Bidirectional communication between tissues regulating morphogenesis in a *Drosophila* model of wound healing

by

Xi Chen

B.Sc., Simon Fraser University, 2008
B.Sc., Northeast Forestry University, 2004

Thesis Submitted In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

In the
Department of Molecular Biology and Biochemistry
Faculty of Science

© Xi Chen 2014

SIMON FRASER UNIVERSITY
Summer 2014

All rights reserved. However, in accordance with the Copyright Act of Canada, this work may be reproduced, without authorization, under the conditions for "Fair Dealing." Therefore, limited reproduction of this work for the purposes of private study, research, criticism, review and news reporting is likely to be in accordance with the law, particularly if cited appropriately.
Approval

Name: Xi Chen
Degree: Doctor of Philosophy
Title of Thesis: Bidirectional communication between tissues regulating morphogenesis in a Drosophila model of wound healing
Examining Committee: Chair: Dr. Michael Silverman
Associate Professor, Dept. of Biological Science

Dr. Nicholas Harden
Senior Supervisor
Professor

Dr. Esther Verheyen
Supervisor
Professor

Dr. David Baillie
Supervisor
Professor

Dr. Barry Honda
Internal Examiner
Professor
Dept. of Molecular Biology and Biochemistry

Dr. Tony Harris
External Examiner
Associate Professor,
Department of cell & systems biology
University of Toronto

Date Defended/Approved: May 28, 2014
Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the non-exclusive, royalty-free right to include a digital copy of this thesis, project or extended essay[s] and associated supplemental files (“Work”) (title[s] below) in Summit, the Institutional Research Repository at SFU. SFU may also make copies of the Work for purposes of a scholarly or research nature; for users of the SFU Library; or in response to a request from another library, or educational institution, on SFU’s own behalf or for one of its users. Distribution may be in any form.

The author has further agreed that SFU may keep more than one copy of the Work for purposes of back-up and security; and that SFU may, without changing the content, translate, if technically possible, the Work to any medium or format for the purpose of preserving the Work and facilitating the exercise of SFU’s rights under this licence.

It is understood that copying, publication, or public performance of the Work for commercial purposes shall not be allowed without the author’s written permission.

While granting the above uses to SFU, the author retains copyright ownership and moral rights in the Work, and may deal with the copyright in the Work in any way consistent with the terms of this licence, including the right to change the Work for subsequent purposes, including editing and publishing the Work in whole or in part, and licensing the content to other parties as the author may desire.

The author represents and warrants that he/she has the right to grant the rights contained in this licence and that the Work does not, to the best of the author’s knowledge, infringe upon anyone’s copyright. The author has obtained written copyright permission, where required, for the use of any third-party copyrighted material contained in the Work. The author represents and warrants that the Work is his/her own original work and that he/she has not previously assigned or relinquished the rights conferred in this licence.

Simon Fraser University Library
Burnaby, British Columbia, Canada

revised Fall 2013
Abstract

*Drosophila* embryonic dorsal closure is a developmental epithelial fusion event that resembles vertebrate processes such as neural tube closure and wound healing. Dorsal closure involves the migration of two epidermal sheets to close an epidermal hole occupied by a tissue called the amnioserosa. The successful completion of this process requires a signalling network that involves communication between the amnioserosa and the dorsal epidermis, and the Dpp and JNK pathways are two central participants. Dorsal closure is driven in part by myosin-dependent cell shape change in both the dorsal epidermis and the amnioserosa. Consistent with this, myosin heavy chain expression from the *zipper* (*zip*) gene is upregulated in these two tissues in a Dpp-dependent manner and we believe that *zip* regulation is a critical component of dorsal closure.

We have taken a candidate gene approach to identify new participants in *zip* regulation during dorsal closure. We have established that Egfr acts as a brake on dorsal closure in that it inhibits the expression of both *dpp* and *zip*. Egfr is itself negatively regulated by the non-receptor tyrosine kinase Ack, which may be reducing Egfr levels by promoting its endocytosis. Previous work suggests that a diffusible signal from the amnioserosa regulates gene expression in both the amnioserosa and the dorsal epidermis. We have established that this diffusible signal is the steroid hormone ecdysone, which cooperates with JNK signalling to turn on *zip* expression. We have also determined that Dpp secreted from the dorsal epidermis turns on ecdysone production in the amnioserosa by promoting expression of the ecdysone biosynthetic enzyme Spook. Thus, we have uncovered reciprocal signalling between two tissues leading to coordinated morphogenesis. We have further established that ecdysone is acting in a non-canonical manner in its cooperation with JNK signalling in this process. We provide genetic and immunohistochemical evidence that the ecdysone receptor turns on *zip* by complexing with the JNK-activated transcription factor AP-1. Using the proximity ligation assay, we visualize this complex in the nuclei of amnioserosa and dorsal epidermal cells during dorsal closure. Our findings may provide clues to the signalling events occurring during other epithelial fusions. What we found in this project could help to better understand the bidirectional communication that occurs between the amnioserosa and dorsal epidermis.
at the level of signaling network allowing coordinated morphogenesis of the two tissues through the regulation of actomyosin contractility.

**Keywords:**  *Drosophila; dorsal closure; zipper; epithelial morphogenesis; Dpp; Egfr; ecdysone; JNK*
This thesis is dedicated to my parents and my husband, for their great support in my work.
Acknowledgements

In the first place I would like to express my deepest appreciation for my supervisor Dr. Harden who gave me the opportunity to join his lab. His inspiration, encouragement, patience and mentorship guide me through the graduate study.

I gratefully acknowledge Dr. Verheyen and Dr. Baillie for their advice, supervision and tremendous support in my research, which include letting me use their equipments and many precious reagents.

I am especially grateful to Dr. Weiping Shen for his tremendous help and guidance in the beginning of my graduate study. My special gratitude goes to Dr. Simon Wang for his valuable advice in science discussion and his great effort to help me edit my thesis and make it more readable. I want to thank all the members, past and present, of the Harden lab. They made the Harden lab a wonderful place to work in and I am glad to be part of this great team.

In addition, I would like to thank our collaborator Dr. Bruce Reed (University of Waterloo), for his help and amazing live imaging results.

I feel thankful that I have made many friends during these years of study. Their support and friendship made my graduate school life exciting and wonderful.

Lastly, I thank my parents and my husband for their unconditional support.
# Table of Contents

Approval ................................................................................................................................. ii
Partial Copyright Licence .................................................................................................... iii
Abstract ................................................................................................................................ iv
Dedication ............................................................................................................................. vi
Acknowledgements ........................................................................................................... vii
Table of Contents ............................................................................................................. viii
List of Tables ..................................................................................................................... x
List of Figures .................................................................................................................... xi

1. **Introduction** ............................................................................................................ 1
   1.1. *Drosophila* embryonic dorsal closure ................................................................. 1
   1.2. Jun-N terminal kinase cascade in *Drosophila* ...................................................... 7
   1.3. Transforming growth factor pathway in *Drosophila* ........................................... 13
   1.4. Role of zipper gene in dorsal closure ................................................................... 17
   1.5. The Rho subfamily of small GTPases in dorsal closure ......................................... 19
   1.6. *Drosophila* activated Cdc42-associated kinase .................................................. 21
   1.7. Egfr signaling in *Drosophila* ......................................................................... 22
   1.8. Ecdysone signaling ............................................................................................. 26
   1.9. Goals of this thesis .............................................................................................. 30

2. **Material and Methods** ............................................................................................. 32
   2.1. Fly stocks ............................................................................................................... 32
   2.2. cDNAs .................................................................................................................... 32
   2.3. Antibodies ............................................................................................................. 32
   2.4. Cuticle preparations .............................................................................................. 33
   2.5. Embryo fixation .................................................................................................... 33
   2.6. Standard molecular techniques ............................................................................ 34
   2.7. Generation of digoxigenin-labeled RNA probes .................................................... 35
   2.8. Fluorescent *in situ* hybridization ..................................................................... 35
   2.9. Immunostaining of *Drosophila* embryos ............................................................ 37
   2.10. 20 hydroxyecdysone incubation of *Drosophila* embryos .................................... 38
   2.11. Proximity ligation assay .................................................................................... 38

3. **Results part I: Dpp cooperates with ACK to regulate myosin levels during dorsal closure** .................................................................................................................. 40
   3.1. Dpp signaling is necessary but not sufficient for *zip* expression in the DME cells and amnioserosa .......................................................... 41
   3.2. Ack cooperates with Dpp signaling in regulating *zip* expression ....................... 44
   3.3. Effects of excessive Dpp signaling in the embryo and wing are suppressed in a Ack mutant background ................................................... 54

4. **Results part II: Role of Egfr signaling in dorsal closure** ...................................... 63
   4.1. Egfr is required for normal dorsal closure ............................................................ 64
   4.2. Ras/Raf/MAPK cascade is active during dorsal closure ......................................... 69
   4.3. Egfr signaling represses *dpp* expression ............................................................. 71
4.4. Egfr negatively regulates zip expression ................................................................. 75
4.5. Egfr inhibits apoptosis in the amnioserosa ............................................................ 78
4.6. Ack negatively regulates Egfr levels in the amnioserosa through endocytosis ............................................................................................................. 81
4.7. Search for transcription factors regulating zip expression downstream of Egfr ................................................................. 85

5. Results part III: Cooperation between ecdysone and JNK signaling in regulation of zip expression during dorsal closure ......................................................... 92
5.1. Ecdysone promotes zip expression during dorsal closure ...................................... 92
5.2. Dpp signaling regulates ecdysone production ......................................................... 95
5.3. Ecdysone signaling cooperates with JNK signaling to promote zip expression ................................................................................................. 97
5.4. Evidence that EcR acts in a complex with Jun in amnioserosa and DME nuclei to regulate zip expression .............................................................. 106
5.5. Testing for AP-1 regulation by other nuclear receptors ......................................... 113
5.6. Testing candidate ligands and receptors for a role in JNK activation .................... 116

6. Discussion .............................................................................................................. 121
6.1. A reciprocal signaling network regulating zip expression by mediating communication between the amnioserosa and epidermis ........................................ 121
6.2. Future directions .................................................................................................. 124
   6.2.1. How does ecdysone co-operate with the JNK pathway in driving gene expression? ................................................................................................. 124
   6.2.2. The mechanism of Egfr repression of reciprocal signaling during dorsal closure .................................................................................................. 125
   6.2.3. How is the JNK cascade activated during dorsal closure? ....................... 126
6.3. Is the signaling network controlling dorsal closure conserved in other epithelial fusion events? ....................................................................................... 126

7. Conclusion .......................................................................................................... 131

References ................................................................................................................... 132
List of Tables

Table 3.1  Genetic interaction between Ack and Dpp signaling in regulation of dorsal closure ........................................................................................................ 46

Table 5.1  Information on candidate genes regulated by EcR/AP-1 complex ................. 112
List of Figures

Figure 1.1  Dorsal closure of the Drosophila embryo ..................................................... 3

Figure 1.2  Contribution of contraction of cells occupying hole to epithelial closure. ......................................................................................................... 4

Figure 1.3  Simplified schematic illustration of the JNK signaling pathway in Drosophila embryonic dorsal closure ......................................................... 12

Figure 1.4  Simplified schematic illustration of the Dpp signaling pathway in Drosophila .................................................................................................. 16

Figure 1.5  zip expressions in wild-type embryos ............................................................................................................................ 18

Figure 1.6  Simplified schematic illustration of the Egfr signaling pathway in Drosophila .................................................................................................. 24

Figure 1.7  Simplified schematic of canonical ecdysone signaling in Drosophila ........................................................................................................... 28

Figure 1.8  Schematic of amnioserosa and DME cells showing JNK signaling and Dpp signaling as central components of dorsal closure ...................... 31

Figure 3.1  zip expression in the amnioserosa and dorsal epidermis is regulated by Dpp and ACK.......................................................................................... 43

Figure 3.2  Ack cannot activate the Dpp pathway ............................................................................................................................ 48

Figure 3.3  Ack is not a target of the Dpp pathway ............................................................................................................................ 49

Figure 3.4  Molecular characterizations of Ack and PR2 mutations ............................................................................................................................ 52

Figure 3.5  Cuticle phenotypes associated with perturbations of Dpp signaling and ACK function .................................................................................. 53

Figure 3.6  Loss of Ack suppresses wing phenotype resulting from expression of tkvQ199D in enhancer piracy line TAJ3 ............................................................. 56

Figure 3.7  Model for coordinated regulation of zip expression in the amnioserosa and DME by Dpp and ACK .......................................................................... 59

Figure 4.1  Cuticle preparations of embryos with mutant Egfr alleles or dominant negative Egfr transgene .................................................................................. 67

Figure 4.2  Morphogenetic defects of embryos with impaired Egfr ............................................................................................................................ 68

Figure 4.3  Strong immunoreactivity of phospho-MAPK was observed in wild-type embryos during dorsal closure ........................................................................ 70
Figure 4.4  Egfr signaling represses dpp expression .......................................................... 73
Figure 4.5  Egfr does not regulate Wg or phyllopod levels during embryogenesis .......................................................... 74
Figure 4.6  Egfr signaling negatively regulates zip transcription .......................................................... 77
Figure 4.7  Egfr inhibits apoptosis and morphogenesis in the amnioserosa ................................. 80
Figure 4.8  Ack may antagonize Egfr in zip regulation ...................................................................... 82
Figure 4.9  Evidence that ACK down-regulates Egfr through endocytosis ........................................ 84
Figure 4.10  zip FISH of embryos with disruption of candidate transcription factors .......................................................... 86
Figure 4.11  Model for Egfr acting as a brake on dorsal closure .......................................................... 89
Figure 5.1  Ecdysone promotes zip but not dpp expression during dorsal closure .................................................. 94
Figure 5.2  Dpp signaling and Hnt regulate spo expression in the amnioserosa ..................................... 96
Figure 5.3  JNK signaling promotes IMP-L1 and IMP-E1 expression ............................................. 98
Figure 5.4  JNK signaling promotes zip expression .............................................................................. 100
Figure 5.5  JNK signaling cooperates with ecdysone to regulate target gene expression .......................................................... 101
Figure 5.6  spo FISH of embryos with mis-expression of Ack and Egfr signaling components .......................................................... 103
Figure 5.7  Evidence that EcR regulates zip expression independent of the canonical ecdysone response element .................................................. 105
Figure 5.8  EcR is in a complex with Jun .......................................................................................... 108
Figure 5.9  PLA signals are reduced in zip deficient embryo .......................................................... 110
Figure 5.10  Testing of zip regulation by nuclear receptors .............................................................. 115
Figure 5.11  Activated kinases promote zip expression ........................................................................ 118
Figure 5.12  Schematic of reciprocal signaling mediating myosin expression in the amnioserosa and DME cells during dorsal closure .................................................. 120
Figure 6.1  Schematic of reciprocal signaling mediating myosin expression in the amnioserosa and DME cells during dorsal closure .................................................. 123
Figure 6.2  Parallels between Drosophila dorsal closure and mammalian eyelid closure ........................................................................................................... 130
1. Introduction

1.1. *Drosophila* embryonic dorsal closure

*Drosophila* embryonic dorsal closure is a developmental morphogenetic epithelial fusion event analogous to vertebrate processes such as neural tube closure and palate fusion. Dorsal closure occurs at the end of *Drosophila* embryogenesis between embryonic stages 12 (~9 hours after egg lay at 25°C) and 16 (~12 hours after egg lay at 25°C) after germband retraction and coincident with head involution (Harden 2002). Following germband retraction, a hole is left in the dorsal epidermis of the embryo occupied by a single layer of flat epithelial cells, called the amnioserosa, which lies over the yolk sac. During dorsal closure, the two epithelial sheets surrounding the amnioserosa migrate from both sides toward the dorsal midline to close the hole. With the subsequent fusion and zippering, the end of dorsal closure is marked by the seamless joining of the two epidermal flanks at the dorsal midline (Figure 1.1). Coordinately, the amnioserosa constricts apically to reduce its surface area and eventually disappears through cell extrusion and apoptosis as the flanking epidermis converges dorsally (Belacortu and Paricio 2011; B. G. Fernandez et al. 2007; Jacinto et al. 2002) (Figure 1.2 A). Failure of dorsal closure is lethal, as it typically leaves a large hole in the dorsal epidermis through which the gut is extruded.

Results from various systems indicate that developmental epithelial fusions are essentially wound healing events; indeed, the *Drosophila* embryo uses the same signaling “cassettes” to close inflicted wounds as it does to achieve dorsal closure (Martin and Parkhurst 2004; Ramet et al. 2002; Wood et al. 2002). More specifically, striking parallels are found between developmental morphogenetic fusions and wound healing in terms of the events at the leading edge of the migrating epithelial flanks, including the use of actomyosin contractile purse strings and lamellipodial/filopodial-mediated cell migration and matching, AP-1 activity and TGF-β signaling (Grose and Martin 1999; Jacinto et al. 2000; Jacinto and Martin 2001; Martin and Parkhurst 2004;
Song et al. 2002; Zeitlinger et al. 1997). In addition, the presence and requirement of a "connective tissue", for example amnioserosa in dorsal closure and fibroblasts in vertebrate adult wound, is conserved between the two types of events (Figure 1.2).

In the case of external injuries and physiological dysfunction, wound healing is one of the important mechanisms to maintain the structural and functional integrity of the organism. Improving wound healing is particularly important for victims of burns or blasts and people with slow healing skin lesions. Furthermore, wound closure is the most important step after surgery which contributes to the success or failure of the surgical intervention. A more critical finding showed that healing skin shares gene expression pattern with malignant tumors through DNA microarray analyses (Chang et al. 2004; Iyer et al. 1999); thus non-healing wounds could be at risk of malignant transformation (Schafer and Werner 2008).

*Drosophila* dorsal closure represents an ideal model for wound healing studies, because it possesses many advantages when compared with other animal models. With great conservation of the fundamental cellular processes as well as many genes and signaling pathways in common with vertebrates, it has a simpler genome and less genetic redundancy than the vertebrate systems. In addition the many research tools used in dorsal closure such as genetic screening, live imaging, and use of mutants and transgenes, are more difficult and costly in vertebrate model studies. The application of the yeast *GAL4/UAS* system in *Drosophila* allows the study of the effects of over-expression or inactivation of any gene in a selected tissue and developmental stage (Brand and Perrimon 1993). Overall, what is learned from dorsal closure will be broadly applicable to other epithelial fusion events, and will be of interest to those studying birth defects involving failed epithelial fusions such as spina bifida and cleft palate, and researchers elucidating the mechanisms of wound healing or seeking improved treatments for wound repair.
Figure 1.1  Dorsal closure of the Drosophila embryo

All embryos have their anterior to the left in this and subsequent figures unless otherwise specified. (A-D) Confocal fluorescent images (from N. Harden) of the progression of dorsal closure. The embryos were stained with anti-phosphotyrosine (PY) antibody to show closure of the dorsal opening occupied by the large flat amnioserosa cells. Note that these embryos are shown in dorsal views, whereas in most of the other figures they are shown in dorsolateral views.
Figure 1.2 Contribution of contraction of cells occupying hole to epithelial closure.

(A) Cross-sectional view of a Drosophila embryo undergoing dorsal closure. Apical constriction of AS cells (blue) contributes to closure. (B) Vertebrate embryonic wound healing. Connective tissue (blue) contraction contributes to closure. (C) A vertebrate adult wound occupied by fibrin clot (red). Fibroblasts (blue) migrate into the wound and differentiate into contractile myofibroblasts.
There are a number of mechanical forces responsible for dorsal closure, where the main force-generating tissues contributing to dorsal closure have been determined through laser ablation experiments (Hutson et al. 2003; Kiehart et al. 2000). At the interface between the amnioserosa and the epidermis, called the leading edge, an actin-myosin cable is assembled prior to the beginning of dorsal closure. The “purse string” model suggests that this cable generates contractile force along the length of the cable and thus contributes to the stretching of the dorsal most epidermal (DME) cells in the dorsal-ventral axis and movement towards the dorsal midline (Franke et al. 2005; Harden 2002; Lawrence and Morel 2003). However, another model suggests that the cable acts as a supra-cellular ratchet to prevent the relaxation of amnioserosa cells (Solon et al. 2009).

The amnioserosa provides another major force driving dorsal closure by progressively reducing its surface area in an active process, which is achieved through the apical constriction of its individual cells and both basal and apical extrusion of cells in a random pattern through apoptosis (B. G. Fernandez et al. 2007; Gorfinkiel and Arias 2007; Kiehart et al. 2000; Toyama et al. 2008). The evidence that the amnioserosa can contract in the absence of a fully functional actin-myosin cable, as well as the fact that laser ablation of the amnioserosa slows down the rate of dorsal closure (Gorfinkiel et al. 2009; Laplante and Nilson 2006), indicates that the amnioserosa actively participates in dorsal closure. However, amnioserosa cells don’t continuously decrease their surface area; instead, they undergo fluctuations in their shape through periodic cycles of contraction and expansion (Blanchard et al. 2010; David et al. 2010; Solon et al. 2009). Live imaging of cytoskeletal reporters reveals the appearance of transient foci of actin and myosin across the apical surface of amnioserosa cells in correlation with apical constriction (Blanchard et al. 2010; David et al. 2010). Indeed, these actin-myosin accumulations can be detected as early as germband retraction, and before the beginning of dorsal closure all the cells show transient actin-myosin foci that flow across the apical surface (David et al. 2010). Therefore, changes in the concentration or activity of actin and myosin could affect the contractile behaviour of this tissue.

A third force is provided by the lateral epidermis, which resists the net dorsal ward movement to balance the forces and rate of the cell shape change and tissue movements during dorsal closure. This has been shown in early laser ablation
experiments where the epidermis was observed to recoil ventrally upon lateral epidermal break (Gorfinkiel et al. 2011).

The last force involves the lamellipodia/filopodia-mediated zippering of the leading edge once the two lateral epidermal flanks meet at the anterior and posterior canthi (Hutson et al. 2003). These actin-based cellular projections which also contain tubulin, myosin and other cytoskeletal components (Gates et al. 2007; Jacinto et al. 2000; Jankovics and Brunner 2006; R. Liu et al. 2008) are important for the matching of cells from the same segment as the leading edges meet up. It has been shown that the filopodia from one cell can recognize the filopodia from the partner cell even when they are misaligned, and finally bring the opposing matching cells together (Millard and Martin 2008). In addition to the guidance of matching, the filopodia also provide pulling force, demonstrated by the slowdown of the zippering rate in situations where the number and length of filopodia are compromised (Gates et al. 2007). However, the mechanism behind the zippering starting from the canthi at the anterior and posterior ends of the dorsal hole is mostly unknown.

The question arises as to how the forces driving dorsal closure are generated and regulated in individual cells at the molecular level. Intensive genetic studies have found a great number of components that are involved in dorsal closure regulation (Harden 2002). It is fascinating to see a picture emerging of the signaling network composed of all the molecules controlling dorsal closure. Dorsal closure requires coordinated cell shape changes within the amnioserosa and the lateral epidermis. It is shown that events in the DME cells are a vital component of dorsal closure, as many signaling components participating in dorsal closure show elevated levels in this tissue. The amnioserosa also plays structural and signaling roles during dorsal closure, as suggested in experiments which showed that genetic ablation of the amnioserosa leads to dorsal closure defects caused by disruption of the mechanical and molecular signals (Scuderi and Letsou 2005). In a vertebrate wound healing system, it has been shown that there is crosstalk between keratinocytes and the fibroblasts occupying the hole that leads to transcriptional responses in both cell types (Werner et al. 2007). We speculate that similarly the amnioserosa and the dorsal epidermis cooperate through reciprocal communications to regulate the precise execution of dorsal closure. For this task, cells
rely on signaling pathways that transduce extracellular signals into intracellular changes, and allow communication between cells as well as tissues.

1.2. Jun-N terminal kinase cascade in Drosophila

The Jun N-terminal kinase (JNK) cascade is one of the mitogen-activated protein kinase (MAPK) superfamily cascades which is conserved throughout the eukaryotes (Qi and Elion 2005). Members of the MAPK signaling pathways are key molecules that link extracellular signals to modified gene expression and intracellular changes. The major feature of this type of pathway is the central component of three sequential phosphorylations conducted by the three core kinases: the MAPK kinase kinase (MAPKKK), which is a ser/thr kinase that phosphorylates and activates a MAPK kinase (MAPKK), which is a dual-specificity kinase phosphorylating a TXY motif in the target the MAPK. MAPKs are ser/thr kinases whose nuclear and cytoplasmic targets are mainly transcription factors, but also include cytoskeleton-associated proteins and other kinases. Generally, there is greater diversity at the level of MAPKKK than at the level of MAPKK or MAPK, the MAPKKs exhibit great specificity for their cognate MAPK but are regulated by many MAPKKKs (Qi and Elion 2005). MAPK pathways are conserved both structurally and functionally in all eukaryotic cells studied in the regulation of a wide number of processes including cellular differentiation, proliferation, apoptosis, stress responses and morphogenesis (Bogoyevitch and Kobe 2006; Brown and Sacks 2008; Raman et al. 2007; Widmann et al. 1999).

The JNK pathway has been extensively studied in Drosophila, because of the powerful genetic tools, and little or no redundancy. It has been shown this pathway is required for follicle cell morphogenesis, embryonic dorsal closure, thoracic closure and genital disc rotation/closure (Agnes et al. 1999; Dobens et al. 2001; Macias et al. 2004; Martin-Blanco et al. 2000; Rousset et al. 2010; Zeitlinger and Bohmann 1999), all processes with requisite cell shape changes. Many participants of the JNK pathway in Drosophila were identified through genetic screens, where they all displayed similar “dorsal open” cuticle phenotypes (Jurgens et al., 1984; Nusslein-Volhard et al., 1984). Mutations in any of the JNK pathway components result in a similar range of dorsal closure defects including defects in DME cell elongation, disruption of the actomyosin
cable at the leading edge, reduced expression of target genes in the DME cells, and failure of the epidermal flanks to meet up (Harden 2002).

