A search for new players in the signalling pathways regulating dorsal closure of the *Drosophila* embryo

by

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B.Sc., Simon Fraser University, 2004

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Abstract

Dorsal closure in *Drosophila* is a widely used model system to study developmental epithelial fusions and wound healing, and had been shown to require coordinated cell morphogenesis and reciprocal communication between different tissue types. In this thesis, I focused on identifying new components of the signalling pathways regulating dorsal closure.

First, I extended the characterization of Epidermal Growth Factor Receptor (EGFR) signalling, which is an important regulator of dorsal closure, and established new leads in the search for components upstream and downstream of EGFR during dorsal closure.

I have also identified the ligand Folded gastrulation (Fog) as a candidate upstream regulator of signalling during dorsal closure. Fog appears to regulate the transcript levels of *decapentaplegic* and *zipper*, two important genes known to participate in dorsal closure.

Finally, I initiated studies to explore if regulators of cell adhesion at the neuromuscular junction are conserved regulators of cell adhesion during dorsal closure.

**Keywords:** Dorsal closure; *Drosophila*; Egfr; Signal transduction; Folded gastrulation
Dedicated to my parents who never stopped believing, and for their unconditional love and support
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<th>Description</th>
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<tbody>
<tr>
<td>CaMKII</td>
<td>Calcium/Calmodulin-dependent Kinase II</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>Dpp</td>
<td>Decapentaplegic</td>
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<tr>
<td>Egfr</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>Fog</td>
<td>Folded Gastrulation</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>Hts</td>
<td>Hu-li-tai-shao</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase cascade pathway</td>
</tr>
<tr>
<td>Lgl</td>
<td>Lethal giant larvae</td>
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<tr>
<td>Mmy</td>
<td>Mummy</td>
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<tr>
<td>PAR1</td>
<td>Partition defective 1</td>
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<tr>
<td>Put</td>
<td>Punt</td>
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<tr>
<td>Pvf</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>Pvr</td>
<td>Platelet-derived Growth Factor Receptor</td>
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<tr>
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<td>Phospho-Tyrosine</td>
</tr>
<tr>
<td>Scrib</td>
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<tr>
<td>Tkv</td>
<td>Thickvein</td>
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Chapter 1. Introduction

From bacteria to humans, structural integrity is vital to survival, whether it is to resist environmental insults or accommodate growth needs. This has led to complex, evolutionarily-conserved signalling networks involved in developmental tissue fusions and wound repair, with the two processes sharing many signalling components (Martin & Parkhurst, 2004). During development of the human embryo, collective epithelial cell migration and fusion underlies critical events such as the formation of the brain and spinal cord, the cranial cavity, jaws, and reproductive organs. In the wound healing process, epithelial cells migrate over the wound cavity area and reform the barrier between external and internal environments (Reviewed in Harden, 2002). In both scenarios cells migrate in a directed fashion, and there is a large amount of communication between cells. Indeed, some researchers claim that the process of wound healing recapitulates the developmental process of tissue fusion (Grose & Martin, 1999; Wood et al., 2002). Malfunction in these coordinated processes has profound impact on human well being, as it can cause developmental defects such as spina bifida and cleft palate (Reviewed in Kang & Svoboda, 2005; Mitchell et al., 2004). With regard to wound healing, myofibroblast contraction failure and failed re-epithelialization, such as is seen in diabetics, often leads to prolonged open wounds or even amputations due to infection (Reviewed in Degen & Gourdie, 2012). Prolonged wound healing is also a risk for malignant transformation, as it has been shown that gene expression patterns in cells participating in wound healing are similar to that seen in malignant tumours (Chang et al., 2004; M. Schafer & Werner, 2008). Furthermore, the healing process also determines the success of any kind of surgical procedure. All these factors provide strong motivation to further our knowledge on the field of epithelial fusions.
1.1. *Drosophila* dorsal closure as a model for studying epithelial fusion defects

Studies in epithelial repair signalling pathways span much of the spectrum of eukaryotes from mice and frogs to flies and worms, where researchers use various scenarios of induced or natural hole closure as models (Brock, Midwinter, Lewis, & Martin, 1996; Davidson, Ezin, & Keller, 2002; Reviewed in Harden, 2002; Martin & Parkhurst, 2004; Reviewed in Simske & Hardin, 2001). Prior research indicates that the underlying mechanisms are conserved across species, and the knowledge gained in one system may be applicable to others (Brock et al., 1996; Davidson et al., 2002).

One of the most studied models for wound healing is dorsal closure in the fruit fly *Drosophila melanogaster* (Reviewed in Harden, 2002). During development of the *Drosophila* embryo, a hole occurs naturally on the dorsal side of the epidermis. This hole is covered by a flat layer of epithelial cells called the amnioserosa, which acts as a temporary film to prevent leakage of embryonic material. Dorsal closure occurs between developmental stage 12 and 16 (Figure 1.1A), and involves the dorsal most epidermal (DME) cells from the two lateral sides of the hole co-ordinately migrating dorsal-wards and over the amnioserosa towards each other (Reviewed in Harden, 2002). The epidermis advances from both sides of the amnioserosa while the amnioserosa cells contract, closing the distance between the epidermal sheets. The sheets eventually meet at the dorsal midline of the embryo and fuse, sealing the hole and forming a continuous epidermis. The amnioserosa and DME cells communicate during dorsal closure and the amnioserosa undergoes apoptosis during and after the dorsal closure process. If the epidermal migration is disrupted or the final sealing of the two sides do not occur, the embryo dies due to failed formation of a proper outer “casing”.

Research into dorsal closure indicates remarkable similarities to vertebrate wound healing (Martin & Parkhurst, 2004; Martin & Wood, 2002). In both processes epithelial cells form sheets and migrate co-ordinately to cover an opening, while the cell shapes change accordingly. The fibroblasts in the wound constrict, similar to amnioserosa cells during dorsal closure, to facilitate migration of the keratinocyte/epithelial sheet. An actin string forms around the opening to further assist
closing of the space, and much inter-cell communication occurs between the epithelial cells and the fibroblasts being covered, which also occurs during dorsal closure. There is also the presence of filopodia at the leading edge during both processes that allows epithelial cells to crawl over the connective tissue or amnioserosa, and assists in sealing of the two epithelial sheets. The rationale for using dorsal closure as a study model for healing is further supported by the fact that in *Drosophila* itself, wound healing uses the same set of signalling components as in dorsal closure (Martin & Parkhurst, 2004). In combination with low genetic redundancy, fast generation time, well characterized genome, and efficient genetic tools available (Reviewed in Belacortu & Paricio, 2011; Reviewed in Harden, 2002), *Drosophila* dorsal closure presents a powerful study model for wound healing and epithelial morphogenesis.

1.2. Actomyosin regulation during dorsal closure

During the dorsal closure process, a diverse range of signalling and morphogenesis events takes place. In order to migrate across the amnioserosa, DME cells first elongate dorsally, while amnioserosa cells undergo pulsatile constriction. Constriction and elongation are facilitated by accumulation of actomyosin cable at the leading edge of the DME cells (Figure 1.1B), which acts as a “ratchet” for DME cells to migrate as a firm, coordinated front, and which reduces the relaxation range of amnioserosa cells during their pulsatile contractions. The presence of actin along the leading edge also assists dorsal closure by a ‘purse string’ mechanism (Reviewed in Franke, Montague, & Kiehart, 2005; Harden, 2002; Lawrence & Morel, 2003; Rousset, Almeida, & Noselli, 2003; Tan, Stronach, & Perrimon, 2003; Young, Richman, Ketchum, & Kiehart, 1993). The pulsed constriction of amnioserosa cells, that reduces the distance DME cells need to travel, also depends on an organized actin filament network across the apical cell surface (Figure 1.1C) (Franke et al., 2005; Kiehart, Galbraith, Edwards, Rickoll, & Montague, 2000; Peralta et al., 2007). Furthermore, filopodia and lamellipodia formed from actin- and myosin-rich membrane protrusions provide locomotive capability for the DME cells, adhesion capability for traction during migration, and “zippering” activity for when the two epidermal sheets join (Jacinto et al., 2000; Jacinto et al., 2002; Mitchison & Cramer, 1996; Wood & Jacinto, 2005).
Figure 1.1. Stages of development in *Drosophila melanogaster* and main regions of myosin distribution regulating dorsal closure

A: Diagram showing the stages of development in the *Drosophila melanogaster* embryo with the stages during dorsal closure outlined. B & C: Figures showing accumulation pattern of actin at the leading edge (B) and amnioserosa (C) with arrows indicating forces produced. Figure adapted from *Atlas of Drosophila Development*, Volker Hartenstein, Cold Spring Harbor Laboratory Press (1993). D: FISH image of *zipper* showing myosin transcription pattern during dorsal closure.
Studies in chick embryos have also indicated the dependence on an actin purse-string and epithelial edge “zippering” in incision wound closures. With the actin cables forming at the wound edge, Myosin II, a non-muscle myosin, is also recruited to the cable to provide tensile strength and contractive force to assist closure of the opening (Brock et al., 1996). The *Drosophila* myosin II gene *zipper (zip)* is a critical component for *Drosophila* embryogenesis, and mutations in *zip* result in head involution and dorsal closure defects (Franke et al., 2005).

All of these studies indicate the importance of actin/myosin regulation during the closure process. As current evidence suggests, accumulation and polymerization of the actin cable in the DME cells, as well as zippering of the epidermal sheets, depends on activation of the JNK-MAPK cascade (Homsy et al., 2006; Jacinto et al., 2000; Jasper et al., 2001; Kaltschmidt et al., 2002; Ricos, Harden, Sem, Lim, & Chia, 1999).

1.2.1. JNK regulation of dorsal closure

Jun N-terminal kinases (JNKs), also called Stress-Activated Protein Kinases, were originally identified as stress response enzymes. The Jun N-terminal kinase cascade (hereafter described as JNK pathway) is a subgroup of the mitogen-activated protein kinase (MAPK) pathways, which consists of highly conserved enzymes in eukaryotes involved in stress pathways, cell integrity, survival, proliferation and differentiation (Reviewed in Bogoyevitch & Kobe, 2006; Reviewed in Hindley & Kolch, 2002; Reviewed in Qi & Elion, 2005).

The *Drosophila* JNK is coded by the gene *basket (bsk)*, and activation of JNK pathway in *Drosophila* involves sequential phosphorylation of the JNK kinase-kinase-kinase Misshapen (Msn), JNK kinase-kinase Slipper (Slpr), JNK kinase Hemipterous (Hep), then the *Drosophila* JNK, Basket (Bsk). Phospho-activated Bsk phosphorylates Jun-related antigen (Jra), the *Drosophila* Jun, and causes dimerization of Jra with Kayak (Kay), forming the transcription factor AP-1 (Ciapponi & Bohmann, 2002; Martin & Parkhurst, 2004; Stronach, 2005; Stronach & Perrimon, 2002). AP-1 then activates transcription of target genes in the nucleus (Figure 1.2). Disruptions in this pathway lead to a range of dorsal open phenotypes in embryos, suggesting an important role of JNK in dorsal closure (Reviewed in Harden, 2002). Aside from that, JNK had also been shown
to be involved in filopodia and lamellipodia modelling and epithelial migration during dorsal closure in *Drosophila* (Kaltschmidt et al., 2002; Sluss et al., 1996), further highlighting JNK’s importance.

During dorsal closure, JNK is activated in the DME cells, leading to elevated expression of JNK target genes. One of these is *puckered* (*puc*), which codes for a MAPK phosphatase that de-phosphorylates Bsk and contributes to negative feedback regulation of JNK activity (Glise, Bourbon, & Noselli, 1995; Suzanne, Perrimon, & Noselli, 2001). Another target gene is *decapentaplegic* (*dpp*), which codes for a diffusible morphogen found to be involved in regulation of *zip* (Zahedi et al., 2008).

### 1.2.2. The Dpp pathway and JNK regulation of *dpp*

Dpp is a diffusible morphogen used as both a short range and long range messenger for cellular communication (Nellen, Burke, Struhl, & Basler, 1996), and a *Drosophila* homolog of vertebrate Bone Morphogenic Protein (BMP). BMPs are highly conserved proteins that belong to the TGF-β cytokine family of growth factors, consisting of soluble, extracellular signalling proteins important for developmental regulation in both vertebrates and invertebrates. Maintenance of TGF-β gradient is crucial for tissue specification and patterning, such as D-V axis determination, cartilage and bone formation, bone repair and maintenance, and regeneration. TGF-β has also been identified as a key player in the healing process in a multitude of tissues and organs including skin, bone and heart (Reviewed in R. A. F. Clark, 1996; Degen & Gourdie, 2012). When TGF-β binds to its receptor, the receptor’s intracellular serine-tyrosine kinase domain is activated, which in turn activates corresponding Smad proteins that translocate into the nucleus and regulate transcription (Reviewed in Raftery & Sutherland, 1999).

