Plasma Membrane Dynamics in the Drosophila Embryo

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PLASMA MEMBRANE DYNAMICS IN EMBRYONIC DROSOPHILA

DEVELOPMENT

A Thesis

Presented to

The Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Amelia E. Zommer

June 2011

Advisor: Todd Blankenship
ABSTRACT

Convergent extension is a highly conserved process among mammals, in which the tissue narrows in one axis, and extends across another. Tissue elongation is directed by the regulation of cell interface behaviors, which guides cell intercalation and rosette formation. Rosette formation occurs through the contraction of vertically oriented cell interfaces, and the subsequent elongation of new horizontal interfaces. It has been shown that actomyosin-generated tension functions to direct rosette formation. In this thesis, I have tested the function of regulators of F-actin networks, as well as endocytic and exocytic mechanisms, to identify new components that control interface behaviors and cell shape. I have performed a screen of F-actin regulators and nucleators, and pinpointed the specific actin nucleator dPod-1 as a candidate protein that is localized to vertical interfaces during tissue elongation. Furthermore, I have probed the function of endocytosis using the Shibire mutation, and demonstrated that endocytosis is required for vertical interface shrinking. Finally, I have used mutations in components of the Exocyst Complex and the associated protein RalA to inhibit exocytic mechanisms, in order to address their function in directing cell and tissue morphologies.
Table of Contents

Introduction .......................................................................................................................... 1
  Overview and embryonic development................................................................. 1
  Endocytosis and tissue morphology...................................................... 5
  Actin nucleation and organization.............................................. 6
  Exocytosis and new membrane addition.................................... 7
  Actin and plasma membrane dynamics........................................ 9
  Thesis specific aims ........................................................................ 10

Materials and Methods .................................................................................................. 11
  Scoring .......................................................................................... 11
  Live imaging, RNAi ................................................................. 12
  Injections ...................................................................................... 13
  Confocal microscopy, immunohistochemistry.................................. 14
  Hand peeling fixation .................................................................. 15
  Heat fixation, methanol popping, PCR ........................................... 16
  Testis dissection ................................................................. 19
  Fly stocks created/used ............................................................... 20

Results ............................................................................................................................. 22
  F-actin organization and nucleation in GBE........................................ 22
  dPod1 localization in GBE .............................................................. 28
  Shibire transheterozygotes disrupt GBE ........................................ 29
  Shibire disrupts localization of Armadillo ......................................... 33
  Shibire localizes to vertical interfaces during GBE ............................ 34
  Shibire interface dynamics ................................................................. 35
  RalA is required at very early stages of development ....................... 43
  RalA mutants cause a unique nuclear phenotype ......................... 45
  Mutations in the Exocyst Complex affect membrane addition in cytokinesis...48

Discussion ....................................................................................................................... 56
  Overall conclusions ................................................................. 56
  Actin regulation in GBE .............................................................. 57
  Shibire and vertical interface orientation ........................................ 58
  RalA and the Exocyst Complex .................................................. 60
  A model for the control of interface behavior .................................. 63

Works Cited ..................................................................................................................... 68
INTRODUCTION

Overview

Drosophila embryonic development is a highly organized process. While many aspects of this process are intriguing, the novel tissue morphologies that direct convergent extension movements are remarkable. The formation of an elongated body axis during embryonic development is a highly conserved process, and yet the proteins and pathways that define this process are not well understood. Of particular interest are the plasma membrane dynamics during elongation, as large-scale changes to cell and tissue shape occur. My work seeks to explore the relationship between plasma membrane and cytoskeletal architecture, and to shed light on the molecular basis of this fundamental process.

Embryonic Development

Different from the embryonic development of most organisms, once a Drosophila embryo is fertilized, its nucleus divides in the absence of cytokinesis, and the embryo develops as a syncytial tissue (Sokac et al., 2008). When the embryo reaches the 10th round of mitosis (cycle 10), the nuclei migrate to the periphery of the embryo and arrange themselves in a monolayer. After three more divisions, cellularization, or the subdivision of the nuclei into discrete cells, takes place. During cellularization, plasma
membrane surface area increases dramatically and invaginates around each nucleus (Schematic I). Cellularization precedes gastrulation, in which major body and tissue layers will be established through the invagination of mesoderm and endoderm. Also during gastrulation, the embryonic epithelium will double in length in a process called germ band extension (GBE). The major driving force behind this extension is cell intercalation (Blankenship et al., 2008). At the onset of gastrulation, epithelial cells arranged in a hexagonal array begin to intercalate between one another, moving away from their original neighbors. During this event, unique “rosette” structures are observed. Rosette formation begins when columnar pairs of cells contract their vertical interface to meet at a common vertex (Schematic II). Aside from the apparent reorganization/reduction of plasma membrane, F-actin and Myosin II localize to these shrinking interfaces. When rosette cells share a common vertex, the rosette undergoes a resolution step that builds a new interface along the horizontal axis. During the formation of this new interface, a burst of filamentous actin occurs along with the deposition of new junctional complexes (Blankenship et al., 2006). Rosette structures are observed stochastically throughout the epithelial sheet. After tissue elongation, the embryo goes on to develop internal organs and a central nervous system.
Schematic 1: Diagram of Cellularization (from The Cell Cycle: Principles of Control by David Morgan.

A) Plasma membrane begins to increase its surface area and push between adjacent nuclei. B) A furrowing event propels plasma membrane invagination deeper, starting to separate nuclei. F-actin and Myosin II become concentrated at the furrow canal, the leading edge of the cellularization front. C) Budding of plasma membrane forms a bottle neck structure. D) Scission of plasma membrane resulting in individual cells.
**Schematic 2. Multicellular Rosette Formation.** A-D show the process of constriction of internal interfaces of a group of vertically oriented cells (A, A' and B, B'). Formation of the vertex occurs (C & C’). Structure is resolved by formation of horizontal interfaces (D & D’). Adapted from Blankenship et al., 2006
Endocytosis and Tissue Morphology

The role of endocytosis in cell shape change is not a new principle. Tissue morphology often requires the redistribution or utilization of Myosin, F-actin, Microtubules, Cadherins, Integrins, Rab proteins and membrane trafficking pathways, all of which control membrane behaviors. For example, migratory cells utilize F-actin polymerization and Myosin II motors to push the leading edge forward, while microtubules, adhesion molecules, and the recycling of membrane via endocytosis and exocytosis are essential for cell body retraction and lagging edge movements (Mitchison et al., 1996).

Two main mechanisms of endocytosis exist: clathrin-dependent endocytosis and clathrin-independent endocytosis. In both forms, extracellular proteins or peptides bind to surface receptors and are rapidly internalized. Both events can occur constitutively or as a response to certain stimuli (Goldstein et al., 1979). However, in clathrin-dependent endocytosis, clathrin initiates the formation of a budded vesicle from the cytosolic face of the plasma membrane. Once the clathrin “bud” has rounded into a near vesicle shape, it undergoes a scission event from the membrane with the help of Dynamin. Dynamin forms a helical collar around the neck of an invaginating clathrin-coated vesicle, where it is thought to “pop” the vesicle off of the membrane (Takei et al., 2001). Once the vesicle is freed, it loses its clathrin coating and proceeds to the early endosome, where proteins and lipids may then be further sorted to a variety of different destinations. Alternative mechanisms of endocytosis independent of clathrin also exist, and are carried out with the help of actin structures or the protein caveolin. While the types of proteins or lipids
internalized may differ, as well as their fate once inside the cell, it is important to note they many of these pathways (but not all) require the function of Dynamin.

