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A Role for Dynamin-Dependent Endocytosis During Drosophila Gastrulation

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A ROLE FOR DYNAMIN-DEPENDENT ENDOCYTOSIS DURING *DROSOPHILA* GASTRULATION

A Thesis

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Abstract

Gastrulation, a process conserved among many higher organisms, is the directed migration of cells into layers that will establish various tissues targeted to become anatomical structures. This process is accomplished through another conserved morphogenetic event, known as cell intercalation. Early in development, this movement of cells within an organized tissue leads to unique cellular arrangements where neighboring cells contract their shared interfaces in order to meet at a shared vertex. In this thesis, I present work that demonstrates a requirement for Dynamin-dependent endocytosis during these contraction events. Using quantitative analysis, I have identified varied cell behaviors during experiments which knockdown the function of dynamin. In addition, I demonstrate the existence of an antagonistic relationship between Dynamin and the Myosin II motor protein. Lastly, localization and functional studies I performed for this work suggest a role for Sorting Nexin proteins during plasma membrane reorganization required for Dynamin-dependent endocytosis.
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Introduction

Morphogenesis is the process by which an organism develops its organs, body compartments and adult body plan through a series of cues which direct cell proliferation, migration and differentiation. In early mammalian development each cell receives the same set of developmental signals, known as determinants, so that cues determining cell fates are directed by cell-cell interactions in a process known as regulative development. Insect development cues, namely in the model organism Drosophila melanogaster, differ in the way which cells establish their eventual function. In Drosophila, cell determination begins prior to fertilization with an egg chamber supported by fifteen nurse cells that share a continuous cytoplasm with the oocyte. These nurse cells provide the oocyte with specific sets of determinants that are maternally, rather than zygotically, contributed. The mother deposits distinguishing mRNAs into the nurse cells that, in turn, dispense the mRNAs locally to the egg, allowing for distinct expression translated into anterior-posterior/dorsal-ventral (AP/DV) axes used to establish directional body plan cues. This mosaic development allows for early patterning in the Drosophila embryo upon fertilization and directs changes in cell shape and behavior which, after a series of metamorphosis events, will lead to the advancement of an adult body plan (Raven et al., 2005).
Planar Cell Polarity

The localized nature of maternally contributed mRNA allows for the manifestation of patterned gene expression and the establishment of polarity pathways within the Drosophila embryo. The Bicoid protein is produced from mRNA provided by the nurse cells and is one of the first proteins involved in patterning. Bicoid diffuses throughout the egg forming a gradient where high concentrations are found within the anterior region (those denoting the future head and thorax) and has also been found to establish later segmentation patterning events. More than two hours after fertilization, Bicoid initiates a signal cascade involving six zygotically transcribed Gap genes which will be responsible for dividing the embryo into large blocks. Gap genes encode for transcription factors that act as molecular switches by activating eight classic pair-rule genes. These pair-rule genes initiate the production of spatially isolated transcription factors corresponding to an accumulation of factors in specific regions, or stripes, and the loss of pair-rule products in the intervening stripes (Nusslein-Volhard and Wieschaus 1980; Carroll, 1990; Raven et al., 2005). The importance of these transcriptional regulators has been demonstrated via a large-scale mutagenesis screen. This screen, the effort of which was awarded a Nobel Prize, identified over 100 important genes encoding transcriptional regulating proteins. Two of these pair-rule genes, Even-skipped and Runt, were ectopically expressed in mutant flies leading to issues in the polarity of adjacent cells. This misexpression resulted in cellular reorientation leading to mistaken directional cues and eventual lethality at later stages (Irvine and Wieschaus, 1994; Zallen and
Wieschaus, 2004). The resulting seven regions designated by pair-rule genes are again divided into segmented sections which will result in the development of 14 compartments, each of which will develop into specific body regions denoting the head, thorax and abdomen (Raven et al., 2005). In total, it is these early patterning events that establish the planar cell polarity (PCP) pathway and which will guide cells in their behaviors during embryogenesis and metamorphosis.

Early Drosophila Embryogenesis

Early development of Drosophila melanogaster has been studied extensively and pioneering researchers have developed a series of stages, known as Brownes Stages, to explain the development process which is unique to fruit flies. Shortly after male and female pronuclei fuse (stage 1), a series of 13 mitotic divisions begins within the preblastoderm (stage 2). This series of synchronous nuclear divisions occurs within the first 130 minutes of development (stages 2–4), and is unique because they occur in the absence of cytokinesis, leading to a multinucleated single cell known as a syncytial blastoderm. At the onset of nuclear cycle 8 these nuclei begin migrating from the center of the blastoderm towards the periphery of the embryo in a microtubule-dependent process (Sullivan et al., 1993; von Dassow and Schubiger, 1994). During mitotic division 9 a subset of 1-5 nuclei commence migration from the center of the embryo towards the posterior germplasm. These nuclei divide again and eventually form individual cells, after which they are extruded to form exterior polar buds which give rise to the germline (stage 3) (Foe and Alberts, 1983; Williamson and Lehmann, 1996; Deshpande et al.,
2005). By early cycle 10, the remaining centrally localized nuclei embark on a journey towards the periphery of the embryo to form a homogenous nuclear monolayer (stage 4) (Foe and Alberts, 1983). At the onset of stage 5 the roughly 6,000 resulting somatic nuclei begin the synchronous process of plasma membrane addition at the apical-most region between each nuclei. The process, known as cellularization, is driven by actin polymerization and Myosin II contraction that directs furrow ingression from the apical region towards the base of what will eventually become individual cells. Microtubules aid in providing apical-basal directionality for actin polymerization, during which time membrane is provided via recycling endosomal compartments, facilitated in large part by a series of Rab GTPase proteins (Foe et al., 2000; Pelissier et al., 2003). Upon the completion of cellularization these cells begin to exhibit complex polarities and rearrangements in which neighboring cells work together to become functional units important for the growth and development of the organism.

**Apicobasal Polarity**

Working in concert with planar cell polarity is the establishment of an apical-basal polarity scheme within each cell. Similarly to the PCP pathway, a vast number of proteins work to initiate, inhibit and antagonize other proteins in the distinction between apical and basal domains. Research performed in both mammals and *Drosophila* characterizes the integrated activity of three groups of proteins found to initiate apicobasal polarity cues in epithelial cells. These groups include the PAR-3/PAR-6/aPKC apical domain group (known as the Bazooka/PAR-3 complex in flies), the Crumbs/Discs
Lost/Stardust group (the Crumbs (Crb) complex) and the basal domain group Lethal Giant Larvae (Lgl) (Gibson and Norbert, 2003). While not entirely understood yet, these proteins can act in complex or individually to localize and regulate counterpart proteins. For instance, studies in *Drosophila* neuroblasts suggest that basolateral Lgl proteins work to counteract the apicalization activity of the Crb complex, as identified by a mutant screen for *Crb* enhancers, demonstrating a negative regulatory interaction (Tanentzapf and Tepass, 2003). Like proteins involved in the PCP pathway, many aspects of the initial apicobasal polarity machinery are maternally contributed (Gibson and Norbert, 2003). It is the proteins which establish the apical-basal polarity network which will accommodate certain remodeling events required for gastrulation, including actin polymerization, adherens disassembly and reorganization, as well as the initiation of plasma membrane addition during cellularization steps.

*Germband Extension*

Gastrulation encompasses the directed migration of cells into layers that will establish tissues targeted to become various anatomical structures. It is from this process that the layers of cells created will begin to differentiate their roles and behaviors through varied gene expression. Five fundamental events occur during *Drosophila* gastrulation. These events entail the formation of a cephalic furrow separating the head and body, formation of a ventral furrow to internalize the mesoderm (future musculature), convergent extension movements and the development of the amnioserosa (tissue used to guide the embryo through later development) (Leptin, 1999). While many of these
detailed changes differ when compared to mammals, some of the events are conserved among higher organisms. Convergent extension movements aid in the production of a symmetrical 2.5 fold increase in length along the anterior-posterior axis of the *Drosophila* embryo, and a simultaneous narrowing along the dorsal-ventral axis (Campos-Ortega and Hartenstein, 1985; Irvine and Wieschaus, 1994; Keller et al., 2000; Wallingford et al., 2002; Nikolaidou and Barrett, 2004; Solnica-Krezel, 2005; Blankenship et al., 2006). In *Drosophila*, this convergent extension process, known as germband extension (GBE). During GBE cells within the germband move from a ventral position along the embryo around the posterior end and extend along the dorsal side of the embryo towards the cephalic furrow. These germband cells crowd between neighboring cells and eventually give rise to the ectoderm and later epithelial tissue (Schock and Perrimon, 2002). In *Drosophila* the way in which these as of yet undifferentiated cells prepare themselves for their future function is through the redistribution of specific cortical proteins, cell shape changes and neighbor exchanges which will aid in the eventual elongation of the embryo.
Figure 1: WT germband extension events.
Images depict the progression of germband extension in wild type embryos. Black arrowheads represent the ends of the germband located along the ventral side of the embryo. The open arrowhead represents the area for cephalic furrow development and orientation is represented with anterior on the left, posterior on the right, dorsal on the top and ventral on the bottom. (A) An embryo prior to germband extension (B) the same embryo 10 minutes later shows how the germband moves around the posterior end (C) and after 20 minutes begins pushing dorsally towards the cephalic furrow. Germband cells continue to move dorsally for (D) 30 minutes, (E) 40 minutes, (F) 1 hour, (G) 1.5 hours, (H) 2 hours and (I) 2.5 hours after the beginning of germband extension. Image adapted from Irvine and Wieschaus, 1994.
**Intercalation**

Cellular movements that facilitate convergent extension are achieved through yet another conserved process, known as cell intercalation. In *Drosophila*, cells within the ventrolateral ectoderm move within the epithelial sheet and rearrange themselves into new neighbor relationships, so that cells that were not previously neighbors now share specific interfaces. Two types of intercalation events have been identified as integral components in the overall extension of the *Drosophila* embryo along its anterior-posterior axis. The simplest of these rearrangements, known as a Type1-Type2 transition, is when four cells share a common isolated interface (T1 arrangement) which runs parallel with the DV axis (otherwise known as a vertical interface). Over a period of roughly ten minutes, this vertical interface undergoes a process of contraction so that each of the four cells eventually share a common vertex; a T2 arrangement. The second type of intercalation event that aids in elongation is a unique cellular arrangement where 5-11 cells share a series of linked vertical interfaces. Like the T1-T2 transition, these linked interfaces will also undergo contraction events leading to a meeting of each of the 5-11 cells at a common vertex, forming what is known as a multicellular rosette structure. As intercalation precedes it is important that certain cellular interfaces (vertical) are dismantled, while new interfaces (horizontal; those which delineate the dorsal-ventral axis) are formed so that these cellular rearrangements can resolve in a direction that propagates the extension of the entire embryo (Blankenship et al., 2006; Zallen and Blankenship, 2008).
Figure 2: Cellular reorganization during intercalation events.
Images depict intercalation movements involving isolated (A-C) and linked (D-F) interfaces in a live embryo expressing a plasma membrane marker-GFP fusion. Orientation remains anterior to the left, posterior to the right, dorsal on top and ventral on the bottom. (A) In a T1-T2 neighbor exchange four cells share a common isolated vertical interface (positioned between anterior/posterior cells). (B) The shared interface has contracted to achieve a common vertex for four neighboring cells and (C) upon full vertical interface contraction a new horizontal interface (positioned between ventral/dorsal cells) is created to resolve along the axis of embryo elongation. (D) Seven cells align to share four linked vertical interfaces. (E) The linked interfaces eventually fully contract to share a common interface and form a seven-cell rosette. (F) After vertical interface contraction has completed a new horizontal interface forms and resolves in the direction of embryo elongation. Image adapted from Zallen and Blankenship, 2008.
Cytoskeletal Reorganization

