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RalA and Rab8 Regulate Drosophila Embryogenesis

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RALA AND RAB8 REGULATE *DROSOPHILA* EMBRYOGENESIS

A Thesis
Presented to
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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Advisor: James T. Blankenship
Abstract

Pseudocleavage furrow dynamics initiate at cycle 10 to help with mitotic divisions that lack cytokinesis. Permanent furrows then form at the beginning of cellularization (cycle 14). Cytoskeletal networks such as F-actin and non-muscle Myosin II have been demonstrated to be required for furrow formation and provide anchor points for chromosomal segregation. However, what drives the onset of furrow formation and membrane addition remains largely unclear. In my thesis, I have explored RalA and Rab8 function during furrow formation at metaphase and cellularization stages. These furrows are critical for correct chromosomal segregation, while disruption of furrow formation results in severe developmental defects and lethality. Polyclonal antibodies against both RalA and Rab8 were also generated to test the endogenous protein function and degree of the transgenic over-expression.

Through the spatial regulation of cell polarity genes and tension generated by an actomyosin network, intercalation establishes the Drosophila body axis during germband extension (GBE). Previous evidence has shown that interfaces undergo stepwise contraction with a stabilization period between each shrinking interval. However, little is known about how horizontal interface resolution occurs after vertical interface contraction. I have performed time-lapse live-imaging of embryos by spinning disk confocal microscopy, in order to address the potential interface extension mechanism.
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Introduction

Overview of The Early Embryogenesis in *Drosophila melanogaster*

*Drosophila melanogaster* has become a classic model organism for both genetics and molecular biology since Charles W. Woodworth was credited for the first breeding during early 1900s. A huge number of scientific inquiries have used this small organism due to the small number of chromosomes, short lifespan, and ease of stock maintenance. *Drosophila* embryos take about 4 hours after fertilization to complete the fast phase of the GBE. During the first 3 hours after egg laying, cells inside of the embryos experience both syncytial and mitotic divisions to form a uniformly distributed epithelium. Gastrulation occurs right after the cellularization process with the formation of the cephalic furrow and GBE. GBE makes the embryo extend 2.5 fold of its original length along the anterior-posterior axis (Hartenstein and Campos-Ortega, 1985; Irvine and Wiechaus, 1994).

However, rather than getting the extension force from mitosis, cell intercalation becomes the major driving force for the embryonic extension (Irvine and Wiechaus, 1994). With all of the complicated cellular mechanisms mentioned above, it is obvious that *Drosophila* early embryonic development requires to be regulated in a very organized and delicate way. However, the actual regulators of these behaviors and how
they interact with each others are still largely unknown. My work specifically focuses on exploring the possible protein regulators for furrow dynamics before the onset of gastrulation, and the mechanisms of interface horizontal extension during GBE.

**RalA Regulates Transient Furrow Dynamics during Metaphase Stages**

The *Drosophila* embryo develops as a syncytium during the first three hours after egg laying. In this period, cells in the fertilized eggs go through 13 cycles of syncytial divisions without cytokinesis and become a large, single-celled embryo that has approximately 6000 nuclei in a common cytoplasm (Foe and Alberts, 1983; Zalokar and Erk, 1976). The first nine rounds of division occur deep in the interior of the embryo, while divisions proceed directly beneath the plasma membrane and are associated with transient metaphase furrows during cycle 10 to 13 (Kotadia et al., 2010). The presence of metaphase furrows create anchoring points for the microtubule spindle and serve to separate adjacent nuclei during mitosis. Both actin and myosin (nonmuscle myosin II and myosin IV) networks are believed to provide the major driving force for the invagination of furrows (Mermall and Miller, 1995; Foe et al., 2000; Royou et al., 2003). If actin dynamics are observed during the metaphase stages, actin is seen to localize at the top of each nucleus (actin cap) during interphase and then transform into ring structures at mitosis and during pseudocleavage furrow formation.

Other than the major cytoskeletal element of F-actin and astral microtubules, previous research has also identified additional proteins that are required for furrow formation in the early stage of the embryo. Other studies have also found that various
factors such as spectrin, coflin, ARP, anillin, septins, and formins are present at the metaphase furrow (Riggs et al., 2003; Miller and Kiehart, 1995; Stevenson et al., 2002). Also, membrane addition is another important factor for the furrow formation since it gives these furrows their identity. However, the actual mechanism of how metaphase furrow dynamics are regulated is poorly understood.

RalA belongs to the Ras-related small GTPase family (Ohta et al., 1999). As a GTPase, RalA is active in its GTP state, while expression of GDP-locked inactive RalA delays border cell migration during Drosophila oogenesis (Lee et al., 1996). Here, we observe that disruption of rala function by either RNAi knockdown through in vivo injection or maternal knockout by applying germline clone techniques results in severe defects in chromosomal segregation and causes the hatching failure of embryos. I have also observed that RalA marks the future furrow formation site and the absence of rala function results in a complete absence of metaphase furrows.

Exocytosis is believed to depend on the function of the exocyst complex, which tethers vesicles to the cell cortex. The Drosophila exocyst complex has eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. The SNARE proteins then mediate the actual fusion of the vesicle to the plasma membrane. Previous evidence has also shown that a complex is formed between active, GTP-bound RalA and the Ral-binding domain (RBD) of Sec5 (Fukai et al., 2003). The loss of function of Sec5, Sec6, and Sec15 in Drosophila epithelial cells causes the enlargement of Rab11 recycling endosomes and prevents the transportation of E-cadherin to the plasma membrane (Langevin et al., 2005). Therefore, it is possible to predict a mechanism, in which RalA
acts as a regulator and controls the transient metaphase furrow dynamics through the exocyt complex.

**Rab8 Regulates Furrow Formation during Cellularization**

Cellularization happens during mitotic cycle 14 and it takes about fifty minutes from start to finish. Within this time frame, permanent furrows start to form between cortical nuclei and isolate these nuclei inside of their own individual cytoplasm. According to the velocity and length of furrow ingression, cellularization is divided into a slow phase and a following fast phase. The slow phase takes about 35-40 minutes from the onset of cellularization and furrows in this stage invaginate to a depth of 10 μm from the embryo surface. The fast phase then initiates and furrows reach about 30 μm deep from the surface of the embryo (Lecuit et al., 2002). The final result of cellularization is to form an evenly distributed epithelial cell sheet. To achieve this goal, the plasma membrane must rapidly increase at least 30 fold to satisfy the needs of these forming furrows (Lecuit et al., 2002). The furrow invagination process during metaphase and cellularization are believed to be mechanically related to cytokinesis and both of them have cytoskeleton rearrangements and Myosin II has been observed at the forming furrows (Anne et al., 2003). Similarly, the regulators and mechanisms that guide furrow formation is an unsolved puzzle.