The *Drosophila* homolog of Dynamin is “*Shibire*”. In flies, loss of function of *Shibire* is characterized by reversible paralysis at both adult and larval stages when placed at non-permissive (NP) temperatures over 28 degrees Celsius (Poodry *et al.*, 1979). This was found to be due to the blocking of endocytosis. While the highest levels of *Shibire* are found in the central nervous system, it is also expressed during embryonic development (Chen *et al.*, 1992). *Shibire* mutations have also been found to specifically disrupt vesicular trafficking in the *Drosophila* embryo during cellularization (Kawasaki *et al.*, 2000, Lecuit *et al.*, 2002). Temperature sensitive mutations in *Shibire* have been identified. *Shi*<sup>ts1</sup> and *Shi*<sup>ts2</sup> have an optimal permissive temperature of 22° C. When raised to the NP temperature, found to be around 31-32° Celsius, the *Shibire* protein becomes inactive, most likely due to instability of the protein structure. This system is especially helpful in examining the role of endocytosis during specific stages of development. Since *Shi*<sup>ts1</sup> and *Shi*<sup>ts2</sup> are viable at the permissive temperature, flies will develop normally until the desired stage, making *Shi* temperature sensitive mutations a very useful tool.

**Actin Nucleation and Organization**

Actin is a dynamic globular protein that is in constant flux between a monomeric and polymeric form. Vital to many processes during development and tissue formation, actin plays a central role in cell morphology. Actin is organized in both branched and unbranched forms. Branched actin networks form under the nucleating activity of the Arp2/3 complex, while members of the Formin family direct unbranched actin
polymerization (Keheller et al., 1995). Additional actin effectors, for example Rho GTPase family members, can also regulate actin organization, through modulating Arp2/3 and Formin activity.

There are a variety of roles for actin in early Drosophila development. One of the earliest roles of actin in development is prior to cellularization, when the nuclei are undergoing cycles of mitotic division. A halo of F-actin has been proposed to organize the syncytial nuclei and maintain proper nuclear spacing (Blankenship et al., 2001). Also, as previously mentioned, F-actin functions in new interface growth during rosette formation (Blankenship et al., 2006). Finally, F-actin has been implicated in directing numerous endocytic and exocytic pathways.

There are several populations of F-actin that are observed within the embryonic epithelium during gastrulation. First, there is a population of F-actin that localizes with Myosin II to vertical interfaces to promote constriction of membrane surfaces. This behavior is observed during the vertex generation step of rosette formation. An actomyosin network also functions on the ventral surface of the embryo to drive ventral furrow formation (Leptin et al., 1994). A second population of F-actin is localized in short bursts at sites of newly initiated cell surfaces during rosette resolution and is not associated with Myosin. Finally, there is a third population of F-actin located in basal regions of the cell in large stress fiber-like accumulations.
Exocytosis and New Membrane Addition

Cell secretion is a fundamental process in cell homeostasis. Similar to clathrin participating in multiple pathways of endocytosis, exocytosis also employs the help of special proteins. The last steps of exocytosis are the tethering and fusion of secretory vesicle to the target membrane. Tethering plays a critical role in the specificity of vesicle targeting, and protein complexes identified as being involved are called tethering factors (Whyte et al., 2002). Two main classes of proteins essential to exocytosis are the tethering complex proteins and the SNARE proteins. A v-SNARE on the membrane of a vesicle binds a complimentary t-SNARE at the sites of desired membrane targeting, facilitating the final fusion of lipid bilayers (Rothman, 1994). One key plasma membrane tethering complex, is called the Exocyst Complex. The Exocyst Complex (EC) is composed of eight proteins, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Members of the EC were first implicated in vesicular trafficking in a screen in which Exocyst mutant cells accumulated secretory vesicles in the cytoplasm (Lipshutz et al., 2002). This finding supports the current model, where the EC is critical in directing vesicles to the plasma membrane and localizing them there for eventual fusion (Lipshutz et al., 2002). Regulators of the EC have been shown to be small GTPases (Cai et al., 2007). Small GTPases of the Ras-like (Ral) family are central in many signaling pathways. Not surprisingly, the EC has been associated with sites of newly forming membrane, and in such processes as growth cone development in synapses, and cytokinesis in several cell types (Wang et al., 2004). In Drosophila, the Exo84 component of the EC is vital to apical epithelial identity, as transmembrane proteins required for polarity are mislocalized due to lack of the polarity protein Crumbs being
secreted properly (Blankenship et al., 2007). This finding implicates the EC in the secretory regulation of important proteins during development to establish polarity.

RalA directly interacts with two members of the EC, Sec5 and Exo84, and cells lacking RalA have a compromised stability of the Exocyst Complex. As I am interested in identifying pathways contributing to new membrane insertion, I have chosen to explore whether Ral proteins are involved in early Drosophila development.

A member of the Ras family of small GTPases, Ral regulates cellular processes depending on its GTP-state, which controls protein-protein interactions (M. van Dam et al., 2006). A few Ral effector proteins have been established, such as GEFs (guanine nucleotide exchange factors) and Calmodulin. Additionally, some Ral effectors possess GAP domains that may regulate the GTPase activity of small G proteins in the Cdc42 and Rac family. These proteins have been implicated in cytoskeletal rearrangements and may provide a link between membrane trafficking events and cytoskeletal function (M. van Dam et al., 2006).

**Actin and Plasma Membrane Dynamics**

Many studies have indicated a role for F-actin in both endocytosis and exocytosis. For example, fluid uptake levels in cells treated with Latrunculin A decreases, demonstrating that F-actin is necessary for endocytosis to function properly (Baggett et al., 2003, Shen et al., 2005). We now know more about the cytoskeleton’s role in endocytosis, as microtubules transport cargo between organelles, and F-actin rearrangements help facilitate vesicle intake at specific sites of membrane (Apodaca et al., 2001, Verrey et al., 1999). F-actin also appears to have an essential function in
exocytosis. For example, in yeast cytokinesis, actin cables have been shown to direct secretion to the bud neck. This mechanism allows targeted delivery of new membrane and proteins required for the additional surface area needed for cell division. Members of the Exocyst Complex, such as Sec3, are also localized to this region during cell division (Amberg et al., 1997). It is clear that cell surface remodeling requires a collaborative effort between membrane trafficking components and cytoskeletal architecture.

**Thesis Specific Aims**

The goal of my work is to examine the intersection of F-actin and cytoskeletal function with plasma membrane addition and removal to drive changes in cell and tissue shape. In *Drosophila*, there are ten categories of known actin regulating proteins; some are involved with nucleation and regulation, while others are associated with cross-linking and severing mechanisms. The first aim of my thesis is to examine these known regulators of F-actin function and determine if they direct cell shape changes during GBE and early development. The second aim of my thesis is to probe the role of endocytosis and exocytosis in regulating cell shape. I have used the *Shibire* temperature-sensitive mutations to disrupt endocytosis during GBE with the goal of determining the effects of such disruption on changes in cell and tissue shape. I have probed the function of exocytosis in directing tissue morphologies by investigating RalA and Exocyst Complex function, and the roles they may have during new membrane addition in vital developmental stages.
MATERIALS AND METHODS

Scoring

Embryos may be "scored" to investigate a possible phenotype or disruption in development. Both male and female flies were placed in a plastic breathable cup fitted with an apple juice agar plate and allowed to mate and lay embryos for an allotted period of time. Embryos were then coated (still on the plate) with Halocarbon 27 oil, which allowed the outer membrane of the egg to become translucent. Embryos can develop normally under this oil, and gross embryonic morphologies are clearly visible. In addition, embryos can be subjected to temperature-shift treatments as necessary (Eg; Shibire). Counting the number of deformed or delayed embryos and dividing them by the total number of embryos scored yields percentages of a particular phenotype in a given genetic background.

Live Imaging

Fixed imaging of tissue may have limitations in its analysis. Therefore, transgenic fly lines (either created in lab or acquired from outside sources) may be used to map the characteristics of a mutation in 4D (3D plus time). Embryos from these fly lines were obtained in a similar manner to what was described in the “scoring” section of
Materials and Methods. These embryos were then removed from their chorion with a 50/50 bleach-H2O solution for two minutes, rinsed repeatedly, and mounted onto a gas permeable membrane in Halocarbon Oil. A slide with a small amount of tape holds the embryos in place and they are visualized via confocal microscopy.

