Characterization of signaling pathways enabling coordinated morphogenesis of tissues during *Drosophila* dorsal closure

by

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Abstract

Drosophila dorsal closure (DC) is the best-characterized model system for studying wound healing. During DC, a hole in the dorsal epidermis, covered by an epithelium called the amnioserosa (AS), is sealed by migration of the epidermal flanks. Seamless closure is achieved through coordinated morphogenesis of the AS and epidermis, which is facilitated by communication between the two tissues via bidirectional signaling networks. To better understand this crosstalk, three diffusible signals present during DC were analyzed, and their signaling roles were identified: 1.) Folded gastrulation (Fog), which may act as an upstream activator of a JNK pathway in the epidermis; 2.) the TGF-β ligand, Decapentaplegic (Dpp), which regulates production of the steroid hormone, 20-hydroxyecdysone (ecdysone) in the AS; 3.) ecdysone, which interacts with the transcription factor AP-1 to regulate gene transcription in the AS. Signaling via these molecules ultimately regulates myosin contractility necessary for morphogenesis of both tissues during DC.

Keywords: *Drosophila*; dorsal closure; bidirectional communication; Fog; Dpp; ecdysone

To my parents and my sister who have supported me through this long journey with their unconditional love

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List of Acronyms

AS	Amnioserosa
BMP	Bone Morphogenetic Protein
Brk	Brinker
Bsk	Basket
Cka	Connector of Kinase to AP-1
Cta	Concertina
Dad	Daughters against Dpp
DC	Dorsal Closure
Dib	Disembodied
DME	Dorsal Most Epidermal
Dpp	Decapentaplegic
Ecdysone (20E)	20-hydroxyecdysone
EcR	Ecdysone Receptor
EcRE	Ecdysone Response Element
FISH	Fluorescent *in situ* Hybridization
Fog	Folded Gastrulation
GAP	GTPase-activating Proteins
GEF	Guanine-nucleotide Exchange Factor
GPCR	G protein-coupled Receptor
Hep	Hemipterous
Jar	Jaguar
JNK	Jun N-terminal Kinase
Jra	Jun-related Antigen
Kay	Kayak
Mad	Mothers against Dpp
MAPK	Mitogen-activated Protein Kinase
MAPKK	MAPK Kinase
MAPKKK	MAPK Kinase Kinase
Med	Medea
MLK	Mixed Lineage Kinase
Msn	Misshapen

Nvd	Neverland
Phm	Phantom
PLA	Proximity Ligation Assay
Puc	Puckered
Put	Punt
pY	Phospho-Tyrosine
Rho GTPases	Rho Family Small GTPases
ROK	Rho-associated Kinase
Sad	Shadow
Shd	Shade
Spo	Spook
Spok	Spookier
TGF-β	Transforming Growth Factor β
Tkv	Thickveins
Usp	Ultraspiracle
Zasp52	Z-band Alternatively Spliced PDZ-motif Protein 52
Zip	Zipper

Chapter 1. Introduction

1.1. Dorsal closure as a model system for studying epithelial migration and fusion

The migration and fusion of epithelial sheets occur during several human developmental processes including the formation of the neural tube (i.e. the precursor to the brain and spinal cord), palate (i.e. the roof of the mouth) and cloaca (i.e. the precursor to the openings of the urinary, intestinal and genital tracts) (Pai, Abdullah et al. 2012; Ray and Niswander 2012; Heisenberg and Bellaiche 2013). To understand how these morphogenetic processes work, researchers study epithelial migration and fusion events in other animals such as dorsal closure (**DC**) in *Drosophila melanogaster*, the common fruit fly. DC is a late embryonic developmental event, occurring approximately 9-13 hours after egg laying, i.e. between developmental stages 12 and 16. Following germband retraction, a hole is naturally left in the dorsal epidermis of the embryo. The hole is occupied by a flat layer of epithelial tissue called the amnioserosa (**AS**), which covers and contains the underlying yolk sac. DC begins when the epidermal flanks that surround both sides of the hole migrate dorsalward, up and over the AS towards each other. The advancing flanks eventually meet, starting at the anterior and posterior canthi (i.e. the corners of the “eye-shaped” opening), and fuse at the dorsal midline, sealing the hole completely to form a continuous epidermis (**Figure 1.1**) (Campos-Ortega and Hartenstein 1985; Harden 2002). As a consequence, the AS is internalized and degraded through a process of apoptosis (Jacinto, Woolner et al. 2002; Reed, Wilk et al. 2004; Fernandez, Arias et al. 2007; Toyama, Peralta et al. 2008; Belacortu and Paricio 2011; Shen, Chen et al. 2013; Saias, Swoger et al. 2015). If DC fails to complete, the embryo will die since the AS does not secrete a cuticle necessary for insect life. This makes identifying genes required for the process simple as mutations in these genes, i.e. the “DC genes”, often result in a visible dorsal hole in the embryo (Harden 2002).

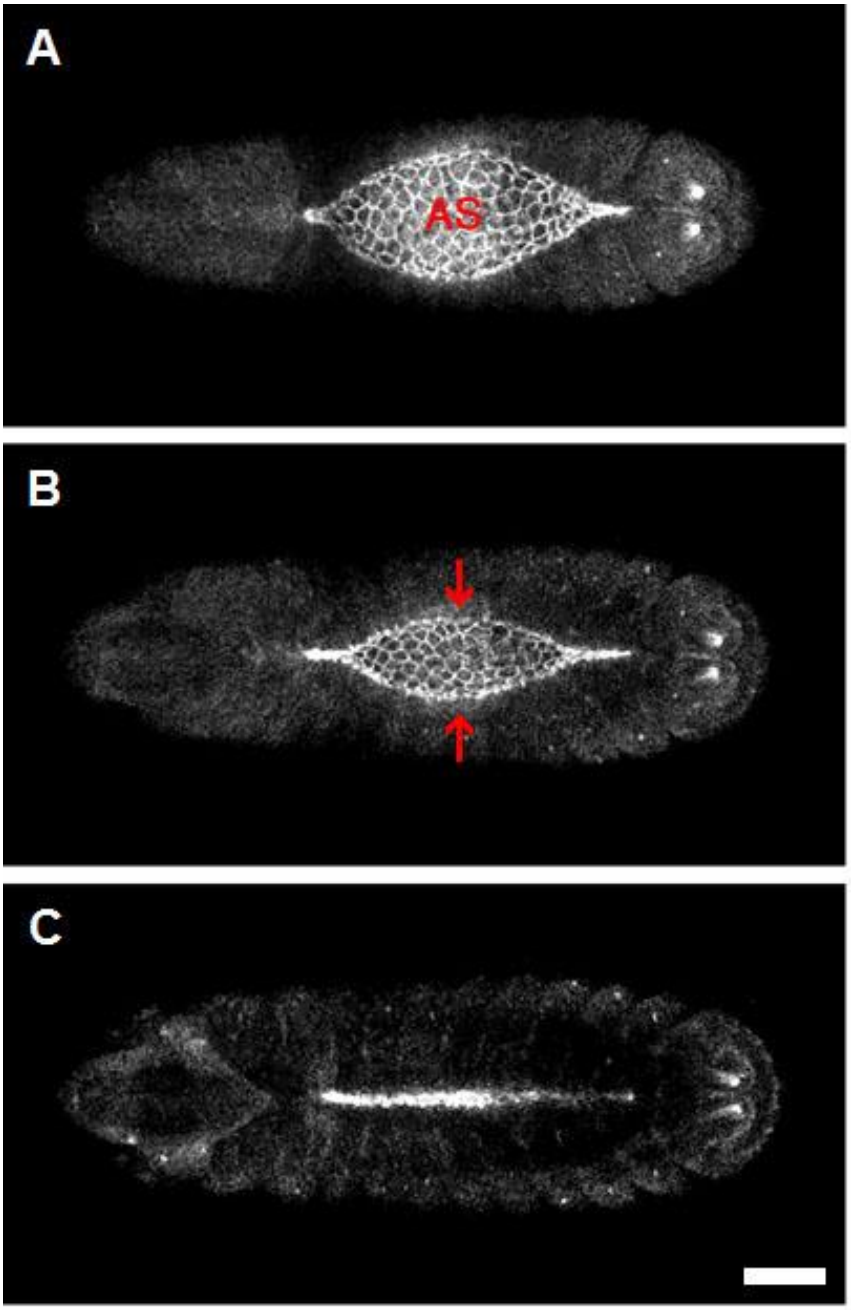

Figure 1.1. Dorsal closure of the *Drosophila* embryo.

