanogaster*

Abstract

One of the most fundamental changes in cell morphology is the formation of a plasma membrane furrow. The *Drosophila* embryo undergoes several cycles of rapid furrow ingression during early development, which culminates in the formation of an epithelial sheet. Previous studies have demonstrated the requirement for intracellular trafficking pathways in furrow ingression; however, the pathways that link compartmental behaviors with cortical furrow ingression events have remained unclear. This research shows that Rab8, a small GTPase associated with late exocytic trafficking events, demonstrates striking dynamic behaviors *in vivo*; transitioning from punctate structures to a stable association with the cortex during furrow formation. Active, GTP-locked Rab8 is primarily associated with dynamic membrane compartments and the cortical array, while inactive, GDP-locked Rab8 forms large cytoplasmic aggregates. After early furrow initiation, Rab8 transitions to a cortical location that coincides with known regions of directed plasma membrane addition, with Sec5, a subunit of the exocyst and RalA, a small GTPase implicated in directed membrane trafficking.

Disruption of Rab8 function results in a complete failure of furrow formation in the early embryo, due to a failure of proper F-actin cytoskeletal dynamics and localization, which ultimately results in the failure to create an epithelial sheet. Additionally, an intact microtubule network is required for proper Rab8 dynamics during early furrow formation, but is dispensable once a Rab8 cortical array has formed. These studies suggest that active, membrane-bound Rab8 populations prefigure and initiate furrow ingression, and direct membrane trafficking behaviors *in vivo* in the *Drosophila* embryo. The work presented here demonstrates a novel role regarding the function of Rab8 and the Rab family of proteins' requirement in complex developmental processes. By elucidating new mechanisms of furrow formation, and ultimately cell division, this work will help to enhance the general knowledge of potential therapeutic targets in such diseases as cancer, developmental defects and other scenarios in which polarized membrane addition is required for the formation of discreet cell and tissue types.

Document Type

Dissertation

Degree Name

Ph.D.

Department

Biological Sciences

First Advisor

James Todd Blankenship, Ph.D.

Keywords

Drosophila, Furrow formation, Rab8

Subject Categories

Cell Biology | Developmental Biology

Publication Statement

Copyright is held by the author. User is responsible for all copyright compliance.

This dissertation is available at Digital Commons @ DU: <https://digitalcommons.du.edu/etd/1393>

Rab8 directs tubulation and furrow ingression during epithelial formation in

Drosophila melanogaster

A Dissertation

Presented to

The Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Lauren M. Mavor

June 2015

Advisor: Dr. James Todd Blankenship

Author: Lauren M. Mavor
Title: Rab8 directs tubulation and furrow ingression during epithelial formation in *Drosophila melanogaster*
Advisor: Dr. James Todd Blankenship
Degree Date: June 2015

ABSTRACT

One of the most fundamental changes in cell morphology is the formation of a plasma membrane furrow. The *Drosophila* embryo undergoes several cycles of rapid furrow ingression during early development, which culminates in the formation of an epithelial sheet. Previous studies have demonstrated the requirement for intracellular trafficking pathways in furrow ingression; however, the pathways that link compartmental behaviors with cortical furrow ingression events have remained unclear. This research shows that Rab8, a small GTPase associated with late exocytic trafficking events, demonstrates striking dynamic behaviors *in vivo*; transitioning from punctate structures to a stable association with the cortex during furrow formation. Active, GTP-locked Rab8 is primarily associated with dynamic membrane compartments and the cortical array, while inactive, GDP-locked Rab8 forms large cytoplasmic aggregates. After early furrow initiation, Rab8 transitions to a cortical location that coincides with known regions of directed plasma membrane addition, with Sec5, a subunit of the exocyst and RalA, a small GTPase implicated in directed membrane trafficking.

Disruption of Rab8 function results in a complete failure of furrow formation in the early embryo, due to a failure of proper F-actin cytoskeletal dynamics and localization, which ultimately results in the failure to create an epithelial sheet. Additionally, an intact microtubule network is required for proper Rab8 dynamics during

early furrow formation, but is dispensable once a Rab8 cortical array has formed. These studies suggest that active, membrane-bound Rab8 populations prefigure and initiate furrow ingression, and direct membrane trafficking behaviors *in vivo* in the *Drosophila* embryo. The work presented here demonstrates a novel role regarding the function of Rab8 and the Rab family of proteins' requirement in complex developmental processes. By elucidating new mechanisms of furrow formation, and ultimately cell division, this work will help to enhance the general knowledge of potential therapeutic targets in such diseases as cancer, developmental defects and other scenarios in which polarized membrane addition is required for the formation of discrete cell and tissue types.

ACKNOWLEDGEMENTS

The work presented here would've not been made possible were it not for my Ph.D. advisor and mentor, Dr. J. Todd Blankenship. Todd, I owe you so much gratitude and respect for accepting me into your lab and entrusting me to be your first Ph.D. student (to this day, I still don't feel completely worthy of the honor). I have learned so much about myself: of whom I am as an individual, and a scientist. Your mentorship and leadership have shown me how to push the limits of our knowledge and that perseverance--and maybe bull-headedness on my part--can often lead to amazing results (I'm looking at you, CRISPR). Most importantly, you have expanded my love and passion for science in ways that I never would've thought possible, and I so look forward to the days when hopefully I can inspire someone as much as you have inspired me. To my graduate committee, Dr.'s Joe Angleson, Scott Barbee and Michelle Knowles, thank you for your advice and expertise, your input has helped me a great deal with my scientific work and helped me succeed as a researcher.

To my parents, you've always told me that anything was possible as long as I worked hard. Thank you so much for always pushing me while also helping me keep a level head in whatever passion I've decided to follow. To Keith, my partner in crime and best friend, you have been such an amazing support through my time in grad school. You've always shown me the importance of shaking off the negative and keeping my attitude positive. You've been an amazing bolster of confidence and encouragement. Thank you for always believing in me, and showing me to believe in myself. And especially, thank you for pretending you know what I'm talking about when I nerd-out.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION.....	1
Furrow formation and the early development of <i>Drosophila melanogaster</i>	1
Furrow formation requires active cytoskeletal networks	4
Cellularization is responsible for establishing apical-basal polarity	9
Membrane requirements during cellularization	10
Rab protein function and roles in developmental processes and disease	11
Identifying Rab8 as the link between intracellular membrane stores and the delivery of membrane to ingressing furrows	15
 CHAPTER 2: RESULTS	 16
Rab8 displays dynamic behaviors in the <i>Drosophila</i> embryo	16
The Rab8 cortical array forms during the fast phase of cellularization	21
Disruption of Rab8 leads to a failure of furrow ingression and F-actin network formation	27
Rab8 associates with secretory pathways and the exocyst during cellularization	31
Rab8 function is required for dynamic membrane addition.....	34
Rab8 localization and dynamics are GTPase activity dependent.....	36
Rab8 populations are largely distinct from recycling endosomes and the Golgi.....	39
Sec5 and Rab11 function are required for targeting of Rab8 to the cell cortex	43
Rab8 requires RalA in order to target to the cortex	46
Rab8 tubule structures extend basally and precede F-actin	48
Rab8 dynamics are microtubule-dependent and F-actin independent	53
Development of a Rab8 antibody in three methods	58
 CHAPTER 3: DISCUSSION	 60
Rab8 dynamics <i>in vivo</i> are spatiotemporally regulated and require GTPase activity ...	60
Rab8 regulates furrow formation and functions in an exocytic pathway.....	61
Rab8 regulates polarized membrane addition downstream of the RE	62
Rab8 requires the MT but not F-actin cytoskeletal network to behave dynamically....	63
A model for polarized membrane addition regulated via Rab8 complexes	65
Future directions and conclusions	67

CHAPTER 4: MATERIALS AND METHODS	70
Fly Stocks and Genetics	70
Construction of endogenous promoter driven GFP:Rab8 transgenic line.....	71
Construction of CRISPR GFP:Rab8 transgenic line.....	71
PCR-Verification of CRISPR GFP:Rab8 transgenic line	72
Primers designed for cloning, guideRNAs and PCR verification of CRISPR GFP:Rab8 insertion.....	72
Confocal microscopy and time-lapse imaging	74
Embryo fixation and immunostaining.....	75
Fixed and time lapse embryo analysis and quantification of vesicle colocalization.....	75
siRNA Preparation	77
RNAi Treatments	78
Drug Treatments.....	78
 REFERENCES.....	 80

TABLE OF FIGURES

Figure 1. Early development of the <i>Drosophila</i> embryo.....	3
Figure 2. Cellularization requires dynamic F-actin reorganization and polarized membrane addition.....	8
Figure 3. Rab family proteins exhibit GTPase activity and associate with specific intracellular vesicular compartments.	13
Figure 4. Effects of Rab GTPase Activity Mutants on <i>Drosophila</i> Development	18
Figure 5. Rab8 exhibits dynamic behaviors associated with transient furrow ingression <i>in vivo</i>	20
Figure 6. Rab8 dynamics during cellularization resemble those seen during syncytial divisions.	22
Figure 7. Rab8 expressed at endogenous levels displays dynamic transition from puncta to cortical association.....	25
Figure 8. Successful insertion of GFP into the <i>Drosophila</i> genome displays dynamic transition from puncta to cortical association during furrow formation.	26
Figure 9. Knockdown of Rab8 via RNAi leads to early developmental defects in embryos.....	29
Figure 10. Knockdown of Rab8 severely disrupts furrow ingression in the early embryo.	30
Figure 11. Rab8 compartments demonstrate colocalization with newly delivered membrane and the exocyst during cellularization.	33
Figure 12. Disruption of Rab8 results in failure of proper membrane trafficking to ingressing furrows.....	35
Figure 13. Rab8 cortical behaviors are GTPase activity dependent <i>in vivo</i>	38
Figure 14. Rab8 forms a distinct compartment from the RE and Golgi body.	41
Figure 15. Rab8 localization with RE and Golgi is GTPase activity-dependent.	42
Figure 16. Rab8 requires the exocyst and RE to behave dynamically and regulate membrane addition during furrow formation	45
Figure 17. Rab8 requires RalA to behave dynamically and regulate membrane addition during furrow formation.	47
Figure 18. Rab8 tubules localize to sites of furrow ingression and precede the F-actin network.	50
Figure 19. Rab8 tubules precede F-actin ingression <i>in vivo</i>	52
Figure 20. Disruption of microtubule networks produces distinct affects on Rab8 dynamics.	55
Figure 21. Rab8 does not require F-actin to behave dynamically.	57
Figure 22. Rab8 regulates membrane addition and furrow formation during cellularization.....	64

CHAPTER 1: INTRODUCTION

Furrow formation and the early development of *Drosophila melanogaster*

Ingression of a plasma membrane furrow is an essential cell shape change that occurs in all eukaryotic organisms. Furrow ingression is also an obligatory step in animal cells during cytokinesis. Understanding how cells are able to dynamically remodel plasma membrane topologies and form a stable furrow is an unresolved question. The early *Drosophila* embryo forms thousands of furrows, with some being generated in as little as three minutes. Fly embryos make both transient and permanent furrows over the course of development. Following fertilization and the fusion of the male and female pronuclei, the zygotic nucleus undergoes thirteen rounds of replication in the absence of cytokinesis to generate a single-celled syncytium with approximately 5,000 nuclei (Fig. 1; Hartenstein, 1993). The first nine rounds of replication occur deep in the yolk of the embryo; however, at cycle 10, nuclei migrate to the periphery and the plasma membrane begins forming furrows in order to separate mitotic nuclei in the syncytium. These furrows form during metaphase and are transient, extending to their maximum extent during telophase where they compartmentalize and anchor nuclear spindles before then regressing. Embryos undergo four rapid rounds of transient furrow formation (cycles 10-13, Fig. 1C), followed by a final fifth round of permanent furrow ingression which results in the packaging of nuclei into individual cells and the formation of an

epithelium through a process known as cellularization (Fig. 1D). During cellularization, nuclei are arrested in interphase and the ingression of furrows is regulated by both actin and microtubule cytoskeletal networks (Fig 2). Initially, furrow ingression proceeds at a slow ingression rate (slow phase, Fig. 2E, F) before transitioning to a fast phase of ingression (about 50 minutes after initiation, Fig. 2G) during which furrow formation is completed and membrane is pinched off to encapsulate individual nuclei (Fig. 2H; Lecuit and Wieschaus, 2000).

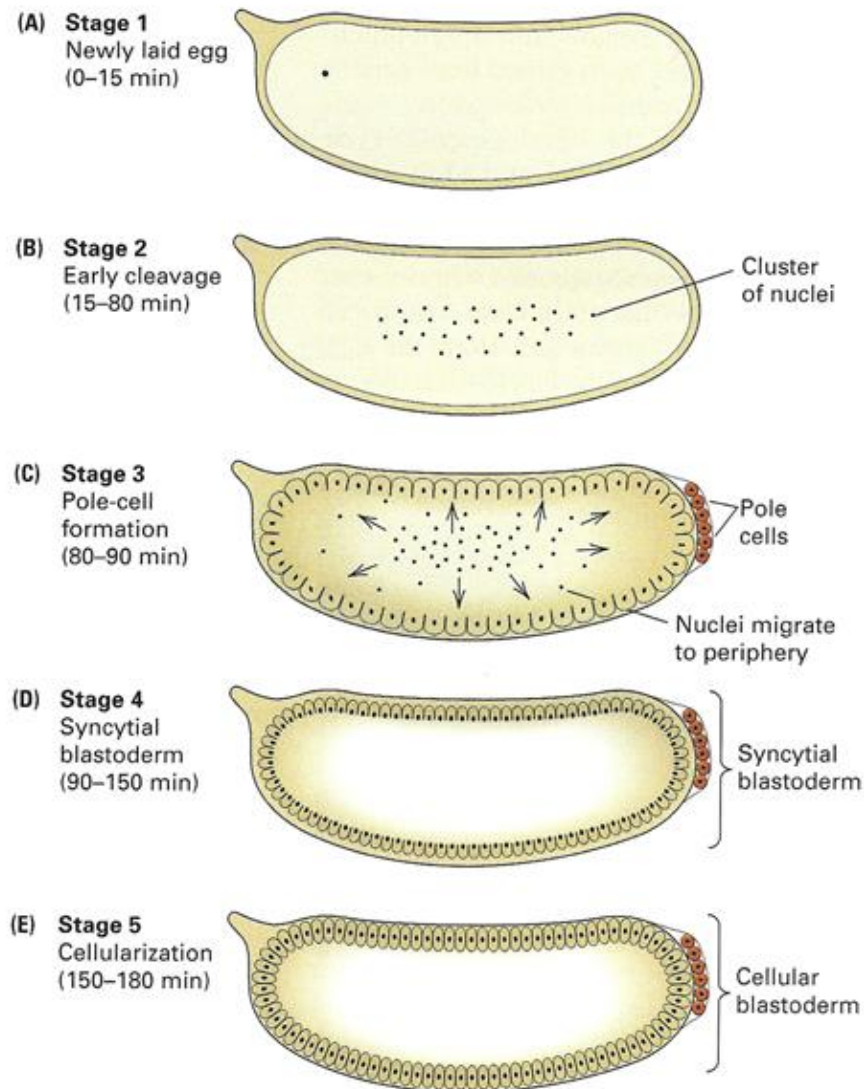


Figure 1. Early development of the *Drosophila* embryo.

(A) Fusion of male and female pronuclei creates a fertilized embryo. (B) Nuclear division cycles 1-9 occur deep in the yolk of the embryo. (C) At nuclear division cycle 10, nuclei have reached roughly 5,000 in number and migrate to the periphery of the syncytium. Transient furrows form during cycles 10-13 (C and D), serving as an anchor point for mitotic nuclei and prevent chromosomal mixing. (E) After cycle 13, nuclear division halts and permanent furrows ingress during cellularization to form the embryonic epithelial sheet. Figure adapted from Stauzer, 2013.

Furrow formation requires active cytoskeletal networks

Both transient and permanent furrows require the function of filamentous actin (F-actin) and microtubule (MT) networks. During formation of transient metaphase furrows, F-actin is concentrated in cortical caps above the nuclei, which rearrange into rings at the onset of metaphase to create shallow furrows which serve to separate the mitotic microtubule spindles and prevent chromosomal collision (Foe and Alberts, 1983). It is also known that the classical non-muscle Myosin-II (MyoII) associates with these furrows and is required for ingression (Foe et al., 2000).

During cellularization, F-actin transitions to a hexagonal array (Fig. 2A) and permanent furrow ingression begins via the formation of a bulbous Furrow Canal (FC) which is directed basally (Fig. 2E; Schejter and Wieschaus, 1993). MyoII associates with the tip of the FC and its function is temporally regulated to prevent premature encapsulation of nuclei (Fig. 2E-H). Once the FC has reached its basal-most ingression (about 30 μ m), pinching of membrane, directed by MyoII, functions to encapsulate each nucleus within a continuous plasma membrane, transitioning the embryo from a single celled syncytium to a multicellular embryo surrounded by an epithelium of roughly 5,000 cells (Fig. 2H).

The actomyosin network is tightly regulated during the cellularization process by three classically identified proteins: nullo, serendipity-alpha ($sry-\alpha$), and bottleneck (bnk) (Rose and Wieschaus, 1992; Schwiesguth et al., 1990, Schejter and Wieschaus 1993). During slow-phase, nullo and $sry-\alpha$ function to regulate the actomyosin hexagonal array by localizing to discrete positions along the furrow. Nullo localizes in puncta along the

hexagonal array, with strong localization at vertices and the apical position of ingressing furrows, whereas *sry-α* positions in a “line” along the hexagonal array and to areas just above the FC (Postner and Wieschaus, 1994). Disrupting the function of either protein results in very similar defects to ingressing furrows: ingression proceeds normally initially, but results in the sporadic disruption of the actomyosin network and the formation of multinucleate cells (Schwiesguth, et al., 1990; Rose and Wieschaus, 1992). It has also been shown that proper function and localization of *nullo* is independent of *sry-α*, but *sry-α* localization requires the function of *nullo*, suggesting that these two proteins may interact in a common pathway with *sry-α* functioning downstream of *nullo* to regulate proper actomyosin formation and thus furrow ingression during cellularization (Postner and Wieschaus, 1994).

At the onset of fast phase, the actomyosin network shifts from a hexagonal array to a ring structure reminiscent of the cytokinetic ring seen in dividing cells (Fig. 2 C, D). As ingression proceeds, these rings shrink until they have completely pinched off membrane and encapsulated nuclei in a continuous membrane (Schejter and Wieschaus 1993). It is during this time that *bnk* functions as a timing mechanism which regulates the switch from hexagonal array to ring structure as well as the contractile activity of MyoII. *Bnk* has been shown to localize to the embryo surface and transient membrane furrows during syncytial cycles, and strongly associates with the leading edge of the FC during the slow phase of cellularization. This localization as well as *bnk* protein is depleted when F-actin fully reconfigures into ring structures. Loss of *bnk* results in a premature closure of the actomyosin network, resulting in the pinching of nuclei to form

“bottleneck” structures. Thus the depletion of bnk at the onset of fast phase regulates the reconfiguration of actomyosin from a structural to contractile network (Schejter and Wieschaus 1993).

The Rho family of proteins has also been implicated in regulating the actin hexagonal array during cellularization. Rho protein is a member of the p21 small GTPases, proteins that cycle between being bound to GTP, during which time they are active, or GDP, at which time they are inactive. Rho family proteins have been implicated in initiating intracellular responses and regulating cytoskeletal rearrangements (reviewed in Chircop, 2014; Crawford et al., 1998). During cellularization, active Rho is required for the maintenance of the actin hexagonal array and this regulation is antagonized by Cdc42, another GTPase within the Rho family (Crawford et al., 1998).

Alongside F-actin, the microtubule network is required throughout the cellularization process in two main ways: to control the elongation of nuclei during cellularization, and to provide a directional cue for the ingression process (Fullilove and Jacobsen, 1971; Foe et al., 2000). Microtubules are organized as an “inverted basket” structure, with minus ends oriented at the apex of nuclei and plus ends oriented basally, forming a basket structure around individual nuclei (Fig. 2; Fullilove and Jacobsen 1971; Foe and Alberts, 1983; Cermelli et al., 2006). It is believed that this basket serves to control the elongation of nuclei which occurs during the slow phase of cellularization (Fig. 2E-H; Fullilove and Jacobsen, 1971).

During furrow formation, it has been demonstrated that there is cross-talk and regulation between actomyosin and microtubular networks (Foe et al., 2000; Goode et al.,

2000) During cellularization, loss of an intact MT array leads to a failure of proper furrow formation (Fig. 17B; Foe et al., 2000), and it has been demonstrated that a plus-end directed motor, Pav-KLP (a kinesin-6), is required for crosslinking microtubules and contributing to actin furrow formation. Pav-KLP is responsible for providing a driving force during the slow phase of cellularization and regulates transport of actin- and membrane-containing vesicles to the furrow during fast phase. Loss of Pav-KLP results in a failure to form furrows and changes in the distribution of actin during cellularization (Sommi et al., 2010). These results demonstrate a requirement of the MT network in not only organizing and regulating actomyosin dynamics during permanent furrow formation, but in providing a driving force for initial ingression during slow phase.