In *Drosophila*, the JNK pathway is a canonical MAPK signaling module remarkably similar to the mammalian stress-activated protein kinase (SAPK) pathway, which results in phosphorylation of Jra (Jun-related antigen, hereafter referred to as Jun) on several residues in its N-terminal domain (Riesgo-Escovar et al. 1996; Sluss et al. 1996). Jun, together with Fos, encoded by the *kayak (kay)* locus, constitute the AP-1 transcription factor, whose activity is stimulated by phosphorylation (Kockel et al. 2001) (Figure 1.3).

There is only one JNK in *Drosophila*, which is coded by *basket (bsk)* (Sluss et al. 1996) (Figure 1.3). The activity of Bsk is regulated through phosphorylation by upstream JNKKs. Two JNKKs have been identified, the homolog of mammalian M KK7 encoded by *hemipterous (hep)* (Figure 1.3), and the *Drosophila* homolog of M KK4. Bsk has been shown to be the substrate of Hep, however, the requirement for M KK4 in vivo remains unclear (W. Chen et al. 2002b; Glise et al. 1995; Han et al. 1998; Holland et al. 1997). The Hep-Bsk-Jun/Kay interactions appear to be scaffolded together at the core of the JNK signal transduction pathway, since this module can be coimmunoprecipitated from embryonic protein extracts with a scaffold protein (H. W. Chen et al. 2002a).

Hep is the substrate for upstream JNKKKs. Consistent with the greater diversity at the level of MAPKKK mentioned previously, six kinases that could function at the level of JNKKK to phosphorylate and activate Hep were found in the *Drosophila* genome (W. Chen et al. 2002b; B. Stronach and Perrimon 2002). Among the characterized mutants, only the *slipper (slpr)* locus, encoding mixed lineage kinase (MLK), is required to transduce the signal to the core pathway during dorsal closure (Sathyanarayana et al. 2003; B. Stronach and Perrimon 2002) (Figure 1.3). Other JNKKKs are involved in other processes regulated by JNK, for example, Tak1 mediates an immune response (Vidal et al. 2001), and Mekk1 is activated by heat and osmotic stress (Inoue et al. 2001). Furthermore, there is unlikely to be an additional JNKKK family member required for JNK activation in dorsal closure, because the phenotype of the *slpr* mutant is as severe as *hep, bsk* and *jun* null mutants (B. Stronach 2005). Note these results raise the possibility that the specificity of the pathway occurs at the level of JNKKK, which
functions as a link between different upstream signals and the core module of the JNK pathway (B. Stronach 2005).

JNKKKs can be activated by small GTPases of the Ras superfamily (W. Chen et al. 2002b; Teramoto et al. 1996), JNKKK kinases of the Sterile-20 superfamily including misshapen (msn) (Leung and Lassam 2001; Su et al. 1998), or adapter proteins that link membrane receptors to the kinase module, such as tumor necrosis factor (TNF)-receptor associated factors (Traf) (H. Liu et al. 1999). It is suggested through various genetic and biochemical experiments that Slpr is activated by Rac1 GTPase and the JNKKKK Msn in both Drosophila and mammalian systems (Gallo and Johnson 2002; Kiefer et al. 1996; Leung and Lassam 2001; B. Stronach and Perrimon 2002; Su et al. 1998) (Figure 1.3).

The extracellular signaling molecules and membrane receptors leading to JNK pathway activation during dorsal closure are still unidentified (Rios-Barrera and Riesgo-Escovar 2013). The most upstream acting molecules identified for JNK signaling during dorsal closure are non-receptor tyrosine kinases, including the Src family proteins: Src42A, Src64B and Btk29A (Tateno et al. 2000), and Shark (R. Fernandez et al. 2000), as well as the adapter protein Dok which convey signals between non-receptor tyrosine kinases (Biswas et al. 2006) (Figure 1.3).

During dorsal closure, activation of the JNK pathway occurs specifically in the DME cells and leads to a transcriptional response in the nucleus. Two transcriptional target genes were initially reported to respond to the activation of the JNK pathway: decapentaplegic (dpp) and puckered (puc) (Jackson and Hoffmann 1994) (Figure 1.3). Dpp is a major ligand for the activation of the TGF-β pathway in Drosophila, and a major role for JNK pathway activation during dorsal closure is maintaining dpp expression in the DME cells (Noselli and Agnes 1999). The Dpp ligand produced in the DME cells is secreted to both the amnioserosa and lateral epidermis to induce cell shape changes (B. G. Fernandez et al. 2007; Wada et al. 2007). puc, an immediate early gene of the JNK pathway, encodes a dual specificity MAPK phosphatase that down-regulates JNK/Bsk activity through de-phosphorylation and serves as a negative feedback control in the JNK pathway during dorsal closure (Martin-Blanco et al. 1998) (Figure 1.3). Overexpression of puc shares a similar phenotype with loss of the JNK pathway including loss of dpp expression in the DME cells and loss of the actomyosin contractile
apparatus (Harden 2002). The expression of dpp and puc have been widely used as readouts of JNK signaling in different genetic backgrounds (Rios-Barrera and Riesgo-Escovar 2013).

More transcriptional targets of JNK/AP-1 signaling have been identified through genomic and proteomic approaches such as the serial analysis of gene expression (SAGE) and microarray analyses in the embryo (Jasper et al. 2001) and in adults subjected to septic injury (Boutros et al. 2002), as well as in tissue culture (Boutros et al. 2002; Silverman et al. 2003). For instance, JNK signaling also activates expression of chickadee, encoding the Drosophila homolog of vertebrate profilin (Jasper et al. 2001). Considering profilin’s well-known function as a regulator of the actin cytoskeleton, this finding is consistent with the role of the JNK pathway in controlling cell shape change. Other JNK/AP-1 target genes include cytoskeletal genes like integrin, matrix metalloproteinase, stress-related genes and ecdysone responsive genes, such as IMPL-1 and IMPE-1 (Homsy et al. 2006; Stevens and Page-McCaw 2012; Wang et al. 2003).

As a highly functionally conserved and fundamental pathway, JNK signaling has been shown implicated in a wide range of processes, including tissue morphogenesis (Harden 2002), wound healing (Ramet et al. 2002), immune response (Sluss et al. 1996), planar cell polarity (Boutros et al. 1998), programmed cell death (Adachi-Yamada et al. 1999) and synaptic plasticity (Sanyal et al. 2002). Therefore, the activity of this pathway requires precise spatial and temporal controls. Besides the positive contributions from the core components like Hep, Bsk, Jun and Fos, there are several levels of negative restrictions that modulate the final output of this pathway. A basal “on” repressed state model has been proposed, which provides a more rapid response in case of injury or stress, in which process the JNK pathway was first identified (Rios-Barrera and Riesgo-Escovar 2013). The best characterized inhibitor is the Bsk-specific phosphatase encoded by puc, who is a target gene of the JNK pathway as mentioned previously. The mechanism that activation of the JNK pathway produces Puc to form a negative feedback loop is used in different processes such as follicle cell morphogenesis, dorsal and thoracic closure and stress responses (Karkali and Panayotou 2012). Although the extracellular and the membrane-associated components upstream of JNK are unknown in dorsal closure, the existence of the secreted protease Scarface (Scaf) which is also a target gene of JNK implies another negative feedback at
the extracellular level, as shown by the dorsal closure defects and ectopic puc expression in scaf mutants (Rousset et al. 2010). A pioneer protein Raw, counteracting the JNK pathway, has been positioned downstream of Bsk and upstream of Jun through epistasis analysis, however its mechanism of action remains to be determined (Bates et al. 2008). At the level of target gene promoters, JNK pathway inhibition is implemented by transcriptional repressors; one example being the Anterior Open (Aop). Mutants for aop have been shown to enhance JNK effects in differentiation of the epidermis and proliferation of its precursors (Riesgo-Escovar and Hafen 1997; Rogge et al. 1995). Finally, there is another inhibition carried out specifically in the amnioserosa by Hindsight (Hnt), which turns off JNK signaling by impeding Jun/Fos accumulation in the nucleus (Reed et al. 2001). The Hnt expression in the amnioserosa which is controlled by the early Dpp expression gradient is also important for survival and differentiation of this tissue (Frank and Rushlow 1996; Lamka and Lipshitz 1999; B. E. Stronach and Perrimon 2001).
Figure 1.3  Simplified schematic illustration of the JNK signaling pathway in Drosophila embryonic dorsal closure

The extracellular signaling molecules and membrane receptors leading to JNK pathway activation during dorsal closure are unknown. Src family proteins, Dok and Shark are the most upstream components found to activate JNK pathway. There is no evidence showing direct interaction between Src/Dok/Shark and Msn/Rac1. Activation of JNK pathway leads to assembly of AP-1 transcriptional factor, which then regulates target gene transcription in the nucleus. dpp and puc are two of the well-known target genes. puc codes for a Bsk specific phosphatase which forms a negative feedback loop to modulate JNK pathway activity.
1.3. Transforming growth factor pathway in *Drosophila*

Dpp, a secreted protein in the transforming growth factor β (TGFβ) family, is a *Drosophila* homolog of vertebrate bone morphogenetic proteins 2/4 and serves as a morphogen to deliver cell signals over a long distance (Raftery and Sutherland 1999). Once Dpp reaches its target cells, it binds to the TGFβ type II receptor, Punt (Put), which is a constitutively active kinase, on the cell surface. The Dpp-Put complex then recruits the TGFβ type I receptor, Thickvein (Tkv) (Shimmi and Newfeld 2013). The constitutively active kinase function in the intracellular domain of Put phosphorylates and activates the Tkv receptor kinase, which subsequently recruits and phosphorylates its cognate substrate Mothers against Dpp (Mad), the only known fly homolog of vertebrate receptor-regulated Smads (R-Smad). Phosphorylated Mad forms a complex with the common-mediator Smad (Co-Smad), Medea, which is an ortholog of vertebrate Smad 4 (Inoue et al. 1998; Wisotzkey et al. 1998). The Mad-Medea complex then translocates into the nucleus. Once in the nucleus, the Smads directly regulate transcription of their target genes, which may require assistance of additional transcription factors (Figure 1.4). This basic scenario is shared among the whole TGFβ family, although each pathway varies in the participating proteins (Shimmi and Newfeld 2013).

Activation of the TGFβ signaling pathway may lead to positive or negative changes in the transcription of several hundred genes with spatial and temporal availability of the transcriptional factors determining which genes are transcriptionally regulated in different cell types and at different times (Kang 2006). In *Drosophila*, one of the mechanisms restricting the Dpp signaling output is the negative feedback loops provided by Daughters against dpp (Dad) and Brinker (Brk), which are targeted products of this pathway (Campbell and Tomlinson 1999; Inoue et al. 1998; Minami et al. 1999) (Figure 1.4). However, they provide the feedback differently. Dad competes with Mad for receptor binding in the cytoplasm, thus, functioning like vertebrate inhibitor Smad (I-Smad) and Dad is a homolog of human I-Smad 6 and 7 (Marquez et al. 2001; Newfeld et al. 1999). Brk acts as a transcriptional repressor that suppresses transcription of Mad-Medea target genes. The repressive control of Brk on Dpp signaling is limited by Schnurri (Shn), which is a zinc finger protein and another component of the TGFβ
pathway promoting target gene transcription through repression of \textit{brk} expression (Dai et al. 2000; Udagawa et al. 2000; Yao et al. 2006) (Figure 1.4).

During dorsal closure, the epidermal expression of \textit{dpp} is concentrated in two stripes, one in the DME cells, the other one in several rows of ventral lateral cells. The high level of \textit{dpp} expression in DME cells is maintained by the JNK signaling pathway (Jackson and Hoffmann 1994). Roles for the TGFβ pathway in dorsal closure have been demonstrated through gain-of-function and loss-of-function assays of TGFβ pathway components. Ectopic expression of \textit{dpp} or a constitutively active form of the TGFβ type I receptor, Tkv are able to substantially rescue dorsal closure defects caused by mutations in JNK pathway components (Sluss and Davis 1997). Loss-of-function mutations in the TGFβ pathway components \textit{tkv, put, mad} and \textit{shn} all lead to dorsal closure defects (Affolter et al. 1994; Brummel et al. 1994; Dai et al. 2000; Letsou et al. 1995; Penton et al. 1994; Ruberte et al. 1995; Udagawa et al. 2000). All this evidence indicates that the TGFβ pathway plays important roles in dorsal closure downstream of the JNK pathway. In embryos deficient for TGFβ pathway components, the DME cells bend along the anterior-posterior axis, and epidermal cells ventral to the DME cells fail to elongate, leading to a typical “bunching” phenotype where the dorsal ends of the segments come together and the dorsal hole fails to close (Ricos et al. 1999; Riesgo-Escovar and Hafen 1997). However, how the TGFβ signaling guides the direction of DME cell movement is still unclear.

Although Dpp acts downstream of JNK signaling, as elevated Dpp signaling activity in the DME cells can substantially rescue dorsal closure defects resulting from the loss of JNK signaling (B. G. Fernandez et al. 2007; Glise and Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997; Sluss and Davis 1997), it is not just a linear relationship because the dorsal closure defects caused by the loss of the two pathways are different. Elongation of the DME cells is absent in JNK pathway mutants, but still occurs in Dpp signaling mutant embryos, indicating that the JNK pathway does not mediate effects in dorsal closure only through Dpp signaling (Ricos et al. 1999; Riesgo-Escovar and Hafen 1997). One supporting example is that Rho1 regulates DME cell elongation independent of Dpp signaling (Lu and Settleman 1999).
Conversely, JNK signaling is not the only input for *dpp* expression in the DME cells during dorsal closure as a zinc finger transcription factor, Pannier (Pnr), is also required (Herranz and Morata 2001). The expression of *pnr* overlaps with *dpp* in the DME cells, and *dpp* expression is diminished in these cells in *pnr* mutant embryos. Furthermore, *puc* expression remains wild-type in *pnr* mutant (Herranz and Morata 2001). These results indicate the JNK signaling and Pnr regulate *dpp* expression independent of each other. The action of Pnr and JNK signaling is limited to the DME cells, since the ventral lateral stripe of accumulated *dpp* expression is not affected by the alteration of Pnr or JNK signaling (Herranz and Morata 2001; McEwen et al. 2000). The Wingless (Wg) pathway is required for both stripes of *dpp* expression, as in strong *wg* mutant embryos, *dpp* expression in both stripes is greatly reduced (McEwen et al. 2000; Morel and Arias 2004). However, it is also been suggested that the function of the Wg pathway is in the maintenance of the pattern of *dpp* expression but not the establishment (Morel and Arias 2004).
Figure 1.4  Simplified schematic illustration of the Dpp signaling pathway in
Drosophila

Once Dpp reaches the target cell, the active receptor complex forms and activates Mad. The
activated Mad form a complex with Medea, which then translocate to the nucleus to regulate the
transcription of target genes, such as \textit{Dad} and \textit{Brk}. Both \textit{Dad} and \textit{Brk} provide negative
feedbacks to Dpp pathway, with \textit{Dad} inhibiting Mad binding with the receptor complex, and \textit{Brk}
being a transcriptional repressor that blocks the binding between Mad-Medea transcription factor
and target genes.
1.4. Role of zipper gene in dorsal closure

Dorsal closure is driven in part by myosin-dependent cell shape change in the epidermis and the amnioserosa. The accumulation of non-muscle myosin II together with F-actin at the leading edge forms a cable that constricts the DME cells to elongate in a dorsal ward direction; furthermore, lack of Myosin II in single amnioserosa cells slows down their apical surface area reduction showing that apical constriction is an active process driven by Myosin II (Franke et al. 2005; Young et al. 1993). Thus, as part of the force generator, loss of non-muscle myosin II heavy chain expression from the zipper (zip) gene makes a significant contribution to dorsal closure failure. In wild-type embryos, zip is highly expressed in both the amnioserosa and DME cells during germband retraction, however, around the time that dorsal closure commences, the expression in amnioserosa is shut off, while the DME expression remains at a high level (Figure 1.5).

zip mutant embryos have dorsal closure defects, at least due in part to disruption of the actomyosin “purse-string” operating in the DME cells (Franke et al. 2005). The restoration of Myosin II through expression of a zip transgene in either the epidermis or the amnioserosa can suppress the dorsal closure defects of zip mutant embryos, indicating a role for myosin in both tissues. Loss of zip expression in the DME cells of tkv mutant embryos suggests that zip expression is dependent on Dpp signaling (Arquier et al. 2001). However, ectopic activation of the TGFβ pathway in the epidermis does not result in ectopic zip expression, which implies other independent input(s) might be required for Dpp-mediated zip expression.
Figure 1.5  

zip expression in wild-type embryos

zip FISH on wild-type embryos (A-B) embryos in early germband retraction (A) or mid-germband retraction (B) stage showing upregulation of zip expression in both the amnioserosa and DME cells. (C) Embryo at dorsal closure stage showing zip expression remains high in DME cells but has shut off in the amnioserosa. Scale bar: 50 µm.
1.5. The Rho subfamily of small GTPases in dorsal closure

The small GTPases serve as “molecular switches” cycling between an “on” and “off” status by binding to GTP and GDP, respectively. It has been found that all the *Drosophila* Rho family members Rac, Cdc42 and Rho function in dorsal closure (Harden 2002). Embryos expressing a dominant negative version of *Rac1*, *Rac1N17*, show loss of the cytoskeleton at the leading edge of the DME cells, failure of cell elongation in the epidermis and failure to close the dorsal hole (Harden et al. 1995; Harden et al. 1999). The dorsal closure defects in *Rac1* dominant negative mutants are similar to those caused by the loss of JNK pathway members and can be partially rescued by the expression of a constitutively active form of *Jun* (Hou et al. 1997). In addition, expression of the constitutively active version of *Rac1*, *Rac1V12* induces ectopic expression of *puc* and *dpp* in a *hep*-dependent manner (Glise et al. 1995). The fact that expression of constitutively active *jun* only partially suppresses the dorsal closure defects in embryos expressing *Rac1N17* suggests that the JNK pathway is not the only route of *Rac1* activity in dorsal closure. Embryos in which all three *Drosophila* Rac genes (*Rac1*, *Rac2* and *Mtl*) were knocked out show little or no actin accumulation at the leading edge of the DME cells and lack filopodia and lamellipodia (Hakeda-Suzuki et al. 2002). Additionally, expression of constitutively active *Rac1* in the amnioserosa elevates F-actin and non-muscle myosin in the amnioserosa, which leads to premature apical constriction of the amnioserosa cells (Harden 2002).

*RhoA* deficient embryos either carrying a loss-of-function mutation or expressing a dominant negative *RhoA* transgene have dorsal closure defects (Harden et al. 1999; Magie et al. 1999). RhoA is probably required for contractility of the leading edge cytoskeleton through its effects on myosin (Harden 2002). RhoA signaling, via Rho-kinase, activates non-muscle myosin II in two ways: direct phosphorylation of myosin regulatory light chains and inactivation of myosin phosphatase (Halsell et al. 2000; Mizuno et al. 2002). The phosphorylated myosin regulatory light chains can be inactivated through dephosphorylation by myosin phosphatase which consists of a catalytic subunit, protein phosphatase 1c, the myosin-binding subunit and two non-catalytic subunits (Alessi et al. 1992; Hartshorne 1998; Totsukawa et al. 2000), whereas
Myosin phosphatase can be inactivated through phosphorylation of its myosin-binding subunit by Rho-kinase (Totsukawa et al. 2000; Winter et al. 2001).

Similar to the expression of constitutively active Rac1, expression of constitutively active Cdc42 in the DME cells can also induce ectopic activation of the JNK cascade during dorsal closure, which implies that Cdc42 is an upstream activator of the JNK cascade (Glise and Noselli 1997). However, reduction of Cdc42 during dorsal closure produces phenotypes different from that of Rac1-deficient embryos, indicating that Cdc42 has roles distinct from Drac1 in dorsal closure. Due to a requirement for Cdc42 in oogenesis, it is not possible to look at dorsal closure in embryos mutant for both maternal and zygotic Cdc42. Nevertheless, Cdc42-deficient embryos with dorsal closure defects can be derived from mothers bearing a heteroallelic combination of weak and strong Cdc42 alleles (Genova et al. 2000). In addition, embryos expressing a dominant Cdc42 transgene, Cdc42N17, display dorsal closure defects. Partial loss of the leading edge cytoskeleton in Cdc42N17-expressing embryos suggests that Cdc42 is involved in the establishment and/or maintenance of the leading edge cytoskeleton, similar to Rac1 (Harden et al. 1999; Riesgo-Escovar et al. 1996). However, several studies indicate Cdc42 may also function downstream of the TGFβ pathway. The "bunched" segment phenotype frequently seen in TGFβ pathway mutants is also found in Cdc42N17 expressing embryos (Harden et al. 1999; Ricos et al. 1999). However, dpp transcription in the DME cells of Cdc42 deficient embryos is not altered, indicating that Cdc42 does not act upstream of TGFβ signaling in generating the "bunched" segment phenotype and that Cdc42 is not a major activator of the JNK cascade. Importantly, dorsal closure defects in tkv7 embryos can be suppressed by expressing constitutively active Cdc42 suggesting that Cdc42 interacts with TGFβ signaling somewhere downstream of the Tkv receptor (Ricos et al. 1999). These data suggested that Cdc42 may be a component of the TGFβ signaling pathway or that Cdc42 and the TGFβ pathway may share the same target gene(s)/protein(s). Whether Cdc42 participates in mediating dorsal closure through the TGFβ pathway or in a parallel manner to the TGFβ pathway is still elusive. Identifying downstream effectors of Cdc42 in dorsal closure will provide us with clues to solve this problem.
1.6. *Drosophila* activated Cdc42-associated kinase

Activated Cdc42-associated kinases (Ack) are a family of non-receptor tyrosine kinases. ACK family tyrosine kinases specifically associate with Cdc42 and cannot bind to other small GTPases, such as Rac1 and RhoA (Elliot-Smith et al. 2005; Mott et al. 1999). The *Drosophila* ACK family has two members, Ack and PR2 (Sem et al. 2002). A typical Ack protein contains three functional domains: the amino terminal tyrosine kinase catalytic domain, a SH3 domain and Cdc42/Rac interactive binding (CRIB) domain at the carboxyl terminal. The CRIB domain was identified by sequence comparisons between various Cdc42/Rac effector proteins, but in the ACKs does not appear to bind to Rac (Burbelo et al. 1995).

Previous studies in the Harden lab demonstrated that Ack encodes a 1,073-amino-acid protein with a molecular weight of 118 kDa (Sem et al. 2002). Embryo whole mount *in situ* hybridization and Northern blot experiments indicated a single Ack transcript of 4.75kb that is widely expressed during embryogenesis with enriched transcription in the head and the DME cells during dorsal closure. The second *Drosophila* ACK member, PR2, encodes two predicted protein isoforms of 1,274 and 1,356 amino acids which differ at the N-terminus. Like Ack, PR2 is also highly expressed in the DME cells during dorsal closure and is enriched in the developing embryonic tracheal system (Baharak Zahedi, Ph.D. dissertation (Zahedi 2005)).

There is evidence indicating that Ack is a downstream effector for Cdc42 during dorsal closure. For example, transcription of Ack during dorsal closure responds to alterations in Cdc42 signaling by expression of dominant negative or constitutively active versions of Cdc42. Furthermore, Ack overexpression can suppress the dorsal closure defects of embryos expressing dominant negative Cdc42 ((SEM et al. 2002); Baharak Zahedi, Ph.D. dissertation (Zahedi 2005)). Although Cdc42 cannot bind to Ack, the results mentioned above suggest that it can regulate Ack via transcription, and in a tissue-specific manner. Global expression of Cdc42 in the embryo leads to transcriptional up-regulation of Ack specifically in the amnioserosa, indicating that Ack could be an effector for Cdc42 in this tissue. Ack loss-of-function mutant females survive to adulthood and produce progeny with only a very low frequency of dorsal closure defects, suggesting possible redundancy with PR2.
There is little known about substrates, binding partners or downstream effectors for the fly Acks. Most studies on Acks come from cell culture-based research and might provide some clues for Ack substrates and binding partners that may provide insight in further understanding how Ack is involved in the regulation of dorsal closure. Studies on the mammalian ACK family indicate that it is involved in mediating endocytosis. Clathrin is the main structural component of clathrin-coated vesicles and clathrin-mediated endocytosis plays a critical role in receptor internalization. Both mammalian ACK1 and ACK2 were reported to co-localize with and bind to the clathrin heavy chain (Teo et al. 2001). Co-expression of mammalian ACK2 and its binding partner SNX9 significantly reduce epidermal growth factor receptor (Egfr) levels upon Egf stimulation, and implies that ACK2 together with SNX9 may promote the degradation of Egfr (Q. Lin et al. 2002). This hypothesis is supported by another study, which demonstrated that ARK-1, the ACK protein in C. elegans, inhibits Let-23-mediated ovulation (let-23 encodes an Egfr homolog) (Hopper et al. 2000). In addition, a direct interaction between the carboxyl terminus of ACK1 and tyrosine-phosphorylated Egfr was reported (F. Shen et al. 2007). Knocking-down expression of ACK1 through ACK1-RNAi inhibits endocytotic degradation of Egfr. Taken together, these studies indicate that ACK plays an important role in regulation of Egfr degradation. ACK1 has been identified as an ubiquitin-binding protein (F. Shen et al. 2007). ACK1 is able to bind both poly- and mono-ubiquitin at its ubiquitin-association domain at the carboxyl-terminal. Ectopic expression of ACK1 with deletion of the ubiquitin-association domain blocks ligand-induced degradation of Egfr. The results suggest that ACK regulates Egfr degradation through endocytosis and ubiquitinization. If Ack is regulating dorsal closure, it is of interest to consider if the Egfr pathway is involved in this process.