Stills from a movie showing the live imaging of a *Drosophila* embryo expressing DE-cadherin-GFP, a cell adhesion marker, globally expressed under the ubiquitin promoter. Note that the figure shows a dorsal view of the embryo, with the anterior end (head) facing to the left and the posterior end (tail) facing to the right. **(A)** Following germband retraction, a hole is naturally left in the dorsal epidermis of the embryo, which is occupied by the amnioserosa (AS). **(B)** DC occurs when the epidermal flanks that surround both sides of the hole migrate up and over the AS towards each other (see red arrows for directionality). **(C)** The advancing flanks eventually meet at the dorsal midline and fuse, closing the hole shut to form a continuous epidermis. As a result, the AS is internalized and degraded. Scale bar represents 50μm.

Results from various studies have indicated that many of the proteins regulating other epithelial migration and fusion events are also implicated in DC, and the underlying mechanisms are conserved across species. What is learned about DC is thus extensively applicable to other organisms, helping researchers better understand how embryos develop in general (Jacinto, Martinez-Arias et al. 2001; Harden 2002). Most importantly, DC is of great medical interest as it is the best-characterized model system for the study of wound healing (Kiehart 1999; Jacinto and Martin 2001; Jacinto, Martinez-Arias et al. 2001). Wound healing shares strikingly similar morphogenetic and molecular characteristics with DC. Regarding events occurring at the leading edge of the migrating epidermal flanks, the same molecular "cassettes" are engaged in both processes. These include the use of a contractile actomyosin cable, filopodial/lamellipodial-mediated cell protrusions, JNK/AP-1 signaling, and TGF-β signaling (**Figure 1.2**) (Zeitlinger, Kockel et al. 1997; Harden 2002; Martin and Parkhurst 2004). In addition, the connective tissues, i.e. the AS in DC and fibroblasts in vertebrate wound healing contribute similarly to the dynamics of closure (**Figure 1.2**). Therefore, studying DC can improve our understanding into wound closure, and may one day lead to the development of new drugs and treatments to help those with compromised healing such as burn victims and sufferers of persistent skin lesions due to diabetes. Moreover, DC can provide significant insights concerning birth defects that result from failed epithelial fusions such as cleft palate, spina bifida and anencephaly (Mitchell, Adzick et al. 2004; Kang and Svoboda 2005; Pai, Abdullah et al. 2012).

1.2. Coordinated actomyosin regulation during DC

There are several mechanical forces that are responsible for DC, and the main force-generating tissues contributing to DC have previously been identified by laser ablation experiments (Kiehart, Galbraith et al. 2000; Hutson, Tokutake et al. 2003). These experiments, along with detailed genetic studies, have determined that coordinated actomyosin regulation in both the epidermis and the AS is critical for DC to proceed properly. In order to migrate across the AS, the dorsal most epidermal (**DME**) cells (i.e. the first row of epidermal cells that directly flank the amnioserosa and which contain the leading edge) constrict along the anterior-posterior axis, consequently stretching in the

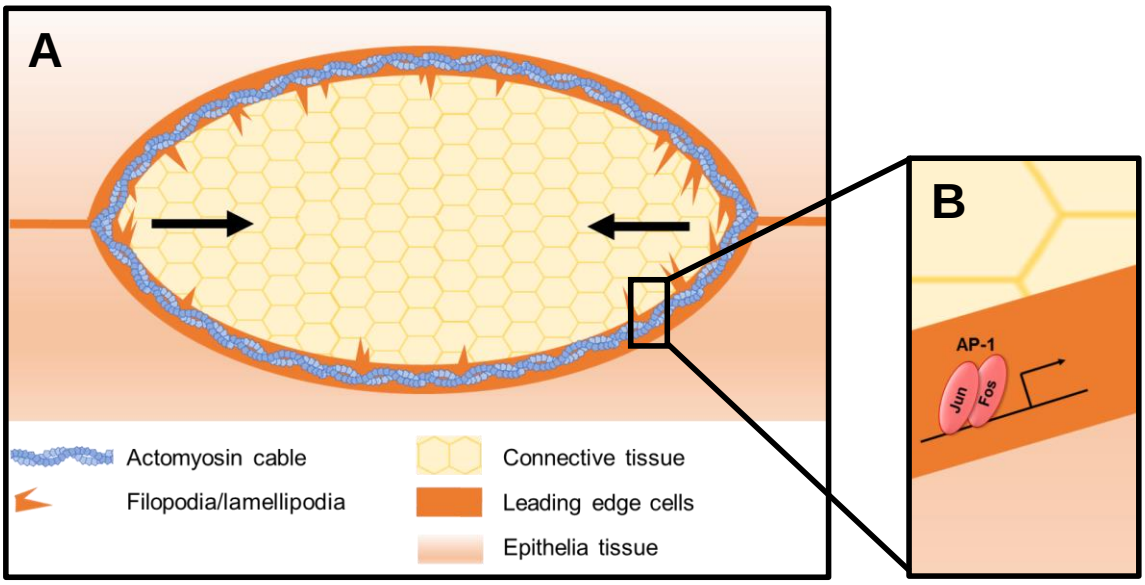

Figure 1.2. *Drosophila* DC and vertebrate wound healing are morphogenetically and molecularly similar processes.

(**A)** Simplified illustration of the hole, which can represent either the *Drosophila* dorsal hole or the wound in vertebrate, surrounded by the epithelial tissue. (**A**) Zippering starts from the corners/ends of the hole, as indicated by black arrows. An actomyosin cable (blue) surrounds the hole. Filopodia/lamellipodia extend from the leading edge. (**B**) The AP-1 transcription factor is upregulated at the leading edge.

dorsal-ventral direction. This is facilitated by the accumulation of an actomyosin cable along the leading edge membranes of the DME cells, where the action of the cable has been likened to a "purse-string" that enhances the closure dynamics (Young, Richman et al. 1993; Kiehart, Galbraith et al. 2000; Harden 2002; Jacinto, Woolner et al. 2002; Lawrence and Morel 2003; Franke, Montague et al. 2005; Rodriguez-Diaz, Toyama et al. 2008). An alternative model suggests that the actomyosin cable acts as a supra-cellular ratchet to support the forces mediated by the oscillating AS cells, counteracting ventral-ward relaxation of the epidermis (Solon, Kaya-Copur et al. 2009). Nevertheless, as the two mechanisms of the cable have been proposed based on laser ablation experiments, there are some issues regarding the technique in a sense that both the cable and the cells themselves are affected (Kiehart, Galbraith et al. 2000; Hutson, Tokutake et al. 2003). Therefore, the specific action of the cable has remained controversial.

In a search for the specific function of the actomyosin cable, two independent studies have been conducted recently, both of which exclude the previously proposed mechanisms of the cable at the leading edge cells. In one study, the investigation of actin cable contribution was been done by selectively depleting myosin, a component of the actomyosin cable, in either the AS or the leading edge of the epidermis (Pasakarnis, Frei et al. 2016). Their data have shown that DC is blocked when myosin is depleted specifically in the AS while it is completed when myosin is efficiently inactivated in the leading edge, suggesting that it is AS cell constriction but not epidermal actin cable that can autonomously drive DC. However, their interpretations have yet to be analysed further to entirely discard the pulse string model as there are some uncertainties. For example, when myosin function is eliminated in the AS, the geometry of the dorsal hole is disrupted, with no canthi formed properly. This aberrant morphology thus may have caused a perturbed purse string curvature, thereby DC not being expected to proceed in the first place. In addition, embryos with depleted myosin in the leading edge exhibit various degrees of puckering and anteriorly ruptured epidermis. Moreover, several abnormal characteristics are reported when functional myosin in the leading edge is lacking, including the slowed closure rate, longer and wider lamellipodia and filopodia from the leading edge and aberrant zippering at the end of DC. All of the above are thus quantitatively consistent with the pulse-string mechanism of the epidermal actin cable that contributes the force at least partially to DC in wild-type embryos (Hutson, Tokutake et al.