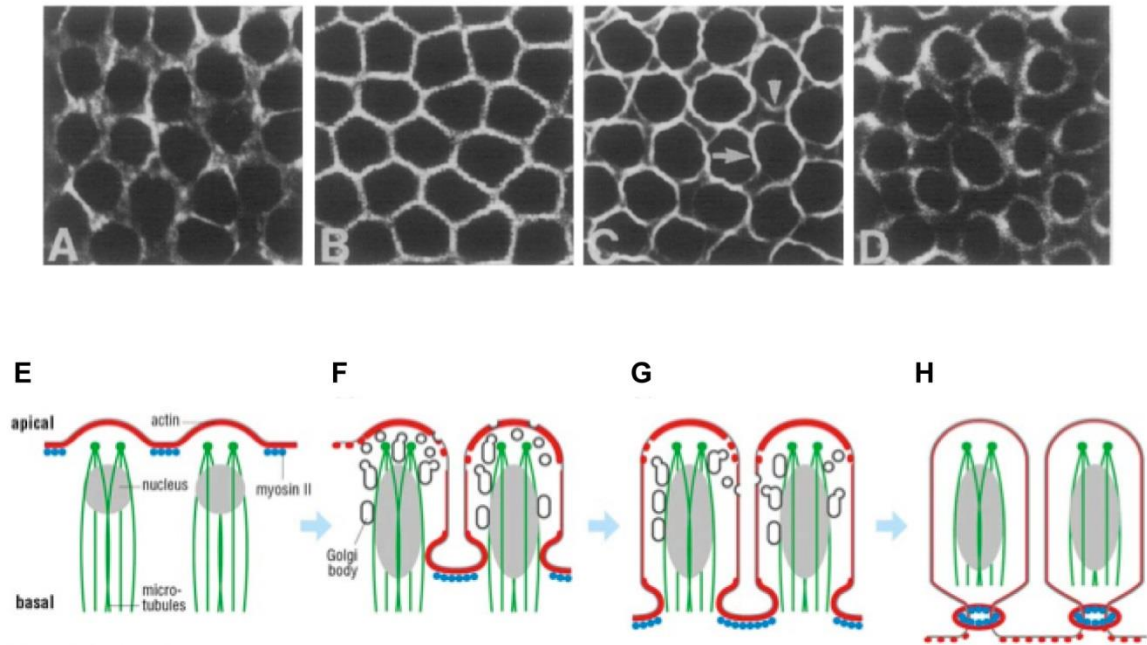


Figure 2. Cellularization requires dynamic F-actin reorganization and polarized membrane addition. (A-D) Planar views of fixed embryos stained for Phalloidin (F-actin). During the slow phase of cellularization F-actin appears as a hexagonal array (A, B). At the onset of fast phase, the actomyosin network transitions to a ring structure (C), and these rings gradually decrease in diameter as membrane is pinched by MyoII (D). (E-H; cartoon model, apical-basal views) (E) At the onset of cellularization, microtubules (green) serve to orient the furrow system (apical at the surface, basal into the interior of the embryo). (F) F-actin (red) along with myosin (blue) and associated plasma membrane (black) form the FC which ingresses basally. During slow phase, membrane addition (black) occurs at apical portions of the furrow. (G) At the onset of fast phase, the FC has reached the basal portion of the nuclei (grey) and ingression speed increases. Membrane addition transitions to an apical-lateral position of the furrow. (H) Once fast-phase is complete, actomyosin networks work to pinch membrane and encapsulate individual nuclei in plasma membrane, ultimately creating the epithelial sheet. (A-D) Adapted from Schejter and Wieschaus 1993. (E-H) Adapted from Morgan, 2007.

Cellularization is responsible for establishing apical-basal polarity

The columnar epithelial cells which are formed via the cellularization process require a very specific polarity along their apical-basal axis in order to function as an intact tissue. Proteins must be properly localized along the axis of individual cells in order to maintain tissue integrity, and it has been demonstrated that this polarization of proteins is established during cellularization. Cell-cell contacts are formed during the furrow ingression process, with the *Drosophila* homologue of β -catenin, Armadillo (Arm), localizing to the leading edge of furrows (Müller and Wieschaus, 1996). Once cellularization has completed, Arm is relocated from the basal-most region of the lateral membrane and a second population arises during mid-cellularization at the apical-lateral portion of furrows where it will form spot-junctions which eventually give rise to the Zonula Adherens during gastrulation (Hunter and Wieschaus, 2000). The basal junctions and later apicolateral junctions also demonstrate the recruitment of other classical zonula adherens proteins including E-cadherin and α -catenin (Hunter and Wieschaus, 2000). Additionally, nullo is required for the localization of basal-lateral Arm during cellularization and ectopic expression of nullo prevents the apical localization of Arm during mid-cellularization (Hunter and Wieschaus, 2000), demonstrating that nullo regulates the actomyosin array during furrow ingression, as well as the proper localization of adhesion proteins.

The protein Slow-as-molasses (Slam) has been shown to associate with furrows during the slow phase of cellularization and regulates the proper accumulation of junctional components Arm, Discs-lost (Dlt) and MyoII. Slam localizes to the FC and to

basal adherens junctions during cellularization and this localization is controlled by the recycling endosome (Acharya et al., 2014). Loss of Slam results in a failure of the recruitment of adhesion proteins and a failure of proper ingression movements (Lecuit et al., 2002). It has also been suggested that the accumulation of adhesion junctions is responsible for providing the proteins that connect the plasma membrane with actin cytoskeleton dynamics. Taken together, these results show that the localization of adhesion proteins occurs simultaneously with furrow ingression and that this polarization of furrows is required both for the cellularization process as well as the maintenance of tissue integrity during epithelial cell formation.

Membrane requirements during cellularization

While regulation of a contractile cytoskeleton is predominantly required to provide the force behind furrow ingression, the 30-fold increase of surface area during cellularization requires a large supply of membrane material. Early studies demonstrated the presence of membrane ruffles at the surface of embryos and the eventual smoothing of these ruffles during the ingression process of cellularization (Fullilove and Jacobsen, 1971; Turner and Mahowald, 1976). However, it has been demonstrated that these ruffles do not provide enough of the membrane required for furrows to ingress to their full depth (Figard and Sokac, 2014). Intracellular stores of membrane are required and this membrane is added in a directed fashion at apical portions of ingressing furrows during slow phase, which shift to an apical-lateral position during the fast phase (Fig. 2B, C; Lecuit and Wieschaus, 2000; Lecuit, 2004), which also coincides with shifts in adhesion

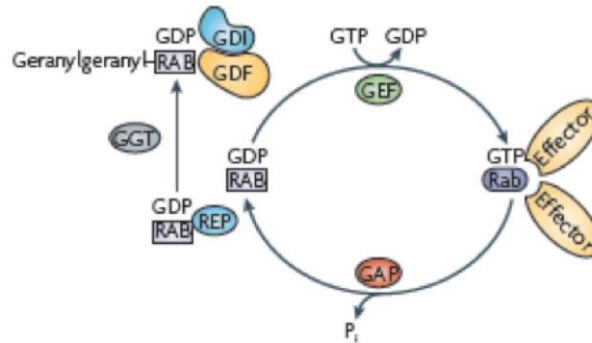
protein localization (Lecuit et al., 2002). This newly added membrane has been shown to be derived from both the recycling endosome (RE) and Golgi compartments. Disrupting the function of either of these intracellular compartments results in the failure in furrows to reach their full extension (Lecuit and Wieschaus, 2000; Sisson et al., 2000; Pelissier et al., 2004). While both Golgi and RE pathways have been shown to be required for furrow ingression, and membrane marking experiments have indicated regions where directed membrane addition is likely to occur (Lecuit and Wieschaus, 2000; Sisson et al., 2000; Pelissier et al., 2003), markers for Golgi and RE compartments such as Lava Lamp (Lva) and Rab11 show relatively static behaviors, and do not move in a polarized fashion towards the developing furrow. This lack of dynamic behavior towards the furrow led to the desire to identify new putative trafficking pathways that demonstrate an active association and function with membrane addition during furrow formation.

Rab protein function and roles in developmental processes and disease

The Rab-family proteins are key mediators of membrane trafficking and cytoskeletal dynamics. Rab proteins are small GTPases involved in many cellular processes associated with vesicular trafficking (Fig. 3A; Ali and Seabra, 2005). Rab proteins localize to specific vesicular compartments and mediate their mobility, targeting and fusion to other compartments including the plasma membrane (Fig. 3B; Zerial and McBride, 2001). When in an active GTP-bound state, Rab proteins orchestrate membrane compartment dynamics through the recruitment of tethering and trafficking effectors (Pfeffer, 2005; Grosshans et al., 2006; Horgan and McCaffrey, 2012). At the plasma

membrane Rab proteins have been shown to bind to the targeting and tethering exocyst (Sec6/8) complex. During lumen formation and ciliogenesis in mammalian cells, Rab8 binds to the Sec15 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), while mutations in Rab proteins are associated with a variety of diseases and developmental disorders (Baskys et al., 2007; Mitra et al., 2011; Recchi and Seabra, 2012; Hardiman et al., 2012). A newly emerging theme on Rab protein function is the ability of Rabs to act as potent regulators of the cytoskeleton (Riggs et al., 2003; Hattula et al., 2006; Cao et al., 2008; Zhang et al., 2009; Peranen, 2011).

A



B

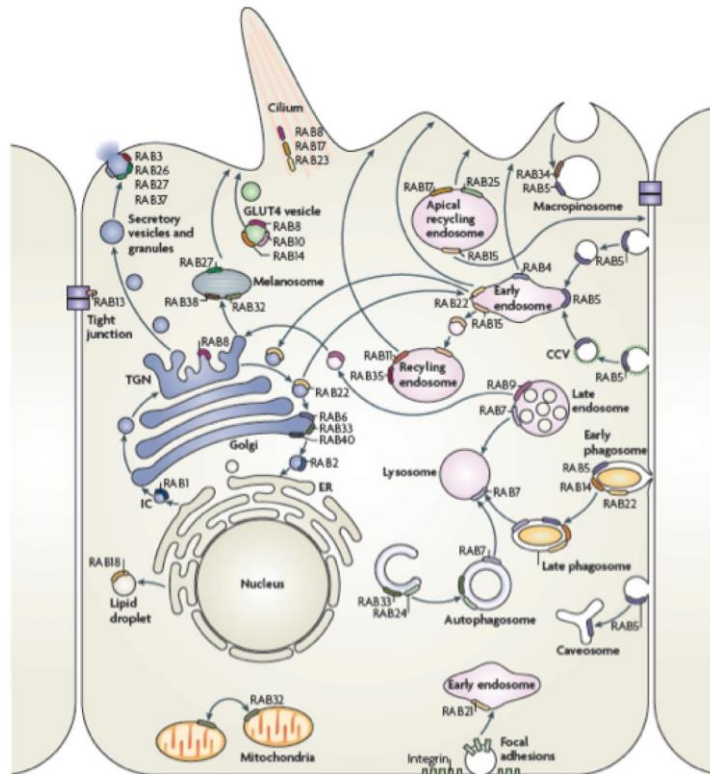


Figure 3. Rab family proteins exhibit GTPase activity and associate with specific intracellular vesicular compartments.

(A) Rab proteins cycle between being bound to GTP (active state) and GDP (inactive state). These states are modulated by a variety of effectors and activators resulting in high levels of spatiotemporal regulation. (B) Rab proteins associate with membrane-bound compartments within the cell in a very specific manner and are responsible for regulating the identity, trafficking, targeting and fusion of these compartments. Figure adapted from Stenmark, 2009.

Within *Drosophila*, there are 33 known Rab proteins, which are directly homologous to mammalian Rabs. Screening for the requirement of Rab proteins and their associated trafficking compartments during early embryonic development is possible as a complete collection of YFP-tagged wild-type (WT), constitutively active (CA, GTP-locked) and dominant negative (DN, GDP-locked) lines of all known *Drosophila* Rab proteins have been constructed (Zhang et al., 2007). An additional collection of large genomic fragment-driven Rab Gal4s permits the systematic profiling of the cellular and subcellular expression of Rab GTPases (Chan et al., 2011).

The Rab8 protein has been identified within the Rab family as associating and giving identity to late exocytic vesicles. This protein has been identified as directing polarized trafficking events during ciliogenesis, neuron development, regulated secretion and cytokinesis (Yoshimura et al., 2007; Nachury et al., 2007; Huber et al., 1995; Chen et al., 2001; Kaplan and Reiner 2011), possibly through the ability to reorganize actin and microtubules (Peranen et al., 1996). Defects in Rab8 function have been implicated in trafficking diseases including retinal degeneration, neuronal overgrowth phenotypes, cell polarity defects, and irregular tissue formation (Moritz et al., 2001; West et al., 2015; Satoh et al., 2007; Huber et al., 1995). The work presented here describes a novel role for Rab8 in the directed addition of membrane during furrow ingression and ultimately epithelial tissue formation.

Identifying Rab8 as the link between intracellular membrane stores and the delivery of membrane to ingressing furrows

One puzzling aspect of furrow formation in the *Drosophila* embryo has been the absence of identified dynamic membrane compartment behaviors that are directed towards the furrow. This work establishes Rab8 as the mediator of active membrane addition and a possible link from the Golgi and RE to the plasma membrane surface. Rab8 demonstrates dynamic behaviors that are specifically associated with furrow ingression. Here, it is shown that Rab8 exists in both punctate and cortical associations as well as apical-basal tubular structures. Rab8 tubules prefigure ingression of both syncytial furrows and the cellularization front, and Rab8 localizes both temporally and spatially to regions of rapid membrane addition to the furrow. Rab8 is required during these furrow ingression stages as knockdown of this protein results in an abolishment of proper F-actin behavior and localization, failure of furrow formation and thus loss of the ability to create an epithelial sheet. Rab8 requires an intact MT network in order to behave dynamically, but this network is dispensable once Rab8 is cortically associated with nascent furrows. The work presented here aims to elucidate a new mechanism in which Rab8 functions as the key mediator of directed membrane addition and putative regulator of cytoskeletal dynamics during furrow formation and tissue development.

CHAPTER 2: RESULTS

Rab8 displays dynamic behaviors in the *Drosophila* embryo

An initial screen was performed to determine which of the *Drosophila* Rab proteins were involved in early developmental processes. Either GDP-dissociation deficient, dominant negative (DN) or GTPase deficient, constitutively active (CA) Rab proteins were expressed in embryos and scored under oil for tissue-level defects in early embryonic development. From this screen, three Rabs, Rab8, Rab11 and Rab40 showed phenotypes in over 20% of embryos scored, while six Rabs displayed phenotypes in 10-20% of embryos (Fig. 4). Of the three Rabs which demonstrated phenotypic defects in more than 20% of embryos, Rab8 was of particular interest. Rab8 is relatively uncharacterized in *Drosophila*, and Rab8 DN created the highest percentage of mutant embryos (28-35%). Expression of Rab8 DN resulted in embryos displaying an uneven epithelium and a loss of later gastrulation movements, suggesting that Rab8 is required in early morphological processes (Fig. 4).

Rab5 and Rab11 showed mutant phenotypes ranging from 5-25% of embryos (Fig. 4) and are well-described regulators of endocytic trafficking. Rab5 has been shown to regulate and associate with early endocytic vesicles, whereas Rab11 is known to associate with the RE (reviewed in Zerial and McBride, 2001). Interestingly, both Rab5

and Rab11 have been described as functioning to regulate furrow formation, and ultimately epithelial morphology in early embryos. Rab5 has been shown to regulate the establishment of apical-basal polarity (Lu and Bilder, 2005), as well as the remodeling of apical membrane ruffles during the fast phase of cellularization (Fabrowski et al, 2013). Rab11, through its regulation of the RE has also been well established as regulating both membrane addition and actin cytoskeletal dynamics throughout the furrow formation process (Lecuit and Wieschaus, 2000; Riggs et al, 2003; Pelissier et al., 2004; Cao et al., 2008; Achayra et al., 2014). Due to this well-defined role of Rab5 and Rab11 in endocytic trafficking, alongside the strong defects seen in Rab8, our focus turned towards examining Rab8 dynamics and function to elucidate new trafficking pathways involved in early development.

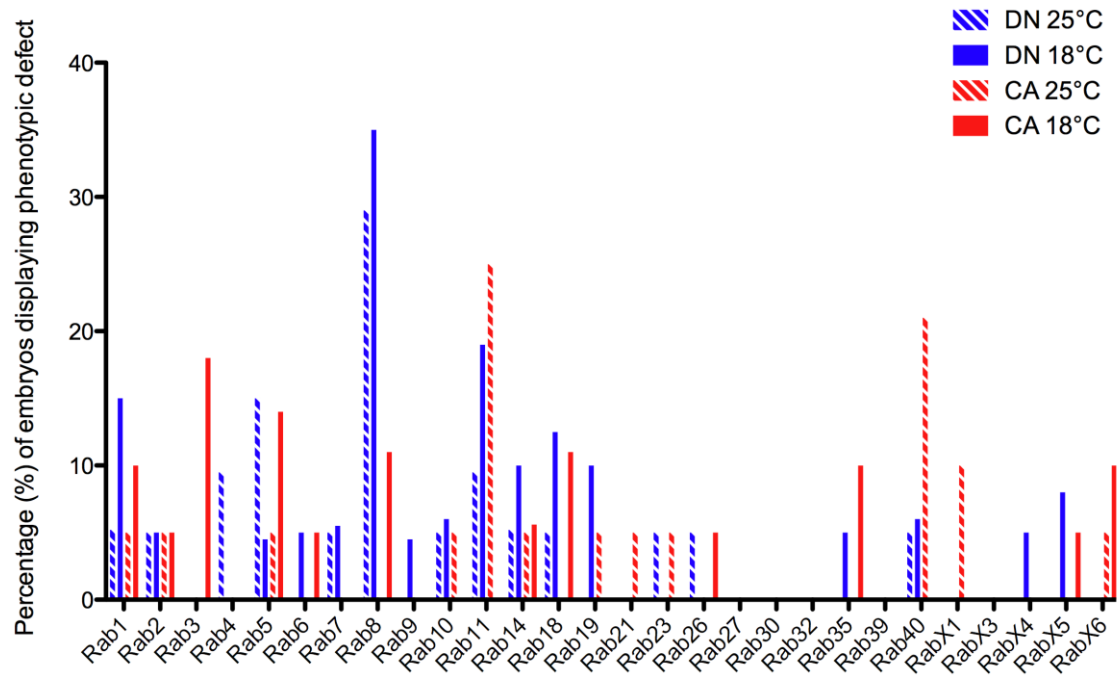


Figure 4. Effects of Rab GTPase Activity Mutants on *Drosophila* Development

GDP-locked, Dominant Negative (DN) or GTP-locked, Constitutively Active (CA) Rab mutants were expressed in embryos at either 25°C or 18°C. Colder temperatures roughly double developmental time, resulting in greater amount of protein expression. Embryos were scored for tissue-level developmental defects under oil using light microscopy. It can be noted that expression of Rab8 DN at both temperatures (25 °C and 18 °C) resulted in the highest percentage (28-35%) of phenotypic defects.

These results led to the examination of the dynamics of Rab8 in early embryos. Embryos expressing YFP:Rab8 fusion proteins (Zhang et al., 2007) were imaged using a spinning disc microscope. Prior to the movement of nuclei to the embryonic periphery at cycle 10, Rab8 exists in small, relatively inactive, puncta. However, once furrow formation is initiated in the syncytial divisions, Rab8 displays striking behaviors (Fig. 5A). As the division cycle proceeds, Rab8 puncta become more numerous (Fig. 5A-A'). These puncta then begin to send out small tubule-like projections (Fig. 5A'). Tubule formation increases and then Rab8 transitions to a continuous array (Fig. 5A''). This array persists through the remainder of the division cycle (Fig. 5A'''). The formation of the continuous array is rapid and occurs within a few minutes (Fig. 5A-A'').

To identify how changes in Rab8 populations are correlated with the cell cycle, Rab8 localization was examined in both fixed embryos stained for YFP:Rab8 and DNA (Fig. 5B-E) and over time in embryos expressing YFP:Rab8 and Histone:RFP (His:RFP; Fig. 5F). During interphase cycles, Rab8 exists in a punctate population (Fig. 5B, F) and transitions to a cortical population at the onset of furrow formation and mitosis (Fig. 5C, F'). These structures persist through the mitotic division (Fig. 5D, F'') and return to a punctate population at the onset of the next interphase (Fig. 5E, F'''). These results suggest that Rab8 dynamics are cell cycle regulated and may direct changes in membrane or cytoskeleton structure during these division cycles and metaphase furrow formation.

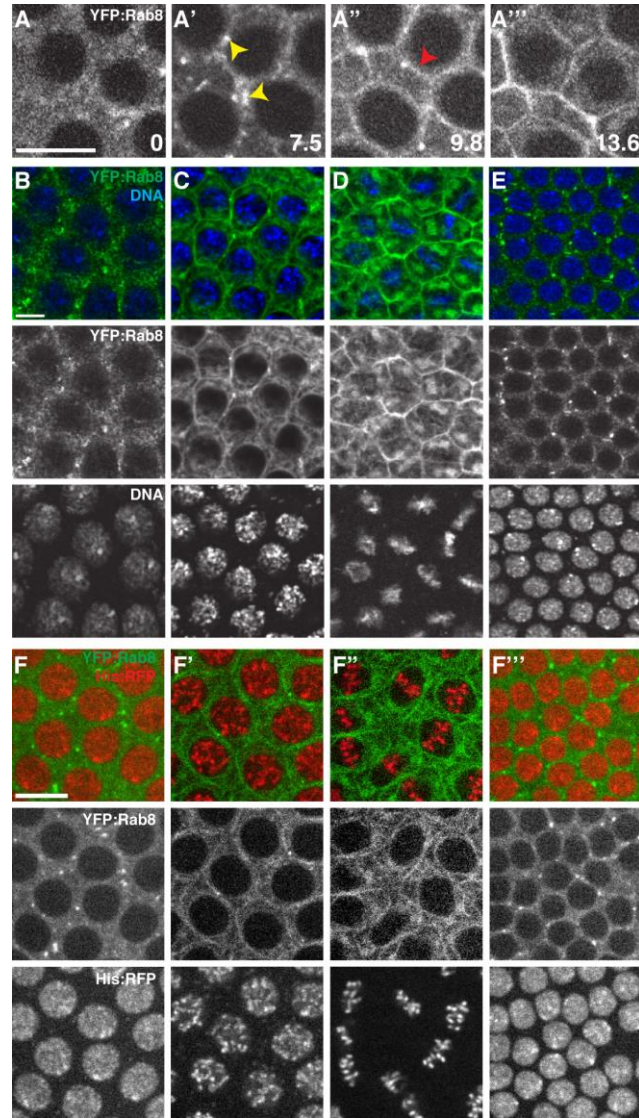


Figure 5. Rab8 exhibits dynamic behaviors associated with transient furrow ingression *in vivo*.

(A-A''') Still-frames measured from the beginning of cycle 13 of a whole mount embryo expressing YFP:Rab8. (A) At early cycle 13, Rab8 exists in punctate populations. (A') As the cycle proceeds, these puncta become more numerous and begin to send out projections (yellow arrowheads). (A'') Rab8 transitions into a continuous cortical array (red arrowhead). (A''') The cortical array persists for the remainder of the division cycle. (B-E) Syncytial, fixed embryos expressing YFP:Rab8 (anti-GFP, green; DNA, Hoescht, blue). (B) During interphase cycles, Rab8 exists in punctate structures. (C) Early in mitosis, Rab8 transitions to a cortical population. (D) This population remains present during metaphase and anaphase stages. (E) Rab8 returns to a punctate state at the onset of the next syncytial cycle. (F-F''') Embryo expressing YFP:Rab8 (green) and His:RFP (DNA, red). *In vivo* live imaging shows the transition from puncta during interphase (F) to a cortical array that arises at metaphase (F') and persists through anaphase (F''). Rab8 returns to a punctate state at the interphase of the next division cycle (F'''). (A) Time indicated in min. Scale bars in A and F are 10 μ m. Scale bar in B is 5 μ m.