Previous work has shown F-actin to be the first of many regulating proteins that localize asymmetrically to establish AP polarity. This asymmetry indicates subcellular architectural remodeling prior to the necessary cell changes in shape, location and adhesion within the tissue (Blankenship et al., 2006). Prior to intercalation, cells acquire a uniform hexagonal topology, yet shortly after intercalation begins these cells experience cycles of apical contraction and expansion (as initiated by a dynamic actomyosin meshwork), which alters their morphology to a diverse array of shapes (Fristrom, 1988; Irvine and Wieschaus, 1994; Bertet et al., 2004; Zallen and Zallen, 2004; Zallen and Blankenship, 2008; Vichas and Zallen, 2011). Through the use of immunocytochemistry experiments performed prior to intercalation movements, F-actin and Myo-II show a symmetrical localization to both AP and DV interfaces. Immediately preceding intercalation initiation, F-actin and then Myo-II begin to preferentially localize cortically along AP interfaces within the planar tissue (Blankenship et al., 2006). This alignment of cytoskeletal architecture proteins along contracting interfaces suggests a role for these proteins in a process whereby local tension-mediated cytoskeletal reorganization initiates changes in cellular morphology, leading to directional resolution of multicellular arrays along the AP body axis. In fact, fluorescence recovery after photobleaching (FRAP) experiments have demonstrated that in regions of high tension there is a decrease in the dissociation of Myo-II from the cortex (Fernandez-Gonzalez et al., 2009; Vichas and Zallen, 2011). This suspected inhibition of Myo-II lateral diffusion suggests a positive feedback loop in which Myo-II generates its own tension, and in turn, tension recruits
more Myo-II to the cortex. A model has been suggested whereby this recruitment of Myo-II and its associated increase in tension represent a contractile wave, passing from cell to cell throughout the entirety of intercalation events (Vichas and Zallen, 2011). However, it is not currently understood if these forces are acting directly on Myo-II or indirectly via changes in cytoskeletal or junctional remodeling. These tension-dependent changes in Myo-II localization may represent the coordination of cell rearrangements in a polarized and directional manner (Fernandez-Gonzalez et al., 2009; Vichas and Zallen, 2011) or possibly the stabilization of contractile cables involved in the directional advancement of the epithelial sheet (Kiehart et al., 2000; Franke et al., 2005; Vichas and Zallen, 2011).

Adherens Junction Reorganization

The apicobasal polarity established during cellularization events is important for numerous future changes that the epithelial cells will undergo. One such event is the change in adhesion at apical adherens junctions (AJs). Adherens junctions, sometimes known as spot junctions or the zonula adherens, are areas where distinct proteins dimerize with groups of proteins on adjoining cells. This homophilic coupling allows for the formation of intercellular contacts that provide a stabilizing force for individual cells within an epithelial sheet or tissue. Problems in adhesion can result in cellular fallout leading to structural issues or even metastasis, as found in certain types of cancer. Perhaps the most well known AJ protein is E-cadherin, known as Shotgun in flies, which is the transmembrane protein involved in dimerization and the initial localization of
which is regulated by the Bazooka/PAR-3 complex (Harris and Peifer, 2004; Harris and Peifer, 2005). In late stage 6 embryos (late cellularization) Bazooka is cortically localized homogeneously throughout apicolateral cell surfaces. Immediately prior to intercalation Bazooka becomes preferentially localized to horizontal DV interfaces opposite of Myo-II. At the onset of intercalation, Shotgun also becomes asymmetrically localized to DV interfaces (Blankenship et al., 2006). This spatio-temporal relationship between Bazooka, Shotgun and Myo-II during intercalation events suggests their involvement in cellular rearrangements important in gastrulation. In order to allow for the movement of cells within the tissue it is likely that AJ proteins, like Shotgun, need to be dismantled or reorganized to accommodate these movements. In fact Bazooka, along with Myo-II, has been shown to enable junctional disassembly at the apical margin of cells involved in Drosophila dorsal folding (Wang et al., 2012). Endocytic recycling continues to be a likely candidate for the redistribution of adhesive membrane components, as Rab11 has also been shown to coordinate vesicular trafficking of E-cadherin (Blankenship et al., 2007; Roeth et al., 2009).

*Endocytosis and Dynamin*

Endocytosis is a well-established mechanism for plasma membrane reorganization and is a potential model for vertical interface contraction during intercalation events. Endocytosis allows for the internalization and potential redistribution of various proteins, including transmembrane proteins, receptors, enzymes, ligands and other potential cargos. Clathrin-mediated endocytosis in particular is one of
the major pathways that is utilized in eukaryotes, appearing in not only a variety of biological systems, but also in multiple biological processes. Future sites of endocytosis, known as Clathrin-coated pits (CCP), are invaginations in the plasma membrane that facilitate cargo capture and internalization for the vesicular formation process. These CCPs are nucleated at the plasma membrane via the transmembrane binding of coat and adapter proteins (i.e., Clathrin and AP2) to specific phospholipids (Nunez et al., 2011). While recruitment of cargo and coat proteins is important in the process of CCP formation, other proteins have been shown to affect the efficacy of mature endocytic events. The final stage of endocytosis is the act of fission between the would-be vesicle and the plasma membrane, otherwise known as scission. Scission is performed by a number of proteins, but perhaps the most utilized and well studied of these proteins is Dynamin. Dynamin, the human homolog of Shibire in Drosophila, is a large GTPase that is recruited to CCPs prior to Clathrin at predefined repeated CCP nucleation sites, known as hotspots, and after Clathrin in non-hotspot areas (Nunez et al., 2011). Many forms of Clathrin-dependent endocytosis have been shown to require Dynamin for successful scission of the vesicle from the plasma membrane. In fact, some studies have even suggested that Dynamin performs a rate-limiting step not in scission but rather in late CCP maturation (Soulet et al., 2005; Loerke et al., 2009). Dynamin is so pervasive throughout biological systems that even Clathrin-independent forms of endocytosis rely on the large GTPase (Artalejo et al., 2002).

A role for Dynamin as a monitor of endocytic factors is established through its C-terminal proline/arginine-rich domain, and its subsequent interaction with a number of
proteins that possess an Src homology (SH3) domain (Schmid, 1998). Some of these SH3 domain-binding proteins can even affect the basal GTPase activity and self-assembly properties of Dynamin (Lundmark and Carlsson, 2003; Soulet et al., 2005).

Dynamin exists in the cytoplasm as an assembly-incompetent conformation until membrane binding causes a conformational change, exposing the interface necessary for helical assembly at the neck of invaginated CCPs. Constriction of the neck to achieve scission is triggered by structural changes in the middle GTPase effector domain. These changes allow for tetrameric assembly and a rotation of the bundle-signaling element within the GTPase domain, causing a final conformational change required for constriction at the vesicle neck, referred to as Dynamin’s power stroke (Chappie et al., 2011).

**Shibire**

The study of Dynamin in flies is achieved via mutations at the shibire locus. Mutations that result in a temperature sensitivity in the shibire protein introduce the reversible paralysis of adult flies after a non-permissive (high) temperature shift (Grigliatti et al., 1973). Morphological analysis of nerve terminals in temperature shifted shibire flies demonstrated a depletion of synaptic vesicles and, in turn, an accumulation of membrane invaginations indicative of CCP’s at the cell surface. These findings suggest that disruption of shibire does block Dynamin-dependent forms of endocytosis (Kosaka and Ikeda, 1983). Temperature sensitivity of gene products has been deemed as the consequence of a single amino acid substitution in a polypeptide. This substitution
alters the biological activity of the protein at different non-permissive temperatures (Suzuki, 1970). While previous studies have utilized a permissive temperature of 17°C and a restrictive temperature of 29°C for various proteins (Suzuki, 1970), other research on shibire demonstrated a reversible block of Dynamin endocytosis at 30°C and utilized permissive temperatures of 19°C (Kosaka and Ikeda, 1983).

**Plasma Membrane Reorganization**

The composition of lipids, cholesterol, enzymes, transmembrane proteins and accessory proteins varies depending on how any particular section of a phospholipid bilayer will be utilized for renovation and reorganization purposes. Each component that comprises a bilayer contains specific affinities or signals used to recruit or alter other protein functions. Phosphatidylinositol phosphates (PIPs) are negatively charged lipid chains that act as guidance cues and anchors for accessory proteins required for specific endocytic pathways. PIP2 and PIP3, for instance, have been shown to bind to the accessory protein Sorting Nexin 9 (SNX9) via its N-terminal Phox (PX) homology domain (Shin et al., 2008; Yarar et al., 2008) and its BAR (Bin/Ampiphysin/Rvs) domain (Peter et al., 2004; Nunez et al., 2011). The interaction with phospholipids via its C-terminal BAR domain imparts on SNX9 properties that allow it to generate and sense curvature within the attached plasma membrane (Worby and Dixon, 2002; Peter et al., 2004; Nunez et al., 2011). SNX9 has the ability to bind not only the plasma membrane, but can also bind and initiate known members of endocytic pathways including N-WASP, (Yarar et al., 2007; Badour et al., 2007; Shin et al., 2008; Yarar et al., 2008;
Nunez et al., 2011), Clathrin, AP-2 and Dynamin via its SH3 protein-protein binding domain (Lundmark and Carlsson, 2003; Soulet et al., 2005; Yarar et al., 2007). SNX9 has been shown to be important for endocytosis because of its recruitment of endocytic components, its ability to organize and activate actin nucleation and in its propensity to initiate changes in other required proteins. The interaction between SNX9 and the actin cytoskeleton is initiated through the binding of the SH3 domain of SNX9 to the actin nucleation promoting protein N-WASP, thereby changing the autoinhibitory shape of N-WASP in order to initiate activation of the branched actin nucleator Arp2/3 (Lundmark and Carlsson, 2003; Soulet et al., 2005; Nunez et al., 2011). The activation of Arp2/3 will therefore be initiated in a spatio-temporal manner to portions of membrane that are undergoing membrane curvature in preparation for endocytosis. Previous work in yeast has shown a requirement for branched actin networks to generate force for membrane invagination during Clathrin-mediated endocytosis (Karksonen et al., 2003; Aghamohammadzadeh and Ayscough, 2009); however, specific actin requirements for Dynamin-dependent endocytosis during intercalation events are thus far unknown.
Thesis Aims

Specific Aims

My specific aims are directed towards understanding proteins that are integral for the contraction of vertical interfaces during GBE. Within this thesis the involvement of specific proteins is broken down into three chapters relating to endocytic remodeling of these interfaces: the first chapter studies the involvement of Dynamin, the second chapter is dedicated to cytoskeletal components and the third chapter identifies a role for curvature generating proteins during remodeling.