Rab proteins have a well-known role in cargo delivery during metaphase trafficking. Rab proteins traffic vesicles, recruit motor proteins, and activate docking and fusion of the vesicles to the plasma membrane (Zerial and McBride, 2001; Miaczynska
and Zerial, 2002; Pfeffer, 2005). More than 75 Rab proteins have been identified in vertebrates, while only 33 Rab proteins have been found in Drosophila (Zhang et al., 2007). Similar to RalA, the Rab proteins are small GTPases that have both GTP (active) and GDP (inactive) states. This permits the use of constitutively active (GAP pathway deficient) and dominant negative (GEF pathway deficient) fly lines to identify where Rab proteins function. In the constitutively active mode, the Rab protein remains in GTP-locked state, while the Rab protein remains in a GDP-locked state in the dominant negative mode. By screening the 33 Rab proteins in Drosophila, Rab8 dominant negative embryos were found displaying an uneven epithelium and loss of later gastrulation movements (Lauren Mavor, unpublished data). Additionally, RNAi knockdown of Rab8 indicated the loss of the invagination furrows as well as the absence of F-actin networks (Lauren Mavor, unpublished data). To better resolve the dynamics of the Rab8 protein, I designed a “squish” technique to dissect embryos. The embryonic dissection is able to convert the 3D structures to 2D structures, while cellular processes continue normally. The thinner layer of the cells can provide a better resolution by limiting signal extinction. Using this new technique, embryos from metaphase to late cellularization stages have been dissected and tubule dynamics have been analyzed.

**Cell Interface Dynamics during GBE**

Convergent extension movements occurs in order to shape the body axis and GBE forces are generated through the spatial regulation of intercellular adherens junctions (Blankenship et al., 2006; Zallen and Blankenship, 2008). GBE occurs after gastrulation initiates and it takes less than two hours to complete (Irvine and Wiechaus, 1994). Similar
to the cellularization stage, GBE is also divided into fast and slow phases. The fast phase of the GBE occurs during the first 30-40 minutes and it achieves about 80% of the extension at the end through cell rearrangement rather than mitosis (Blankenship et al., 2006; Hartenstein and Campos-Ortega, 1985). The slow phase takes place after the fast phase and completes the rest of the GBE for the following time period. We are most interested in the fast phase because it drives of the major amount of elongation during GBE.

The intercalation mechanism is highly regulated by the genes that establish the body axis. The planar polarization of the PAR3 scaffolding protein, E-cad, and α-catenin occurs at horizontal interfaces, the planar polarization of F-actin pattern at vertical interface is the first symmetry-breaking event (Bertet et al., 2004; Blankenship et al., 2006; Zallen and Wieschaus, 2004). All of these proteins mentioned above provide the polarization guidance cues to the intercalating cells. For the actual shrinking of the interface, evidence has shown that it is a biphasic motion with stabilization events between each contraction (Rauzi et al., 2010). Interface length measurements from our lab on rosette formation also demonstrated the similar biphasic contraction patterns (Ashley Motlong, unpublished data). Medial Myosin II and junctional Myosin II are found as two distinct populations at the contraction interface of the T1-T2 transformation in *Drosophila* and they were suggested to regulate interface contraction and stabilization (Rauzi et al., 2010). Both the isolated interface and linked interface behaviors reach a common vertex and then resolve horizontally to finish the intercalation process during GBE. However, even though F-actin was observed to localize briefly (~150s) on the
newly growing horizontal interfaces, no other evidence has shown if there is any difference between the shrinking of the vertical interfaces and growing horizontal interfaces (Blankenship et al., 2006).

**Thesis Specific Aims**

The goals of this thesis are to understand how the RalA and Rab8 proteins regulate furrow dynamics during early embryonic development prior to gastrulation, and to observe the potential mechanism that drives interface growth during GBE. To examine the first aim, I have laser confocal microscopy to test the localization and expression of the endogenous and ectopically expressed proteins in both wild-type and mutant fly lines. I have also utilized germline clone techniques to exam the maternal knockout of RalA during the metaphase stages. To better visualize the furrow dynamics for the Rab8 protein, squished embryo dissection technique was developed and live-imaged by a spinning disk confocal microscope. Several cell components such as Golgi, plasma membrane, and late endosome were tested to see the potential sources of membrane addition during the invagination of furrows. For the second aim, a fly line with plasma membrane markers was live-imaged during GBE. Both interfaces from the T2-T3 transformation and rosette resolution were measured manually with Photoshop and more precise measurements were obtained with Matalab (Tim Vanderleest).
Material and Methods

Confocal Microscopy and Time-lapse Live Imaging

Immunofluorescence specimens were first fixed and then acquired under an oil 40X objective with 1.35NA on an Olympus Fluview FV 1000 confocal laser-scanning microscope. The time-lapse live images were acquired under an oil 63X objective with 1.40NA on a Ziess XA10 spinning confocal microscope. To prepare the live imaging slide, right-staged embryos were dechorionated with 50% bleach and immersed with Halocarbon 27 oil before the placement of the cover glass. Both the Argon 488 and Argon 561 lasers were adjusted to the 25% of their maximum power with EM Gain of 90. All of the time-lapse live images were acquired with an exposure of 150.

Drug Treatment and Embryonic Injection

Embryos were line-up on the apple juice plate and glued to the cover glass. After 11 minutes dehydration in the dehydration chamber, the embryos were immersed in Halocarbon 100 oil. Lectrunculin B (10mM, Sigma) was injected into the ventral part of the embryo through the needle.
**Embryo Dissection and Squish Movies**

Embryos were prepared with 50% bleach and qualified embryos were separated in single cover glass with Halocarbon 27 oil. The selected embryo was dissected with two pairs of sharp forceps. The cover glass was then inverted and placed on the slide with biofolie film (hydrophobic side faces the embryo). Live images were acquired under the spinning disk confocal microscope described above.

**Fly Stocks and Genetics**

Fly Stocks are maintained in the 25°C incubator and all UAS fly stocks were crossed to mataTub-Gal4VP16 67C; 15 (D.St. Johnson). The F2 generation from the cross was collected.