The Rab8 cortical array forms during the fast phase of cellularization

Similar to the earlier formation of metaphase furrows, Rab8 shows a transition from punctate structures to a continuous array during cellularization. At early cellularization stages, Rab8 exists in punctate structures (Fig. 6A). As cellularization proceeds, these puncta become more numerous and begin to send out small tubule projections (Fig. 6A', F). Rab8 then makes a transition to a tight cortical array (Fig. 6A''). These cortical arrays persist throughout the remainder of cellularization (Fig. 6A'''). This transition from punctate to cortical association also represents a transition from the slow phase (Fig. 6B, C) to the fast phase of cellularization (Fig. 6D, E), which is marked by the F-actin furrows reaching the basal most portion of nuclei (Fig. 6E). During fast phase, furrow ingression rapidly increases and membrane addition is targeted to an apicolateral location (Lecuit and Wieschaus, 2000). Rab8 localizes to this area of rapid membrane addition (Fig. 6D, E) and transitions to a cortical association.

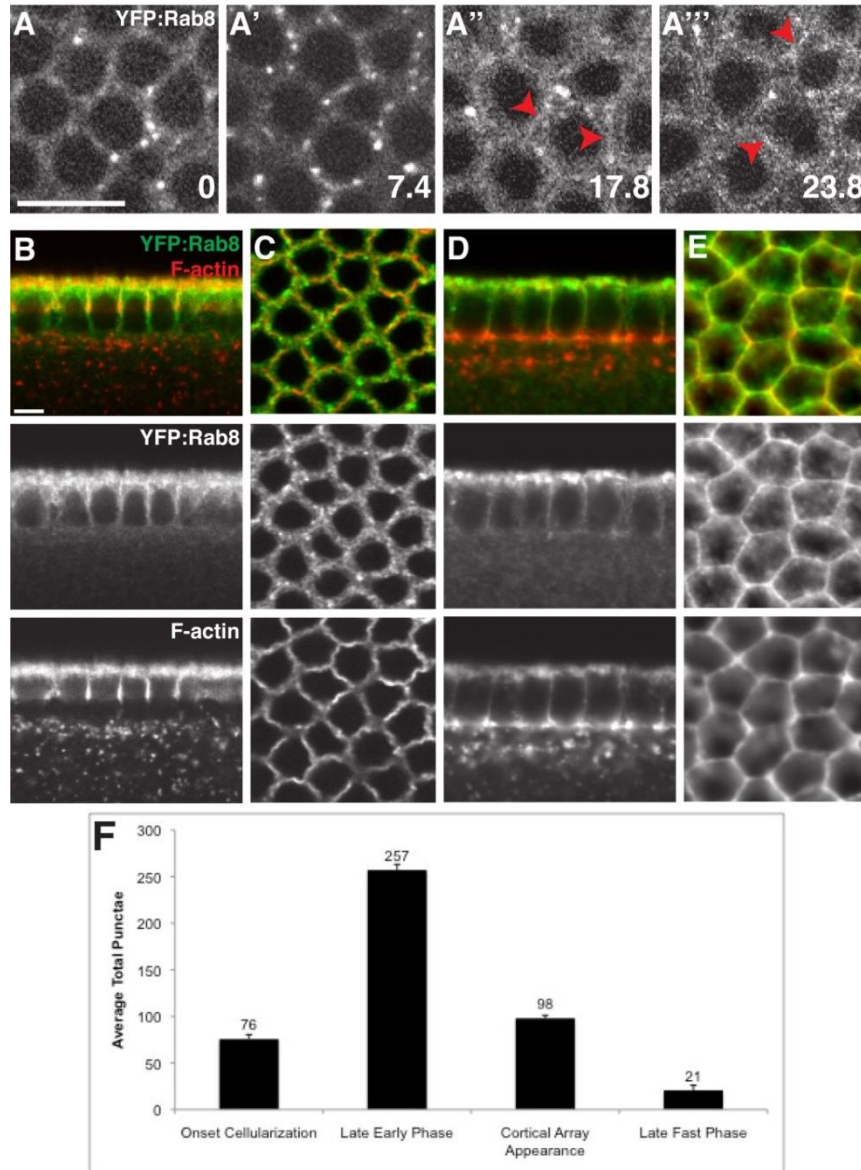


Figure 6. Rab8 dynamics during cellularization resemble those seen during syncytial divisions.

(A-A''') Still-frames measured from the onset of interphase 14 of the same embryo shown in Fig. 1A. (A) Rab8 exists in punctate structures at the onset of cellularization. (A') These puncta become more numerous and send out small tubular projections. (A'') At mid-cellularization, Rab8 transitions to a cortical association (red arrowheads). (A''') The cortical array persists through cellularization (red arrowheads). (B-E) Localization of YFP:Rab8 (anti-GFP, green) and F-actin (Phalloidin, red) in fixed, cellularizing embryos. (B and C) during early cellularization, Rab8 exists in apical punctate populations (B, viewed along apical-basal axis; C, planar view of the same embryo). (D and E) As fast-phase begins, Rab8 remains apically associated (D, apical-basal view) and transitions to a cortical population (E, planar view of the same embryo). (F) Quantification of vesicle counts, including s.e.m., shows that puncta number increases during cellularization and depletes at the appearance of a cortical array. (A) Time indicated in min. Scale bar in A is 10 μ m. Scale bar in B is 5 μ m.

In order to determine that the dynamics displayed in YFP:Rab8 embryos were not a result of overexpression of the UASp-YFP:Rab8 transgene, three approaches were taken to observe Rab8 dynamics at, or near endogenous expression levels. First, YFP:Rab8 was expressed under the control of Gal4 driven by the Rab8 promoter (Fig. 7A, B). Gal4 is responsible for binding the UAS promoter region and activating expression of UASp-YFP:Rab8 (Brand and Perrimon, 1993). The data shown in Figures 5 and 6 used Gal4 expressed by the maternally driven α Tubulin-Gal4 system (67;15), a potent promoter which drives high expression of Gal4 and ultimately strong expression of UASp-YFP:Rab8. Therefore, by placing Gal4 under control of the Rab8 promoter, it can be ensured that ectopic UASp-YFP:Rab8 is expressed at similar time periods as endogenous Rab8 although total expression levels may be enhanced in comparison to endogenous Rab8 expression.

A second ectopic transgenic line expressing GFP:Rab8 directly under the control of a genomic portion of the Rab8 promoter was created. To this end, 1.5kb upstream (5') of the Rab8 ATG start site was cloned into the pCasper4 plasmid (Addgene), followed by the coding sequence for GFP as well as the coding region of Rab8 plus 1.5kb downstream (3') of the Rab8 stop codon (Fig. 7C-J). In total, 5.04kb of the genomic Rab8 locus alongside the 718bp coding region of GFP was cloned and expressed in flies.

A final third expression line, with GFP directly inserted into the endogenous *Drosophila* Rab8 locus using the CRISPR-HDR technique (Gokcezade et al., 2014) was developed (Fig. 8A-H). The same GFP:Rab8 construct described above was cloned into the pBluescript plasmid (Addgene). Two short guideRNA sequences were also generated.

These guideRNA sequences are required to guide the Cas9 nuclease enzyme to specific genome locations. Cas9 then cuts the genomic DNA and these cut regions were designed to directly flank the Rab8 ATG start site. If the genome then repairs itself via homologous recombination, then GFP:Rab8 genetic material may be introduced to this site. Successful recombination events were scored by positive GFP fluorescence in embryos using a spinning-disk microscope. In total, 345 possible recombinant lines were generated. One line of the 345 was shown to successfully have inserted GFP and fluoresce (Fig. 8). Insertion of GFP into the Rab8 locus was verified via genomic PCR (described in methods) through the use of three primer pairs designed to amplify a product only when GFP was present downstream of the Rab8 promoter. PCR was performed in both CRISPR GFP:Rab8 and wild-type, OreR embryos to verify proper recombination had occurred (Fig. 8I, J). CRISPR GFP:Rab8 is fully functional and flies that are homozygous for CRISPR GFP:Rab8 are viable and fertile.

It can be seen in all three endogenous expression backgrounds that Rab8 dynamics are similar: Rab8 initially localizes to a dynamic punctate population and transitions to a cortical array during the formation of both syncytial and cellularization furrows (Fig. 7, 8). These results demonstrate that these dynamics reflect the behaviors displayed by endogenous Rab8 protein. Additionally, the similarity in Rab8 behavior during both syncytial divisions and cellularization suggests that both these processes may use Rab8-dependent machinery to initiate furrow formation.

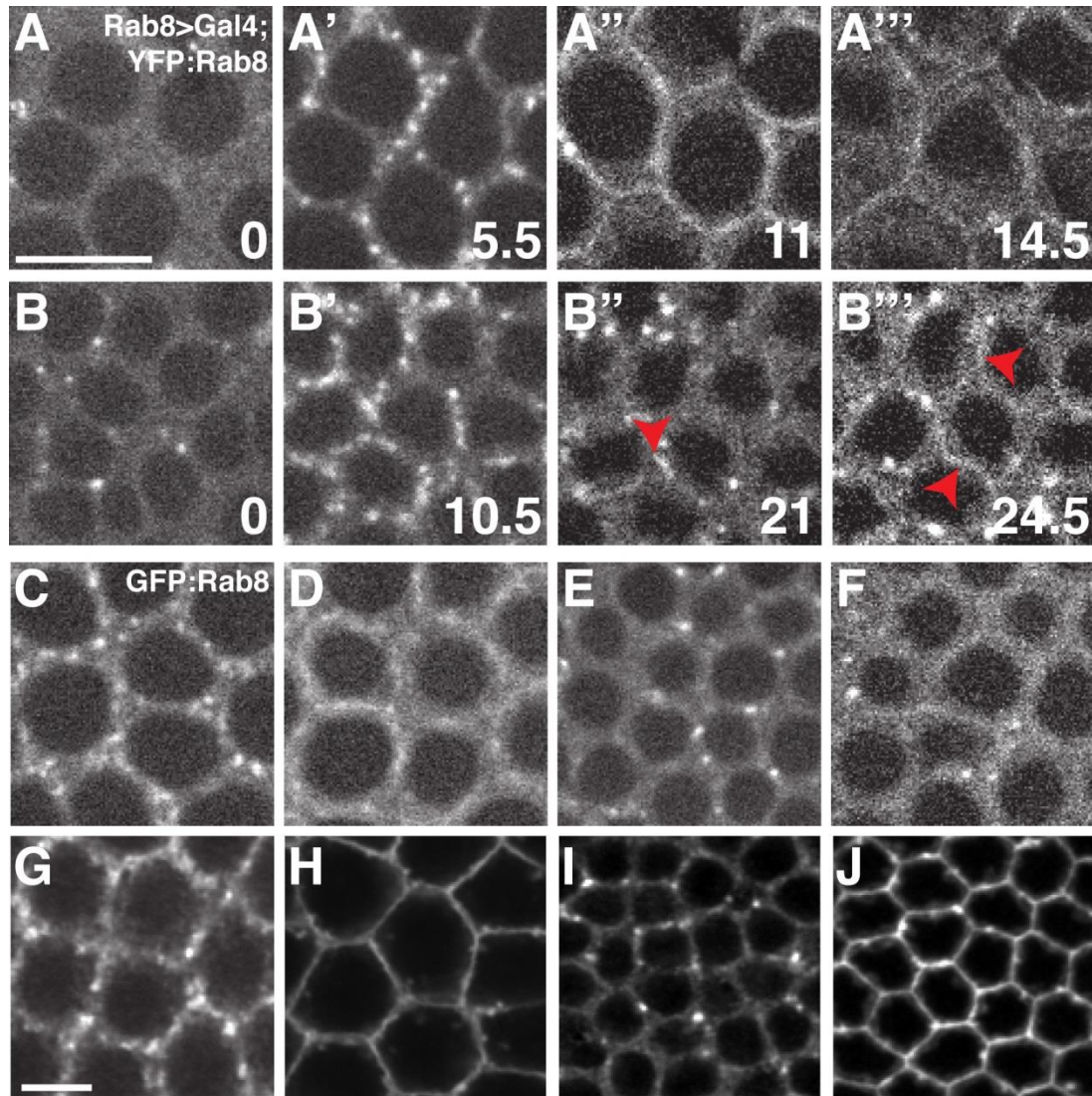


Figure 7. Rab8 expressed at endogenous levels displays dynamic transition from puncta to cortical association.

(A) Live-imaging of embryo expressing YFP:Rab8 driven by the Rab8-Gal4 promoter shows increase of puncta (A') and transition to stable cortical array (A'' and A''') during syncytial divisions. (B) The same embryo from (A) displays similar transition from punctate to cortical association during cellularization stages. Puncta increase in number over time (B, B') and begin to send out projections (B'', red arrowhead), before transitioning to a cortical array (B''', red arrowhead). (C-J) Stills of embryos expressing GFP:Rab8 under control of the Rab8 promoter from live-imaging (C-F) or fixed preparations (anti GFP, G-J). Transition from punctate (C, E, G, I) to cortical association (D, F, H, J) can be seen during both syncytial (C, D, G, H) and cellularization (E, F, I, J) cycles. (A, B) Time indicated in min. Scale bar in A is 10 μ m; scale in G is 5 μ m.

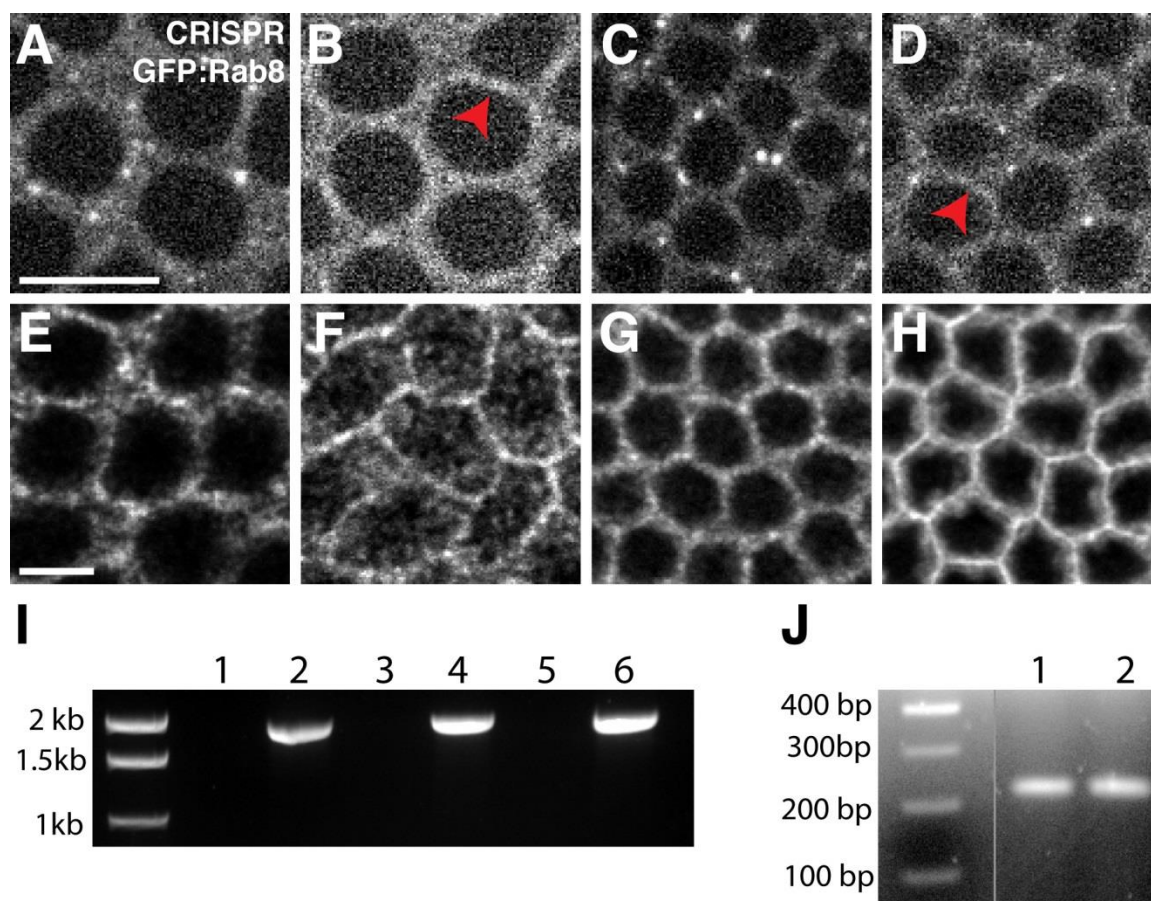


Figure 8. Successful insertion of GFP into the *Drosophila* genome displays dynamic transition from puncta to cortical association during furrow formation.

(A-D) Live imaging stills of embryos expressing CRISPR GFP:Rab8 shows transition from puncta (A, C) to cortical array (B, D; red arrowheads) in syncytial (A, B) and cellularization (C, D) stages. (E-H) Fixed images of embryos expressing CRISPR GFP:Rab8 (anti-GFP) show same transitions in syncytial (E, F) and cellularization (G, H) stages. (I) PCR verification of GFP:Rab8 insertion into the genome. Three primers pairs (lanes 1 and 2, 3 and 4, or 5 and 6) were designed with the 5' primer binding upstream of the cloned 1.5kb 5' of the Rab8 ATG start site present within the pBluescript KS plasmid. 3' primers were designed to bind to regions of the GFP coding sequence. Successful amplification of these regions can be seen in CRISPR GFP:Rab8 expressing embryos (lanes 2, 4 and 6), but not OreR control embryos (lanes 1, 3 and 5), demonstrating that GFP was properly inserted downstream of the Rab8 promoter. (J) Control PCR against primers located in the Rab35 gene locus demonstrate loading control of OreR control (lane 1) and CRISPR GFP:Rab8 (lane 2) genomic DNA products. Scale bar in A is 10µm; scale bar in E is 5µm.

Disruption of Rab8 leads to a failure of furrow ingression and F-actin network formation

Both fixed and live imaging of Rab8 structures demonstrate dynamic behaviors and a strong association with the development of membrane furrows. This led to the examination of the effects of Rab8 knockdown on furrow formation. Complete knock-out of Rab8 via the creation of germline clones could not be performed as the Rab8 and FRT chromosomal locations are not far enough apart from one another to elicit the genetic recombination required to generate mutant clones. As a result, knock-down experiments using RNAi targeted against the Rab8 gene were performed. dsRNA was introduced by microinjection into a WT background and embryos were scored for defective phenotypes (Fig. 9). Two different dsRNAs directed against Rab8 were used. Both dsRNAs demonstrated a severe disruption in embryonic development (Fig. 9). Importantly, the majority of dsRNA injected embryos arrested at stages requiring furrow ingression. 40% of embryos injected with SNAPDRAGON-designed (DRSC, Harvard) Rab8 dsRNA arrested during syncytial divisions (Fig. 9, n=89), while 56% arrested during cellularization (Fig. 9).

In order to examine the effects of Rab8 on furrow formation, RNAi of Rab8 was performed in embryos expressing Histone:RFP and GFP fused to the actin-binding-domain of Moesin (GFP:MoeABD). This allowed the visualization of nuclei and the ingressing furrows concurrently during development. As compared to water-injected control embryos, Rab8 RNAi has a dramatic effect on the formation of furrows. In water-treated embryos, metaphase furrows form and ingress during cycles 10-13 to prevent

chromosomal mixing during nuclear divisions (Fig. 10A). In Rab8 knockdown embryos, furrow formation was disrupted and ingression furrows were not formed (Fig. 10B). Cortical F-actin that would normally associate apically with ingressing furrows is completely disrupted in these severely affected embryos (Fig. 10B). Nuclei aggregated from improper division cycles and subsequently dropped from their subcortical positions (Fig. 10B). The defects in these syncytial cycles were severe, and resulted in embryos with no formation of an epithelial sheet (Fig. 10B). These knockdown experiments demonstrate that Rab8 plays a vital role directing formation and ingression of furrows in the early embryo.

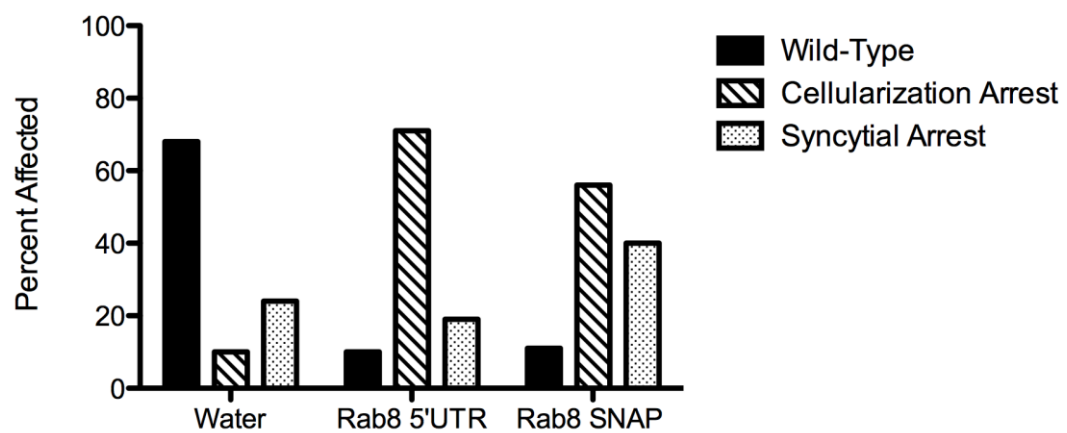


Figure 9. Knockdown of Rab8 via RNAi leads to early developmental defects in embryos.