Hypotheses

The work in this thesis is performed in an effort to understand the role of Dynamin-dependent endocytosis in the contraction of vertical cellular interfaces during intercalation remodeling. By instituting a reduction in shibire function via temperature sensitive mutants and classifying the resulting gross morphological disruption phenotypes, I aim to demonstrate a requirement for Dynamin-dependent endocytosis during gastrulation events in germband epithelial cells. If shibire function is disrupted
and the subsequent cell-cell interactions are monitored and analyze, I expect that endocytosis will be blocked and germ band cells will subsequently lose their ability to contract their vertical interfaces. In addition to showing that Dynamin is required for endocytic recycling events at lateral cell interfaces I aim to investigate the possibility of a positive feedback-loop existing between Dynamin and components of the cytoarchitecture, including F-actin (observed via its binding partner Moesin) and the regulatory light chain of Myo-II (known as Spaghetti Squash (Sqh) in flies). A loss of function in Dynamin that induces a disruption in the localization or function of either actin or Myo-II suggests that these proteins are downstream of Dynamin. I hypothesize that if shibire function is disrupted, and if F-actin and Myo-II are monitored and their localization analyzed, this will result in a failure of cellular morphology and gastrulation events. Lastly, I will explore three Sorting Nexin proteins (SNX2, SNX6 and SNX9) which are ideal candidates to act as intermediaries between the actin cytoskeleton and the plasma membrane during Dynamin-dependent endocytic events because of their structure, as well as their identified roles in actin polymerization and the recruitment of endocytic proteins. I predict RNAi knockdown of SNX proteins will result in a stagnation of Dynamin-dependent endocytosis and the resulting phenotypes will be similar to those observed during temperature-sensitive shibire experiments. The following work is presented in an effort to display the importance of these proteins in their regulation of intercalation events in early embryogenesis.
Materials and Methods

Molecular Cloning – Constructs

Construct Design

Molecular cloning constructs were designed for N-terminus fusion of green fluorescent protein (GFP) tagged to one of three Sorting Nexin proteins (SNX2, SNX6 and SNX9). Work by Brand and Perrimon has described a protocol that enables users to generate viable fly lines that allow for targeted gene expression in a tissue-specific manner. The protocol utilizes two separate transgenic lines in order to separate the target gene from its transcriptional activator. Upon crossing these two fly lines the target gene is activated in the resulting F1 progeny and expression of the desired gene can be studied. In order to ensure that only the targeted gene is activated, a fly line that encodes a transcriptional activator that has no endogenous targets in the Drosophila system is required. GAL4, which is an activator in the yeast system, has been shown to activate transcription in flies from promoters that contain GAL4 binding sites. In order to produce GAL4 to achieve binding, and in doing so activate transcription of the desired gene, virgin females from an enhancer trap line in which the GAL4 gene has been randomly inserted into the genome was used. This fly line (known as 67; 15) allows for GAL4 expression under the control of the α-tubulin promoter. In order to create a GAL4-
responsive transgenic line that contains the gene targeted for overexpression a P-element insertion vector, pUAST, was utilized. The gene of interest was subcloned into this vector by utilizing specific restriction enzyme cut sites which are located behind a specific upstream activating sequence (UAS) promoter containing 5 tandem GAL4 binding sites to allow for transcriptional activation (Brand and Perrimon, 1993). Special characteristics of this pUAST vector include an antibiotic resistance gene to select for resistant colonies containing the newly subcloned gene of interest, a TATA box downstream of the UAS, an artificial polyadenylation site downstream of specified restriction enzymes and the expression of a mini-white gene marker which will induce a red eye phenotype in all flies which contain the transgene.

Polymerase Chain Reaction (PCR)

In order to amplify the desired segment of DNA to be introduced to specific restriction enzyme sites (those which will not cleave the protein of interest) a PCR protocol was followed to obtain billions of copies of the desired DNA sequence to ensure adequate material for introduction to the plasmid vector. Primers were designed to target cDNA sequences upstream of the start and stop codons for the protein of interest and its GFP tag, as well as sequences downstream of the stop codon for the protein of interest and its GFP counterpart. Primer design was accomplished by finding the coding sequence (CDS) for each gene of interest on FlyBase.org, pasting the resulting sequence into a nucleic acid file available in the MacVector program and analyzing the sequence to
identify non-cutting restriction enzymes. The same analysis was performed for the GFP tag and three matching pUAST compatible enzymes were selected for use. Using the New England Biolabs catalog the sequences for chosen enzymes were acquired, as were the results for necessary base pair (bp) addition at the beginning of each enzyme sequence for assured effectiveness. For 5’ primer design the restriction enzyme sites were added to the beginning of each target protein sequence. The same design was performed for the 3’ primer except that the cDNA for the target gene was also reversed and complemented using MacVector to account for the 5’-3’ directionality of DNA synthesis.

In order to ensure continuous translation of the target N-terminis GFP-SNX gene fusion the stop codon was removed from the GFP resulting in a 3’ primer created just upstream of the non-included stop codon. cDNA for SNX2 (BDGP clone RE32163), SNX6 (clone LD22082) and SNX9 (clone LD47602) were purchased from DGRC (*Drosophila Genomics Resource Center*). Annealing temperature for individual primers was calculated using an ACGT calculator provided by http://sabina.anzlovar.com/calc/acgt.cgi. PCR reactions were prepared for a total volume of 20µl as specified in the instructions included in the Expand High Fidelity PCR System kit purchased from Roche. An MJmini Personal Thermal Cycler from BIORAD was utilized for amplification and all reactions were performed under the “standard” thermal profile using an elongation temperature of 72°C (≤3kb) as developed for the Applied Biosystems GeneAmp PCR System 9600.
For the GFP-SNX2 construct the amplification primers were as follows:

5’ GFPEcoRI primer - GGAATTCATGGTGAGCAAGGGCGAGGAGC
3’ GFPBglII primer - GAAGATCTCTTTGTACAGCTCGTCCATGCC
5’ SNX2BglII primer - GAAGATCTATGGAGGGTGGAAAGCCCAGGAACAC
3’ SNX2XbaI primer – GCTCTAGATAATACGATTTCTCGAGGCAAAAC

For the GFP-SNX6 construct the amplification primers were as follows:

5’ GFPXhoI primer - CCGCTCGAGATGGTGAGCAAGGGCGAGGAGCTG
3’ GFPKpnI primer - GGGGTACCCCTTGTACAGCTCGTCCATGCCG
5’ SNX6KpnI - GGGGTACCATGATGGACGGCAGGATGA
3’ SNX6XbaI - GCTCTAGATCAGCAATCTCTTTTGAGCGCTA

For the GFP-SNX9 construct the amplification primers were as follows:

5’ GFPEcoRI primer - GGAATTCATGGTGAGCAAGGGCGAGGAGC
3’ GFPXhoI primer - CCGCTCGAGCTTGTACAGCTCGTCCATGCCG
5’ SNX9XhoI primer - CCGCTCGAGATGACCTCGTACGTGCCTGACCGTGCCTG
3’ SNX9XbaI primer - GCTCTAGACTACTCAATCTTGACCGCCTGCCTG

Restriction Enzyme Digest and Ligation

In order for the desired DNA to be inserted into the cloning vector both the PCR product and the plasmid must posses the same restriction enzyme cut sites. After the
DNA has been amplified using PCR, the resulting product (which contains the proper sequence for the desired non-cutting enzyme) can be inserted into the vector by creating two tandem digestion reactions each with the same encoded restriction enzyme: one reaction contained the enzyme mixed with the pUAST vector while the other reaction contained the enzyme mixed with the purified PCR product mentioned previously.

Plasmid reactions were mixed for 20µl total volume while insert reactions were mixed in a 30µl total reaction (using 20µl of PCR product while only 10µl of plasmid was used). Each reaction also included the two enzymes matching the primers for the PCR reaction, a buffer which possesses the highest activity for both enzymes (as determined by New England Biolabs) and water. Reactions were placed in a heating block at 37°C and allowed to incubate overnight. The resulting products were run on a thick 1.7% agarose gel using a BIO RAD PowerPac Basic Electrophoresis machine for one hour at 110V. Bands of the size corresponding to the insert and plasmid were then extracted using an OMEGA e.Z.N.A. Gel Extraction kit. By cutting both the DNA and the plasmid at the appropriate sites “sticky ends” were created which allowed the PCR product to be annealed to the plasmid upon the introduction of both products with T4 DNA Ligase from New England Biolabs.

**Transformation**

Upon successful ligation, DNA that encodes both the cloning vector as well as the gene of interest was created and must be amplified. The DNA resulting from the ligation
was introduced to a competent, non-pathogenic *E. Coli* strain (available from New England Biolabs - One Shot MAX Efficiency DH5α-TIR) which will accept and amplify the circular plasmid DNA containing the target gene(s) during the normal growth cycle of the bacteria. The ampicillin resistance gene that has been cloned into the pUAST vector ensures that when the bacteria are grown on an ampicillin containing media that only the desired resistant colonies will grow.

**Sequencing**

Sequencing primers were designed to allow for analysis performed by a third party consultant at the University of Colorado Sequencing and Analysis Core to ensure the accuracy of the DNA sequence subcloned into the plasmid. Sequencing primers were each designed to be 20 bp long and were created by finding sequences within each protein’s CDS at a period of 300 or 400 bp downstream of the start codon in the forward direction, 700 or 800 bp downstream of ATG in the forward direction, 1,200 bp downstream of ATG in the forward direction (if applicable) as well as a GFP primer in the reverse direction located 400 bp downstream of ATG.

*For the GFP-SNX2 fusion construct the sequencing primers were as follow:*

- GFP 300 forward - CATCTTCTTCAAGGACGACG
- GFP 400 reverse - CCCAGGATGTTGCCCCTCCTC
- GFP 700 forward - GGGATCACCTCCTGGCATGGA
- SNX2 400 forward - CATCATACCGCCGCGCCCTC
SNX2 800 forward - CATGAAATCGCTGGTCACGT
SNX2 1,200 forward - CGACGACATTTCGCGGGAAA

For the GFP-SNX6 fusion construct the sequencing primers were as follows:

GFP 300 forward - CATCTTCTTCAAGGACGACG
GFP 400 reverse - CCCAGGATGTTGCGTTCCTC
GFP 700 forward - GGGATCACTCTCGGCATGGA
SNX6 400 forward - ATTATTCGCCCCTGTCCACCT
SNX6 800 forward - CCACGGCCACTTGCGCGAGG
SNX6 1200 forward - AATCTATCTCTGCATGTGGA

For the GFP-SNX9 fusion construct the following sequencing primers were as follows:

GFP 300 forward - CATCTTCTTCAAGGACGACG
GFP 400 reverse - CCCAGGATGTTGCGTTCCTC
GFP 700 forward - GGGATCACTCTCGGCATGGA
SNX9 400 forward - TGCGGGACAGTCGGCTCGCG
SNX9 800 forward - GCACAGTCGACTGGCTCGCG
SNX9 1,200 forward - AAAGGAGTTCCAGCGCATG
SNX9 1,600 forward - CGGGATAACGCATTTGAAGCA

Using the MacVector program, the actual DNA sequence was then compared to the desired sequence to ensure that no mutations had occurred.
**DNA Library Cloning**

Sequencing of the cDNA for SNX2 found a single adenine-guanine bp mismatch at position 1,476 resulting in the transformation of a Glutamic acid to a Glycine. As no other SNX2 cDNA was available for purchase, cDNA was created utilizing a DNA library that was prepared by undergraduate researcher Aparna Kailasam. She prepared the library by collecting tissue samples from hand-peeled GBE OreR (WT) embryos and prepared using QIAGEN RNeasy mini kit. Primers for creating SNX2 cDNA were designed using the same method as SNX6 and SNX9 except they contained no restriction sites.

*For the GFP-SNX2 construct the following new amplification primers were as follows:*

5’ SNX2BglII primer - ATGGAGGTGGAAAGCCCGGAACAC

3’ SNX2XbaI primer – TTAAACGATTTTCGCGGGCAAAC

Due to the vastness of working with an entire genome as opposed to a specific cDNA, the original PCR reaction was set up using 4µl of the GBE cDNA library provided by Aparna Kailasam instead of the standard 0.4µl totaling a 20µl total reaction. The resulting PCR product was run on a 2% gel for one hour (to ensure adequate band separation) using 1X TAE buffer at 110V with 25µl of PCR product ran alongside a 1Kb Fisher BioReagent exACTGene DNA ladder. Resulting ultraviolet (UV) Chemidoc photographic analysis of the gel displayed a band around 14,000 bp, the size of SNX2
and another band <500 bp. A subsequent PCR reaction using yields from the first cDNA library PCR was performed using the original 5’ SNX2 forward primer (with BglII restriction site) and the original 3’ SNX2 reverse primer (with XbaI restriction site). The PCR reaction was purified using OMEGA e.Z.N.A. Cycle Pure kit and ran 25µl of product and 10µl of 1Kb ladder was run in a thick 1.7% gel at 110V for 1 hour to allow for adequate band separation (SNX2 band and unknown smaller band). To ensure amplification of SNX2 cDNA alone, the top band believed to represent the Sorting Nexin protein based on size analysis was excised from the gel using the QIAGEN QIAquick Gel Extraction kit. After purification of the cDNA 5µl of the purified product was ran on another gel to check for any erroneous bands, of which there were none.