UASp-YFP:Rab8

UASp-YFP:Rab8 T22N (Dominant negative)

UASp-YFP:Rab8 Q67L (Constitutively active)

Jup:GFP; mCh:Rab8

117; UASp-mCh:Rab8

UASp-GFP:RalA (#8/Tm3)

117; 95-1

Gap43:mCh; Rab8:YFP
Jup: GFP
Oregon R

OvoD

RalA PL56-FRT19A

RalA: GLC; Jup: GFP

RalA: GLC; Moe: GFP

RalA: GLE; His: RFP

Moe: GFP

Moe: mCh; UASp-YFP: Rab8

Germline Clone Maternal Knockout

To knockout the maternal protein contribution to the embryos, germline clone technique was applied with a dpodΔ96 deletion. RalA homozygous is lethal and OvoD FRT mutant is dominant female-sterile. RalA PL56-FRT19A/FM7 virgin females were crossed to the OvoD FLP- FRT males in the bottle for three days before the parental generation was transferred to a new bottle. Embryos were incubated in 25°C for another 24 hours before performing a 2-hour heat-shock in 37 Celsius water bath. Repeat the heat-shock process after another 24 hours. Adult female virgin flies were collected after they hatched. Virgin females were then crossed to OreR males and placed in a collection cup and the embryos from this cup were collected for further analysis.
**Handpeeling Embryo Fixation**

Embryos were collected in an apple juice plate over a designated time period (usually 2-4 hours) and collected with a stainless steel water-net after 2 minutes soaking with 50% bleach solution on a shaker (240 rpm). The collected embryos were then rinsed 5 times with DI water and placed in a 10mL fixative solution (5mL Heptane, 0.625mL Paraformaldehyde, and 4.375mL 1×PBS) for 1.25 hours. Retrieved the embryos from the fixative solution and rinsed them for another 5 times of DI water. Embryos were then glued on a Petri-dish with a small paint-brush. Added 1×PBS+1%BSA on the lid of the plates to prevent dehydration of embryos. With the indirect light on the dissection scope, vitelline membrane of the correct stage of the embryos were removed with a capillary needle. The freed embryos were then transferred into a 1.5mL centrifuge tube by using a pasteur pipette. Solution in the centrifuge tube was exchanged with blocking buffer and then placed on a nutator at 4°C for overnight or 2 hours in room temperature.

**Immunohistochemistry**

In order to visualize the specific protein under laser confocal microscope, fixed embryos were then transferred into primary antibody solution on the nutator for either 2 hours in room temperature, or 4°C for overnight. The primary antibody was then washed off by using PBT (50mL 1×PBS+1%BSA and 50μL of Tx-100) solution 4 times within one hour period. The embryos were then placed into the secondary antibody solution and incubated for 45min to 1 hour. Washed away the secondary by using PTW (50mL 1×PBS+1%BSA and 50μL of Tween-100) solution 4 times within one hour period.
Mounted the embryos with Prolong gold with or without DAPI (Invitrogen) on a glass slide. The slide was able to observe under laser confocal microscope 4 hours later.

Antibody used: Rabbit α-GST (1:500, Santa Cruz Biotechnology), rabbit α-GFP (1:1000, Life Technologies), mouse α-GFP (1:100, Life Science), mouse α-lamin (1:1, ADL), rabbit α-l avalamp (1:250, Sissionetal., 2000), Hoescht (1:500, Sigma), mouse α-HRS (1:500), α-mouse 488 (1:500, Life Sciences), α-guinea pig 488 (1:500, Life Sciences), α-rabbit 488 (1:500, Life Sciences), and Phalloidin 546 to mark F-actin (1:250, Molecular Probes).

**Methanol Popping Embryo Fixation**

Embryos were collected and fixed the same way as the hand-peeling technique described above. However, rather than a 75 minute fixation, only 30min in the fixative solution was required. After the fixation, the embryos were transferred with a pasteure pipette in to a new conical vial that contains 5mL of Heptane. Quickly added 5mL of methanol into the conical vials that contained embryos and capped the vial with lid. A 30 second vigorous shaking was required and all the popped embryos were sunk to the bottom. Transferred the embryos at the bottom of the conical vial to a 1.5mL centrifuge tube and rinse it 5 times with pure methanol. Embryos in the methanol can either store in a -20°C freezer until use, or placed in the blocking buffer and followed the protocols of hand-peeling method described above.
PCR Cloning and Protein Chromatography

1. PCR Cloning

Primer with targeted cDNA sequence of Rab8 and RalA were designed and a standard 20μL PCR reaction was prepared with Roche expand high fidelity system. The PCR machine we used was from BioRad and the entire process usually took around 2 hours. Reaction product was then purified with Cyclepure kit (Omega Biotek) and 5 μL sample was ran on a 1.7% agarose gel to confirm the purity of the sample. Enzyme double digest (NEB) was then performed after the purification and the entire post-digest sample was run on a 1.7% agarose gel. Specific bands were collected and Gel extraction kit (Omega Biotek) was used to recycle the purified bands. Plasmid was purified with the Plasmid purification kit (Omega Biotek) and digested with the restriction enzymes. The digested cDNA and plasmid were ligated by using the T4 ligase with cutsmart buffer (Both from NEB). The ligated sample was transformed in to E.coli (NEB) for future analysis. Restriction enzyme used: BamHI HF (NEB), NdeI (NEB), and SalI HF (NEB).

2. Transformation to Protein Expression E.coli

Cells were lysed and plasmids were extracted with Plasmid purification kit (Omega Biotek). The purified plasmids were then transformed to the protein expression E.coli (NEB C2523H).

3. Protein Expression and Column Purification

The bacteria were planted in a 20mL (in 150mL Elenmeyer flask) rich broth by using a P-2 pipette tip and cultured in a shaker at 37℃ for overnight. Poured the 20mL
solution into a 2L Erlenmeyer flask contained 1L of rich broth during the morning of the second day and cultured at 37°C in the shaker until the A600 reached 0.5. The IPTG was then added into the solution and the bacteria were cultured for an additional 3 hours. Transferred the solution into the 1L centrifuge bottle and centrifuged for 20 minutes at 4,000g. Decanted the supernatant and then re-suspended the cell pellet with 25mL of the buffer solution (1×PBS+10% Glycerol). The sample was left in -20°C overnight. Took out the sample from the freezer and let it thawed in room temperature. Sonication was followed after the bacteria were thawed to break the cell membrane and the mixture was placed in a 50mL centrifuge tube and centrifuged for 20 minutes at 10,000g. Pour out the supernatant and diluted it at 1:5 ratios with column buffer. Prepared the column (usually 2mL of the bedding volume) and wash the column with 1×water adn 1×column buffer. Ran the sample through the column either by using batch method, gravity method, or spinning method. Washed the column two more times with full column volume of column buffer and eluted with designated volume of the elution buffer.