Embryos were injected with water or Rab8 dsRNA prepared against two different regions of the Rab8 gene and phenotypic defects were quantified. Water (n=71), Rab8 5'UTR (1.3 μ g/ μ L; n=70), Rab8 SNAP (1.4 μ g/ μ L; n=89). $P < 0.0001$ for all changes in proportions across groups. $P < 0.0001$ for each treatment group when compared to proportions seen in water injected control embryos

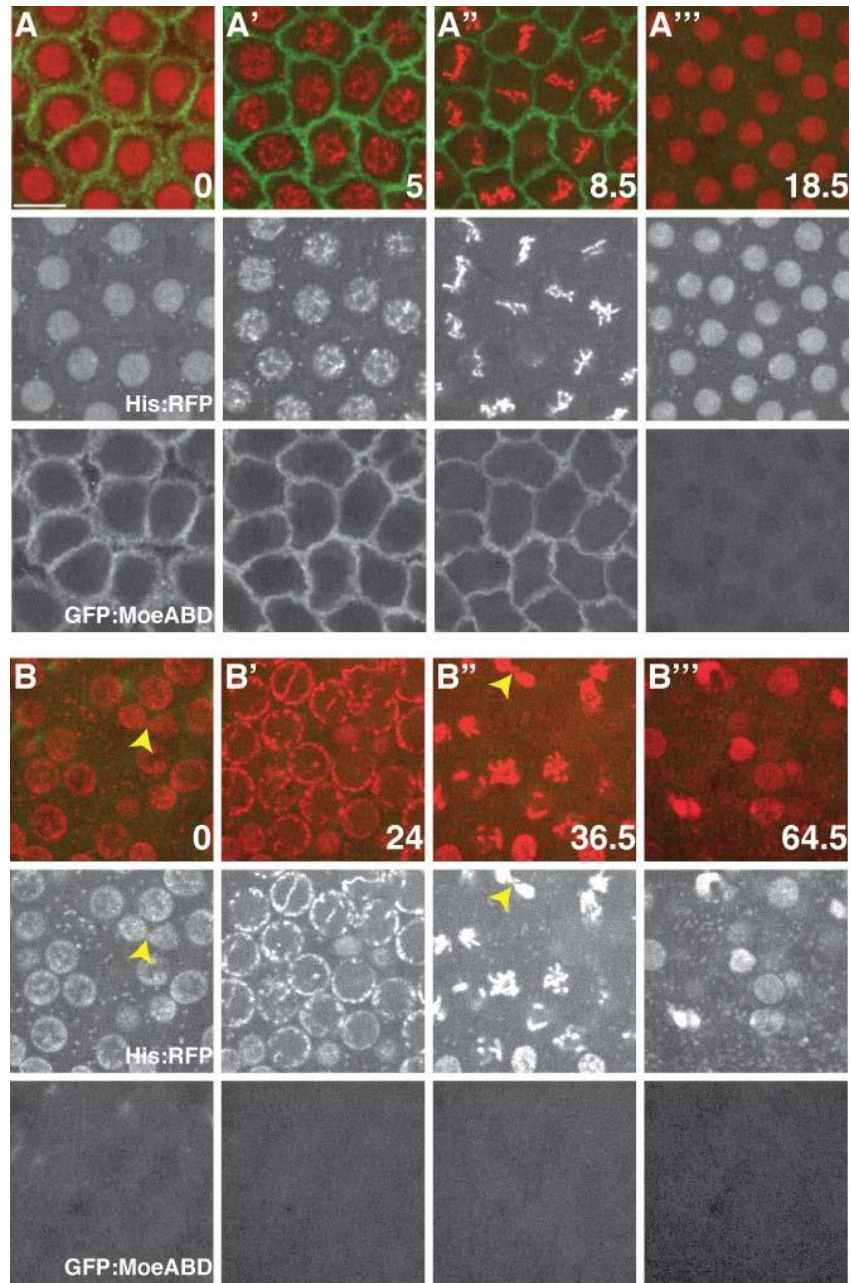


Figure 10. Knockdown of Rab8 severely disrupts furrow ingression in the early embryo.

(A-B) Whole-mount embryos expressing Histone:RFP (His;RFP; DNA, red) and GFP:MoeABD (F-actin, green). (A-A'') Still-frames of water-injected embryos imaged from a planar orientation. (A) Control embryos show proper formation of F-actin furrows at the onset of division cycle. (A' and A'') F-actin furrows form during condensation of DNA. (A'') Post-division, F-actin furrows retract. (B-B'') Still frames of Rab8 RNAi injected embryos imaged in a planar orientation. (B) F-actin furrows fail to form and result in incomplete nuclear divisions (yellow arrowhead). (B'-B'') F-actin furrows are absent and nuclei show irregular spacing and failure of proper separation (B'', arrowhead). Aberrant nuclei undergo nuclear fallout and lose attachment, depleting the cortical nuclear layer (B'', B''). Time indicated in min. Scale bar is 10 μ m.

Rab8 associates with secretory pathways and the exocyst during cellularization

The dynamic localization of Rab8 and the protein's association during fast-phase in the apical portions of ingressing furrows led to examine if Rab8 is responsible for delivering the membrane that is necessary for furrow ingression to occur. In order to address this question, immunohistochemistry was performed against YFP:Rab8 and Neurotactin (Nrt), a transmembrane protein that transits through the exocytic pathway and is delivered to the plasma membrane (Pelissier et al., 2003; Murthy et al., 2010). Immunostaining reveals a strong level of coincidence between Rab8 and Nrt in both early and late cellularization stages (Fig. 11A, B). Rab8 and Nrt coincide apically at the cortex of cells, but also show a high degree of colocalization in cytoplasmic compartments (Fig. 11A, B, white arrowheads). In early cellularization stages, 53% (n=107) of Nrt compartments stained positive for Rab8, whereas 45% (n=107) of Rab8 puncta were positive for Nrt (Fig. 11A, E). Association of cytoplasmic Nrt and Rab8 remained high during mid-cellularization stages with 65% (n=103) of Nrt vesicles coinciding with Rab8, and 48% (n=109) of Rab8 puncta colocalizing with Nrt (Fig. 11B, E). These data demonstrate that Rab8 localizes with cargo of the exocytic trafficking pathway and may be responsible for delivering new membrane to the cortex.

The association of Rab8 with the apicolateral cell cortex and Neurotactin puncta led us to determine if Rab8 colocalized with the exocyst complex. The exocyst is an eight member protein complex implicated in the delivery and docking of vesicles to the plasma membrane, and localizes to the apicolateral cortex where membrane addition occurs (Lecuit and Wieschaus, 2000; Murthy et al., 2010). Immunostaining against YFP:Rab8

and the exocyst member, Sec5, was performed to examine colocalization between these proteins. Fixed imaging revealed a strong association between Rab8 and Sec5 at the cortex as well as in cytoplasmic compartments (Fig. 11D) during the fast-phase of cellularization. Colocalization of Rab8 and Sec5 in cytoplasmic puncta was also observed in early cellularization (Fig. 11C). 38% (n=95) of Sec5 positive compartments co-stained for Rab8, whereas 20% (n=107) of Rab8 positive puncta were also positive for Sec5 (Fig. 11C, E). However, Sec 5 puncta are largely absent within the cytoplasm by mid-cellularization and colocalization with Rab8 occurs primarily at the cortex (Fig. 11D). These data not only confirm Rab8's localization in late exocytic trafficking pathways but also suggest a possible interaction between Rab8 and the exocyst in the delivery of membrane to ingressing furrows.

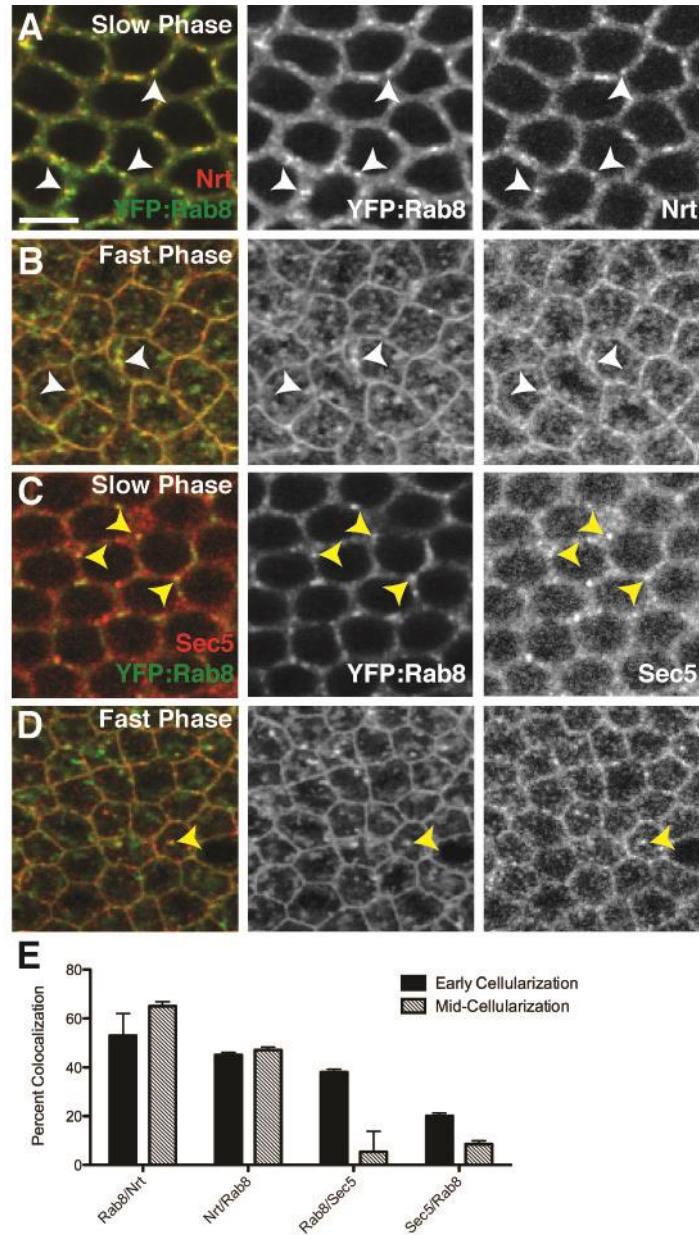


Figure 11. Rab8 compartments demonstrate colocalization with newly delivered membrane and the exocyst during cellularization.

(A, B) YFP:Rab8 (anti-GFP, green); Neurotactin (Nrt), (anti-Nrt, red). (A) Fixed embryo in early cellularization demonstrates high level of overlap between YFP:Rab8 puncta populations and Nrt puncta (white arrowheads). (B) Fixed embryo in mid-cellularization shows strong colocalization between Rab8 and Nrt in both cytoplasmic compartments (arrowheads) and at the cortex. (C, D) YFP:Rab8, (anti-GFP, green); Sec5 (anti-Sec5, red). (C) YFP:Rab8 puncta and Sec5 compartments show high level of coincidence in early cellularization (yellow arrowheads). (D) During mid-cellularization, Rab8 and Sec5 demonstrate coincidence in both cytoplasmic compartments (yellow arrowhead) and at the cortex. (E) Quantification of percentage of colocalization between Rab8, Nrt and Sec5 compartments in early and mid-cellularization including standard error. Scale bar is 5 μ m.

Rab8 function is required for dynamic membrane addition

To examine if Rab8 regulates membrane addition during furrow ingression, RNAi was performed in a Gap43:mCherry background. This Gap43 marker consists of a 20 amino acid peptide containing a double palmitoylation sequence which directs mCherry to the plasma membrane and possibly intracellular compartments via raft association (Fig. 12A). Upon knockdown of Rab8, trafficking of Gap43 and associated membrane is severely disrupted; Gap43 resides in cytoplasmic puncta and never associates with the cortex of ingressing furrows (Fig. 12B). These results suggest that Rab8 indeed functions as a regulator of exocytic trafficking of membrane during furrow formation.

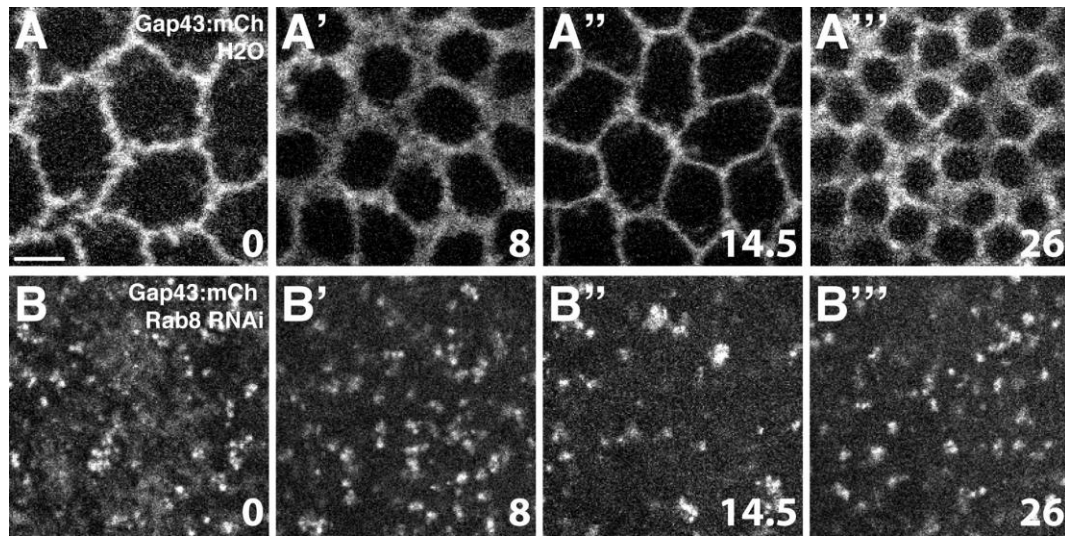


Figure 12. Disruption of Rab8 results in failure of proper membrane trafficking to ingressing furrows.

(A-A''') Water-injected control embryo expressing Gap43:mCherry. Gap43 associates with the cortex of furrows during syncytial divisions (A and A''') and in cytoplasmic puncta during retraction phases of transient furrows (A', A''). (B-B''') Rab8 RNAi treated embryo expressing Gap43:mCherry. Knockdown of Rab8 results in Gap43 existing in cytoplasmic puncta and a failure to localize cortically. Time indicated in min. Scale bar is 10 μ m.

Rab8 localization and dynamics are GTPase activity dependent

To examine how the GTP state of Rab8 directs its activity, the localization of GTPase or GDP-dissociation deficient mutants was observed. It was hypothesized that GTP-bound Rab8 represented the active population and would associate with those compartments in which Rab8 is directing membrane dynamics. Likewise, GDP-bound Rab8 would most likely represent inactive pools of the protein. To this end, both live imaging and immunostaining against these activity-deficient mutants of Rab8 was performed in order to examine how the GTP state affects the localization of Rab8 in relation to other compartments involved in furrow ingression.

CA or DN versions of YFP-tagged Rab8 were expressed in embryos to examine changes in protein behavior over time. Immunostaining revealed that Rab8 CA forms similar structures to Rab8WT (compare Fig. 13A, B and 6B, C). However, it can be noted that the cortical array arises earlier, during the slow phase of cellularization, as opposed to the fast-phase in WT embryos (compare Fig. 13B and 6E). Large cytoplasmic aggregates of Rab8 DN form and these aggregates localize to apical portions of the cell (Fig. 13C). Very low levels of Rab8 DN can also be seen in the cortical array when stained embryos are imaged at high gain settings (Fig. 13D).

Effects of GTPase activity on Rab8 behavior were also examined in whole mount embryos expressing either Rab8 CA or Rab8 DN over time. Again, Rab8 CA showed similar behaviors to WT. During early cellularization, Rab8CA exists in punctate populations before transitioning to a cortical array which persists until the end of cellularization (Fig. 13E-E'''). Expression of Rab8 DN in whole embryos shows the

progressive appearance of apical cytoplasmic aggregates, which grow and persist throughout the cellularization process (Fig. 13F-F’’’). The above results suggest that active, GTP-bound Rab8 localizes to punctate and cortical array Rab8 populations while inactive, GDP-bound Rab8 is cytoplasmic.

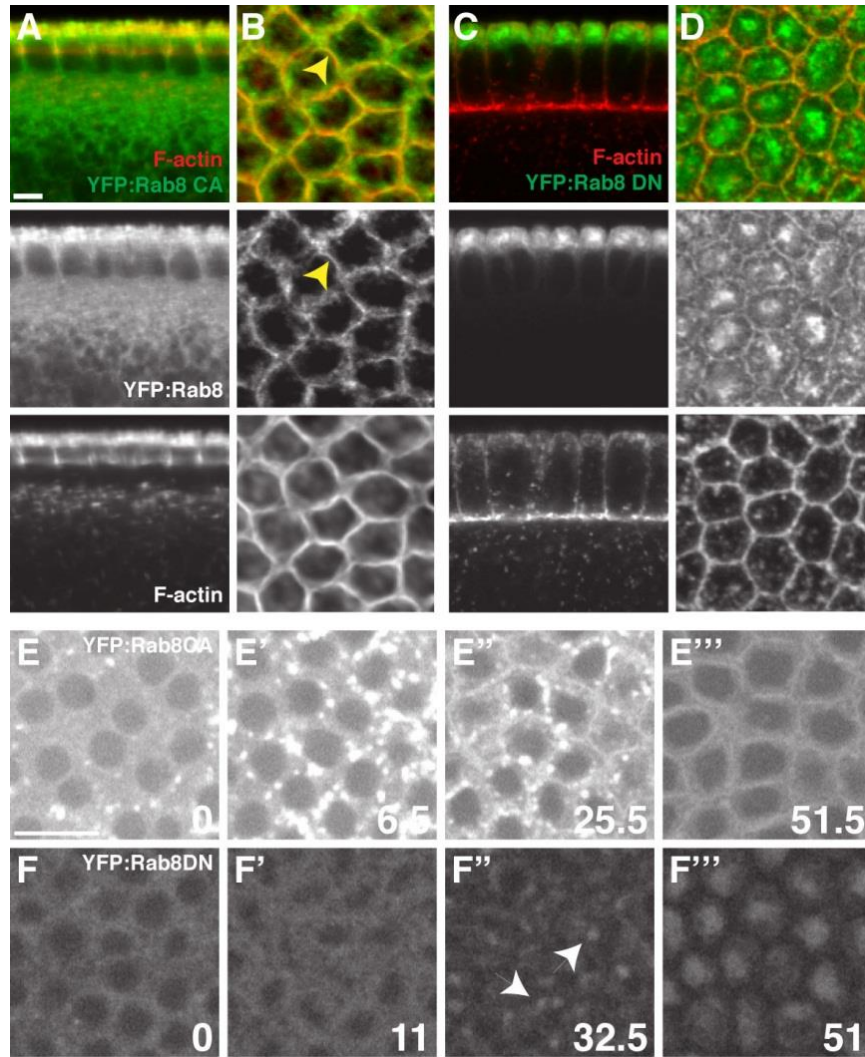


Figure 13. Rab8 cortical behaviors are GTPase activity dependent *in vivo*.

(A and B) YFP:Rab8 CA (anti-GFP, green); F-actin, (Phalloidin, red). Fixed images show that Rab8 CA localizes apically in early cellularization stages (A, apical-basal view) and prematurely forms a cortical array (B, arrowhead, planar view of the same embryo). (C and D) YFP:Rab8 DN (anti-GFP, green), F-actin, (Phalloidin, red). Large aggregates of Rab8 DN form above the nuclei in the cytoplasm (C, apical-basal view; D, planar view, same embryo). (E-E''') Still-frames of live-imaged, YFP:Rab8 CA embryo measured from the onset of interphase 14. (E) Initially, Rab8 CA exists in punctate structures. (E') These puncta become more numerous and send out tubule structures. (E'') Rab8 CA transitions to a cortical array. (E''') Cortical arrays persist through cellularization. (F-F''') Still-frames of live-imaged, YFP:Rab8 DN embryo measured from the onset of interphase 14. (F, F') Punctate structures are lacking in Rab8 DN embryo at the onset (F) and early (F') stages of cellularization. (F'') As cellularization proceeds, cytoplasmic aggregates begin to form (arrowheads). (F''') In later stages, aggregates become larger and denser. Embryos in (E) and (F) were leveled via photoshop at same levels to demonstrate differences in protein expression for comparison. (E, F) Time indicated in min. Scale bar in A is 5 μ m. Scale bar in E is 10 μ m.

Rab8 populations are largely distinct from recycling endosomes and the Golgi

Previous reports have shown the functional requirement of the recycling endosome (RE) and the Golgi during furrow ingression in the early embryo (Pelissier et al., 2003; Sisson et al., 2000). This led us to determine whether Rab8 structures are coincident with the RE or Golgi. Immunostaining against YFP:Rab8 and Rab11, an RE marker, show that these proteins are largely present in distinct compartments (Fig. 14A, B). When viewed from a planar orientation, Rab11 and Rab8 display minimal overlap as both punctate and cortical Rab8 populations demonstrate little coincidence with Rab11 during both the slow (Fig. 14A) and fast phases (Fig. 14B) of cellularization. However, at a low frequency there are Rab8 puncta that also immunostain for Rab11. Quantitation of fixed mid-cellularization embryos, when fast phase initiates, demonstrated that 6.6% of Rab11 vesicles localize with Rab8 (n=105), and 15.3% of Rab8 vesicles coincided with Rab11 vesicles (n=93; Fig. 14G). Intriguingly, compartments that colocalize Rab8 and Rab11 occurred with a higher frequency in early cellularizing embryos (Figure 14A). Quantitation of early, slow phase embryos showed that 18% of Rab11 vesicles were coincident with Rab8 (n=94, Fig. 14A, G), and 10% of Rab8 vesicles coincided with Rab11 vesicles (n=107; Fig. 14B, G). It may be that these numbers reveal a transient interaction between Rab11 and Rab8 compartments, and that this interaction is more prevalent during early cellularization stages when cytoplasmic membrane stores are mobilized.

Rab8 also forms a distinct compartment from the Golgi. Rab8 vesicle populations show little association with Golgi compartments (Fig. 14C, D). In mid-cellularization

stages, 10% of Golgi compartments were coincident with Rab8 vesicles (n=108; Fig. 14D, G) compared to 14% of Rab8 vesicles coinciding with Golgi compartments (n=91; Fig. 14D, G). Converse to what was observed with Rab11, quantitation of early cellularizing embryos showed that 1.9% of Golgi compartments localized with Rab8 puncta (n=92, Fig. 14C, G), whereas 0% of Rab8 puncta coincided with the Golgi (n=108; Fig. 14C, G) at this stage. Once again, the slight colocalization between Rab8 puncta and Golgi would be consistent with a possible transient interaction between Rab8 and the Golgi that occurs with higher frequency in mid-cellularizing embryos. The colocalization of Rab8 with the RE and Golgi likely represents a real association, as Rab8 vesicles display minimal colocalization with Hrs (2.8%), an early lysosomal compartment marker (n=103, Fig. 14E, F and G) during both phases of cellularization.

The effects GTPase activity on the localization of Rab8 in relation to the RE and Golgi were also examined. Rab8 CA forms distinct compartments from both the RE and Golgi (Fig. 15A, D), comparable to Rab8 WT (Fig. 14B, D). Rab8 DN also does not appear to associate with Golgi compartments (Fig. 15E, F). However, Rab11 compartments appear enlarged in embryos expressing Rab8 DN when compared to those in Rab8 CA and WT (Fig 15B, C). This enlargement is apparent both in apical planes (Fig. 15B) and more basal sections (Fig. 15C). This would be consistent with a requirement for Rab8 GTPase activity to traffic membrane from the RE to the ingressing FC and further supports that these two compartments may interact with each other.