2003; Peralta, Toyama et al. 2007). In another complementary study, the role of the actin cable during DC was analyzed in embryos homozygous mutant for Zasp52, an upstream regulator of actin cable formation (Ducuing and Vincent 2016). The authors have also concluded that the actin cable is dispensable in driving DC as the acute removal of the actin cable at the leading edge does not affect the kinematics of DC based on their observations. Nevertheless, the closure rate overall appears reduced in embryos mutant for Zasp52, and the mutant embryos also display irregular and misshapen leading edges. Hence, a detailed investigation needs to be carried out further to confirm their conclusions. Even though it has been exclusively studied, the specific action of the actin cable remains still elusive; however, the actin cable at the leading edge certainly plays an essential role in DC.

Furthermore, the DME cells extend actin- and myosin-rich filopodia and lamellipodia that contribute to locomotion, traction over the AS, and ''zippering'' when the two epidermal flanks fuse together starting at the canthi (Hutson, Tokutake et al. 2003; Eltsov, Dube et al. 2015; Takacs, Jankovics et al. 2017). These epidermal protrusions, also containing tubulin and other cytoskeletal components, are critical for the matching/pairing of the cells during DC, resulting in a seamless closure with each segment from the two sides of the dorsal hole properly aligned (Jacinto, Wood et al. 2000; Jankovics and Brunner 2006; Gates, Mahaffey et al. 2007; Liu, Woolner et al. 2008; Millard and Martin 2008). Beside their roles in segment aligning, filopodia also provide pulling forces, demonstrated by the slowed speed of epithelial zippering in circumstances where the filopodial number and length are reduced (Gates, Mahaffey et al. 2007). Nevertheless, the detailed mechanism for the initiation of zippering at each canthus needs to be further investigated.

The last, but not the least, force that generates DC is the AS. A series of morphological rearrangements take place as early as germband elongation and retraction, which consequently provide the embryo with mechanical forces necessary for proper epidermal migration. With their mechanical contributions, the AS cells have been determined to play a critical role during these developmental stages (Lynch, Crews et al. 2013; Lacy and Hutson 2016). In terms of events occurring in DC, the AS cells undergo contractions that constrict their apical surfaces, a mechanism that depends on an

organized actomyosin network across the apical membrane (Kiehart, Galbraith et al. 2000; Fernandez, Arias et al. 2007; Gorfinkiel and Arias 2007). A high-resolution live imaging study has shown that there are dynamic cell shape fluctuations through the process of contractions and expansions of the AS cells (Blanchard, Murugesu et al. 2010; David, Tishkina et al. 2010). These pulsed contractions by the actomyosin cytoskeleton then generate forces that promote DC and counterbalance the tension mediated by the epidermis (Young, Richman et al. 1993; Hutson, Tokutake et al. 2003; Saias, Swoger et al. 2015; Coravos, Mason et al. 2017). At the same time that the cells in the AS continue to oscillate, they also begin to ingress into the embryo interior to prevent DC from being impeded (Kiehart, Galbraith et al. 2000; Sokolow, Toyama et al. 2012) – such ingression processes are considered as the major mechanism for decreasing apical area in the AS, thereby contributing additional forces to DC. Furthermore, at the end of DC, the AS tissue becomes apoptotic (Rodriguez-Diaz, Toyama et al. 2008). Apoptosis has been characterized to play a mechanical role during DC, demonstrated by the slowed closure rate when apoptosis is suppressed. Conversely, the closure dynamics are increased when apoptotic genes are overexpressed (Toyama, Peralta et al. 2008; Muliyil, Krishnakumar et al. 2011).

All of these investigations have emphasized the significance of actomyosin regulation in both the epidermis and the AS during DC. The question then arises as to how these forces are generated and regulated in individual cells. Previous studies have shown that the *Drosophila* non-muscle myosin II heavy chain gene, *zipper* (***zip***), plays a central role in the regulation of actomyosin cytoskeleton as part of the force generator. In wild-type embryos, *zip* is highly expressed both in the AS and the DME cells, starting as early as germband retraction. *zip* expression in the DME cells persists longer than that in the AS, but it eventually fades as DC progresses (**Figure 1.3**). *zip* is essential for *Drosophila* embryonic development, as *zip* mutations result in head involution and DC defects (Franke, Montague et al. 2005). Current evidence indicates that the transcription of *zip* depends on the activation of multiple signaling pathways, including JNK-Dpp cascade, signaling through steroid hormone ecdysone and Rho GTPases.

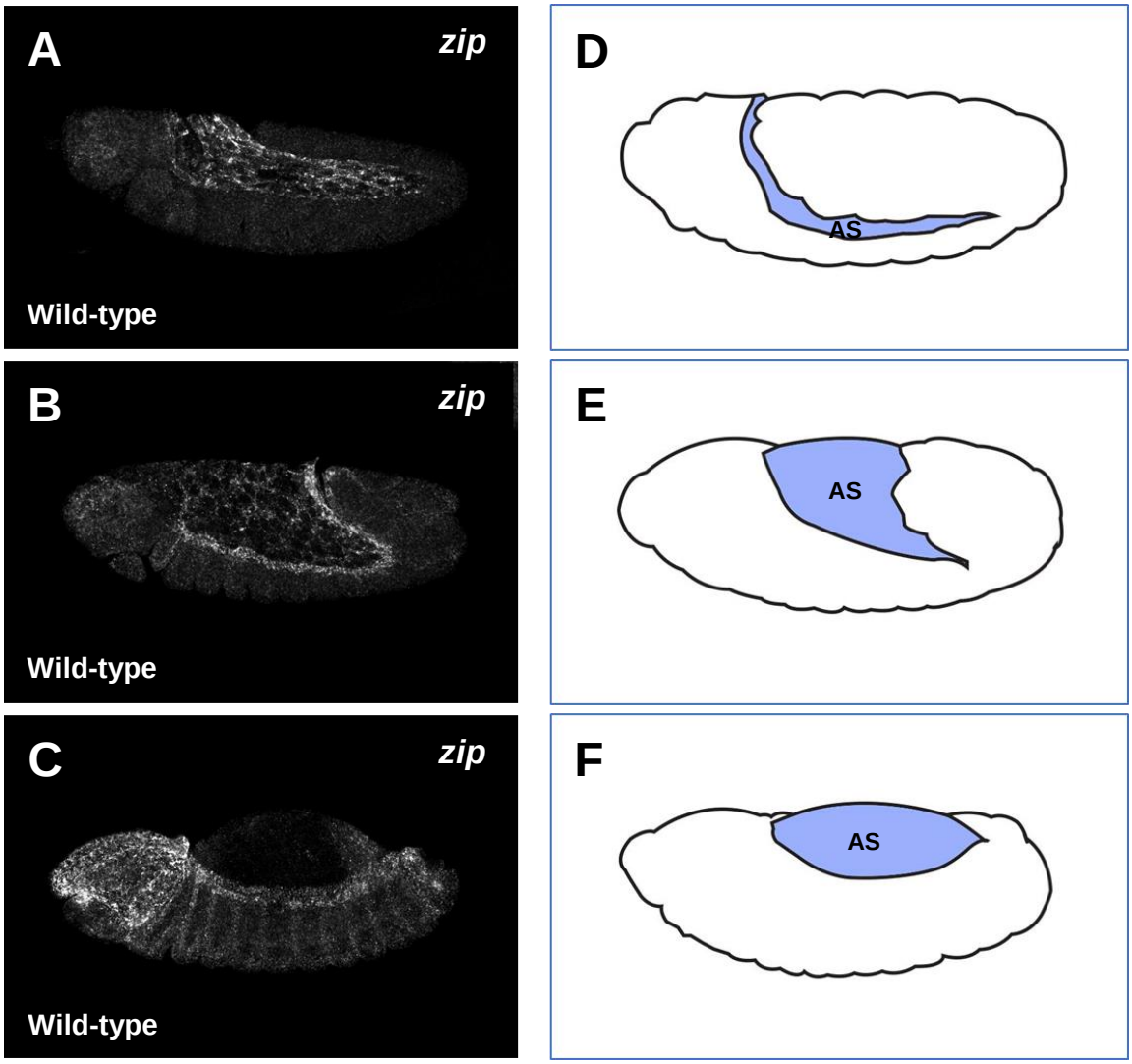

Figure 1.3. *zip* expression before and during DC.

zip FISH on wild-type embryos (**A-C**) in early germband retraction (**A**) or mid-germband retraction (**B**) show up-regulation of *zip* expression in both the AS and DME cells. *zip* FISH during DC (**C**) shows *zip* expression remains high in DME cells but has shut off in the AS.