During wound healing TGF-β signalling mediates communication between keratinocytes and fibroblasts, where wound edge keratinocyte expression of TGF-β promotes fibroblast differentiation into myofibroblasts (Reviewed in Werner, Krieg, & Smola, 2007). This bears resemblance to the relationship between DME cells and amnioserosa cells during dorsal closure, as *dpp* is also expressed at the wound margin,
amnioserosa cells contract using actomyosin in a similar fashion to myofibroblasts, and *dpp* mediates communication between amnioserosa and DME cells (Glise & Noselli, 1997; Gorfinkiel, Blanchard, Adams, & Martinez Arias, 2009; Martin-Blanco, Pastor-Pareja, & Garcia-Bellido, 2000; Zahedi et al., 2008).

During dorsal closure, JNK-induced persistent *dpp* expression in the DME cells activates signalling events through the Dpp receptors Thickveins (Tkv) and Punt (Put) (Figure 1.2) (Glise & Noselli, 1997; Hou, Goldstein, & Perrimon, 1997; Riesgo-Escovar & Hafen, 1997). Both Tkv and Put are critical for the coordination of dorsal closure between the epidermis and amnioserosa (Zahedi et al., 2008). When Dpp binds to its receptor Tkv, Put is recruited by Tkv, where Put’s intracellular kinase domain phosphorylates Tkv, activating Tkv’s kinase function. Phospho-activated Tkv then recruits and activates Mothers against dpp (Mad) by phosphorylation. Phospho-activated Mad forms a complex with Smad and Medea, and the Mad-Medea complex moves into the nucleus to mediate transcription either directly or indirectly through negative regulation of the transcription repressor *brinker* (*brk*) (Reviewed in Hamaratoglu, Affolter, & Pyrowolakis, 2014). Mad-Medea mediated transcription can lead to positive feedback by inducing *dpp* transcription, or negative feedback by producing Daughters against *dpp* (Dad) and Brinker (Brk), suppressing the Dpp pathway by competitive binding with Mad to Tkv in the cytoplasm, and transcriptional suppression of Smad-Medea targets in the nucleus, respectively (Campbell & Tomlinson, 1999; Inoue et al., 1998; Minami, Kinoshita, Kamoshida, Tanimoto, & Tabata, 1999). This complex regulation provides robust control over expression of patterning and morphogenetic genes.

Mutations in Dpp pathway components lead to dorsal closure defects similar to JNK mutants (Affolter, Nellen, Nussbaumer, & Basler, 1994; Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1996; Penton et al., 1994; Ruberte, Marty, Nellen, Affolter, & Basler, 1995). On top of that, ectopic expression of Dpp or constitutively active Tkv is capable of rescuing dorsal closure defects in JNK mutants, further supporting a relationship between Dpp and JNK pathways where Dpp acts downstream of JNK (Riesgo-Escovar & Hafen, 1997; Sluss & Davis, 1997; Su, Treisman, & Skolnik, 1998).
Figure 1.2. JNK and Dpp pathway interaction

Expression of \textit{dpp} during dorsal closure (stage 13) is confined in a dorsal stripe in the DME cells, a ventral stripe along the ventral-lateral epidermis, and a patch of expression in the midgut. Of these regions, only the DME expression is regulated by JNK (Reviewed in Harden, 2002; Jackson & Hoffmann, 1994; McEwen, Cox, & Peifer, 2000; St Johnston & Gelbart, 1987). Similarly, DME expression of \textit{zip} at the leading edge is greatly enhanced during dorsal closure (Franke et al., 2005; Zahedi et al., 2008). This \textit{zip} expression pattern is found to be at least partially dependent upon \textit{dpp} expression, as \textit{tkv} mutants show reduction of Zip both in the amnioserosa and DME, from germband retraction stage to dorsal closure (Arquier, Perrin, Manfruelli, & Semeriva, 2001; Fernandez, Arias, & Jacinto, 2007; Zahedi et al., 2008). At the same time, Dpp also auto-regulates itself to maintain \textit{dpp} expression in the DME cells during dorsal closure (Johnson, Bergman, Kreitman, & Newfeld, 2003). Furthermore, \textit{dpp} is also required for proper actin cytoskeleton arrangement during filopodia extension at the leading edge (Martin-Blanco et al., 2000), demonstrating the important regulatory role of \textit{dpp} during dorsal closure.

\subsection*{1.2.3. Pvr as an activator of JNK}

A known activator of JNK is the \textit{Drosophila} Platelet Derived Growth Factor Receptor (PDGFR) homolog, PDGF/VEGF-Receptor related (Pvr) protein. Pvr activates JNK during thorax closure in \textit{Drosophila}, which is a process similar to dorsal closure (Ishimaru, Ueda, Hinohara, Ohtani, & Hanafusa, 2004). Expression of the activated form of Pvr, $\lambda$Pvr, leads to expression of JNK target genes including an increase in \textit{zip} expression in the \textit{Drosophila} embryo during dorsal closure. This supports the claim of Pvr being an activator of JNK during embryonic development (Harden, unpublished data). However, introduction of a dominant negative form of Pvr does not have an obvious effect on \textit{zip}, suggesting other inputs compensating for the lack of Pvr signal for \textit{zip} expression (Harden, unpublished data). Nevertheless, the increase in Pvr activity does seem to affect \textit{zip} expression, and Pvr likely plays a part in the control of dorsal closure. To further investigate Pvr's role in dorsal closure, the obvious candidate genes are the three known Pvr ligands, Pvf1-3 (Figure 1.3A) (Cho et al., 2002). Of these, Pvf1 is minimally expressed and noticeable only past stage 11 in the salivary gland and malpigian tubule. Pvf3 is heavily expressed globally from early on in development.
(stage 4), and especially concentrated at the malpighian tubule, vent midline, hindgut and optic lobe. Pvf2 on the other hand has a more refined expression pattern, showing up in the foregut, ring gland and Malpighian tubule. Interestingly, Pvf2 expression is also present along the leading edge during dorsal closure, making it the most promising candidate to study (Berkeley Drosophila genome Project: Hammonds et al., 2013; Tomancak et al., 2002; Tomancak et al., 2007).

1.2.4. Epidermal Growth Factor Receptor and dorsal closure

Besides JNK, Egfr is another player that affects the key dorsal closure genes dpp and zip. Originally discovered around thirty years ago, Epidermal Growth Factor Receptor (Egfr) is a receptor tyrosine kinase from a family of highly conserved signal transduction molecules. Egfr activates a well conserved growth control signalling pathway involved in proliferation, survival and development, and is necessary for proper embryonic development and survival in mammals (Dackor, Caron, & Threadgill, 2009; Dackor, Li, & Threadgill, 2009; Plowman et al., 1990; Yamamoto et al., 1986; Zeineldin & Hudson, 2006). Furthermore, it was shown that expression of EGF in mice enterocytes improves healing of intestinal damage and survival rate post septic peritonitis (J. A. Clark et al., 2009). Hyper-activation of Egfr activity is found in a multitude of tumour types, and increasing levels of expression correlates with increased proliferation and metastasis capability of tumours (Bracher et al., 2013; De Jong et al., 1998).

The Egfr family proteins share a common composition consisting of an extracellular ligand binding region, an intracellular region which contains a tyrosine-kinase domain, and a segment of hydrophobic trans-membrane region in between them (Livneh, Glazer, Segal, Schlessinger, & Shilo, 1985). This makeup of the protein allows it to propagate signals from extracellular ligands into the cell, often leading to transcriptional control of target genes. In Drosophila, there is only one Egfr gene, torpedo (Clifford & Schupbach, 1992). Activity of Egfr depends upon the phosphorylation state of an intracellular tyrosine residue, although how this is achieved has not been completely elucidated.
Normally in a monomeric state, ligand bound Egfr dimerizes and trans-activates its binding partner by phosphorylation at the partner’s tyrosine residue (Arkhipov et al., 2013; Ushiro & Cohen, 1980). Phosphorylation at the intracellular c-terminal of Egfr also allows docking of Src homology 2 containing molecules, allowing activation of signalling pathways such as Ras, PI3K, and PKC pathways (Zenz et al., 2003). Previous studies have identified a requirement for Egfr in both amnioserosa and epidermis for proper dorsal closure, where it represses expression of both dpp and zip (W. Shen et al., 2013). The repression of zip is likely mediated through a diffusible signal, as amnioserosa-specific alterations of Egfr function affect expression of zip not only in the amnioserosa but in the epidermis as well. On top of that, Egfr also represses apoptosis in the amnioserosa, which is also required for proper dorsal closure (W. Shen et al., 2013). These results suggest that Egfr is an important factor in the process of dorsal closure, acting as a “brake” on the process.

Aside from ligand activation, Egfr can also be trans-activated by other routes, such as integrin-mediated cell attachment during fibroblast attachment to fibronectin (Moro et al., 1998). In addition, the Ras-MAP kinase pathway can also be activated by integrin-mediated cell adhesion through induction of formation of a Shc/Grb2/Sos complex (Wary, Mainiero, Isakoff, Marcantonio, & Giancotti, 1996). Given that the JNK pathway is a MAP kinase cascade it is possible that JNK might be activated this way too. Furthermore, it had been shown that Jun can regulate Egfr and EGF expression in vivo, suggesting complex regulatory interactions between Egfr and JNK signalling that control cell differentiation and actin deposition (Zenz et al., 2003).

Egfr also was shown to regulate integrin-dependent activation of PI3K activity (Tiganis, Kemp, & Tonks, 1999). This observation was strengthened by a study that showed a dependency of adherence mediated Akt activation on Egfr activity (Marcoux & Vuori, 2003). Activated PI3K is capable of restoring Rac activity in Egfr inhibited, non-adherent cells, and dominant negative PI3K is capable of blocking Rac activity in adherent cells (Marcoux & Vuori, 2003). The Rac downstream pathway also leads to the activation of JNK, and in all, these results suggest a complex interplay between Egfr signaling, integrin-mediated adhesion, and JNK signalling (Figure 1.4).
Schematic representation the hypothesised Pvr signalling pathway (A) and Egfr signalling pathway (B) regulating dpp and zip during dorsal closure. Pvf: Platelet-derived Growth Factor. Pvr: Pvf Receptor.
Figure 1.4. Hypothesised Egfr pathway controlling \textit{dpp} and \textit{zip} expression

Figure showing possible ways Egfr may be activated and how it may regulate \textit{dpp} and \textit{zip} expression both cell autonomously and non-autonomously. GPCR: G protein coupled receptor. MMP: Matrix metalloproteinase. ADAM: a disintegrin and metalloprotease. Egf(r): Epidermal growth Factor (Receptor). Mmy: Mummy. JNK: Jun kinase.
1.2.5. Mummy as a regulator of Dpp

While transcription of \textit{dpp} had been studied extensively, it is still unclear how the Dpp gradient is established - an important issue since differential gradients of Dpp initiate different cell fates (Campbell & Tomlinson, 1999; Humphreys et al., 2013; Irish & Gelbart, 1987; Nellen et al., 1996; Teleman & Cohen, 2000; Wharton, Ray, & Gelbart, 1993). To this end, \textit{mummy} (\textit{mmy}), the only UDP-N-acetylglucosamine pyrophosphorylase in \textit{Drosophila}, had recently been identified as such a regulator in the \textit{Drosophila} embryo (Humphreys et al., 2013).

Originally isolated as a lethal mutation that affects cuticle pattern, \textit{mmy} is involved in biosynthesis of UDP-N-acetylglucosamine (UDP-GlcNAc), a major component of chitin. Not only that, \textit{mmy} is also crucial for the synthesis of the glycosylphosphatidylinositol (GPI) linker, which acts as an anchor for a multitude of cell surface proteins at the plasma membrane that regulate signalling, including the Dpp pathway (Araújo, Aslam, Tear, & Casanova, 2005). Further evidence for the importance of \textit{mmy} in development besides chitin synthesis comes from observations that \textit{mmy} mutants demonstrate more severe developmental phenotypes than the other chitin synthase, \textit{krotzkopf verkehrt} (Araújo et al., 2005).

Further evidence supporting involvement of \textit{mmy} in dorsal closure comes from cuticle phenotype studies. \textit{mmy} is a member of the \textit{raw}-group genes, which cause failed dorsal closure and ectopic transcription of \textit{dpp} when mutated. Among these genes, \textit{mmy} is unique in that while the other \textit{raw} group genes exert effects on \textit{dpp} expression through the JNK/AP1 pathway, \textit{mmy} operates in a JNK-independent manner, possibly downstream of JNK, and may affect Dpp feedback regulation of \textit{dpp} levels (Humphreys et al., 2013). This is independent of the chitin synthesis function of \textit{mmy}, as chitin synthesis is unaffected by some \textit{mmy} hypomorphs that show dorsal closure defects (Humphreys et al., 2013).