RNAi

RNAi was used to address the function of the dPod1 gene. Double-stranded RNA was transcribed based off of a 500 bp portion the dPod1 coding sequence. The region used was between base pairs 1021-1500, with the 5’ primer reading TAATACGACTCACTATAGGGgtcataccca tcatgtacca (annealing temperature of 58.63°C) and the 3’ primer reading (3’) TAATACGACTCACTATAGGGGcagttctcc gatgactcag (annealing temperature 58.73°C). dPod1 cDNA was cloned and PCR was performed as described in “PCR” of Materials and Methods. Following PCR purification, dPod1 was transcribed in vitro using MEGAscript® T7 High Yield Transcript kit and from a that solution was prepared as follows: 2 ul of ATP, GTP, UTP and CTP, 2 ul buffer, 8 ul PCR product, and 2 ul enzyme. Reaction went overnight at 37 degrees Celsius. After incubation, 1 ul of DNase was added, and the RNA Cleanup protocol from the Qiagen RNAeasy® Mini Kit was performed.
**Injections**

Embryos were collected on an apple juice plate for one hour so that injections were done prior to the onset of zygotic transcription. After the one-hour collection, the chorion of the embryos was removed with a 50% bleach:50%dH₂O solution for 2 minutes. The embryos were rinsed and dried repeatedly to clear them of any bleach residue that may interfere with development, and transferred via paintbrush to a portion of an unused apple juice plate surface. Under the dissection microscope, the embryos were oriented in two vertical lines, head to tail, with the ventral side exposed to the outer edge. After the embryos are positioned, a 24x20 glass slide is prepared with heptane glue, and gently placed on top of the embryos. A paintbrush lightly swiped the top of the slide to ensure that the embryos adhered to the slide, and the slide was then lifted up. The embryos were then desiccated for 10-12 minutes. After desiccation, Halocarbon oil 27 was lightly applied to the embryos and they were ready to be injected via capillary needle. Approximately 2 ul of transcribed RNA was added to the open end of the needle, and after the needle tip was broken, the embryos were each injected with small amounts of RNA, with water injected embryos serving as a control. Following injection, the embryos on the slide were transferred to a warmed apple juice plate and kept at 25 degrees Celsius and their development was tracked under oil.

**Confocal Microscopy**

Embryos stained with actin/membrane regulators were imaged on the confocal microscope with 40x objective. Whole embryo pictures were taken to establish staging and orientation. Subcellular resolution was achieved by zooming in by 5x while at the
40x objective. At this zoom, the cells were fitted in a z-slice from the apical to basal regions of the cell, 1 um apart, typically allowing a maximum of 15 slices. The gain was adjusted according to the intensity of the antibody signal and the laser was kept at the lowest levels possible while still achieving high resolution without photo bleaching.

**Immunohistochemistry**

In order to visualize the localization and distribution of various membrane regulators, the embryos of various fly lines were fixed according to one of three specific protocols. All methods begin with the embryos being collected over a span of 1-5 hours on an apple juice plate. The embryos were then introduced to a 50% bleach solution in order to dissolve their outer membranes. The embryos in the bleach solution were then poured into a mesh net where they were rinsed and dried repeatedly with ddH₂O water to remove any residing bleach.

**Antibodies used:** mouse anti-Neurotactin (1:1, DSHB), rabbit anti-Armadillo (1:200, Wieschaus lab), rat anti-DE Cadherin (1:100, DSHB), mouse anti-Adducin (1:5, 1:50 DSHB) mouse anti-chickadee (1:5, 1:50, DSHB), mouse anti-disabled (1:5, 1:50, DSHB), mouse anti-enabled(1:5, 1:50, DSHB), mouse anti-fascin(1:5, 1:50, DSHB), mouse anti-spectrin (1:5, 1:50, DSHB), mouse anti-synapsin (1:5, 1:50, DSHB), mouse anti-synaptogamin (1:5, 1:50, DSHB), mouse anti-peanut (1:5, 1:50, DSHB), mouse anti-villin (1:5, 1:50, DSHB), mouse anti-crumbs(1:5, 1:50, DSHB), mouse anti-Shot (1:5, 1:50, DSHB), mouse anti-3A6( 1:5, 1:50, DSHB), guinea anti-SCAR (1:500, Zallen lab),
guinea anti-WAVE (1:1000), guinea anti-WASP (1:1000), Rho1 (1:5, 1:50, DSHB), anti-Dynamin (1:1000, Ramaswami lab), Hoechst stain (1:5), anti-Abi (1:100), Rabbit anti-mCherry (1:200), guinea pig anti-dPod1 (1:2000, YN Jan lab)

**Hand Peeling** (manual removal of vitelline membrane):

Embryos are placed in a 10 ml fixative solution (5 ml Heptane, 4.375 ml 1X PBS, 625 ul 32% Paraformaldehyde) for 75 minutes. In the meantime, a small petri dish lid was prepared for hand peeling by the addition of a small amount of heptane glue on its surface. The embryos were removed from the interface of the fixative solution, repeatedly rinsed, and then transferred via paintbrush to the glue portion of the dish. A solution containing PBS +1 % BSA was poured over the embryos to prevent dehydration. The plate containing the adhered embryos was moved to a dissection microscope, where the embryos were gently nudged out of their vitelline membranes with a capillary needle. The freed embryos were collected with BSA treated pasteur pipette and put into a blocking solution (84.5ml 1X PBS, 1.5 ml BSA, 50 ul Tx-100) overnight at 4 degrees Celsius or for two hours at room temperature. Embryos were then put in a primary antibody solution, prepared in PBS + 1% BSA overnight at 4 degrees Celsius or for two hours at room temperature. The embryos were rinsed 6 times over 2 hours with PBT (50 ml 1X PBS and 50 ul Tx-100) and introduced into 500 ul of secondary solution @ 1:500 dilution prepared in PBS + 1% BSA for 45 minutes at room temperature. After application of the secondary solution the embryos were rinsed 2x with PTW (50 ml 1X PBS and 50 ul Tween-20) followed by four rinses with PBT over a 2 hour period.
Embryos were mounted in 80 ul of Prolong Gold® antifade DAPI reagent on glass slides with 24x40mm cover slips and allowed to harden before being imaged.

**Heat Fixation**

Heat fixed embryos were first bleached with 50% bleach 50% ddh$_2$O water solution, rinsed and dried. They were then placed in a vial with 10 ml of Arno’s Heat Fix Solution (1.5mL TritonX-100, 20 g NaCl) which was put in a distillation plate filled halfway with water. The solution was heated until boiling and the embryos were submerged in the vial for 10 seconds. After 10 seconds, cold heat fix solution was rapidly added to the vial and the embryos were freed of their outer membrane via Methanol popping (See Below).