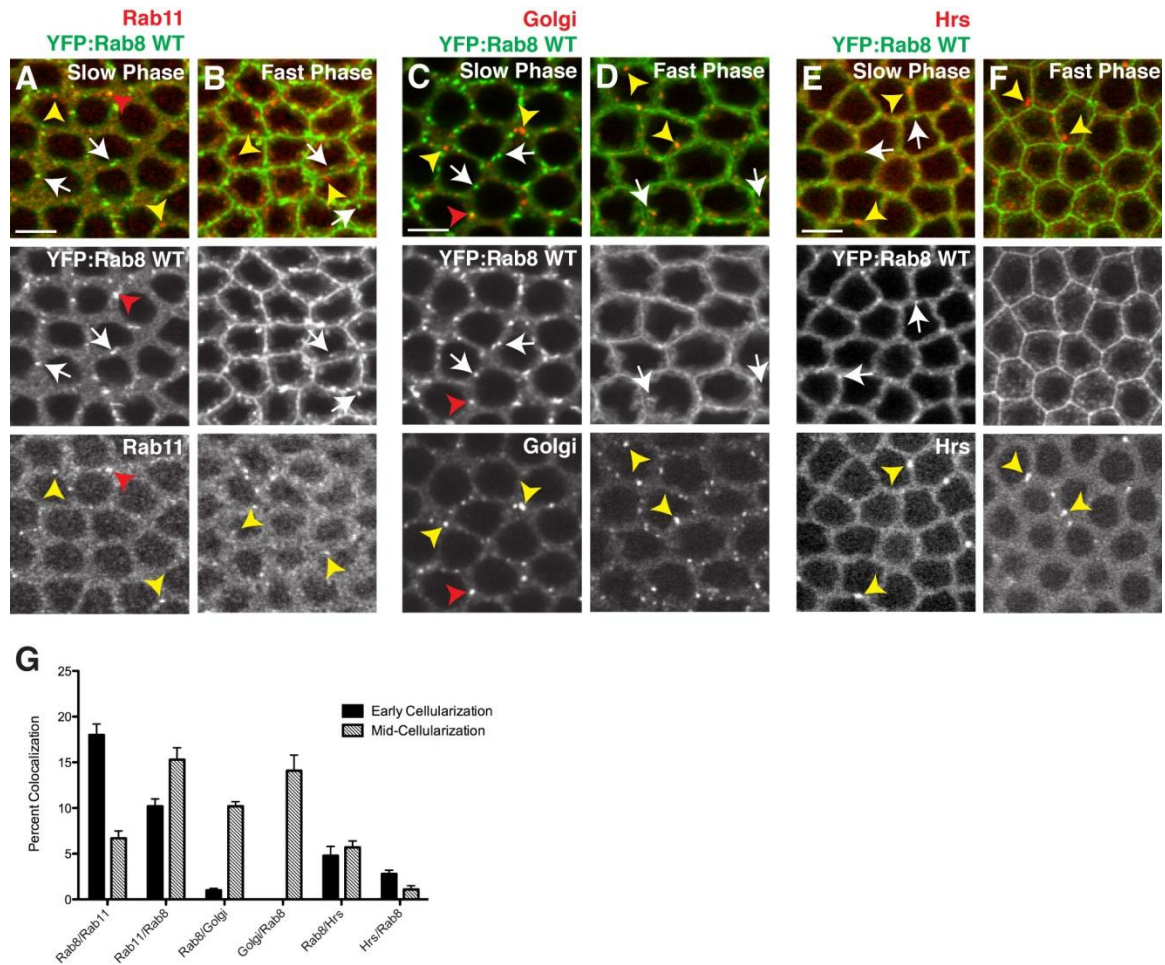


Figure 14. Rab8 forms a distinct compartment from the RE and Golgi body.

(A-B) YFP:Rab8 (anti-GFP, green); RE (anti-Rab11 red). (A) Early cellularization embryo. Rab8 WT puncta (arrows) show little colocalization with the RE (yellow arrowheads), however occasional overlap between compartments is seen (red arrowhead). (B) During mid-cellularization, Rab8 puncta (arrow) are distinct from the RE (yellow arrowheads). (C-D) YFP:Rab8 (anti-GFP, green); Golgi (anti-Lva, red). (C) Rab8 WT (arrows) and Golgi compartments (yellow arrowheads) form distinct populations and show little colocalization in early cellularization. (D) Rab8 WT (arrows) and Golgi compartments (yellow arrowheads) remain distinct in late cellularization. (E-F) YFP:Rab8 (anti-GFP, green); Late endosome (anti-Hrs, red). (E and F) Rab8 and late endosomes (yellow arrowheads) form distinct compartments in early (E) and late cellularization (F) stages. (G) Quantitation of percentage of colocalization including standard error between Rab8, RE, Golgi and late endosomal compartments in early and mid-cellularization. Scale bars are 5µm.

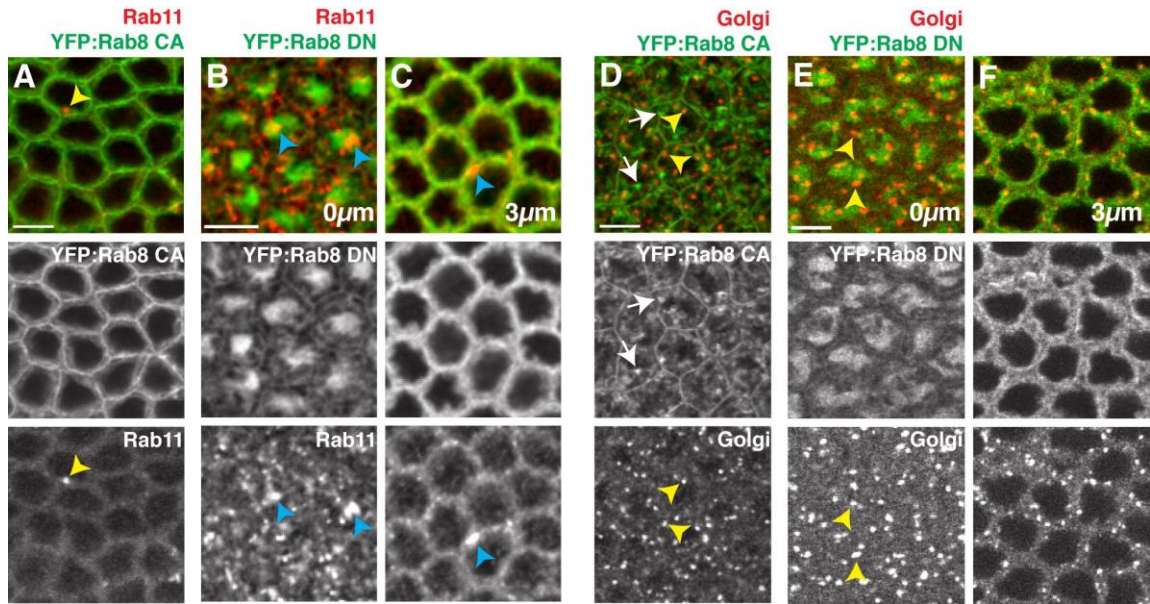


Figure 15. Rab8 localization with RE and Golgi is GTPase activity-dependent.

(A-C) YFP:Rab8 (anti-GFP, green), RE (anti-Rab11, red). (A) Rab8 CA puncta remain distinct from RE compartments (yellow arrowhead). (B) Rab8 DN forms large aggregates at the apical surface; these aggregates remain largely distinct from RE puncta (yellow arrowheads), however, the RE is enlarged in Rab8 DN expression (blue arrowheads). (C) At more basal levels (3μm below F) Rab8 DN is largely cytoplasmic and RE compartments are enlarged (blue arrowheads). (D-F) YFP:Rab8 (anti-GFP, green), Golgi (anti-Lva, red). (D) Rab8 CA (arrows) and Golgi compartments (yellow arrowheads) also form separate populations and show little overlap (D, compare arrows to arrowheads). (E and F) Rab8 DN aggregates do not colocalize with the Golgi (arrowheads); Rab8 DN expression does not cause an enlargement of the Golgi compartment at apical (E, 0μm) or more basal levels (F, 3μm from E). Scale bars are 5μm.

Sec5 and Rab11 function are required for targeting of Rab8 to the cell cortex

Due to the high level of coincidence seen between Rab8 and Sec5 in fixed images (Fig. 11B, C), we asked if Rab8 required the exocyst to behave dynamically in order to deliver membrane. RNAi against Sec5 was performed in a YFP:Rab8 background and imaged over time. These experiments resulted in a failure of Rab8 to make the transition from punctate to cortical array (Fig 16B-B'') as compared to water injection controls (Fig. 16A-A''), suggesting that Rab8 may be utilizing the exocyst to properly localize to the plasma membrane.

Despite lower levels of overlap than that seen with transmembrane proteins or Sec5, results still suggested that Rab8 and the RE might be transiently interacting to regulate membrane addition during cellularization. Thus, RNAi was performed against Rab11 to ask if disruption of the RE resulted in dynamic changes in Rab8 protein behavior. Similar to Sec5 knock-down, disruption of Rab11 led to a loss of Rab8 puncta to cortical array dynamics over time (Fig. 16C-C''). Interestingly, when RNAi of Rab8 is performed in a YFP:Rab11 background, compared to water controls (Fig. 16D-D''), Rab11 puncta dynamics are not affected (Fig. 16E-E'') but recycling endosomes appear slightly enlarged (Fig. 16E', E'') . Taken together, these data along with the Sec5 RNAi data suggest that Rab8 requires both the exocyst and RE in order to dynamically transition from punctate to cortical localization. It is possible that Rab8 requires the exocyst as a localization cue in order to properly associate with the cortex, while functioning downstream of the RE and requiring Rab11 to activate and dynamically reorganize to the cortex. The slight enlargement of RE puncta in a Rab8 RNAi

background also strengthens the possibility that Rab8 functions with the RE to deliver membrane material to the plasma membrane during furrow ingression.

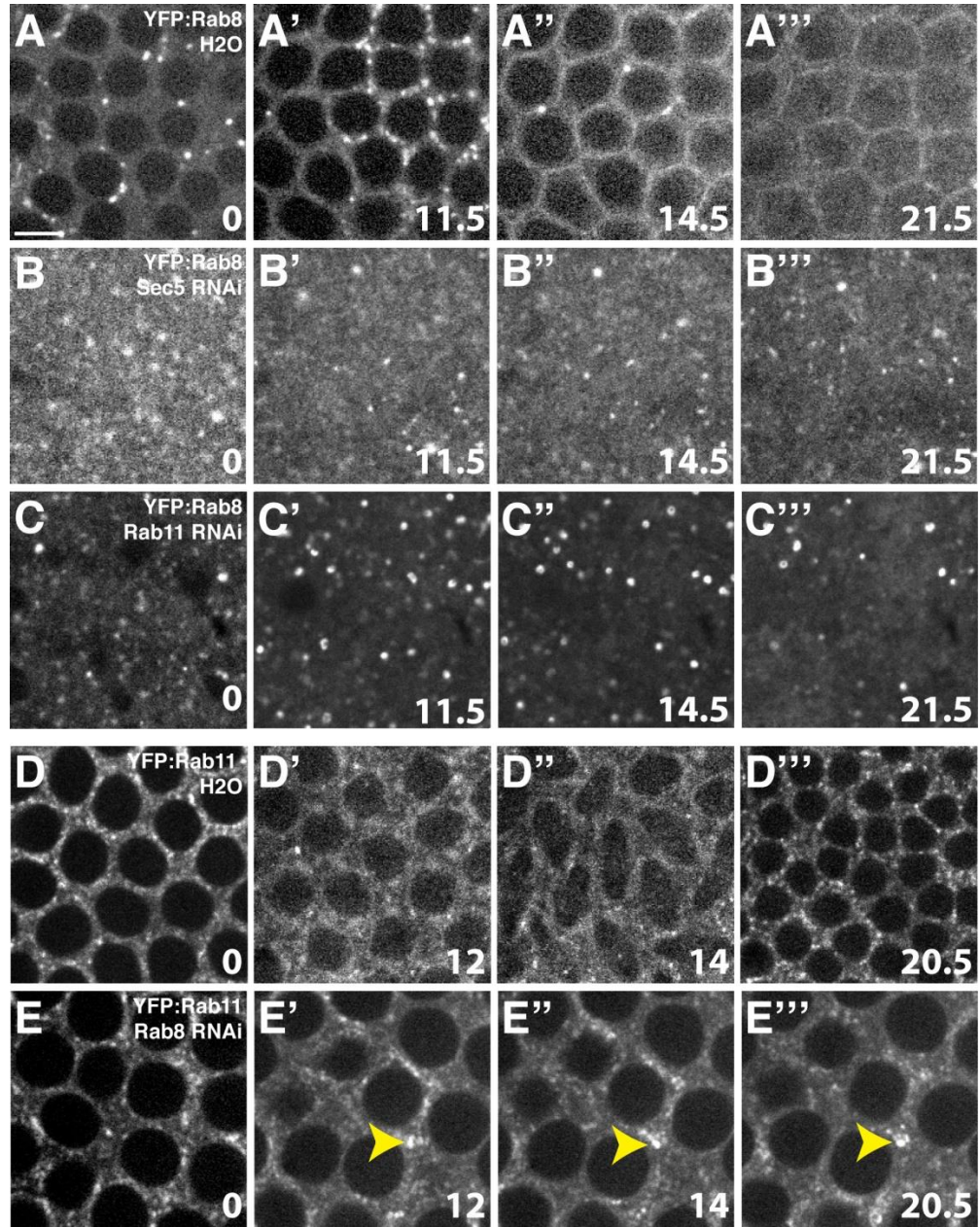


Figure 16. Rab8 requires the exocyst and RE to behave dynamically and regulate membrane addition during furrow formation

(A-A'') Water-injected control embryo expressing YFP:Rab8. Rab8 shows dynamic transition from punctate to cortical association over time. (B-B'') Sec5 RNAi treated embryo expressing YFP:Rab8. Disruption of Sec5 results in a failure for Rab8 to adopt cortical localization over time. (C-C'') Rab11 RNAi treated embryo expressing YFP:Rab8. Disruption of the RE also results in a failure of Rab8 to localize cortically. (D-D'') Water-injected control embryo expressing YFP:Rab11. Rab11 exists in punctate population over time during syncytial division cycles. (E-E'') Rab8 RNAi treated embryo expressing YFP:Rab11. Disruption of Rab8 does not appear to affect Rab11 population dynamics, but compartments become enlarged over time (yellow arrowhead). Time indicated in min. Scale bar is 10 μ m.

Rab8 requires RalA in order to target to the cortex

Ras-like protein A (RalA), is a small GTPase which has been demonstrated to function through exocyst subunits Sec5 and Exo84 to control polarized membrane addition (Moskalenko et al., 2002). Recent studies performed in our own lab have shown that RalA is a critical regulator of membrane addition during both transient and permanent furrow formation (Holly et al., In Review). Due to its function during furrow formation and interaction with exocyst subunits, we analyzed effects of RalA knock-down on Rab8 behaviors. RalA RNAi treatments result in a drastic loss of Rab8 activity (Fig. 17B-B’’’). Rab8 remains in punctate compartments and fails to associate with the cortex, comparable to Sec5 RNAi treatments (Fig. 17B). Conversely, while Rab8 RNAi results in a loss of proper furrow formation (Fig. 17D’’, D’’’), proper RalA behaviors appear to remain unperturbed at early stages of knockdown treatment (Fig. 17D, D’). These results taken with effects of Sec5 knockdown further strengthen a model in which Rab8 functions downstream of the exocyst to deliver membrane in a directed fashion during furrow ingression.

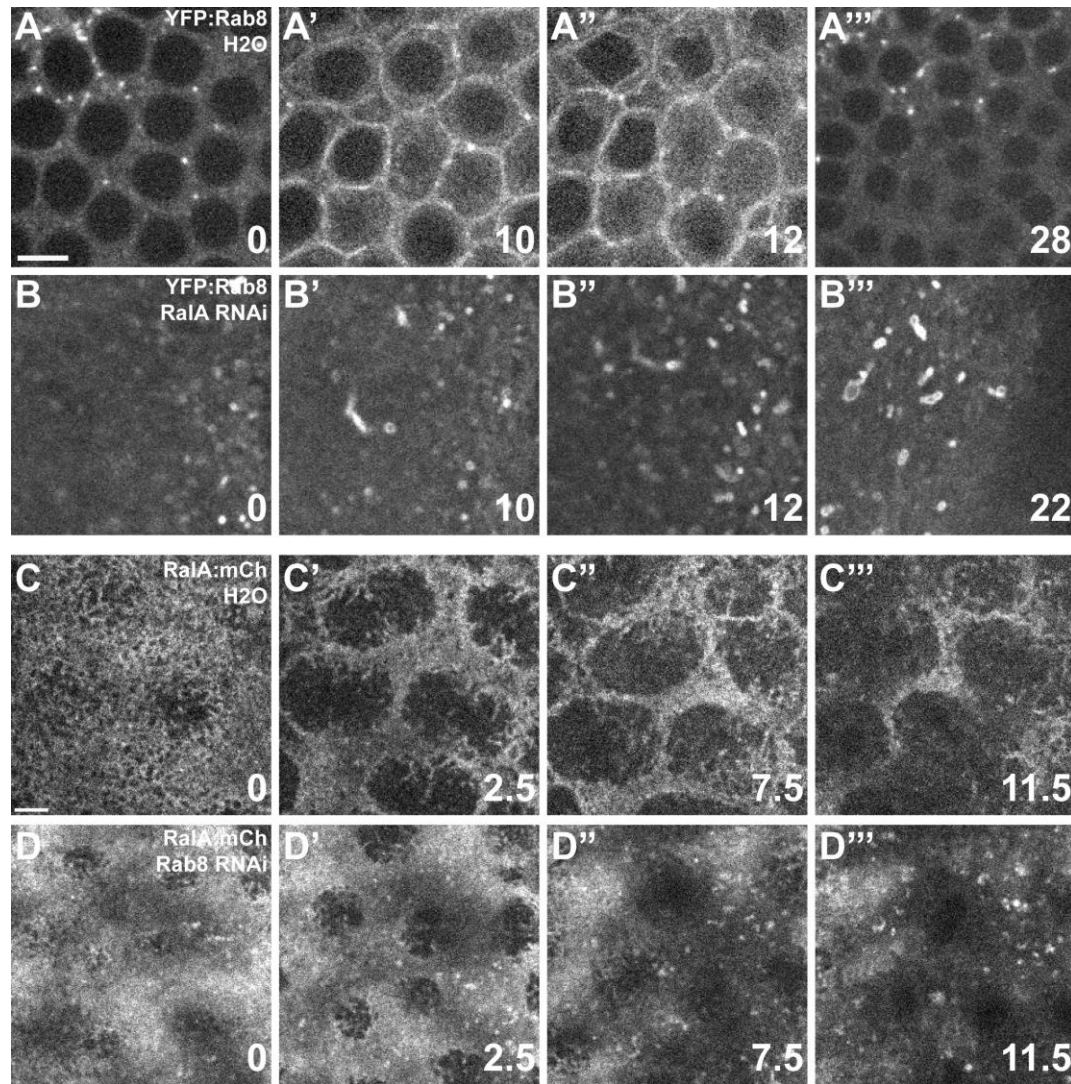


Figure 17. Rab8 requires RalA to behave dynamically and regulate membrane addition during furrow formation.

(A-A''') Water-injected control embryo expressing YFP:Rab8. Rab8 shows dynamic transition from punctate to cortical association over time. (B-B''') RalA RNAi treated embryo expressing YFP:Rab8. Disruption of RalA results in a failure for Rab8 to adopt a cortical localization over time. (C-C''') Water-injected control embryo expressing RalA:mCh. RalA is able to dynamically associate with furrows during transient furrow formation. (D-D''') Rab8 RNAi treated embryo expressing RalA:mCh. Disruption of Rab8 leads to a loss of furrow formation, however RalA localization remains unchanged at early stages (D, D'), suggesting Rab8 does not affect RalA dynamics. Time indicated in min. Scale bars are 10 μ m. Figure adapted from Holly et al., In Review.

Rab8 tubule structures extend basally and precede F-actin

The transition from punctate structures to cortical association by Rab8 as well as its function during furrow ingression displays dynamic new protein behaviors within the Rab protein family. Moreover, these behaviors suggest that Rab8 actively interacts with cytoskeletal components. To better understand the dynamics of Rab8 *in vivo*, embryos expressing YFP:Rab8 were dissected and spread onto a coverslip. This technique allows for better optical resolution and a similar approach has been used to image microtubule-dependent movements of lipid droplets in the early embryo (Welte et al., 1998). Live imaging of these dissected preparations revealed that Rab8 is capable of forming striking tubular structures, which display dynamic behavior and are capable of extending many microns over short time periods (Fig. 18A, B). We then asked how these Rab8 tubules relate to the furrow canal and ingression of the F-actin network during furrow formation.

Immunostaining of YFP:Rab8 revealed the presence of long, basally-directed Rab8 tubules. These tubules precede the contractile F-actin network during furrow ingression, and first arise in syncytial blastoderm embryos during cortical divisions (Fig. 18C). During syncytial nuclear divisions, Rab8 forms projections that are directed towards the interior of the embryo and mark future sites of furrow ingression (Fig. 18C). As the F-actin front proceeds inward it overtakes the Rab8 tubules, and F-actin and Rab8 colocalize (Fig. 18D). Similar tubular structures arise during cellularization, although they do not extend as far basally as they do during earlier cycles. Prior to the onset of cellularization, Rab8 tubules project interiorly between nuclei (Fig. 18E). By mid-

cellularization, Rab8 and F-actin fully overlap along the length of the ingression furrow and Rab8 no longer precedes the cellularization front (Fig. 18F).

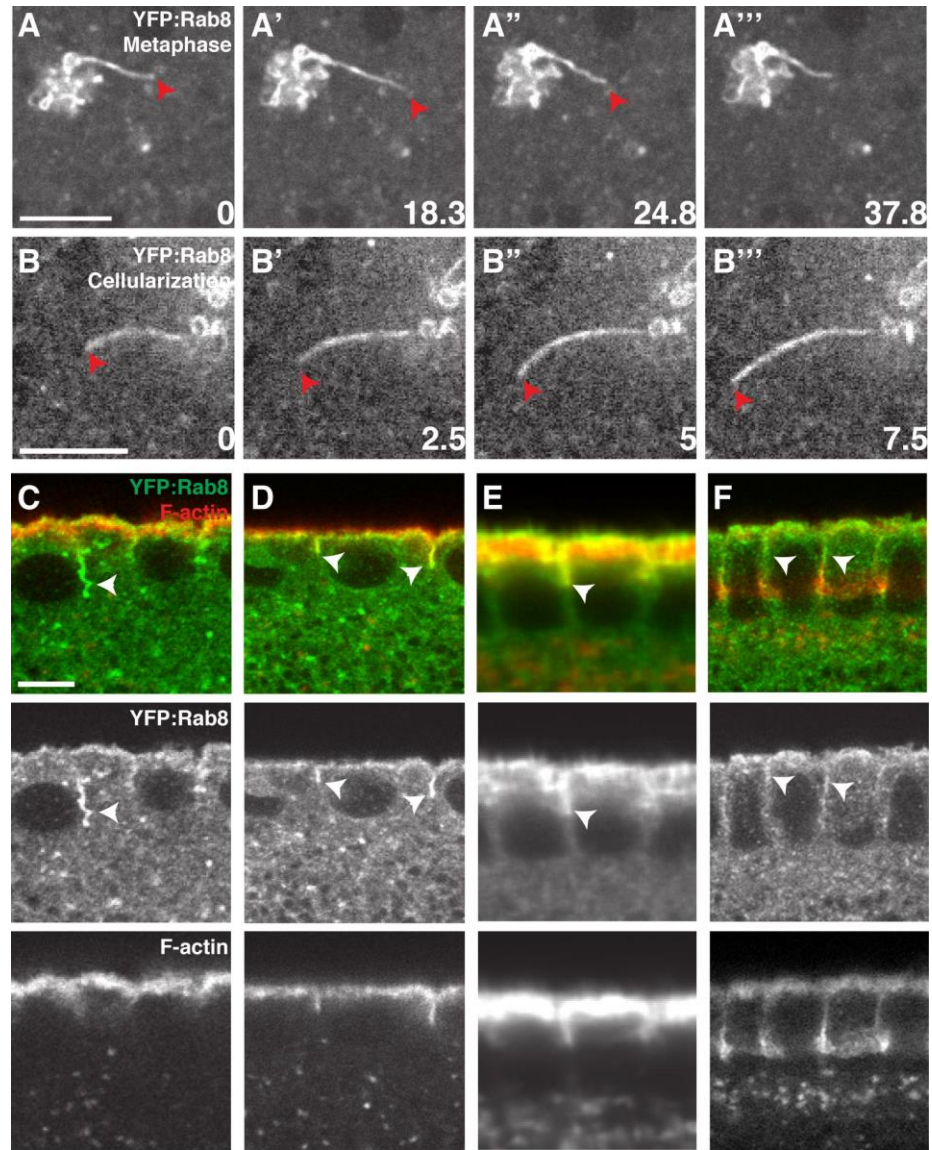


Figure 18. Rab8 tubules localize to sites of furrow ingression and precede the F-actin network.