Interestingly, the phenotypes from \textit{mmy} mutants are consistent with mutants in \textit{Egfr}, which downregulates \textit{dpp} in a JNK-independent manner. \textit{mmy} mutants show ectopic expression of \textit{dpp} during dorsal closure in the epidermis, which is also observed in \textit{Egfr} mutants. Furthermore, \textit{mmy} hypomorph mutant cuticles failed development.
around the germband retraction stage, resulting in curled, shrimp-like cuticles with anterior (head) holes in the cuticle (Humphreys et al., 2013); this is also what was observed in Egfr mutants (W. Shen et al., 2013). These observations suggest overlap between Egfr and mmy signalling, where mmy may be the as yet unidentified effector through which Egfr regulates \textit{dpp} expression during dorsal closure (Figure 1.3B).

### 1.2.6. Rho family small GTPases and dorsal closure

A further mechanism through which \textit{zip} can be regulated is found through studies on the Rho family GTPases (hereon referred to as Rho GTPases) (Reviewed in Harden, 2002). The Rho family proteins consist of well conserved small GTPases that act as molecular "switches" which cycle between a GTP-bound "On" state and GDP-bound "Off" state, catalyzed by associated guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively (Reviewed in Harden, 2002). Activity of Rho GTPases is also controlled via binding to guanine-nucleotide dissociation inhibitors, which form a complex with Rho GTPases, restricting their presence to the cytosol and preventing interaction with GEFs/GAPs (Reviewed in Garcia-Mata, Boulter, & Burridge, 2011). Rho GTPases are expressed widely throughout \textit{Drosophila} embryogenesis, with Rho1 and Rac2 expressed in many different tissues, supporting the idea that Rho GTPases are important morphogenetic regulators (Reviewed in Lu & Settleman, 1999b). The three main subgroups of Rho GTPases, Cdc42, Rho, and Rac, are all involved in dorsal closure. Cdc42, Rac1 and Rac2 are all found to activate JNK cascade and cause a corresponding increase in \textit{dpp} expression in \textit{Drosophila}, though they are not the sole contributing activation signals and how they activate JNK is not completely understood (Glise et al., 1995; Reviewed in Harden, 2002; Hou et al., 1997). There is also preliminary evidence that shows phospho-activation of upstream kinases by Rac, but no conclusive data on such interaction as of yet (Reviewed in Harden et al., 2002; Sharma, Urano, & Jaeschke, 2012).

Rho1, on the other hand, affects myosin accumulation at the leading edge but does not affect \textit{dpp} in a JNK dependent manner (Reviewed in Harden, 2002). Studies on Rho1 have demonstrated its requirement during dorsal closure, where loss-of-function mutants and dominant negative Rho1 expression both cause dorsal closure
defects consisting of uneven constriction along the leading edge, and disruptions in the leading edge myosin accumulation (Reviewed in Harden, 2002; Harden, Ricos, Ong, Chia, & Lim, 1999). Furthermore, in a *Drosophila* embryo-based single cell laser ablation study, it was found that Rho1, Cdc42, and Rac are all rapidly recruited to the edge of the wound into overlapping zones, with Rho1 being the first to arrive, accumulating in a tight band along the wounding edge (Abreu-Blanco, Verboon, & Parkhurst, 2014). These results stress the importance of Rho1 in activation of myosin II to associate with actin, forming the actomyosin contractile apparatus.

The relationship between Rho1 and myosin does not seem to require JNK signalling, however, as JNK dependent *dpp* expression at the leading edge is not disrupted (Lu & Settleman, 1999a). This suggests a separate pathway though which Rho1 interacts with myosin. Indeed, genetic and molecular evidence have shown that Rho1 regulates myosin through Rho-associated Kinases (ROKs), most likely through ROK phosphorylation of myosin regulatory light chain. Phosphorylation at Ser19 of myosin activates myosin’s ATPase activity and promotes formation of actomyosin filaments (Reviewed in Bresnick, 1999). Activated myosin can then, with cooperation with Ras, activate the JNK cascade (Khoo, Allan, Willoughby, Brumby, & Richardson, 2013).

Although we know that Rho1 is activated by RhoGEF2, further upstream signaling components during dorsal closure are not clear. In an attempt to elucidate this signal, we turn to another developmental process in *Drosophila* development where major actomyosin rearrangement and cell constriction takes place – gastrulation.

### 1.2.7. G protein coupled receptors and JNK / Egfr

The gastrulation process consists of invagination of cells forming the ventral furrow and is caused by apical cell constriction, driven by cytoplasmic myosin accumulation and cytoskeletal rearrangements (Costa, Wilson, & Wieschaus, 1994). For this process to proceed properly the gene *folded gastrulation (fog)*, which codes for a secreted protein, is essential (Costa et al., 1994). It was found that the presence of Fog exactly precedes regions of apical constriction, and the recruitment of Fog receptor
on the cell surface is thought to recruit actomyosin for cell contraction (Dawes-Hoang et al., 2005). This receptor for Fog was uncharacterized at the beginning of my study, although it is suspected to be a G protein-coupled receptor (GPCR), since an intracellular response element for Fog was identified to be the Ga protein Concertina (Cta) (Costa et al., 1994; Parks & Wieschaus, 1991). One receptor has since been characterized as the GPCR Mist (Manning, Peters, Peifer, & Rogers, 2013).

GPCRs are cell receptors found only in eukaryotes, which regulate cellular response through coupling extra cellular signals to intracellular response elements. Ga proteins are part of the GPCR complex, and are located on the intracellular side to mediate intracellular signaling functions. Cta belongs to the Ga12/13 class of Ga proteins, which are known to activate JNK signaling, and regulate focal adhesion assembly and transcription of cell growth/migrational genes (Reviewed in Dhanasekaran & Dermott, 1996). Concertina itself is known to activate the small GTPase Rho1 through activation of RhoGEF2 and promotes actin rearrangements (Barrett, Leptin, & Settleman, 1997), suggesting Fog’s role as a diffusible signal that can act from a distance to induce cytoskeletal and migrational changes (Figure 1.5B). More importantly, the above evidence makes Fog a likely upstream signal for Rho mediated actomyosin accumulation during dorsal closure.

Furthermore, in cancer cells Ga is involved in stabilizing membrane localization of Egfr to promote cell migration (Ghosh et al., 2010). Since Egfr has also been shown to be transactivated by GPCR ligands in the absence of Egf (Chan & Wong, 2004; B. Schafer, Gschwind, & Ullrich, 2004), this would suggest a scenario where Fog activates downstream Egfr pathways in the absence of EGF. fog’s interaction with JNK and Egfr pathways, as well as its ability to trigger Rho1 signalling, makes it a strong candidate as a regulator for dorsal closure.
Figure 1.5. Schematic representation of hypothesised Ack regulation of \textit{dpp/zip}, Fog regulation of cellular processes, and Hts regulation of Dlg delocalization.
A: Hypothesised Ack regulation of dpp and zip through endocytosis regulation of Egfr. B: Illustration of hypothesized Fog’s participation in pathways involving cell migration and adhesion. Fog. It is known that Fog can activate Rho pathway through the receptor which is hypothesized to be Mist. I hypothesize that though this and subsequent activation of ROK, Fog signalling may lead to cellular contraction, actin depolymerisation, and/or loss of Dlg at the cell attachment junctions. ROK: Rho kinase. MLC: Myosin light chain. MHC: Myosin heavy chain. LIMK: Lim Kinase. Dlg: Discs large. C: Current view on Hts regulation of Dlg localization through CaMKII and PAR-1. It is unknown yet if this process is through transcriptional regulation, protein stability, or post-translational modifications.
1.3. Cell-cell adhesion during Dorsal Closure

Besides actomyosin arrangements, proper cell-cell adhesion is also an important component of dorsal closure (Reviewed in Harden, 2002). The DME cells have to break their original adhesion with amnioserosa cells in order to migrate across the amnioserosa, and new connections have to be made to fuse with opposing DME cells as the two sides meet up at the dorsal midline (Bahri et al., 2010; Reviewed in Baum, Settleman, & Quinlan, 2008). This adhesion in *Drosophila* is mediated in part by cell-cell junctions called septate junctions, which incorporates the Scribble complex consisting of the proteins Scribble (Scrib), Discs large (Dlg), and Lethal giant larvae (Lgl) (Humbert et al., 2008). Studies in mammalian cell cultures have shown that Scrib is required at the migration front of cells in both wound healing and developmental epithelial fusions (Humbert, Dow, & Russell, 2006), where it recruits a wide range of molecules to the leading edge. These molecules include Dlg, the GTPases Cdc42 and Rac (Dow et al., 2007), their respective GEFs (Osmani, Vitale, Borg, & Etienne-Manneville, 2006), and the Cdc42/Rac effector kinase PAK (Nola et al., 2008).

1.3.1. Dlg is delocalized during dorsal closure

Dlg is a member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins, acting as a “connector joint” for multiple proteins with its multi-protein interaction domains (Reviewed in Funke, Dakoji, & Bredt, 2005). Known to be involved in cell polarity determination and septate junction integrity, Dlg is a strong candidate for dorsal closure regulation since dorsal closure also requires extensive cytoskeletal control and cell adhesion regulation. In the *Drosophila* embryo Dlg is present in the epithelial cells and the nervous system (Woods & Bryant, 1991). During early dorsal closure, when the DME cells starts to elongate, Dlg is lost from the leading edge and stays absent as dorsal closure progresses. This absence is consistent with the lack of other septate junction proteins at the leading edge (Bahri et al., 2010). As dorsal closure proceeds to completion, Dlg and the septate junction are reconstituted shortly after contact of the two opposing DME cells, facilitating “zippering” of the two DME cells (Bahri et al., 2010; Jacinto et al., 2000; Koh, Popova, Thomas, Griffith, & Budnik, 1999). Disruption in Dlg and Scrib complex restoration at the leading edge
results in failed dorsal closure (Bahri et al., 2010). These results highlights the importance of precise loss and reconstitution of Dlg at the leading edge for proper dorsal closure, but how this is achieved is not known.

1.3.2. Hts delocalizes Dlg during neuromuscular junction remodeling

From previous studies in mammalian cells, it was found that Adducin (Add), a three domain cytoskeletal protein, is involved in cell proliferation and adhesion. The myristoylated alanine-rich C Kinase substrate (MARCKS) domain on Add mediates membrane localization (Reviewed in Matsuoka, Li, & Bennett, 2000), and in Drosophila, the orthologue of Add is coded by the hu-li-tai-shao (hts) locus. Originally identified as a mutation in which females produce egg chambers with less than the normal number of nurse cells, hts is also expressed throughout Drosophila embryonic development in the embryonic central nervous system (Zaccai & Lipshitz, 1996). At the neuromuscular junction (NMJ) and in epithelia, Hts co-localizes and exist as a complex with Dlg (S. Wang et al., 2011). Over-expression of Hts leads to delocalization of Dlg from the NMJ (S. Wang et al., 2011). This disruption of Dlg localization by Hts in the NMJ shows similarity to previous studies where over-expression of two protein kinases, Partitioning-defective 1 (PAR-1) and Calcium/calmodulin-dependent protein kinase (CaMKII), results in delocalization of Dlg from the NMJ (Figure 1.5C) (Koh et al., 1999; Y. Zhang et al., 2007).

PAR-1 is a polarity determining protein which codes for a Serine/Threonine kinase, and is found in a variety of tissue types while also conserved across eukaryotes (Guo & Kemphues, 1995). Previous studies have shown that PAR-1 regulates phosphorylation of Serine797 on Dlg at the Drosophila NMJ, resulting in delocalization of Dlg from the synapse (Y. Zhang et al., 2007). CaMKII is also a serine/threonine kinase that is ubiquitously expressed and enriched in the CNS of many organisms (Silva, Paylor, Wehner, & Tonegawa, 1992). Similar to PAR-1, CaMKII also negatively regulates localization of Dlg to the postsynaptic membrane of NMJ in Drosophila, although it achieves that by phosphorylating Dlg at Serine48 (Koh et al., 1999). Hts appears to control Dlg localization, at least in part, by regulating the levels of these two kinases (S.
Wang et al., 2011). It is not known if CaMKII and PAR-1 have the same activity on Dlg during dorsal closure, but since the same signalling cassettes are often reused during development this is an interesting idea to test.

1.3.3. Possible routes via which Hts regulates CaMKII and PAR-1 levels

Although evidence points toward Hts regulation of Dlg by controlling the presence of PAR-1 and CaMKII, how this is achieved is not yet known. There are a few ways by which protein levels can be controlled. Firstly, transcription of mRNA of the protein could be altered, which will result in less protein being produced. It was known that human adducin can interact with nuclear proteins that in turn alter gene expression (Boito et al., 2005). Furthermore, adducin is also found to translocate into the nucleus when cell-cell junctions are lost (C. L. Chen, Lin, Lai, & Chen, 2011), where it may be able to interact with nuclear proteins to affect transcription. It is therefore possible that Hts in *Drosophila* has transcriptional control over CaMKII and PAR-1.