1.7. Egfr signaling in Drosophila

Epidermal growth factor receptor (Egfr) signaling is one of the key signaling pathways participating in the development of metazoans. In Drosophila, it is used in many developmental processes such as spermatogenesis, oogenesis, embryogenesis, and larval, pupal and adult development, where it functions to direct cell fate, cell division, cell survival and migration (Shilo 2003).
The Egfrs are a subfamily of receptor tyrosine kinases in the ErbB family of receptors. Unlike humans, where there are multiple members, there is only one Egfr in the fly genome (Shilo 2003), which was identified by Stanley Cohen as a 170 kD membrane glycoprotein composed of an extracellular region, a hydrophobic transmembrane region and an intracellular region (Ushiro and Cohen 1980). The extracellular region containing two highly conserved cysteine rich domains is responsible for ligand binding. The tyrosine kinase domain resides in the intracellular cytoplasmic region, and is followed by the carboxyl-terminal tail with regulatory tyrosine residues. Egfr is activated through ligand binding, otherwise, the inactive Egfr stays mostly as a monomer. The binding between ligand and the amino-terminal extracellular portion of Egfr leads to dimerization of the receptors, which allows the cytoplasmic tyrosine kinase domains to cause trans-auto-phosphorylation on the tyrosine residues within the carboxyl-terminus (R. Clifford and Schupbach 1994). The activated Egfr then transduces the signal into the cell through further phosphorylation of and/or physical association with downstream signaling pathway components.

Despite the diverse roles of Egfr signaling, its principle downstream cascade is the Ras/Raf/MEK/MAPK pathway which in most cases appears to be unbranched (Shilo 2005) (Figure 1.6). A primary consequence of Egfr activation is the docking of the adapter protein Downstream of Receptor Kinase (DRK; known as GRB2 in vertebrates) on the carboxyl-terminal phospho-tyrosine residue of the receptor. Following that, DRK recruits the guanine nucleotide exchange factor Son of Sevenless (SOS), which activates the GTPase Ras. The molecular switch Ras, which is now in the GTP-bound on state, recruits and activates the serine/threonine kinase Raf. Once activated, Raf phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK (Figure 1.6). The main event after that is the translocation of MAPK from the cytosol to the nucleus, where it phosphorylates and modulates the activity of specific transcription factors, thus adjusting gene expression (Sundaram 2006).
Figure 1.6  Simplified schematic illustration of the Egfr signaling pathway in Drosophila

Spitz is the main ligand for the Drosophila Egfr pathway and plays roles in many different tissues. Spitz attaches to the extracellular domain of Egfr leading to its dimerization. In the canonical Ras/MAPK route, Egfrs phosphorylate each other within the dimer and activate the guanine exchange factor, SOS, which may require forming a complex with the adaptor protein DRK. SOS then activates Ras which subsequently phosphorylates its downstream effector, Raf, followed by Raf activating MEK. Eventually, MAPK is phosphorylated and transported into nucleus, where it mediates the expression of target genes of Egfr signaling.
A key mechanism by which the single receptor can perform such versatile roles is through the presence of various ligands that activate the pathway. Vein is produced as a secreted protein that activates Egfr in a restricted manner, and also serves as a positive feedback to reinforce the initial activation of Egfr by other ligands (Golembo et al. 1999; Schnepp et al. 1996). Keren, Gurken and Spitz are three other ligands for Egfr that are produced as inactive membrane-bound precursors requiring cleavage to release the extracellular EGF-containing domain as the active ligand (Shilo 2005). Keren is shown to complement Spitz in certain tissues, and Gurken is found function specifically in oogenesis, whereas Spitz is broadly expressed (Neuman-Silberberg and Schupbach 1993; Reich and Shilo 2002; Rutledge et al. 1992). Spitz represents the primary ligand that is used in most tissues and is itself regulated through an intracellular trafficking and processing machinery to achieve the spatial- and temporal- specific activation of the Egfr pathway (Shilo 2005). Argos is an inhibitory Egfr ligand which provides negative feedback by competing with Spitz for receptor binding or by sequestration of Spitz (Jin et al. 2000; Klein et al. 2004).

The negative regulation of Egfr signaling is also provided by Kekkon, Sprouty and YAN that target the Egfr pathway at different levels. Kekkon is a membrane protein and binds to the extracellular domain of Egfr and attenuates receptor dimerization (Ghiglione et al. 1999; Ghiglione et al. 2003). Sprouty is an intracellular protein that has been shown to not only target the receptor but also cytoplasmic components to repress Egfr signaling (Casci et al. 1999). Finally, the transcription repressor, YAN (also known as Aop), represses Egfr at the level of transcription of its target genes by masking the DNA binding site for transcriptional activators (Gabay et al. 1996). Unlike the ubiquitously expressed Argos, the negative regulation provided by these inhibitors is tissue specific.

Another strategy to accomplish the multiple outcomes of the Egfr pathway is via interaction with other signaling pathways. There is evidence suggesting that the Egfr pathway and Wg signaling interact antagonistically in the Drosophila larval epidermis (Szuts et al. 1997). During Drosophila eye development, Egfr pathway interacts with the Notch signaling pathway, where there is both antagonism and cooperation between the two pathways (Doroquez and Rebay 2006). A final example of signaling interactions
with the Egfr pathway involves Dpp signaling, which also has both antagonistic and synergistic interactions (Szuts et al. 1998; Wappner et al. 1997).

A role for the Egfr pathway in dorsal closure hadn’t been specifically addressed by other workers. As reviewed by Ben-Zion Shilo, there is “A thousand and one roles for the *Drosophila* Egfr” (Schweitzer and Shilo 1997), and we are interested to find out if Egfr plays a role during dorsal closure, which is a major morphogenetic event during embryogenesis.

### 1.8. Ecdysone signaling

The steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone or 20E) is a key regulator of cell death and cell shape changes during *Drosophila* metamorphosis and a number of results suggest a role for this hormone in dorsal closure. The amnioserosa is considered a major source of 20E during embryogenesis, and mutants of the Halloween group of genes, which encode enzymes in the 20E biosynthetic pathway, display dorsal closure defects (Chavez et al. 2000; Giesen et al. 2003; Kozlova and Thummel 2003). 20E, which is the only molting hormone in *Drosophila*, is synthesized from dietary cholesterol by a series of terminal cytochrome P450 hydroxylases. Genes encoding the 20E biosynthesis enzymes include *neverland* (*nvd*), *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*) (Rewitz et al. 2006).

The responses to 20E are usually mediated by the transcriptional regulatory functions of a heteromeric nuclear receptor, consisting of the ecdysone receptor (EcR) and the fly orthologue of the vertebrate RXR receptor, Ultraspiracle (Usp) (Riddiford et al. 2000; Thummel 1996). EcR/Usp binding to ecdysone responsive elements (EcRE) can mediate repression in the absence of ligand and activation when the ligand is present (Cherbas et al. 1991) (Figure 1.7). *Drosophila* metamorphosis is the most intensively studied process regulated by ecdysone signaling, where this single steroid hormone transforms tissues in the transition from larva to adult. The ecdysone signal is transduced and amplified via a two-step regulatory hierarchy (Ashburner 1974). Pulses of ecdysone at specific time points during development directly induce a small set of
widely expressed “early” genes, at least three of which encode transcription factors. These early genes both repress their own expression and induce more than 100 “late” secondary response genes (Thummel 1995). *IMP-E1* and *IMP-L1* are two of the ecdysone inducible genes, which were used as indicators of canonical ecdysone signaling activity in my study.
Figure 1.7  Simplified schematic of canonical ecdysone signaling in Drosophila

The pathway is activated when 20E binds to EcR which is in a dimer with Usp. The ligand receptor complex then recognizes the ecdysone responsive element (EcRE) in the target gene, and turns on their transcription.
EcR, the only ligand-dependent nuclear receptor identified to date in *Drosophila*, is induced directly by 20E, suggesting an autoregulatory loop that increase the level of receptor protein in response to its ligand. The gene coding for EcR produces three isoforms, EcR-A, EcR-B1 and EcR-B2, which differ in their amino-terminal sequences but have identical DNA binding domain (DBD) and ligand binding domain (LBD) sequences (Talbot et al. 1993). While Usp is the only known *Drosophila* homolog of the vertebrate RXRs, all three EcR isoforms require heterodimerization with Usp for DNA binding (Henrich et al. 1990; Thomas et al. 1993). Although the 20E binding motif is inside EcR, the heterodimerization with Usp ensures the ligand receptor interaction. Upon ligand binding, the EcR-Usp heterodimer is further stabilized, in addition, this also increases the affinity for binding to ecdysone responsive elements (EcREs) (Horner et al. 1995). All available in vivo evidence indicates that these proteins function as a heterodimer, supporting data including the antibody staining of larval salivary gland polytene chromosomes, where all sites occupied by EcR also showed Usp signal (Talbot et al. 1993). Unlike EcR, other nuclear receptor superfamily members participating in ecdysone regulation do not show direct interaction with ecdysone, such as *seven-up* (*svp*), *knirps-related* (*knrl*) and *DHR38* (Thummel 1995). These nuclear receptors usually affect the ecdysone response by interfering with the ecdysone receptors. For instance, *DHR38* forms an inactive heterodimer with Usp to prevent activation of the target promoter (Sutherland et al. 1995), whereas *svp* forms a heterodimer with EcR to do the same (Zelhof et al. 1995).

One pulse of ecdysone production is required during embryo development which occurs between 6-10 hours after egg laying with a peak at 8 hours, suggesting a role for ecdysone during embryogenesis (Chavoshi et al. 2010). In addition, embryos with loss of ecdysone due to mutations in the Halloween group of genes all fail to close the dorsal hole (Giesen et al. 2003). Consistent with a role for ecdysone in dorsal closure, a GAL4-based ligand sensor system, which assays in vivo activities of the ligand-binding domain of nuclear receptors fused to the GAL4 DNA-binding domain, reveals that embryonic EcR and Usp activity first appears in the amnioserosa (Kozlova and Thummel 2003). Despite the evidence that 20E functions in embryonic development, the roles and mechanism of ecdysone signaling in the embryo and dorsal closure remained poorly defined at the start of this project.
1.9. Goals of this thesis

Dorsal closure is a process where cells and tissues coordinate their morphogenesis and movement to achieve perfect “healing” of the epidermis and communication between cells and tissues is essential. It was apparent at the outset of this work that this involves the cooperation between multiple pathways modulating communication between tissues. Figuring out the signaling network regulating dorsal closure could provide a roadmap for understanding other epithelial fusions, and the overall goal of this thesis was to better understand this network, with a particular focus on the transcriptional modulation of the force generator Myosin II transcribed from the zip gene. Additionally, zip turned out to be a good choice for study as it provided a read out for activation of the JNK pathway, a central component of the signaling regulating dorsal closure.

Previous results from our lab suggested the requirement of inputs in addition to Dpp signaling on zip gene expression. Furthermore, evidence from our lab and others suggested the existence of signaling back and forth between the “wounded” epithelium and the tissue occupying the hole in both dorsal closure and other epithelial fusion events. For example, in vertebrate wound healing there is crosstalk between keratinocytes and the fibroblasts occupying the hole that leads to transcriptional responses in both cell types, including TGF-β signaling from keratinocytes to fibroblasts, which in turn promotes differentiation of the fibroblasts into contractile myofibroblasts, and secretion of growth factors from fibroblasts to stimulate proliferation and differentiation of keratinocytes (Werner et al. 2007). To identify new components of the signaling network, we largely used a candidate gene approach.

In this thesis, three candidates, Ack, Egfr signaling and ecdysone signaling, are studied for their roles as regulators of zip expression and dorsal closure. Efforts have been made to address the relationships between the candidates and well-known dorsal closure participants, such as the Dpp pathway and JNK signaling, thereby positioning the new players into the framework of the existing signaling network (Figure 1.8). What is learned from this study could provide more information on how tissues coordinate their morphogenesis during development and tissue regeneration.
This figure shows part of the dorsal closure signaling network I was working with at the start of my thesis work. For clarity not all components of the cascades are shown. Route of pathway activation upstream of Rac1 is unknown. Dpp is present in DME cells prior to JNK activation, but is maintained in DME cells by JNK pathway during dorsal closure. Data from our lab and other groups indicates that Dpp signals from the DME cells to the amnioserosa. Dpp signaling is required for zip expression in both the amnioserosa and DME cells, but not sufficient for zip expression, thus other inputs need to be identified.
2. Material and Methods

2.1. Fly stocks

The Ack mutant alleles, $\text{Ack}^{1ob}$ and $\text{Ack}^{86}$, were generated by previous graduate students in the lab through imprecise excisions of the P element, $\text{KG00869}$ (Xing Xu, M.Sc. thesis (XU 2005), Baharak Zahedi, Ph.D. dissertation (ZAHEDI 2005)). $\text{UAS-Ack}$, and $\text{UAS-KD-Ack}$ transgenes were made by previous graduate students in the lab (Sem et al. 2002). $\text{PBc02472}$, a $\text{PR2}$ hypomorphic allele was obtained from S. Artavanis-Tsakonas, $\text{UAS-tkv}^{Q199D}$ (TAJ3), $\text{UAS-tkv}^{DN}$ from A. Garcia-Bellido, $\text{UAS-dpp-GFP}$ from SM Cohen, $\text{ush}^2$ and $\text{hn}^{204a}$ from H. Lipshitz, $\text{Egfr}^{2c82}$, $\text{Egfr}^{1F26}$, $\text{Egfr}^{A.2}$ and $\text{Egfr}^{A.4}$ from T. Schupbach, $\text{UAS-Egfr}^{EGFP}$ from J. Duffy, $\text{UAS-sSpi}$ from B. Shilo, $\text{spo-Gal4}$ and $\text{spo}^{2339}$ were gifts from M. O’Connor, $\text{LE-Gal4}$ from S. Noselli, $\text{Hs-Gal4}$ from J. Roote and $\text{UAS-svp}$ from Y. Hiromi. $\text{UAS-Ack-RNAi}$ and $\text{UAS-PR2-RNAi}$ were originally from National Institute of Genetics, Japan. $\text{Dad-lacZ}$, $\text{salm-lacZ}$ and $\text{brk-lacZ}$ were from E. Verheyen. All other stocks were obtained from the Drosophila Stock Centre at Bloomington, Indiana.

2.2. cDNAs

cDNAs for $\text{dpp}$ (RE20611), $\text{Ack}$ (GH10777), $\text{Dad}$ (GH23534), $\text{spo}$ (RE13908), $\text{IMP-E1}$ (IP15635), $\text{IMP-L1}$ (LP06390), $\text{svp}$ (GH08189), $\text{phyllopod}$ (RH04401), $\text{pvf2}$ (RH40211) and $\text{wg}$ (RE02607) were obtained from Canadian Drosophila Microarray Centre. $\text{zip}$ (LD21871) was obtained from Open Biosystems.

2.3. Antibodies

Mouse anti-phosphotyrosine (P-Tyr-100, #9411) was from Cell Signaling; rabbit anti-Ack was produced in the Harden lab (SEM et al. 2002); mouse anti-HA (sc-7392)
and rabbit anti-phosphotyrosine (sc-18182) were purchased from Santa Cruz Biotechnology; rabbit anti-GFP (G1544) was from Sigma; rabbit anti-jun (sc-25763) was purchased from Santa Cruz Biotechnology; mouse anti-β-galactosidase (Z278A) was from Promega; mouse anti-EcR (DDA2.7) was from the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-pMad was generously provided by Dr. E. Verheyen.

2.4. Cuticle preparations

Embryos at desired stage were collected on grape juice agar plates, and aged at least 30 hours in order to allow enough time for the secretion of the cuticle. After being transferred into a collecting basket, embryos were dechorionated in the dechorionation solution for 3 minutes. Then embryos were rinsed three times with washing solution. The dechorionated embryos were mounted on slides in Hoyer’s medium. Embryos were baked at 65°C in Hoyer’s until the embryos were cleared.

Reagents:

Dechorionation solution: 50% household bleach in 0.01 % Triton-X.

Wash solution: 0.01 % Triton-X in double distilled water.

Hoyer’s medium: 30 g of Gum Arabic was dissolved in 50 ml of water. Then, 200 g of chloral hydrate was added, followed by 16 ml of glycerol while stirring. The mix was centrifuged at 5000 rpm to 10000 rpm for 20 minutes to separate and remove the sediment. The medium was stored in the dark at room temperature.

2.5. Embryo fixation

Embryos at desired stage were collected on grape juice agar plates and rinsed off using washing solution into a collecting basket. The embryos were dechorionated for 3 minutes in dechorionation solution, followed by two 5 minute washes in the washing
solution. Then, the embryos were transferred into 10ml of fixing solution in a 20ml scintillation vial. The embryos were fixed by shaking the vial vigorously for 25 minutes at room temperature. After fixation, the bottom layer (paraformaldehyde and PBS) was removed. 10ml of 100% methanol (Caledon Laboratories Ltd, 6700-1) was added to the vial and the sample was shaken vigorously for 1 minute. After the embryos settled down, the top layer (heptane) was removed, and embryos were washed twice with methanol before storage at -20°C. Because some antibodies are sensitive to methanol treatment, methanol was substituted with 80% ethanol in those cases.

Reagents:

10X Phosphate buffered saline (PBS): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄ in 1000 ml distilled H₂O and adjusted to pH 7.4

20% Paraformaldehyde (PFA) stock solution: 10 g paraformaldehyde (Anachemia, UN-2213) was added to 35 ml of double distilled water with 0.5 ml of 1 N NaOH in a 50 ml Falcon tube. The solution was stirred and heated to 65°C until the paraformaldehyde dissolved. 5 ml of 10X Phosphate buffered saline and double distilled water were added to bring the final volume to 50 ml. The solution was stable at 4°C for one month.

Fixing solution: 5 ml heptane (Caledon Laboratories Ltd, 5400-1), 1 ml of 20% paraformaldehyde (freshly diluted from 20% paraformaldehyde stock solution), and 4 ml of 1X PBS. Storage solution: 100% methanol (Caledon Laboratories Ltd, 6700-1) or 80% ethanol.

2.6. Standard molecular techniques

cDNA clones were first streaked on LB plates with the appropriate antibiotic and incubated for 16-20 hours at 37°C. A single colony was picked up for liquid culture the next day. Plasmid was purified from overnight liquid culture using the Qiagen Plasmid Mini Preparation Kit. Purified cDNA clones were verified either by restriction digestion mapping or DNA sequencing. Bacterial strains carrying cDNA clones were stored in glycerol at -80°C. cDNA was dissolved in ddH₂O and stored at -20°C.
**LB medium**: for 1L of medium, Add 10g Bacto-tryptone, 5g yeast extract and 10g NaCl to 800ml H₂O. Adjust pH to 7.5 with NaOH, and volume to 1L with dH₂O. Sterilize by autoclaving.

### 2.7. Generation of digoxigenin-labeled RNA probes

The cDNA sequence of the gene of interest which was in a vector with T3, T7 or SP6 promoters was linearized at the 5’ end with an appropriate restriction enzyme to prevent transcription of the vector DNA. Anti-sense probe was synthesized using RNA polymerase and digoxigen-labeled NTP according to manufacturer’s instructions (all reagents from Roche). After purification using the MicroSpin S-200 HR columns (GE Healthcare, 27-5120-01), the probe were aliquoted and stored at -20°C.

### 2.8. Fluorescent *in situ* hybridization

The fluorescent *in situ* hybridization (FISH) was performed according to (Lecuyer et al. 2007). All reactions and washes were conducted at room temperature, and all washes and rinses utilized 1ml of solution unless specified. The fixed embryos in Eppendorf tubes were rehydrated through a series of rinses with methanol only, 1:1 mix of methanol and PBTween, and then PBTween alone. The rehydrated embryos were then post-fixed in 4% PFA for 20 min, followed by several rinses with PBTween. After post-fixation, the embryos were incubated with 3µg/ml proteinase K solution for 2 min on a rocking platform, and then left to stand in ice for 1 hour. To remove the proteinase, the embryos were rinsed twice with 2mg/ml glycine. Another round of post-fixation was performed to strengthen the overall structure of the embryos. The embryos were rinsed five times with PBTween to remove any remaining fixatives, and then prepared for incubation in the RNA hybridization solution by rinsing first with 1:1 of hybridization solution and PBTween, and then with the hybridization solution alone. The embryos were pre-hybridized with fresh pre-boiled hybridization solution for 2 hours at 56°C in an oven with rotation. During this time, the probe solution was prepared by adding around 100ng of the probe stock into 100µl of hybridization solution and heating up to 80°C for 3
min followed by cooling down on ice for 5 min. For overnight hybridization, the embryos were incubated in the probe solution without rotation in the 56°C oven.

After hybridization with the RNA probe, the hybridization solution was removed and embryos put through a series of washes including 20 min in fresh and pre-warmed (56°C) hybridization solution; 15 min in 3:1, 1:1, 1:3 mixes of pre-warmed hybridization solution and pre-warmed PBTween, and then four times 5 min in pre-warmed PBTween. The embryos were then blocked in 3% BSA for 10 min. The embryos were then incubated with 1:200 anti-dig-POD antibody (and in some cases with an appropriately diluted primary antibody against a protein of interest) in 3% BSA for 2 hours, followed by several 10 min washes with 3% BSA. After 2 hours of incubation with appropriate secondary antibodies in 3% BSA, the embryos were washed once with PBTween, and twice with PBS. The last step was the enzymatic substrate reaction between the POD and tyramide by incubation of the embryos with 1 in 100 dilution of cyanine tyramide substrate solution for 2 hours. Six washes with PBS were conducted to remove background signal, and then the embryos were re-suspended in Vectorshield mounting medium (Vector Laboratories, Inc. H-1000) overnight at 4°C. The embryos were imaged on either a Quorum WaveFX/Carl Zeiss Spinning disc confocal microscope or a Nikon A1R laser scanning confocal microscope.

**Reagents:**

**PBTween:** PBS containing 0.1% Tween-20.

**4% paraformaldehyde (PFA):** freshly diluted from the 20% paraformaldehyde stock solution in PBTween.

**3 µg/ml of proteinase K:** freshly diluted from 3 mg/ml of proteinase K (Sigma P6556) stock in PBTween.

**2 mg/ml of glycine:** 20 mg glycine (BioShop, Canada; GLN001.500) was dissolved in 10 ml PBTween (can be stored at 4°C for one month).

**Hybridization solution:** 50% deionized formamide, 4X SSC, 1X Denhardts, 0.1% Tween-20, 5% dextran sulphate, 250 g/ml salmon sperm DNA and 50 g/ml heparin, stored at -20°C.
3% Bovine serum albumin (BSA): 3g BSA (BioShop; ALB 001) was dissolved in 100 ml PBTweeen.

Anti-DIG antibody: Anti-digoxigenin-POD (Roche; 11207733910) was diluted in 3 % BSA before use.

Cyanine 3 tyramide substrate: 1:100 dilution of Cyanine 3 tyramide reagent (PerkinElmer, SAT705A) in amplification diluent supplied by the manufacturer.

2.9. Immunostaining of Drosophila embryos

Immunostaining was conducted as described (Harden et al. 1996). All incubation and wash steps were performed at room temperature with rotation, using 1ml of solution unless otherwise stated. Fixed embryos were rehydrated using three times 20 min washes in PB Triton. Then embryos were blocked in 1 % BSA for 1 hour. Appropriately diluted primary antibodies were added into the embryos and the incubation was usually done for 2 hours at room temperature or overnight at 4°C. After removal of the primary antibody, the embryos were washed for three times 20 min in 1 % BSA. The desired secondary antibody conjugated with a fluorescent label was incubated with embryos for 2 hours at room temperature. Note that the secondary antibody incubation was conducted in the dark. Sometimes, a biotin-conjugated secondary antibody (1:200) was used, followed by streptavidin-FITC or Texas Red (1:1000) to amplify signal. Once the fluorescently-labeled secondary antibody incubation was done, three times 10 min washes with PBS were applied before resuspending embryos in Vectashield mounting medium. The embryos were imaged on either a Quorum WaveFX/Carl Zeiss Spinning disc confocal microscope or a Nikon A1R laser scanning confocal microscope.

Reagents:

PBTriton: 0.01 % Triton-X in PBS.

1% BSA: 1g of bovine serum albumin (BioShop, ALB 001) was dissolved in 100 ml PBTriton.
2.10. 20 hydroxyecdysone incubation of Drosophila embryos

The 20 hydroxyecdysone (20E) incubation of Drosophila embryos was conducted according to (Kozlova and Thummel 2003). Embryos 3-7 hours after egg laying were collected and dechorionized in 50% bleach. After several rinses with water, the embryos were transferred to a scintillation vial containing 6ml heptane and 2ml Modified Basic Incubation Medium (MBIM) with 5 x 10⁻⁶ M 20E (powder from Sigma, dissolved in ethanol) and permeabilized for 2 min with gentle swirling. After carefully removing the heptane without taking up the embryos, the last bit of solvent was allowed to evaporate before adding another 2ml of MBIM with 20E. The embryos were then soaked in this 20E medium for 4 hours at 25°C in a moist chamber. Finally, the embryos were fixed according to the embryo fixation protocol, and stored at -20°C for further immunostaining or FISH experiments. The control embryos were treated exactly the same, except for using ethanol alone instead of 20E dissolved in ethanol.

MBIM: for 100 ml of medium, add 0.22 g MgCl₂·6H₂O, 0.297 g MgSO₄·7 H₂O*, 0.042 g MaH₂PO₄*, 1.21 g Glutamic acid*, 0.605 g Glycine*, 0.066 g Malic acid*, 0.0027 g Sodium acetate, 0.22 g Glucose* into 86 ml double distilled H₂O. Adjust pH to 6.8 using a mixture of equal amounts of 5% NaOH and 5% KOH, then add 0.099 g CaCl₂, and double distilled H₂O up to 100 ml. Filter sterilize through a 0.2 µm filter. Items identified by an asterisk must be tissue culture reagent grade.