(A-A'') Still frames of dissected embryo expressing YFP:Rab8 during syncytial furrow formation. Rab8 forms long tubular extensions which are capable of extending and retracting over time (red arrowhead). (B-B'') Still frames of dissected embryos expressing YFP:Rab8 during cellularization. Rab8 forms long tubular extensions which extend many microns over time (red arrowhead). (C-F) Fixed embryos expressing YFP:Rab8 (anti-GFP, green), F-actin (Phalloidin, red) viewed from an apical-basal orientation. (C) Syncytial embryo shows YFP:Rab8 tubules (arrowhead) which mark future sites of furrow ingression. (D) As metaphase furrows form, F-actin and YFP:Rab8 tubules (arrowheads) colocalize at the furrow. (E) In early cellularization stages, Rab8 tubules (arrowheads) extend basal to the F-actin front. (F) At mid-cellularization, F-actin colocalizes with Rab8 and Rab8 appears cortically associated (arrowheads). (A, B) Time indicated in sec. Scale bars in A and B are 10 μ m. Scale bar in C is 5 μ m.

To look more closely at the dynamics of Rab8 and the cytoskeleton, we focused on live *in vivo* imaging of Rab8 and F-actin. Embryos expressing YFP:Rab8 and mCh:MoeABD revealed that Rab8 tubules precede F-actin and initially lead the basal-most portion of the FC (Fig. 19). At the onset of cellularization, Rab8 tubules become numerous and begin to descend basally between nuclei and mark sites of furrow ingression (Fig. 19A, A'). As furrow ingression initiates, F-actin then coalesces apically while Rab8 tubules extend basally (Fig. 19B, B'). The F-actin network follows Rab8 tubules downward causing FC ingression to proceed (Fig. 19B). Rab8 tubules clearly lead the basal-most portion of the FC, as is seen when an early cellularization embryo is viewed at two different depths (Fig. 19B, B', C and C'). During the fast phase of cellularization, tubules are not apparent and basal Rab8 appears punctate and cortically associated (Fig. 19D).

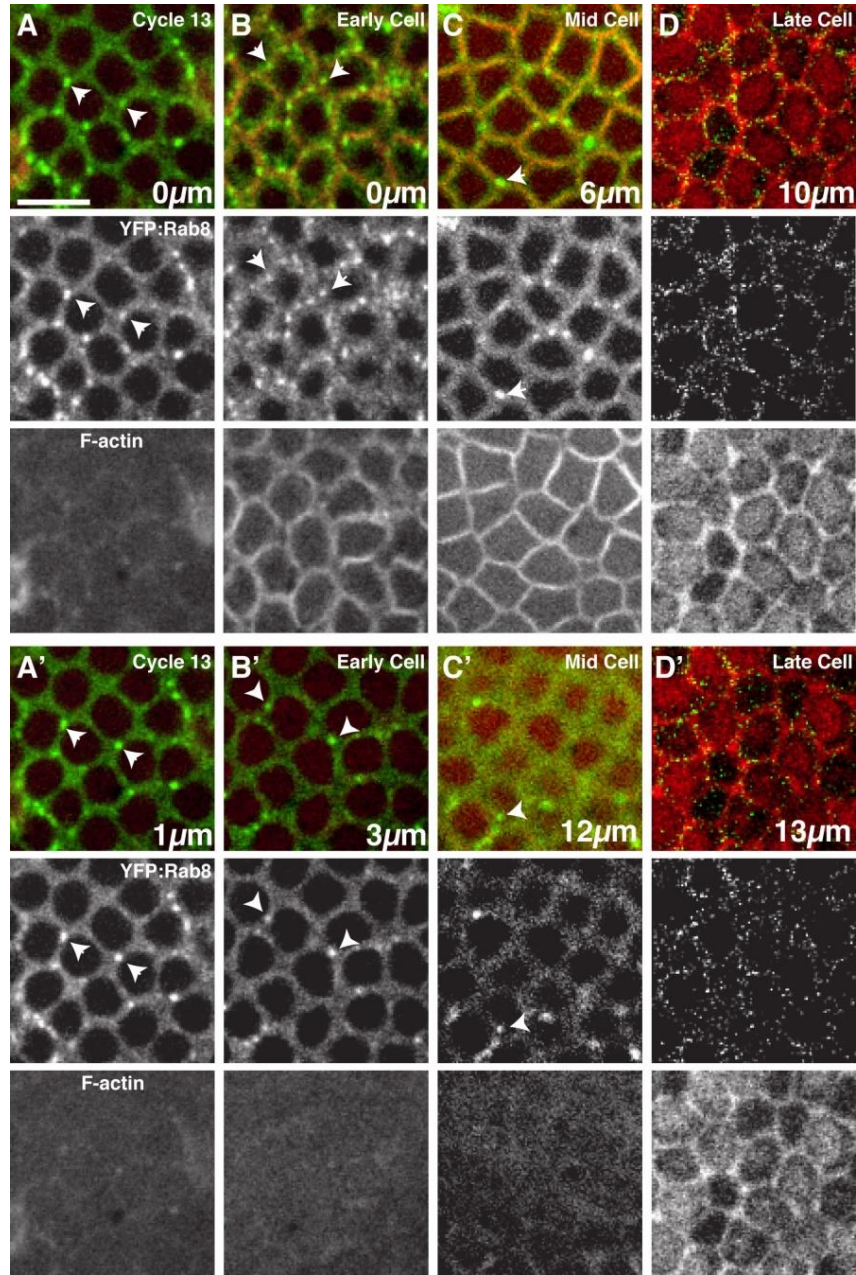


Figure 19. Rab8 tubules precede F-actin ingress *in vivo*.

(A-D) Embryo expressing mCh:MoeABD (F-actin, red) and YFP:Rab8 (green) at the onset of interphase 14 (A) and early (B), mid (C), and late (D), stages of cellularization viewed from a planar orientation. (A) Rab8 tubules (arrowheads) precede F-actin and ingress basally (A, 0 μ m; A', 1 μ m). (B) As slow-phase proceeds an F-actin array forms (B, red) and Rab8 tubules (B, arrowheads, 0 μ m) extend past the contractile array (B', arrowheads, 3 μ m). (C) In mid-cellularization stages, Rab8 tubules (C, arrowheads, 6 μ m) continue to extend basal to the F-actin array (C', arrowheads, 12 μ m). (D) At late stages, F-actin furrows extend into the embryo and Rab8 becomes diffuse basally. Scale bar in A is 10 μ m.

Rab8 dynamics are microtubule-dependent and F-actin independent

Our live imaging studies demonstrate that Rab8 not only exists in multiple populations, but that these populations are extremely dynamic, existing in a punctate population before associating with the cell cortex. Our knockdown studies also suggest that Rab8 dynamically regulates formation of F-actin structures during furrow formation (Fig. 10). The microtubule network has been shown to provide a directional cue during the early stages of cellularization (Foe et al., 2000). Microtubules extend basally into the yolk from centrosomes that are apical to the nuclei (Fig. 20A). Additionally, it has been demonstrated that membrane stores derived from the Golgi require MTs in order to mediate membrane addition at ingressing furrows (Sisson et al., 2000). Microtubule tracks are therefore positioned to potentially guide Rab8 tubule growth (Fig. 20A). This led us to determine which cytoskeletal network Rab8 uses to regulate its dynamics and whether disruption of either F-actin or the microtubule networks affected Rab8 behavior.

Embryos expressing either Jupiter:GFP (Jup:GFP), a microtubule marker, or YFP:Rab8 were injected with colchicine and imaged to visualize changes in Rab8 dynamics *in vivo* when microtubules are disrupted. When treated with water, both YFP:Rab8 and Jup:GFP embryos displayed wild type dynamics (Fig. 20B, C). Rab8 showed the proper transition from punctate to cortical array (Fig. 20B), while microtubules displayed proper division dynamics (Fig. 20C). When treated with colchicine, both microtubule and Rab8 populations were severely disrupted (Fig. 20D, E). In the presence of colchicine, Rab8 failed to display any dynamic behaviors. Some punctate populations were visible, but were immobile (Fig. 20D). Likewise, microtubules

displayed a complete loss of function, failing to create spindles or show division behaviors (Fig. 20E). However, when colchicine was injected into fast-phase embryos, Rab8 was able to still localize to the cortical array (Fig. 20F). This suggests that Rab8 primarily uses the microtubule network to perform dynamic behaviors and create tubules during furrow ingression, but does not require microtubules once the cortical array has been formed.

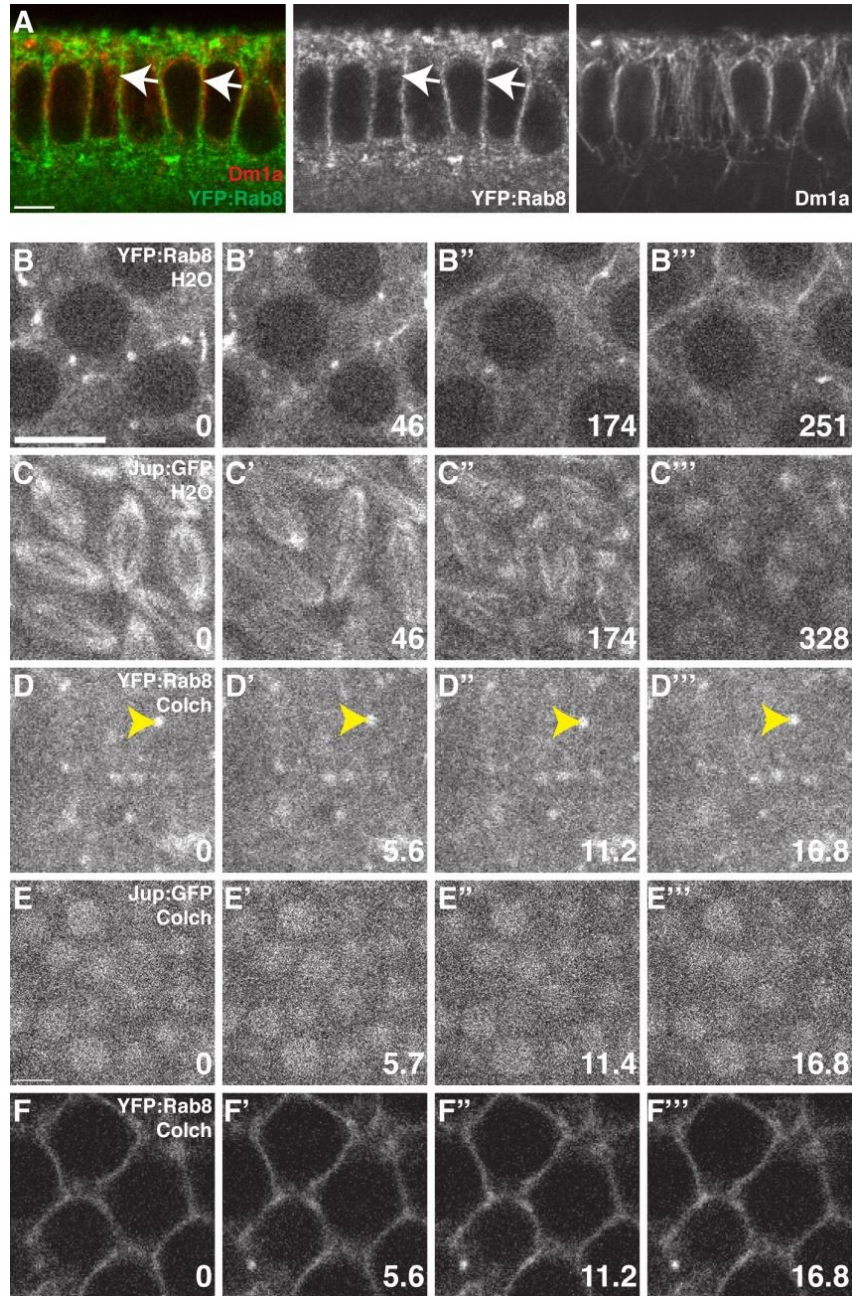


Figure 20. Disruption of microtubule networks produces distinct affects on Rab8 dynamics.

(A) Microtubules (anti-Dm1a, red), YFP:Rab8 (anti-GFP, green). Rab8 shares common orientation and localization with microtubule networks (arrows). (B-B''') YFP:Rab8 water-injected embryo displays WT dynamics. (C-C''') Jupiter:GFP (microtubule) water-injected embryo displays proper spindle formation and division dynamics. (D-D''') YFP:Rab8 colchicine injected embryo displays severe disruption of dynamics. Tubules do not form and puncta are immobile (arrowhead). (E-E''') Jupiter:GFP embryo injected with colchicine shows complete disruption of microtubule network. (F-F''') YFP:Rab8 embryo injected with colchicine during cellularization; cortical array remains intact when injected during fast phase. (B, C) Time indicated in sec, (D-F) time indicated in min. Scale bar in A is 5 μ m. Scale bar in B is 10 μ m.

The converse experiments were performed using Latrunculin B (Lat-B) to disrupt F-actin networks in embryos expressing mCh:MoeABD and YFP:Rab8. Lat-B functions to sequester monomeric actin (G-actin) and thus prevent polymerization of F-actin, ultimately resulting in a failure in furrow formation. Control embryos treated with DMSO showed wild type behaviors; the F-actin network remained intact and Rab8 tubule dynamics were unchanged (Fig. 21A). Latrunculin B injection severely disrupted F-actin, causing a loss of F-actin furrows and a disorganization of Rab8 tubules. However, despite these gross defects in embryonic organization, Rab8 still formed dynamic tubules (Fig. 21B). It can be noted that due to the extensive cross-talk between F-actin and MT networks (Foe et al., 2000), disruption of F-actin by Lat-B may result in misorganization of MT networks, which ultimately causes the disorganized Rab8 tubules seen in Figure 21. Nonetheless, these results demonstrate that Rab8 does not use the F-actin network to create tubules and behave dynamically.

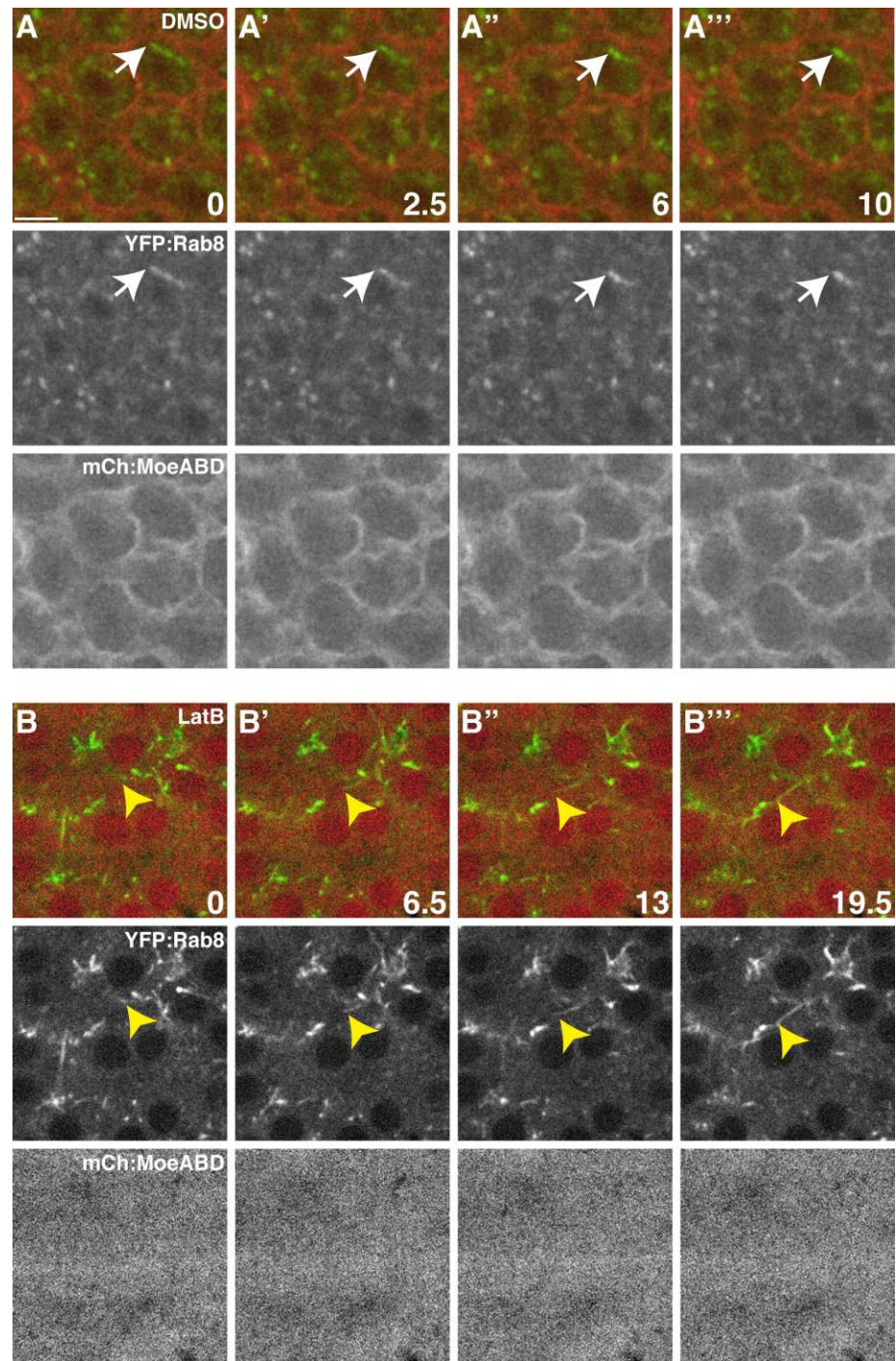


Figure 21. Rab8 does not require F-actin to behave dynamically.

(A and B) Embryos expressing mCherry:MoeABD (F-actin, red) and YFP:Rab8 (green) injected with DMSO (A) or LatB (B). (A-A''') Control embryos injected with DMSO display proper F-actin organization and YFP:Rab8 forms tubules (arrow). (B-B''') Latrunculin B injected embryos display loss of F-actin and misorganization of furrows; however, Rab8 is still capable of forming dynamic tubules (arrowhead). (A, B) Time indicated in sec. Scale bar in A is 10µm.

Development of a Rab8 antibody in three methods

In an attempt to examine endogenous localization in fixed embryos, three different approaches to generate antibodies against Rab8 were undertaken. Peptide antibodies targeted against a region of Rab8 predicted bioinformatically to be hydrophilic and antigenic were generated. In addition, two antibody approaches targeted against the full length of Rab8 fused to Maltose Binding Protein (MBP) were performed. Antibody production was performed in rabbit or guinea-pig backgrounds (Rab8 peptide), or rabbit-only backgrounds (full-length antibodies).

Full-length protein isolation was done using traditional cloning methods. A protein construct of the Rab8 amino acid sequence fused to MBP was generated and expressed in a protein expression strain of *E. coli*. MBP-Rab8 was purified out of cells using sonication and column-purification techniques and this technique typically yielded ~1mg protein per 4L *E. coli* growth culture. This MBP-tagged vector was originally designed to have a Factor-Xa protease cleavage site serving to separate MBP from Rab8 prior to exposure in animals. However, Rab8 was found to precipitate out of solution once this cleavage was performed. Consequently, MBP-Rab8 was used for antibody production. Two antibody production methods were performed: a fast-track one-month boosting regimen or a classical three-month boosting regimen.

Immunohistochemistry was performed using all three antibodies (Rab8 peptide, MBP-Rab8 1-month and MBP-Rab8 3-month), and all three exhibited non-specific cytoplasmic localization in fixed embryos. To ensure this non-specific binding was not due to the presence of MBP-specific antibodies, affinity purification of the full-length 3-

month antibody against a fusion protein of GST and 50% of the Rab8 protein measured from the C-terminus (GST-Cterm50Rab8) was performed and immunohistochemistry was repeated. This method proved ineffective at improving localization quality as localization remained non-distinct and cytoplasmic.

CHAPTER 3: DISCUSSION

Rab8 dynamics *in vivo* are spatiotemporally regulated and require GTPase activity

This research identifies a new trafficking pathway that directs furrow ingression in the early embryo of *Drosophila melanogaster*. Through a screen of known Rab proteins, Rab8 was identified as a critical component in the establishment of the embryonic epithelium. Live imaging studies demonstrate that Rab8 behaves in a strikingly dynamic manner, existing in small punctate structures which transition to a cortical array over time at apical portions of putative cells. This transition correlates with a switch from interphase to mitosis in syncytial embryos as well as the onset of the fast phase of cellularization. When viewed in lateral cross-section or dissected embryo preparations, it can be noted that Rab8 creates potentially long, basally directed tubules which precede F-actin ingression dynamics. This precession can be seen in both syncytial and cellularization furrows as demonstrated in both fixed and live imaging preparations.

Rab8 GTPase activity is essential for proper protein function and furrow formation. A GDP-locked form of Rab8 results in a complete loss of protein dynamics and structure, indicating that the GTP-bound form of Rab8 regulates proper protein localization and activity. Indeed, when expressed in a GTP-locked state, it can be noted that the transition from punctate to cortical associated Rab8 occurs

earlier, during the slow phase of cellularization, demonstrating that the GTPase activity of Rab8 may be tightly regulated in a temporal manner to control Rab8 and furrow dynamics.