**Transgenic Injection**

Transgenic injection services were solicited from the third party company BestGene, Inc. The company required a DNA concentration of 50µg so midi-prepped cDNA was prepared via standard dilution with Milli-Q water (MQH2O). Sample concentration was determined using the NanoDrop ND1000 Spectrophotometer. Transgenic flies were injected by BestGene and we received the resulting larvae. After expansion of transgenic stocks flies were sorted to retain the miniwhite gene (and in doing so the P-element insertion.) Stocks then underwent chromosome balancing in order to prevent homozygous lethal inheritance of the mutant allele, prevent loss of the
mutation within the stocks as well as track which chromosome the P-element has been inserted into.

*Phenotypic Scoring*

Control and *shibire* mutant embryos were collected overnight at 18°C in fly cups with yeasted apple juice collection plates. Apple juice plates were removed and subsequently replaced for further collections. Any remaining yeast was removed from the overnight apple juice plate and bleached (2.5% working concentration) for two minutes to remove outer chorion membranes. The bleach and embryos were then dispensed into a fine mesh collection net and rinsed thoroughly with deionized (DI) water. To observe development, embryos were removed from the net using a paintbrush and placed on an apple juice media square. Embryos were manipulated with Dumont #5 tweezers, were chosen at the earliest developmental stages available, including early cellularization (stages 1-5), were lined up in rows along an apple juice square and covered in halocarbon oil 27 under indirect light on standard Zeiss AX10 white light microscope. Embryos on apple juice squares were then placed in petri dishes with lids and made to float in a Fisher Scientific ISOTEMP 210 temperature controlled water bath at the nonpermissive temperature of 29.5°C. Every half an hour embryos were removed from the water bath and the developmental stages were monitored and recorded over the course of 4 hours to allow for full GBE. Scoring at permissive temperatures was achieved by preparing embryos in the same manner described above but were incubated at 18°C and monitored.
for an 8 hour period. Results were charted as one of five potential phenotypes: (I. Complete Germband Extension II. Germband Extension Arrest III. Cellularization Arrest IV. Syncytial Arrest and V. Reversal of Development). Each category represents either the wild-type development of complete germband extension (I) or a lack of full development describing an embryo that has failed to develop beyond a certain stage (II, III, IV and V).

*Spinning Disk Confocal Microscopy*

**Embryo Collection and Slide Preparation**

Fly cups kept at 18˚C overnight were collected for both control and mutant embryos. Apple juice plates were removed and embryos were bleached, poured into a fine mesh net and rinsed thoroughly in DI water. Embryos were transferred from the net onto a plastic live imaging slide. The live imaging slide was covered in In Vitro Systems biofilm with the hydrophobic side facing up to allow for proper ventilation of embryos to sustain live development and the insert was placed inside to create a tight seal. Halocarbon 27 oil was smeared along the hydrophobic side of biofilm and embryos were transferred to the slide using a paintbrush. Embryos at desired developmental stages (mid cellularization through early GBE) were placed at the middle of the slide to allow for optimal imaging. To cover the biofilm an 18X18 inch coverslip was lowered onto the slide. The coverslip was then taped on one corner to prevent movement and the slide was
anointed with a small dot of Olympus immersion oil. Images were collected using a Zeiss STEMI 2000 spinning disk confocal microscope.

**Non-permissive Temperature Shift Experiments**

Both wild type and mutant embryos were subjected to temperature shift experiments for comparison of intracellular dynamics. Embryos were collected and prepared as described above ([Embryo Collection and Slide Preparation](#)) but using a plastic slide which was cut into a 3.3cm diameter circle to allow for use in the temperature controlled stage. Prior to imaging a separate LiveCell Pathology Device temperature controlled chamber was placed into the Zeiss spinning disk microscope stage and plugged into its associated control box. The control box was set to the nonpermissive temperature of 29.5°C and allowed to fully reach temperature before embryos were introduced to the chamber.

**Live Imaging Parameters**

Both slow and fast movies were acquired using the spinning disk confocal (two different settings). Slow movies were used to assess not only overall cellular intercalation dynamics but also to determine characterization at various apical-basal levels within the epithelial tissue. Movies were acquired using the 488 laser at 12-16% laser power and images were retrieved every 20-30 seconds at 300ms exposure. Z-stacks were assigned to 1µm depths and for temperature shift experiments began 15µm above cells to account for
shifts in the focal plane generated by suspected expansion of the embryo upon heating. Images were auto leveled prior to image acquisition and then if necessary the EM gain was increased to allow for enhanced contrast of fluorophores to background.

Immunohistochemistry

**Embryo Collection and Fixation**

Fly cups for 117; 95-1:GFP (membrane markers) control lines were incubated with a newly yeasted apple juice collection plate at 25°C for a period ranging from 2-4 hours and were then removed from the cup and allowed to age for 1-3 hours to in order to acquire desired developmental stages (cellularization through late GBE). Aged apple juice plates were then bleached, poured into a net, rinsed and blotted with a paper towel to dry. Nets containing embryos were then immersed in a scintillation vial containing the following fixative solution: 4.375ml PBS (46%), 5ml Heptane (50%) and 625ml of 32% Paraformaldehyde (4%). After fixation was complete the inner vitelline membrane was removed via a Methanol popping protocol or a hand peeling protocol. For the Methanol popping protocol embryos were fixed for a period of 25-35 minutes, after which the top layer of fixative (Heptane) was removed using a glass pipette and dispensed as liquid waste. Embryos were removed using the same glass pipette and were then transferred into a new scintillation vial containing 5ml Heptane. Next, 5ml Methanol was quickly added
to the new vial containing the embryos and immediately capped and shaken vigorously for 10 seconds. Successfully popped embryos fell to the bottom of the scintillation vial and after removal of Heptane layer embryos were again collected with a glass pipette and transferred to a 1.5ml microcentrifuge tube. After embryos settled to the bottom of the tube the Methanol was removed using a glass pipette and new Methanol was added in order to remove any residual Heptane. Methanol washing was performed four times after which embryos were frozen in Methanol at -20°C for a period of up to three weeks prior to antibody staining. For the hand peeling protocol embryos were fixed for a period of one hour and fifteen minutes after which they were removed from the fixative solution using a glass pipette and transferred into a fine mesh net, rinsed and blotted dry with a paper towel. A hand-peeling platform was created using the lid of a small petri dish plate coated with Heptane glue (made by dissolving adhesive from double-sided scotch tape in Heptane). Fixed embryos were transferred to the hand-peeling platform via a paintbrush and were gently rolled and pressed onto the tacky heptane glue to immobilize them for vitelline membrane removal. The hand peeling platform was then covered with PBS and 1% BSA. Embryos were visualized under a Zeiss AX10 white light microscope using indirect light to allow for developmental stage identification. Embryos of the desired stage were then pushed out of their membranes using glass needles made from 0.5mm (inside diameter) capillary tubes pulled on a Sutter Instrument Flaming/Brown Micropipette Puller at a setting of heat=480 pull=70 velocity=80 and rel=130. Embryos that were successfully liberated from their vitelline membrane floated to the top of the
buffer solution and were subsequently collected using a glass pipette and transferred into a 1.5ml microcentrifuge tube. These embryos were refrigerated at 4°C on a rocker for a period not exceeding two days.

**Antibody Staining**

Embryos which were successfully fixed and de-vitellined were washed one time with PBS and 1% BSA. After washing with PBS/BSA the liquid was removed and a blocking buffer was added to the microcentrifuge tube (PBS +1% BSA and 0.1% Triton detergent to encourage the permeabilization of the membrane for antibody incubation.

Embryos blocked on a rocker either for two hours at room temperature or overnight at 4°C. A primary antibody solution was formulated in 100µl of PBS/BSA and was allowed to incubate on a rocker for either a period of 2 hours at room temperature or overnight at 4°C. The working concentrations of the antibodies used were mouse α-Neurotactin (1:1, DSHB) and rabbit α-GFP (1:1000, Life Sciences). Primary antibody was then removed and embryos were rinsed with PBT (PBS + 0.1% Triton-100) six times over a period of two hours. A secondary antibody solution was then formulated in 500µl of PBS/BSA and was allowed to incubate on a rocker (covered in foil due to light sensitivity) for either a period of 45 minutes to one hour (for the drug Phalloidin). The antibodies used were mouse α-546 1:500, Life Sciences) or α-568 (1:500, Life Sciences) and rabbit α-488 (1:500, Life Sciences). Secondary antibody was then removed and a series of 2 washes with PBT and 4 washes with PTW (PBS+0.1%Tween) ensued for a period of 2 hours.
After washing embryos were mounted on glass imaging slides using Prolong Gold with DAPI (Invitrogen) and allowed to dry in the dark overnight prior to imaging.

**Fixed Imaging Parameters**

Fluorescent imaging was performed on an Olympus 1X81 laser scanning confocal microscope. Images were acquired using 16-26% laser power and adjusted HV levels for each channel on an as needed basis. Embryos were staged using the 10X objective while fixed images were captured on a 40X objective under 5X zoom. Imaging studies were performed on the ventral side of early embryos towards the anterior portion behind the cephalic furrow in order to visualize a heavily intercalated region. Other imaging parameters include: the aspect ratio set 1:1, acquisition intervals set to 4µs/pixel, z-stacks imaged every 1µ, kalman set to 5 and laser line scan was set to sequential.

**RNA Interference (RNAi)**

Double-stranded RNA (dsRNA) was introduced via injection to early *Drosophila* embryos in order to target mRNA transcripts for degradation and in doing so induce sequence-specific gene silencing. The enzyme Dicer cleaves exogenous small interfering RNAs (siRNA) to generate a 19-21 nucleotide long siRNA duplex to be used as a molecular ruler for the identification of endogenous mRNAs. Dicer interacts directly with the RNA-induced silencing complex (RISC) that is known to aid in target recognition and
cleavage of the mRNA of interest. RISC acts by separating dsRNA to capture a “guide” strand that is used to find target RNAs based on their complementary sequence. Upon target identification an Argonaut protein known as Slicer (a component of the RISC) works to cleave the mRNA of interest and targets it for degradation.

**dsRNA Design**

dsRNA was designed using SnapDragon program which was designed and provided by the *Drosophila* RNAi Screening Center at Harvard Medical School. The CDS for the target gene was entered into the system. The SnapDragon program analyzed the data and highlighted promising sequence options for 5’ and 3’ primers within the CDS in which no off-target effects from other similar proteins was predicted. These options were then categorized as to their potential effectiveness and those with the lowest pair-penalty as well as the lowest left and right mismatch penalties were chosen. Each option also provided a list of restriction enzymes that would not cleave the target sequence. The beginning of each 5’ and 3’ primer designed by SnapDragon also received a T7 tag (TAATACGACTCCTATAGGG) to aid in proper replication when using the Life Technologies MEGAscript RNAi kit.
dsRNA Preparation

dsRNA was prepared using a standard PCR reaction with the cDNA of interest and the designed T7 primers. Annealing temperature was calculated using an ACGT calculator provided by [http://sabina.anzlovar.com/calc/acgt.cgi](http://sabina.anzlovar.com/calc/acgt.cgi).

For SNX9 dsRNA the amplification primers were as follows:

5’ TAATACGACTCACTATAGGGTGATACGACTTTACCCGAGGAG
3’ TAATACGACTCACTATAAGGGGATTGGGTATCATCGTTGGG

The PCR product was purified using the e.Z.N.A. Cycle Pure kit and both the purified and unpurified products were run on a gel to ensure accurate size of the product (448 bp). 6µl of PCR product was then incubated with ATP, CTP, GTP, UTP, reaction buffer, RNA free water and an enzyme mix at room temperature overnight as described by the Life Technologies Megascript T7 kit. dsRNA samples were then purified using Qiagen RNeasy Mini kit with 40µl of elutant being passed through the filter membrane twice. Purified samples were then Nanodropped to determine concentration (a minimum of 150µg/µl or 1000ng was required) and samples ranged from 248ng/µl to 1273ng/µl.