4. Protein Purity Check and HPLC

The sample concentration was checked by using a NanoDrop 1000 machine (Scott Barbee lab) and a SDS-PAGE gel (Bio-Rad) was ran to detect the purity of the elution product. A HPLC (Scott Pagen lab) was performed for the elution product to dilute the compounds in the elution buffer to a non-antigen considerable amount. Besides, HPLC could separate the non-specific bands from the target band and exchange the column buffer to the desired buffers.
Western Blot

Western blot was used to detect the specificity of the GSTα Rabbit (Santa Cruz 1:10,000) and the RalA antibodies (Both rabbit and guinea pig) from Procono Rabbit Farm company. 0.2-0.5μg of the sample from protein purification was used per SDS-PAGE gel well (Bio-Rad) and ran at 150V for 1 hour. The gel was transferred to a nitrocellulosal membrane overnight within the transfer buffer (1 × TGS, Bio-Rad) at 30V in a 4°C cold room. The membrane was then blocked with dry milk solution to eliminate non-specific binding sites (195mL DI water and 10g dry milk). The primary and secondary antibodies were then followed the blocking process. To illustrate the result, the developer kit contained HRP (Life Science) was used.
Results

**RalA is required for Metaphase Furrow Dynamics**

Nuclei migrate peripherally to a space beneath the plasma membrane as embryos enter the metaphase stages. At the onset of syncytial division cycle 10, transient metaphase furrow dynamics become observable by confocal microscopy. As previously mentioned, actomyosin networks are known components that localize to metaphase furrow. Therefore, F-actin can be used as a good marker for metaphase furrow (Figure 1 A’ and B’). To illustrate the embryonic location of RalA, fluorescence-tagged transgenic protein fly stocks were created, and the selected embryos were imaged under both live and fixed conditions. Fixed staining images of metaphase stage embryos indicate that RalA localizes at the future pseudocleavage furrow formation sites (Figure 1, A-A”), and partially coincides with F-actin on the furrows during syncytial divisions (Figure 1, B-B”).

We also performed two color live-imaging of mCherry-tagged RalA and GFP-tagged Histone embryos (Ryan Holly, unpublished data). Results from the live embryo experiments supported the immunofluorescence data in Figure 1, and showed the transformation of the RalA population from the cytoplasm to the cell cortex at the onset of syncytial divisions (data not shown). Nucleus structure and nuclear envelope (NE)
morphology from cycle 10 to 13 were also imaged with fixed embryos of both wild-type and RalA mutants. For a wild-type embryo, normal nuclei had a circular-shaped NE during interphase (Figure 2).

In order to test if RalA is required for the furrow dynamics, we tested both RNAi injection with scoring (Ryan Holly, unpublished data) and rala mutation. RNAi knockdown of RalA function caused over 90% of the injected embryos to arrest before GBE. In order to completely knockout the RalA maternal and zygotic expression, germline clones were performed. In these germline clone mutant embryos, nuclear malformation and chromosomal missegregation was observed. Additionally, embryonic lethality occurred before cellularization (Figure 3). To better analyze furrow function and chromosomal segregation, apical lateral view of the embryos were fixed and imaged. We noticed that both wild-type and rala mutant embryos had normal sizes of the actin caps and nuclei shape before the onset of metaphase (Figure 4, A-A”, Figure 5, A-A”). However, metaphase furrows were missing during the entire mitotic phase in the mutant embryos and resulted in nuclear separation defects (Figure 4 and Figure 5).

Two types of the abnormal chromosomal missegregation behaviors were observed in the rala mutant. They were: 1) daughter nuclei chromosomal detachment failure, or 2) adjacent nuclei fusion. In the former type of the behavior, two daughter nuclei fail to form because their chromosomes are still connected (yellow arrows in Figure 3 and Figure 5). For the second type of chromosomal behavior, daughter nuclei fused with the neighboring nuclei (red arrows in Figure 3 and Figure 5). Analysis of live imaging movies acquired by the spinning disk confocal microscope demonstrated that about 22%
of the nuclei (N=110) have chromosomal detachment failure, while 6% of the nuclei fused with their neighbors within the first cycle of the abnormal syncytial division. Nucleus division kept going for one to two cycles until three or more nuclei fused together, which caused embryonic lethality.
Figure 1. RalA marks the future furrow ingression sites. Selected metaphase stage GFP:RalA embryos were fixed and stained with F-actin marker Phalloidin and GFP antibody at the apical lateral view. Arrows (red and yellow) on the top of the figure panel (A-A’”) indicate the sites where RalA precedes F-actin and mark the future ingression furrows. At the bottom part of this image (B-B’”), the formed metaphase furrows show colocalization of RalA and F-actin. Images were acquired at a 40x objective, scale bar is 5μm.
Figure 2. Normal chromosomal segregation during metaphase. OreR embryos were fixed and stained with the DNA marker Hoechst, and nuclear envelope (NE) antibody Lamin. Planar view of the embryos from cycle 10 to 13 (A-D”) were then selected and imaged under laser confocal microscope with a 40x objective. Images were acquired on a 40x objective, scale bar is 5μm.
Figure 3, *rala* mutant disrupts normal chromosomal segregation. Maternal and zygotic knockout *rala* germline clone embryos (RalA-GLC; Moe:GFP) were fixed and stained with the DNA marker Hoechst and a Lamin antibody that labeled the NE. Embryos without RalA experience failure of chromosomal segregation during metaphase (A and B) and cause the failure to hatch. Two types of chromosomal missegregation are showed above: daughter nuclei chromosomal detachment failure (red arrows) or adjacent nuclei fusion (yellow arrows). Images were acquired on a 40x objective, scale bar is 5μm.
Figure 4. Metaphase furrow formation in OreR embryos. OreR wild-type embryos were fixed and stained with F-actin marker Phalloidin and DNA marker Hoechst. Images of metaphase stage embryos were selected and acquired with the laser confocal microscope. Actin caps are formed apically during interphase stages (A-B) and transformed into ring structures that elongate into furrows when nuclei enter the mitotic cycles (C-F’). Initiation of the metaphase furrows formation is labeled with red arrows (C’). Images were acquired on a 40x objective, scale bar is 5μm.
Figure 5. Metaphase furrows are missing in rala mutant embryos. Germline clone embryos (RalA-GLC; Moe:GFP in A-A”, RalA-GLC; Jup:GFP in B-C”) were fixed and stained with F-actin marker Phalloidin and DNA marker Hoechst. Mutant embryos are initially normal at the onset of metaphase stage (A-A”) with normal-shaped actin caps. However, no metaphase furrow is observed throughout the divisions (B’ and C’). Both daughter nuclei chromosomal detachment failure (red arrows in B”) and adjacent nuclei fusion (yellow arrows in C”) show up along the embryonic development. Images were acquired on a 40x objective, scale bar is 5μm.
Abnormal behavior type | Daughter nuclei detachment failure | Adjacent nuclei fusion
---|---|---
Number of the nuclei | 24 | 9
Percentage | 21.8% | 8.4%