**Methanol Popping**

Embryos were fixed in fixative solution (see “Hand Peeling” ) for 25-30 minutes. They were then transferred via pasteur pipette to a clean scintillation vial with 5 mls of heptane. Next, 5 mls of methanol were added to the vial, and it was capped and vigorously shaken for 10 seconds. Embryos that had failed to pop off their outer shield remained at the interface between the Heptane and methanol, while freed embryos sank to the bottom of the methanol. The top and interface layers of solution were removed and the embryos were rinsed 5x with methanol before being put into blocking solution and continuing the staining process.
PCR

Two fusion constructs were created: SNX2:mCherry and RalA:mCherry. Both processes were done with identical cloning schemes, minus the restrictive enzyme sites. Using RalA as an example, primers were designed according to the coding sequence of *Drosophila* RalA found on flybase.org. The left primer was GA AGATCT ATGAGCAAGAAGCCGACAG, with the AGATCT being a BglII restriction enzyme cleavage site (annealing temperature of 58.4750°C). Enzymes were provided from New England Biolabs. The right primer was CCG CTCGAG AAGTAGGGTACACTTAAGTC, with the CTCGAG being a XhoI restriction enzyme site (annealing temperature of 58.5800°C). The coding sequence for mCherry was obtained in a parallel manner. The left primer also shared a XhoI cutting site (CTCGAG) and was CCG CTCGAG ATGGTGAGCAAGGGCGAGGA, and the right Primer had an Xba cutting site (TCTAGA) and was GC TCTAGA CTA CTTGTACAGCTCGTCCATGC. Sequencing primers were designed as follows: I @ 400bp (reverse) CCTTGCGCTTATCAT, II @ 350bp (forward) ACCGTTTCTCTGCTGAT, and III @ 822bp (forward) TACGGCTCCAAGGCC. RalA cDNA was transformed and isolated and used in a PCR reaction that was composed as follows: (20ul solution) 0.4 ul dNTPs, 2 ul 10x Buffer + MgCl2, 0.52 TAQ Hi Fidelity Polymerase, 0.5 ul template DNA, 0.6ul of both left and right primer, and 15.38 ul ddH2O. The Thermo Cycler program was set as follows: a denaturation step at 94 degrees Celsius for 2 minutes, a combined subsequent denaturation step at 94 degrees Celsius for 15 sec, with an annealing step at 58 degrees Celsius for 30 seconds, and a elongation step at 72 degrees Celsius for 1 min. This cycle was repeated 25x. The final
elongation step was done at 72 degrees Celsius for seven minutes. Amplified PCR product was purified with a Fermentas PCR Purification kit and run out on a 1.7 % agarose gel for 45 minutes, with 400 amps at 110 volts. Samples loaded were 5 ul and were combined with 1 ul of 6x loading dye. A Gene Ruler1 kb DNA ladder from Fermentas® was used to determine protein size. Purified PCR of RalA was digested parallel with purified PCR of pUAST (the vector, previously done by Lauren Mavor) with the left and middle restriction enzymes in a 20 ul solution as follows: 7 ul ddH$_2$O, 2 ul 10X Green loading dye, 10 ul DNA template, and 1 ul of Bgl11 and 1 ul of Xho1 enzyme. Digestion went for 2 hours at 37 degrees Celsius, and samples were loaded into a gel with the same parameters as previously described. However, full sample size was loaded and loading dye was adjusted accordingly. Bands seen in the gel, imaged by the BioRad imaging machine, were excised with a razor and purified via the Fermentas Gel Extraction and Purification Kit. Another gel was run to test the efficiency of the extraction, and to make sure that there was ample product remaining to perform a ligation. Ligation was set up as follows: 2 ul pUAST, 10 ul RalA, 2 ul Buff, 1 ul DNA ligase, and 5 ul ddH$_2$O (20 ul solution). Ligation ran overnight at 22 degrees Celsius. Transformation was done using One Shot MAX Efficiency DH5α - T1$^R$ Competent cells, with 5 ul of ligation reaction being added to a vial of competent cells on ice. Reaction sat on ice for 30 minutes and was subsequently heat shocked at 42 degrees Celsius for 30 seconds. Then 250 ul of SOC medium from Invitrogen was added to the ligation/transformation reaction, and cells shook at 225 rpm for 1 hour at 37 degrees Celsius. Cell mixture was plated on LB agar plates with the appropriate antibiotic at volumes of both 150 and 100 ul, and inverted and incubated over night at 37 degrees
Celsius. Colonies were picked the following afternoon and grown up in Luria broth. After miniprep using Omega Plasma Mini Kit I, the samples were re-digested with restrictive enzyme and a gel was run to test whether insertion of gene into vector had successful occurred. Same process was done with mCherry into pUAST:RalA. Ultimately, a QIAGEN® Plasmid Midi Prep kit was used to amplify DNA levels for sequencing. After final sequencing was examined for mutations, finished samples were sent to be injected into embryos.

**Testis dissection**

Male larvae were collected from either Beta-tubulin-GFP/PLC-PH-GFP; Fun/TM6b or Beta-tubulin-GFP/PLC-PH-GFP; Onr/TM6b crosses and put into 0.77M NaCl solution under a dissection microscope. Meanwhile, a live imaging chamber was prepared as follows: a metal rectangular slide with a circular hole in the middle was covered on one side with a 24x40 mm glass slide adhered with tape at both edges. With the metal slide open side facing up, the gaps between the slide and the hole were sealed with vacuum grease. A combination of 10% Halocarbon 700 and 90% Halocarbon 27 is poured into the sealed chamber. Once the chamber has been created, the larvae are patted on a kimwipe and transferred into chamber for dissection, using two scalpels. The testis sacks are extracted from the larvae and spread open onto the slide with the scalpels. They are then imaged on the confocal microscope at 60x zoom with time.

**Fly Lines Created**

\[ Shi^{as1};\text{Resille-GFP} \]

\[ Shi^{as1};\text{ECAD-GFP} \]
Shi<sup>ts1</sup>;Moesin-GFP
RalA;Resille-GFP
RalA;His-GFP
Beta-tubulin-GFP/PLC-PH-GFP; Onr/TM6b
Beta-tubulin-GFP/PLC-PH-GFP; Fun/TM6b

**Fly Stocks Used**

OreR
Ral-a-FRT, OVOD2/xx; L914 F38/hsftp
w; PLC-PH-GFP; Fun/TM6b
w; PLC-PH-GFP; Onr/TM6b
Shi<sup>ts1</sup>
Shi<sup>ts2</sup>
117/117; 95-1/95-1
moe-GFP
Beta-tubulin-GFP; Onr/TM6b
Beta-tubulin-GFP; Fun/TM6b
His-GFP
E-Cad-GFP
Cappuccino
Spire
Rho1
UAS-RalA:mCherry
Mat 67:15

Fm6/Fm6; Sco/Cyo

Fm7/w; Sb/TM3, Ser
RESULTS

F-actin organization and nucleation in GBE

As previously mentioned, actin plays a critical role in several developmental processes. In order to assess F-actin contributions to gastrulation in the Drosophila embryo specifically, a general scan of potential F-actin regulators was performed. The proteins chosen for the screen were selected carefully from each category of F-actin regulators. Aside from actin nucleators, there are G-actin binding proteins, F-actin binding proteins, actin depolymerizing proteins, and actin bundling/cross linking proteins. Since little is known about how F-actin populations are established during gastrulation, a protein from any one of these categories might be of interest. Out of a screen of twenty targets, only a few fixed stains displayed any polarized behavior (Tables 1 and 2). Adducin and dPod1 both had intriguing localizations (Figure 1 and Figure 2, respectively) at vertical interfaces during GBE. Antibody stains were ranked on a scale of planar polarization, with IV being the least planar polarized, and I being the most.
Table 1. Screen of antibodies directed against known membrane regulators and actin nucleators. Protein name is provided in the left column; capital letters indicate panel of immunofluorescence image Figure 1. All proteins were grouped in a class, I-IV, depending on polarization or unique localization (Class I is most polarized, Class IV least). *Adducin revealed the most polarized orientation out of all of those screened.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Known Function(s)</th>
<th>Class (I-IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A6 (Q)</td>
<td>Tyrosine Phosphatase</td>
<td>IV</td>
</tr>
<tr>
<td>Adducin (A)*</td>
<td>Actin regulation and bundling</td>
<td>II</td>
</tr>
<tr>
<td>Chickadee (N)</td>
<td>Actin polymerization protein</td>
<td>III</td>
</tr>
<tr>
<td>Crumbs (F)</td>
<td>Maintains adherens integrity</td>
<td>IV</td>
</tr>
<tr>
<td>Disabled (I)</td>
<td>Signaling Protein</td>
<td>IV</td>
</tr>
<tr>
<td>Enabled (G)</td>
<td>Facilitates F-actin polymerization</td>
<td>IV</td>
</tr>
<tr>
<td>Fascin (D)</td>
<td>Actin Bundling</td>
<td>IV</td>
</tr>
<tr>
<td>Peanut (C)</td>
<td>Cleavage furrow in cytokinesis</td>
<td>II</td>
</tr>
<tr>
<td>Shot (H)</td>
<td>F-actin organization, Filapodia Formation</td>
<td>III</td>
</tr>
<tr>
<td>Spectrin (B)</td>
<td>Forming of Furrow Canals</td>
<td>IV</td>
</tr>
<tr>
<td>Synapsin (L)</td>
<td>Vesicles and F-actin</td>
<td>IV</td>
</tr>
<tr>
<td>Syntaxin (P)</td>
<td>Exocytic SNARE</td>
<td>IV</td>
</tr>
<tr>
<td>Synaptogamin (M)</td>
<td>Exocytic SNARE</td>
<td>IV</td>
</tr>
<tr>
<td>Villin (O)</td>
<td>Form cytoplasmic actin bundles</td>
<td>IV</td>
</tr>
</tbody>
</table>
### Table 2. Second screen of actin nucleators/associated proteins