2.11. Proximity ligation assay

The proximity ligation assay (PLA) was performed according to the manufacturer’s instructions (Sigma-aldrich, Duolink in situ PLA kit). The fixed embryos of the desired genotype were first incubated with mouse anti-EcR and rabbit anti-Jun primary antibodies as described in section 2.9, Immunostaining of Drosophila embryos. After three times 10 min washes using PBTriton, the embryos were then incubated for 1.5 hours at 37°C with Duolink anti-rabbit plus probe and anti-mouse minus probe (tagged with plus and minus oligonucleotides, respectively) diluted 1:5 in the 1X diluent provided in the kit. The secondary antibodies were removed using two washes of 1X
wash buffer A (provided in the kit) at room temperature. The ligation reaction was performed by adding the ligation solution and then incubating for 1 hour at 37°C, during which time the two oligonucleotides from the ligation stock will hybridize with the two PLA probes and join to form a closed circle if they are in close proximity. The embryos were washed in 1X wash buffer A again to remove the ligase and other reagents. Then the PLA signal was generated by incubating in the amplification solution, which contains nucleotides and cyanine3 fluorescently labeled oligonucleotide probes, for 2 hours at 37°C. Note that starting from this step, the reaction was light sensitive, so the embryos had to be kept in the dark. The embryos were then incubated with FITC- and Texas Red fluorescently-labeled mouse and rabbit secondary antibodies for another 2 hours at room temperature to reveal the staining of the two primary antibodies. After washes with 1X buffer B and 0.01X buffer B (provided in the kit), the embryos were mounted in Duolink II Mounting Media with DAPI overnight before imaging on a Nikon A1R laser scanning confocal microscope.

Reagents:

**Ligation solution**: dilute the ligation stock 1:5 and ligase 1:40 in high purity water and mix. E.g. for a 40 µl reaction take 8 µl of the 5x Ligation stock and 1 µl of ligase to 30 µl of high purity water.

**Amplification solution**: dilute the amplification stock 1:5 and polymerase 1:80 in high purity water and mix. E.g. for a 40 µl reaction take 8 µl of the 5x amplification stock and 0.5 µl of ligase to 30.5 µl of high purity water.
3. Results part I: Dpp cooperates with ACK to regulate myosin levels during dorsal closure

Work from this section was published in “Leading edge-secreted Dpp cooperates with ACK-dependent signaling from the amnioserosa to regulate myosin levels during dorsal closure” by Baharak Zahedi, Weiping Shen, Xing Xu, Xi Chen, Meena Mahey, and Nicholas Harden (Developmental Dynamics 237:2936-2946, 2008). My contribution was in the zip FISH, Dad FISH, anti-HA, anti-Ack, anti-GFP and anti-PY immunostaining experiments, and preparation of GFP negative homozygous mutant embryos for cuticle preparations.

We previously showed that expression of a constitutively active version of the small GTPase Cdc42 can suppress the dorsal closure defects associated with a tkv loss-of-function allele, tkv<sup>−</sup> (Ricos et al. 1999). A likely route by which Cdc42 drives the suppression of the tkv phenotype is through activation of a downstream effector protein. Ack and PR2 are the two Drosophila members of the activated Cdc42-associated kinase (ACK) family of non-receptor tyrosine kinases (Burbelo et al. 1995; Clemens et al. 2000; Manser et al. 1993; Sem et al. 2002; Worby et al. 2002). Throughout this chapter, the general term ACK is used to refer to any or all members of the ACK family. When a point is made about a particular ACK protein, its specific name is used. Ack over-expression can suppress the dorsal closure defects caused by expression of a dominant negative version of Cdc42, and expression of constitutively active Cdc42 causes elevation of Ack transcript levels in the amnioserosa during dorsal closure (Sem et al. 2002). These results make the ACK proteins promising candidates for Cdc42-effectors impacting Dpp signaling, and here we show that ACK-dependent signaling from the amnioserosa cooperates with Dpp secreted from the leading edge to drive zip expression in these two tissues. As the zip product is required in both tissues for cell shape change (Franke et al. 2005), this transcriptional regulation may provide a means for coordinating morphogenetic events during dorsal closure.
3.1. Dpp signaling is necessary but not sufficient for zip expression in the DME cells and amnioserosa

In addition to controlling morphogenesis of the epidermis, the Dpp pathway is required for apical cell constriction in the amnioserosa, but the route by which Dpp regulates this cell shape change is not clear (B. G. Fernandez et al. 2007). To determine if the amnioserosa cells are exposed to Dpp ligand produced in the DME cells, we expressed GFP-tagged, biologically active Dpp (Teleman and Cohen 2000) using the LE-Gal4 driver, which is expressed in a subset of DME cells (Glise and Noselli 1997) (Figure 3.1 A), and stained embryos with anti-GFP antibodies to reveal Dpp-GFP over the amnioserosa (Figure 3.1 B). Dpp secreted from the DME cells signals back into these cells to promote their morphogenesis, at least in part by driving zip expression, and we wondered if Dpp migrating over the amnioserosa might similarly drive zip expression in the amnioserosa cells to promote apical constriction (Arquier et al. 2001; Franke et al. 2005). One way to ensure coordinated morphogenesis of neighboring tissues would be to have a signal from one tissue transcriptionally regulating a required component of morphogenesis in another, and we looked for Dpp-dependent zip expression in the amnioserosa. Using high sensitivity fluorescent RNA in situ hybridization (FISH) with tyramide signal amplification, we detected zip transcription in the amnioserosa that was highest at the beginning of germ band retraction and had disappeared by the beginning of dorsal closure (Figure 3.1 C-E). In contrast, DME expression of zip was weak during germ band retraction but high during dorsal closure. To assess the requirements for Dpp signaling in the regulation of zip expression, we performed zip FISH on tkv\textsuperscript{-} mutant embryos and saw a reduction in zip levels in the amnioserosa during germ band retraction and a reduction in zip levels in the DME cells during dorsal closure (Figure 3.1 F, G). The latter finding confirms previous results using
digoxigenin *zip in situ* hybridization (Arquier et al. 2001). The Dpp pathway can be ectopically activated in the embryo through expression of a constitutively active version of the Tkv receptor, Tkv<sup>Q199D</sup>, or a *Dpp* transgene (Dorfman and Shilo 2001; Hoodless et al. 1996). Ectopic activation of Dpp signaling by expression of Tkv<sup>Q199D</sup> with *prd-Gal4* did not result in *zip* transcript elevation in *prd* stripes (data not shown), and we conclude that Dpp signaling is necessary but not sufficient for *zip* transcription. This result demonstrates a requirement for an additional input or inputs for transcriptional regulation of *zip*. 
Figure 3.1  zip expression in the amnioserosa and dorsal epidermis is regulated by Dpp and ACK
3.2. Ack cooperates with Dpp signaling in regulating zip expression

Given our previous finding that over-expression of Ack can suppress the dorsal closure defects caused by impaired Cdc42 signaling, and that activation of Cdc42 signaling could suppress the dorsal closure defects of tkv7 embryos, we tested to see if Ack over-expression would suppress the effects of loss of Dpp signaling (Ricos et al. 1999; Sem et al. 2002). Ubiquitous over-expression of Ack suppressed the dorsal defects of tkv7 embryos by around 40% (Table 3.1). The dorsal closure defects of tkv mutant embryos can be significantly suppressed by amnioserosa-specific expression of TkvQ199D and we wondered if similarly over-expressing Ack in this tissue alone would suppress Dpp pathway defects, particularly considering that global activation of Cdc42 signaling causes an upregulation of Ack expression specifically in the amnioserosa (B. G. Fernandez et al. 2007; Sem et al. 2002). Expression of Ack using the amnioserosa-specific driver AS-Gal4C381 (Manseau et al. 1997) suppressed the defects of tkv7 embryos by roughly 30% (Table 3.1). To see if Ack function had any bearing on zip
transcription in the amnioserosa, we expressed wild-type and kinase-dead (KD) Ack transgenes (Sem et al. 2002) using AS-Gal4\textsuperscript{C38f} and performed FISH. Surprisingly, both forms of Ack caused a dramatic increase in zip expression not only in the amnioserosa but also in the dorsal epidermis (Figure 3.1 I-L). When these transgenes were expressed with prd-Gal4 (the striped expression of which extends into the amnioserosa, see Figure 3.2 I), elevated zip expression was seen throughout the amnioserosa and dorsal epidermis, but not in prd stripes in the epidermis (Figure 3.1 M, N). We found that with either Gal4 driver, KD-Ack had a stronger effect on zip levels than wild-type Ack, and that both forms of Ack were capable of promoting zip expression in the amnioserosa during germband retraction (Figure 3.1 I, L and data not shown) and during dorsal closure (Figure 3.1 M, N and data not shown), when the amnioserosa normally shows no zip expression (compare Figure 3.1 M to E). The two Gal4 drivers, the expression patterns of which overlap only in the amnioserosa, caused essentially the same pattern of zip elevation, and our interpretation of these results is that Ack functions in the amnioserosa in a kinase-independent and cell non-autonomous manner to promote zip transcription on the dorsal side of the embryo. Based on these results, Ack overexpression could suppress the dorsal closure defects of tkv mutant embryos by restoring some zip expression in cooperation with residual dorsal Tkv activity, and DME and amnioserosa zip transcription in tkv\textsuperscript{7} mutant embryos over-expressing Ack was restored to near wild-type levels (Figure 3.1 H and data not shown).
Table 3.1  Genetic interaction between Ack and Dpp signaling in regulation of dorsal closure

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Dorsal defects in progeny of cross used to create indicated genotype*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) tkv^7, hs-Gal4/tkv^7</td>
<td>16.7 (66.8)</td>
<td>927</td>
</tr>
<tr>
<td>(B) tkv^7, hs-Gal4/tkv^7, UAS- Ack</td>
<td>9.9 (39.6)</td>
<td>707</td>
</tr>
<tr>
<td>(C) tkv^7, hs-Gal4/tkv^7</td>
<td>77.2</td>
<td>101</td>
</tr>
<tr>
<td>(D) tkv^7, hs-Gal4/tkv^7, UAS- Ack</td>
<td>47.6</td>
<td>42</td>
</tr>
<tr>
<td>(E) tkv^7/tkv^7, UAS- Ack (29°C)</td>
<td>25.3 (100)</td>
<td>572</td>
</tr>
<tr>
<td>(F) tkv^7/tkv^7, UAS- Ack; Gal4^{C381/+} (29°C)</td>
<td>16.7 (66.8)</td>
<td>534</td>
</tr>
<tr>
<td>(G) +/+; Ack^{10b}/Ack^{10b}</td>
<td>0.7 (1.4)</td>
<td>2632</td>
</tr>
<tr>
<td>+/+; Ack^{29b}/Ack^{29b}</td>
<td>0.4 (0.8)</td>
<td>258</td>
</tr>
<tr>
<td>+/+; Ack^{86}/Ack^{86}</td>
<td>5.4 (10.8)</td>
<td>1116</td>
</tr>
<tr>
<td>+/+; Ack^{86}/Ack^{86}</td>
<td>10.2 (20.4)</td>
<td>147</td>
</tr>
<tr>
<td>(H) dpp^{hr27/+}; Ack^{86}/Ack^{86}</td>
<td>1.5</td>
<td>336</td>
</tr>
<tr>
<td>dpp^{hr27/+}; +/TM3Sb</td>
<td>14.5 (29)</td>
<td>400</td>
</tr>
<tr>
<td>dpp^{hr92/+}; Ack^{86}/Ack^{86}</td>
<td>2.6</td>
<td>1480</td>
</tr>
<tr>
<td>(I) tkv^7/+; Ack^{10b}/Ack^{10b}</td>
<td>4.8 (19.2)</td>
<td>249</td>
</tr>
<tr>
<td>(J) tkv^7/+; +/+</td>
<td>0</td>
<td>1271</td>
</tr>
</tbody>
</table>

(A) tkv^7, hs-Gal4/CyO females x tkv^7/CyO males; (B) tkv^7, hs-Gal4/CyO females x tkv^7, UAS- Ack/CyO males; (C) tkv^7, hs-Gal4/CyO-twist-GFP females x tkv^7/CyO-twist-GFP males; (D) tkv^7, hs-Gal4/CyO-twist-GFP females x tkv^7, UAS- Ack/CyO-twist-GFP males. For both (C) and (D), tkv^7 homozygotes were selected prior to cuticle preparation based on absence of expression of twist-GFP reporter on CyO balancer, and result is representative of three separate experiments. (E) tkv^7/CyO females x tkv^7, UAS- Ack/CyO males; (F) tkv^7/CyO; Gal4^{C381}/Gal4^{C381} females x tkv^7, UAS- Ack/CyO males. Done at 29°C to maximize Gal4 expression; (G) Ack / Ack females x Ack/TM3Sb males (Ack homozygous males are sterile); (H) Initial cross: dpp/CyO females x Ack^{66}/TM3Sb males. For genetic interaction crosses, F1 males of genotype dpp^{+}/+; Ack^{86}^{+} were crossed to Ack^{86}/Ack^{86} females. For controls, F1 males of genotype dpp^{+}/+; TM3Sb^{+}/+ were crossed to w^{1118} females; (I) tkv^7/CyO; Ack^{10b}/TM3Sb females x Ack^{10b}/TM3Sb males. Note that tkv^7/CyO; Ack^{10b}/Ack^{10b} flies do not survive to adulthood; (J) tkv^7/CyO females x w^{1118} males. *Estimated % dorsal defects in homozygous mutant individuals are shown in brackets.
Our data suggest that Ack acts in parallel to Dpp signaling rather than functioning as either a component or a target of the Dpp pathway, and we did several experiments to address this further. First, we evaluated Ack for its ability to activate the Dpp pathway at either the receptor level or at the level of transcription of a Dpp-responsive gene other than zip. Activation of the Dpp pathway at the level of the receptor complex can be visualized by staining embryos with an antibody (anti-pMad) that recognizes the receptor-phosphorylation of Mad (Persson et al. 1998) (Figure 3.2 A, B). As a likely Dpp-responsive gene during dorsal closure, we chose Daughters against dpp (Dad), a Dpp target in the wing that is expressed in the amnioserosa and dorsal epidermis (Tsuneizumi et al. 1997; Wada et al. 2007) (Figure 3.2 D, E). We determined that the Dpp pathway is necessary and sufficient for Dad expression in embryos during dorsal closure. tkv\textsuperscript{7} embryos lost Dad expression, whereas expression of Dpp using prd-Gal4 caused an elevation of Dad levels in prd stripes (Fig 3.2 G, H). Over-expression of a wild-type Ack transgene failed to induce either pMad or Dad accumulation in prd stripes (Figure 3.2 C, I’). Similarly, Ack could not drive ectopic expression of spalt major (salm), another target of Dpp signaling that shows a similar expression pattern to Dad during dorsal closure (Lecuit et al. 1996; Nellen et al. 1996) (Figure 3.2 F and data not shown). We then checked to see if Ack was a target of the Dpp pathway, at the level of regulation of the quantity or activity of Ack protein. prd-Gal4 expression of either Dpp or Tkv\textsuperscript{Q199D} did not result in an increase in Ack protein levels (Figure 3.3 A’ and data not shown). Increased Ack tyrosine kinase activity due to over-expression of a Ack transgene in a tissue-specific manner can be visualized as an increase in phosphotyrosine levels in that tissue (Sem et al. 2002) (Figure 3.3 B’). This assay did not detect increased Ack tyrosine kinase activity in embryos expressing Dpp in prd stripes (Figure 3.3 C’).
Figure 3.2  Ack cannot activate the Dpp pathway

All embryos are at stage 13, (A-C) stained with anti-pMad, (D, G, H) hybridized with a Dad riboprobe, (E, F, I) stained with anti-β-galactosidase, (I) stained with anti-Ack. (A) Wild-type embryo showing pMad accumulation in dorsal epidermis (arrowhead) and ventrolateral epidermis. (B) prd-Gal4/UAS-dpp embryo, showing ectopic pMad in prd stripes. (C) Embryo expressing Ack in prd stripes showing wild-type pMad distribution. (D) Wild-type embryo showing Dad transcripts in dorsal epidermis. (E) Dad-lacZ embryo showing nuclear Dad reporter gene expression in dorsal epidermis and amnioserosa. The failure to detect amnioserosa Dad transcripts by in situ in (D) may be due to their being distributed throughout the large amnioserosa cells. Dad-lacZ embryo showed nuclear Dad reporter gene expression in both the dorsal epidermis and amnioserosa. (F) salm-lacZ embryo showing nuclear salm reporter gene expression in dorsal epidermis and amnioserosa. (G) tkv embryo showing loss of Dad mRNA expression. (H) prd-Gal4/UAS-dpp embryo, showing ectopic Dad transcripts in prd stripes. (I, I) UAS-Ack/+;Dad-lacZ/prd-Gal4 embryo, showing wild-type Dad-lacZ expression pattern.
Figure 3.3  Ack is not a target of the Dpp pathway

(A, A´) prd-Gal4/UAS-tkvQ199D embryo in which tkv is HA-tagged, stained with anti-HA (A) and anti-Ack (A´) to show lack of ectopic Ack expression where Dpp signaling is activated.  (B, B´) prd-Gal4/UAS-Ack embryo subjected to Ack FISH (B) and anti-phosphotyrosine (PY) staining showing elevated PY where Ack is over-expressed.  (C, C´) prd-Gal4/UAS-dpp-GFP embryo stained with anti-GFP (C) and anti-PY (C´) showing no elevation of PY where Dpp signaling is activated.
To characterize further the interaction of Ack with Dpp signaling, we generated loss-of-function alleles of Ack. Through excision of a P element, KG00869 (Bellen et al. 2004), inserted 5 base pairs upstream of the start point of transcription of the longer of the two Ack transcripts, we created Ack alleles that were homozygous viable and showed no detectable Ack transcripts on Northern blots of adult flies but retained expression of flanking genes (Figure 3.4 A, B). Cuticle preparations revealed that Ack mutant embryos had a low frequency of dorsal defects (Table 3.1) and we tested to see if reducing Dpp signaling at either the ligand or receptor level would have an effect on this phenotype. When Ack mutant embryos were made heterozygous for either of two embryonic lethal dpp alleles, dpp^{hr27} and dpp^{hr92} (St Johnston et al. 1990; Wharton et al. 1993), or tkv^{7}, the frequency of dorsal defects increased significantly (Table 3.1).

zip expression levels appeared near normal in Ack mutant embryos (data not shown), and we wondered if this was due to overlapping function with the other ACK, PR2. There are no null alleles available in PR2, but we determined that a piggyBac transposon insertion in the first intron of PR2 (Thibault et al. 2004) led to decreased PR2 transcript levels but did not affect transcription of neighboring genes (Figure 3.4 C, D). We found that PR2 Ack double mutant embryos showed a loss of zip transcription in the dorsal embryo (Figure 3.1 O). Amnioserosa-specific expression of RNAi transgenes targeting Ack and PR2 also resulted in loss of dorsal zip expression (Figure 3.1 P). Expression of either RNAi transgene alone did not cause a detectable decrease in zip levels (data not shown). Thus we conclude that Ack and PR2 cooperate in regulating zip expression. We could not quantify the effects of reducing ACK function on dorsal closure, as double mutant embryos, as well as embryos with ACK knocked down by RNAi, either in the amnioserosa or globally by heat shock using an Hs-Gal4 driver,
showed a high frequency of failures to secrete cuticle. However, many ACK-deficient embryos that secreted cuticle but did not hatch into larvae showed defects in the dorsal surface including holes in the cuticle and germband retraction failures (Figure 3.5 C-F). Failure to secrete cuticle, dorsal holes and germband retraction defects are also seen with Ack over-expression in the embryo (Sem et al. 2002). An interesting distinction was noted between the dorsal defects of embryos bearing RNAi knockdowns of both ACKs compared to embryos in which PR2 function was knocked down with RNAi and Ack function impaired with an Ack allele, in that the former tended to have large dorsal holes extending to the anterior end (indicative of defects in both dorsal closure and head involution), whereas the latter tended to have small anterior holes (indicative of head involution defect only) (Compare Figure 3.5 D, E with Figure 3.5 C, F). Ack RNAi could be eliminating maternal Ack transcripts that would be retained in a zygotic Ack mutant, and the result suggests that head involution may be more sensitive to loss of ACK than dorsal closure.
Figure 3.4 Molecular characterizations of Ack and PR2 mutations

(A) Schematic diagram (not to scale) of Ack genomic region showing the insertion site of KG00869 and flanking genes Fit1 and Chd64. Deletions in four Ack alleles are shown. Deletions were evaluated by Southern and Northern analysis, and in the case of Ack^{10b} using PCR amplification of genomic DNA. Some P element sequences are retained in Ack^{10b}. (B) Northern blots of total RNA from homozygous Ack mutant and w^{1118} adult flies showing that the two Ack transcripts are lost in Ack mutants, but transcripts of neighbouring genes are retained, with the exception of Ack^{52a} in which Chd64 transcripts are lost (other data for Ack^{52a} not shown). In our hands the Chd64 probe consistently hybridized to a streaked pattern of transcripts extending down to the rp49 control band, suggesting significant transcript degradation. This streaked pattern is not seen with Ack^{52a}. (C) Northern blot of total RNA from homozygous PR2^{pBC02472} mutant and w^{1118} adult flies showing that PR2 transcripts are reduced in PR2^{pBC02472} mutants, but transcription of neighbouring genes Cap-G and TppII is maintained. A probe against ribosomal protein 49 (rp49) was used as a loading control for blots in (B) and (C). (D) Schematic diagram (not to scale) of PR2 genomic region showing the insertion site of pBC02472 and flanking genes Cap-G and TppII. PR2 resides in an intron of Cap-G.
Figure 3.5  Cuticle phenotypes associated with perturbations of Dpp signaling and ACK function
(A) Wild-type embryo. (B) prd-Gal4/UAS-dpp embryo, showing defects in dorsal surface including dorsal anterior hole and failure of germband retraction. (C) Embryo from PR2pBC02472/CyO; Ack10b/TM3 stock showing dorsal anterior hole. (D) Embryo from cross in which PR2-RNAi and Ack-RNAi transgenes were induced by heat shock using Hs-Gal4 showing dorsal hole extending to the anterior end and germband retraction failure. (E) Embryo from cross in which PR2-RNAi and Ack-RNAi transgenes were induced in amnioserosa using AS-Gal4C381 showing large dorsal hole extending to the anterior end. (F) Embryo from cross in which PR2-RNAi transgene had been induced in amnioserosa using AS-Gal4C381 in an Ack10b mutant background showing dorsal anterior hole. (G) Chart showing frequencies of cuticle defects in progeny of crosses used to create embryos deficient in ACK signaling. Chart shows percentage of progeny that failed to hatch into larvae and distribution of three cuticle phenotypes among these dead embryos: wild-type cuticle, failure to form cuticle and cuticle with dorsal defect. High frequency of lethality in crosses producing PR2pBC02472/PR2pBC02472; Ack10b/Ack10b embryos can be explained by lethality of balancer chromosome homozygotes. Crosses in which only one ACK gene was targeted by RNAi showed a very low frequency of lethality. The following crosses were performed to create the indicated genotypes: PR2pBC02472Ack10b: PR2pBC02472/CyO; Ack10b/TM3 females X males. Hs-Gal4>PR2-RNAi,Ack-RNAi; Hs-Gal4/Hs-Gal4 females X PR2-RNAi/Cyo; Ack-RNAi/TM3 males. AS-Gal4C381>PR2-RNAi,Ack-RNAi: AS-Gal4C381/AS-Gal4C381 females X PR2-RNAi/Cyo; Ack-RNAi/TM3 males. AS-Gal4C381>PR2-RNAi,Ack-RNAi: AS-Gal4C381/AS-Gal4C381 females X Ack-RNAi/TM3 females X Ack10b/TM3; AS-Gal4C381/AS-Gal4C381 males. Hs-Gal4>PR2-RNAi: Hs-Gal4/Hs-Gal4 females X PR2-RNAi/Cyo males. Hs-Gal4>Ack-RNAi: Hs-Gal4/Hs-Gal4 females X Ack-RNAi/TM3 males.

3.3. Effects of excessive Dpp signaling in the embryo and wing are suppressed in a Ack mutant background

We have demonstrated that ACK function positively affects the output of Dpp signaling on the dorsal side of the embryo and we checked to see if reducing ACK function could suppress the effects of excessive Dpp signaling in this region. Overexpression of Dpp with prd-Gal4 caused dorsal anterior holes in the cuticle and germband retraction defects, indicating effects on morphogenetic events that are also sensitive to changes in ACK signaling (Figure 3.5 B). These phenotypic effects were completely blocked when Dpp-over-expressing embryos were made homozygous for the Ack10b allele (data not shown).