Table 1. Percentages of different types of chromosomal missegregation in *rala* mutant embryos during metaphase. Nuclei were counted from two individual RalA-GLC; Histone:RFP live imaging embryo movies. Every nucleus from these two movies was classified either under two categories with the relative percentage calculated within one syncytial division (N=110).
**Rab8 Dynamics during Cellularization**

Previous experiments from our lab indicated that Rab8 transformed from punctuate structures to a cortical array when syncytial divisions occurred (Lauren Mavor, unpublished data). Instead of transient metaphase furrows, Rab8 starts to form a tightly organized cortical array from the punctuate structure at the beginning of cellularization. Both punctuate structures in the cytoplasm and furrow structures at the cortical array persist throughout the entire cellularization stage (Figure 6). Additionally, Rab8 tubules extended first from the apical surface of the embryo at the beginning of the slow phase of the cellularization (Figure 6 A’ and A”). By the end of slow phase, F-actin co-localized with Rab8 and cellularization furrows had enclosed each nucleus into their individual cytoplasm (Figure 6 D-D”).

To further examine Rab8 dynamics and their tubular behavior, constitutively active (GTP-locked) and dominant negative (GDP-locked) version of the Rab8 small GTPase were expressed and imaged by laser confocal microscopy (Figure 7). Both tubular and punctuate cytoplasmic localized structures were found in wild-type and constitutively active embryos (A-B), while dominant negative Rab8 remained as large aggregates at the top of nuclei throughout the cellularization stage (C-D). However, neither dominant negative nor constitutively active transgenic lines caused lethality of the embryos.

Golgi and endosomal compartments were demonstrated by previous studies to be required for cellularization furrow formation (Sisson et al., 2000; Pelissier et al., 2003).
Therefore, we tested the localization of Rab8 with various endocytic and exocytic cellular component markers (Lava Lamp for Golgi, Hrs for late endosome, and Rab11 for recycling endosome) by laser confocal microscopy. Rab8 showed minimal colocalization with Rab 11 (less than 18%) during cellularization (Graph 1). Intriguingly, Rab11 and Rab8 colocalized a little bit more during the early cellularization (18%) than at the mid-cellularization stage (6.6%). For the Golgi and late endosomes, Rab8 showed minimal colocalization with these two compartments during cellularization (Graph 1, Figure 8).

**Embryo Dissection Reveals the Tubular Dynamics of Rab8 Tubules**

To better analyze the Rab8 dynamics during Drosophila early embryogenesis and to reduce the light diffraction due to the thickness of the embryos, I developed an embryo dissection technique. As a test of this technique, we compared microtubules in intact and in dissected embryos. Only microtubule bundles and MTOC can be visualized in intact embryos (Figure 9A). However, if the embryo was dissected and imaged under the same conditions, individual microtubule dynamics were readily detectable (Figure 9B). With the success of the squished movie technique when imaging the microtubule network, I extended the work to Rab8. Time-lapse images were acquired with the spinning disk confocal microscope at a rate of 500ms per frame. Results from the experiments indicated that wild-type Rab8 embryos formed small projections at the onset of the cellularization (Figure 10A), and tubule dynamics were observable during the mid-cellularization stage (Figure 11A).
As the cortical array formed at mid- to late cellularization, squished embryos also displayed similar networks at similar stages (Figure 12A). Constitutively active Rab8 dissection movies indicated similar dynamics of Rab8 as in the wild-type controls, except that tubular structures were readily to see during early cellularization stage in this GTP-locked state (Figure 10B-12B).

With the default presumption that GDP-locked state was considered as inactive, dominant negative embryonic dissections showed no tubule dynamics throughout the entire cellularization stage. Rather, large aggregates were observed and they remained static throughout cellularization (Figure 10C-12C). These aggregates are highly similar to the cytoplasmic punctuate structures that reside above the nuclei from intact immunofluorescence stained images (Figure 7C and 7D). Additional experiments were also performed to examine the source of membrane for tubule formation in dissected embryos (2 color imaging with: 117; mCh:Rab8, Gap43:mCh; Rab8:YFP). However, plasma membrane components did not show co-localization with Rab8. I have also tested F-actin to see if it behaved similar to Rab8 tubular behaviors and the results were also negative (Moe:mCh; UASp-YFP:Rab8).
Figure 6. Rab8 forms a cortical array during cellularization. Wild-type YFP:Rab8 embryos were fixed and stained with F-actin marker Phalloidin and GFP antibody. At the onset of cellularization (A), Rab8 is near the apical surface. By mid- (B) and late (C) cellularization, Rab8 has become cortically localized. Rab8 starts to form furrows that precede the F-actin during the slow phase (red arrow in A’ and A’’). Both cortical array and punctuate structure population are showed (D-D’’). Images were acquired on a 40x objective, scale bar is 5μm.
Figure 7. GTP-locked and GDP-locked states of Rab8 embryos at mid-cellularization. CA (A-B) and DN (C-D) embryos were fixed and stained under the same condition in Figure 7. Images were acquired on a 40x objective, scale bar is 5μm.
Figure 8. Rab8 populations are largely distinct from the late endosome and the Golgi. Wild-type YFP:Rab8 embryos were collected and stained against GFP antibody and either Golgi (Lva) or late endosome (Hrs). Rab8 does not show much colocalization for either Golgi (A-B) or the late endosomal compartments (C-D). Images were acquired on a 40x objective, scale bar is 5μm.
Graph 1. Colocalization percentage between Rab8 and various cellular compartments. Quantitation of the colocalization percentages, with standard errors for Rab8, recycling endosome (Rab11), Golgi, and late endosomal compartments during early and mid-cellularization (Lauren Mavor, unpublished data).
Figure 9. Embryo dissection gives detail of individual microtubule structure. Similar stages of syncytial division embryos (Jupiter:GFP) were selected and live-imaged by spinning disk confocal microscope at 500ms interval for 50s. Microtubule-organizing centers (MTOC) dynamics are able to be observed in the intact embryo (A). With the embryonic dissection, individual microtubule structures are able to be seen with similar conditions (B). Images were acquired under a 63x objective and scale bar is 10μm.
Constitutively active Rab8 shows tubule dynamics at the onset of the cellularization. Wild-type, constitutively active, and dominant negative YFP:Rab8 embryos were dissected and live-imaged on a spinning disk confocal microscope. Small projections extend from punctuate structures of wild-type embryos (yellow arrowheads, A-A”). For constitutively active embryos, tubules are formed and project out from similar punctuate structures (yellow arrowheads, B-B”), while dominant negative embryos remain as large aggregates (C-C”). Images were acquired at a rate of 500ms for 50s under a 63x objective. Scale bar is 10μm.
Figure 11. Rab8 tubule dynamics at mid-cellularization. Dissected YFP:Rab8 embryos at mid-cellularization were imaged under the same condition in Figure 11. Both wild-type (yellow arrowheads, A-A") and constitutively active (yellow arrowheads, B-B") embryos show tubule dynamics, while dominant negative embryos remain in large aggregates (C-C"). Images were acquired at a rate of 500ms for 50s under a 63x objective. Scale bar is 10μm.
Figure 12. Tubule structures stabilize at late cellularization. Tubules form static structures at the end of the cellularization stage in wild-type (A-A") and constitutively active (B-B") embryos. However, dominant negative embryos (C-C") form even larger aggregate structures compared to mid-cellularization. Images were acquired at a rate of 500ms for 50s under a 63x objective. Scale bar is 10μm.
Protein Purification and Antibody Generation