1.3. A Jun N-terminal kinase cascade as a central component of DC

The Jun N-terminal Kinase (**JNK**) cascade is a subgroup of the mitogen-activated protein kinase (**MAPK**) pathways that consist of highly conserved proteins involved in stress pathways, survival, cell cycle progression and differentiation. To regulate these vital cellular processes, the MAPK pathways, activated by diverse range of stimuli, phosphorylate and activate various proteins including transcription factors, kinases and cytoskeletal components. The centre of the MAPK pathway is a sequential activation of three kinases – a MAPK kinase kinase (**MAPKKK**), MAPK kinase (**MAPKK**) and MAPK – by phosphorylation. The MAPKKKs are serine/threonine kinases that phosphorylate and activate MAPKKs, the dual-specificity kinases that subsequently activate MAPKs by phosphorylation at Thr-X-Tyr motifs. The MAPKs then phosphorylate their downstream substrates on serine and threonine residues, with most of the substrates identified to be transcription factors. The JNK cascade, after the sequential activation of three kinases (i.e. JNKKK, JNKK and JNK), results in the phosphorylation and activation of a leucine zipper transcription factor called Jun, as the name of cascade suggests (Harden 2002; Qi and Elion 2005) (**Figure 1.4**).

In *Drosophila*, numerous studies have indicated that the JNK pathway is required not only for embryonic DC, but also for various processes including follicle cell morphogenesis, thorax closure and imaginal disc morphogenesis (Agnes, Suzanne et al. 1999; Zeitlinger and Bohmann 1999; Martin-Blanco, Pastor-Pareja et al. 2000; Dobens, Martin-Blanco et al. 2001; Macias, Romero et al. 2004). Regarding the events occurring in DC, the central players of the JNK pathway have been identified through intense genetic studies. Mutations in components of the JNK cascade lead to DC defects exhibiting a characteristic “dorsal open” phenotype. In addition to the dorsal open phenotypes, a number of other DC defects have been observed ranging from the disruption of DME cell elongation and actomyosin cable to the reduction of target gene expression in the DME cells, all of which are demonstrated by failure of DC to proceed to completion (Harden 2002).

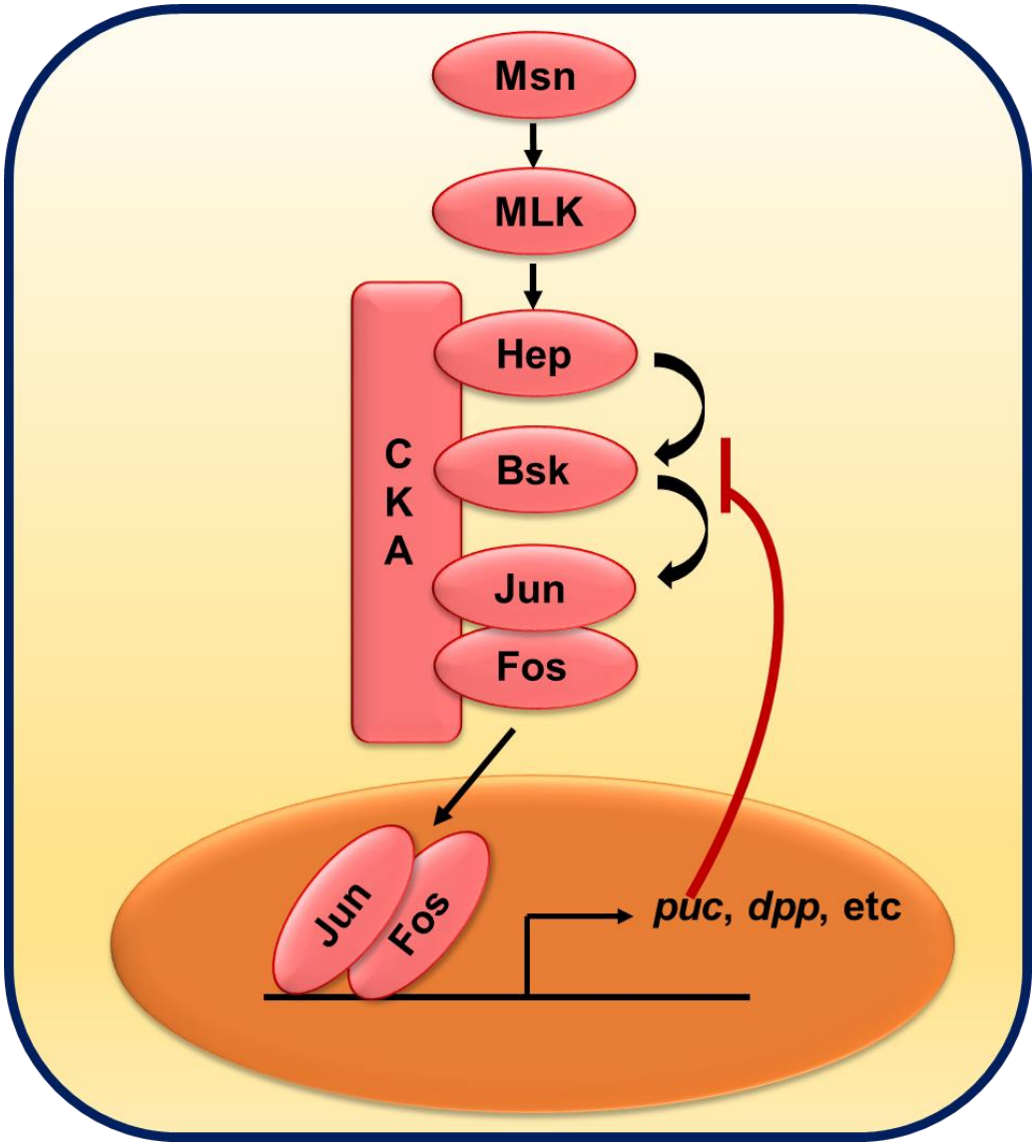

Figure 1.4. A simplified schematic representation of the JNK cascade.

The sequential phosphorylation and activation of components in the pathway lead to the assembly of the AP-1 transcription factor that consists of Jun and Fos. The AP-1 transcription factor then translocates into the nucleus to regulate the transcription of target genes, such as *puc* and *dpp*. *puc* codes for a Bsk specific phosphatase which forms a negative feedback loop to down-regulate the activity of the JNK pathway. Msn: Misshapen. MLK: Mixed lineage kinase. Hep: Hemipterous. Bsk: Basket. *puk*: *puckered*. *dpp*: *decapentaplegic*.

In the *Drosophila* JNK pathway, the sequential activation of kinases results in the phosphorylation of Jun-related antigen (**Jra**, the *Drosophila* Jun; hereafter referred to as **Jun**). The prevalent functional form of Jun is to form the AP-1 transcription factor by heterodimerizing with Fos, a leucine zipper protein encoded by *kayak* (***kay***). The AP-1 transcription factor then translocates into the nucleus, where it activates its target genes (Ciapponi and Bohmann 2002; Harden 2002).