Does ACK impact Dpp signaling elsewhere during development? Dpp signaling has been extensively studied in wing development, during which expression of TkvQ199D in the enhancer piracy line TAJ3 causes ectopic vein formation between L3 and L4...
(Hoodless et al. 1996) (Figure 3.6 B). When TAJ3 flies were made homozygous for the \textit{Ack}^{10b} allele, the ectopic vein phenotype was significantly suppressed, again indicating a positive input of ACK on Dpp signaling (Figure 3.6 C).
Figure 3.6   Loss of Ack suppresses wing phenotype resulting from expression of tkv$^{Q199D}$ in enhancer piracy line TAJ3

(A) Wild-type wing showing L3 and L4 veins.  (B) TAJ3 wing showing ectopic vein tissue between L3 and L4 (arrowhead).  (C) Wing of TAJ3 fly homozygous mutant for Ack$^{10b}$ showing reduced ectopic vein tissue.
Our results on the regulation of $zip$ expression by Dpp are consistent with the model of Fernandez and colleagues in which two rounds of $dpp$ expression in the DME cells regulate dorsal closure (B. G. Fernandez et al. 2007). In the first round of $dpp$ expression, prior to completion of germband retraction, Dpp signals from the DME cells to the amnioserosa. This signaling can be visualized by pMad staining in the amnioserosa, which is obvious during germband retraction but fades away by the beginning of dorsal closure, a pattern paralleled by $zip$ expression. In the second round of signaling, occurring during dorsal closure, Dpp signals to the dorsal epidermis, as demonstrated by robust pMad in this tissue (see Figure 3.2 A) and high $zip$ levels in the DME cells. We have demonstrated that Dpp signaling and ACK function are both necessary but not sufficient for $zip$ expression in the embryo during dorsal closure. Activation of either Dpp signaling or ACK function in $prd$ stripes does not lead to ectopic $zip$ expression indicating that in each case additional inputs are required. Ack is able to elevate $zip$ expression only on the dorsal side of the embryo in regions where $zip$ is normally expressed, indicating that required additional inputs are present; we propose that one such input is Dpp signaling. When Ack is over-expressed in the amnioserosa, either in $prd$ stripes or throughout the whole tissue, its effects on $zip$ expression are non-cell autonomous, leading to up-regulation of $zip$ expression throughout the dorsal side of the embryo. With regard to the $zip$ expression pattern we see with $prd$-Gal4-driven Ack transgenes, a diffusible signal emitted from $prd$ stripes in the amnioserosa could attain a fairly uniform distribution over the dorsal side of the embryo. We propose that Dpp secreted from the DME cells cooperates with a diffusible signal from the amnioserosa (regulated by ACK in a kinase-independent manner) to drive coordinated $zip$ expression in these two tissues (Figure 3.7). ACK appears to make the larger input into $zip$ expression as Ack over-expression results in a clear elevation in $zip$ levels on the dorsal
side of the embryo, but this is not seen with excessive Dpp signaling. Simultaneously
activating Dpp signaling and over-expressing Ack with prd-Gal4 is not sufficient to
promote ectopic zip expression (for example in the ventral epidermis) (W. S,
unpublished observations), indicating that other components are required for zip
expression, consistent with Ack operating through downstream signaling events.
Figure 3.7  Model for coordinated regulation of zip expression in the amnioserosa and DME by Dpp and ACK

A DME cell is shown at bottom and an amnioserosa (AS) cell at top. zip expression in both cell types requires the Dpp pathway (triggered by Dpp secreted from the DME) and a second unknown pathway regulated by "X" secreted from the amnioserosa in an ACK-dependent manner. Loss-of-function and over-expression data indicate that ACK makes a greater contribution to zip expression than Dpp.
It is well established that communication between the amnioserosa and the epidermis is critical for embryonic morphogenesis (Conder et al. 2004; B. G. Fernandez et al. 2007; Glise and Noselli 1997; Lamka and Lipshitz 1999; Reed et al. 2001; Scuderi and Letsou 2005; B. E. Stronach and Perrimon 2001; Wada et al. 2007) and we have identified the zip locus as one target of such crosstalk, with zip transcription in both tissues dependent on signals secreted by both tissues. A diffusible signal from the amnioserosa to the epidermis has been proposed in the regulation of germband retraction by Hindsight, a transcription factor that is member of the U-shaped group of genes expressed in the amnioserosa (Frank and Rushlow 1996; Lamka and Lipshitz 1999). How could ACK tie into transcriptional regulation of a diffusible signal from the amnioserosa to the epidermis? There is evidence that ACK functions in clathrin-mediated receptor endocytosis in a kinase-independent manner (Hopper et al. 2000; Teo et al. 2001; Yang et al. 2001; Yeow-Fong et al. 2005), and it is possible that ACK regulates by receptor endocytosis a pathway in the amnioserosa that leads to a transcriptional response. Our data suggest that the kinase activity of Ack may actually impair its ability to drive zip expression, as KD-Ack promoted higher zip levels than wild-type Ack.

We had previously found that global activation of Cdc42 signaling in tkv mutant embryos could suppress the dorsal closure defects caused by a reduction in Dpp signaling, and our present results indicate a major route of action for this suppression is ACK (Ricos et al. 1999). The activation of Cdc42 throughout the embryo leads to increased expression of Ack specifically in the amnioserosa, and we have shown here that over-expressing Ack in this tissue can suppress tkv dorsal closure defects (Sem et al. 2002). Our results indicate a tissue-specific regulation of Ack levels by Cdc42 that
may be part of a sophisticated signaling network enabling the coordinated morphogenesis of tissues in the embryo. Ack does not bind Cdc42 but PR2 does and Cdc42 may also regulate ACK function during dorsal closure through direct interaction with PR2 (Burbelo et al. 1995; Sem et al. 2002).

We see head involution defects and germband retraction failures in ACK-deficient embryos and loss of Ack can suppress the head involution defects and germband retraction failure caused by over-expression of Dpp. These results suggest that Dpp signaling and ACK cooperate in the regulation of these morphogenetic events in addition to dorsal closure. A review has highlighted the parallels between dorsal closure and head involution in terms of morphogenetic events and the genes required, with both involving epithelial sheet migration, zip expression and Dpp signaling (VanHook and Letsou 2008). Ack over-expression leads to excessive zip levels in the head and it is likely that ACK and Dpp signaling work together to provide myosin for head involution. That signaling from the amnioserosa is involved in regulating head involution is supported by our earlier finding that impairing dPak function in the amnioserosa causes failures in this process (Conder et al. 2004).

Does ACK impact Dpp signaling other than at the level of zip transcription? Homozygosity for an Ack allele suppresses the ectopic wing vein phenotype caused by excessive Dpp signaling. It is likely that this phenotype is caused by something other than misregulation of zip expression, and ACK could be regulating the expression of a subset of Dpp target genes (other than dad or salm) or may be interacting with the Dpp pathway at another level.
TGF-β family signaling is a central regulator of dorsal closure and other epithelial fusions, but how Dpp controls dorsal closure has not been well-defined. We have shown that regulation of zip expression in cooperation with the Drosophila ACKs constitutes a major route of action of Dpp during dorsal closure. These findings may be relevant to vertebrate wound healing, in which closure of the wound involves both epithelial movement and TGF-β-dependent contraction of connective tissue in the wound (Grose and Martin 1999).
4. **Results part II: Role of Egfr signaling in dorsal closure**

Some of the work from this section was published in “Modulation of morphogenesis by Egfr during dorsal closure in *Drosophila*” by Weiping Shen, Xi Chen, Olga Cormier, David Cheng, Bruce Reed and Nicholas Harden (PLoS ONE 2013; 8(4): e60180). Dr. Weiping Shen and I contributed equally to this publication. The live imaging and apoptosis experiments were done by Dr. Reed’s group and the rest of the work done by the Harden group. Note that work done by others is credited throughout the text.

As detailed in the previous chapter, we demonstrated that two members of the ACK family of nonreceptor tyrosine kinases, Ack and PR2, co-operate with Dpp to regulate myosin levels in the amnioserosa and epidermis during dorsal closure, probably by regulating production of a diffusible signal emanating from the amnioserosa (Zahedi et al. 2008). Cell shape change in both the amnioserosa and the epidermis requires the zip product, myosin heavy chain, and this signaling network may help coordinate morphogenetic events during dorsal closure (Franke et al. 2005). There is considerable evidence that a major function of ACK is negative regulation of Egfr through effects on the endocytosis and/or ubiquitination of Egfr (Grovdal et al. 2008; Hopper et al. 2000; Q. Lin et al. 2002; Q. Lin et al. ; F. Shen et al. 2007), and Egfr is therefore a likely participant in ACK-mediated regulation of dorsal closure.

The Egfr pathway is used repeatedly throughout *Drosophila* development and regulates processes such as cell proliferation, cell differentiation, apoptosis, cell motility and adhesion regulation (reviewed in (Schweitzer and Shilo 1997; Shilo 2003)). While it has long been recognized that Egfr has multiple roles in embryonic morphogenetic events including germband retraction, there has not been an effort to specifically look for a role in dorsal closure (R. J. Clifford and Schupbach 1989; R. Clifford and Schupbach 1992).
4.1. Egfr is required for normal dorsal closure

To determine whether Egfr is required for dorsal closure, we examined by embryonic cuticle preparation three mutant Egfr alleles previously shown to disrupt embryogenesis: Egfr\textsuperscript{f2}, an amorphic allele, Egfr\textsuperscript{2C82}, a hypomorphic allele, and Egfr\textsuperscript{1F26}, a temperature conditional allele (Figure 4.1 A-D). To exclude the possibility of second site mutations in the genetic background, we also examined embryos heteroallelic for Egfr\textsuperscript{f2} and Egfr\textsuperscript{2C82} (Figure 4.1 E-F). Consistent with previous findings (R. Clifford and Schupbach 1992), all of the mutant alleles exhibited the “curled” phenotype, with the posterior end of the embryo in close proximity to the head, indicating a defect in germband retraction (Figure 4.1 C, E). In embryos less curled up, we were able to observe holes in the dorsal surface (Figure 4.1 F). However, with these severe Egfr mutant alleles, most embryos died at germband retraction or earlier stages before dorsal closure, making it impossible to analyze dorsal closure. Note homozygous or heteroallelic embryos were selected by absence of a GFP balancer chromosome.

The different types of embryonic phenotypes observed by cuticle preparation indicated that Egfr is also important for processes other than dorsal closure, such as head involution and germband retraction. In order to distinguish the effects of Egfr on dorsal closure from earlier developmental stages, we took advantage of the temperature sensitive allele, Egfr\textsuperscript{1F26}. The Egfr\textsuperscript{1F26} embryos show no defects when raised at the permissive temperature of 18°C, but have strong defects when grown at the restrictive temperature of 29°C (Figure 4.1 D). To demonstrate when Egfr\textsuperscript{1F26} would affect dorsal closure, Weiping Shen shifted the embryos from 18°C to 29°C to shut off Egfr function at various time points during embryonic development. As a result, embryos transferred to 29°C at around embryonic stages 10/11, which is before dorsal closure initiation, showed the most dorsal closure defects, while temperature shifting at earlier stages resulted in the head and germband retraction defects observed with other severe Egfr alleles. No significant dorsal closure defects were seen in Egfr\textsuperscript{1F26} embryos when they were shifted to 29°C at stage 12 or later (Weiping Shen, Ph.D. dissertation (Shen 2008)).

To study the tissue specific requirement of Egfr, we knocked down Egfr function through expression of a dominant negative version of Egfr, Egfr\textsuperscript{DN}. Egfr\textsuperscript{DN} lacks the
cytoplasmic portion containing the tyrosine kinase domain necessary for signal transduction, and blocks the Egfr signaling by competing with endogenous Egfr for ligand binding. When \textit{Egfr}^{DN} was expressed in most of the epidermis using the epidermal drivers, 69B-Gal4 and patched (\textit{ptc})-Gal4 (Brand and Perrimon 1993), holes were observed on the dorsal surface of the embryos, however, head development was near normal and the tail was less curled compared to embryos with severe loss-of-function alleles (Figure 4.1 G, H). Due to the important role of the DME cells in dorsal closure, we induced \textit{Egfr}^{DN} expression specifically in these cells using the \textit{LE}-Gal4 driver, which is active in the first two rows of dorsal epidermal cells flanking the amnioserosa during dorsal closure. Knock down of Egfr function in this subset of cells resulted in the puckered phenotype, a mild dorsal closure defect (Figure 4.1 J). In addition, under control of the amnioserosa specific driver, \textit{AS}-Gal4\textsubscript{C381}, embryos also showed a dorsal hole phenotype (Figure 4.1 I). Therefore, Egfr activity in both the amnioserosa and epidermis is required for normal dorsal closure.

We also observed the morphology of Egfr mutant embryos by antibody staining against phosphotyrosine (PY) which reveals the cell outline. Mutant embryos showed mixed types of defects to the overall morphology. The common phenotypes seen among all the mutant alleles included the curled tail, disorganized leading edge cells and the bunching of the epidermal segments, which were considerably consistent with the phenotypes seen in the cuticle preparations (Figure 4.2 B-E, G, I). The bunched segment phenotype was also seen when Egfr was only removed from the DME cells through expression of \textit{Egfr}^{DN} by the \textit{LE}-Gal4 driver, which suggests that Egfr loss from the DME cells is responsible for the morphological defect of these cells (Figure 4.2 H).

In addition to the results of the fixed embryos, our collaborator Dr. Bruce Reed and his graduate student Olga Cormier also analyzed the \textit{Egfr} mutant defects by live imaging. The cell membranes of the live embryos were outlined by the ubiquitous expression of a GFP tagged adhesion protein, \textit{Ubi-DEcadherin-GFP}. Consistent phenotypes were observed, including abnormal shape and degradation of amnioserosa cells, failure of germband retraction, loss of head tissue, and bunching of the dorsal epidermis. More interestingly, the amnioserosa cells were found constricting in the dorsal-ventral direction instead of the normal anterior-posterior direction (W. Shen et al. 2013). Some of the phenotypes observed above suggested that the Egfr loss affected
dorsal closure through disruption of actomyosin contractility which is a key regulator of cell shape change during dorsal closure (B. G. Fernandez et al. 2007).
Figure 4.1  Cuticle preparations of embryos with mutant Egfr alleles or dominant negative Egfr transgene.

(A) Dorsal-lateral view of wild-type embryo showing intact cuticle.  (B, C)  Egfr\textsuperscript{2} embryo (B) and Egfr\textsuperscript{2C82} embryo (C) showing severe cuticle defects.  (D) Embryo from temperature-sensitive Egfr\textsuperscript{1F26} stock that had been developed at 18°C before shifting to the restrictive temperature of 29°C at around stage 10 showing large dorsal hole (white arrow head) and the bowed appearance (black arrows).  (E) Lateral view of an Egfr\textsuperscript{2C82}/Egfr\textsuperscript{2} embryo showing “curled up” phenotype.  (F) Dorsal view of an Egfr\textsuperscript{2C82}/Egfr\textsuperscript{2} embryo showing large cuticle hole (white arrow head).  (G-J) Embryos in which the dominant negative Egfr transgene (Egfr\textsuperscript{DN}) was expressed in particular tissues using tissue-specific Gal4 drivers showing similar phenotypes including small dorsal hole or scab (arrow head) and the bowing (black arrows).  Scale bar: 50 µm.
**Figure 4.2 Morphogenetic defects of embryos with impaired Egfr**

Confocal images of embryos stained with anti-phosphotyrosine (PY) antibody. (A, F) Wild-type embryos showing well organized DME cells and fairly smooth leading edge. (B-E, I) Embryos mutant for Egfr gene showing disrupted cell organization, abnormal cell shape in both amnioserosa and dorsal epidermis, and the bunching of segments (white arrow head). (G) An embryo in which Egfr\textsuperscript{DN} was expressed under control of the LE-Gal4 driver showing the bunching of segments (white arrow-head). Scale bar: 50µm (A-E) (F-I)
4.2. Ras/Raf/MAPK cascade is active during dorsal closure

A principle route Egfr goes through to transduce the signal and induce the cellular response is the Ras/Raf/MAPK cascade. The status of this cascade can be indicated by the phosphorylation level of MAPK which can be detected by the anti-pMAPK antibody. Such staining in wild-type embryos showed that there was strong pMAPK activity in the center of amnioserosa and lateral epidermis, but weak signal in the peripheral amnioserosa cells and dorsal epidermis (Figure 4.3). The amnioserosa pMAPK signal was eliminated when $Egfr^{DN}$ was expressed under the amnioserosa driver $AS-Gal4^{C381}$, indicating that Egfr is indeed activating the pathway in this tissue (data not shown). The low levels of pMAPK in the amnioserosa periphery and dorsal epidermis suggest negative regulation of the pathway in these two tissues.
Figure 4.3  Strong immunoreactivity of phospho-MAPK was observed in wild-type embryos during dorsal closure

(A) Anti-phospho-MAPK staining of a wild-type showing strong immunoreactivity in the center of the amnioserosa and lateral epidermis but little staining in dorsal epidermis and cells at periphery of the amnioserosa.  (A’) Same embryo as shown in A with cell outlines revealed by anti-PY staining.  (A’’) Merged image of A and A’ (pMAPK in red, PY in green).  Scale bar: 50 μm.
4.3. Egfr signaling represses dpp expression

A mechanism for Egfr signaling to achieve different outcomes in different developmental processes is through cooperation with other signaling pathways. Dpp signaling has been reported to genetically interact with the Egfr pathway in either an antagonistic or synergistic manner (Freeman 1998). We were interested in whether these two pathways interact during dorsal closure. The first question we asked was whether Egfr signaling controls the expression of the dpp gene. With FISH, we were able to see the endogenous dpp mRNA distribution in wild-type embryos. During dorsal closure, epidermal dpp transcripts were organized into two stripes on each lateral side along the anterior-posterior axis: one stripe in the DME cells and one stripe in the ventral lateral epidermis (Figure 4.4 A). As dorsal closure proceeds, dpp expression declines. In Egfr2$^{2c82}$/Egfr$^{f2}$ embryos, we were not able to compare the intensity of the dpp stripes to the wild-type embryos, because the severe morphogenetic defects made it difficult to stage the embryos. However, it was clear that there was ectopic dpp mRNA signal in the lateral epidermis expanding beyond the DME cells, which was never seen in wild-type (Figure 4.4 B). Similar results were seen when Egfr was locally removed through expression of the Egfr$^{DN}$ using various epidermal drivers (W. Shen et al. 2013) (Weiping Shen, Ph.D. dissertation (Shen 2008)).

To study the effect of excessive Egfr signaling on dpp expression, we used three Egfr transgenes: UAS-Egfr$^{EGFP}$, expressing biologically active Egfr tagged with enhanced GFP (EGFP); UAS-Egfr$^{A2}$, a constitutively active version of Egfr; and UAS-sSpi, expressing secreted Spitz (active) which is a ligand for Egfr. When inducing ectopic Egfr signaling in every other segment using prd-Gal4, the dpp stripes became patchy with expression in prd stripes greatly decreased (Figure 4.4 C, D). Furthermore, activating Egfr signaling more broadly using PTC-Gal4 caused a stronger effect, with both the dpp stripes showing breaks between segments (Figure 4.4 E, E’). Consistently, when expressing the constitutively active version of the downstream Egfr signaling component Ras, UAS-Ras$^{V12}$ using PTC-Gal4, similar dpp down-regulation effect was found (Figure 4.4 F).

These results suggested that the Egfr signaling represses dpp expression in the epidermis. It is well established that the DME dpp stripe but not the ventral lateral stripe
is dependent on JNK signaling (Jackson and Hoffmann 1994; Sluss and Davis 1997). Therefore, we can exclude the possibility that Egfr signaling in the epidermis inhibits dpp expression through inhibition of JNK, as excessive Egfr signalling in the epidermis affects both dpp stripes.

We were curious as to how Egfr regulates dpp expression. Wingless (Wg) is a diffusible signal required for proper dpp expression in both the DME and ventral lateral stripes during dorsal closure (McEwen et al. 2000). Furthermore, it is demonstrated that in the eye imaginal disc, Egfr negatively regulates Wg levels by transcriptionally regulating phyllopod (phyl), which encodes a cytoplasmic adapter protein that blocks the Wg pathway by trapping the pathway components in early endocytic vesicles (Nagaraj and Banerjee 2009). In an attempt to test if this relationship is true in dorsal closure, we looked at wg and phyllopod levels in dorsal closure-staged embryos with altered Egfr signaling. However, we observed no apparent effect on either the phyllopod expression or the wg levels (Figure 4.5). Therefore, the mechanism of Egfr regulation of dpp expression during dorsal closure remains unclear.
Figure 4.4  Egfr signaling represses dpp expression

All images except (F’) are confocal microscopic images of dpp FISH of embryos during dorsal closure stage.  (A) Wild-type embryo showing endogenous dpp expression pattern.  (B) Egfr^{2CS2}/Egfr^{12} embryo showing abnormal dpp signal in lateral epidermis (white arrow).  (C-D) Embryos in which Egfr signaling was ectopically activated in paired stripes showing reduction of the dpp signal in those stripes.  Note the particularly strong effect of expression of secreted (active) Egfr ligand Spitz, UAS-sSpi, with the dpp signal reduced in both DME and ventral lateral stripes.  (E, F) Embryos in which the Egfr signaling was ectopically activated using the PTC-Gal4 driver showing reduction of expression in both dpp stripes.  (E’) Anti-GFP immunostaining of the same embryo as in (E) revealing the pattern driven by PTC-Gal4 driver.  (E’’) Merged image of (E) and (E’) showing dpp FISH in red and anti-GFP immunostaining in green.  The white arrowheads in (E – E’’) indicate the reduction of dpp signal where the UAS-Egfr^{EGFP} was expressed.  Scale bar: 50µm.
Figure 4.5  Egfr does not regulate Wg or phyllopod levels during embryogenesis

(A-C) Anti-Wg antibody staining of embryos during dorsal closure showing comparable Wg immunosignal between wild-type embryo (A) and the embryo with loss of Egfr signaling (B) or gain-of-function of Egfr signaling (C).  (D-E) wg FISH of wild-type embryo (D) and embryo with UAS-EgfrEGFP expressed in prd stripes (E) showing similar wg expression.  (F-H) phyllopod FISH of embryos during germband retraction showing comparable phyllopod expression between wild-type embryo (F) and the embryo with loss-of-function of Egfr signaling (G) or gain-of-function of Egfr signaling (H).  Scale bar: 50µm.
4.4. Egfr negatively regulates zip expression

Having proved that Egfr is required for dorsal closure, we were interested in whether Egfr regulates the zip gene, since Egfr was originally considered as a candidate component for zip regulation. In wild-type embryos, there are two big waves of zip expression: one is in the amnioserosa stretching from early germ band retraction to the beginning of dorsal closure; the other one is in the DME cells persisting through both germband retracton and dorsal closure (Figure 4.6 A, B). We first looked at the zip mRNA levels in Egfrf2 embryos, and ectopic zip signal was observed in the lateral epidermis beyond the wild-type distribution pattern. In addition, the zip signal in DME cells where zip mRNA accumulates in wild-type embryos showed slightly higher intensity (Figure 4.6 C compare to B). Furthermore, expressing UAS-EgfrDN under control of epidermal drivers, such as LE-Gal4 and 69B-Gal4, also caused excessive epidermal zip expression (Weiping Shen, Ph.D. dissertation (Shen 2008), and data not shown). zip expression in the amnioserosa could not be reliably evaluated in Egfrf2 embryos, because the amnioserosa cells were generally disrupted. Expression of UAS-EgfrDN in the amnioserosa led to persistence of zip expression in this tissue, suggesting that Egfr signalling represses zip in the amnioserosa (Figure 4.6 D). Furthermore, expression of dominant negative version of Ras, UAS-RasN17, in the amnioserosa phenocopied the result of UAS-EgfrDN, with strikingly high levels of zip expression in the amnioserosa during dorsal closure (Figure 4.6 E). These results indicate that Egfr inhibits zip expression through the Ras/Raf/MAPK pathway.

To determine if excessive Egfr signaling would have the opposite effect on zip expression, we expressed Egfr transgenes in the amnioserosa. In general, ectopic Egfr signaling achieved by expressing UAS-Egfr4.2, UAS-Egfr4.4, UAS-EgfrEGFP, UAS-sSpi or the downstream effector Ras, UAS-RasV12, resulted in reduction of zip transcription in both the germ band retraction and dorsal closure stages (Figure 4.5 F-J). A cell non-autonomous pattern was consistently noticed, since amnioserosa removal of Egfr signaling led to an increase of zip signal in both the amnioserosa and DME cells. The more striking cell non-autonomous effects were seen in the embryos with increased Egfr signaling, where both the amnioserosa and DME zip levels were dramatically reduced.
In conclusion, Egfr signaling from both the amnioserosa and epidermis was required for maintaining normal zip levels during dorsal closure. Furthermore, Egfr signaling represses zip transcription in a cell non-autonomous manner.
Figure 4.6  Egfr signaling negatively regulates zip transcription
All images except (I’) are confocal microscopic image of zip FISH. (A, B) Wild-type embryos showing endogenous zip expression pattern in germband retraction stage (A) and dorsal closure stage (B). (C) Egfr\textsuperscript{iz} embryo showing ectopic zip signal in epidermis (white arrow). (D) Embryo expressing Egfr\textsuperscript{DN} in amnioserosa showing cell non-autonomous elevation of zip expression. (E) Embryo expressing dominant negative Ras in amnioserosa showing similar change of zip expression as (D). (F-H) Embryos expressing activated Egfr in amnioserosa at germband retraction stage (F) and dorsal closure stage (G, H) showing reduction of zip level in both amnioserosa and DME cells. (I) Embryo expressing UAS-Egfr\textsuperscript{EGFP} in amnioserosa showing reduction of zip signal in both the amnioserosa and DME cells. (I’) same embryo as in (I) stained with anti-GFP antibody to reveal the UAS-Egfr\textsuperscript{EGFP} expressing cells. (I’’) Merged image of (I) and (I’) showing zip FISH in red and anti-GFP staining in green. (J) Embryo expressing constitutively activated Ras showing very similar result as (I). Scale bar: 50\(\mu\)m.

4.5. Egfr inhibits apoptosis in the amnioserosa

The amnioserosa contributes to dorsal closure not only by undergoing cell shape changes but also by undergoing apoptosis, and regulation of cell death in the amnioserosa could be an important component of dorsal closure (Toyama et al. 2008). We noticed that the amnioserosa cells in Egfr mutant embryos are prematurely lost, suggesting an early onset of apoptosis, similar to the mutant phenotypes of the U-shaped group of genes which are required for maintenance of the amnioserosa (Frank and Rushlow 1996). Consistent with this, there is evidence of Egfr being involved in apoptosis regulation. For example, expression of a constitutively active version of Ras, RasV12, in the amnioserosa causes the tissue to persist longer than wild-type, which indicates a negative regulatory role for the Egfr pathway (Mohseni et al. 2009). Furthermore, based on acridine orange staining of embryos with global gains or losses of Ras signaling, it was concluded that Ras signaling negatively regulates apoptosis throughout the embryo (Kurada and White 1998).