Embryo Preparation and Live Injection

Embryos were collected for a period of one hour at 25°C on an apple juice media. Early embryos were bleached, transferred to a net and rinsed. Embryos were aligned on an apple juice square in the same orientation to allow for viable injection into the ventral
side and were manipulated using Dumont #5 tweezers. The embryos were then glued to a 18X18mm coverslip using Heptane glue and taped to a glass slide. Embryos were allowed to dry in a desiccation chamber (with Dri-Rite) for a period ranging from 9-12 minutes. Upon adequate desiccation the embryos were coated with viscous halocarbon oil 700 and injected with thawed (on ice) dsRNA using Olympus CX21 white light microscope and a Narishige IM-58 oil microinjection apparatus. Glass needles were pulled from capillary tubes (using the same settings described for hand peeling needles) and were loaded with 0.8µl of sample. After breaking the needle on stacked glass imaging slides the embryos were injected with the pressure setting on the injection apparatus at a ¼ turn. Stages of embryos and the status of injection success was recorded and monitored over a period of 4 hours for initial scoring. In order to live image injected embryos the coverslip containing the glued embryos was removed from the glass slide and placed onto a plastic live imaging slide with biofilm (coated in more halocarbon oil 700). Images for dsRNA and MQ water (control) injections were obtained on a Zeiss STEMI 2000 spinning disk confocal microscope at an exposure of 300ms, an interval of 15 seconds with an average of 35 z-stacks using 1µ spacing. Laser power was set to an average of 13% and EM gain values were adjusted as necessary.
Automated Analysis

Segmentation

Quantitative analysis of global interface dynamics was made possible by collaboration with the Loerke lab. Thanks to Dr. Loerke and doctoral student Tim Vanderleest automated analysis was designed and performed for both control (117; 95-1:GFP) and mutant (shiT5; 117:GFP) live imaging data. Using MatLab software, seeded watershed transformation algorithms were adjusted and performed for segmentation analysis. Seeds were created to mask the interior of the cell so that the watershed lines could be forced in between seeds. The watershed technique identifies lines of image intensity (for control and mutant lines a 117:GFP membrane marker was used) and therefore delineated cell outlines. Data that was extracted from segmentation include the position of interface nodes, the lengths between nodes and their corresponding angle. Segmentation of the time series of images was automated (with some manual corrections) and cells were assigned unique numbers through cell tracking. Vertical interfaces were manually set at a 90° angle (as determined by the anterior/posterior axis) and variation within this set parameter was accepted up to a 15° limit on either side of the standard. Linked interfaces were identified only at the beginning of each movie and were cataloged as either disappearing (contracted) or sustained (no contraction) while T1 isolated interfaces were recognized and identified throughout the duration of live imaging data. Gaussian filters were applied to raw images to help with stability of the segmentation.
Statistics

Statistical significance was determined by processing data using Prizm software to compare phenotypic scoring results between control groups and experimental groups. In order to compare the proportions of non-quantitative phenotypic categories amongst independent mutant or WT fly lines a series of 2-dimensional contingency tables were organized and analyzed using Chi-squared analysis between observed and expected values (as determined by WT proportions of phenotypic categories). Contingency tables were first organized to identify significance between separate fly lines and their proportions of developmental phenotypes with a null hypothesis of evenly spread proportions amongst developmental categories and an alternate hypothesis of differing proportions of phenotypes within individual fly lines. Subsequent contingency tables were organized to identify significance between the proportion of phenotypes as compared between different fly lines with a null hypothesis of individual fly lines possessing the same proportions of phenotypes (i.e., WT=shit$^{TS}$; 117 A3) or an alternate hypothesis of each fly line possessing differing phenotypic proportions. The second round of 2D contingency analysis involved comparing each mutant fly line against WT lines/conditions and determining their significance via Chi-squared test where $\alpha=0.05$. 

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**Fly Lines Used**

*OreR*

w; 117;95-1 (Resille:GFP, Spider:GFP)

w; 117/Dr; TM3

w; Sp/Cyo; Dr/TM3

w; ShiWT:GFP

w; $shi^{TSI}$

w; $shi^{TS2}$

w; $shi^{TSI}$:GFP

w; $shi^{TS2}$:GFP

w; $shi^{TSI}$; 117:GFP

w; $shi^{TSI}$; Moe:GFP

w; $shi^{TSI}$; Sqh:GFP

**Fly Lines Created**

w; pUAS-GFP:SNX2

w; pUAS-SNX2:mCherry/Cyo; +/TM3

w; pUAS-SNX2:mCherry/Cyo; Dr/TM3

w; pUAS-Sp/Cyo; SNX2:mCherry/TM3

w; pUAS-+/+; SNX2:mCherry/TM3

w; pUAS-+/Cyo; SNX2:mCherry/TM3
w; pUAS-GFP:SNX6
w; pUAS-GFP:SNX6/Cyo; +/+ 
w; pUAS-GFP:SNX6/Cyo; +/Cyo 
w; pUAS-Sp/Cyo; GFP:SNX6/TM3 
w; pUAS-GFP:SNX9 
w; pUAS-GFP:SNX9/Cyo; Dr/TM3 
w; pUAS-GFP:SNX9/Cyo; +/+ 
w; pUAS-Sp/Cyo; GFP:SNX9/TM3 
w; pUAS-+/-Cyo; GFP:SNX9/TM3 
w; pUAS-SNX6:mCherry/Cyo; Dr/TM3 
w; pUAS-SNX6:mCherry/Cyo; +/TM3 
w; pUAS-Sp/Cyo; SNX6:mCherry/TM3
Results

Chapter I: Dynamin-Dependent Endocytosis During GBE

Observations on changes in cell shape and behavior led to the discovery of neighbor exchange events during intercalation, whereby interfaces located between anterior and posterior cells undergo shrinkage, facilitating the formation of a shared vertex. A mechanism with the potential for reorganizing plasma membrane, and therefore changes in cell shape and behavior, is endocytosis. Dynamin-dependent endocytosis has been shown to be a common mechanism in a variety of systems including Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana and mammals (Praefcke and McMahon, 2004). In order to determine if Dynamin-dependent endocytosis is a regulator of vertical interface contraction during GBE both localization and functional studies must be performed. Transgenic flies with GFP tagged Shibire (the fly homolog of Dynamin) were utilized in experiments to determine Dynamin localization. ShibireWT:GFP embryos were collected, fixed and stained using both a GFP antibody and the drug Phalloidin, which binds F-actin. Flies that facilitate the imaging of individual cells (117;
95-1) were also stained as controls. These control flies have two GFP tagged plasma membrane proteins known as Resille (117:GFP) and Spider (95:GFP) and have been shown to undergo wild type development. Imaging using laser scanning confocal microscopy revealed both a cortical and cytoplasmic localization of Shibire during cellularization (Figure 3B). During early GBE, Shibire transitions to a tighter cortical array (Figure 3D); however, in neither of these stages does the protein show any planar polarization. It is not until late GBE when Shibire displays a preference for vertical interfaces during T1-T2 transitions and rosette formation (Figure 3E). While Shibire localizes to areas outside of the key interests in this thesis, it is important to note that it does appear at areas of vertical interface contraction during GBE; it is therefore a candidate for plasma membrane remodeling.

After showing that shibire is localized to vertical interfaces, it is important to determine if Dynamin-dependent endocytosis plays a functional role during remodeling. Through the use of temperature-sensitive shibire mutants (shiTS), a reversible block of Dynamin-dependent endocytosis was achieved and developmental defects were assessed. To ensure that Dynamin was actually affected during development, shiTS embryos were scored under oil both at the permissive (18°C) and nonpermissive temperature (29.5°C). The proportions of developmental defects were broken down into five potential development phenotypes: 1) complete GBE, 2) arrest during attempted GBE, 3) arrest during attempted cellularization, 4) arrest at the syncytial stage, or 5) an attempt at cellularization that resulted in reversion back to the syncytial stage (Figure 4).
Proportions of the occurring phenotypes were compared between functional *shibire* (permissive) and nonfunctional *shibire* mutants (nonpermissive) using Chi-squared analysis. Changes in proportion of *shi^TS* as compared to its wild type counterpart at the permissive temperature displayed a significant difference. This indicates that when *shi^TS* embryo is raised to the nonpermissive temperature of 29.5°C development, including GBE, is negatively affected.

To ensure that the *shibire* mutation will block Dynamin-dependent endocytosis in a variety of other mutant backgrounds, *shi^TS* lines were created with other GFP tagged proteins including the Moesin Actin Binding Domain (ABD), Myosin light chain (Sqh) and the 117 plasma membrane protein. Embryos for each of these lines were harvested and scored every 15 minutes during development at the nonpermissive temperature (Figure 5). Results from these temperature shift experiments were compared to wild type controls (117; 95-1), which are unaffected by increased temperatures, during development. The proportions of developmental stages achieved were tallied and compared to control proportions to determine the significance of differences between *shi^TS* and 117; 95-1. Significant differences in proportion of developmental stages was displayed for every line when compared to the control, the most significant of which were MoesinABD:GFP and MyosinII:GFP lines (p<0.0001). The difference in proportions for *shi^TS* flies in a 117 background was also found to be significant (p<0.05). This screen determined that a variety of *shi^TS* fly lines could be utilized to administer functional blocks during Dynamin-dependent endocytosis.
While temperature shift experiments have shown a down regulation of *shibire* function, it is important that actual cell interface dynamics are monitored to determine what the exact role of Dynamin is during GBE. To do this, embryos expressing the 117:GFP in a *shi<sup>TS</sup>* background were monitored at the nonpermissive temperature. High resolution spinning disk confocal microscopy allowed for live imaging of cell behaviors during blocks in *dynamin* function (Figure 6). When compared to 117; 95-1:GFP controls *shibire* embryos displayed two loss of function phenotypes. The most severe phenotype, termed ‘frozen’, displays very little movement of cells within the tissue (Figure 6C). Both T1-T2 transitions and rosette formation occur rarely, but happen at slower and reduced rates. In one frozen movie that was analyzed using computational algorithms, the number of disappearing (shrinking) vertical interfaces was 5 out of 83 interfaces (6%). The other *shibire* phenotype, termed ‘nonfrozen’, represents the seemingly less affected embryos. These embryos may not have as deep a functional disruption of Dynamin function. In nonfrozen embryos, cells are able to move within the tissue quickly in a manner reminiscent of control embryos. As with frozen embryos the formation of both types of multicellular structures occurs in nonfrozen embryos (Figure 6D-E), but takes longer than in controls (Figure 6A-B). In both *shi<sup>TS</sup>* phenotypes, a fraction of embryos also arrest during GBE (never reaching the full 60% of total egg length as seen in wild type development). Unexpectedly, the occurrence of disappearing (shrinking) vertical interfaces in nonfrozen embryos occurs at a higher rate than in control embryos (67% as compared to 25% for controls). Other interesting behaviors also occur in these *shi<sup>TS</sup>*
backgrounds, as vertical interfaces that are actively attempting contraction display abnormal periods of interface growth. Occasionally, the vertical interfaces which are contracting to form multicellular structures experience popping out of an already contracted interface, which we refer to as ‘wobble’ (Figure 7A). Interfaces which are either already fully contracted or have begun contraction can experience a rapid increase in plasma membrane length before contraction re-initiates (Figure 7A). Frozen embryos also display yet another occasional event in which already formed rosettes undergo a full reversal; this is never observed in WT embryos. During normal development rosettes form so that multiple cells meet at a common vertex, after which a new horizontal interface is created to resolve along the anterior-posterior axis. During reversal these already formed rosettes fail to follow a rosette developmental program, and change their positions within the tissue without directional resolution (Figure 7B). Despite differences in cell behavior both shibire phenotypes are believed to represent a common set of behaviors, as suggested by their gross morphological developmental defects.