1.4. Aims of the study

The purpose of my research project was to identify new players in the process of dorsal closure and how they interact with existing pathways. More specifically, there are three major components: 1) Further characterization of Egfr function in dorsal closure including searching for target genes using a candidate gene and genetic modifier screen approach. 2) Determining if Fog and Pvf2 are ligands regulating dorsal closure. 3) Looking for evidence that Hts/CamKII/PAR-1 might be regulating Dlg during dorsal closure.

Dorsal closure is a good starting point for cell migration and epithelial fusion studies as many factors involved in the process are conserved between humans and flies. On top of that, dorsal closure is also of considerable medical interest as it provides a model for wound healing. Findings from these studies may provide insight to birth defects due to abnormal epithelial migration or fusion, such as cleft palate, spinal bifida and anencephaly. Furthermore, it may also aid the development of new treatment
methods or drugs to assist patients with increased healing burden or impaired healing abilities, such as burn victims and diabetics.
Chapter 2. Materials and Methods

2.1. Fly Stocks

UAS-Ack was made by previous graduate students in the Harden lab (Sem et al., 2002). UAS-tkv$^{Q199D}$ (TAJ3) was a gift from M. O’Connor. UAS-tkv$^{DN}$ was a gift from A. Garcia-Bellido. UAS-Fog$^{6}$, UAS-Fog$^{8}$ and UAS-Fog$^{12}$ were gifts from Eric F. Wieschaus. Egfr$^{1F26}$ and UAS-Egfr$^{4,4}$ were gifts from T. Schupbach. UAS-dpp-GFP was a gift from SM Cohen. Hs-Gal4$^{M4}$ was from J. Roote, UAS-sSpi was from BZ Shilo. UAS-PLC$\gamma$PH-GFP was a gift from Susan M. Parkhurst. All other stocks were obtained from the Drosophila Stock Center at Bloomington, Indiana.

2.2. cDNAs

cDNAs for dpp (RE20611), zip (LP037037), CaMKII (IP15240), par-1 (RE47050), ush (SD10668), cta (LD04530), pvl2 (RH40211), pvl3 (RE18107), fog (SD02223), and mmy (LD24639) were originally isolated by the Berkeley Drosophila Genome Project. cDNA clones were obtained from Drosophila Genomics Resource Center.

2.3. Antibodies

Mouse anti-phosphotyrosine (P-Tyr-100, #9411) was from Cell Signalling; rabbit anti-DAck was produced in the Harden lab (Sem et al., 2002); rabbit anti-PAK and rabbit anti-PAK3 were produced by Dr. Nicholas Harden; mouse anti-GFP (G6539) was from Sigma Aldrich; rabbit anti-phosphotyrosine (sc-18182) and goat anti-Egfr were from Santa Cruz Biotechnology; mouse anti-β-galactosidase (Z278A) was from Promega; rabbit anti CaMKII was a gift from Dr. Leslie Griffith; rat anti-Fog was generously provided by Dr. Eric F. Wieschaus.
2.4. Standard molecular techniques

Standard molecular techniques were performed according to Sambrook et al. (Sambrook, Fritsch, & Maniatis, 1989). cDNA clones were streaked on LB plates with appropriate antibiotics and incubated for 18~22 hours at 37°C. Single colonies from plates were picked and cultured individually in liquid culture for 18~22 hours at 37°C and plasmid was extracted using Qiagen Plasmid Mini Preparation Kit. Verification of purified cDNA clones was carried out by restriction digestion mapping. Restriction enzyme digestions and Polymerase Chain Reactions (PCR) were performed according to Sambrook et al. (Sambrook et al., 1989) and the manufacturer’s Instructions.

LB:

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<tr>
<th>10g</th>
<th>tryptone</th>
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<tr>
<td>5g</td>
<td>yeast extract</td>
</tr>
<tr>
<td>10g</td>
<td>NaCl</td>
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Top up to 1L with ddH₂O, autoclave. Can be stored at 4°C for 2 months.

2.5. Embryo Fixation

- **10x Phosphate Buffered Saline (PBS):**

  8 g NaCl
  0.2g KCl
  1.44g Na₂HPO₄
  0.24g KHPO₄

  Dissolved in 1 L de-ionized H₂O and adjusted to pH 7.4

- **20% Paraformaldehyde stock solution:**

  10 g paraformaldehyde (Anachemia UN-2213)
  35ml de-ionized distilled H₂O
  0.5ml 1 N NaOH

  Dissolved at 65°C in 50ml Falcon tubes, and then add
5ml 10X PBS
top up with ddH₂O to a final volume of 50ml, Can be stored at 4°C for 4 months

- **Washing solution:**
  0.01% Triton-X (Sigma Aldrich, T8787) in ddH₂O

- **Dechorionation solution:**
  50% disinfecting bleach (Final sodium hypochlorite concentration 3.08%) in 0.01% Triton-X

- **Fixing solution:**
  5ml heptane (Caledon Laboratories Ltd., 5400-1)
  1ml 20% paraformaldehyde
  4ml 1x PBS
  Mixed and made fresh for each collection

- **Storage solution:**
  100% methanol or 80% ethanol

   Flies were allowed to lay eggs on grape juice agar plates for 16~18 hrs and the eggs/embryos were washed off into a collecting basket using washing solution. Embryos in the basket were dechorionated for 5 minutes in dechorionation solution, washed three times for 3 minutes each in washing solution, then transferred into 10 ml fixing solution in 20ml scintillation vial. Embryos were agitated vigorously for 25 minutes at room temperature. After agitation, the bottom aqueous layer was discarded, and 10 ml storage solution was added to the remaining organic layer. The embryos were then manually agitated for 1 minute. After the embryos settled to the bottom of the vial, both the top organic layer and bottom aqueous layer were removed, and the embryos were washed with fresh storage solution before extraction and storage at -20°C.

### 2.6. Cuticle Preparations

- **Dechorionation solution:** See section 2.5
• **Washing Solution**: See section 2.5

• **Hoyer’s medium**:

<table>
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<tr>
<th></th>
<th>Gum Arabic</th>
<th>chloral hydrate</th>
<th>glycerol (Caledon, 5350-1-45)</th>
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<tr>
<td>30g</td>
<td></td>
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<td>200g</td>
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<td>16ml</td>
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Gum Arabic was dissolved in 50ml water, and then chloral hydrate and glycerol are added to the mixture while stirring. Mixture was centrifuged at 12,000g for 3 hours to remove impurities. Prepared medium was stored at room temperature in amber bottles.

After 16~20 hrs of egg laying agar plates were aged for at least 48 hours to ensure enough time for cuticle secretion. Embryos were collected, dechorionated, and washed as described for embryo collection. After washing, excess water was dabbed off on a paper towel and the dechorionated embryos together with larvae were mounted on slides in Hoyer’s medium. The slides were incubated at 65°C until the larvae and embryos were clear. This is a modified protocol from Ashburner (Ashburner, 1989). Prepared slides were stored at room temperature and viewed using phase contrast optics on a Nikon TMS inverted microscope.

Classification of wild-type and mutant embryos was in these categories: Normal cuticle, normal larvae, minor closure defects, major closure defects, and no cuticle/early stage lethal.

### 2.7. Digoxigenin-and Biotin-labelled RNA probe generation

Plasmids containing cDNA of gene of interest were linearized at the 5’ end using appropriate restriction enzymes to prevent transcription of vector DNA. Anti-sense RNA probes were synthesized by incubation at 37°C for 4 hours using appropriate polymerases, and appropriately labelled UTP. RNA synthesis was performed according to manufacturer’s instructions (Roche).Synthesized RNA probes were purified using MicroSpin S-200 HR columns (GE Healthcare, 27-5120-01). The purity of the probes was then checked using agarose gel electrophoresis, and quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific). Purified probes were stored at -80°C. The
only difference between production of digoxigenin- and Biotin- labelled probes was the labelled UTP used; digoxigenin-labelled UTP was used for digoxigenin-labelled probes, and biotin-labelled UTP was used for biotin-labelled probes.

2.8. **Fluorescent in-situ hybridization**

- **4% paraformaldehyde:**
  
  diluted fresh from 20% paraformaldehyde stock as described in section 2.5

- **10% Tween-20:**
  
  Diluted from 100% Tween-20 (Fisher Scientific, BP337-500)

- **10X PBS:** See section 2.5

- **Methanol:**
  
  100% methanol

- **1X PBTween:**
  
  1X PBS with 0.1% Tween-20
  
  diluted fresh from 10X PBS and 10% Tween-20 stock

- **3µg/ml Proteinase K:**
  
  Freshly diluted from 3mg/ml stock (Sigma, P6556)

- **2 mg/ml Glycine:**
  
  20mg Glycine (BioShop, GLN001.500)
  
  10ml 1X PBTween
  
  Stored at -20°C

- **50X Denhardt’s Reagent:**
  
  2.5g Ficoll
  
  2.5g Polyvinylpyrrolidone
  
  2.5g Bovine Serum Albumin (BioShop, Alb001.500)
Dissolve and top up to 250ml with water. Filter sterilize with 2.5um filter discs. Stored at -20°C

- **Hybridization buffer:**
  - 50% Formamide (Alfa Aesar, A11076)
  - 4X SSC (Sambrook et al., 1989)
  - 1X Denhardt’s Reagent (Sambrook et al., 1989)
  - 0.1% Tween-20
  - 5% Dextran sulphate
  - 250 µg/ml Salmon sperm DNA
  - 50 µg/ml Heparin

  Made and used within 2 months, stored at -20°C. For pre-boiled buffer, hybridization buffer was boiled for 5 minutes at 100°C, and then cooled on ice for at least 5 minutes.

- **RNA probe solution:**
  - 100ng RNA probe
  - 100µl Hybridization buffer

  Prepared fresh just before hybridization of embryos. Prepared solution was heated at 80°C for 3 minutes then cooled on ice for at least 5 minutes.

- **Anti-Dig antibody:**
  - 1:200 Anti-digoxigenin-POD (Roche, 11207733910)

- **3% Bovine Serum Albumin:**
  - 3g BSA (BioShop, ALB 001)
  - 100ml 1X PBTween

- **Cyanine 3 Tyramide:**
  - Cyanine 3 Tyramide (Perkin Elmer, SAT705A)

- **Mounting solution:**
  - Vectashield mounting medium (Vector Laboratories, H-1000)

Hybridization was performed on collected embryos using a protocol from Lecuyer et al. (Lécuyer, Parthasarathy, & Krause, 2008). Except when specified, all incubations
and washes were performed with rotation at room temperature. Fixed embryos were rinsed in methanol, a 1:1 mixture of methanol:PBTween, then twice in PBTween. Embryos were then post-fixed for 20 min in 4% paraformaldehyde before being washed three times in PBT for 2 min each. After that embryos were incubated in 3µg/ml proteinase K for 2 minutes then placed on ice for 1 hr. At the end of the incubation proteinase K was removed and digestion stopped by washing twice with 2mg/mL glycine for 2 minutes each. Glycine was removed by 2 washes of PBTween for 2 minutes each. Embryos were post-fixed again in 4% paraformaldehyde for 20 minutes, rinsed 5 times in PBTween for 2 minutes each, then rinsed in 1:1 PBTween:RNA hybridization buffer and 100% RNA hybridization buffer. The embryos were then pre-hybridized in pre-boiled RNA hybridization buffer for 2 hrs at 56°C. After incubation pre-hybridization buffer was removed and replaced by RNA probe solution, and then incubated at 56°C for 16-20 hrs. After incubation RNA probe solution was removed, and the embryos were rinsed once with pre-warmed 100% hybridization buffer at 56°C, then washed with pre-warmed 100% hybridization buffer, 3:1, 1:1, 1:3 hybridization buffer:PBTween mixture for 20 minutes each, all at 56°C. After that the embryos were washed 3 times with pre-warmed PBTween at 56°C for 20 minutes each, then once with pre-warmed PBTween at room temperature for 20 minutes. After washes the embryos were blocked by incubating with 3%BSA in PBTween for 10 minutes before incubation in anti-DIG-POD antibody and other required primary antibody for 2 hrs. The embryos were then washed 6 times with PBTween for 10 minutes each, then incubated with appropriate secondary antibody in 3% BSA in PBTween for 14-20 hrs at 4°C. For this step and all steps afterwards the tubes were shielded from light to reduce photobleaching. After secondary antibody incubation the embryos were washed in PBTween 6 times for 10 minutes each, followed by PBS washes 3 times at 5 minutes each. When the washes were completed embryos were incubated with 50µl of 1/50 Tyramide reagent in provided buffer for 2 hrs. After incubation the embryos were washed with PBS 6 times for 10 minutes each. All PBS was then removed, and 3 drops (around 100µl) Vectashield was added to each tube. The embryos were gently resuspended in Vectashield and stored in 4°C for at least 24 hrs before mounting on platform slides for imaging on a Nikon A1R confocal microscope. Images from the confocal microscope were merged stacks from sequential image slices.
2.9. Immunostaining of *Drosophila* embryos

**PBTriton:**

0.01% Triton-X in PBS

**1% BSA:**

3g Bovine Serum Albumin (BioShop, ALB001) Dissolved and topped up to 100ml with 1X PBTriton

All washes and incubations were performed at room temperature with rotation unless otherwise noted. Embryos were rehydrated by washing 3 times for 5 minutes each in PBTriton before blocking with 1% BSA for 1hr. After blocking embryos were incubated 16-20 hrs at 4°C with appropriated primary antibodies diluted in1% BSA, or for 2hr at room temperature. Then, the embryos were washed 3 times for 30 minutes each in PBTriton before incubation with secondary antibody for 2 hrs at room temperature. From this onwards all tubes are shielded from light to prevent photobleaching. After incubation with secondary antibody, the embryos were washed 3 times for 10 minutes each in PBTriton, then incubated in Vectashield for at least 24 hrs before mounting on platform slides for imaging on a Nikon A1R confocal microscope. For cases where tertiary antibody was used, the embryos were instead washed 3 times for 10 minutes each with PBS, and incubated for 2hrs in appropriated tertiary antibody diluted in PBS. After tertiary antibody incubation the embryos were washed 3 times for 10 minutes each in PBS, before incubation in Vectashield for at least 24 hrs and mounting on slides.