Six additional actin regulators were screened via live and fixed imaging for unique polarization or localization. Protein is provided in the left column; capital letter indicates panel of immunofluorescence image in Figure 1 (NP = not pictured). *dPod1 revealed the most polarized localization and is shown in Figure 2.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Known Function(s)</th>
<th>Class (I-IV)</th>
</tr>
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<tbody>
<tr>
<td>Abi (K)</td>
<td>Acts upstream of WAVE, binds WASP to stimulate WASP dependent F-actin polymerization</td>
<td>II</td>
</tr>
<tr>
<td>Capu (NP)</td>
<td>Actin nucleators /organizes actin mesh network</td>
<td>II</td>
</tr>
<tr>
<td>dPod1*</td>
<td>Crosslinks actin/reorganizes actin cytoskeleton</td>
<td>I</td>
</tr>
<tr>
<td>SpirC</td>
<td>Actin nucleators /organizes actin mesh network</td>
<td>IV</td>
</tr>
<tr>
<td>Wasp (J)</td>
<td>Activate Arp2/3 complex</td>
<td>IV</td>
</tr>
<tr>
<td>Wave (E)</td>
<td>Activate Arp2/3 complex</td>
<td>III</td>
</tr>
</tbody>
</table>
Figure 1. Immunofluorescence staining for potential membrane regulators or actin nucleators. First three panels show an example of polarization seen in F-actin populations (stained with Phalloidin) during early, middle, and late germ band extension. A) Adducin B) Spectrin C) Peanut D) Fascin E) WAVE. All stains are shown in grayscale and are not co-stained for other proteins. Scale bar = 10 um.
Figure 1 cont’d. Immunofluorescence staining for potential membrane regulators or actin nucleators. F) Crumbs G) Enabled H) Shot I) Disabled J) Wasp K) Abi L) Synapsin M) Synaptogamin. All stains are shown in grayscale and are not co-stained for other proteins.
Figure 1 cont’d. Immunofluorescence staining for potential membrane regulators or actin nucleators. N) Chickadee O) Villin P) Syntaxin Q)3A6 – Tyrosine Phosphatase. All stains are shown in grayscale and are not co-stained for other proteins.
Figure 2.  *dPod1* is largely localized to vertical interfaces during GBE

a) embryo oriented with anterior at left at low magnification (10x) with actin in red and *dPod1* in green.  b) 5x zoom, 40x objective (scale = 10um) of the embryo in (a) demonstrating colocalization of actin (in red) and *dPod1* (in green). Both are present at vertically oriented interfaces.  c) grayscale actin d) grayscale *dPod1*.
Shibire heteroallelic crosses cause a functional disruption of gastrulation but not cellularization

In order to determine whether Shibire was required at either stage of development (GBE or cellularization), embryos of the temperature sensitive allele (Shi<sup>ts1</sup>) were scored under oil when raised to the non-permissive temperature (31.5°C) for 45 minutes. No phenotype was witnessed, raising the possibility that homozgyous stocks may have accumulated a suppressor mutation. With this in mind, the two alleles of Shibire were crossed, the males of one allele crossed to a female of the other allele, and vice versa. The embryos from these parents were scored both at the non-permissive and permissive temperatures. By generating a heteroallelic mutant embryo, a deep disruption in epithelial function was ultimately seen during gastrulation but not during cellularization of Shi<sup>ts1</sup> females crossed to Shi<sup>ts2</sup> males (Figure 3).
Figure 3. *Shibire* ts^1^ embryos developed normally during both cellularization (n=30) and gastrulation (n=21) when raised to the NP temperature (31.5°C) for 45 minutes. Gray = wild type development, Black = developmental defect.
Figure 3 cont’d. Embryos from *Shibire* ts¹ females crossed to *Shibire* ts² males showed a marked defect in gastrulation (n=62) but not cellularization (n=59) when raised to the non-permissive temperature of 31.5°C for 45 minutes. Gray = wild type development, Black = developmental defect.
Figure 3 cont’d. Embryos from *Shibire* ts² females crossed to *Shibire* ts¹ males also developed normally during cellularization (n=68) and gastrulation (n=88) despite being raised to the non-permissive temperature (31.5° C) for 45 minutes. Gray = wild type development, Black = developmental defect.
*Shibire* embryos raised to 31.5°C show disruption of Adherens Junctions

In order to assay the state of adhesion and to gain more insight on the localization of the key adhesion molecule Armadillo during GBE in a *Shibire* mutant, embryos collected from Shi\(^{ts1}\) females crossed to Shi\(^{ts2}\) males were raised to the non-permissive temperature (NP) for 45 minutes and co-stained with Neurotactin (Nrt, a membrane marker) to visual cell outlines.

![Figure 4](image1.png)

**Figure 4. Shibire mutations cause mislocalization of Armadillo.** a) Control embryo undergoing GBE stained for Nrt (red) and Armadillo (green), Armadillo is localized punctately between each cell. b) A mutant embryo raised to the NP temperature for 30 minutes shows somewhat intact Armadillo localization c) A mutant embryo raised for 45 minutes at NP temperature (5x zoom) shows cytoplasmic Armadillo localization d) A low magnification view of a mutant embryo raised for 45 minutes at the NP temperature.
Shibire localizes to select vertical interfaces during GBE in fixed staining

While Shibire mutations were shown to disrupt GBE, more information about its localization and behavior during this stage was needed. Wild type embryos were fixed and stained for anti-Dynamin and Phalloidin (F-actin) after a five hour collection, and the GBE embryos were imaged on the confocal microscope. Intriguingly, Dynamin was seen to localize to short vertical interfaces in the germ band epithelium.

![Figure 5. Shibire localizes to short vertical interfaces during GBE.](image)

Dynamin is seen enriched at short vertical interfaces during tissue elongation (shown in green, arrows) and is associated with internal vertical T1 and Rosette interfaces. Dynamin orientation can be computationally quantified as vertical (Dinah Loerke, bottom panels). Scale bar = 5um.
Live imaging of *Shibire* embryos reveals unique vertical interface behaviors

As *Shibire* can disrupt gastrulation at the gross level, live imaging was used to follow cell behaviors as embryos were shifted to the NP temperature. A *Shi*\textsuperscript{ts1};Resille-GFP stock was created, and the resulting females were crossed to *Shi*\textsuperscript{ts2} males. Resille-GFP is a general plasma membrane marker. Live embryos were imaged either at the permissive or NP temperature and interface dynamics were recorded. Consistent with my hypothesis, vertical interface contraction was defective at the NP temperature. Additionally, it appears that it is the contraction of linked interfaces characteristic of rosette structure that is disrupted.

![Vertical Interface Dynamics in *Shibire*\textsuperscript{ts1x2};Resille-GFP](image)

**Figure 6.** *Shibire* embryos at the permissive temperature contract almost all vertical interfaces while those at the NP temperature do not.
Although interface analysis revealed that nearly half of vertical interfaces in the *Shibire* embryos raised to the NP temperature still contracted, these interfaces were found to be associated with T1 processes. TI processes are alternative cellular structures seen during gastrulation that are composed of four cells and only require minimal vertical interface shrinking during formation. On the other hand, rosette structures had deep failures in interface contraction in mutant embryos. Both T1 and Rosette vertical interfaces contracted at the permissive temperature.