As mentioned earlier, the use of computer-based algorithms that can monitor and calculate cell behaviors are important for the analysis of Dynamin function during GBE. The tracking of individual cells was possible through the use of seeded watershed transformation algorithms (Figure 8). Cell tracking allowed for the identification of individual cells during live imaging experiments so that the behaviors of those cells and their corresponding interfaces could be quantitatively measured (Figures 9-15). Vertical interface lengths were measured in control embryos (117; 95-1:GFP) as well as both
frozen and nonfrozen $shi^{TS}$ embryos. Automated analysis of vertical interfaces in control embryos revealed an unexpected biphasic regulation of contraction events. During remodeling, vertical interfaces undergo periods of fast contraction that are followed by periods of little to no contraction, referred to as stabilization. These biphasic contraction events occur in isolated interfaces during T1-T2 transitions (Figure 9) and in linked interfaces during rosette formation (Figure 10). In addition, linked interfaces involved in rosette formation appear to alternate so that while one linked interface is undergoing contraction the other linked interface is more passively undergoing stabilization (Figure 10). While these dual phases are cyclic in nature, there as of yet appears to be no defined duration of each phase.

To further investigate the role of Dynamin during contraction events, the same algorithm was used for cell tracking and interface length measurements over time in $shi^{TS}$ embryos. In the less severely affected nonfrozen phenotype, a complete breakdown of biphasic contractions was apparent (Figure 11). As shown earlier, T1-T2 transformation can occur in nonfrozen embryos (Figure 6), but interface length analysis reveals that these neighbor exchanges are less efficient. Vertical interfaces are able to undergo fast cycles of contraction, but they cannot undergo the stabilization phase (as no plateaus in length measurements were observed). The same inefficient attempts at contraction are true for rosette formation in nonfrozen $shi^{TS}$ embryos (Figure 12). For both T1-T2 transitions and rosette formation, interface lengths are erratic and support the identification of wobble events as seen in both frozen and nonfrozen embryos during live
imaging (Figure 7). Because many vertical interfaces in frozen embryos do not undergo contraction, a length analysis was performed for a non-contracting vertical interface. As expected, interface length analysis supported findings of largely unchanged interface length for the duration of the live imaging experiment. Length measurements demonstrated the inability of these interfaces to undergo either fast cycles of contraction or stabilization phases (Figure 13). Vertical interfaces that were able to undergo contraction in frozen phenotypes showed similar behavior as nonfrozen embryos. During T1-T2 transitions, frozen embryos experience a breakdown of biphasic contraction, as fast cycles of contraction are followed by a lack of stabilization (Figure 14). The same breakdown of stabilization phases was true of linked interfaces during rosette formation, as was the breakdown of communication between linked interfaces (Figure 15). Similar to results shown in Figure 6, the formation of both T2 intermediates and rosettes appeared to take longer in both nonfrozen and frozen embryos (Figures 11-12 and 14-15). Perhaps the only potential difference revealed by length analysis between nonfrozen and frozen rates of contraction is that nonfrozen embryos seemed to undergo more rounds of fast contraction than frozen embryos; however more concrete definitions of contraction and stabilization are necessary to appropriately demonstrate this potential difference.
Figure 3: ShibireWT localization.
Immunohistochemistry experiments were performed in transgenic embryos expressing ShibireWT:GFP and control 117;95-1:GFP embryos during cellularization and GBE events using a GFP antibody and the drug Phalloidin. (A-A’’) Control embryo displays cortical localization of plasma membrane and F-actin during cellularization. (B-B’’) ShibireWT:GFP embryo displays apical nonpolarized cortical and diffuse cytoplasmic populations during cellularization. (C-C’’) Control embryo displays cortical localization of plasma membrane and F-actin during early GBE (EGBE). (D-D’’) ShibireWT:GFP embryo displays apical nonpolarized cortical population during cellularization. (E-E’’) Control embryo displays cortical localization of plasma membrane during late GBE (LGBE). (F-F’’) ShibireWT:GFP embryo displays increased apical vertical polarization of cortical population during late GBE. Scale bar is 5µm.
Figure 4: Comparison of morphological defects observed under WT development and under shibire loss of function
Embryos were scored under oil for morphological defects during wild type development of *shibire* at the 18°C permissive temperature and hindered development of *shibire* at the 29.5°C nonpermissive temperature. Phenotypes representing stages of development include complete GBE (wild type), GBE arrest, cellularization arrest, syncytial arrest and reversal (embryo began cellularization but reverted back to its syncytial stage). Significant differences in proportions for mutants were compared to permissive non-mutants and significant difference was determined using a Chi-squared analysis with α=0.05. Differences in phenotype proportions between nonpermissive and permissive temperature were significant as **** represents p<0.0001.
Figure 5: Gross morphological defects comparing control and multiple shibire loss of function lines. Embryos were scored under oil for morphological defects during development at the 29.5°C nonpermissive temperature for shibire. Fly lines scored include 117; 95-1 (control), shibire with MoesinABD:GFP, shibire with 117 and shibire with Sqh:GFP. Phenotypes representing stages of development include complete GBE (wild type), GBE arrest, cellularization arrest and syncytial arrest. Significant differences in proportions for mutants were compared to wild type embryos (117;95-1) and analysis was performed using a Chi-square with α=0.05. Differences in phenotype proportions between nonpermissive shibire and wild type controls were significant as * represents p=0.0477 and **** represents p<0.0001.
**Figure 6:** *shibire* germband extension defects.
Control and *sh*TS embryos were imaged using high temporal resolution spinning disk confocal microscopy during nonpermissive temperature shifts (29.5°C) during germband extension. (A) Control (117; 95-1:GFP) embryos are able to undergo normal vertical interface contraction resulting in a (A-A’) T1 to (A’’) T2 transition. The T2 structure is further able to develop by (A’’) building a new horizontal interface that resolves along the axis of anterior-posterior elongation. (B-B’’) A control embryo is able to form multicellular rosette structures by (B-B’) contracting its linked vertical interfaces (B’’) and eventually a rosette is formed when all six cells share a common vertex. (B’’) Control rosettes are also able to resolve directionally. (C-C’’) A frozen *sh*TS embryo fails to undergo contraction of its isolated interface while attempting a T1-T2 transition. (D-D’’) A nonfrozen *sh*TS embryo takes longer than controls to contract its isolated vertical interface during its T1-T2 transition (E-E’’) A nonfrozen *sh*TS embryo takes longer than controls to contract its linked vertical interfaces during multicellular rosette formation. For all panels red arrowheads represent actively contracting interfaces while the yellow arrowhead represents a completely contracted interface where four cells share a common vertex. Scale bar is 5µm.
Figure 7: Wobble and rosette reversals occur during shibire function knockdown. In a shiTS embryo that experiences a nonpermissive temperature shift (29.5°C), linked vertical interfaces experience a popping-out (wobble) of a previously contracted linked interface. (A) The topmost linked vertical interface is completely contracted between the purple and green cell when (A’) half a minute later the interface wobbles out of its contracted state. (A’’) Wobble experienced by the vertical interface persists until (A’’) the topmost vertical interface is able to fully contract again. (B) A nonfrozen shiTS embryo is able to form a multicellular rosette (B’) but after five minutes rather than resolving directionally by the formation of a new horizontal interface along the anterior-posterior axis the rosette experiences a reversal where the five cells lose their shared vertex and ‘fall out’ of their rosette structure. Scale bar in A is 5µm and scale bar in B is 10µm.
Figure 8: Watershed segmentation algorithm allows for tracking and measuring individual cells and their interfaces during intercalation. Segmentation analysis allows for the tracking of individual cells within the epithelial sheet. The image on the left represents cell tracking at the 5th frame (2.5 seconds post acquisition initiation) of a control (117; 95-1:GFP) embryo during temperature shift experiments. The image on the right represents cell tracking at the 2400th frame (20 minutes) of the same control embryo. Cell #2 has been pseudocolored to allow for visualization of an individual cell and how it moves over time within the tissue. Orientation of the above images is anterior in the upper right-hand corner, posterior in the lower left-hand corner, dorsal along the bottom edge of the pictures and ventral along the top edge of the pictures.
Figure 9: Interface length analysis reveals biphasic contractions during WT T1-T2 transitions. Cell tracking and segmentation analysis of live imaging data allows for precise measurement of isolated vertical interfaces undergoing a T1-T2 transition. Tracking is performed for three individual transitions over time in a single control (117; 95-1:GFP) embryo experiencing a temperature shift to 29.5°C (nonpermissive for shiTS). Rather than a single contraction event leading to a complete decrease in interface length, two different phases can be seen. Vertical interfaces undergo a period of fast contraction preceded or followed by a period of stabilization where overall contraction length is limited. Yellow shading represents manual adjustments in the focal plane during image acquisition.
Figure 10: Interface length analysis reveals biphasic contractions during WT rosette formation. Interface measurements are performed for linked vertical interfaces during the formation of a six-cell rosette. The top red trace represents one linked vertical interface and the bottom purple trace represents another linked vertical interface in the same rosette. Length analysis also shows a dual-phase contraction where interfaces undergo periods of fast contraction and periods of stabilization where contraction is unable to occur as efficiently. In addition, linked interface traces reveal potential communication between the two interfaces where one interface undergoes fast contraction while the other interface experiences a lack of contraction.
Figure 11: Interface length analysis reveals breakdown of biphasic contractions during nonfrozen *shibire* T1-T2 transformations.

Interface length analysis of nonfrozen *shibire* embryos experiencing a nonpermissive temperature shift (29.5°C) show a lack of alternation between periods of fast contraction and periods of non-contraction in isolated interfaces. The stabilization phase is missing or greatly reduced. Each trace represents a different T1-T2 transition in the same embryo and traces reveal fast periods of contraction, but little to no plateaus, during which interface length is stable. Isolated interface traces also reveal a potential wobble phenotype where an interface attempts to contract but then interface length expands. Yellow shading represents manual adjustments in the focal plane during image acquisition and black arrow indicates one incidence of wobble.
Figure 12: Interface length analysis reveals breakdown of biphasic contractions during nonfrozen shibire rosette formation.