2.10. Mating and selection scheme for Egfr modifier screen

All crosses for the modifier screen were carried out in 25°C incubator. For the modifier screen the following crossing scheme was used:

**Parental Cross:**

Egfr^{1F26} / Cyo-t-GFP  X  Deficiency / Balancer

1/4 of the F1 progeny is expected to show no Cyo and no appropriate balancer phenotype and bear the following genotype:
Egfr^{1F26} / + ; Deficiency / +

Crossing the selected F₁ Progeny to Egfr^{1F26} / Cyo stock would result in 1/4 of the F₂ genotypes being homozygous Egfr^{1F26} mutants, and half of them (1/8 of total F₂) also heterozygous for the deficiency.
Chapter 3.  Results

3.1.  Egfr function during dorsal closure

3.1.1.  Ack leads to internalization of Egfr in the amnioserosa during dorsal closure

Previous results in the Harden lab suggested that the kinase Ack is involved in the regulation of dorsal closure through regulation of \textit{dpp} and \textit{zip} expression (Zahedi et al., 2008), although how this was achieved was not identified. As Ack is involved in the endocytosis and down-regulation of Egfr in other organisms (Grøvdal, Johannessen, Rødland, Madshus, & Stang, 2008; F. Shen, Lin, Gu, Childress, & Yang, 2007), it is reasonable to suspect that Ack performs similar functions in \textit{Drosophila}. Indeed, further investigations by the Harden lab identified that Egfr is required in the DME cells and amnioserosa for dorsal closure, and that Egfr represses \textit{dpp} and \textit{zip} expression and amnioserosa apoptosis, thus acting as a “brake” on dorsal closure by repressing the morphogenesis caused by actomyosin contractility and cell death (W. Shen et al., 2013). We speculated that Ack could promote dorsal closure by down-regulation of Egfr and looked for evidence of this in the large, flat cells of the amnioserosa. Embryos in which Ack was over-expressed in \textit{prd} stripes using \textit{prd-Gal4} were stained for Egfr and the early-endosome marker Rab5. As shown in Figure 3.1, Ack over-expressing cells (identified by a corresponding increase in pY (Sem et al., 2002)) showed a decrease of Egfr staining along cell outlines and a corresponding increase in vesicular Egfr staining in the cytoplasm, suggesting endocytosis of Egfr following Ack expression. Consistent with this, Ack expression caused an increase in the number of Egfr-positive vesicles that were also positive for Rab5, and we propose that Ack promotes, at least in part, dorsal closure by down-regulating Egfr activity.
Figure 3.1. Ack internalizes Egfr in the amnioserosa during dorsal closure
Figure shows amnioserosa cells and part of leading edge during dorsal closure. A: Cells that express Ack are identified by an increase in pY due to the kinase function of Ack. B: Egfr expression. Note a shift in Egfr localization from cortical to cytoplasmic in cells over-expressing Ack (refer to panel A). C: Rab5 staining showing early endosomes. There is an increase in number of early endosomes in the Ack over-expressing cells compared to WT cells. D: A larger number of Egfr puncta co-localize with Rab5 in cells over-expressing Ack (arrows) than in wild-type cells (arrow heads). E: Pathway figure summarizes how Ack may regulate dpp and zip through down-regulation of Egfr through endocytosis.
3.1.2. Egfr does not seem to repress dpp transcription in the epidermis by regulating mmy transcription

Egfr signalling represses dpp transcription in both the dorsal and ventrolateral stripes, indicating that it does not rely on the JNK pathway to regulate dpp, as the ventrolateral stripe of dpp expression is JNK-independent (W. Shen et al., 2013). A recent study identified the gene mmy as a JNK-independent repressor of dpp expression (Humphreys et al., 2013), and we wondered if Egfr might regulate dpp through mmy.

In wild-type embryos mmy is globally expressed in a low-level during dorsal closure with enrichment in the trachea. Gains or losses of Egfr function in prd stripes did not affect epidermal levels of mmy in prd stripes (Figure 3.2). Ectopic expression of the Egfr activating ligand sSpi, however, resulted in major disruption of tracheal mmy expression (Figure 3.3 A, B). This effect on tracheal mmy is unlikely to be relevant to dorsal closure, and in any case we would predict that Egfr should be promoting mmy expression during dorsal closure. We have not tested the possibility that Egfr could be regulating mmy post-transcriptionally, but to test this will require a Mmy antibody or tagged mmy transgene.

3.1.3. Modifier screen for Egfr interacting partners during dorsal closure

In order to identify other potential interacting partners for Egfr, we performed a deficiency screen by scoring cuticle phenotypes of homozygous Egfr mutants which also carry a certain deletion in the genome, and comparing the phenotype to that of the homozygous Egfr mutants. If the gene that is deleted interacts with Egfr, we expect to see a change in the cuticle phenotype compared to Egfr mutants without the deletion. To this end, a sensitized Egfr mutation which gives a mild phenotype is required for us to be able to see both rescuing and worsening effects. The temperature sensitive mutant, Egfr\textsuperscript{1F26}, was chosen for this purpose. When Egfr\textsuperscript{1F26} flies are raised at 18°C, majority of the embryos survive, and the cuticles collected show no obvious defects. In contrast, a significantly larger number of cuticles collected from 29°C cultures show large head and dorsal holes, and a majority of them failed development at the germband retraction stage, resulting in curled up, shrimp-like cuticles. Cuticles collected from 25°C cultures
displays an intermediate phenotype, with many cuticles displaying minor head or dorsal holes and a small number failing germband retraction. The phenotypes were scored using the following categories: No visible cuticles (Unfertilized), larvae or no observed cuticle defects (wild-type), small dorsal or head holes with little to no curling of embryo (Mild), or curled and shrunken cuticle or holes larger than 1/3 of the embryo (Severe) (Figure 3.4). “Unfertilized” category is not included for the phenotype ratio calculation. Mutant for the Dpp receptor Punt was used in place of the deficiency stocks as a positive control to confirm validity of the assay. Since not all of the collections produced enough embryos for analysis, different collections of the same crosses were pooled together. Even so, some collections do not yield enough cuticles and had to be left out from the figure. The cut-off for this screen is set at <10 total cuticles, and <50 total specimens (larvae + cuticle). Full data table is in the Appendix.

The 25°C Egfr^{1F26} control resulted in 20% of specimens showing defects, and 4% is of the “Severe” category. This defect ratio is close to the expected ratio of 1/4, the expected homozygous Egfr^{1F26} mutant ratio. The reason why this is not exactly 25% may be that some homozygous mutants may have successfully eclosed at this semi-permissive temperature. At 29°C the overall defect ratio stays the same, while almost all observed defects are of the “Severe” category. At 18°C almost all of the specimens observed are wild-type. Punt mutation in homozygous Egfr^{1F26} mutants caused an increase of the “Severe” phenotype compared to the 25°C background, from about 4% to 8%). Other deficiency crosses show varied effects on the phenotype as illustrated in Figure 3.5.

For an alternate visualization of the phenotype changes, the ratio of each defect category is compared to ratio of the corresponding category in the 25°C control (Appendix, Figure A2). Worsening of the genotype is visualized as an increase in “Severe” ratio to more than 1, as evident for the 29°C control and Punt cross. Crosses 1990, 2596, 2597, 6367, 7676, 7680, 7681, 7737, 7983, 8029, 8967, 9204, 9227, 24516, and 26580 also show similar increase in the “Severe” ratio, suggesting a worsening genetic interaction as well. A rescue effect is shown as a reduction in the overall defect ratio, as in the 18°C control. Crosses 3486, 3547, 7413, 7443, 7633, 7634, 7675, 8105, 8957, 8965, 9208, 9347, 25390, and 26529 demonstrate this effect. Crosses 7675,
9208 and 9347 will also be classified as rescuing, as the increase in “Mild” ratio is accompanied by a corresponding decrease in “Severe” ratio, suggesting an improvement of the phenotype. The crosses 1990, 7983, 9204, 9227, and 26529 are difficult to categorize due to the lack of major difference and lack of a calculated confidence interval.
mmy transcript levels do not appear to be affected by changes in Egfr signalling

![A](UAS-EGFR-GFP / Cyo-t-GFP) ![A'](mmy)  

mmy FISH in embryos expressing Egfr-GFP, dominant negative form of Egfr (Egfr^{DN}), or activated Egfr (Egfr^{Alop4.4}). Embryos bearing the Cyo-t-GFP chromosome from the Prd/Cyo-t-GFP x Egfr^{Alop4.4} cross were used as the control embryo to show wild-type mmy expression. A: GFP stain demonstrating presence of Cyo-t-GFP chromosome. A': Same embryo as in A showing wild-type mmy expression. B: GFP signals showing ectopic Egfr expression pattern in Prd / UAS-Egfr-GFP embryos. B': mmy expression in the same embryo as in B. C, D: mmy expression in embryos expressing either Egfr^{Alop4.4} or Egfr^{DN} in prd stripes.
Figure 3.3. Ectopic sSpi disrupts mmy expression in the tracheal region

Embryos expressing sSpi in paired stripes show major disruption in tracheal mmy expression (arrowheads). A-A': Embryos expressing sSpi in paired stripes. B-B'': Higher magnification of the embryo in A. Note the irregularity in epidermal segment arrangement common in embryos expressing sSpi.
Figure 3.4. Cuticle phenotype classification scheme
Classification scheme used to assign cuticle phenotype for the deficiency screen. A&B: wild-type cuticles. Note the properly formed mouth hooks, fully extended body, and neatly arranged denticle belts. C&D: Mild defect. Note the deformed mouth in C and small head hole in D. A small degree of curling is present in C. E&F: Severe defect. Note major shrinkage of cuticles indicative of major loss of embryonic material. A large hole in the dorsal-head area is present in F. G&H Severe defect. These embryos are similar to the mild defect cuticles except the additional closure defects present the ventral and posterior sides in G, and the larger sized dorsal-head hole in H. A hole 1/3 the size of the embryo or larger is used as the cut-off for "severe" classification.
Figure 3.5. Cuticle phenotypes analysis graphs

% cuticle defect to total specimen

Graph showing percentage of cuticle phenotypes of the controls and deficiency crosses. Empty cuticles are not included in calculation. Crosses with less than 10 cuticles or less than 50 total specimens are not included in the graph. Refer to Appendix A1 for full data table. Y axis represent Percentage of phenotypes in relation to total number of cuticles. X axis represent control or Deficiency line used. Numbers inside bracket indicate total number of specimens (cuticle plus larvae) counted. Punt cross was used as a positive control. Note that for clarity wild-type section for each bar is not shown, but will make up rest of the percentage bar.
3.2. Characterization of Pvf2, a possible ligand activating the JNK pathway during dorsal closure

As mentioned in the introduction, an activated form of Pvr was found to cause elevated expression of zip and dpp during dorsal closure, suggesting that it could be a JNK pathway activator, and we were interested in identifying potential ligands for Pvr during dorsal closure (Ishimaru et al., 2004; Macias et al., 2004). Three ligands for Pvr have been identified, Pvf1-3, and interestingly, one of these, Pvf2, is expressed by a subset of DME cells, suggesting that this could be a JNK-activating ligand during dorsal closure (Berkeley Drosophila Genome Project: (Hammonds et al., 2013; Tomancak et al., 2002; Tomancak et al., 2007) (Figure 3.6 A’). To determine if ectopic expression of Pvf2 could activate the JNK pathway, dpp FISH was performed on embryos expressing Pvf2 in prd stripes. From the data obtained, there was no obvious change in dpp expression level or pattern between the control and Pvf2-expressing embryos, and no difference between adjacent segments in Pvf2-expressing embryos (Figure 3.6). We conclude that over-expression of Pvf2 alone does not activate the JNK cascade. This does not necessarily mean that it is not contributing to JNK activation during dorsal closure as it may be cooperating with additional inputs.