Rab8 regulates furrow formation and functions in an exocytic pathway

It is shown here that Rab8 function is crucial for furrow ingression; knock-down of Rab8 by RNAi results in a severe loss of furrows and an inability to develop an embryonic epithelium. In the most severe phenotypes, embryos were unable to complete proper nuclear divisions due to a complete loss of F-actin furrow dynamics in syncytial stages, resulting in the ultimate failure of cellularization. Immunostaining studies reveal a 67% colocalization between Rab8 and Neurotactin, a transmembrane protein that must pass through the secretory pathway to reach the plasma membrane. Disruption of Rab8 by RNAi also abolishes the ability of another transmembrane protein, Gap43, from being delivered to the plasma membrane in live-imaging studies.

In fixed images, a 42% colocalization between Sec5 cytoplasmic compartments and Rab8 during early cellularization and a strong association between these proteins at the cortex from mid-cellularization onwards was observed. Disruption of Sec5 by RNAi leads to a failure of Rab8 to demonstrate a transition into a cortical association. Rab8 is additionally shown to act downstream of RalA, a GTPase which has been implicated in the regulation of directed membrane addition through interactions with the exocyst (Holly et al., in review). These results suggest that Rab8 functions within the exocytic

trafficking pathway to provide an essential factor that directs membrane addition to the furrow.

Rab8 regulates polarized membrane addition downstream of the RE

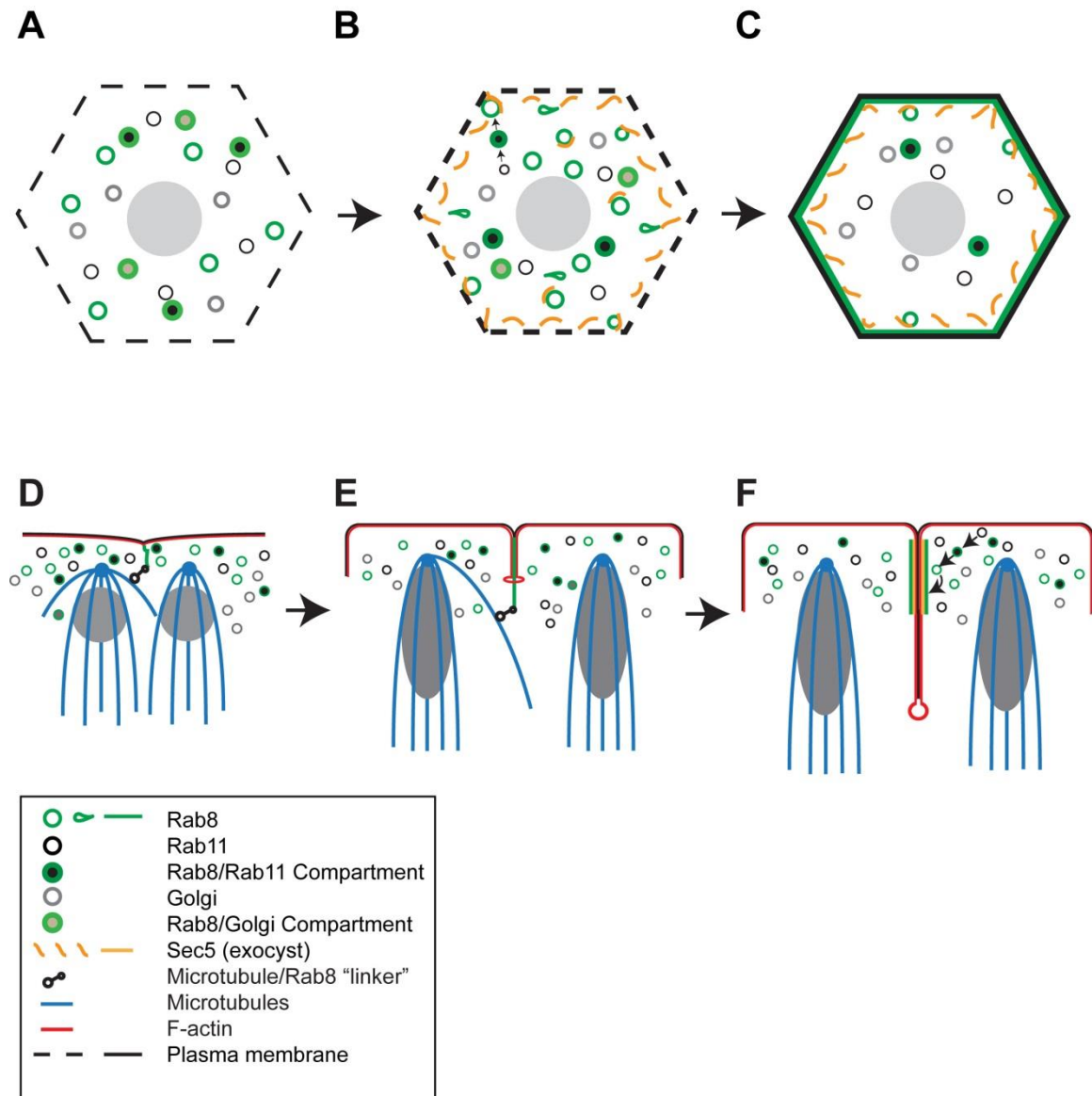
New membrane added during cellularization is provided through the RE and Golgi trafficking pathways (Sisson et al., 2000; Pelissier et al., 2002). Immunostaining against either of these structures and Rab8 indicated that Rab8 largely exists in distinct compartments. However, quantification of colocalization revealed that Rab8 does display a small, but reproducible coincidence with the RE and Golgi (~10% of vesicles quantified). It is therefore possible that these populations have transient interactions with each other. The fact that a small population of Rab8 colocalizes with the RE at a higher frequency in early versus late stages of cellularization, as well as the expression of Rab8 DN and Rab8 knockdown via RNAi leading to an expansion of RE compartments, suggests that Rab8 may direct trafficking events from this compartment to the plasma membrane. It was seen in this study that while disruption of Rab8 function caused a slight enlargement of RE compartments, RNAi of Rab11 completely abolished the transition from punctate to cortical population of Rab8, suggesting that Rab8 may function downstream of the RE. The development of two-color *in vivo* studies will be required to determine if transient interactions occur between these two compartments. Indeed, Rab trafficking pathways often function through compartments changing their identity by dissociating with one Rab and acquiring another (Rink et al., 2005), and Rab11 has been demonstrated to bind and stimulate a Rab8 GEF, Rabin8, during ciliogenesis (Knodler et

al., 2010). We thus propose a model in which Rab8 functions downstream of the RE and Golgi in order to receive intracellular membrane and uses the exocyst to properly associate with and deliver membrane to the cortex of ingressing furrows (Fig. 22).

Rab8 requires the MT but not F-actin cytoskeletal network to behave dynamically

Fixed embryos treated with Taxol to stabilize MTs demonstrates that Rab8 tubules appear to follow the apical-basal directional cue provided by the MT network during furrow formation (Foe et al., 2000). Disruption of the MT network through colchicine treatment leads to a complete loss of Rab8 tubules and the presence of static Rab8 puncta. However, once Rab8 has transitioned to a cortical association, the loss of the MT network does not disrupt protein localization. This suggests that Rab8 puncta are actively associated with MTs, while cortical Rab8 instead represents a stable association with membrane at ingressing furrows.

Conversely, disruption of F-actin by Latrunculin-B does not perturb Rab8 dynamics. Both puncta and tubules are capable of retaining their activities, demonstrating that Rab8 does not require the actomyosin network to function. This, taken with the fact that Rab8 structures precede F-actin at furrow sites, suggests that Rab8 functions upstream of F-actin during syncytial divisions and cellularization.



A model for polarized membrane addition regulated via Rab8 complexes

How does Rab8 guide furrow ingression? This can occur through two steps, 1) an early polarity process that is essential to the formation of the actomyosin network, and 2) a fast phase mechanism during which Rab8 guides membrane addition to the furrow (Fig. 22). Early in furrow formation, Rab8 puncta appear to prefigure the embryo for ingression of the furrow canal. Rab8 puncta gather membrane material from an intracellular store, most likely RE-derived, and begin to form extensions (Fig. 22B, D). Active, GTP-bound Rab8 localizes to this punctate population. Many Rab proteins bind to effector cytoskeletal motor proteins (Grosshans et al., 2006). Rab8 may therefore function by binding to plus-end-directed microtubule motor proteins, which then mediate the transition to cortical association (Fig. 22).

How then does the transition from puncta to cortical array aid furrow ingression? Ingression of furrows is completely abolished in Rab8 knockdown embryos, suggesting that the cortical array provides an important formative cue for the actomyosin network. By analogy to cytokinetic events, recent studies have demonstrated that cleavage furrow localized membrane compartments may serve as scaffolds for the recruitment of cytoskeletal remodeling factors (Riggs et al., 2003; Connell et al., 2009; Dambournet et al., 2011; Schiel and Prekeris, 2012), and expression of Rab8 has been shown to activate the formation of actin-dependent structures such as filopodia and lamellopodia (Peranen et al., 1996; Hattula et al., 2006). An attractive facet of this model is that Rab8 may provide the critical link between the polarizing microtubule network and the contractile actomyosin array, and thus suggest a molecular explanation for the requirement of

microtubule function solely during early cellularization (Fig. 22; Foe et al., 2000; Royou et al., 2004).

During the fast phase of cellularization, Rab8 shifts from a directional cue for the cellularization front, to aiding the rapid addition of membrane to the ingressing furrow (Fig. 22). Again, a confounding aspect of the known membrane pathways that are required for furrow ingression (Golgi, RE) has been the lack of observed association with, and movement to, the furrow. In contrast, Rab8 is found during fast-phase at the apicolateral membrane, the region in which membrane addition to the furrow is known to occur. Rab8 mediation of membrane trafficking to the plasma membrane may occur through its binding and tethering of vesicles to the exocyst complex (Fig. 22). During lumen formation and ciliogenesis in mammalian cells, Rab8 binds to the Sec15 subunit of the exocyst complex (Bryant et al., 2010; Knodler et al., 2010; Feng et al., 2012). As described above, this function and interaction may be preserved during cellularization as the Sec5 subunit of the exocyst complex is required for cellularization, and Sec5 localizes to the apicolateral region of the cellularizing furrow in the area where membrane addition and Rab8 localization occurs (Murthy et al., 2010). Additionally, disruption of the Exo84 exocyst subunit in the fly embryo leads to an enlargement of Rab11 REs, which resembles the defects observed in Rab8 DN expressing embryos (Blankenship et al., 2007). In summary, Rab8 may direct vesicular traffic from the RE/Golgi to exocyst tethering complexes on the ingressing furrow and thus function as the final and necessary step in regulating polarized membrane addition during epithelial cell formation.

Future directions and conclusions

It has become increasingly clear with the current body of research that plasma membrane, rather than being a passive agent during cell formation is revealing itself to be a highly dynamic entity during the regulation of cell growth and cytoskeletal dynamics. The work presented here provides a novel role for intracellular trafficking in the regulation of the actomyosin network during furrow formation and raises questions in regards to the interplay between Rab8 compartments and cytoskeletal components.

Of future interest is the identification of an interaction between Rab8 and a microtubule-based motor. Data described herein demonstrates the requirement of an intact microtubule network for proper Rab8 dynamics, which demonstrates an interaction between Rab8-positive vesicles and microtubules. As Rab8 tubules display an apical to basal ingression, which follows the minus- to plus-end orientation of the microtubule network, it is hypothesized that Rab8 will demonstrate an association with a Kinesin motor. Specifically, it is proposed that Rab8 interacts with Kinesin-1, which has been shown to be active on the basal ingression of lipid droplets during these developmental stages (Cermelli et al., 2006; Shubeita et al., 2008). Functional studies using RNAi and point-mutations against Kinesin-1 will allow for *in vivo* studies of Rab8 dynamics in a plus-end motor-deficient background. It is expected that Rab8 dynamics will be lost in this background. Additionally, biochemical studies via co-immunoprecipitation could be performed to conclude if a direct Kinesin-Rab8 interaction exists to allow for this regulation.

Imaging and functional studies performed here suggest that Rab8 functions upstream of F-actin dynamics to prefigure future furrow sites and regulate ingression. Identifying how Rab8 regulates F-actin during these developmental time-points is of great interest. It can be hypothesized that Rab8-positive vesicles, while required for the regulation of polarized membrane addition, might also be required for delivering regulators of the actomyosin network to putative furrow sites.

The idea that regulation of actomyosin dynamics at the cortex via modification of lipids within membranes has become increasingly evident (Mayer et al., 1993; McLaughlin et al., 2002; Moss et al., 2012; Reversi et al., 2014). Recently, it was shown that phosphoinositide presence at the plasma membrane plays a role in regulating furrow ingression dynamics. Specifically, PI(3,4,5)P₃ is required for both the maintenance of the actomyosin network and the recruitment of bnl to ingressing furrows during slow phase, while PI(4,5)P₂ promotes the assembly and contractility of the actomyosin network during the fast phase of cellularization (Reversi et al., 2014). Thus it would be interesting to determine if Rab8 exocytic vesicles contain and are responsible for the delivery of specific phosphoinositides to putative furrow sites or if Rab8 is responsible for regulating the balance of these lipids during furrow ingression. Phosphoinositide sensors have been used to identify specific compositions of membranes, and these could be used in two-color imaging studies with Rab8 to determine if colocalization between these sensors and Rab8 vesicles occurred during furrow ingression.

As mentioned above, the protein Slam is required for specification of the furrow and regulation of actomyosin assembly during cellularization (Acharya et al., 2014) and

slam localization has been demonstrated to be regulated by the RE during this process. Additionally, a novel protein Disrupted-underground-network (Dunk) has been identified to localize to furrows and regulate MyoII localization and contractility during cellularization (He and Wieschaus, 2015). Embryos double mutant for the loss of both *slam* and *dunk* result in a complete failure of furrow ingression, reminiscent of the severe Rab8 knock-down phenotypes seen in this research. These similar phenotypes alongside the requirement of the RE to regulate slam localization, raise the question of whether Rab8 is required in the delivery of either of slam or dunk proteins to nascent furrows and ultimately regulation of the actomyosin network via their delivery during epithelial formation.

The interplay between membranes and the cytoskeleton is becoming complex. One emerging viewpoint in cell biology is that, in some modalities, membrane may have place in precedence to the cytoskeleton. Plasma membrane is not merely a passive structure on which the cytoskeleton exerts forces, but instead can function to recruit protein complexes and modulate cytoskeletal behaviors and force-driven dynamics. Here it is shown that Rab8 and associated intracellular compartments may link membrane dynamics to the formation of a contractile network and provide direct evidence for putative regulation of actomyosin via membrane delivery dynamics.

CHAPTER 4: MATERIALS AND METHODS

Fly Stocks and Genetics

Flies were maintained at 25°C by standard procedures. All UAS transgenic flies were crossed to $\text{mat}\alpha\text{Tub-Gal4VP16}^{67C;15}$ (D. St. Johnson) maternal driver females or $\text{Rab8}>\text{Gal4}$ (Fig. 1, Bloomington) and second generation embryos were analyzed.

Fly Stocks from Bloomington Stock Center:

UASp-YFP:Rab8

UASp-YFP:Rab8 T22N

UASp-YFP:Rab8 Q67L

UASp-YFP:Rab11

sqh-Histone:RFP

Private Stocks:

sqh-GFP:MoesinABD (Kiehart et al., 2000)

sqh-mCh:MoesinABD (Millard and Martin, 2008)

Jupiter:GFP (Buszczak et al., 2007).

Gap43:mCh (Martin et al., 2010)

UASp-RalA:mCh (Holly et al., In Review)

Rab8-GFP:Rab8

CRISPR GFP:Rab8

Construction of endogenous promoter driven GFP:Rab8 transgenic line

To create transgenic lines of GFP:Rab8 driven by Rab8 promoter elements, genomic DNA comprising the 1.5 kb upstream of the Rab8 ATG start site was cloned into pCasper4 (Addgene), followed by the coding sequence of GFP and the remainder of the Rab8 gene plus 1.5 kb 3' of the Rab8 stop codon. In total, 5.038 kb of the genomic Rab8 locus was cloned and inserted into the pCasper4 plasmid, along with the 718 bp CDS of eGFP, using standard PCR and cloning protocols.

Construction of CRISPR GFP:Rab8 transgenic line

Plasmid design for GFP:Rab8 was followed as described above for the endogenous promoter line using the pBluescript KS plasmid (Addgene). Additionally, guideRNA sequences required to guide Cas9 nuclease enzyme activity to specific genome cut locations were designed as according to Gokcezade et al. (2014). These were all sequence-verified (midi-prep, Qiagen, UC Denver Core Sequencing).

Guide RNAi (100ng/μl) and GFP:Rab8 plasmid (150ng/μl) were co-injected into Cas9 flies by BestGene. Surviving adults were crossed to sp/Cyo;Dr/Tm3 flies in a series of crosses to create 345 possible recombination lines. Embryos from these individual

lines were tested for fluorescence using a spinning-disk microscope. From this, one line was determined to properly express GFP:Rab8 and proper insertion into the genome was verified by PCR (see below).

PCR-verification of CRISPR GFP:Rab8 transgenic line

PCR primers to verify correct insertion of GFP into the *Drosophila* genome via CRISPR were designed in the following manner. The 5' primer was targeted against a region (1.7kb upstream of Rab8 start codon) outside of that which was cloned into the GFP:Rab8 plasmid and the 3' primer was targeted against a region within the GFP coding sequence. Thus, successful PCR product could only be produced if both DNA sequences were present within the CRISPR line. 3 primer pairs were designed in this manner (NIH, Primer Design Tool) and PCR was performed in both CRISPR GFP:Rab8 flies and OreR flies for control.

Primers designed for cloning, guideRNAs and PCR verification of CRISPR GFP:Rab8 insertion

The following primers were used in the creation of the endogenous GFP:Rab8, CRISPR GFP:Rab8, CRISPR guide RNA's and PCR verification of genomic GFP insertion under the Rab8 promoter:

Cloning primers GFP:Rab8 and CRISPR GFP:Rab8

5'EndogRab8(1)	BamHI	CGGGATCCAAATGCTCCCCTTTCATTAT
3'EndogRab8(1)	SpeI	GGACTAGT TTTGTGTGCTTTTGCGGTAG
5'EndogGFP	SpeI	GGACTAGTATGGTGAGCAAGGGCGAGGA
3'EndogGFP	SacII	TCCCCGCGGCGCCCCGCCCTGCCACTCAT
5'EndogRab8(2)	SacII	TCCCCGCGGATGGCCAAAACCTACGACTA
3'EndogRab8(2)	KpnI	GGGGTACCTTACGGGACTTTTCTTTAAT
5'CRISPRRab8(1)	SpeI	GGACTAGTAAAATGCTCCCCTTTCATTAT
3'CRISPRRab8(1)	BamHI	CGGGATCCTTTGTGTGCTTTTGCGGTAG
5'CRISPRGFP	BamHI	CGGGATCCGTTGTACAGCTCGTCCATGC
3'CRISPRGFP	SalI	ACGCGTCGACCGCCCCGCCCTGCCACTCAT
5'CRISPRRab8(2)	SalI	ACGCGTCGACATGGCCAAAACCTACGACTA
3'CRISPRRab8(2)	KpnI	GGGGTACCTATATAAATAAAATGTTTGT

Guide RNA's for CRISPR GFP:Rab8

5'chiRNA-1	CTTCGAACAGATAGTCGTAGGTTT
3'chiRNA-1	AAACAAACCTACGACTATCTGTTC
5'chiRNA-2	CTTCGCCCCGATAATAGTGCTTAGC
3'chiRNA-2	AAACGCTAACGACTATTATCGGGC

PCR Verification of CRISPR GFP:Rab8 insertion

5'PCR-1	TGACATGCAGAATTAAAAGCCC
3'PCR-1	CGGACACGCTGAACTTGTG
5'PCR-2	ATGACATGCAGAATTAAAAGCCCA
3'PCR-2	GTCAGCTTGCCGTAGGTGG
5'PCR-3	TGACATGCAGAATTAAAAGCCCAAT
3'PCR-3	GCTGAACTTGTGGCCGTTTAC

Confocal microscopy and time-lapse imaging

Confocal images were acquired on an Olympus Fluoview FV1000 confocal laser scanning microscope with 40x/1.35NA or 60x/1.42NA objectives for fixed specimens. Time-lapse imaging was performed on a spinning disk confocal from Zeiss and Solamere Technologies Group with 40x/1.3NA or 63x/1.4NA objectives. Embryos were imaged after dechoriation and placement on a gas permeable membrane in Halocarbon 27 oil. For dissection preparations, embryos were positioned on a coverslip and dissected apart using two sharp forceps. The coverslip was then inverted and placed on a gas permeable membrane. Live imaging was performed using exposure settings of 150-500msec and images were acquired at either fast (< 1/sec) or slow (1/30sec) rates.

Embryo fixation and immunostaining

Embryos were dechorionated in 50% bleach solution and fixed for 1 hr 15 min at the interface of heptane and 3.7% formaldehyde in 0.1M sodium phosphate buffer (pH 7.4) before being manually devitellinized and stained with rabbit anti-GFP (1:1000, Invitrogen), mouse anti-GFP (1:100, Molecular Probes), rabbit anti-lava (1:250, Sisson et al., 2000), rabbit anti-Rab11 (1:1000, Satoh et al., 2005), Hoescht (1:500, Sigma), mouse anti-Nrt (1:1) or mouse anti-Sec5 (1:35; Murthy et al., 2010).

Embryos stained for microtubules were first treated with 10 μ M Taxol (Sigma) in 1:1 PBS/Heptane for 90 sec and fixed for 30 min at the interface of heptane and 3.7% formaldehyde in 0.1M sodium phosphate buffer (pH 7.4) before being devitellinized by osmotic shock and stained with mouse anti-Dm1a (1:500, Sigma) and rabbit anti-GFP (1:1000, Invitrogen). Conjugated secondary antibodies Alexa-546 phalloidin (1:200, Molecular Probes), Alexa-488, Alexa-546 or Alexa-568 were used at 1:500 (Molecular Probes). Embryos were mounted in Prolong Gold with DAPI (Molecular Probes).

Fixed and time lapse embryo analysis and quantification of vesicle colocalization

Confocal and spinning disk images were edited using Adobe Photoshop. Channels for fixed images were uniformly leveled for optimal channel appearance. Time-lapse images were leveled with Photoshop to show optimal protein populations, or were processed with identical settings to permit comparisons in protein levels (Figure 13).