Interface length analysis for a nonfrozen shi\textsuperscript{T5} embryo shows that in linked vertical interfaces dual-phase contraction events do not exist, or are greatly reduced. The red trace plots one linked interface, while the purple trace plots the other linked interface in the same six-cell rosette. Traces show that only periods of fast contraction occur and that periods of little-to-no contraction are absent. Length plots also display a breakdown in the communication shared between the two linked interfaces within the same rosette so that contractions are occurring at the same time in both interfaces. Black arrow indicates one incidence of wobble.
Figure 13: Interface length analysis of a non-contracting vertical interface in frozen *shibire* T1-T2 transformation. Length analysis in a frozen *sh/Ts* embryo undergoing a nonpermissive temperature shift (29.5°C) highlights the severity of the frozen phenotype when vertical interfaces, which should contract, fail to do so. The above trace is of a vertical interface in a T1 structure that should undergo contraction to form an intermediate T2 structure but does not.
Figure 14: Interface length analysis reveals breakdown of biphasic contractions during frozen *shibire* T1-T2 transformations. Length analysis of a frozen *shibire* embryo demonstrates how an isolated vertical interface is able to undergo fast periods of contraction but this occurs in the absence of stabilization periods of little-to-no contraction. Multiple periods of wobble also exist in frozen T1-T2 transitions. Black arrow indicates one incidence of wobble.
Figure 15: Interface length analysis reveals breakdown of biphasic contractions during frozen shibire rosette formation. Analysis of interface lengths in a frozen \textit{shiT}\textsuperscript{TS} embryo reveals a failure of dual-phase contraction events as seen in control embryos. The top blue trace represents one of the linked interfaces in a five-cell rosette while the green trace represents the other linked interface in the same rosette. Length plots demonstrate not only a lack of stabilization period in contracting interfaces but also an occurrence of interface wobble similar to nonfrozen \textit{shiT}\textsuperscript{TS} embryo dynamics (which is not observed in controls). Black arrow represents one incidence of wobble.
Chapter II: Feedback Between the Cytoskeleton and Dynamin-Dependent Endocytosis

A role for the actin cytoskeleton during endocytic processes was initially demonstrated with budding yeast defective in fluid-phase endocytosis. These growing yeast were not only defective in one form of endocytosis but also showed mislocalized actin populations, indicating a potential link between actin and endocytosis (Kübler and Riezman, 1993; Raths et al., 1993; Smythe and Ayscough, 2006). Subsequent studies have suggested a specific role for actin during the scission process of endocytosis, though current research does not indicate whether actin acts directly or indirectly. To determine a role for actin during Dynamin-dependent endocytosis, actin was visualized during shibire disruption. By using a GFP tagged version of the MoesinABD (an F-actin binding partner) in a shi\textsuperscript{TS} background, live imaging experiments were conducted at the nonpermissive temperature. Spinning disk confocal microscopy revealed that F-actin localization is the same in both control (MoeABD:GFP) embryos and shi\textsuperscript{TS} mutants (shi\textsuperscript{TS}; MoeABD:GFP). During late cellularization Moesin exists in a tight cortical array with no indication of planar polarization in either controls or mutants (Figure 16A and B). At the initiation of early GBE, F-actin adopts a more punctuate localization with large globules apparent at vertices, interface nodes and occasionally at intermodal regions (Figure 16 A’ and B’). F-actin localization does not change as early GBE continues (Figure 16A’’ and B’’); however, it is likely significant that the appearance of these puncta are relegated apically within the cells (data not shown). While this data does not disprove a role for actin during endocytosis, it does suggest that actin regulation is not
downstream of Dynamin. Further localization and functional studies for the F-actin network would be appropriate to determine a specific role for F-actin during Dynamin-dependent endocytosis.

While it seems that F-actin regulation is not downstream of Dynamin, it is possible that Myosin II may be. In order to determine a role for Myosin II during Dynamin-dependent endocytosis, Myosin II was visualized during shibire knockdown. By raising a Sqh:GFP fusion in a shiTS background to the nonpermissive temperature, Myosin II localization was determined using confocal microscopy. In both control (Sqh:GFP) embryos and mutant (shiTS; Sqh:GFP) embryos, Myosin II displayed planar polarization during GBE events (Figure 17). Even though both fly lines showed the same asymmetric AP localization, the differences in expression are highly noticeable. Both control and mutant embryos received the same treatment (nonpermissive temperature, same imaging parameters, etc.) and, interestingly, the shiTS mutants actually required lower EM gain adjustments than the control. The observed differences in intensity indicate that when shibire is down regulated that Myosin II is upregulated. While Dynamin is not required for planar polarization of Myosin II, an antagonistic relationship may exist as we see an increase in Myosin II levels during shibire knockdown.
**Figure 16:** F-actin exhibits WT localization during *shibire* loss of function. Images acquired using high resolution spinning disk confocal microscopy reveal no changes in actin localization during *shibire* knockdown. In late cellularization stages (A and B), embryos display a tight cortical array in both control and mutant backgrounds respectively. (A’ and B’) After the initiation of germband extension (10 minutes after initial image acquisition) F-actin is still localized cortically but acquires a more punctate appearance. (A’’ and B’’) At the end of image acquisition (late GBE), F-actin retains its punctate form in both controls and mutants. Scale bar is 10µm.
Figure 17: Dynamin antagonizes Myosin II during intercalation. Images acquired using high resolution spinning disk confocal microscopy reveal changes in Myosin expression during shibire knockdown. (A-A'' and B-B'') In GBE, embryos display a planar polarized AP enrichment in both control and mutant backgrounds. (B-B'') Although asymmetric localization is retained, there is a noticeable increase in expression of Myosin when compared to WT controls (A-A''). Image acquisition settings for both control and shibire experiments were the same for laser power, exposure, interval and temperature. Adjustments for EM gain values were made manually; Sqh:GFP EM=189 and Shi^{TS}; Sqh:GFP EM=123. Scale bar is 10µm.
Chapter III: Relationship Between Dynamin-Dependent Endocytosis and Three Candidate SNX Proteins

According to the National Library of Medicine, SNX proteins are identified as a large family of phosphatidylinositol phosphate-binding proteins. Members of this family are known to be involved in mediating intracellular transport and protein sorting by utilizing a variety of endocytic pathways. While the number of SNX proteins varies by organism, a large-scale yeast screen has identified a conserved sequence that is related to the PX lipid-binding domain (Teasdale et al., 2001). Given the ability of these proteins to associate with the plasma membrane, and their suspected role as membrane curvature/generating proteins, SNX proteins make ideal candidates as facilitators of Dynamin-dependent endocytosis during GBE. Based on sequence homology and predictions made by the Blast NCBI database, SNX2, SNX6 and SNX9 in particular may play an important role during the contraction of vertical interfaces. To determine if SNX2, SNX6 or SNX9 display localization appropriate for a role in endocytosis and intracellular transport, transgenic constructs were created. The fusion of a GFP tag to the N-termini of each SNX CDS was achieved utilizing a pUAS vector and expression was driven by the production of Gal-4. Early embryos for each of these constructs were harvested, fixed, stained and imaged using laser scanning confocal microscopy. GFP:SNX2 was stained with anti-GFP as well as plasma membrane marker anti-Neurotactin (Nrt). Findings identify that SNX2 accumulates in cortically associated puncta during cellularization and early GBE (Figure 18). Imaging at various tissue depths
demonstrates SNX2 aggregates that preferentially localize to apical surfaces within cells in early development (Figure 18 A, C, E, G, I and K). Imaging of late GBE embryos, indicates that SNX2 loses its punctuate appearance and shows a preferential planar polarization towards vertical interfaces, similar to what was seen in (Figure 3 and Figure 18 M, O and Q). While some SNX2 protein remains cortically associated, others initiate a transition towards a more diffuse cytoplasmic localization (Figure 18 M, O and Q). Due to indications that SNX proteins play a role in intracellular trafficking, punctuate structures shown in SNX2 may represent endosomal compartments.

The SNX6 construct was also stained with anti-GFP and anti-Nrt; however results are less suggestive of a role in endocytosis. Like SNX2, SNX6 also showed cortical localization during cellularization and GBE, however puncta were not readily apparent (Figure 19 B and D). In addition, SNX6 also appeared to have a more diffuse cytoplasmic localization than SNX2 during cellularization (Figure 19 B). While SNX6 did adopt a tighter cortical array during GBE, there was no indication of planar polarization or of discrete structures (Figure 19 D).

Previous localization and functional studies have indicated a role for SNX9 during various forms of Clathrin-dependent endocytosis, including Dynamin-dependent forms. Staining of GFP:SNX9 embryos with anti-GFP and the F-actin marker Phalloidin display the potential for endocytic involvement in this system. During cellularization, SNX9 remains largely cortically associated in the form of puncta, and like SNX2 and SNX6, additionally show some cytoplasmic localization (Figure 20 B). In early GBE
SNX9 transitions from its punctuate location to a cortical array and continues to demonstrate a cytoplasmic localization (Figure 20 D). While SNX2 and SNX9 localization studies show the most promise as mediators of Dynamin-dependent endocytosis due to their presence in punctuate structures, stains from each of the SNX proteins tested suggest separate pools of protein (cytoplasmic versus cortical), which suggests SNX proteins may have more than one defined role in this system.

In order to investigate a role for SNX9 in Dynamin-dependent endocytosis, functional studies directing the knockdown of SNX9 have been performed. RNAi knocks down the function of SNX9 using dsRNA targeted against a piece of the CDS for SNX9. Early (syncytial) OreR (WT) embryos were injected with SNX9 dsRNA, while control embryos were injected with MQ water. Injected embryos were scored over a four-hour period to determine if any developmental defects occur. Similar to shi<sup>Ts</sup> scoring, the proportions for five potential phenotypes were tallied for each treatment (Figure 22). The proportions of phenotypic effects in SNX9 knockdown embryos were compared to those of control embryos. Using Chi-squared analysis, the proportions between the two treatments was found to be significantly different with p=0.0007, suggesting that the knockdown of SNX9 does cause developmental defects (including in GBE). Live imaging of 117; 95-1:GFP embryos undergoing SNX9 knockdown confirm the existence of GBE defects. Panels of spinning disk confocal microscopy imaging show a SNX9 defect in GBE (Figure 21), which phenocopies the frozen phenotype seen in shibire (Figure 6C). While these functional experiments suggest a requirement for SNX9 during Dynamin-dependent
endocytosis, further studies are required for precise identification of the role for SNX proteins during GBE.
Figure 18: SNX2 localizes cortically within discrete puncta. Images from embryos fixed and stained for GFP and the plasma membrane marker Neurotactin (Nrt). Transgenic embryos expressing GFP:SNX2, as well as 117:95-1:GFP controls, were stained during cellularization, early and late GBE. Images from GFP:SNX2 expressing embryos show a cortical localization of puncta during cellularization (A’) with the majority of puncta localized apically. Scale bar is 5µm.
Figure 18 Cont’d: SNX2 localizes cortically within discrete puncta. Images acquired during early GBE show a continuation of punctate localization (G’) with the majority of puncta localized apically.
Figure 18 Cont’d: SNX2 localizes cortically within discrete puncta. During late GBE SNX2 localization remains largely apical (M’) but losses its punctate form and becomes more diffuse (M’, O’ and Q’) suggesting cytoplasmic localization. Planar polarization of SNX2 is not apparent until late GBE.
Figure 19: SNX6 shows both cortical and cytoplasmic localization. Transgenic GFP:SNX6 and control (117;95-1;GFP) embryos were fixed and stained with GFP and Nrt antibodies. (B-B’) During cellularization SNX6 displays a largely cytoplasmic array with some aggregation suggestive of puncta. (D-D’) In GBE embryos SNX6 observes a less diffuse localization and puncta are not readily apparent. Scale bar is 5µm.
Figure 20: SNX9 localizes cortically within puncta and cytoplasmically. Embryos expressing GFP:SNX9 were stained alongside control (117;95-1:GFP) embryos during (A-B) cellularization (C-D) and GBE. (B-B’) During cellularization embryos displayed both a slightly cytoplasmic localization and a more cortical punctate localization. (D’) During GBE SNX9 persists with a cytoplasmic and cortical array but little to any puncta exists and (D) there is little overlap with the plasma membrane. SNX9 localization does not appear to be planar polarized during either developmental stage. Scale bar is 5μm.
Figure 21: RNAi against SNX9 shows gross morphological defects during development. Cellularizing embryos with GFP tagged plasma membrane markers were injected with either MQH2O (control) or dsRNA targeted against SNX9. Subsequent imaging was performed using spinning disk confocal microscopy for over an hour to determine if mutated embryos display defective development and panels represent the onset of imaging during late cellularization at time 0, 10 minutes, 30 minutes and one hour post injection. Control embryos display wild type development during (A-A’) cellularization (A”-A”’) and are able to undergo effective GBE. (B-B”’) Embryos experiencing knockdown of SNX9 phenocopy shibire frozen development.
Figure 22: Comparison of morphological defects observed between control and SNX9 RNAi microinjections. Early (syncytial) wild type embryos were injected with either control MQH20 or dsRNA targeted against SNX9 and development was scored under oil over a period of four hours. Developmental stages reached by each embryo were compiled into one of five phenotypes; complete GBE was considered wild type behavior while other embryos arrested their development while attempting to undergo GBE or cellularization. Some embryos were unable to achieve cellularization (syncytial arrest) and some embryos attempted cellularization but reverted back to their syncytial stage (reversal). Proportions of phenotypes for SNX9 mutants were compared to proportions for controls via Chi squared analysis using $\alpha=0.05$. Differences in proportions between control and SNX9 were found to be significant. *** Represents $p=0.0007$. 
Discussion