Pvf2 is also of interest as it represents a new gene showing specific upregulation in the DME cells. The expression of Pvf2 in a subset of DME cells is reminiscent of JNK-independent dpp expression in the dorsal epidermis before dorsal closure and we wondered if Pvf2 could be a target for the Dpp pathway. To test this idea, we looked at pvf2 transcription in embryos in which the Dpp pathway was either activated or impaired in prd stripes using a Dpp-GFP transgene, an activated form of the Dpp receptor Tkv, TkvTAJ3, or a dominant negative form, TkvDN. From the images obtained, there were no obvious differences in Pvf2 expression pattern between internal control and dpp over-expressing embryos, and no differences between neighbouring segments (Figure 3.7). No difference was observed either for embryos expressing TkvDN or TkvTAJ3 (Figure 3.8).
Figure 3.6. Pvf2 over-expression does not affect dpp expression

*dpp* FISH. Presence of GFP in the embryo in A indicates no ectopic expression of Pvf2, and was used as internal control. A-A‴: Cyo-t-GFP bearing embryo used as internal control. B-B‴: Embryo expressing Pvf2 in paired stripes.
Figure 3.7.  *dpp* expression does not affect *Pvf2* levels during dorsal closure

*pvf2* FISH in wild-type and *dpp* over-expressing embryos.  A-A": *pvf2* transcription profile in wild-type embryo.  B-B": non UAS-Dpp-GFP expressing embryo used as internal control for C.  C-C": embryos expressing UAS-Dpp-GFP in paired stripes.
Figure 3.8.  Gains or losses of Tkv function do not affect expression of Pvf2 during dorsal closure

Pvf2 FISH of embryos expressing constitutively active (Tkv\textsuperscript{TAJ3}) or dominant negative (Tkv\textsuperscript{DN}) forms of the Dpp receptor Tkv.  A-A’: Cyo-t-GFP bearing embryo acting as internal negative control for B and C.  B-B’: embryo expressing Tkv\textsuperscript{TAJ3}.  C-C’: embryo expressing Tkv\textsuperscript{DN} in paired stripes.  For both of these embryos there is no obvious change in Pvf2 expression.
3.3. Fog is a regulator of dpp and zip

While many of the signaling pathways operating during dorsal closure are known, we still do not know what the major upstream activating signals are. As described in the Introduction, two pathways that are major regulators of actomyosin contractility during dorsal closure are the JNK cascade, which regulates myosin transcription from zip, and the Rho pathway regulating myosin activation (Fernandez et al., 2007; Reviewed in Harden, 2002; Khoo et al., 2013). The Rho pathway activating myosin has best been described in gastrulation, where it lies downstream of the secreted ligand Fog, the Gα subunit Concertina, and RhoGEF2 (Barrett et al., 1997; Reviewed in Dawes-Hoang et al., 2005; Nikolaidou & Barrett, 2004). Recent results have demonstrated the participation of RhoGEF2 in amnioserosa morphogenesis (Baek, Kwon, Lee, & Choe, 2010; L. Zhang & Ward, 2011), raising the possibility that the pathway activating cell shape change in gastrulation might be acting in dorsal closure. As JNK has been shown to be activated by Gα12/13 proteins (Dermott, Ha, Lee, & Dhanasekaran, 2004; Jho, Davis, & Malbon, 1997) it is possible that this pathway could be used to activate both Rho1 and JNK signalling during dorsal closure. I used FISH and anti-Fog antibody to see if this ligand was expressed during dorsal closure, and found that strikingly Fog is expressed in both the amnioserosa and DME cells (Figure 3.9).

3.3.1. Fog over-expression in the epidermis represses dpp and zip expression

To determine if Fog might be activating the JNK pathway during dorsal closure, ectopic Fog was expressed in prd stripes and dpp and zip transcript levels were evaluated by FISH. Transcript levels of dpp and zip in embryos expressing ectopic Fog were markedly decreased when compared to control embryos (Figure 3.10, Figure 3.11). These effects were non-cell autonomous, which is perhaps not surprising given the diffusible nature of Fog. As seen in Figure 3.10B, the Fog expressing stripes were not very distinguishable from non-expressing stripes, and the Fog stain seemed to spread to the other segments. Amnioserosa-specific expression of Fog, on the other hand, did not alter the levels of dpp or zip noticeably (Figure 3.11, Figure 3.12). In contrast, RNAi knockdown of fog in either prd stripes or the amnioserosa resulted in an increase in dpp
transcript level (Figure 3.13), opposite to what was seen with over-expression phenotypes.

These results indicate that Fog is not an activating signal for the JNK cascade during dorsal closure, but may in fact be a negative regulator of gene expression during dorsal closure. In this regard it is similar to Egfr, and future work should address possible interactions between Fog and Egfr. In preliminary results, gains or losses of Egfr in the amnioserosa did not affect Fog expression in this tissue (Figure 3.14). We have not addressed effects of Fog on Rho1 signaling in detail, but this should be worthwhile, given Fog’s enrichment in the amnioserosa and DME cells.
Figure 3.9. *fog* is expressed at the leading edge and amnioserosa from germband retraction stage through dorsal closure.

*fog* FISH and Fog antibody stain of wild-type embryos. Note the relative high level of *fog* transcript and Fog protein at the leading edge and amnioserosa. A-C: *fog* FISH. A: Germband retraction stage (stage 11) embryo. B: Early dorsal closure (stage 13) embryo. C: Mid dorsal closure stage (stage 14) embryo. D: Fog antibody stain of wild-type embryos during early dorsal closure (stage 13). Arrows indicate expression in the amnioserosa; arrowheads indicate expression along the leading edge.
Figure 3.10. Expression of Fog in alternate segments globally decreases *dpp* expression

A-A'': Embryo not expressing ectopic Fog. This is used as an internal control to B. B-B'': Embryo expressing ectopic Fog in paired stripes. Note the diffuse Fog signal (arrowheads) extending beyond the *prd* stripes (arrow). The level of *dpp* expression in B' is greatly reduced globally compared to A'.
Figure 3.11. Amnioserosa specific expression of Fog has no effect on zip expression, but prd expression of Fog seems to reduce zip transcription.

This figure shows the effect of ectopically expressed Fog on zip expression along the leading edge during dorsal closure. A-A” show embryos expressing Fog in the amnioserosa; B-B” show embryo expressing Fog in paired stripes. Note the diffusion of Fog through the embryo and knockdown of zip along the whole leading edge.
**Figure 3.12. Amnioserosa over-expression of Fog has no effect on dpp expression**

*dpp* FISH in embryos over-expressing UAS-Fog using amnioserosa specific c381-Gal4 driver. UAS-Fog*6* and UAS-Fog*12* transgenes used in crosses were heterozygous over the Cyo-t-GFP balancer. c381-Gal4 driver used in cross was homozygous. A-A“ and C-C“: Embryos not expressing ectopic Fog, used as internal control for embryos over expressing Fog*6* (B-B“) and Fog*12* (D-D“) respectively. B-B“: Embryos over-expressing Fog*6* in the amnioserosa. D-D“: Embryos over-expressing Fog*12* in the amnioserosa.
Expression of Fog RNAi in the amnioserosa causes ectopic expression of dpp. A: dpp expression pattern of stage 13 wild-type embryo. Note the two lateral lines of expression along the leading edge on the dorsal side and along the ventral epidermis. No other dpp expression is observed in the epidermis except for patched expression around the midgut and posterior. B: Stage 12 Embryo expressing FogRNAi using amnioserosa specific driver. Note ectopic dpp expression present in between the dorsal and ventral stripes along the embryo. C: Wild-type stage 11 embryo probed for dpp expression. Note the refined expression along the leading edge and band of expression along the ventral epidermis. No other dpp expression is present in the epidermis. D-F: dpp expression in stage 11 embryos expressing FogRNAi in prd stripes. Note increased dpp in the epidermis.
Figure 3.14. Amnioserosa-specific changes in Egfr signalling do not affect Fog level during dorsal closure

Fog antibody stain of wild-type embryos and embryos expressing Egfr-GFP, dominant negative form of Egfr (Egfr<sup>DN</sup>), or constitutively active Egfr (Egfr<sup>λtop4.4</sup>). A-A': wild-type. B-B'', C-C'', D-D'': embryos expressing Egfr-GFP, Egfr<sup>λtop4.4</sup>, or Egfr<sup>DN</sup> in the amnioserosa, respectively.
3.4. Regulation of Dlg during dorsal closure

3.4.1. CaMKII is expressed in the amnioserosa cells bordering the leading edge during early dorsal closure

As discussed in the Introduction, we are interested in determining if Dlg regulation at the leading edge of the DME cells is similar to Dlg regulation in the larval NMJ. In both systems Dlg needs to be removed from cell-cell junctions to enable plasticity during development (S. Wang et al., 2011). In the case of dorsal closure, Dlg and other septate junction proteins need to be removed from the leading edge at the beginning of dorsal closure in order to break adhesion with the neighboring amnioserosa (Bahri et al., 2010; Perrimon, 1988). In the case of the NMJ, Dlg needs to be removed from the NMJ to allow synapse expansion during larval growth (S. Wang et al., 2011). This Dlg removal from the muscle membrane is achieved, at least in part, by Dlg phosphorylation by the CaMKII and PAR-1 kinases (Koh et al., 1999; Y. Zhang et al., 2007). CamKII distribution during dorsal closure was examined by anti-CamKII antibody staining of embryos. Strikingly, at the beginning of dorsal closure, CaMKII was expressed at high levels in the peripheral amnioserosa cells, which are the amnioserosa cells in contact with DME cells (Figure 3.15A’). There also appeared to be some accumulation of CaMKII at the leading edge of the DME cells (Figure 3.16). These results support the hypothesis that CaMKII is involved in the process of dorsal closure, and might function to remove Dlg from junctions between the amnioserosa and DME cells. It is interesting to note that as dorsal closure progresses, CaMKII distribution becomes more cortical in the amnioserosa cells (Figure 3.17), which raises the possibility of down-regulation of adhesion between amnioserosa cells, which are known to delaminate to facilitate dorsal closure (Muliyl, Krishnakumar, & Narasimha, 2011). In contrast, PAR-1 showed a uniform distribution during dorsal closure (S. Wang, unpublished data), suggesting some differences in how PAR-1 and CaMKII might function in dorsal closure.
Figure 3.15. CaMKII is expressed at periphery of the amnioserosa and at the leading edge during early dorsal closure.

Stage 13 wild-type embryo showing CaMKII antibody staining around the first row (peripheral) amnioserosa cells and at the leading edge. A: pY stain marking outer edge of cells. A': CaMKII antibody stain. A'': Merge of A and A'.
Figure 3.16. CaMKII localization at the amnioserosa and leading edge changes as dorsal closure proceeds

CaMKII antibody stain of wild-type embryos undergoing dorsal closure. A-A’: Early germband retraction stage (stage 11) embryo. Note the high level of CaMKII in the amnioserosa cells, with possible leading edge accumulation and patchy CaMKII throughout the epidermis. B-B’: Early dorsal closure stage (stage 13) wild-type embryo. Note the accumulation of CaMKII at the leading edge and periphery of amnioserosa. C-C’: Mid dorsal closure (stage 14) wild-type embryo. Note the increase in cortical CaMKII in the amnioserosa cells.
Figure 3.17. CaMKII accumulation in the amnioserosa cells changes during dorsal closure

High magnification images of CaMKII protein localization during dorsal closure. A-A': high magnification image of a section of early dorsal closure (stage 13) embryo section from Figure 3.16 B. B: high magnification image of a section of mid dorsal closure (stage 14) embryo section from Figure 3.16 C. Note the increase in cortical amnioserosa cell CaMKII levels as dorsal closure proceeds.
3.4.2. **Hts does not appear to affect Dlg phosphorylation during dorsal closure through transcriptional control of CaMKII or PAR-1**

Previous work in the Harden lab demonstrated that Hts, *Drosophila* adducin, could regulate membrane localization of Dlg in both the NMJ and amnioserosa cells by controlling the levels of the CaMKII and PAR-1 kinases. Preliminary results from the NMJ suggested that Hts might affect CaMKII and PAR-1 at the transcript level (S. Wang et al., 2011). I performed FISH for *CaMKII* and *PAR-1* in embryos over-expressing Hts in paired stripes, and the results showed no obvious difference in *CaMKII* (Figure 3.18) or *PAR-1* (Figure 3.19) transcript levels between Hts over-expressing and non-over-expressing stripes. This would suggest regulation of CaMKII and PAR-1 on a different level than transcription quantity.