![Interface Analysis at Permissive Temperature (22 C)](image)

**Figure 7.** Vertical interfaces contract in both T1 and Rosette processes during GBE in *Shh* exhibition embryos raised at the permissive temperature. Vertical interfaces were followed for 10 minutes and were grouped as a part of a rosette structure, a T1 structure, or undefined.
Figure 8. Vertical interfaces in T1 processes but not rosette structures contract. Although vertical interface contraction was witnessed in the Shi\textsuperscript{ts1ts2} mutant embryos raised to the NP temperature, most contraction was seen in interfaces where the interface length is very small (in comparison to the long linked interfaces observed in rosette structures). In contrast, rosette structures largely failed to contract their interfaces. Another population of verticle interfaces were grouped as “others”, because it was not clear whether they were involved with either process.
Figure 9. Linked vertical interfaces shrink during GBE in Shibire embryos at the permissive temperature. From left to right: Arrow points to interface of interest at 3 minutes of live movie, interface starts to contract at 6 minutes, and finally brings the cells together at a common vertex at 9 minutes.

Figure 10. Linked vertical interfaces show a failure in contraction in Shibire embryos raised to the NP temperature. From left to right: Arrow points to linked interface(s) at the start of GBE, further along development, and at the end of roughly ten minutes. Interface shows slight reduction from box one to box two but overall remains the same length over time.
Figure 11. Automated computational segmentation of *Shibire*; Resille-GFP embryos, $t=0$ min. Using a MatLab algorithm cell outlines have been reconstructed digitally based on images collected during 4D movie creation. This method allows vertical interfaces to be tracked and quantitatively measured for amount of contraction. Shown: Embryo at the permissive temperature (WT) undergoing gastrulation (Dinah Loerke).
Figure 11 cont’d. Automated computation segmentation of *Shibire*; Resille-GFP embryos at 11 minutes after Panel A (Dinah Loerke).
Figure 11 cont’d. Automated computation segmentation of *Shibire*; Resille-GFP embryo at 24 minutes after Panel B (Dinah Loerke).
Figure 12. **Interface contractions in *Shibire* embryos can be monitored over time.** Graph showing average length of interfaces (y axis) against time frame (x axis), majority of interfaces were vertically oriented and were found to slightly contract overall.
**RalA is required at very early stages of development**

To first assess RalA function, germline clones of a *RalA* mutant were generated. These germline clones remove both maternal and zygotic function. Embryos deficient in *RalA* were scored under oil at different time points and were severely disrupted (data not shown). Development was deeply affected, so fixed staining was done to learn more about the nature of this phenotype. With such a strong phenotype, fixed imaging was done to examine the dynamics of DE-cadherin, an adhesion protein, and Phalloidin, a drug that binds actin (Figure 14). Confocal imaging showed that F-actin clustered in aggregates and DE-cadherin was largely mislocalized. Again, live imaging techniques would be the most useful in understanding the nature of this phenotype, as any stage of development could be filmed from start to finish to observe the order in which the phenotype occurs.

RalA:Resille-GFP constructs were created, and introduced into the germline clone as well. Live imaging revealed, once again, a deep defect and absence of normal cellularization behaviors (data not shown). However, this construct only mapped out cell outlines, so if the mutant phenotype occurs very early in development, it may be affecting the nuclear syncytium.
Figure 13. *RalA* disrupts embryos and causes mislocalization of DE-Cadherin and aggregation of the F-actin network during development. Embryos were stained for Phalloidin (in red) and DE-Cadherin (in green) and were found to be severely disrupted.
Figure 14. RalA mutants cause a unique nuclear defect in early development.

(a)-(d) show mitotic cycles 10-13 in a wild type background. Nuclei divide in an organized fashion and are even spaced apart by actin mesh networks (not shown) (e)-(h) are RalA mutant embryos grossly correlated to controls based on number of nuclei since obvious developmental markers normally present have been abolished. Nuclei clump together and fail to move to the periphery of embryo. DAPI is in blue, Phalloidin is in red, and Tubulin (e only) is pictured in green.
**RalA:mCherry construct creation**

While examining the effects of mutant *RalA* was helpful in determining its role during development, I also wanted to learn more about the nature of the endogenous protein. As no antibodies to RalA exist, I created a RalA:mCherry construct and transgenic *Drosophila* line (Figure 13). The RalA:mCherry construct would be a good tool to study the behavior of the protein aside from a mutant background, via fixed staining and live imaging. Disappointingly, live imaging of RalA:mCherry displayed a diffuse, ubiquitous localization in cellularizing and gastrulating embryos. Fixed stains with anti-mCherry antibody also revealed the same results (data not shown). This could represent the behavior of endogenous RalA, or it could be a result of tagging an mCherry sequence onto the protein.
Figure 15. Schematic of RalA:mCherry fusion construct. RalA:mCherry was created in order to observe the endogenous localization and behavior of RalA.
Mutations in components *Sec8* and *Exo84* of the Exocyst complex effect elongation during cell division

Although in a different stage of development, another fundamental elongation event that widely occurs is during mitosis. In cytokinesis, the stage of mitosis where the cell yields two daughter cells, the membrane surface area undergoes a large increase. In testis cells of males deficient in either *Sec8* or *Exo84* there is a dramatic reduction of membrane addition and a failure to complete division. In Figure 16, a simulation of what is expected in *Sec8* or *Exo84* mutants versus wild type cell divisions is depicted. While wild type cells successfully expand their membrane surface and yield two daughter cells during mitosis, my hypothesis is that the mutants will fail to do so. The control testis division (Figure 17) shows a nice elongation, pinching of the cytokinetic furrow, and subsequent division of two daughter cells.

The average circumference increase in wild type cells was found to be 27% from prophase to cytokinesis. On the other hand, Figures 18 and 19 show that *Sec8* and *Exo84* mutants both had a reduced elongation event, and never successfully complete the final stages of mitosis. In fact, quantitative measurements revealed a dramatic reduction in circumference values, with those of the mutant cells being smaller than what the cell initially started out with (Figure 21).
Figure 16. Simulation of expectations during testis germline cell division in Exocyst Complex mutant cells. On the top part of the diagram, in a wild type background, a cell undergoes and maintains an elongation event until it divides into two resulting daughter cells. A mutant cell, depicted in the lower half of the diagram, fails to elongate due to a failure in membrane addition.
Figure 17. A wild type testis cell undergoes division and yields two daughter cells. Live cell mounts imaged on the confocal microscope, cells are outlined with PLC-PH-GFP and Tubulin-GFP to visualize cell outlines and mitotic spindle, respectively. (a)-(d) are prophase, metaphase, anaphase, and telophase, respectively. Scale = 10 μm.
Figure 18. Testis cells lacking the sec8 (funnel cakes) component of the Exocyst Complex fail to complete the final stages of cytokinesis and do not undergo anaphase elongation. Live cell mounts imaged on the confocal microscope, cells are labeled with PLC-PH-GFP and β-tubulin-GFP to visualize cell outlines and mitotic spindle. While the centrosomes are localized correctly and a mitotic spindle forms, there is little evidence of a cleavage furrow or any lasting membrane addition. (a)-(d) are prophase, metaphase, anaphase, and telophase, respectively. Scale = 10 um.
Figure 19. Testis cells lacking the Exo84 (onion rings) component of the Exocyst Complex fail to complete the final stages of cytokinesis and do not undergo anaphase elongation. Live cell mounts imaged on the confocal microscope, cells are labeled with PLC-PH-GFP and β-tubulin-GFP to visualize cell outlines and mitotic spindle. While the centrosomes are localized correctly and a mitotic spindle forms, there is little evidence of a cleavage furrow or any lasting membrane addition. (a)-(d) are prophase, metaphase, anaphase, and telophase, respectively. Scale = 10 um.
Table 3. *onr* and *fun* mutants fail to finish cytokinesis and show reduced levels of elongation compared to wild type cell divisions. Going from left to right: type of cell being measured, length across cell at the start of prophase, length across the cell at its longest elongation, length of the cell (or both cells) at the completion of cytokinesis, length of the spindle during anaphase.