The activity of Jun is regulated by phosphorylation of the JNK, which is encoded by *basket* (***bsk***). In *Drosophila*, Bsk is the only JNK that has been identified to date (Sluss, Han et al. 1996; Harden 2002). The upstream regulator of Bsk is the JNKKs. Previous studies have so far identified two JNKKs: *Drosophila* homologues of MKK7 and MKK4. Of the two JNKKs, the *Drosophila* ortholog of MKK7, encoded by *hemipterous* (***hep***), is reported to phosphorylate and activate Bsk. Mutants for *Drosophila* MKK4 have not been isolated, and its functional contribution to JNK signaling cascade during DC therefore remains unknown (Glise, Bourbon et al. 1995; Holland, Suzanne et al. 1997; Han, Choi et al. 1998; Han, Enslen et al. 1998; Chen, White et al. 2002). The Hep-Bsk-Jun/Fos interactions appear to be regulated by the multidomain protein CKA, encoded by *connector of kinase to AP-1* (***cka***). CKA acts as a potential scaffolding protein that forms a complex with kinases and transcription factors to regulate transcriptional activity of the JNK cascade (Chen, White et al. 2002).

The JNKK Hep is activated by upstream JNKKKs. At the level of JNKKK, six kinases in the *Drosophila* genome have been identified that could function as regulators of Hep (Chen, White et al. 2002; Stronach and Perrimon 2002). Based on genetic and cell biological analysis, only one of six kinases is shown to be required for transduction of signals to the JNK cascade during DC: a mixed lineage kinase (**MLK**), encoded by the *slipper* (***slpr***) locus (Stronach and Perrimon 2002; Sathyanarayana, Barthwal et al. 2003). As the severity of *slpr* mutant phenotype is greatly similar to those of *hep*, *bsk*, and *jun* mutants, MLK is likely the central member of the JNK signaling pathway during DC (Stronach 2005). Several biochemical and genetic evidences have also revealed that the JNKKK MLK is likely to be activated by the JNKKKK Misshapen, encoded by *misshapen* (***msn***), which is a *Drosophila* homolog of Sterile-20 kinase (Kiefer, Tibbles et al. 1996; Su, Han et al. 1997; Su, Treisman et al. 1998; Leung and Lassam 2001).

With the central components identified as noted above, the JNK signaling during DC is up-regulated specifically in the DME cells, resulting in the elevated expression of its target genes. One of the identified target genes is *puckered* (***puc***), which codes for a dual specificity MAPK phosphatase that down-regulates Bsk, mediating a negative feedback loop (Martin-Blanco, Gampel et al. 1998; Harden 2002). Another well-characterized target gene is *decapentaplegic* (***dpp***), which encodes for a diffusible ligand for the activation of the TGF-β pathway (Noselli and Agnes 1999).

1.4. A transforming growth factor β pathway in DC, acting downstream of the JNK cascade

Dpp, a *Drosophila* homolog of vertebrate Bone Morphogenetic Protein (**BMP**) 2/4, is a secreted morphogen in the transforming growth factor β (**TGF-β**) family that serves as a messenger to deliver cellular signals locally and over a long-range distance (Nellen, Burke et al. 1996; Raftery and Sutherland 1999). BMPs are highly conserved proteins across species, and their roles are critical for bone and cartilage formation and repair, cell proliferation and differentiation in embryonic development, and homeostasis of bone in the adult. Signal transduction studies have discovered that the association of the BMP ligand with the extracellular domains of BMP receptors leads to the initiation of signaling transduction. BMP receptors are serine/threonine kinase receptors, classified into two subgroups: type I and type II TGF-β receptors. Upon the activation of the receptors, the corresponding Smad proteins are phosphorylated and activated, which subsequently translocate into the nucleus to regulate the transcription of their target genes (Bragdon, Moseychuk et al. 2011; Carreira, Alves et al. 2014).

When the Dpp ligand, induced by the JNK cascade at the leading edge cells during DC, reaches its target cells, it binds to the TGF-β type II receptor called Punt (**Put**), which is a constitutively active kinase. Put then recruits and phosphorylates the TGF-β type I receptor, Thickveins (**Tkv**) (Jackson and Hoffmann 1994; Letsou, Arora et al. 1995; Ruberte, Marty et al. 1995; Hou, Goldstein et al. 1997; Riesgo-Escovar and Hafen 1997). The activated Tkv next recruits and phosphorylates its substrate Mothers against Dpp (**Mad**), a *Drosophila* Smad. Subsequently, the phosphorylated Mad forms a complex with the common-mediator Smad, Medea (**Med**), which in turn translocates into the nucleus to

mediate expression of their target genes (Affolter, Marty et al. 2001). The Dpp pathway is controlled both positively and negatively depending on its temporal- and spatial-specific necessities. One of the target genes is *dpp* itself, which consequently up-regulates the pathway by forming a positive feedback loop. On the other hand, the down-regulation of the Dpp signaling is accomplished by negative feedback loops that are generated by its two target products: Daughters against dpp (**Dad**) and Brinker (**Brk**). While Dad competes with Mad for binding to Tkv in the cytoplasm, thereby suppressing the Dpp signaling, Brk acts as a transcription repressor that inhibits the transcription of Mad/Med target genes (Inoue, Imamura et al. 1998; Campbell and Tomlinson 1999; Minami, Kinoshita et al. 1999). These feedback systems maintain the robust control over the Dpp signaling during morphogenesis (**Figure 1.5**).

Previous genetic studies have determined a relationship between the JNK pathway and the Dpp pathway during DC. Mutations in the Dpp signaling components, including *tkv*, *put*, and *mad*, all exhibit the DC defects that are similar to the JNK mutant phenotypes (Affolter, Nellen et al. 1994; Brummel, Twombly et al. 1994; Penton, Chen et al. 1994; Letsou, Arora et al. 1995; Ruberte, Marty et al. 1995; Hudson, Podos et al. 1998). Moreover, either ectopic expression of Dpp or expression of constitutively active Tkv is substantially capable of rescuing the DC defects caused by impaired JNK signaling (Hou, Goldstein et al. 1997; Riesgo-Escovar and Hafen 1997; Sluss and Davis 1997; Su, Treisman et al. 1998). All the evidence indicates that the Dpp signaling is required for DC, acting downstream of the JNK pathway.

During DC, the expression of *dpp* occurs in two distinct stripes, DME cells and the other in ventrally located epidermal cells. Of the two, only *dpp* expression in the DME cells is JNK-dependent (Jackson and Hoffmann 1994; Harden 2002). Given that *zip* expression is also up-regulated specifically in the DME cells, Dpp could be involved in the regulation of *zip* in that region. Indeed, the JNK-mediated Dpp is shown to be responsible for inducing *zip* expression at the leading edge during DC. As *tkv* mutant embryos lead to a significant reduction in *zip* transcription both in the AS and the DME cells, from germband retraction to DC, the JNK-Dpp pathway accounts at least partially for modulating the expression of *zip* during DC (Arquier, Perrin et al. 2001).