3.4.3. **Over-expression of Fog in prd stripes causes a corresponding increase in CaMKII protein**

During *Drosophila* development, Rho1 regulates the cell adhesion regulator Hts through Rho kinase (ROK) (Verdier, Guang Chao, & Settleman, 2006). This phosphorylation event stabilizes Hts’s association with the plasma membrane, resulting in persistence of Hts on the cell membrane and continued Hts activity. Elevated presence and activity of Hts had been implicated to enhance presence of CaMKII and PAR1, which phosphorylates the cell adhesion molecule Dlg (S. Wang et al., 2011). Phosphorylation of Dlg detaches it from the plasma membrane, which compromises the Scrib adhesion complex, resulting in loss of cell adhesion. This loss of cell adhesion might facilitate movement of DME cells over the amnioserosa during dorsal closure. It is therefore interesting to test if Fog has an effect on Hts mediated Dlg removal, as Fog is upstream of Rho1 and ROK during gastrulation. Fog over-expression in prd stripes appears to cause a modest increase in CaMKII levels (Figure 3.20), suggesting that it might tie into Dlg regulation.
Figure 3.18. Hts does not affect transcript levels of CaMKII during dorsal closure

Embryos expressing Hts in paired stripes. A & B: Hts protein antibody stain showing Hts protein levels. A’ & B’: CaMKII FISH showing transcript levels of CaMKII. No change in CaMKII transcript levels (A’ & B’) is observed between wild-type segments and segments over-expressing Hts.
Figure 3.19. Hts does not affect transcript levels of PAR-1 during dorsal closure

PAR-1 FISH of embryos expressing Hts in paired stripes. A & B: Hts protein antibody stain showing Hts protein levels. A’ & B’: PAR-1 FISH showing transcript levels of PAR-1. No change in PAR-1 transcript level (A’ & B’) is observed between wild-type segments and segments over-expressing Hts.
Figure 3.20. Fog expression in prd stripes results in corresponding elevation of CaMKII expression.

Embryos over-expressing Fog in prd stripes co-stained for Fog and CaMKII proteins. A, B, C: Fog antibody stain. A’, B’, C’: CaMKII antibody stain of corresponding embryos. Note the corresponding increase in CaMKII staining along segments where Fog is being over-expressed.
Chapter 4. Discussion

4.1. Ack controls dorsal closure through down regulation of Egfr by internalization

Our results suggest that Ack increases the internalization of Egfr, as is evident from the reduction in cortical staining and increase in cytoplasmic Egfr puncta in Ack over-expressing cells. This is apparently through endocytosis of Egfr, as there is an increase in co-localization of cytoplasmic Egfr puncta with Rab5. Aside from that, the overall increase in Rab5 signal also suggests that Ack over-expression leads to a general increase in intracellular vesicular trafficking. It is interesting to note that many of the cytoplasmic Egfr puncta do not correlate with Rab5 signals, suggesting that they are not in early endosomes. It is likely that this Egfr is in multivesicular bodies, as activated Egfr undergoes sorting and processing in multivesicular bodies prior to degradation (Eden, White, & Futter, 2009).

It is not known however, if Ack specifically targets Egfr, or if Egfr is non-specifically internalized due to the increased rate of endocytosis. To answer this, the experiment needs to be repeated to see if other receptors expressed in the amnioserosa are internalized when Ack is over-expressed. It may also be possible to perform dextran uptake assays (Entchev, Schwabedissen, & González-Gaitán, 2000) on embryos to see if Ack over-expression has a non-specific affect on endocytosis.

One other limitation of my experiment is that only the early endosome marker was used, so the final fate of internalized Egfr was not confirmed. Endocytosis is known to perform a multitude of regulatory roles during morphogenesis such as promoting cell shape change (Mateus, Gorfinkiel, Schamberg, & Martinez Arias, 2011), modulating focal adhesion components, and modulating cell surface receptors (Reviewed in P. Caswell & Norman, 2008). Endocytosis not only performs inhibitory functions but can
also promote signalling by concentrating receptors on a specific region of the cell membrane for polarized signalling and assists directed migration during morphogenesis. This positive regulation of internalized receptors is mediated by targeted recycling, as shown by findings that the level of the recycling endosomal GTPase Rab11 positively correlates with invasiveness of cancer cells (Cheng et al., 2004). Mutations in Rab11 also greatly reduce cell migration and invasion capabilities in a 3D matrix (Reviewed in P. Caswell & Norman, 2008; P. T. Caswell et al., 2007). In *Drosophila*, the directional migration of border cells during oogenesis is also mediated in part by an endo-exocytic cycle of Egfr, where Egfr is first internalized then re-targeted to the plasma membrane at the migration front (Assaker, Ramel, Wculek, Gonzalez-Gaitan, & Emery, 2010). This prevents dilution of receptors due to lateral diffusion, and concentrates receptors to the migration front (Reviewed in Jones, Caswell, & Norman, 2006). Alternatively, endocytosis can selectively maintain association between receptors and downstream signalling molecules, leading to prolonged receptor signalling and/or selective pathway activity (Reviewed in P. Caswell & Norman, 2008). It had also been shown that internalized inactive Egfr can persist in endosomes (Lai, Cameron, Doherty, Posner, & Bergeron, 1989; Y. Wang, Pennock, Chen, & Wang, 2002).

These greatly different fates of endocytosed receptors make it important to identify if the endocytosed Egfr is degraded or recycled. Although it is true that membrane staining of Egfr is reduced in Ack over-expressed cells, it is not clear if this is due to permanent removal of Egfr from the membrane, or increased turn-over leaving little static Egfr on the membrane. It is also not clear if Egfr signalling is maintained in the endosomes or if they are degraded soon after. To elucidate this, other Rab proteins, such as the recycling endosome marker Rab11, or the degradation marker Rab7, can be co-stained with Egfr in Ack over-expressing cells. This should clarify the fate of the endocytosed Egfr. Alternatively, quantification of Egfr may be used to identify if the level of Egfr is generally lower in Ack over-expressing cells, which will also support Ack mediated degradation of Egfr.

In any case, since previous results suggest that pMAPK is reduced along the peripheral amnioserosa (W. Shen et al., 2013) it is most likely that in this scenario
internalization of Egfr does indeed result in down-regulation of Egfr signalling. The previously mentioned experiments should further confirm the validity of this hypothesis.

4.2. *mmy* is regulated by sSpi in the trachea

Egfr regulation of dorsal closure is not dependent upon the well known JNK-pathway as over-expression of Egfr in the epidermis is also capable of down-regulating the JNK independent, ventral-lateral stripe of *dpp* expression (W. Shen et al., 2013). How Egfr regulates *dpp* expression during dorsal closure remains unclear, although this does not seem to proceed through transcriptional regulation of *mmy*. The results obtained show no changes in *mmy* expression between segments expressing constitutively-active or dominant-negative Egfr compared to wild-type segments. It was however surprising to find that, in contrast, over-expression of the Egfr ligand sSpi had a negative impact on *mmy* transcription in the trachea. This may be due to sSpi activating other receptors than Egfr, or the ligand being more effective at turning on the pathway than over-expressing receptors.

In any case, the effect of sSpi signalling on *mmy* expression was observed in the trachea, and may not be relevant to dorsal closure. In the study I did not observe significant *mmy* expression at the leading edge or other parts of the epidermis. In order to determine if Mmy protein is present in the epidermis a good Mmy antibody would be required. Further projects to pursue possible contributions of *mmy* during dorsal closure will investigate post-transcriptional controls of *mmy*, but a good *mmy* antibody or *mmy*-tagged transgene will be necessary.

More recent studies have now suggested Egfr inhibition of signalling by the steroid hormone ecdysone as the method of controlling *dpp* and *zip* expression (X. Chen, 2014), making ecdysone the preferred candidate for future studies in Egfr regulation of dorsal closure over *mmy*. 


4.3. New candidate Egfr interactors from the deficiency screen

From the screen, 15 lines had been classified as being worsening, and 14 lines had been classified as rescuing for Egfr<sup>F26</sup> mutation. The next step will first involve confirmation of the result and identifying deleted genes using the deficiency database. Flanking deficiencies will be used to narrow down the candidate genes. It is interesting that known the endocytosis related Rab7 and Rab23 genes are deleted in the identified rescuers for Egfr mutant. The deficiency screen system employed has the advantage of being relatively fast, but at the same time harbours some inherent flaws and disadvantages. With the way the screen was setup, it is expected that 1/4 of the specimens collected will be homozygous mutant for Egfr<sup>F26</sup>, and at most only half of these, or 1/8 of the total specimens, will be carrying the deficiency at the same time. Therefore, the most drastic effect we expected to see was a 12.5% change in cuticle phenotype. However, as the graph in Figure 3.5 shows, many crosses show drastic changes of more than 12.5%. This may be due to the sensitivity of this assay to genetic background in this setup, where background mutations in the deficiency lines have effects on dorsal closure as well. To remedy this, it will be necessary to correct the collected data with cuticles collected from the deficiency lines alone.

Another issue about the screen setup is that since only 1/8 of the progeny are of the proper genotype, it is important to obtain a large sample pool to minimize statistical errors. Because of this, some crosses require pooling of sample counts from separate collections, making it difficult to calculate the confidence interval. Without the confidence interval it is difficult to tell if an observed effect is true. The small sample size also necessitates that the embryos be given only broad classifications, with no differentiation between refined characteristics such as dorsal hole versus head/ventral hole, and puckering of the leading edge. This makes it difficult to distinguish between dorsal closure-related and other morphogenesis defects. Although more detailed classification will be more informative, and had been attempted during the early stage of the screen, it greatly reduced the sample size of each category, and was also not realistic to perform in the timeframe available.
Lastly, embryos with no noticeable cuticles were classified as unfertilized and not included in ratio calculation, when they may in fact be early stage lethals, failing development prior to formation of cuticle. This may cause underestimation of potential strong genetic interactions.

To remedy these issues, the experimental scheme will need to be altered so that the embryos are laid and incubated in permissive temperature until just prior to dorsal closure stage, and then moved to semi-permissive temperature until ready for cuticle collection. Also, multiple collections will need to be performed for each cross in order to calculate standard error for more accurate statistical estimation. Although worth pursuing in the future, this was not realistic to perform within the timeframe and resources available for the study.

Even with these shortfalls, the deficiency screen nevertheless provided starting points for future studies into Egfr regulation of dorsal closure. The screen should be improved with larger, multiple sample collections and a temperature shift protocol to better serve as system for more detailed screens in the future, addressing Egfr’s role in dorsal closure.

4.4. Fog negatively regulates dpp and zip during dorsal closure, but is likely not downstream of Egfr

As a candidate that may affect multiple regulatory pathways during dorsal closure, Fog was found to negatively regulate dpp and zip when expressed in the epidermis. It is interesting to note that the same negative effect on dpp and zip is not observed when Fog is over-expressed in the amnioserosa using the c381 driver. It is possible that it is not merely the presence, but the Fog gradient difference between tissues that drives the regulatory process. As observed in Figure 3.9, wild-type Fog expression during dorsal closure is concentrated at the peripheral amnioserosa cells, with the highest level observed at the leading edge. Fog level diminishes gradually as one move dorsally in the amnioserosa. In contrast, if one crosses the leading edge ventrally from the amnioserosa to the DME cells, there is a sharper decrease in Fog protein level, resulting in a noticeable boundary. Such gradient differences had been known to play a role in
polarization of cell structure, such as in dpp regulation of wing morphogenesis, where
the combination of receptor and ligand gradient results in complex regulation of organ
formation (Reviewed in Hamaratoglu et al., 2014; Reviewed in Schwank & Basler, 2010).

It is also likely that the difference is due to the natural level of Fog protein in the
embryo. There is a large amount of Fog protein in the amnioserosa in wild-type
embryos (Figure 3.9D), so it may be speculated that in wild-type embryos Fog is
saturated in the amnioserosa. Additional Fog will then not make a noticeable difference
in signalling. Knockdown of Fog, on the other hand, will alter the normal level of Fog
and results in observable effect. Additionally, alteration of Fog has a stronger effect in
the epidermis than amnioserosa. This suggests that the proximity of Fog, a diffusible
ligand, may also have a role in the observed effects, due to the amnioserosa Fog
needing to diffuse into the epidermis, while Fog being expressed in the epidermis can
function in situ.