<table>
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<th>Finish (μm)</th>
<th>Spindle (μm)</th>
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<table>
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<td>20.618</td>
<td>17.874</td>
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</table>
Figure 20. Cell diameter increases more in wild type dividing cells versus cells mutant for either *exo84* or *sec8*. On average, the cell diameter recorded at the start of mitosis compared to the longest elongation reached was higher than that of testis cells mutant for *Exo84* or *Sec8*. WT n=6, average percentage = 19, std dev = 0.075. Mutant n=7 (5 *fun* mutants/2 *onr* mutants), average percentage = 7, std = 0.079.
Figure 21. Circumference greatly increases in wild type dividing cells versus exocyst mutant testis cells (Exo84 or Sec8). In order to evaluate membrane from the start of division to the end, circumference was calculated (2πr) at the start and end of mitosis for both wild type and mutant cells. Wild Type cells yielding two daughter cells had combined circumference calculations. Wild type percentage increase = 27, std dev = 3.71, mutant percent change onr = -7.38, std dev = 7.2, mutant percent change fun = -4.35, std dev = 2.61.
DISCUSSION

Overall Conclusions

This research has examined multiple pathways that are critical to the regulation of the F-actin cytoskeleton, exocytosis, endocytosis, and analyzed their involvement in the processes of early *Drosophila* development. A general screen of actin regulators has sorted through 20 potential F-actin regulators to show that *dPod1* has a striking localization to multiple F-actin networks in the early embryo. *dPod1* displayed a polarized localization and a high degree of colocalization with F-actin in the extending germ band. I have also found that functional disruption of *Shibire* demonstrates the importance of coordinating actomyosin contractions with endocytosis. Dynamin is enriched in short vertical interfaces and loss of Dynamin function led to failure in rosette formation. Finally, analysis of exocytic components such as the Exocyst Complex and the associated regulatory protein RalA, confirmed the requirement for exocytosis in developmental processes. All in all, this work has provided evidence on the mechanisms by which these three major cellular processes guide the generation of cell and tissue morphologies.

Actin Regulators

While the protein Adducin was the first to show a strong localization to cortical interfaces during GBE (Figure 1), the degree of planar polarization appeared
to be weak. However, the protein dPod1 showed a strong polarized localization (Figure 2). The dPod-1 protein was initially explored because of its binding to F-actin. However, Pod-1 does not appear to be required for overall integrity of the cytoskeleton (Rothenberg et al., 2003). dPod-1 possesses Coronin repeats, which mediate the regulation of the Arp 2/3 branched network family (Rothenberg et al., 2003). Studies have also shown that dPod1 displays cross linking activity between actin and microtubules in axon targeting (Rothenberg et al., 2003). During GBE, dPod1 was localized to short vertical interfaces. There are two potential hypotheses for dPod1 function. One hypothesis for dPod1 function is that it positions F-actin for actomyosin contraction. A second hypothesis of its activity at those interfaces is that it could be participating with F-actin in active endocytosis of extra plasma membrane resulting from rapid shrinking of cell interfaces. This could be due to an F-actin dependent endocytic pathway in which dPod1 recruits activators of the Arp2/3 complex to aid in a late step in vesicle formation. Key activators of the Arp 2/3 complex, Abi and WASP, also showed a somewhat polarized distribution during fixed staining. Studies show that Abi binds WASP and acts as a strong enhancer of WASP dependent F-actin formation (Bogdan et al., 2005). The resolution of which of these two hypotheses is correct will be determined once a functional disruption of dPod1 is achieved.

In Drosophila oocyte development, F-actin nucleators such as Cappuccino and SpirC create a mesh F-actin network, which has downstream effects on the organization of other crucial cytoskeletal structures (Dahlgaard et al., 2007). Both of these nucleation factors possess Rho binding domains, which act as upstream
regulators of the cytoskeleton. However, live and fixed imaging of GFP constructs of
*Cappuccino*, *SpirC*, and *Rho1* revealed a diffuse localization in the embryo and were
not further pursued (results not pictured). These results suggest that many of the
regulators of unbranched F-actin networks are not present at F-actin populations in
the developing embryo.

**Shibire is required at shrinking vertical interfaces during gastrulation**

The *Drosophila* homolog of Dynamin, *Shibire*, directs scission of vesicles
from the plasma membrane during endocytosis. During gastrulation, when rosette
structures form, interfaces shrink along the vertical axis. I have hypothesized that
endocytic up-regulation will likely be required for this process because linked
interfaces in rosette structure encompass a lengthy amount of shared membrane that
would most likely ruffle or bleb without active endocytosis. I therefore tested if
*Shibire* function is essential to GBE cell movements.

The ultimate test of this hypothesis was to perform live imaging of *Shibire*
deficient embryos via confocal microscopy while utilizing a GFP construct to mark
cell outlines. However, *Shibire* grows at a lower temperature than other flies (18° C)
and generation of GFP constructs is a lengthy process. While these crosses were
being established, fixed imaging of Shibire protein was done (Figure 5). Using a
*Shibire* antibody on wild type embryos it was discovered that in a fixed background
*Shibire* localized to vertical interfaces involved with both rosette and T1 processes.
As actomyosin contraction is believed to generate forces that drive interface
contraction, the enrichment of Shibire at contracting interfaces suggests a
coordination of endocytosis with cytoskeletal function. This coordination may go even deeper, as F-actin polymerization may facilitate the pushing force that is required to pop a vesicle off from membrane, which would indicate a direct role for F-actin in vertical interface endocytosis. In fact, this principle is not unfamiliar, as inhibition of actin with cytochalasin D abolished apical endocytosis in epithelial cells, and an intact F-actin cytoskeleton has been shown to be required for bulk cellular endocytosis (Gottlieb et al., 1993).

Given the polarized localization of endogenous Shibire, I turned to a functional analysis of Shibire. I first determined if disruption of Shibire function had gross morphological effects on development. In Figure 4, it is clear that after 45 minutes epithelial polarity in the embryo is lost. Wild type localization of Armadillo is found at spot adherens junctions that link neighboring cells and Neurotactin nicely outlines the epithelial sheet. In Shibire mutants it is clear that the cells, although somewhat intact, seem to be disorganized, and Arm localization is abolished. Instead, Arm is seen to aggregate into a cytoplasmic pool. This result suggests that polarized endocytosis of apicolateral membrane proteins may be inhibited, and that this endocytosis is critical to the maintenance of Armadillo and adherens junctions.

Given the strong functional disruption observed after prolonged periods at non-permissive temperature, I asked what would happen to interface behaviors during GBE on an acute removal of Shibire function. Shibire\textsuperscript{ts1x2};Resille-GFP embryos were used to determine a) the starting point or nature of the phenotype, and b) to examine whether vertical interface contraction was inhibited in embryos raised to the non-permissive temperature. Once permissive and NP movies were generated from these
crosses, two different methods of data quantification for vertical interface dynamics was performed. First, ten randomly chosen interfaces from each embryo filmed were manually followed over time and scored with a “yes” or “no” as to whether they contracted or not over the developmental time frame. Second, movies were uploaded onto MatLab software that generated an in silico representation of the cell outlines and tracked their interface behaviors by automated computational analysis. These approaches both indicated that vertical interface contraction in Shibire mutant embryos does not occur as normal (Figure 11).