To visualize the effects of losses or gains of Egfr signaling on amnioserosa apoptosis in live embryos, Olga Cormier from Dr. Reed’s lab used the caspase sensor Apoliner (Bardet et al. 2008; Cormier et al. 2012). Apoliner consists of a monomeric red fluorescent protein (RFP) tethered to EGFP by a specific and efficient caspase-sensitive linker (Bardet et al. 2008). Furthermore, the design of the Apoliner construct includes a transmembrane domain that precedes the RFP component while the EGFP component includes a nuclear localization signal (NLS). The whole construct is generated as a single UAS-transgene, which makes it easy to achieve the spatial and temporal control of Apoliner expression. In live cells with no caspase activity, the two fluorophores co-
localize to membranes resulting in a merge colour. Upon caspase activation in live cells, the fluorophores are separated due to the break of the caspase-sensitive linker, this leaves the Apoliner-RFP remaining at membranes while Apoliner-EGFR is translocated to the nucleus.

At the beginning of germband retraction in wild-type embryos, there was little nuclear EGFP signal in the amnioserosa, indicating minimal caspase activity (Figure 4.7 A-A'). However, there was strong nuclear EGFP signal in the amnioserosa of Egfr<sup>1a15/Egfr<sup>2</sup> heteroallelic mutant embryos at the same stage (Figure 4.7 B-B'). As dorsal closure proceeded nuclear EGFP signal accumulated in the amnioserosa of wild-type embryos, indicating the onset of apoptosis in this tissue (Figure 4.7 C-C'). Expression of the baculovirus caspase inhibitor p35 (Clem et al. 1991) caused the elimination of nuclear EGFP signal due to the blocking of apoptosis (Figure 4.7 D-D'). To find out if ectopic Egfr signaling could block apoptosis, either sSpi or RasV12 was expressed in the amnioserosa using the LP1-GAL4 driver. In both cases, cells showed little nuclear EGFP even late in dorsal closure, similar to what was seen with p35 expression (Figure 4.7 E-F). Thus, we conclude that Egfr signaling inhibits caspase activation in the amnioserosa, which is consistent with Egfr's role as negative regulator of apoptosis in previous findings. As expected, a robust increase in Egfr levels in the amnioserosa through expression of Egfr<sup>EGFP</sup> using the double amnioserosa driver combination GAL4<sup>NP3312</sup> + GAL4<sup>N5328</sup> resulted in a failure of the amnioserosa to properly complete morphogenesis. In addition, the amnioserosa of these embryos persisted beyond the normal time of amnioserosa programmed cell death, as compare to the embryos expressing only GFP showing normal progress of amnioserosa morphogenesis (Figure 4.7 G-I, and (W. Shen et al. 2013)).
Figure 4.7  Egfr inhibits apoptosis and morphogenesis in the amnioserosa

(A–F) Apoliner signals in the amnioserosa. Apoliner reporter had been expressed either globally with the tub-Gal4 driver or in the amnioserosa using the LP1-Gal4 driver. For each embryo RFP, EGFP signals and merge are shown. On the right side of each panel is a higher power view of amnioserosa cells. In the absence of caspase activity, RFP and EGFP co-localize at various membranes and there is little EGFP signal in the nucleus. In the presence of caspase activity, EGFP is cleaved away from RFP and moves into the nucleus. (A–A’’) amnioserosa of wild-type embryo prior to germband retraction showing co-localization of RFP and EGFP signals and weak EGFP signals in the nucleus. (B–B’’) amniorsosa of Egfr mutant embryo prior to germband retraction showing strong EGFP signals in the nucleus. (C–C’’) amnioserosa of wild-type embryo during dorsal closure showing strong EGFP signals in the nucleus. (D–D’’) amnioserosa of p35-expressing embryo during dorsal closure showing weak EGFP signals in the nucleus. (E–E’’) amnioserosa of sSpi-expressing embryo during dorsal closure showing weak EGFP signals in the nucleus. (F–F’’) amnioserosa of RasV12-expressing embryo during dorsal closure showing weak EGFP signals in the nucleus. (G) Still from live imaging showing amnioserosa of stage 15 wild-type embryo in which GFP had been expressed with the Gal4NP3312 amnioserosa driver, showing narrow, tube-like amnioserosa. (H, I) Stills from live imaging showing amnioserosa of stage 15 embryos in which Egfr-EGFP and GFP-NLS had been expressed with the double driver combination Gal4NP3312+ Gal4NP5328 showing failure of amnioserosa morphogenesis. The amnioserosa in panel H has failed to narrow throughout while that in panel I has failed to narrow at the anterior end. Scale bars: 50 µm (A–B); 10 µm (C–I).
4.6. Ack negatively regulates Egfr levels in the amnioserosa through endocytosis

One mechanism of Egfr down-regulation is degradation through clathrin-mediated endocytosis (Eden et al. 2009). We suspected that this mechanism was also being used in the amnioserosa during dorsal closure, since when expressing UAS-Egfr\textsuperscript{EGFP} in embryos, in addition to the cortical localized Egfr, much of the protein appeared to be accumulating in cytoplasmic vesicles in the amnioserosa (Figure 4.8 A, B) (W. Shen et al. 2013). A conserved function of Ack family tyrosine kinases is the down regulation of Egfr by endocytosis and subsequent degradation (Q. Lin et al. 2010). Given our previous result that Ack promotes zip transcription during dorsal closure, we were interested to know if the Ack controls zip through down regulation of Egfr. To make it easy to examine both increases and decreases in the zip signal, we chose embryos in their late germ band retraction stage for comparison. Consistent with previous observations, overexpressing Ack in the amnioserosa resulted in a dramatic increase in zip levels (Figure 4.8 D compares to C). Co-expression with UAS-Egfr\textsuperscript{EGFP} resulted in the zip levels dropping back to wild-type (Figure 4.8 E compare to C). As a control, co-expression with UAS-lacZ did not affect the elevated zip levels (Figure 4.8 F). This result supported our hypothesis that Ack controls zip through down regulation of Egfr.
**Figure 4.8  Ack may antagonize Egfr in zip regulation**

(A-B) Anti-GFP immunostaining of embryos expressing UAS-Egfr\textsuperscript{EGFP} in the amnioserosa under AS-GAL\textsubscript{C381} or NP3312 driver showing vesicular accumulation in addition to being at the plasma membrane.  
(C-F) zip FISH of embryos in late germ band retraction stage.  
(C) Wild-type embryo showing endogenous zip accumulating in the amnioserosa and DME cells.  
(D) Embryo in which wild-type Ack was expressed in the amnioserosa showing dramatically elevated zip level in the amnioserosa.  
(E) Embryo with co-expression of wild-type Ack and UAS-Egfr\textsuperscript{EGFP} in the amnioserosa showing wild-type level of zip expression (compare to (C)).  
(F) Control embryo in which wild-type Ack was co-expressed with UAS-lacZ instead of UAS-Egfr\textsuperscript{EGFP} showing comparable zip level as in (D). Scale bars: 10 µm in A-B; 50µm in C-F.
We went on to look at the effect of excessive Ack on the level of Egfr. Over-expression of wild-type Ack resulted in an increase of the level of phosphotyrosine. Taking advantage of this fact, in prd-Gal4 driven UAS-Ack embryos, we relied on the anti-PY immunostaining to mark the Ack expressing cells (Figure 4.9 A). In cells with the over-expression of wild-type Ack, Egfr showed less accumulation in the cell membrane, and more puncta in the cytoplasmic region (Figure 4.9 A'). Some of the Egfr puncta were positive for Rab5 immunostaining, a marker for early endosomes, suggesting the Egfr was going through endocytosis (Figure 4.9 A'''). Many of Egfr-positive puncta were Rab5-negative and we suspect that they may be multivesicular bodies, where endocytosed Egfr is known to accumulate (Eden et al. 2009). Furthermore, there was a generally higher level of Rab5 signal in the Ack-expressing cells, suggesting the Ack tyrosine kinase as a general promoter of endocytosis (Figure 4.9 A''''). Note that this set of stainings was done by David Cheng.

In support of this is an observation made when trying to examine the effects of Ack on apoptosis using the Apoliner reporter. Here with the expression of kinase-dead Ack, which is more effective than wild-type Ack at inducing zip expression (Zahedi et al. 2008), a highly punctate distribution of membrane-localized Apoliner-RFP signal was observed in contrast with the typical homogeneous distribution displayed by wild-type control embryos at the same stage of dorsal closure (Figure 4.9 B-C) (W. Shen et al. 2013). We interpret this difference as reflecting the ability of Ack to promote a general increase in intracellular vesicular traffic, and that this function is independent of the kinase activity of this protein. In summary, we found that the Ack tyrosine kinase appears to promote zip expression through down regulation of Egfr, possibly by facilitating the endocytosis of Egfr for degradation.
Figure 4.9   Evidence that ACK down-regulates Egfr through endocytosis

(A) *prd-Gal4* driven *UAS-WTACK* embryo stained with anti-PY antibody to reveal the Ack expressing cells (outlined with dotted lines). (A’) Anti-Egfr staining of the same embryo in (A) showing less cortical signal (white arrow) compared to neighboring wild-type cells (white arrow head), and more cytoplasmic puncta. (A’’) Anti-Rab5 (an early endosome marker) staining of the same embryo in (A) showing a general increase of endocytosis in the Ack expressing cells. (A’’’) Merged image of (A’) and (A’’). Egfr is shown in red, and Rab5 is shown in green. Note that there is co-localization of the two proteins in both the Ack over-expressing cells (white arrow) and the wild-type cells (white arrow head). (B) Amnioserosa cells in embryo in which Apoliner has been expressed with *LP1-Gal4* driver showing localization of Apoliner-RFP signal to membranes. (C) Amnioserosa cells in embryo in which Apoliner and kinase-dead Ack have been co-expressed with *LP1-Gal4* driver showing punctate localization of Apoliner-RFP signal. Scale bar: 10µm.
4.7. **Search for transcription factors regulating zip expression downstream of Egfr**

To draw a more complete picture, we were interested to find out which transcription factors might be acting downstream of Egfr to control zip expression. Mutations in the U-shaped group of amnioserosa-specific transcription factors encoded by *u-shaped (ush)*, *hindsight (hnt)*, *serpent (srp)* and *tail-up (tup)* produce embryonic phenotypes remarkably similar to *Egfr* alleles (Frank and Rushlow 1996). In addition, the ETS family transcription factor *pointed (pnt)* and *anterior open (aop)* have been demonstrated to function downstream of Egfr (Vivekanand and Rebay 2006). *ush* and *hnt* were tested by Weiping Shen and appear to be inhibitors of zip expression, as mutants had elevated zip levels (Weiping Shen, Ph.D. dissertation (Shen 2008)). We looked at zip FISH in embryos with disruption of other candidate genes. With the exception of *srp*, which showed a reduction of zip signal in the amnioserosa during the germ band retraction stage, all other mutants showed similar zip FISH levels as wild-type (Figure 4.10). In conclusion, *srp* may be another transcription factor regulating zip transcription, and in contrast to the *ush* and *hnt* which are working as inhibitors, *srp* functions as a promoter.
Figure 4.10 zip FISH of embryos with disruption of candidate transcription factors

(A, E) zip FISH of wild-type embryo in dorsal closure stage. (A) and germband retraction stage (E). (B-D) zip FISH in aop mutant embryo (B), tup mutant embryo (C), and embryo expressing tup RNAi driven by prd-Gal4, showing similar zip levels as wild-type embryo. (F) zip FISH of srp mutant embryo showing elimination of zip signal from the amnioserosa. Scale bar: 50µm.
We have determined that Egfr is required in both the amnioserosa and epidermis for dorsal closure to proceed normally, and our results suggest that Egfr signaling has at least three distinct roles in dorsal closure, all of which act to repress morphogenesis (see model in Figure 4.11). First, Egfr is a negative regulator of \textit{dpp} expression in the epidermis. \textit{dpp} is expressed in two stripes during dorsal closure, one composed of the DME cells and the other running along the ventrolateral epidermis, where \textit{dpp} expression in the DME cells, but not the ventrolateral stripe, is dependent on a JNK MAPK cascade (Glise and Noselli 1997; Hou et al. 1997; Jackson and Hoffmann 1994; Riesgo-Escovar and Hafen 1997; Sluss and Davis 1997; St Johnston and Gelbart 1987). Consistent with the notion that Egfr functions as a negative regulator of \textit{dpp} expression, activation of the Egfr pathway can repress \textit{dpp} expression in either stripe. The down regulation of \textit{dpp} expression in both stripes, however, supports the view that Egfr does not reduce \textit{dpp} transcription by impacting the JNK pathway, in which case we would expect to observe down regulation of \textit{dpp} expression only in the DME stripe.

The defects in morphogenesis seen in embryos with impaired Egfr signaling are likely at least in part due to misregulated actomyosin contractility. A recurring phenotype associated with various circumstances of Egfr impairment is the bowed embryo phenotype, where segments are bunched together at the leading edge of the epidermis during dorsal closure. We suspect this is due to uneven actomyosin contractility in the dorsal epidermis resulting from excessive \textit{zip} expression.

While it is likely that increased levels of Dpp in \textit{Egfr} mutant embryos contribute to the elevated \textit{zip} levels, our results with manipulation of Egfr signaling support the interpretation of a separate route for \textit{zip} regulation that involves signaling from the amnioserosa to both the amnioserosa and the epidermis. Thus, we consider this \textit{zip} regulation a second distinct role for Egfr in dorsal closure and we believe this signaling is the same as that regulated by Ack in its control of \textit{zip} expression. Consistent with this, gains or losses of Ack do not affect the Dpp pathway, supporting the view that Ack operates in parallel to Dpp signaling (Sem et al. 2002; Zahedi et al. 2008). The Ack/Egfr-regulated signal could be a diffusible ligand ("X" in Figure 4.11) produced in the amnioserosa cells that activates a pathway in the amnioserosa and DME cells, thereby driving \textit{zip} expression (Zahedi et al. 2008). Alternatively, Egfr could be promoting production of a signal that negatively regulates the pathway required for \textit{zip} expression.
The signaling events regulating zip expression may be occurring at a stage prior to the stage at which the amnioserosa is lost in Egfr mutants, i.e. before initiation of germband retraction. As discussed in the next chapter, we believe that Dpp acts in parallel to produce a second diffusible ligand (“Y” in Figure 4.11) that activates a second pathway contributing to zip expression. Thus, the interplay between Egfr and Dpp during dorsal closure is complex, involving multiple pathways and bidirectional communication between two tissues, and this complex signaling arrangement may function to ensure the coordinated morphogenesis of the amnioserosa and epidermis.
Figure 4.11 Model for Egfr acting as a brake on dorsal closure

Egfr negatively regulates the production and/or secretion of a diffusible signal “X” in the amnioserosa (AS) and is itself negatively regulated by Ack through endocytosis. “X” signals into both the amnioserosa and the DME cells where it activates a pathway promoting transcription of myosin from the zip locus. Previous work from our group and others, and unpublished results from our group, suggest that Dpp from the DME cells diffuses to the amnioserosa where it regulates production of a second diffusible signal “Y” providing a parallel input into zip transcription. Myosin produced through the cooperation of the two pathways then drives morphogenesis of the amnioserosa and DME cells. Egfr additionally regulates this signaling network by negatively regulating dpp transcription in the epidermis, including the DME cells. Egfr further regulates amnioserosa morphogenesis by inhibiting apoptosis in this tissue.
A third major role for Egfr in dorsal closure is as a negative regulator of apoptosis in the amnioserosa. Enhancement of apoptosis accelerates dorsal closure whereas suppression of apoptosis slows it, indicating that apoptosis, similar to actomyosin contractility, provides a force for morphogenesis (Toyama et al. 2008). Thus, down regulation of Egfr in the amnioserosa during dorsal closure provides two means to accelerate the process: increased myosin expression and increased cell death. The “tweaking” of Egfr function in the amnioserosa could constitute an important regulatory mechanism for controlling the rate of closure. We have provided evidence that endocytosis, promoted by Ack, is a route by which Egfr signaling is controlled in the amnioserosa cells. Our results suggest that Ack would have a pro-apoptotic role in the amnioserosa through promotion of Egfr endocytosis. This in contrast to the Drosophila eye in which Ack has an anti-apoptotic function that is independent of Egfr (Schoenherr et al. 2012).

A recent study has demonstrated that endocytosis in the amnioserosa is required for its correct morphogenesis during dorsal closure, but this work focused on the role of endocytosis in removing membrane to promote cell shape change (Mateus et al. 2011). Our results indicate that another route of action for endocytosis in the amnioserosa is in regulation of Egfr signaling. It has been suggested that endocytosis could act as a rheostat in which membrane area is adjusted in response to actomyosin contractility (Mateus et al. 2011); such a rheostat could also be used to adjust Egfr signaling throughout dorsal closure.

Additional avenues for Egfr regulation during dorsal closure could be control of ligands binding to Egfr and feedback inhibition (Freeman 2000; Segatto et al. 2011), but we have yet to address these. In summary, we have identified Egfr signaling as an inhibitor of morphogenesis during dorsal closure that acts at several distinct levels. Having a single pathway control multiple aspects of this complex process may simplify feedback regulation, ensuring that morphogenesis occurs in a coordinated fashion. In essence, Egfr signaling acts as a brake that can be applied when required to ensure that closure proceeds smoothly. Dorsal closure shows striking parallels to the healing of induced wounds in the Drosophila embryo, with the two processes using similar cytoskeletal and signaling machineries (Campos et al. 2010; Martin and Parkhurst 2004; Wood et al. 2002). Egfr has recently been shown to be required for healing of induced
wounds in the embryo and it will be of interest to determine if it uses similar routes of action in this as we have shown in dorsal closure (Geiger et al. 2011).
5. **Results part III: Cooperation between ecdysone and JNK signaling in regulation of zip expression during dorsal closure**

As outlined in the previous two chapters, we have evidence for the existence of a diffusible signal or signals produced in the amnioserosa which activates zip expression in both the amnioserosa and the dorsal epidermis. A candidate signal is the steroid hormone, ecdysone, which is a key regulator of cell death and cell shape changes during *Drosophila* metamorphosis. The amnioserosa is considered a major source of 20E during embryogenesis, and mutations in members of the “Halloween” group of genes encoding enzymes in the ecdysone biosynthetic pathway have dorsal closure defects (Chavez et al. 2000; Giesen et al. 2003; Kozlova and Thummel 2003).

5.1. **Ecdysone promotes zip expression during dorsal closure**

We wondered if ecdysone was required for zip expression, and tested this by looking at embryos with reduced ecdysone levels. Ecdysone is synthesized from cholesterol by a biosynthetic pathway that includes the enzymes, Spook (Spo) and Disembodied (Dib) (X. Huang et al. 2008). We performed zip FISH on *spo*\(^{Z339}\) and *dib*\(^{2}\) mutant embryos in which ecdysone production was disrupted and saw a dramatic loss of zip transcription during dorsal closure (Figure 5.1 B-C). Consistent with this result, a reduction of zip FISH signal was also found in *ecdysoneless*\(^{I}\) (*ecd*\(^{I}\)) embryos raised at 29°C (Figure 5.1 D). *ecd*\(^{I}\) is a temperature-sensitive allele that disrupts ecdysone synthesis at the restrictive temperature of 29°C (Garen et al. 1977; Henrich et al. 1987).

To assess the effects of excessive ecdysone signaling on zip expression, wild-type embryos were treated with exogenous 20E. Strikingly, zip expression was elevated in the epidermis, extending ventrally down the segment borders, but amnioserosa and head zip levels remained similar in comparison to wild-type (Figure 5.1 E-F). These
results demonstrate that ecdysone is required for zip expression. However, it is not sufficient to drive zip expression globally throughout the embryo. We were curious to see if ecdysone affected the expression of another key dorsal closure gene, dpp, but saw no change in dpp expression in spo mutant embryos, (Figure 5.1 G-H).
Figure 5.1  Ecdysone promotes zip but not dpp expression during dorsal closure

(A-F) zip FISH on embryos at dorsal closure stage. (A) Wild-type embryo showing high level of zip expression in the DME cells. (B, C) spo (B) and dib (C) mutant embryos showing lack of zip expression in DME cells. (D) ecd¹ mutant embryo, collected after transferring the parents to restrictive temperature (29°C) for 30 hours, showing reduced zip expression. (E, F) 20E treatment on wild-type embryos. (E) Control embryo treated with ethanol (F) Embryo treated with 20E in ethanol showing ectopic zip expression in stripes along segmental grooves. G-H) dpp FISH on wild-type embryo (G) and spo mutant embryo (H) showing similar levels of dpp expression. Scale bar: 50 µm.
5.2. Dpp signaling regulates ecdysone production

Having established that 20E is required for zip expression in the amnioserosa and DME cells, we looked for upstream regulators of 20E production. The timing of 20E production in the amnioserosa could be controlled by having all members of the ecdysone biosynthetic pathway present at the dorsal side of the embryo, with the exception of one or a few enzymes that would be expressed in the amnioserosa at the appropriate time. Dpp signals from the epidermis to the amnioserosa, and is required for zip expression and morphogenesis in these two tissues (Zahedi et al. 2008). spo is the only gene encoding a member of the 20E biosynthetic pathway shown so far to actually be transcribed in the amnioserosa (Figure 5.2 B). Thus, we speculated that the ecdysone pulse in the amnioserosa during germband retraction was dependent on spo.

We looked at spo expression in.tkv mutant embryos, which lack one of the Dpp receptors, and found that spo expression in the amnioserosa was markedly reduced in homozygous mutant embryos (selected by the absence of GFP signal) compared to their heterozygous siblings (Figure 5.2 A-B). We postulated that the lack of zip expression previously reported in tkv mutant embryos was due to reduced spo expression and the resulting reduction in 20E production. Consistent with this hypothesis, treatment of tkv mutant embryos with exogenous 20E was found to restore zip expression to near wild-type levels (Figure 5.2 C-D).

In the search for candidates that might function downstream of Dpp signaling to regulate spo expression, we focused on Hindsight (Hnt). Hnt is a nuclear zinc-finger protein required for dorsal closure, and its expression in the amnioserosa is dependent on Dpp signaling (B. G. Fernandez et al. 2007). hnt mutant embryos were found to display elevated spo expression in the amnioserosa compared to heterozygous sibling controls (Figure 5.2 E-F), and as predicted, they also showed elevated zip expression in the dorsal epidermis (Figure 5.2 G-H). Hnt may be functioning in a negative feedback loop during dorsal closure, being turned on by Dpp signaling and then terminating ecdysone production by down regulation of spo expression.
**Figure 5.2**  **Dpp signaling and Hnt regulate spo expression in the amnioserosa**

(A, B, E, F) spo FISH on embryos at the beginning of germband retraction. (C, D, G, H) zip FISH on embryos during dorsal closure. (A, B) tkv mutant embryo (A) and heterozygous sibling (B) showing that spo expression is greatly reduced in the absence of Dpp signaling. (C) tkv mutant embryo showing disruption of zip expression. This embryo has been treated with ethanol the same as (D) but with no 20E. (D) tkv mutant embryo treated with 20E in ethanol showing restoration of zip expression to near wild-type levels. (compare to Fig. 1F). (E, F) hnt heterozygous embryo (E) and hemizygous sibling (F) showing that loss of Hnt leads to elevated spo expression. (G, H) hnt mutant embryos showing elevated zip expression in the epidermis. Scale bar: 50 µm.
5.3. Ecdysone signaling cooperates with JNK signaling to promote zip expression

Embryos cultured with 20E showed a restricted pattern of ectopic zip expression, although a previous study showed that the ecdysone receptor (EcR) is widely activated by such treatment (Kozlova and Thummel 2003). This result suggests that 20E signaling is not sufficient to activate zip expression and requires additional inputs. The JNK cascade which leads to the activation of the AP-1 transcription factor represents a likely input, as it is required for dorsal closure as a central component of signaling in the DME cells. Interestingly, a study using SAGE analysis identified two ecdysone-responsive genes, IMP-E1 and IMP-L1, as being regulated by JNK in the embryo (Jasper et al. 2001). Furthermore, the expression of both genes in the embryonic epidermis during dorsal closure has been reported to be dependent on the Halloween group genes (Chavez et al. 2000).