To demonstrate the endogenous protein function and the degree of the over-expression from transgenic lines, antibody generation became a priority. In order to generate polyclonal antibodies, antigen was first created with IPTG triggered expression in *E. coli*. cDNAs of Rab8, Rab10, Rab35, and RalA were first amplified by PCR with specific primers that contained restriction enzyme recognition sequences (Figure 13). Following a standard protocol of cloning, these cDNA sequences were fused to plasmid vectors and eventually transformed into a protein expression strain of *E. coli*. Three vectors that have the affinity tag were used for protein purification and they were GST, Histidine, and Maltose Binding Protein (MBP). Controls of these three tags were done with either the empty plasmid vectors or proteins that were known to have good yields (Figure 14). Antigen yields varied from less than 1mg to 35mg per liter of rich broth. MBP tagged vector was originally designed to have a Factor-Xa protease cleavage site that could separate the MBP protein from the protein of interest. However, both Rab8 and RalA precipitated out of column buffer once protease cleavage began. Hence, MBP-Rab8 and MBP-RalA (both proteins are about 70kDa) were sent for antibody production. We tested the generated antibodies by western blot. However, to date, specific binding has not been observed (Figure 18).

GST-RalA and GST-Rab8 has also been expressed and purified with affinity columns. Only GST-RalA was able to generate an amount of 5mg protein per 1 liter of rich broth (Figure 15), while GST-Rab8 had a yield less than 1mg per 1 liter of rich broth. Western blotting technique was applied to identify the unknown second band that showed
at the size of either MBP or GST in the elution line on the SDS-PAGE gel. Results from the western blot demonstrated that the unknown bands were actually GST and MBP. In order to exam the reason for the low yield of the GST-Rab8 and to potentially create a protein that specifically attaches to the antibody, fractional GST-Rab8 proteins were created. These fractions contained a GST tag with either N- or C-terminal portion of Rab8 (50% or 75% of the Rab8 cDNA sequence; Figure 16). Only the C-terminal 50% GST-Rab8 had a significant yield (4mg per liter of rich broth), while the other three Rab8 constructs had minimal yield (Figure 17). Overnight room temperature culturing with IPTG was also tested to improve the folding and solubility of the proteins. However, no significant increase of yield was observed.
Figure 13. **Affinity column tagged cDNA sequences.** Rab8, Rab10, Rab35, and RalA cDNA sequences were PCR amplified with specifically designed primers for each protein expression vector. The base-pair ladder is showed on the left of the EtBr gel. MBP-Rab8 and MBP-RalA are not shown in this gel, but the results were positive.
Figure 14. GST protein expression and purification control. PGEX-6p-1 vector was expressed without any inserted protein of interest. SDS-PAGE image above indicates a successful IPTG–triggered protein expression and purification. Both Elution 1 and Elution 2 lanes showed specific GST band (~28kDa) with a good amount of the yield (~36mg per liter of rich broth). MBP and His protein are not shown in this image, but the results were positive.
Figure 15. Protein purification of the GST-RalA. Protein expression of the GST tagged RalA were expressed in *E.coli* and passed through an agarose Glutathione affinity column. Protein ladder, pre-column extract, flow-through (FT), and eluted product are run on the SDS-PAGE gel. Destained image indicates the position of the desired protein and a second band is the GST.
Figure 16. Test digest of the fractional Rab8 DNA. Fractional Rab8 cDNA PCR was performed for 20 cycles and ligated into the GST (PGEX-6p-1) plasmid. Test digest was done for two hours and run out on a 1.7% EtBr Agarose gel.
Figure 17. **Fractional protein expression (50%) of the GST-Rab8.** N-terminal 50% and C-terminal 50% of the GST-Rab8 was expressed and purified with the protein column. The SDS-PAGE image above indicated that only C-50% elution has expression at a level of 3-5mg per liter of rich broth. Pre-col = total protein extract from the bacteria. Wash = flow through. N-50% = N-terminal 50% of the GST-Rab8 elution. C-50% = C-terminal 50% of the GST-Rab8 elution. MW_{50\%GST-Rab8} is about 40kDa.
Figure 18. Western blot analysis of antibody bleeds. MBP-RalA polyclonal antibodies were tested by the Western blot. The sources of proteins is from whole-embryo extract (OreR and GFP:RalA). Each lane was loaded with 20μg of the proteins. In panel A, Rabbit anti-RalA antibody is used to blot. Red marks indicate the expected results from RalA antibodies in general. Two guinea pig anti-RalA antibodies were tested in-parallel under the same conditions (B and C). However, none of them are able to show the correct-size bands.
**Horizontal Extension of Cell-Cell Interfaces during GBE**

GBE allows the embryo to establish the body axis and increases its body length by 2.5 fold through cell-cell intercalation. GBE utilizes a network of planar polarized proteins that direct interface behaviors of contraction and growth. Isolated interface contraction is also defined as a T1 transformation (Weaire and River, 1984), while linked interface contraction is defined as “rosette” behaviors (Blankenship et al., 2006). Both T1 and rosette behaviors shrink vertical interfaces to generate a common shared vertex (Figure 19). However, we noticed that it only took about 5 minutes for an isolated interface to finish its contraction (Figure 19B), while linked interfaces needed about 15 minutes to reach to common vertex (Figure 19A). As previously mentioned, T1-T2 transformation was observed to have stabilization periods within the contraction intervals (Rauzi et al., 2010). Similar results were obtained from our lab during the process of rosette formation (Ashley Motlong and Tim Verderleest). In order to observe if the horizontal interfaces could also be described by a ratcheting model, I examined T2-T3 transformations and rosette resolution (Figure 20). One suggestion that two distinct mechanisms might drive interface growth comes from the finding that F-actin is enriched at the newly formed horizontal interfaces for about 150 seconds (Blankenship et al., 2006). I would therefore be interesting to see if there were any changes in interface growth rate between the first 150s and the rest of the resolution process.