Interestingly, it was noticed that vertical contractions in the Shibire-compromised embryos were limited to T1 processes. T1 processes are four cell vertices that are also observed during cell intercalation, however the shared vertical interface is very short (Bertet et al., 2004). The reason that vertical interface contraction was still observed in mutant embryos within T1 processes, may be that while major modes of endocytosis are inhibited, another form of membrane remodeling competent enough for small membrane surface areas is being used. This would explain the failure for vertical interfaces involved with rosette structures to contract, as the greater length of the interface is dependent on large-scale endocytosis. Once more computational analysis is finished on the live movies, the relationship between interface length and the ability to contract will be better defined.

**RalA and components of the Exocyst Complex mediate membrane addition**

In GBE, rosette resolution is the ultimate step that propels lengthening of the epithelial sheet. I hypothesized that membrane addition during this stage might be a
major contributor to this due to the increase in interface length. As previously stated, F-actin bursts are seen during this elongation event. However, the role of exocytosis had not been addressed. The initial goal of the RalA experiments was to use it to address the function of exocytosis in driving interface behaviors, however the initial phenotype observed was so severe that it was difficult to draw conclusions on gastrulation-stage movements. To address where and when RalA may be functioning, I created a RalA:mCherry fusion construct (Figure 15). However live imaging and fixed imaging both revealed a diffuse and ubiquitous localization throughout the embryo (data not shown). This lack of specificity may be a result of adding the mCherry sequence onto the RalA protein, which may have caused it to become defective. A RalA:mCherry fusion at the N-terminal end is currently being produced.

RalA function was knocked down through the use of ovoD germline clone techniques. Maternally loaded genes allow an otherwise mutant embryo to survive early developmental stages in which zygotically produced protein is not yet used or required (Selva et al., 2007). For example, if maternal function of RalA was not knocked down, the embryos might be able to make it through early developmental stages, and only show disruption in stages where zygotic transcription of RalA is required. Since fixed imaging of RalA mutants showed a nuclear phenotype, generation of a RalA-HisGFP has been initiated in order to visualize nuclear displacement over time. The grouping together of the nuclei could be due to an inappropriate interaction between adjacent spindles in the syncytial embryo that occurs because the F-actin-dependent spacing of nuclei is abberant.
The fixed imaging of tight embryo collections showed that nuclear organization early in development was highly disrupted (Figure 14). In addition, F-actin mesh network populations were also aggregated and not correctly localized. In wild type embryos, as the nuclei divide, they space themselves out accordingly. It is interesting that in the earliest cycles, the phenotype does not appear to be as devastating as it is at the latest cycle (compare e and h in Figure 14). Completion of the RalA;HisGFP construct will help determine the evolution of this nuclear phenotype though live imaging.

Mitosis is another cellular processes that require a crucial elongation event. Mutations in the Sec8 and Exo84 components of the EC were found to inhibit the final stages of cytokinesis during cell division (Figures 18 and 19). Dividing cells mutant for Exocyst components displayed some amount of membrane addition, however it was not long lasting and the cell fails to yield daughter cells. Furthermore, the circumference of the cell was found to be either the same or less than what it was at the beginning of prophase (Figure 21). Since wild type cells yield two separate daughter cells, a way to compare the amount of membrane at the end of mitosis to the mutant cells was desired. However, adding together the measurements of the two daughter cells and comparing them to the final measurement of a mutant cell that had failed to divide did not make mathematical sense. Therefore, the circumference of starting values and ending values (done for each daughter cell and added together) were used in addition to diameter calculations (Figure 21). Analysis of circumference more accurately depicted the amount of membrane remodeling that wild type cells undergo compared to mutant cells.
One reason behind the failure of Exocyst mutant cells to elongate during cell division could be the involvement of the EC in tethering vesicles during exocytosis. As reported in the introduction, these components work cooperatively to aid in the final tethering of a vesicle to its target membrane. Since mutant cell elongation is short lived, one hypothesis is that the fusion event is not facilitated correctly, and therefore is not stable. The more obvious hypothesis is that exocytic function is compromised as a whole during this process, and alternative modes of membrane remodeling are attempting to add membrane its absence. In addition, being that RalA is shown to interact with the exocyst to initiate crucial cellular signaling, a compromised EC might interrupt this interaction. Clearly the EC plays a pivotal role in membrane addition during vital lengthening events.

A model for the control of interface behaviors

My work has led to various conclusions about the behavior of cell interfaces during GBE. For starters, I have shown that the actin nucleator dPod1 may be directing branched F-actin network formation at vertical interfaces, to work in concert with Myosin II to promote vertical constriction. It was previously determined that a Rok is responsible for the activation of Myosin II, which is thus localized to the F-actin network and promotes the contractile event (Bertet et al., 2004). Briefly mentioned earlier were the T1 structures that are present within the same population as rosette structures. I hypothesize, based off of the quantitative results that I obtained from Shi:Resille-GFP live imaging, that the mechanism by which T1 processes regulate their shrinking vertical interfaces differs from that of rosette
structures. In the proposed model below, it is speculated that in T1 interface contraction, actomyosin behaviors are sufficient contract an interface (Part A), and directed exocytosis via regulation of the Exocyst complex may be responsible for new growing interfaces. On the other hand, in Part B, it is hypothesized that an up-regulation of endocytosis is required, in addition to actomyosin contraction, in order to avoid plasma membrane blebbing or ruffling. One reason that longer linked interfaces may require an up-regulation of endocytosis could be that once the interface has reached a certain amount of contraction, a signal to raise Dynamin production is activated. The opposite order of events is also plausible, such that the onset of endocytosis could precede and activate contraction. While I have implicated endocytosis as the major mechanism controlling shrinking interfaces, I have also proposed that the deposition of plasma membrane and adhesion molecules at new interface growth is regulated by the EC. Although my studies of the EC were done in a cell division background, it has previously been shown that the EC is required for the adhesion molecule DE-Cadherins’ trafficking to the plasma membrane during GBE as well (Blankenship et al., 2007). In the end, my thesis work provides a strong mechanism by which plasma membrane morphologies are regulated during Drosophila gastrulation.
Proposed Model: Interface dynamics during GBE
Future Directions – Actin

Since localization of dPod-1 has been determined, the next step is to address its function during GBE. While I have started to work on this aspect of the project, it is in the very preliminary stages. In order to address function, RNAi of dPod-1 needs to be accomplished. Injecting dsRNA into embryos prior to cellularization will knock down dPod-1 function during the stages of cellularization and GBE and show whether a disruption is witnessed. Results from this experiment will determine whether dPod-1 should be further pursued.

Future Directions – Endocytosis and Shibire

The future goal with Shibire is to finish the automated analysis on the live Shibire<sup>ts1x2</sup>:Resille-GFP movies, both for wild type and mutant embryos. In addition, a Shibire<sup>ts1x2</sup>:E-Cad-GFP construct has been created and is in the final stages of growth. Doing live imaging with this construct will help establish if adhesion molecule localization in wild type and Shibire mutant embryos is affected.

Future Directions – The Exocyst Complex and RalA

The role of the EC in events requiring massive membrane remodeling has been successfully established, however the numbers for the Exo84 mutants are fairly low (n=2). In order to draw concise conclusions about mutant versus wild type cell behaviors, additional Exo84 mutant cells must be imaged.

The RalA project will be better understood when the RalA:His-GFP construct is ready for use. Live imaging of these embryos will highlight the nuclear
organization during beginning stages of development, and pinpoint the exact timing and nature of the developmental defect. Similar to the Shibire constructs created, it was initially thought that RalA in an E-Cadherin-GFP background would be an interesting addition to the experiments. However with the mutation being so severe, the epithelial sheet does not appear to ever be created.

Conclusions

All in all, my thesis work has allowed me to make a few general conclusions about tissue morphology and the mechanisms involved during GBE. As for the F-actin aspect of my thesis, I have identified a candidate F-actin nucleator that localizes to unique vertical interfaces during GBE. As previously mentioned, the aim of this part of the project was to understand more in depth why F-actin networks were established at vertical interfaces in the first place. Below is a proposed model given to support my findings:
WORKS CITED