When we activated JNK signaling by overexpressing an activated version of the small GTPase Rac1, Rac1<sup>V12</sup>, which activates JNK signaling using the prd-GAL4 driver, we observed an elevation of IMP-E1 expression in paired stripes (Figure 5.3 C compares to A). Rac<sup>V12</sup> over-expression causes significant disruption of the embryo and we decided to try activating the JNK pathway by expressing a constitutively active form of the JNKK, hep<sup>CA</sup> (Weber et al. 2000) using the prd-GAL4 driver. These embryos were less disrupted but expression of both IMP-E1 and IMP-L1 were elevated in prd stripes (Figure 5.3 D compares to A, F compares to E), indicating that these two genes can indeed be activated by JNK signalling during dorsal closure (Giesen et al. 2003).
**Figure 5.3** JNK signaling promotes IMP-L1 and IMP-E1 expression

(A-C) IMP-E1 FISH on embryos during dorsal closure. (A) Wild-type embryo showing robust IMP-E1 expression in epidermis. (B, C) Embryos in which JNK signaling was activated through overexpressing UAS-Rac1\(^{V12}\) (B) or UAS-hep\(^{CA}\) (C) in prd stripes showing elevated IMP-E1 expression in the epidermis. (D) Cartoon schematic showing the expression pattern of prd-Gal4 transgene (pink segments). (E-F) IMP-L1 FISH on embryos during dorsal closure. (C) Wild-type embryo showing IMP-L1 expression in epidermis with similar pattern as IMP-E1. (F) Embryos in which JNK signaling was activated through overexpressing UAS-hep\(^{CA}\) in prd stripes showing elevated IMP-L1 expression in the epidermis. Scale bar: 50 µm.
To determine if activation of JNK signaling similarly promoted zip expression, we again expressed hep$^{CA}$ or Rac1$^{V12}$ in prd stripes and found that both elevated zip transcript levels in the epidermis and amnioserosa (Figure 5.4 B-D). To test if endogenous JNK signaling is required for expression of zip in wild-type embryos, we impaired the JNK pathway in prd stripes through the expression of dominant negative JNK (encoded by basket; bsk), bsk$^{DN}$ (Weber et al. 2000), which resulted in a loss of zip transcripts from the DME cells in prd stripes (Figure 5.4 E-F). 20E treatment did not restore zip transcription in bsk$^{DN}$-expressing embryos, indicating that 20E induction of zip expression is JNK-dependent (Figure 5.5 A-B).

To further address the requirement for 20E in promoting JNK/AP-1 signaling dependent gene expression, we again expressed hep$^{CA}$ in prd stripes, but in the spo mutant background where 20E levels are reduced (Chavez et al. 2000). While hep$^{CA}$ could still drive strong IMP-L1 elevation in the absence of 20E (Figure 5.5 E-F), it was less effective at driving expression of zip (Figure 5.5 C-D) and could barely drive any elevation of IMP-E1 expression (Figure 5.5 G-H), suggesting variation in the cooperation between JNK/AP-1 and 20E on the expression of these three genes. These various results indicate that 20E produced in the amnioserosa cooperates with JNK signalling in the expression of genes during dorsal closure. We speculated in the previous chapter that Ack and Egfr might be regulating a diffusible signal from the amnioserosa that was required for zip expression in the amnioserosa and DME cells. Our results in this chapter suggest that this signal could be 20E. We did a preliminary experiment to see if Ack required the spo gene to drive ectopic zip expression. Ack over-expression in prd stripes in the amnioserosa causes ectopic zip accumulation in the amnioserosa (Mateus et al. 2011) (Figure 5.5 I), but this ectopic zip is significantly blocked when Ack is over-expressed in a spo mutant background (Figure 5.5 J). This result suggests that Ack (and therefore Egfr) might regulate zip expression through 20E. However, this regulation is not carried out through controlling spo expression, since spo levels remained wild-type in embryos mis-expressing Ack or Egfr pathway components (Figure 5.6). Nonetheless, the mechanism by which Ack and Egfr pathway regulate the availability of 20E will be an interesting focus for future research.
Figure 5.4  JNK signaling promotes zip expression

zip FISH on embryos during dorsal closure. (A) Wild-type embryo showing zip expression accumulating in DME cells. (B-D) Activation of JNK signaling through expression of Rac1$^{V12}$ (B) or hep$^{CA}$ (C, D) in prd stripes results in ectopic zip expression. Higher power view in (D) reveals that ectopic zip extends into the amnioserosa in prd stripes (white arrow). (E, F) Inhibition of JNK signaling through expression of bsk$^{DN}$ in prd stripes results in loss of zip expression in DME cells within the prd stripes. High power view is shown in (F). Scale bar: 50µm.
Figure 5.5  JNK signaling cooperates with ecdysone to regulate target gene expression
(A-D) *zip* FISH on embryos during dorsal closure. (A) Embryo in which JNK signaling is blocked in *prd* stripes by expressing *bsk*DN showing gaps in DME *zip* expression. (B) Incubation with 20E cannot restore *zip* expression lost in the gaps due to expression of *bsk*DN in *prd* stripes (white arrow). (C, D) Embryo in which the JNK signaling is activated through expression of *hep*CA in *prd* stripes with wild-type background (C) or *spo* mutant background (D), showing the *spo* mutant reduces the ability of *hep*CA to promote robust *zip* expression. (E, F) *IMP-L1* FISH on embryos same as (C) and (D) showing that the *spo* mutant background does not prevent induction of *IMP-L1* expression by *hep*CA. (G, H) *IMP-E1* FISH on embryos with same genotype as (C) and (D) showing that the *spo* mutant background greatly reduced the ability of *hep*CA to promote *zip* expression. (I-J) *zip* FISH on dorsal closure embryos. (I) Embryo in which wild-type Ack was expressed in *prd* stripes ectopic *zip* in the amnioserosa. (J) Embryo in which the wild-type Ack was expressed in *prd* stripes in *spo* mutant background showing no ectopic *zip* in the amnioserosa. Scale bar: 50µm.
**Figure 5.6**  *spo* FISH of embryos with mis-expression of Ack and Egfr signaling components

*spo* FISH of embryo in early germband retraction stage. (A) Wild-type embryo showing *spo* expression in the amnioserosa. (B-C) Embryos in which wild-type (B) or kinase-dead (C) Ack was expressed in *prd* stripes showing wild-type level of *spo* expression. (D-F) Egfr mutant embryo (D) or embryos in which dominant negative version of Egfr (E) or Ras (F) was expressed in the amnioserosa showing similar *spo* levels as wild-type embryo. Scale bar: 50µm.
To address whether ecdysone regulates expression of the three JNK-responsive genes through the canonical ecdysone signaling pathway, we tested the requirement for the ecdysone receptor (EcR). Normally, once it has diffused into the target cell, 20E binds to the EcR, which forms a heterodimer with the retinoid X receptor homologue Ultraspireacle (Kozlova and Thummel 2000) to form a functional transcription factor that then binds to ecdysone responsive elements in target genes. Two dominant negative versions of EcR, EcR-F645A and EcR-W650A are thought to block endogenous EcR by dimerizing with Ultraspireacle and repressing transcription at ecdysone response elements (Cherbas et al. 2003). These two dominant negative transgenes were expressed in prd stripes, and we assessed the effect on expression of zip, IMP-L1 and IMP-E1. We found reduced expression of IMP-L1 within the prd stripes, but zip was unaffected (Figure 5.7 A-F). Surprisingly, the expression of IMP-E1 was also unaffected (Figure 5.7 G-I), which was not what we expected, since the IMP-E1 was reported as an ecdysone inducible gene (Chavez et al. 2000). These results suggested that the 20E does not regulate zip and IMP-E1 through ecdysone responsive elements.

We then went on to further address requirements for EcR in zip expression by studying EcR mutant embryos. Due to a requirement for EcR in oogenesis, it is not possible to create embryos completely devoid of EcR (Carney and Bender 2000). Homozygous mutant embryos for the EcR loss-of-function allele EcRM554fs display dorsal closure defects (Chavoshi et al. 2010), but showed normal expression of zip (data not shown). The temperature sensitive allele EcRA483T, which produces a non-functional EcR at restrictive temperature 29°C due to a conformational change of the protein (Carney and Bender 2000), enabled us to achieve a significant knock down of EcR in the embryo. Embryos heteroallelic for EcRM554fs and EcRA483T raised at 29 °C showed a substantial reduction in zip expression (Figure 5.7 J). Our various results indicate that while 20E does act through EcR in its regulation of zip expression, EcR may not be functioning at ecdysone response elements.
Figure 5.7  Evidence that EcR regulates zip expression independent of the canonical ecdysone response element
(A–C) *zip* FISH on embryos at dorsal closure stage. (A) Wild-type embryo showing *zip* expression in epidermis. (B, C) Embryos in which canonical EcR function was blocked in prd stripes through expression of dominant negative versions of EcR showing wild-type levels of *zip* expression. (D–F) *IMP-L1* FISH on embryos at dorsal closure stage. (D) Wild-type embryo showing *IMP-L1* expression in epidermis. (E, F) Embryos showing elimination of *IMP-L1* expression in prd stripes due to the expression of dominant negative versions of EcR. (G–I) *IMP-E1* FISH on embryos at dorsal closure stage. (G) Wild-type embryo showing *IMP-E1* expression in epidermis. (H, I) Embryo in which dominant negative versions of EcR were expressed in prd stripes showing no effect on *IMP-E1* expression. (J) *zip* FISH on embryo heteroallelic for EcR<sup>M554fs</sup> and EcR<sup>A483T</sup> (temperature sensitive) raised at 29°C, showing dramatically reduced *zip* expression during dorsal closure. Scale bar: 50µm.

### 5.4. Evidence that EcR acts in a complex with Jun in amnioserosa and DME nuclei to regulate *zip* expression

Having established that ecdysone and EcR are both required for *zip* expression, we wanted to further characterize the interaction between JNK and ecdysone signaling pathways during dorsal closure. There is evidence in mammals that the estrogen receptor, which is a steroid hormone receptor similar to EcR, can bind to the JNK-activated transcription factor AP-1 and regulate gene expression, independently of ecdysone response elements (Bjornstrom and Sjoberg 2004; Jakacka et al. 2001). Additionally, a study mapping binding regions for EcR in 20E-treated Kc167 cells identified a 4 kb region of the *zip* gene that is bound by EcR (Gauhar et al. 2009). This region is largely intronic and contains five sequence elements matching the AP-1 consensus binding site (TGANTCA) but no consensus ecdysone responsive element ((A/G)(G/T)TCANTGA(A/C)(C/T)). Therefore, we hypothesized that EcR functions in a complex with AP-1 to drive *zip* transcription in the amnioserosa and DME cells.

To look for evidence that EcR was complexing with AP-1 in the embryo, we first immunoprecipitated EcR from embryo lysates and looked for co-immunoprecipitation of Jun, which together with Fos makes up the AP-1 transcription factor. Despite repeated attempts, an interaction could not be detected. This failure to detect an interaction could be due to the fact that most EcR in the embryo is likely acting independently of AP-1. Thus, we decided to look for interactions between EcR and Jun *in situ* using proximity ligation assay (PLA), a technique recently used to detect protein complexes in the nuclei of *Drosophila* embryos (Petruk et al. 2012; Soderberg et al. 2006). PLA reveals
interactions between two endogenous proteins by detecting close proximity (<40nm) of antibodies against the two proteins (which must have been raised in two different species) using commercially available oligonucleotide-tagged secondary antibodies, which, together with two added oligonucleotides, form a substrate for an in situ rolling circle DNA replication reaction that is detected using fluorescently-labelled probes.

In antibody staining of embryos during dorsal closure, although we could not obtain a good Fos antibody, the mouse anti-EcR and rabbit anti-Jun antibodies clearly revealed the presence of these proteins in the nuclei of both amnioserosa and epidermal cells (Figure 5.8 A-A', B-B'). Therefore, we used these two antibodies for PLA (Figure 5.8). The PLA signal demonstrated complexes of EcR and Jun in amnioserosa nuclei during germband retraction (Figure 5.8 A'') and persisting through to dorsal closure (Figure 5.8 B''). Negative controls in which PLA was performed with the anti-EcR antibody omitted (Figure 5.8 C-C'') or with anti-Jun antibody replaced by anti-pMad, which detects another transcription factor functioning in the epidermis and amnioserosa during dorsal closure (Figure 5.8 D-D''), showed very low background. PLA signals were most obvious in the amnioserosa and there are likely a number of reasons for this. First the levels of both 20E and the EcR (Figure 5.8 A, B) are highest in this tissue. Second, the amnioserosa is polyploid, so there are more copies of a given gene for EcR and Jun to bind to than elsewhere in the embryo (Buchenau et al. 1997).
Figure 5.8  EcR is in a complex with Jun
(A-A’’) Views of embryo during germband retraction. (B-D’’) Views of embryos during dorsal closure. (A, B) Wild-type embryos showing EcR immunostaining in nuclei of the amnioserosa and epidermal cells. (A’, B’) wild-type embryos showing high level of Jun immunostaining in nuclei of the amnioserosa and DME cells. (A’’, B’’) Wild-type embryos showing EcR/Jun PLA signals in both amnioserosa and epidermal cells. Note the PLA signals in the amnioserosa are clustered. (C-C’’) Negative control with EcR antibody omitted showing background level. (D-D’’) Negative control with Jun antibody replaced by pMad antibody showing background level. Scale bar: 50µm. (E) Schematic of zip locus. Coding exon sequences are shown as orange boxes and non-coding exon sequences as grey boxes. Consensus AP-1 binding sites are shown as magenta boxes marked by an asterix. The previously identified 4 kb zip intronic sequence bound by EcR is labeled. Note the cluster of AP-1 binding sites in this region. Scale bar: 50 µm (A-D).

If some of the PLA signal in the amnioserosa is the result of EcR/Jun complexes binding to the zip gene, then the number of complexes should be reduced in embryos lacking the zip locus. Indeed, in embryos homozygous for the deficiency, Df(2R)BSC608, which deletes the entire zip locus, we saw an average of 7 PLA puncta per amnioserosa nucleus compared to an average of 14 PLA puncta per amnioserosa nucleus in wild-type embryos (Figure 5.9 A’, B’ and C). For wild-type, 43 nuclei in 6 embryos were counted and for Df(2R)BSC608 46 nuclei in 6 embryos were counted. This result strongly supports the idea that EcR complexes with Jun at sequences in the zip locus in the amnioserosa, and furthermore that there should be other genes regulated in the amnioserosa in this manner. Our model is that zip is regulated by EcR/Jun in both the amnioserosa and DME cells. There were only scattered PLA signal in the epidermis that at first glance appeared to be just background. However, the average number of PLA puncta per DME nucleus was significantly reduced in Df(2R)BSC608 embryos (~0.1 PLA puncta per DME cell) compared to wild-type (~0.28 PLA puncta per DME cell) after the counting of 285 nuclei in 6 wild-type embryos and 311 nuclei in 6 Df(2R)BSC608 embryos (Figure 5.9 A’’, B’’ and D), consistent with EcR/Jun complexes at zip gene in the DME cells. These findings established dorsal closure as a system to study steroid hormone receptor/AP-1 interactions in the regulation of gene expression.
Figure 5.9  PLA signals are reduced in zip deficient embryo
(A-A’’) wild-type embryo showing Jun immunostaining (A), PLA signals in the amnioserosa and epidermis (A’), and merge of the two signals (A’’). (B-B’’) zip deficiency (Df(2R)BSC608) embryo showing “thinned out” PLA signals (B’), counter staining with Jun antibody shown in (B), and merged image shown in (B’’). White arrows mark the PLA signals in DME cells. Scale bar: 50µm. (C) Quantification of PLA signals in amnioserosa of wild-type and Df(2R)BSC608 embryos. Puncta were counted in embryos at beginning of dorsal closure when amnioserosa cells are flat and unfolded. For wild-type 43 nuclei in 6 embryos were counted and for Df(2R)BSC608 46 nuclei in 6 embryos were counted. P<0.0001. (D) Quantification of PLA signals in DME cells of wild-type and Df(2R)BSC608 embryos. For wild-type 285 nuclei in 6 embryos were counted and for Df(2R)BSC608 311 nuclei in 6 embryos were counted. P<0.0002.

Our results suggest at least one other gene is bound by EcR/Jun in the amnioserosa as half the PLA puncta persist in embryos with the zip locus deleted. We were interested to find out what other genes might be regulated by non-canonical ecdysone signaling. With the assistance of Dr. Jack Chen’s lab, we screened through a collection of 502 genomic sequence segments bound by EcR for candidate genes regulated jointly by AP-1 (Gauhar et al. 2009). We looked for genes that had a minimum of five AP-1 consensus elements but no consensus EcR binding sequence in the EcR-bound region. We found 24 chromosomal regions that met these requirements and which were in introns or adjacent to gene (Table 5.1).

In addition to zip, several other genes in this list, such as u-shaped (ush), enabled, and the insulin-like receptor, have been shown to be involved in dorsal closure (Brogiolo et al. 2001; Gates et al. 2007; Lada et al. 2012). In addition, the gene coding for EcR was also found in our search. ush, encoding a C2H2 zinc finger transcription factor, is of particular interest as expression of ush in the AS is dependent on Dpp signalling. As Dpp is required for ecdysone production, this would be consistent with ush being an ecdysone target in the amnioserosa. Interestingly, ush is an important participant in dorsal closure, being required for morphogenesis of both the amnioserosa and epidermis, and is involved in signaling from the amnioserosa to the DME cells. Remarkably, located in an intron of ush, but transcribed in the opposite direction, is a second amnioserosa-expressed dorsal closure participant, cabut (cbt), which also encodes a C2H2 zinc finger transcription factor. Cbt is required for morphogenesis of the DME cells, and its expression in the amnioserosa is JNK-dependent. Given that enhancers are orientation independent, an interesting possibility is that the EcR/AP-1 complex activates expression of both ush and cbt in the AS. In summary, we have identified interesting candidate genes for regulation by EcR/AP-1 during dorsal closure.
Table 5.1  Information on candidate genes regulated by EcR/AP-1 complex

<table>
<thead>
<tr>
<th>Chr.</th>
<th>start</th>
<th>stop</th>
<th>position</th>
<th>Flybase ID</th>
<th>gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2L</td>
<td>490336</td>
<td>496335</td>
<td>Intergenic 5-prime end</td>
<td>FBgn0043364</td>
<td><em>cabut</em></td>
</tr>
<tr>
<td>2L</td>
<td>518336</td>
<td>524335</td>
<td>Intergenic 5-prime end</td>
<td>FBgn0003963</td>
<td><em>u-shaped</em></td>
</tr>
<tr>
<td>2L</td>
<td>16517917</td>
<td>16521916</td>
<td>Intron</td>
<td>FBgn0032587</td>
<td>CG5953</td>
</tr>
<tr>
<td>2L</td>
<td>18621918</td>
<td>18633917</td>
<td>Intron</td>
<td>FBgn0032694</td>
<td>Misexpression suppressor of ras 3</td>
</tr>
<tr>
<td>2L</td>
<td>19135918</td>
<td>19141917</td>
<td>Intron</td>
<td>FBgn0010300</td>
<td><em>brain tumor</em></td>
</tr>
<tr>
<td>2R</td>
<td>2006638</td>
<td>2012637</td>
<td>Intron</td>
<td>FBgn0000546</td>
<td>ecdysone receptor</td>
</tr>
<tr>
<td>2R</td>
<td>7548793</td>
<td>7554792</td>
<td>Intron</td>
<td>FBgn0044020</td>
<td><em>Roc2</em></td>
</tr>
<tr>
<td>2R</td>
<td>8734792</td>
<td>8740791</td>
<td>Intron</td>
<td>FBgn0033777</td>
<td>CG17574</td>
</tr>
<tr>
<td>2R</td>
<td>9780792</td>
<td>9786791</td>
<td>Intron</td>
<td>FBgn0013733</td>
<td><em>short stop</em></td>
</tr>
<tr>
<td>2R</td>
<td>15031786</td>
<td>15037785</td>
<td>Intron</td>
<td>FBgn0000578</td>
<td>enabled</td>
</tr>
<tr>
<td>2R</td>
<td>20191956</td>
<td>20197955</td>
<td>Intron</td>
<td>FBgn0003888</td>
<td>β-Tubulin at 60D</td>
</tr>
<tr>
<td>2R</td>
<td>20887954</td>
<td>20891953</td>
<td>Intron</td>
<td>FBgn0005634</td>
<td><em>zipper</em></td>
</tr>
<tr>
<td>3L</td>
<td>4115375</td>
<td>4125374</td>
<td>Intron</td>
<td>FBgn0035499</td>
<td>Chd64</td>
</tr>
<tr>
<td>3L</td>
<td>8672391</td>
<td>8686390</td>
<td>Intergenic 3-prime end</td>
<td>FBgn0001168</td>
<td><em>hairy</em></td>
</tr>
<tr>
<td>3L</td>
<td>14050221</td>
<td>14056220</td>
<td>Intron</td>
<td>FBgn0052138</td>
<td>CG32138</td>
</tr>
<tr>
<td>3L</td>
<td>15584222</td>
<td>15592221</td>
<td>Intron</td>
<td>FBgn0036518</td>
<td>Rho GTPase activating protein at 71E</td>
</tr>
<tr>
<td>3L</td>
<td>17908222</td>
<td>17922221</td>
<td>Intergenic 5-prime end</td>
<td>FBgn0036772</td>
<td>CG5290</td>
</tr>
<tr>
<td>3L</td>
<td>22935823</td>
<td>22943822</td>
<td>Intron</td>
<td>FBgn0037207</td>
<td>Mesoderm-expressed 2</td>
</tr>
<tr>
<td>3R</td>
<td>1102004</td>
<td>1108003</td>
<td>Intron</td>
<td>FBgn0013576</td>
<td><em>mustard</em></td>
</tr>
<tr>
<td>3R</td>
<td>5423985</td>
<td>5427984</td>
<td>Intron</td>
<td>FBgn0037720</td>
<td>CG8312</td>
</tr>
<tr>
<td>3R</td>
<td>14737984</td>
<td>14751983</td>
<td>Intron</td>
<td>FBgn0038661</td>
<td>Xrp1</td>
</tr>
<tr>
<td>3R</td>
<td>17021984</td>
<td>17031983</td>
<td>Intron</td>
<td>FBgn0025803</td>
<td>SNF4/AMP-activated protein kinase gamma subunit</td>
</tr>
<tr>
<td>3R</td>
<td>17415037</td>
<td>17427036</td>
<td>Intron</td>
<td>FBgn0013984</td>
<td>Insulin-like receptor</td>
</tr>
<tr>
<td>3R</td>
<td>27230635</td>
<td>27236634</td>
<td>Intron</td>
<td>FBgn0004834</td>
<td>G protein-coupled receptor kinase 2</td>
</tr>
<tr>
<td>X</td>
<td>3321596</td>
<td>3327595</td>
<td>Intergenic 3-prime end</td>
<td>FBgn0029658</td>
<td>CG14269</td>
</tr>
</tbody>
</table>
5.5. Testing for AP-1 regulation by other nuclear receptors

Studies in mammalian cell culture show that AP-1 activity can be controlled at different levels including the abundance of this transcription factor (Angel and Karin 1991), phosphorylation by upstream mitogen activated protein kinases (Wisdom 1999), and finally direct interaction with other proteins which can markedly influence AP-1 activity. Nuclear receptors have been shown to modulate AP-1 activity by a mechanism known as transrepression (Herrlich 2001), and this has may be true in Drosophila. During dorsal closure, the two components of AP-1, Jun and Fos, are expressed relatively uniformly and over-expression of either has essentially no effect (Kockel et al. 1997; Zeitlinger et al. 1997). Seven-up (svp) and knirp-related (knrl), which are nuclear receptors that do not respond to ecdysone have been shown to antagonize AP-1 activity during dorsal closure. Overexpression of the wild-type form either of these two nuclear receptors phenocopied the loss-of-function Fos mutant (kay\sup{1}) phenotype; furthermore, increasing the level of these nuclear receptors in a kay mutant background could enhance the dorsal closure cuticle defect (Gritzan et al. 2002).

While we were testing for the regulation of JNK/AP-1 by EcR, we also wondered whether these two nuclear receptors might inhibit AP-1 activity in the transcriptional regulation of the zip gene during dorsal closure. With no antibody available for these two proteins, I examined the transcript expression pattern using FISH or a lacZ reporter gene.svp FISH showed high levels of transcription during dorsal closure; however the transcripts accumulated in what appeared to be neuronal cells but not in the amnioserosa or epidermal cells (Figure 5.10 A). The expression pattern of knrl was revealed by expressing the UAS-lacZ under the knrl-GAL4 driver, as a result, we saw β-GAL signal in the lateral epidermis and some of the amnioserosa cells (Figure 5.10 B).

To find out ifsvp and knrl affected AP-1’s ability to regulate target gene regulation during dorsal closure, we did dpp and zip FISH on embryos with losses or gains of function of these two genes. In embryos carrying the amorphicsvp allele,svp\sup{1}, neither dpp nor zip expression was affected (Figure 5.10 D-E, F-G). On the other hand, when over-expressing the wild-type form ofsvp and knrl using the prd-GAL4 driver, we could not detect significant changes in either dpp or zip expression in the prd stripes (Figure 5.10 E, H-J). The results above suggest that these two nuclear receptors might
not transrepress AP-1 activity or that this transrepression affects genes other than \textit{dpp} and \textit{zip}.

Although being an orphan nuclear receptor, \textit{DHR38} has been shown to respond to 20E in a ligand sensor system where the GAL4-tagged DHR38 was able to turn on the expression of a \textit{UAS-lacZ} reporter upon the addition of exogenous 20E (Palanker et al. 2006). One hypothesis raised by this finding would be that the EcR and DHR38 might have redundant functions in target gene regulation. As shown above, the reduction in \textit{zip} levels is not as great in EcR mutant embryos as it is in \textit{spo} mutant embryos. Furthermore, expression of RNAi against EcR in \textit{prd} stripes is not effective at knocking down \textit{zip} (data not shown). These results suggest that 20E might act by a route in addition to EcR in its regulation of \textit{zip} expression, with a possible route being DHR38. We tested \textit{zip} expression in embryos with both receptors knocked down by RNAi transgene expression in \textit{prd} stripes. We maximized the RNAi effect by including \textit{UAS-Dicer2 (Dcr2)} and maintaining the mating cage at 29°C. In the positive control FISH, \textit{IMP-L1} expression was reduced in \textit{prd} stripes, similar to what is seen with RNAi against EcR alone (Figure 5.10 J, K). However, we still could not reduce \textit{zip} or \textit{IMP-E1} levels in \textit{prd} stripes (Figure 5.10 L-N). These results suggest that DHR38 does not drive \textit{zip} or \textit{IMP-E1} expression in response to 20E.
Figure 5.10  Testing of zip regulation by nuclear receptors
All the embryos are at dorsal closure stage. (A) svp FISH on wild-type embryo showing accumulation of endogenous svp transcripts in neuronal cells in red, and epidermal cell outlines revealed with anti-PY antibody in blue. (B) Embryo in which the UAS-lacZ reporter transgene was expressed under knrl-GAL4 driver showing the spatial expression pattern of knrl in lateral epidermis and some amnioserosa cells. (C-E) dpp FISH on wild-type embryo (C) and embryos with loss of svp (D) or gain of svp (E) in prd stripes showing similar expression level of dpp. (F-I, L) zip FISH on wild-type embryo (F), embryos with loss of svp (G), gain of svp (H) or knrl (I) in prd stripes, and knocked down of both EcR and DHR38 by RNAi in prd stripes (L), all showing wild-type levels of zip transcription. (J, K) IMP-L1 FISH on wild-type embryo (J) and embryo with knock down of both EcR and DHR38 using RNAi in prd stripes (K). The reduction in IMP-L1 expression is comparable to that seen with knock down of EcR alone (compare to Figure 5.7 E, F). (M, N) IMP-E1 FISH on wild-type embryo (M) and embryo with knock down of both EcR and DHR38 using RNAi in prd stripes showing similar levels of IMP-E1 expression. Scale bar: 50µm.