Similar time lengths for the interface elongation before the final stabilization comparing to the interface contraction was observed ($T_{t2\rightarrow t3} = 3.75\text{min}$, $T_{\text{rosette resolution}} = 13.1\text{min}$). Both types of growing interfaces were graphed either by hand
measurement through the use of the *ruler* tool in Photoshop 5.1, or by automated measurements from MatLab software (Graph 2 and Graph 3). To reduce the background noise and non-specific interface growth, three filters were set at 0.5px (pixel), 1.0px, and 1.5px (Table 2). Four T2-T3 interfaces and three resolving rosettes from time-lapse movies were measured at a rate of every 12.5s for 20 minutes. The interface growth rates were calculated between each two data point and averaged for the specific time intervals listed in the table. Since they were performed by hand measurement, human error was included. Similar growth rates between T2-T3 transformation and rosette resolution were found under the 1 px filter, while all the other filters indicated a difference for at least 0.2 px differences. However, since they were too small to compare with human error pixel size (>1px), no significant difference was observed between T2-T3 and rosette behaviors. Therefore, it appeared that both T2-T3 transformation and rosette resolution followed a constant growth rate of about 3px per 12.5s.
Figure 19. Contraction of linked and isolated interfaces during GBE. 117; 95-1 embryos were live-imaged by a spinning disk confocal microscope. Anterior is left and ventral is down for the images above. Linked interface contraction (A) happens within a time-frame of 15 min, while isolated interface contraction takes about 5 min to finish (B). Images were acquired at a rate of 500 ms for 20 min under a 63x objective. Scale bar is 10 μm (Ashley Motlong).
Figure 20. Horizontal interface elongation during GBE. 117;95-1 embryos were live-imaged under the same conditions as in Figure 14. Both linked (A) and isolated interface (B) horizontal elongation is shown above. Rosette resolution takes about 13-15 min to resolve and T2 to T3 transformation takes about 4-5 min to achieve. Images were acquired at a rate of 500ms for 20min under a 63x objective. Scale bar is 10μm.
<table>
<thead>
<tr>
<th>Pixel Filter and Time Frame.</th>
<th>Rosette Resolution Interface Growth Rate (px/12.5s)</th>
<th>T2-T3 Transformation Interface Growth Rate (px/12.5s)</th>
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</table>

Table 2. Velocity of interface growth with specified noise filter. 117; 95-1 embryos were live-imaged and auto-leveled in Photoshop 5.1. Images were selected every 12.5s and interface lengths were measured. Step-wise velocities were calculated under 0.5px, 1px, and 1.5px noise filters.
Graph 2. **Automated analysis of T2-T3 transformation.** Two isolated interfaces growth were measured with Matlab and the actual growing zone was labeled from 500-1500 (~5min) range for the T2-T3 transformation (Tim Vanderleest).
Graph 3. Isolated and continuous interfaces growth analysis. Both T2-T3 transformation (blue) and rosette resolution (red) interface length growth were measured and plotted in the graph. 6th degree of polynomial regression line is plotted.
Discussion

Overall Conclusions

I examined two main lines of inquiry in this research thesis. Their underlying mechanisms were examined by both fixed and live imaging techniques. For the RalA project, I have found that the critical role of this protein is in the formation of transient metaphase furrows and chromosomal segregation. Also, I have proposed a potential model for RalA function in the regulation of metaphase furrow formation through membrane trafficking and the exocyst complex, rather than the F-actin network.

For the Rab8 project, Rab8 localization from transgenic *Drosophila* embryos were imaged and a new embryonic dissection method was developed. I have shown that Rab8 is a primary regulator of furrow formation during cellularization, which results in the embryonic epithelial sheet. GDP-locked inactive state Rab8 fails to form a cortical array and normal structures during cellularization. Several endosomal components and the Golgi were tested to see if Rab8 regulated furrow membrane addition through any of these pathways. Additionally, I applied an embryonic dissection technique and found striking Rab8 tubule dynamics during early to mid-cellularization. This demonstrates that Rab8 indeed forms tubular structures during the slow phase of the cellularization stage as has been shown in fixed and stained images (Figure 6A'). I have also generated antigens
against Rab8, Rab10, Rab35, and RalA from *E.coli* to test the endogenous function of these GTPases. However, I have not yet successfully imaged with the RalA antibodies.

As a third aim to my thesis, I have also examined the horizontal interface growth after vertical contraction during GBE. Intriguingly, I find that extension appears to occur at a relatively uniform rate, and does not show the obvious ratcheting behaviors observed during interface contraction.