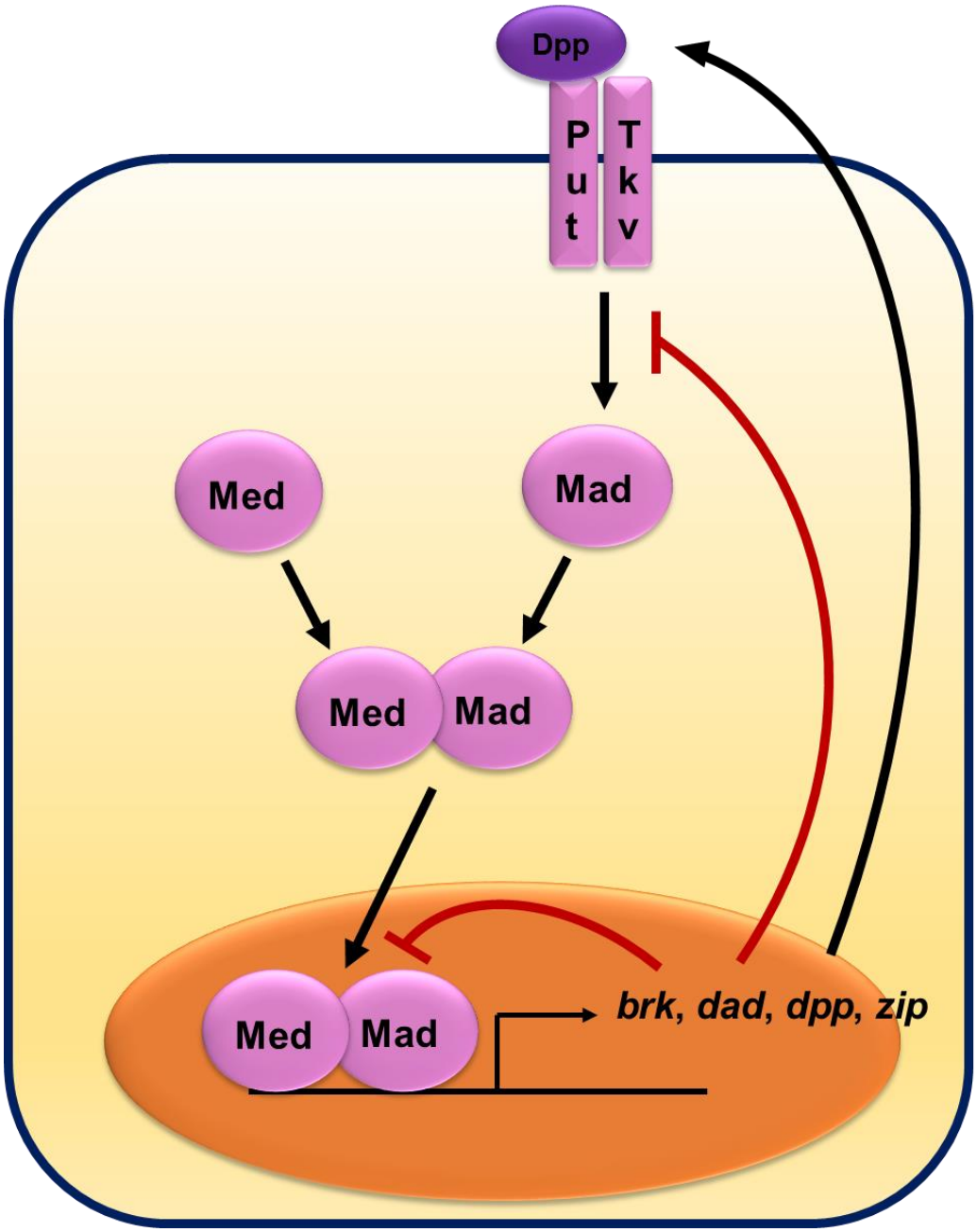

Figure 1.5. A simplified schematic illustration of the Dpp pathway.

Upon binding of Dpp onto its receptor Put, Put in a complex with Tkv activates Mad. The activated Mad then forms a complex with Med, which subsequently translocates into the nucleus to regulate the transcription of target genes, including *brk*, *dad*, *dpp* and *zip*. Both *dad* and *brk* provide negative feedbacks to the Dpp pathway by inhibiting Mad from binding with the receptor complex and by repressing the transcription of Med/Mad target genes, respectively. Dpp: Decapentaplegic. Put: Punt. Tkv: Thickvein. Mad: Mothers Against Dpp. Med: Medea. *brk*: *brinker*. *dad*: *Daughters against Dpp*.

As discussed above, many of upstream activators and downstream effectors of the JNK-Dpp pathway have been characterized in studies on DC. However, the identities of the upstream signals that activate the JNK signaling remain elusive. Several investigations have suggested that numerous inputs may be involved in the activation of the JNK cascade, such as growth factors,cell polarity cues, and/or mechanical stretching (Kushida, Kabuyama et al. 2001; Ramet, Lanot et al. 2002; Igaki, Pagliarini et al. 2006).

1.5. The Rho family small GTPase as an activator of JNK-Dpp pathway

In a search for upstream regulators of the JNK-Dpp pathway, other signaling molecules that are known to regulate *zip* are considered: for example, the Rho family small GTPases (hereafter referred to as **Rho GTPases**). The Rho family proteins consist of well conserved small GTPases that act as molecular "switches" that cycle between a GTP-bound "on" state and GDP-bound "off" state. These “on” and “off” activities of Rho GTPases are catalyzed by associated guanine-nucleotide exchange factors (**GEFs**) and GTPase-activating proteins (**GAPs**), respectively. All the *Drosophila* Rho family proteins, Cdc42, Rac and Rho, have been found to function in DC, through studies such as epistatic analyses of each protein in a relationship with the JNK pathway. Cdc42, Rac1, and Rac2 are all shown to up-regulate the JNK cascade, consequently causing an increase in *dpp* expression; however, they are not the sole activators of the pathway as the mutant phenotypes are not fully rescued by overexpression of the JNK components. It should be noted that Cdc42 and Rac proteins act independently to each other, i.e., they have different roles in the regulation of the JNK cascade, demonstrated by distinct mutant phenotypes (Harden 2002).

In contrast, Rho1 is shown to have effects on myosin accumulation at the leading edge without affecting *dpp* expression in a JNK-dependent manner (Harden 2002). The requirement of Rho1 for DC has been addressed through the loss-of-function mutations in the Rho1 locus and expression of a dominant negative Rho1 transgene, with DC defects observed in both cases. Although the Rho1 mutant embryos and embryos expressing dominant negative form of Rho1 manage to complete DC, a disorganized dorsal surface is seen. Uneven constrictions of cells along the anterior-posterior axis at the leading edge

appear to cause these irregular closures, supporting the idea that Rho1 is required to control the contractility of the leading edge cytoskeleton through the regulation of myosin (Harden, Ricos et al. 1999; Magie, Meyer et al. 1999). Moreover, previous wound assays generated by laser ablation in the *Drosophila* embryo have determined that the three major Rho GTPases, Rho1, Cdc42 and Rac, are all rapidly recruited around the wound and segregated into partially overlapping zones, with each Rho GTPase having a specific contribution to wound repair. Of the three, Rho1 is the one that first appears at the wound edge, co-localizing with myosin II and actin (Abreu-Blanco, Verboon et al. 2014). These results emphasize the importance of Rho1 in the modulation of myosin and actin dynamics, acting as a signaling center that regulates the cytoskeleton.

Rho1, however, might not use the JNK cascade when regulating myosin, as shown by wild-type levels of *dpp* expression at the leading edge in either *rho1* mutant embryos or embryos expressing dominant negative Rho1, but these studies were not that thorough (Lu and Settleman 1999; Magie, Meyer et al. 1999). The interaction of Rho1 with myosin may be controlled by a separate pathway. Several genetic and molecular studies have indeed identified that the Rho-associated kinases (**ROKs**), downstream effectors of Rho1 in mammalian cells, phosphorylate the regulatory light chain of myosin(Bishop and Hall 2000). Phosphorylation of the myosin light chain subsequently activates the myosin ATPase, ultimately promoting the assembly and function of the actomyosin cytoskeleton (Bresnick 1999). This Rho1-ROK-Myosin II activity, in cooperation with Ras, has revealed to up-regulate JNK signaling (Khoo, Allan et al. 2013). Even though the GTP exchange factor RhoGEF2 has been characterized as an activator of Rho1, further upstream signaling components remain unknown during DC.

In order to further identify the upstream signaling components that might affect DC, another developmental process in *Drosophila* morphogenesis is taken into consideration: gastrulation, where major cytoskeletal rearrangement and cell shape changes occur.