5.6. Testing candidate ligands and receptors for a role in JNK activation

Despite the JNK cascade having been long established as a central regulator of dorsal closure, we still do not know how it is activated during this process. There is evidence suggesting the existence of activation by an extracellular signal. For example, the secreted serine protease, Scarface, expressed by the DME cells is thought to repress JNK activation by regulating ligand/receptor interactions in the extracellular space neighbouring the DME cells (Chavoshi et al. 2010; Rousset et al. 2010). Having established that the non-muscle myosin heavy chain gene zip is a target of the JNK pathway during dorsal closure, we have been using transcription of this gene as a read out for JNK pathway activity (Figure 5.11).

Insulin can trigger Rac activation in mammalian cells and the Drosophila insulin receptor homolog Inr is required for dorsal closure (R. Fernandez et al. 1995; Nobes et al. 1995). To determine if Inr could activate the JNK cascade, we expressed a constitutively active version of Inr in prd stripes in the embryo and examined zip expression by FISH. We observed weak ectopic zip expression in prd stripes, suggesting that Inr can modestly activate the JNK pathway during dorsal closure (Figure 5.11 D). However, we could not discern a reduction in zip levels in Inr mutant embryos. This does not necessarily mean that Inr does not have a role as maternal Inr could be sufficient or Inr may contributing to JNK pathway activation in parallel with other receptors.
Pvr, the *Drosophila* homolog of PDGF/VEGF, is required for Rac-mediated activation of the JNK pathway during thorax closure, a process with close parallels to dorsal closure (Ishimaru et al. 2004). When an activated version of Pvr, λPvr, was expressed in the embryo during dorsal closure, it caused ectopic expression of the JNK target gene *puc*, and we confirmed activation of the JNK cascade by λPvr, by showing that it could activate *zip* expression in prd stripes (Ishimaru et al. 2004) (Figure 5.11 E). However, knockdown of Pvr function through expression of a dominant negative transgene did not noticeably affect *zip* levels, once again suggesting redundant receptor function in JNK activation. 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 (Figure 5.11 F). Since both candidate receptors showed the ability to activate the JNK pathway, embryos losing both receptors through double mutant combinations, dominant negative transgenes or RNAi approaches will be needed to test for the requirement of these receptors.
**Figure 5.11 Activated kinases promote zip expression**

(A-E) *zip* FISH on embryos during germ band retraction (A, E) or at the beginning of dorsal closure (B-D). (A) Wild-type embryo during germ band retraction showing *zip* expression in amnioserosa and DME cells. (B) Wild-type embryo at beginning of dorsal closure showing *zip* expression in DME cells. (C) Embryo in which constitutively active Hep has been expressed in *prd* stripes showing elevated *zip* in those stripes. (D) Expression of constitutively active Inr in *prd* stripes causes weak elevation of *zip* in those stripes (examples marked with arrows). Compare with wild-type embryo in (B). (E) Embryo in which constitutively active Pvr had been expressed in *prd* stripes showing elevated *zip* in those stripes (examples marked with arrows). Compare with wild-type embryo in (A). (F) *pvf2* in situ showing that this ligand for Pvr is expressed in a subset of DME cells flanking the dorsal hole. Scale bar: 50 μm.
The work described in this chapter has provided further insight into the bidirectional communication that occurs between the amnioserosa and dorsal epidermis allowing coordinated morphogenesis of the two tissues through the regulation of actomyosin contractility. Dpp signals from the dorsal ectoderm to the amnioserosa, and turns on spo expression, which then enables the production of a diffusible signal from the amnioserosa in the form of 20E. The 20E signal triggers myosin expression in the DME cells as well as the amnioserosa in cooperation with AP-1 (Figure 5.12).

Similar complex bidirectional signaling underlies developmental epithelial closures and wound healing in other systems (Martin and Parkhurst 2004). For example, in vertebrate wound healing there is crosstalk between keratinocytes and the fibroblasts occupying the hole that leads to transcriptional responses in both cell types (Werner et al. 2007). This includes TGF-β signaling from keratinocytes to fibroblasts promoting differentiation of the fibroblasts into contractile myofibroblasts, and secretion of growth factors from fibroblasts that stimulates proliferation and differentiation of keratinocytes. Diffusible signals are clearly a central component of vertebrate wound healing but until the present study Dpp was the only diffusible signal definitively shown to be regulating dorsal closure. In addition to Dpp and 20E it is likely that other diffusible signals are involved such as growth factors activating MAPK signaling, given that an activator of JNK signaling remains to be identified.
Figure 5.12  Schematic of reciprocal signaling mediating myosin expression in the amnioserosa and DME cells during dorsal closure

Prior to commencement of dorsal closure, Dpp diffuses out of the dorsal ectoderm and promotes $spo$ expression in the amnioserosa. $spo$ expression enables production of 20E, which, in cooperation with JNK signaling and the AP-1 transcription factor, promotes myosin expression from the $zip$ gene in amnioserosa and DME cells.
6. Discussion

6.1. A reciprocal signaling network regulating zip expression by mediating communication between the amnioserosa and epidermis

The communication between the amnioserosa and the epidermis is critical for embryonic dorsal closure (Conder et al. 2004; B. G. Fernandez et al. 2007; Glise and Noselli 1997; Lamka and Lipshitz 1999; Reed et al. 2001; Scuderi and Letsou 2005; B. E. Stronach and Perrimon 2001; Wada et al. 2007), and we have focused on the expression of the zip gene which is one target of such communication. zip encodes non-muscle myosin heavy chain, a major component of the myosin motor which together with F-actin forms the actomyosin contractile apparatus (Clark et al. 2007; Young et al. 1993). All four major mechanical forces driving the morphogenetic events of dorsal closure are generated from the actomyosin complex (Iwata et al. 2011). Actomyosin forms different functional structures in different places such as an actomyosin super cable in the leading edge of the DME cells, apical actomyosin structures in the amnioserosa cells and a contractile scaffold for filopodia and lamellipodia extending from the DME cells (Clark et al. 2007; Peralta et al. 2007; Peralta et al. 2008).

Paracrine signaling is a common way by which cells or tissues communicate with each other. Dpp ligand is secreted from the DME cells, as early as prior to germband retraction and maintained during dorsal closure (B. G. Fernandez et al. 2007). The secreted Dpp activates a downstream pathway in the amnioserosa, where it is required for normal zip expression. spo is a gene whose amnioserosa expression is permitted by Dpp signalling and, as a result, the steroid hormone ecdysone is produced in the amnioserosa. Our results demonstrate that Dpp signaling regulates zip levels through controlling the ecdysone production at the level of transcription of the ecdysone biosynthesis enzyme Spo in the amnioserosa. Thus, In response to Dpp signal from the epidermis, the amnioserosa produces ecdysone which signals to epidermis and also
back to the amnioserosa. Ecdysone activates its receptor EcR to turn on various target genes through their ecdysone response element sequence. Our results indicate a non-canonical manner of ecdysone signaling in zip regulation where the ligand bound EcR complexes with JNK-activated AP-1 transcription factor (Figure 6.1). Dpp is also able to shut down this signalling network through production of the zinc-finger transcription factor Hnt, a target gene of Dpp signaling, which is required for the inhibition of JNK activity in the amnioserosa (Wilk et al. 2004). In addition, it appears that Hnt blocks expression of spo and consequently zip.

We have identified Egfr as a repressor of both zip and dpp expression, with the regulation of zip likely occurring through production of a diffusible signal, possibly ecdysone. Apoptosis of the amnioserosa is a contributing force for dorsal closure and we have found that this process is also inhibited by Egfr signaling. Therefore, Egfr could be considered a brake on dorsal closure by being both a key regulator of the reciprocal signaling network and an inhibitor of apoptosis. The levels of this receptor are themselves negatively controlled by Ack, possibly through endocytotic degradation (Figure 6.1).
Figure 6.1  Schematic of reciprocal signaling mediating myosin expression in the amnioserosa and DME cells during dorsal closure.

Prior to commencement of dorsal closure, Dpp diffuses out of the dorsal ectoderm and promotes spo and hnt expression in the amnioserosa. spo expression enables production of 20E, which, in cooperation with JNK signaling and the AP-1 transcription factor, promotes myosin expression from the zip gene in amnioserosa and DME cells. Hnt conducts a forward feedback inhibition on spo expression and JNK activity in the amnioserosa. Egfr serves as a general brake to coordinate the signaling events during dorsal closure by repressing zip and dpp expression and apoptosis of the amnioserosa. Ack negatively controls Egfr through endocytotic degradation. Ack and Egfr together might regulate ecdysone levels to target zip expression possibly through activation of JNK signaling.
6.2. Future directions

6.2.1. How does ecdysone co-operate with the JNK pathway in driving gene expression?

Our results with zip suggest that a non-canonical ecdysone pathway co-operates with AP-1 to drive target gene transcription during dorsal closure. Our PLA and bioinformatical study suggest that the EcR/AP-1 complex targets the AP-1 consensus binding site within zip locus. We believe this is the first example of non-canonical steroid hormone signaling in Drosophila in which EcR is acting independent of ecdysone responsive elements by forming a complex with AP-1 and other proteins at AP-1 sites. It would be more convincing if we could prove the interaction between EcR and Jun using other approaches such as a traditional Co-IP technique. To increase the protein abundance and in turn the possibility of immunoprecipitation, an available TAP-tagged Jun transgene could be used (Matsuda et al. 2013). It would also be nice to directly show the EcR/AP-1 complex binding to the zip locus. Doing PLA on the polytene chromosomes of the Drosophila salivary gland might reveal the complex binding to the cytogenetic position of the zip locus. A definitive demonstration of EcR/AP-1 binding to AP-1 sites in zip could come from use of the chromatin immunoprecipitation method.

In support of our findings, the estrogen receptor, a steroid nuclear receptor similar to EcR, has been shown to activate transcription at AP-1 sites as part of a complex with Jun/Fos and other proteins in mammalian cells (Kushner et al. 2000). The estrogen receptor has been proposed to be linked to AP-1 by p160 proteins and CBP/p300 (Kushner et al. 2000), and it is possible that EcR/AP-1 complex is assembled in a similar fashion. To identify additional components in the complex, we could firstly test whether p160 proteins and CBP/p300 complex with EcR/AP-1 in Drosophila. It has been shown that Tai, the Drosophila p160, bind to EcR directly (Bai et al. 2000).

An interesting possibility is that nuclear receptors other than EcR are involved in zip expression. There are 18 genes encoding nuclear receptors in Drosophila, and our preliminary results showed that three of them, Svp, DHR38 and Knrl, are not involved in zip expression. The rest of the nuclear receptors are worth checking for roles in zip expression.
Our PLA result with the *zip* deficiency embryos, in which half the EcR/Jun PLA puncta persist in the amnioserosa (Figure 5.9) indicates that there is at least one other gene bound by a complex of EcR and Jun in the amnioserosa. Through bioinformatics methods we have narrowed down the number of candidate genes, and the potential regulation of these candidate genes by EcR/AP-1 could be characterized using the same approaches we have already used for *zip*.

### 6.2.2. The mechanism of Egfr repression of reciprocal signaling during dorsal closure

Our results indicate that Egfr represses *zip* expression through the control of a diffusible signal from the amnioserosa. As a diffusible signal from amnioserosa, ecdysone regulates *zip* expression; therefore it is necessary to determine if ecdysone is the signal controlled by Egfr. This effect does not appear to be at the level of *spo* transcription, as we have not been seen any changes in *spo* expression with gains of losses of Egfr in the amnioserosa (Figure 5.6). However, Egfr could affect ecdysone signaling at other levels, for instance, the translational regulation of *spo* or some other aspect of ecdysone biosynthesis. Whether Egfr controls ecdysone production could be revealed by measuring the ecdysteroid titer from embryos with altered Egfr function comparing to wild-type embryos. In addition, if ecdysone regulation is the route of Egfr regulation of *zip*, we would expect to see the restoration of *zip* expression in Egfr-over-expressing embryos by incubation with exogenous ecdysone.

Our results indicate that Egfr signaling regulates *zip* and *dpp* expression using different mechanisms. Further study to characterize the regulation of *dpp* expression by Egfr would help us understanding how a single receptor pathway could achieve multiple outcomes. Our results suggest that *phyllopod* and Wnt signaling are not the route by which Egfr regulates *dpp*. A recently published study suggests the *mummy* (*mmy*) locus, encoding a UDP-N-acetylglucosamine pyrophosphorylase, as a candidate target for Egfr regulation of *dpp* expression during dorsal closure (Humphreys et al. 2013). *mmy* mutant embryos, similar to Egfr mutant embryos, show elevated *dpp* expression in the dorsal epidermis (Humphreys et al. 2013). The relationship between Egfr and *mmy* deserves consideration.
We have shown that Ack controls zip expression through down regulation of Egfr, possibly through clathrin-mediated endocytosis. Ack binds to clathrin to induce endocytosis in mammals, and clathrin-binding sequences are conserved in Drosophila Ack (Teo et al. 2001; Yang et al. 2001). More results are needed to determine if clathrin-mediated endocytosis is required for Ack-mediated regulation of Egfr distribution and zip expression in Drosophila. For example, we can test to see if the distribution of Egfr in Ack over-expressing cells is changed when blocking the endocytosis pathway. We can block the endocytotic pathway globally using mutant alleles of the pathway members, or specifically in the amnioserosa using the RNAi transgenes to determine where endocytosis is required.

6.2.3. How is the JNK cascade activated during dorsal closure?

Despite the JNK cascade having been long established as a central regulator of dorsal closure, we still do not know how it is activated. Our results with Inr and Pvr suggested that the activation of JNK cascade during dorsal closure may be complex, involving more than one ligand/receptor pair. To test this idea, combination of mutations will be needed to determine if multiple ligands/receptors are required for JNK activation and in turn zip expression. JNK signaling has critical roles in development and disease and a thorough understanding of JNK regulation in dorsal closure may be broadly applicable (Sabapathy 2012).

6.3. Is the signaling network controlling dorsal closure conserved in other epithelial fusion events?

Drosophila embryonic dorsal closure has been established as a valuable model for wound healing studies. As pointed out in the introduction, striking parallels have been identified between dorsal closure and wound healing, both morphological and mechanistic. Our studies suggest new parallels, for example the amnioserosa production of a diffusible signal to regulate gene expression in the epidermis in Drosophila dorsal closure is similar to the secretion of growth factors from fibroblasts occupying the hole to stimulate proliferation and differentiation of keratinocytes in vertebrate wound healing (Werner et al. 2007).
As a developmental epithelial fusion event, *Drosophila* dorsal closure also shares many features with analogous mammalian events such as neural tube closure and mouse eyelid closure. Mouse eyelid closure is similar to dorsal closure both morphologically and mechanistically (Zhang et al. 2003). Eyelid closure involves the elongation and migration of epithelial cells at the tip of the eyelid, with the epithelia of upper and lower eyelid fusing to form a closed eye, which remains sealed until postnatal day 12 (Geh et al. 2011).

The parallels between dorsal closure and eyelid closure can be classified into four categories. First, they have similar general morphology (Figure 6.2 A-D). They both show the migration of sheets of epithelial cells, elongation of the leading edge cells of the migrating sheets, and fusion of the epithelial cells from both sides (Geh et al. 2011). In case of closure failure, an open hole phenotype is observed in both organisms: a cuticle hole in the *Drosophila* embryo and eyes open-at-birth in the mouse (Harris 1989).

The second parallel is the high level of JNK activity in the leading edge of the migrating epithelium (Figure 6.2 E). High levels of JNK activity in the DME cell during dorsal closure has been revealed by the transcripts of its target genes, such as *dpp*, *puc*, and *zip* (Reed et al. 2001). Mutations of JNK pathway components in *Drosophila* display a typical dorsal cuticle hole phenotype. In the mouse developing eyelid *Map3k1*, also known as MEKK1, a member of the MAPKKK superfamily, is abundantly expressed in the developing tip of the eyelid epithelium, and mice lacking either the kinase domain or the entire MEKK1 display an eyes open-at-birth phenotype, similar to the phenotype of *Jnk<sup>-/-</sup> Jnk<sup>+/-</sup> mice* (Yujiri et al. 2000; Zhang et al. 2003). MEKK1 is able to activate all three major MAPK pathways, but has a strong preference for stimulation of the JNK pathway through direct interaction and phosphorylation of the JNK activating kinases (A. Lin et al. 1995; Tournier et al. 1997; Z. Wu et al. 1997; Xia et al. 1998; Yan et al. 1994). MEKK1 has been shown to activate JNKs and control cell migration (Xia et al. 2000; Yujiri et al. 2000). However, the *Drosophila* MAPKKK that activates dorsal closure is not a homolog of MEKK1, but that of the mixed lineage kinases, another member of the MAPKKK superfamily (B. Stronach 2005). This further supports the idea that the tissue specificity of JNK regulation is determined at the level of MAPKKK (B. Stronach 2005). In dorsal closure, JNK pathway exerts its function through phosphorylation of Jun which
then forms a functional AP-1 transcription factor with Fos and regulates target gene expression. In eyelid closure, MEKK1 functions through the same route, with expression of kinase domain knockout version of MEKK1 resulting in failure of eyelid closure and reduced c-Jun N-terminal phosphorylation (Zhang et al. 2003).

A third parallel is the requirement of JNK mediated actin polymerization and filopodia/lamellipodia formation (Figure 6.2 E). JNK may regulate F-actin during dorsal closure through both transcriptionally-dependent and independent routes. On one hand, JNK signaling promotes actin polymerization through induction of expression of chickadee, which encodes a homolog of profilin, an essential protein for actin polymerization (Jasper et al. 2001). On the other hand, Drosophila JNK controls F-actin-based filopodia, lamellipodia and actin nucleation through direct modulation of p150-Spir, an F-actin-associated protein homologous to the members of the WASP homology domain 2 (WH2) family of proteins (Otto et al. 2000). MEKK1 ablation in mouse embryos prevents cell shape changes and F-actin formation in the developing eyelid epithelium (Zhang et al. 2003). Furthermore, JNK activation in eyelid closure and in cultured keratinocytes induces actin stress fibers and focal adhesion formation by direct interaction with actin-associated proteins in a transcription independent manner (C. Huang et al. 2003; Xia and Karin 2004).

The last parallel is the involvement of hormones in the process (Figure 6.2 E). A steroid hormone receptor, the glucocorticoid receptor, participates in both vertebrate wound healing and developmental epithelial fusions, processes that also require JNK signaling to AP-1 (Sanchis et al. 2010; Xia and Karin 2004). Administration of cortisone, a steroid hormone that binds glucocorticoid receptor, to the mother during pregnancy can “cure” the eyes open-at-birth phenotype of map3k1 hypomorphic alleles suggesting a cooperation between steroid hormone and JNK signaling similar to what we see in dorsal closure (Harris 1989). Based on this, it will be of interest to test if incubation of exogenous ecdysone could cure the dorsal hole defect of Drosophila embryos with reduced JNK signaling. How cortisone rescues the eyelid open phenotype is unknown. Our data indicating that ecdysone activated EcR cooperates with AP-1 transcription factor in a reciprocal signaling network to regulate zip and dorsal closure could be applicable to the study of the mechanism of cortisone treatment. There are other results showing interplay between steroid hormones and AP-1 in mammalian cells. The
mammalian estrogen receptor, a steroid nuclear receptor similar to EcR, has been shown to activate transcription at AP-1 sites as part of a complex with Jun/Fos and other proteins (Webb et al. 1995). During mammalian vascular development, the glucocorticoid receptor and AP-1 transcription factor synergistically induce notch4 gene expression through direct binding to the notch4 promoter and chromatin domain modification (J. Wu and Bresnick 2007).

Finally, both processes show a requirement for Egfr signaling, however it is being used differently (Figure 6.2 E). In dorsal closure, Egfr functions as a brake on the process through repression of gene expression and amnioserosa apoptosis, whereas, in eyelid closure, TGFα-induced Egfr signaling is responsible for the expression of map3k1 and c-jun (Geh et al. 2011).

Another well-known example of a developmental epithelial fusion event is palate fusion. The signaling mechanisms regulating this process are relatively poorly characterized, except for the intensive study on TGF-β function, which is also a critical component of the reciprocal signaling network regulating dorsal closure (Iwata et al. 2011). It is anticipated that as work progresses on the various developmental epithelial fusions and wound healing events further parallels will emerge and studies will complement each other. In the long term this may lead to new therapeutic interventions for wound healing and the prevention of birth defects.
Figure 6.2  Parallels between Drosophila dorsal closure and mammalian eyelid closure

Live images of Drosophila dorsal closure (A, B) and electron micrographs of mouse eyelid closure (C, D) showing "zippering shut" from both ends of an elliptical opening. (E) A schematic illustration of parallel signaling pathways between Drosophila dorsal closure and mammalian eyelid closure.
7. Conclusion

In this thesis, we have identified new components of the reciprocal signaling network regulating the communication between tissues during *Drosophila* embryonic dorsal closure. Central to this reciprocal signaling we have identified a non-canonical route of ecdysone signaling, in which it cooperates with JNK/AP-1 signaling in the regulation of gene expression. Our findings have revealed further parallels between dorsal closure and wound healing and mammalian developmental epithelial fusions such as mouse eyelid closure.

We believe that further work will reveal additional parallels between dorsal closure and other developmental epithelial fusions such as palate fusion and neural tube closure. We anticipate that future studies on various epithelial fusion events will complement each other, leading to very detailed understanding of how metazoans close epithelial holes during development and wound healing. The insights gained into signaling pathway crosstalk will also be of wide ranging interest to those studying other developmental processes and diseases such as cancer.
References


Bai, J., Uehara, Y., and Montell, D. J. (2000), 'Regulation of invasive cell behavior by taiman, a Drosophila protein related to AI1B1, a steroid receptor coactivator amplified in breast cancer', *Cell*, 103 (7), 1047-58.


--- (1994), 'Molecular analysis of the Drosophila EGF receptor homolog reveals that several genetically defined classes of alleles cluster in subdomains of the receptor protein', *Genetics*, 137 (2), 531-50.


Cormier, O., et al. (2012), 'Autophagy can promote but is not required for epithelial cell extrusion in the amnioserosa of the Drosophila embryo', Autophagy, 8 (2), 252-64.


Elliot-Smith, A. E., et al. (2005), 'Specificity determinants on Cdc42 for binding its effector protein ACK', Biochemistry, 44 (37), 12373-83.


Golembo, M., et al. (1999), 'Vein expression is induced by the EGF receptor pathway to provide a positive feedback loop in patterning the Drosophila embryonic ventral ectoderm', Genes Dev, 13 (2), 158-62.


Henrich, V. C., et al. (1990), 'A steroid/thyroid hormone receptor superfamily member in Drosophila melanogaster that shares extensive sequence similarity with a mammalian homologue', *Nucleic Acids Res*, 18 (14), 4143-8.


Lawrence, N. and Morel, V. (2003), 'Dorsal closure and convergent extension: two polarised morphogenetic movements controlled by similar mechanisms?', *Mech Dev*, 120 (11), 1385-93.


Liu, R., et al. (2008), 'Sisyphus, the Drosophila myosin XV homolog, traffics within filopodia transporting key sensory and adhesion cargos', *Development*, 135 (1), 53-63.


McEwen, D. G., Cox, R. T., and Peifer, M. (2000), 'The canonical Wg and JNK signaling cascades collaborate to promote both dorsal closure and ventral patterning', *Development*, 127 (16), 3607-17.


Ruberte, E., et al. (1995), 'An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo', Cell, 80 (6), 889-97.


Sathyanarayana, P., et al. (2003), 'Drosophila mixed lineage kinase/slipper, a missing biochemical link in Drosophila JNK signalling', *Biochim Biophys Acta*, 1640 (1), 77-84.


--- (2005), 'Regulating the dynamics of EGF receptor signaling in space and time', *Development*, 132 (18), 4017-27.


Solon, J., et al. (2009), 'Pulsed forces timed by a ratchet-like mechanism drive directed tissue movement during dorsal closure', *Cell*, 137 (7), 1331-42.


Stronach, B. (2005), 'Dissecting JNK signaling, one KKKinase at a time', *Dev Dyn*, 232 (3), 575-84.


Wisotzkey, R. G., et al. (1998), 'Medea is a Drosophila Smad4 homolog that is differentially required to potentiate DPP responses', Development, 125 (8), 1433-45.


Xia, Y., et al. (1998), 'JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension', Genes Dev, 12 (21), 3369-81.


Yao, W., et al. (2006), '[Expression level and significance of TGF-beta1, PDGF, CTGF in serum of patients with pneumoconiosis]', Sichuan Da Xue Xue Bao Yi Xue Ban, 37 (5), 754-6, 93.