It is interesting that Egfr is also known to negatively regulate dpp expression in a
JNK independent fashion (W. Shen et al., 2013). The similarity to Egfr naturally directs
our attention to the possibility that Fog may be the missing diffusible link mediating Egfr
down regulation of zip during dorsal closure. However, this does not appear to be the
case, as none of the GFP-tagged functional (Egfr-GFP), dominant negative (EgfrDN), or
constitutively active forms of Egfr (Egfr\textsuperscript{\textit{A}\textit{lop4.4}}) changed the normal
distribution of Fog. This suggests that Fog is not downstream of Egfr, but does not rule out the possibility
that Fog may be acting parallel, or even upstream of Egfr. Indeed, there is evidence
indicating that Egfr can be transactivated by GPCR ligands. This is achieved by GPCR-
mediated phospho-activation of metalloproteinases, which then cleave and release
membrane-tethered EGF proligand (Daub, Weiss, Wallasch, & Ullrich, 1996; Pierce et
al., 2001; Reviewed in Wetzker & Bohmer, 2003).

Besides metalloproteinase-mediated activation, an alternative pathway for Egfr
activation by Fog is through the Rho1 pathway. Studies have shown that RhoGEF2
associates with microtubules and travels close to the cell surface, where activated
concertina recruits and converts RhoGEF2 to the membrane bound, GTP-associated
active form (Rogers, Wiedemann, Hacker, Turck, & Vale, 2004). Activated RhoGEF2
subsequently activates Rho1-ROK pathway. *Drosophila* ROK, the downstream effector of Rho, promotes phosphorylation of *Drosophila* myosin regulatory light chain, Spaghetti squash (Sqh). This is achieved by both direct phosphorylation of Sqh and inhibition of myosin phosphatases, resulting in stabilization of myosin structures (Fox & Peifer, 2007; Rogers et al., 2004). ROK-mediated phosphorylation of MRLC is required in many cell contraction mechanisms, such as the phasic constriction of smooth muscles (Aguilar, Tracey, Zielnik, & Mitchell, 2012; Winter et al., 2001) and the similar process of apical constriction, highlighting its importance in dorsal closure. Recent findings have also suggested the importance of not just phosphorylation itself, but the cycle of phosphorylation/dephosphorylation of myosin to be important for proper apical constriction (Lecuit, Lenne, & Munro, 2011), as in the action of a ratchet. Furthermore, pMRLC can also activate Egfr (Kim, Wang, Conti, & Adelstein, 2012), which may in turn cause the observed negative regulation of *dpp* and *zip*. ROK mediated myosin phosphorylation had also been implicated to be necessary for disruption of nuclear integrity during apoptosis in fibroblast cells (Croft et al., 2005). Since apoptosis of the amnioserosa is also an important part of dorsal closure, this further supports the importance of Fog during dorsal closure.

The Fog results suggest the importance of Rho1-ROK pathway during dorsal closure in terms of cytoskeletal contractility. ROK, and important regulator of cellular processes, is also known to phosphorylate Add leading to actin mesh recruitment, and interacts with *Drosophila* Add, Hts, during morphogenesis (Fukata et al., 1999; Verdier et al., 2006). ROK catalyzes the second phosphorylation at Threonine 445, which promotes Add binding to F-actin and stabilize Add to the plasma membrane (Kimura et al., 1998). In *Drosophila*, the Add homolog Hts is known to delocalize Dlg by regulating its phosphorylation by CaMKII and PAR1 (S. Wang et al., 2011), which destabilizes septate junction and cell-cell adhesion. It is possible that Fog, acting through Rho1 and ROK, activates Hts at the leading edge to cause detachment between DME cells and amnioserosa cells, thereby facilitating epithelial migration. In support of this, CaMKII levels are found to be elevated by ectopic Fog expression. This is does not provide direct proof of activity through Hts however. Further experiments investigating Fog’s relationship to Hts and Dlg localization is necessary to confirm Fog’s contribution in regulation of cell adhesion. This is especially interesting since that embryos mutant for
the adherens junction molecule E-cadherin demonstrate reduced, but not abolished wound healing capability, suggesting a role for other cell adhesion components such as scrib complex in dorsal closure (Reviewed in Abreu-Blanco, Watts, Verboon, & Parkhurst, 2012). If the Fog-Dlg relation is proven true, Fog can be established as a common upstream regulator of two major regulatory forces involved in dorsal closure (Figure 4.1).

4.5. Hts does not regulate CaMKII and PAR-1 by transcription level control in the embryo

From study of the neuromuscular junction in *Drosophila*, it is known that Hts positively regulates the level of the kinases CaMKII and PAR-1 to phosphorylate Dlg. The same was observed at the leading edge during dorsal closure, suggesting conserved functions during these two processes (S. Wang et al., 2011). How Hts mediates positive regulation of CaMKII and PAR-1 is unknown, however. In my study I have confirmed the accumulation of CaMKII along the peripheral amnioserosa cells during dorsal closure in WT embryos, providing further support for CaMKII’s involvement in dorsal closure. I have also found that Hts does not regulate CaMKII and PAR-1 presence via transcription control, as neither of their transcript levels were noticeably changed when Hts was over-expressed in prd stripes in the amnioserosa. Recent unpublished work in our lab suggests that Hts may control the transport of *CaMKII* and *PAR-1* transcripts from the nucleus to the cytoplasm in the muscle (S. Wang, unpublished data). To look for such regulation in the amnioserosa my FISH experiments need to be repeated with a nuclear marker such as DAPI, and number of cytoplasmic versus nuclear CaMKII/PAR-1 puncta calculated and compared.
Figure 4.1. Hypothesized model of Fog regulation of both myosin accumulation and cell adhesion during dorsal closure

This figure illustrates the hypothesized regulatory role of Fog during dorsal closure. The receptor for Fog is still being investigated and may be Mist, Smog or some other undiscovered receptor. It is unknown yet if Fog exerts its effect on dpp and zip through Cta, and if its regulation of Rho plays a major role in dorsal closure.
Chapter 5. Conclusion

For my project I had shown that Egfr is regulated in the amnioserosa by Ack mediated endocytosis during dorsal closure, and may have an effect on mmy during tracheal development. More work will need to be done to elucidate the detailed mechanisms through which Egfr mediates dpp and zip non-autonomously. To this end the deficiency screen was performed. Although the screening methodology used turned out not to be ideal and contains inherent uncertainty in the data, supplementing the screening protocol with temperature shift assay and increasing the number of sample collection will remedy the situation. The updated screening protocol should be able to serve as good basis for future studies on Egfr’s function not only during dorsal closure, but in Drosophila embryo development in general.

I have also identified that Fog interacts with important actomyosin regulation genes dpp and zip during dorsal closure, and may simultaneously control cell adhesion through Rho1. This opens up a new direction for our study of dorsal closure regulators, where Fog may be acting as a two way control mediating both of the important processes for proper dorsal closure and development. With evidence that CamKII is positively regulated by Fog during dorsal closure, we still need to identify if this is a significant contribution in normal embryogenesis in Drosophila, or just a side effect of ectopic expression of Fog. Future work will focus on Fog’s regulation of localization of the known cell adhesion proteins, CaMKII, Hts and Dlg, using both over-expression and knockdowns.

Although we know Hts control Dlg localization through CaMKII and PAR-1 levels, I have shown that Hts does not achieve this through transcription level control of CaMKII and PAR-1. Preliminary data suggest that Hts may act through regulation of CaMKII and PAR-1 transcript transport from the nucleus, and this will be further investigated in the future.
References


Chen, X. (2014). *Bidirectional communication between tissues regulating morphogenesis in a Drosophila model of wound healing.* (Doctor of Philosophy), Simon Fraser University, Burnaby, B.C., Canada.


Appendix.

Raw Data and Other Results

Table A1. Deficiency screen data table

Full data table for the deficiency screen data. % to total specimens is shown in brackets.

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<th>Cross</th>
<th>Wild-type Cuticle (%)</th>
<th>Mild Defect (%)</th>
<th>Severe Defect (%)</th>
<th>Empty Eggs (%)</th>
<th>Total Specimens</th>
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<td>1 (0.01%)</td>
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<td>24971</td>
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<td>4 (0.05%)</td>
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<td>5 (0.06%)</td>
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<td>25019</td>
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<td>10 (0.14%)</td>
<td>4 (0.05%)</td>
<td>16 (0.22%)</td>
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<td>17 (0.16%)</td>
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<td>7 (0.13%)</td>
<td>0 (0.00%)</td>
<td>2 (0.04%)</td>
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<td>29 (0.08%)</td>
<td>15 (0.04%)</td>
<td>61 (0.16%)</td>
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<tr>
<td>26580</td>
<td>167 (82.27%)</td>
<td>3 (0.01%)</td>
<td>20 (0.10%)</td>
<td>13 (0.06%)</td>
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Figure A1. Ratio of cuticle defect to corresponding defect in 25°C control

Graph showing difference in ratio between each cross to the corresponding phenotype of the 25°C control. Empty cuticles are not included in the calculation for both graphs.
Figure A2. Zip expression anomaly during dorsal closure in embryos expressing ectopic fog

A: Prd / UAS-Fog6
B: Prd / UAS-Fog6
C: Prd / UAS-Fog6
D: Prd / UAS-Fog6
E: UAS-Fog6 / + ; c381 / +
F: UAS-Fog6 / + ; c381 / +
G: UAS-Fog6 / + ; c381 / +
H: UAS-Fog6 / + ; c381 / +
Abnormal zip expression is observed in embryos over-expressing fog. A-D: embryos over expressing fog in paired stripes. E-H: embryos over-expressing fog in the amnioserosa. Note failed closure and abnormal openings in the anterior and posterior. In D a ventral hole is evident. There is also increased staining of zip at the anterior part of the embryo in E, G, H. This data suggest dysregulation of zip in these embryos causing constriction related abnormalities.
Figure A3.  Ush is expressed along the leading edge during dorsal closure

Ush FISH of wild-type embryo co-stained for pY. Note the accumulation of Ush in DME cells and canti of the amnioserosa.

Ush is a transcription factor positively regulated by dpp signalling, and was found to be involved in elongation of dorsal most epidermal cells, formation of actin cable at the leading edge, and activation of JNK at the dorsal most epidermal cells (Lada, Gorfinkiel, & Martinez Arias, 2012). Here I present result showing that Ush is transcribed along the leading edge during dorsal closure.
Figure A4. Expression of Cta during dorsal closure

Expression of Cta during dorsal closure in wild-type embryos. The expression seem ubiquitous throughout the embryo. A-A'': wild-type embryos stained for pY and Cta. The bright spot in the amnioserosa may be due to non-specific staining. B-B'': magnified view of embryo in A.

Cta, the downstream effector of Fog receptor, is found to be expressed in both the epidermis and amnioserosa.
Figure A5. PH-PLC does not affect phosphorylation level of Hts
Embryos over-expressing PH-PLC-GFP in prd stripes. There is no obvious difference between alternate segments where PH-PLC is being expressed. A & B: GFP signal showing location of PH-PLC expression. A’& B’: staining of adducin phosphorylated at the MARCKS domain. A” & B”: Merged image of GFP and pAdducin protein stain.

There is data that suggest that over-expression of Hts can affect various signaling pathways by binding and occluding the membrane lipid PIP$_2$, and therefore in parallel I also checked if blocking PIP$_2$, through sequestration via a PIP$_2$ binding elements (PH-PLC) would affect CaMKII and PAR-1 transcript levels. This was done by expression of the PIP$_2$ binding domain of Phospho-Lipase C (PH-PLC), which was successfully used by other researchers to sequester PIP$_2$ in Drosophila (Pinal et al., 2006). The results show that PH-PLC over-expression had no effect on either phosphorylation state of Hts, or CaMKII protein levels. PAR1 protein level was not investigated due to lack of good antibody.
Figure A6. PH-PLC does not affect CaMKII protein level.

CaMKII protein stain in embryos expressing PH-PLC-GFP in prd stripes. There is no obvious change in CaMKII protein level in between wild-type segments and segments over-expressing PH-PLC-GFP. A & B: GFP signal indicating location of PH-PLC-GFP over-expression. A’ & B’: CaMKII protein stain. A’’ & B’’: Merged image of GFP and CaMKII protein stain.
Expression of CalpainA does not have an effect on Hts or CaMKII protein levels. A-A"': embryo over-expressing CalpainA in prd stripes. There is no noticeable change in Hts level between wild-type segments and segments over-expressing CalpainA. B-B"': magnified view of embryo in A-A"'.

CalpainA was speculated to degrade CaMKII and regulate CaMKII’s effect on Dlg localization. This data shows that CalpainA does not have an effect on CaMKII protein level.