1.6. A potential role of G protein-coupled receptors and a secreted protein Fog in DC

During *Drosophila* gastrulation, two populations of cells invaginate, one of which brings posterior endodermal precursor cells into the interior while the other forms the ventral furrow. These morphogenetic migrations are initiated and driven by apical cell constriction and individual cell shape changes, respectively. For gastrulation to proceed properly, zygotic gene *folded gastrulation* (***fog***), which encodes for a secreted protein, is required (Costa, Wilson et al. 1994). The observed colocalization of Fog with the regions where apical constrictions occur suggests that the Fog receptors are recruited on the cell surface around the constriction sites to assemble actomyosin contractile apparatus for the control of cell shape changes (Dawes-Hoang, Parmar et al. 2005). Given that G_{α} protein Concertina (**Cta**) is identified as an intracellular response element for Fog, G protein-coupled receptors (**GPCRs**) have been thought to be the receptors through which Fog transduce signals (Parks and Wieschaus 1991; Costa, Wilson et al. 1994). Indeed, molecular genetic studies have recently identified two GPCRs, Mist and Smog, that couple extracellular Fog to intracellular Cta (Manning, Peters et al. 2013; Kerridge, Munjal et al. 2016).

GPCRs, also known as seven-transmembrane domain receptors, are cell surface receptors found only in eukaryotes. They ultimately modulate cellular responses through linking extracellular molecules to intracellular response elements. As part of this GPCR complex, G_{α} proteins are located on the intracellular side to facilitate downstream signaling functions. Cta belongs to the $G_{\alpha 12/13}$ class of G_{α} proteins, which are reported to mediate cytoplasmic as well as nuclear signaling events, such as JNK signaling. They are also known to regulate focal adhesion assemblies and transcription of specific primary response genes, including growth/migrational genes (Dhanasekaran and Dermott 1996). Cta itself is known to activate RhoGEF2, which subsequently activates Rho1. The activated Rho1 then promotes actin rearrangements during gastrulation (Barrett, Leptin et al. 1997), suggesting Fog's role as a diffusible signal that can act over a short-/long-range distance to induce cytoskeletal changes. The above evidence makes Fog a strong candidate as an upstream signal for Rho-mediated actomyosin accumulation during DC,

given Fog's potential interaction with JNK signaling as well as its ability to trigger the Rho1 pathway (**Figure 1.6**).

As discussed above, the myosin levels depend on the activation of the JNK cascade in addition to signaling through Rho GTPases. Previous work in our lab has demonstrated that communication between the AS and the DME cells is essential to control the myosin levels in both tissues during DC. Dpp, produced in the DME cells, is shown to be involved in the regulation of this reciprocal communication between the two tissues (Zahedi, Shen et al. 2008). This earlier finding also suggests the involvement of another signal(s) from the AS that activate(s) *zip* expression in both the AS and the leading edge cells. A candidate signal is a steroid hormone called 20-hydroxyecdysone.

1.7. Steroid hormone ecdysone during DC

The steroid hormone 20-hydroxyecdysone (hereafter referred to as **ecdysone** or **20E**) plays as a key regulator of programmed cell death and cell shape changes during *Drosophila* metamorphosis. Previous investigations have determined its important role in DC, as well as during germband retraction. The AS is considered as a major source of ecdysone during embryonic development, and the DC defects are observed when mutants in members of the "Halloween" group of genes encoding enzymes in the ecdysone biosynthetic pathway are selectively expressed (Chavez, Marques et al. 2000; Giesen, Lammel et al. 2003; Kozlova and Thummel 2003; Petryk, Warren et al. 2003; Warren, Petryk et al. 2004; Ono, Rewitz et al. 2006; Niwa, Namiki et al. 2010). The synthesis of ecdysone, the only molting hormone in *Drosophila*, from dietary cholesterol is mediated by a series of cytochrome P450 hydroxylases, all of which are encoded by genes in the Halloween family. The Halloween family includes *neverland* (***nvd***), *spook* (***spo***), *spookier* (***spok***), *phantom* (***phm***), *disembodied* (***dib***), *shadow* (***sad***) and *shade* (***shd***) (Rewitz, Rybczynski et al. 2006; Gilbert 2008). Note that experiments were performed on embryos mutant for *spo* or *dib* in my study to determine the role of ecdysone in the regulation of *zip*.

In *Drosophila*, the larval-to-adult metamorphosis is triggered by a pulse of ecdysone, which induces the generation of adult structures from imaginal tissues while

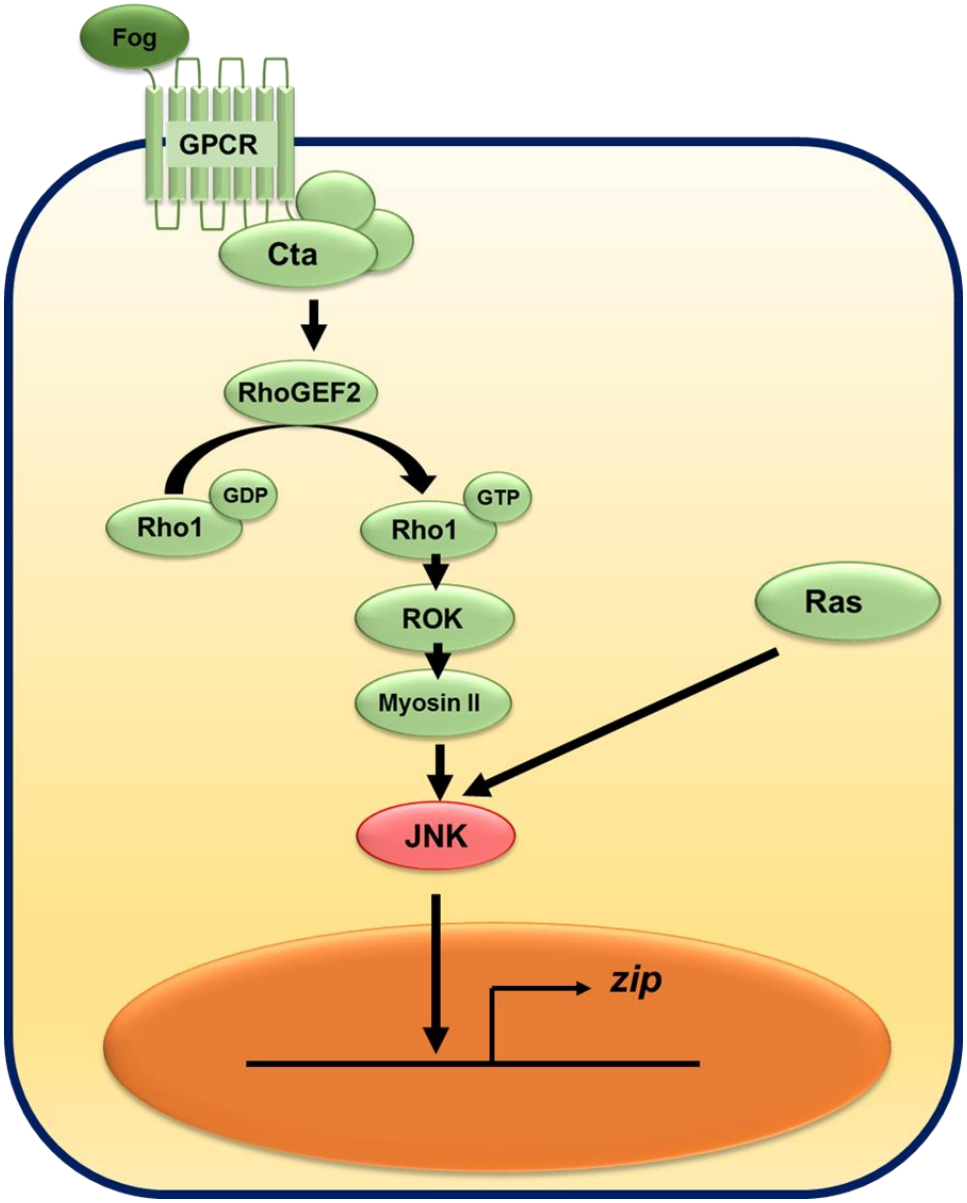

Figure 1.6. A simplified schematic representation of hypothesized Fog regulation of *zip*-encoded myosin II through the GPCR-mediated Rho1-ROK-Myosin-II pathway.