Quantification of overlap between Rab8 vesicle populations and Neurotactin, Sec5, Rab11 RE, Golgi and Hrs populations was performed using Adobe Photoshop and

RGB images obtained from fixed specimens. Quantification was performed by selecting puncta within a 5x5 grid (“grid view” in Photoshop) using the circular selection tool within either the red or green image channel. Puncta always fell within a size of 2-6 pixels in area (1 to 3 pixels across). The selection vector within this 5x5 grid was then overlaid with the second channel (red or green) and puncta which showed overlap by at least 1 pixel inside these selection points was counted as colocalization. Each 5x5 grid was considered a grouping of puncta and this was used to calculate average colocalization by performing a weighted average calculation. Standard error was calculated from a weighted standard deviation. At least 2 separate images were used to count between 80-100 total puncta. This quantification method was used to determine Nrt, Sec5, RE (Rab11), Golgi (Lava) and late endosome (Hrs) colocalization with Rab8. Identical criteria were used for Rab8 puncta as well as to determine Sec5, RE, Golgi and late endosome colocalization with Rab8.

Vesicle counts of YFP:Rab8 during cellularization stages were performed in the same manner as colocalization counts described above. Still frames from the onset of a slow-phase, prior to cortical transition, first appearance of cortical array and persistence of cortical array were chosen and vesicles within 5x5 grids were counted. These grids served as groups of vesicles and all vesicles of a 512x512 pixel image were counted. At least 2 images for each time point described above were used for counting. Average number of puncta was calculated via a weighted average and standard error was calculated using a weighted standard error.

Weighted average and weighted standard error calculations were used to account for variation of puncta number within a 6x6 grouping (i.e. some 6x6 areas had very few puncta, whereas others had many more) due to variation of puncta localization within the overall image.

siRNA Preparation

Primers for siRNA treatments were chosen to represent independent regions of Rab8 (5'UTR) or through the use of the SNAPDRAGON RNAi design program that bioinformatically selects against off-target effects (DSRC, Harvard). siRNA primers for Rab11, Sec5 and RalA were designed also using the SNAPDRAGON system. dsRNA was made using a Megascript T7 transcription kit (Ambion) and purified using Qiagen RNAeasy columns. A final concentration was determined with a NanoDrop ND1000 Spectrophotometer.

siRNA Primers

5'Rab85UTR	T7	TAATACGACTCACTATAGGGCAAACCAACGTAGACTCGCA
3'Rab85UTR	T7	TAATACGACTCACTATAGGGTCCTCTCCGATTCTTGGCTA
5'Rab8SNAP	T7	TAATACGACTCACTATAGGGAGGACAGTTGGTTGTCCAGG
3'Rab8SNAP	T7	TAATACGACTCACTATAGGGTTCGTACACGCTGTCGTCAT
5'Rab11SNAP	T7	TAATACGACTCACTATAGGGAGCAGCTGCAAATCTCCAAT
3'Rab11SNAP	T7	TAATACGACTCACTATAGGGGCTCGGTTCGATATCCTTTG
5'Sec5SNAP	T7	TAATACGACTCACTATAGGGCAGCTACTTGGATTCCCCAA
3'Sec5SNAP	T7	TAATACGACTCACTATAGGGCTCCATCTCGTGATCCCCACT

RNAi Treatments

Embryos were prepared in the same manner as for live imaging and then glued to a coverslip using heptane glue. Embryos were dehydrated for 11 min, and then injected with siRNA against Rab8 (concentration amounts of RNAi treatments are shown in Figure 9), Rab11 (1.3 μ g/ μ l), Sec5 (2.0 μ g/ μ l) or RalA (1.7 μ g/ μ l).

RNAi scoring data (Table 1) was tested for statistical significance using a two-dimensional contingency table with a X^2 test with $\alpha=0.05$. Tests were performed in two ways: (1) All data was tested to determine if changes in phenotypic proportions seen were significant, which resulted in $P<0.0001$. (2) Each RNAi treatment group was tested against water control treatment to determine if changes in phenotypic proportions were significant in relation to control proportions. All tests gave $P<0.0001$.

Drug Treatments

Embryos were prepared as for RNAi treatment and injected with DMSO, water, Latrunculin B (10mM, Sigma) or Colchicine (10mg/mL, Sigma)

Protein Expression and Antibody Production

Peptide antibodies were designed by analyzing the peptide sequence of Rab8 and choosing regions of hydrophilic regions that would potentially be exposed to the cellular environment. These sequences were then used to create protein segments which were then introduced into rabbit or guinea-pig backgrounds (GenScript).

Full-length peptide antibodies were created using traditional cloning methods. A protein construct of the Rab8 coding region fused to Maltose Binding Protein (MBP) was generated and expressed in a protein expression strain of *E. coli* (NEB C2523H). *E. coli* was grown to an A_{500} of 0.5 and 0.3mM IPTG (Gold Biotechnology) was added to induce MBP:Rab8 expression.

MBP-Rab8 was purified out of cells using sonication and column-purification techniques. Cellular extract was run over a column containing a maltose resin bedding and eluted using 10mM maltose. Protein concentration was determined using a NanoDrop ND1000 Spectrophotometer and confirmed via SDS-PAGE (BioRad) and Coomassie stain. This technique typically yielded ~1mg of MBP-Rab8 per 4L *E. coli* growth culture. MBP-Rab8 was purified using HPLC (Glenn Cappodagli, DU) and concentrated to roughly 1mg/ml in PBS + 10% glycerol before being introduced to a rabbit background (PRF+L, one-month protocol; Syd Labs, three-month protocol).

Fixation of embryos was performed as described above and all three antibodies were used at a range of 1:1-1:100 dilution ratios in an attempt to improve image quality.

REFERENCES

- Acharya, S., Laupsien, P., Wenzie, C., Yans, S., Großhans, J. (2014). Function and dynamics of slam in furrow formation in early *Drosophila* embryo. *Dev Biol.* 386, 371-384.
- Ali, B.R. and Seabra, M.C. (2005). Targeting of Rab GTPases to cellular membranes. *Biochem Soc Trans.* 33, 652-656.
- Baskys, A., Bayazitov, I., Zhu, E., Fang, L. and Wang, R. (2007). Rab-mediated endocytosis: linking neurodegeneration, neuroprotection, and synaptic plasticity? *Ann. N. Y. Acad. Sci.* 1122, 313-329.
- Beronja, S., Laprise, P., Papoulas, O., Pellikka, M., Sisson, J., Tepass, U. (2005). Essential function of *Drosophila* Sec6 in apical exocytosis of epithelial photoreceptor cells. *J. Cell. Biol.* 169(4):635-646.
- Blankenship, J. T., Fuller, M. T. and Zallen, J. A. (2007). The *Drosophila* homolog of the Exo84 exocyst subunit promotes apical epithelial identity. *J. Cell. Sci.* 120, 3099-3110.

Brand, A.H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118, 401-415.

Bryant, D. M., Datta, A., Rodriguez-Fraticelli, A. E., Peränen, J., Martín-Belmonte, F. and Mostov, K. E. (2010). A molecular network for de novo generation of the apical surface and lumen. *Nat. Cell. Biol.* 12, 1035-1045.

Buszczak, M., Paterno, S., Lighthouse, D., Bachman, J., Plank, J., Owen, S., Skora, A., Nystul, T., Ohlstein, B., Allen, A., et al. (2007). The Carnegie protein trap library: a versatile tool for Drosophila developmental studies. *Genetics*. 175, 1505-1531.

Cao, J., Albertson, R., Riggs, B., Field, C. M. and Sullivan, W. (2008). Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. *J. Cell. Biol.* 182, 301-313.

Cermelli, S., Guo, Y., Gross, S. P. and Welte, M. A. (2006). The lipid-droplet proteome reveals that droplets are a protein-storage depot. *Curr. Biol.* 16, 1783-1795.

Chan, C-C., Scoggin, S., Wang, D., Cherry, S., Dembo, T., Greenberg, B., Jin, E.J., Kuey, C., Lopex, A., Mehta, S.Q., et al. (2011). Systematic Discovery of Rab GTPases with Synaptic Functions in Drosophila. *Curr. Biol.* 21: 1704-1715.

Chen, S., Liang, M.C., Chia, J.N., Ngsee, J.K., Ting, A.E. (2001). Rab8b and Its Interacting Partner TRIP8b Are Involved in Regulated Secretion in AtT20 Cells. *J. Biol. Chem.* 276: 13209-13216.

Chircop, M. (2014). Rho GTPases as regulators of mitosis and cytokinesis in mammalian cells. 5. pii: e29770. doi: 10.4161/sgtp.29770. Epub 2014 Jul 2.

Connell, J. W., Lindon, C., Luzio, J. P. and Reid, E. (2009). Spastin couples microtubule severing to membrane traffic in completion of cytokinesis and secretion. *Traffic*. 10, 42-56.

Crawford, J.M, Harden, N., Leung, T., Lim, L., Kiehart, D.P. (1998). Cellularization in *Drosophila melanogaster* is disrupted by the inhibitions of Rho activity and the activation of Cdc42 function. *Dev Biol.* 204, 151-164.

Dambournet, D., Machicoane, M., Chesneau, L., Sachse, M., Rocancourt, M., El Marjou, A., Formstecher, E., Salomon, R., Goud, B. and Echard, A. (2011). Rab35 GTPase and OCRL phosphatase remodel lipids and F-actin for successful cytokinesis. *Nat. Cell. Biol.* 13, 981-988.

Fabrowskim P., Necakov, A.S., Mumbauer, S., Loeser, E., Reversi, A., Streichan, S., Briggs, J.A., De Renzis, S. (2013). Tubular endocytosis drives remodeling of the apical

surface during epithelial morphogenesis in *Drosophila*. *Nat. Commun.* 4:2244. doi: 10/1038/ncomms3244.

Feng, S., Knödler, A., Ren, J., Zhang, J., Zhang, X., Hong, Y., Huang, S., Peränen, J. and Guo, W. (2012). A Rab8 guanine nucleotide exchange factor-effector interaction network regulates primary ciliogenesis. *J. Biol. Chem.* 287, 15602-15609.

Figard, L. and Sokac, A.M. (2014). A membrane reservoir at the cell surface: unfolding the plasma membrane to fuel cell shape change. *Bioarchitecture.* 4(2), 39-46.

Foe, V.E., Alberts, B.M. (1983) Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J Cell Sci.* 61, 31-70.

Foe, V. E., Field, C. M. and Odell, G. M. (2000). Microtubules and mitotic cycle phase modulate spatiotemporal distributions of F-actin and myosin II in *Drosophila* syncytial blastoderm embryos. *Development.* 127, 1767-1787.

Fullilove, S.L. and Jacobsen, A.G. (1971) Nuclear elongation and cytokinesis in *Drosophila montana*. *Dev Biol.* 26, 560-577.

Goode, B.L., Drubin, D.G., Barnes, G. (2000). Functional cooperation between the microtubule and actin cytoskeletons. *Curr Op Cell Biol.* 12, 63-71.

Gokcezade, J., Grzegorz, S., Duchek, P. (2014). Efficient CRISPR/Cas9 Plasmids for Rapid and Versatile Genome Editing in *Drosophila*. *G3.* 4(11):2279-2282.

Grosshans, B. L., Andreeva, A., Gangar, A., Niessen, S., Yates, J. R. 3rd, Brennwald, P. and Novick, P.(2006). The yeast Igl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. *J. Cell. Biol.* 172, 55-66.

Hardiman, C. A., McDonough, J. A., Newton, H. J. and Roy, C. R. (2012). The role of Rab GTPases in the transport of vacuoles containing *Legionella pneumophila* and *Coxiella burnetti*. *Biochem. Soc. Trans.* 40, 1353-1359.

Hartenstein, V. (1993). Atlas of *Drosophila* development. (New York: Cold Spring Harbor Laboratory Press).

Hattula, K., Furuholm, J., Tikkanen, J., Tanhuanpää, K., Laakkonen, P. and Peränen, J. (2006). Characterization of the Rab8-specific membrane traffic route linked to protrusion formation. *J. Cell. Sci.* 119, 4866-4877.

He, B. and Wieschaus, E. (2015, March). Dunk stabilizes the tensile actomyosin network at the leading edge of the cleavage furrows during cellularization. *Oral presentation at the annual Genetics of Society of America Drosophila Research Conference, Chicago, IL.*

Holly, R.M., Mavor, L.M., Zuo, Z., and Blankenship, J.T. (2015). A rapid, membrane-dependent pathway directs furrow formation through RalA in the early *Drosophila* embryo. *In Review, Development.*

Horgan, C. P. and McCaffrey, M. W. (2012). Endosomal trafficking in animal cytokinesis. *Front. Biosci.* 1, 1202-1206.

Huber, L.A., Dupree, P., Dotti, C.G., (1995). A Deficiency of the Small GTPase rab8 Inhibits Membrane Traffic in Developing Neurons. *Mol. Cell Biol.* 15(2):918-924.

Hunter, C. and Wieschaus, E. (2000). Regulated expression of nullo is required for the formation of distinct apical and basal adherens junctions in the *Drosophila* blastoderm. *J Cell Biol.* 150, 391-401.

Jafar-Nejad, H., Andrews, H.K., Acar, M., Bayat, V., Wirtz-Peitz, F., Mehta, S.Q., Knoblich, J.A., Bellen, H.J. (2005). Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of *Drosophila* sensory organ precursors. *Dev. Cell.* 9(3):351-363.

Kaplan, A. and Reiner, O. (2011). Linking cytoplasmic dynein and transport of Rab8 vesicles to the midbody during cytokinesis by the doublecortin domain-containing 5 protein. *J. Cell. Sci.* 124: 1-12.

Moritz, O.L., Tam, B.M., Hurd, L., Peränen, J., Deretic, D., Papermaster, D.S. (2001). Mutant rab8 Impairs Docking and Fusion of Rhodopsin-bearing Post-Gliosis Membranes and Causes Cell Death of Transgenic *Xenopus* Rods. *Mol. Biol. Cell.* 12: 2341-2351.

Kiehart, D. P., Galbraith, C. G., Edwards, K. A., Rickoll, W. L. and Montague, R. A. (2000). Multiple forces contribute to cell sheet morphogenesis for dorsal closure in *Drosophila*. *J. Cell. Biol.* 149, 471-490.

Knödler, A., Feng, S., Zhang, J., Zhang, X., Das, A., Peränen, J. and Guo, W. (2010). Coordination of Rab8 and Rab11 in primary ciliogenesis. *Proc. Natl. Acad. Sci. USA.* 107, 6346-6351.

Langevin, J., Morgan, M.J., Sibarita, J.G., Aresta, S., Murthy, M., Schwarz, T., Camonis, J., Bellaïche, Y. (2005). *Drosophila* exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. *Dev. Cell.* 9(3):365-376.

Lecuit, T. (2004). Junctions and vesicular trafficking during *Drosophila* cellularization. *J. Cell. Sci.* 117, 3427-33.

Lecuit, T., Samanta, R., Wieschaus, E. (2002). slam encodes a developmental regulator of polarized membrane growth during cleavage of the Drosophila embryo. *Dev Cell.* 2, 425-436.

Lecuit, T. and Wieschaus, E. (2000). Polarized insertion of new membrane from a cytoplasmic reservoir during cleavage of the Drosophila embryo. *J. Cell. Biol.* 150, 849-860.

Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat. Cell Biol.* 7(12): 1232-1239.

Martin A.C., Gelbart M., Fernandez-Gonzalez R., Kaschube M., Wieschaus E.F.. (2010). Integration of contractile forces during tissue invagination. *J Cell Biol.* 188: 735-749.

Mayer, B.J., Ren, R., Clark, K.L., Baltimore, D. (1993). A putative modular domain present in diverse signaling proteins. *Cell.* 73,629-630.

McLaughlin, S., Wang, J., Bambhir, A., Murray, D. (2002). PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct.* 31, 151-175.

Moss, S.E. (2012). How actin gets the PIP. *Sci Signal.* 5:pe7.
<http://dx.doi.org/10.1126.scisignal.2002839>.

Millard, T. H. and Martin, P. (2008). Dynamic analysis of filopodial interaction during the zippering phase of *Drosophila* dorsal closure. *Development.* 135:621-626.

Mitra, S., Cheng, K. W., and Mills, G. B. (2011). Rab GTPases implicated in inherited and acquired disorders. *Semin. Cell Dev. Biol.* 22, 57-68.

Morgan, D.O. (2007). The Cell Cycle, Principles of Control. *New Science Press.* 297pp.

Moskalenko, S., Henry, D. O., Rosse, C., Mirey, G., Camonis, J. H., White, M. A. (2002). The exocyst is a Ral effector complex. *Nat Cell Biol.* 4, 66-72.

Müller, H.A. and Wieschaus, E. (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J Cell Biol.* 134, 149-163.

Murthy, M., Teodoro, R.O., Miller, T.P. and Schwarz, T.L. (2010). Sec5, a member of the exocyst complex, mediates *Drosophila* embryo cellularization. *Development.* 137, 2773-2783.

Nachury, M.V., Loktev, A.V., Zhang, Q., Westlake, C.J., Peränen, J., Merdes, A., Slusarski, D.C., Scheller, R.H., Bazan, J.F., Sheffiled, V.C., Jackson, P.K. (2007). A Core

Complex of BBS Proteins Cooperates with the GTPase Rab8 to Promote Ciliary Membrane Biogenesis. *Cell*. 129: 1201-1213.

Pelissier, A., Chauvin, J. P. and Lecuit, T. (2003). Trafficking through Rab11 endosomes is required for cellularization during *Drosophila* embryogenesis. *Curr. Biol.* 13, 1848-1857.

Peranen, J. (2011). Rab8 GTPases as a regulator of cell shape. *Cytoskeleton*. 68, 527-539.

Peranen, J., Auvinen, P., Virta, H., Wepf, R. and Simons, K. (1996). Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. *J. Cell. Biol.* 135, 153-167.

Postner, M.A. and Wieschaus, E.M. (1994). The nullo protein is a component of the actin-myosin network that mediates cellularization in *Drosophila melanogaster* embryos. *J Cell Sci.* 107, 1863-1873.

Pfeffer, S. (2005). A model for Rab GTPase localization. *Biochem. Soc. Trans.* 33, 637-630.

Recchi, C. and Seabra, M.C. (2012). Novel functions for Rab GTPases in multiple aspects of tumour progression. *Biochem. Soc. Trans.* 40, 1398-1403.

Reversi, A., Loeser, E., Subramanian, D., Schultz, C., De Renzis, S. (2014). Plasma membrane phosphoinositide balance regulates cell shape during *Drosophila* embryo morphogenesis. *J Cell Sci.* 205, 395-408.

Riggs, B., Rothwell, W., Mische, S., Hickson, G. R., Matheson, J., Hays, T. S., Gould, G. W. and Sullivan, W. (2003). Actin cytoskeleton remodeling during early *Drosophila* furrow formation requires recycling endosomal components Nuclear-fallout and Rab11. *J. Cell. Biol.* 163, 143-154.

Rink, J., Ghigo, E., Kalaidzidis, Y. and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell.* 122, 735-749.

Rose, L.S. and Wieschaus, E. (1992). The *Drosophila* cellularization gene *nullo* produces a blastoderm-specific transcript whose levels respond the nucleocytoplasmic ration. *Genes Dev.* 6, 1255-1268.

Royou, A., Field, C., Sisson, J. C., Sullivan, W. and Karess, R. (2004). Reassessing the role and dynamics of nonmuscle myosin II during furrow formation in early *Drosophila* embryos. *Mol. Biol. Cell.* 15, 838-850.

Satoh, A. K., O'Tousa, J. E., Ozaki, K. and Ready D. F. (2007). Rab11 mediates post-Golgi trafficking of rhodopsin to the photosensitive apical membrane of *Drosophila* photoreceptors. *Development*. 132, 1487-1497.

Schejter, E. D. and Wieschaus, E. (1993). Functional elements of the cytoskeleton in the early *Drosophila* embryo. *Annu. Rev. Cell. Biol.* 9, 67-99.

Schiel, J. A. and Prekeris, R. (2012). Membrane dynamics during cytokinesis. *Curr. Opin. Cell. Biol.* 25, 92-98.

Schweisguth, F., Lepesant, J-A., Vincent, A. (1990). The serendipity alpha gene encodes a membrane-associated protein required for the cellularization of the *Drosophila* embryo. *Genes Dev.* 4, 922-931.

Shubeita, G. T., Tran, S. L., Xu, J., Vershinin, M., Cermelli, S., Cotton, S. L., Welte, M. A. and Gross, S. P. (2008). Consequences of motor copy number on the intracellular transport of kinesin-1 driving lipid droplets. *Cell*. 135, 1098-1107.

Sisson, J. C., Field, C., Ventura, R., Royou, A. and Sullivan, W. (2000). Lava lamp, a novel peripheral golgi protein, is required for *Drosophila melanogaster* cellularization. *J. Cell. Biol.* 151, 905-918.

Sommi, P., Ananthakrishnan, R., Cheerambathur, D.K., Kwon, M., Morales-Mulla, S., Brust-Mascher, I., Mogilner, A. (2010). A mitotic kinesin-6, Pav-KLP, mediates interdependent cortical reorganization and spindle dynamics in *Drosophila* embryos. *J Cell Sci.* 123, 1862-1872.

Stauzer, P. (2013) BIOL 202 Genetics

http://www.discoveryandinnovation.com/BIOL202/notes/images/Jones14_19.jpg

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology.* 10, 513-525.

Turner, F.R. and Mahowald, A.P. (1976). Scanning electron microscopy of *Drosophila* embryogenesis. *Dev Biol.* 50, 95-108.

Welte, M. A., Gross, S. P., Postner, M., Block, S. M. and Wieschaus, E. F. (1998). Developmental regulation of vesicle transport in *Drosophila* embryos: forces and kinetics. *Cell.* 92, 547-557.

West, R.J., Lu, Y., Marie, B., Gao, F.B., Sweeney, S.T. (2015). Rab8, POSH, and TAK1 regulate synaptic growth in a *Drosophila* model of frontotemporal dementia. *J Cell Biol.* 208(7):931-947.

Wu, S., Mehta, S.Q., Pichaud, F., Bellen, H.J., Quirocho, F.A. (2005). Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. *Nat. Struct. Mol. Biol.* 12(10):879-885.

Yoshimura, S-i., Egerer, J., Fuchs, E., Haas, A.K., Barr, F.A. (2007). Functional dissection of Rab GTPases involved in primary cilium formation. *J. Cell Biol.* 178(3):363-369.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol.* 2, 107-117.

Zhang, J., Schulze, K. L., Hiesinger, P. R., Suyama, K., Wang, S., Fish, M., Acar, M., Hoskins, R. A., Bellen, H. J. and Scott, M. P. (2007). Thirty-one flavors of Drosophila rab proteins. *Genetics.* 176, 1307-13022.

Zhang, J., Fonovic, M., Suyama, K., Bogoy, M., Scott, M.P. (2009). Rab35 controls actin bundling by recruiting fascin as an effector protein. *Science.* 325(5945): 1250-1254

Zhang, X.M., Ellis, S., Sriratanana, A., Mitchell, C.A., Rowe, T. (2004). Sec15 is an effector for the Rab11 GTPase in mammalian cells. *J Biol Chem.* 279 (41): 4302-43034.