Overall Conclusions

By inducing nonpermissive temperature shifts to early $shi^{TS}$ embryos, thereby blocking Dynamin function, we have been able to tease apart a mechanism for the formation of multicellular arrays during GBE. Vertical interfaces that define AP neighboring cells require a series of contractions for both the generation of a shared vertex, and the eventual directional resolution of the multicellular structure. By employing high temporal resolution microscopy during shibire temperature shift experiments, we have identified a requirement for Dynamin during the cell intercalation events that drive GBE. Localization studies have supported this finding, as ShibireWT:GFP is found at vertical interfaces both prior to and during GBE. While ShibireWT:GFP does not become planar polarized at AP interfaces until late GBE, it is reasonable to suggest that Dynamin is present and functional during endocytic events necessary for GBE. In addition, we show that actin is not regulated downstream of Dynamin, while at the same time Dynamin may actually antagonize the function of the Myo II motor protein. SNX proteins, specifically SNX2 and SNX9, have been identified as potential modifiers of the plasma membrane behaviors that act as important regulators...
of endocytic vesicle formation required for GBE. By compiling these findings I have generated a model for plasma membrane dynamics during vertical interface contraction (Figure 23).
Figure 23: A model for Dynamin, SNX, Actin and Myosin mediated biphasic vertical interface contraction. This model provides a schematic representation of proposed physical interactions between candidate proteins and the plasma membrane. During periods of fast contraction, the Myosin II motor protein traverses F-actin filaments to generate tension and enable shrinkage of vertical interfaces. During the stabilization phase, Dynamin-dependent endocytosis allows for the uptake of excess plasma membrane produced during contraction remodeling events. SNX adapter proteins facilitate membrane deformations necessary to induce invagination, while Dynamin monomers oligomerize to the necks of budding vesicles, encouraging the liberation of the vesicle from the plasma membrane.
Dynamin Behavior

Two distinct phenotypes arose in shiTS embryos; a severely frozen phenotype as well as a less affected nonfrozen phenotype. The frozen phenotype represents a drastic decrease in the number of completed contraction events, as an almost 80% decrease in completed vertical interface contraction events occurs. These heavily disrupted embryos are hindered not only by their inability to contract all of the necessary interfaces, but also in their ability to maintain multicellular structures once formed. Neighboring cells that share a common vertex are often unable to resolve the formation of a new horizontal interface in a directional manner; instead the cells undergo a reversal where they fall out of formation and appear to reform previously contracted interfaces (Figure 7B). While rosette reversal has only been found to occur in frozen embryos, it is perhaps one example of a larger defect apparent in shiTS embryos; interface wobble. When vertical interfaces in frozen shiTS embryos attempt contraction, they frequently undergo a unique wobbling event not observed in WT development. During wobble, one or more vertical interfaces that have previously contracted will experience a contraction reversal, so that an expansion of the previously shrunken interface occurs (Figure 7A). This wobble event happens both in completely contracted interfaces and in those that are actively undergoing contraction. Both frozen and nonfrozen shiTS embryos contain vertical interfaces that experience wobble, so it likely represents the same mechanistic breakdown in both phenotypes.
In order to characterize WT interface contraction events, and define a specific role for Dynamin, we used segmented watershed analysis to provide reproducible cell tracking in order to obtain quantitative measurements of changes in cell shapes and behaviors. Live imaging of contracting interfaces in control 117; 95-1:GFP temperature shifted embryos confirms the existence of a biphasic contraction mechanism. This biphasic mechanism has been characterized as periods of fast contraction (rapid interface length decline) cycling between periods of little to no contraction, described as stabilization, which are represented by plateaus of interface length. Cycles of contraction and stabilization were found to persist for variable periods of time, although stabilization periods often lasted longer than active contraction, until complete shrinkage was achieved for both isolated and vertical interfaces (Figures 9 and 10). Previous research has demonstrated a similar occurrence for isolated interface contraction during T1-T2 formation, but provided no data on the formation of higher order rosettes (Rauzi et al., 2010). Live imaging of both frozen and nonfrozen shi<sup>Ts</sup> experiments demonstrate that in both phenotypic classes multicellular arrays are able to form via T1-T2 transitions, as well as in the more ordered formation of rosettes (Figure 3). Cell tracking confirms the existence of these structures in both phenotypes and provides insight into what the precise role may be for Dynamin during these events. Interface tracking for both frozen and nonfrozen embryos indicate that cycles of fast contraction persist, while no observable plateaus of interface length characteristic of stabilization periods occurs.
(Figures 12-15). This information suggests a role for Dynamin as an effector of stabilization during interface contraction. It is possible that during vertical interface contraction, Dynamin-dependent endocytosis adopts a stabilizing role as excess plasma membrane is removed during observable plateaus in interface length.

**Contraction Phase**

F-actin localization at interface nodes and internodal regions suggests that a dynamic actin population exists during cellular reorganization events (Figure 16). Taken together with data that indicate a planar polarization of Myosin II at AP interfaces during GBE (Zallen and Wieschaus, 2004; Bertet et al., 2004; Blankenship et al., 2006 and Figure 17), it is likely that the F-actin substrate becomes reorganized during intercalation and that the motor protein Myosin II facilitates contraction movements. This data is consistent with the idea that the mechanical anchoring of the actomyosin network occurs apically in association with AJs in order to generate essential force production during embryonic morphogenesis (Cavey et al., 2008; Rauzi et al., 2010). Contraction events for both isolated and linked vertical interfaces in both shiTS phenotypes show that the ability of interfaces to contract is not diminished; there are multiple dramatic decreases in length over short periods of time until complete contraction is achieved (Figures 11-12, 14-15). F-actin itself, however, does not appear to be affected downstream of Dynamin, as disruptions in shibire fail to mislocalize MoesinABD (Figure 16).
**Stabilization Phase**

We know from live imaging experiments that a decrease in Dynamin function leads to a subsequent decrease in completely contracting interfaces in frozen *shibire* mutants, but surprisingly we also saw an increase in fully contracted interfaces in nonfrozen *shibire* mutants (data not shown). The observation that nonfrozen *shi*<sup>TS</sup> embryos experience almost a 3-fold increase in completed contraction events is the first indicator that an antagonistic relationship may exist between Dynamin and Myosin II. This increase in contraction events is indicative of a system attempting to compensate for a lack in the ability to endocytose plasma membrane, which likely represents a component in the stabilization machinery. If Dynamin-dependent endocytosis is responsible for the uptake of plasma membrane during vertical interface contraction, then the system could be able to sense increased tension (provided by Myo II-dependent contraction), and alleviate strain on the interface by disposing of excess plasma membrane. Further evidence of an antagonistic relationship between Dynamin and Myo II comes from live imaging studies that suggest that upon *shibire* knockdown, expression of Myo II increases (Figure 17). While the nature of this antagonistic relationship is currently unknown, there are many areas for potential interactions. Perhaps competitive binding of WT Dynamin to Myo II exists (via their respective SH3 and SH3-like domains), or maybe there is some unknown relationship between different catalytic domains of either protein (GEF, GTPase, PRD, domains that affect ATP hydrolysis, etc.).
Whatever the interaction may be, it is possible that Myosin II imparts function for both the contraction and stabilization cycles of biphasic vertical interface contraction.

**Directed Plasma Membrane Reorganization**

Previous work has demonstrated a requirement for SNX9 in vesicle formation during Clathrin-dependent endocytosis (Nunez, et al., 2011). Evidence presented in this thesis suggests a role for both SNX2 and SNX9 in endocytic plasma membrane reorganization required for GBE. Localization studies indicate that both SNX2 and SNX9 exist along AP interfaces that are likely undergoing Dynamin-dependent endocytosis. In addition, their preferential aggregation suggests localization at compartments involved in endocytosis, such as recycling endosomes (Figures 18 and 20). The question as to why SNX proteins display punctuate aggregation in cellularization (prior to intercalation) still exists, however, it is possible that these SNX proteins are necessary for endocytic events outside of vertical interface contraction during GBE. Localization experiments for SNX6 may be inconclusive as localization of the GFP:SNX6 construct appeared diffuse throughout development and only slightly cortically localized. This could indicate a potential lack of functionality for the GFP fusion construct, or that SNX6 does not function in the early embryo (Figure 19). The theory that SNX proteins could be involved in Dynamin-dependent plasma membrane remodeling during GBE are further supported by functional studies in which RNAi targeted against SNX9 appear to phenocopy frozen \( shi^{TS} \) embryos (Figures 21 and 22). Other research confirms the likelihood that SNX9 in
particular functions in our system. This research includes the connection between SNX9 and the Arp2/3 branched actin polymerization pathway via N-WASP (Worby and Dixon, 2002; Badour et al., 2007), the ability of SNX proteins with PX domains to bind to PIPs (Shin et al., 2008; Yarar et al., 2008) as well as its demonstrated interaction with actin, Clathrin, AP2 and Dynamin (Lundmark and Carlsson, 2003; Soulet et al., 2005; Yarar et al., 2007).

Future Directions

In order to determine how Dynamin-dependent endocytic events are controlled during GBE, it would be useful to examine the exact roles for both actin and Myosin II during interface contraction. For F-actin, it would be useful to conduct imaging of ShibireWT:GFP during actin polymerization disruption via latrunculin injection to see if Dynamin is affected downstream of actin. Since we suspect that Myosin II is antagonized by Dynamin function it would be useful to continue conducting both live and fixed imaging of Sqh:GFP in both controls and shibire mutants. In order to further tease apart the relationship between Dynamin and Myosin II, future studies in which Myosin II disruption via the drug blebbistatin while visualizing ShibireWT:GFP would also be useful. In this way we could determine if there is a feedback loop between the Myosin II motor protein and Dynamin and if this exists as a reciprocal relationship. In order to further investigate proteins integral in plasma membrane reorganization during GBE it would be ideal to conduct further localization studies of SNX proteins. Co-staining of
SNX proteins and endosomal compartment markers like EEA-1 could be useful in
detailing SNX function and its relation to endocytic recycling events. Along the same
lines, immunoprecipitation experiments could also be conducted with antibodies specific
for SNX2, SNX9, EEA-1 and Shibire to specify what molecular interactions exist. More
functional SNX studies should also be performed; RNAi against SNX2 would be
particularly revealing, especially if it also phenocopies the frozen *shibire* phenotype. In
order to determine if both SNX9 and SNX2 RNAi display the same cell behaviors as the
*shibire* phenotype, more quantitative interface length analysis would be required.


Appendix A: List of Abbreviations Used

ABD – Active binding domain
AJ – Adherens junction
AP – Anterior/posterior
bp – Base pair
CCP – Clathrin coated pits
Crb – Crumbs complex
CDS – Coding sequence
dsRNA – double stranded RNA
DV – Dorsal/ventral
EGBE – Early germband extension
FRAP – Fluorescence recovery after photobleaching
GBE – Germband extension
GFP – Green fluorescent protein
LGBE – Late germband extension
Lgl – Lethal giant larvae
Moe – Moesin
MQH2O – MilliQ water
Nrt – Neurotactin
PCP – Planar cell polarity
PCR – Polymerase chain reaction
PIP – phosphatidylinositol phosphates
PX – Phox homology domain
RNAi – RNA interference
SH3 – Src homology domain
shiTS – shibire temperature sensitive mutant
siRNA – small interfering RNA
SNX – Sorting Nexin
Sqh – Spaghetti Squash
UAS – Upstream activating sequence
WT – Wild type