**RalA Regulates Metaphase Furrow Dynamics**

Metaphase furrow dynamics help the *Drosophila* embryo undergo the syncytial divisions from cycle 10 to 13, when the embryo becomes crowded with nuclei in a common cytoplasm. I have found that a key regulator of furrow dynamics is a small GTPase, RalA. From immunofluorescence stained images (Figure 1), RalA was found localized at future metaphase furrow formation sites and it preceded formation of an F-actin network. In support of the idea that RalA is essential to early embryonic development, both RNAi and a *rala* mutant line were characterized. The results showed that most embryos from the RNAi knockdown arrested before the end of cellularization (Ryan Holly, unpublished data), while the knockdown experiment, which removed both maternal and zygotic contributions of RalA, caused a complete failure of embryos to hatch. In order to examine what actually caused the lethality of the embryos, I performed both time-lapse live imaging and antibody staining techniques to visualize the onset of abnormal behaviors.
Both types of the imaging techniques indicated that chromosomal missegregation was the key factor that arrested development (Figure 3 and Figure 5). Rather than forming the rounded structure of nuclei during syncytial division stages, the rala mutant nuclei showed one of two types of abnormal nuclear behaviors. Nuclei in these mutated embryos either have a chromosomal detachment failure at the anaphase of division, or nuclei fused adjacent with their neighbors (Figure 3 and Figure 5). Interestingly, chromosomal missegregation usually does not occur until cycle 10 or 11 and they are indistinguishable from OreR embryos at the onset of metaphase stages (Figure 4 and Figure 5). I have also found that none of these rala mutant embryos successfully form metaphase furrows during metaphase divisions. As previously mentioned, metaphase furrows associate with the actomyosin network and prevent chromosomal missegregation (Mermall and Miller, 1995; Foe et al., 2000; Royou et al., 2003). Therefore, the lack of furrow formation caused adjacent nuclei to fuse with each other in the mutant embryos. However, no evidence from my experiments or previous studies could explain how RalA may cause daughter nuclei detachment failures. As for the mechanism of membrane addition to the metaphase furrows, structural and biochemical studies have shown that Sec5 and Exo84 from the exocyst complex bind to RalA in a competitive manner (Jin et al., 2005). Therefore, I propose a model that RalA promotes metaphase furrow ingress and membrane addition through the exocyt complex. In this model, RalA promotes exocyst localization to incipient furrows through the potential regulation of complex assembly and/or function.
**Rab8 Acts as a Key Regulator of Epithelium Formation in Drosophila Embryonic Development**

Data from our lab indicate that Rab8 starts to form projections and tubular extensions from punctuate structures as early as metaphase furrow stages (Lauren Mavor, unpublished data). However, my research has focused on the cellularization stage of the development. My data indicates that Rab8 forms tubular structures at the onset of cellularization from small punctuate structures localized apically to the nuclei (Figure 6). These Rab8 tubules precede the ingression of F-actin and mark the future furrow ingression sites during the slow phase of cellularization. F-actin then catches up during the fast phase and extends basally.

My research has also shown that cellularization furrow formation is strongly correlated to the GTPase state of Rab8. Both constitutively active and wild-type Rab8 embryos showed normal punctuate structures and tubular dynamics during cellularization, while dominant negative Rab8 embryos failed to show any normal Rab8 dynamics and structures (Figure 7 C and D). Rab8 RNAi knockdown also showed the failure of epithelium formation and incomplete nuclear divisions (Lauren Mavor, unpublished data). Therefore, Rab8 is an essential regulator for furrow formation during early embryonic development.

Previous studies have indicated that the sources for membrane addition at the furrows during cellularization are from the recycling endosome (RE) and the Golgi trafficking pathways (Sisson et al., 2000; Pelissier et al., 2003). However, the colocalization of Rab8/RE, Rab8/Golgi, and Rab8/late endosomal compartments (Figure
8 and Graph 1) showed a low percentage (less than 10%). This low percentage of colocalization between Rab8 and the other tested components was repeatable in replicated experiments. Hence, it is possible that Rab8 has a transient interaction with these compartments. However, due to the limitation of observing fixed embryo specimens, these transient colocalization periods might not be readily detectable.

I developed a novel embryonic dissection method in order to further understand Rab8 tubular behaviors. Both wild-type and constitutively active embryos showed tubule dynamics at a rate of about 1μm per second, while dominant negative embryos remained in large punctuate aggregates similar to the aggregate structures observed in fixed embryos (Figure 7, Figure 10-Figure 12). To gain a better understanding of how Rab8 relates to F-actin and how Rab8 regulates furrow membrane addition, co-expressed transgenic fly stocks were created that marked the plasma membrane and F-actin. Interestingly, neither of the plasma membrane markers showed colocalization with Rab8 tubules. For F-actin, my data suggest that it remained static and localized adjacent to the Rab8 tubules (data not shown). One possible explanation for the absence of colocalization could be due to the curvature of the plasma membrane. The great curvature in Rab8 tubules may mean that these regions of plasma membrane pose a barrier to lateral diffusion in the plane of the membrane. It could be that transmembrane proteins (such as our marked Gap43) cannot move into tubules.

To address how Rab8 regulates the furrow ingression process, we have proposed a model. Rab proteins have been identified as the master regulators of intracellular trafficking in eukaryotic cells. Rab protein can bind microtubule-based motors and
transport various vesicular cargos with directional control (Horgan and McCaffery, 2011). As injection of the microtubule polymerization inhibitor colchicine caused the failure of furrow formation (Lauren Mavor, unpublished data), it may be that Rab8 interacts with a kinesin motor protein at the onset of cellularization to form tubules beneath the apical plasma membrane and mark the future furrow ingression sites. F-actin then ingresses along the Rab8 tubules, but Rab8 still leads the tubular forming process ahead of the furrow canal. When fast phase arrives, F-actin then replaces Rab8 tubules and Rab8 transforms into cortical arrays. With the possible regulation of the exocyst complex, Rab8 directs membrane addition to form the cellularization furrows.

Horizontal Interface Growth Indicates a Distinguished Pattern during GBE

In addition to the RalA and Rab8 projects, I have also examined the second half of cell intercalation during the GBE stage in *Drosophila*. Compared to vertical interface contraction, interface horizontal growth appeared as a more continuous process. However, a clear definition of the stabilization period has not been developed (Graph 2 and Graph 3). A previous study has also shown that F-actin was enriched at the newly formed horizontal interfaces for about 2.5 minutes (Blankenship et al., 2006). Therefore, I examined if horizontal interface growth has a different rate of extension before and after the 150s mark. With the limitation of the precision with hand measurements, I determined an overall rate of interface growth at approximately 3\(\mu\)m per 12.5s. More accurate measurements can be done with MatLab software, which may permit a better examination of interface growth before and after the transient enrichment of F-actin.
Future Directions

Both the RalA and Rab8 projects need either antibodies or rescue experiments to confirm that the YFP fusion constructs correlate with endogenous protein function. For the embryonic dissection studies, it would be interesting to examine the Rab8 tubule dynamics by performing FRAP experiments. In this case, we could then determine if the Rab8 tubule subunits are adding from an end or if membrane addition may occur anywhere along the tubules. It will also be important to identify what components are actually making these tubular structures. The model of Rab8 tubule formation indicated that kinesin would be a good candidate, since it is a plus end microtubule motor protein that moves away from the apical MTOC during syncytial divisions. For the interface growth project, a more accurate measurement should be applied in order to see if the transient F-actin stabilizes or destabilizes growing interfaces. On the other hand, myosin populations could be examined to see if they indicate a similar pattern to interface contraction during GBE.
References