Fog promotes actomyosin contractility but may also act as an upstream regulator of the JNK cascade, promoting *zip* transcription during DC.

degenerating larval tissues (Talbot, Swyryd et al. 1993). The canonical ecdysone signaling is mediated by binding of ecdysone to the receptor complex, consisted of two members of nuclear receptor family encoded by the *ecdysone receptor* (**EcR**) and the *ultraspiracle* (***usp***) loci. EcR belongs to the same steroid receptor group as the vertebrate liver X receptors and farnesoid X receptor, and Usp is the only known *Drosophila* ortholog of the vertebrate retinoid X receptors. The prevalent functional form of EcR is as a heterodimer with Usp, and in the presence of ecdysone, they together bind DNA in a highly cooperative manner (Henrich, Sliter et al. 1990; Koelle, Talbot et al. 1991; Yao, Segraves et al. 1992; Thomas, Stunnenberg et al. 1993; Yao, Forman et al. 1993; Riddiford, Cherbas et al. 2000; Bonneton, Zelus et al. 2003). The heteromerization of EcR with Usp stabilizes the 20E-binding conformation of EcR, thereby greatly stimulating the interaction between ecdysone and EcR. Ecdysone bound to EcR further stabilizes the structure of the EcR/Usp complex, as well as increases its affinity for binding DNA (Yao, Forman et al. 1993). In order to mediate the transcription of ecdysone-responsive genes, the EcR/Usp complex binds to a specific promoter sequence called ecdysone response element (**EcRE**). There are three EcR isoforms that bind to EcRE: EcR-A, EcR-B1 and EcR-B2, which share common ecdysone-binding and DNA-binding sequences. Different combinations of the isoforms are spatially and temporally controlled, indicating that different isoform mixtures are required to determine cell fates in different ecdysone target tissues at different developmental stages (Cherbas, Lee et al. 1991; Koelle, Talbot et al. 1991; Talbot, Swyryd et al. 1993).

Transduction of the ecdysone signal involves a two-step regulatory hierarchy, classified as a small set of “early” primary 20E-inducible genes and a larger set of “late” secondary response genes. Primary 20E-inducible genes are the earliest genes in the ecdysone cascade to be transcribed, and they are directly regulated by the EcR/Usp heterodimer. The synthesis of these primary genes is then responsible for inducing the expression of secondary 20E-inducible genes. Primary regulatory genes are not only required for the regulation of secondary responsive genes but are also essential for the repression of their own transcription (Ashburner 1973; Thummel 1995; Gonsalves, Neal et al. 2011) (**Figure 1.7**).

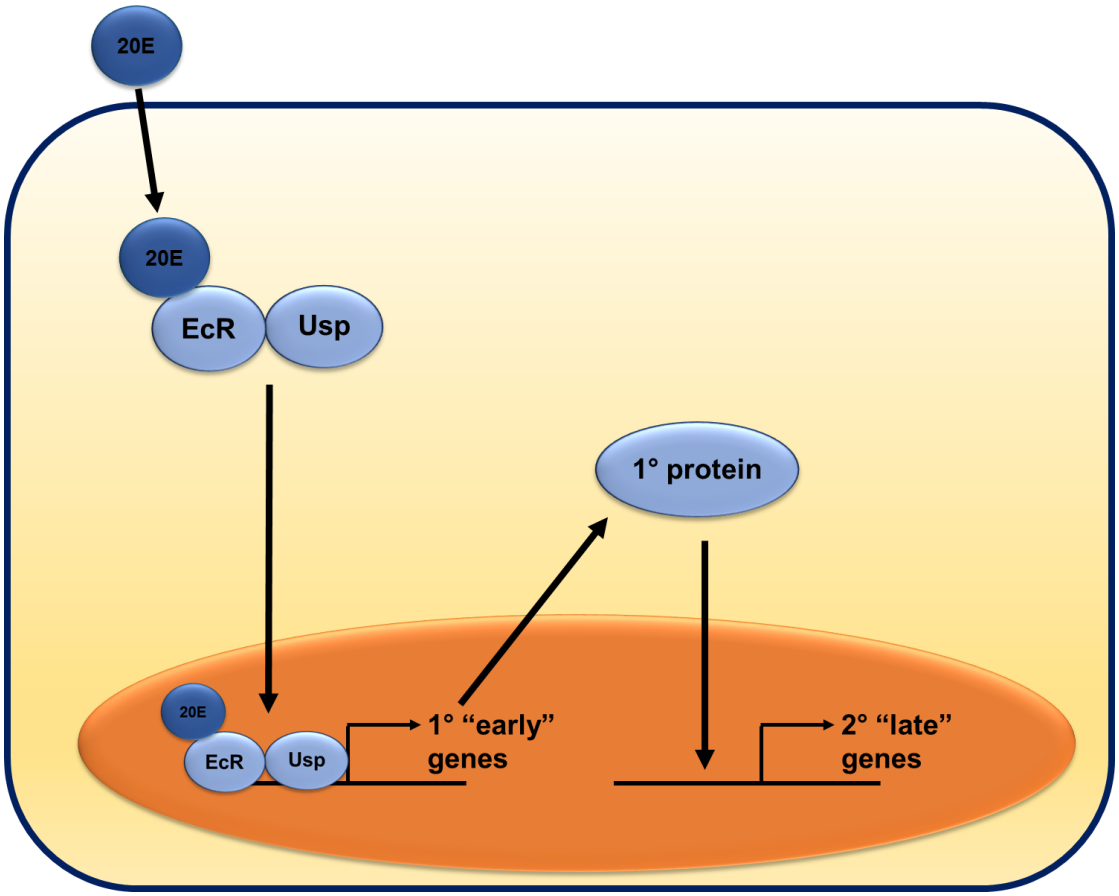

Figure 1.7. A simplified schematic representation of the canonical ecdysone cascade.

The pathway is activated when ecdysone binds to EcR, which is in the form of a heterodimer with Usp. The nuclear receptor complex then recognizes and binds to the EcRE in target genes, turning on the transcription of a small set of primary "early" 20E-inducible genes. Primary regulatory genes then repress their own expression and induce the expression of a larger set of secondary "late" 20E-responsive genes.

Immunofluorescent staining has previously shown that expression of both EcR and Usp is high throughout embryogenesis, and that activation of the two receptors is similar. Before the ecdysone pulse, at stage 11, activation is not detectable in embryos, but is strong in the AS at stage 13 which remains highly active in this tissue through the end of DC at stage 15. These experimental observations indicate that a high concentration of active ecdysone is present in the AS during DC. The embryonic ecdysone titer begins to rise prior to the formation of the ring gland, the major endocrine organ of the fly. This suggests that the embryonic ecdysone pulse is contributed by maternally deposited ecdysone, which is stored as inactive conjugates in the yolk, closely positioned to the AS. These inactive ecdysones are then converted into active forms by the Halloween group of enzymes in the AS, leading to a high concentration of active hormones in this tissue (Kozlova and Thummel 2003). Of the Halloween genes, *spo* has been identified as the only gene encoding enzymes involved in the ecdysone biosynthesis shown so far to actually be transcribed in the AS. Although expression of other members of the pathway is found earlier in nuclei on the dorsal side of the early embryo that will eventually form the AS, none of their transcription in the AS has been demonstrated (Ono, Rewitz et al. 2006). The expression of *spo* in the AS suggests that it is the rate-limiting enzyme for the synthesis of ecdysone. In support of this, ecdysone biosynthesis in the larval prothoracic gland has previously been identified to be induced by prothoracicotropic hormone through activation of Spo (Rewitz, Larsen et al. 2009).

Despite the fact that ecdysone is essential for DC to proceed properly, the detailed mechanism through which ecdysone regulates DC has remained poorly understood.

1.8. Aims of the study

Dorsal closure during embryogenesis is a process where numerous morphogenetic events take place. Coordinated cell shape changes and tissue migrations and fusions are required to achieve this seamless closure of the epidermis. It is now well understood that communication between cells in the same and/or different tissues is mediated by a complex network of signaling pathways critical for this process. A more detailed analysis of the signaling network regulating DC can be applicable to other organisms that require the bringing of tissues together.

The first objective of my research project was to characterize Fog that our lab had recently identified as a potential participant in DC. More specifically, its relationship with the Rho1 pathway and/or the JNK cascade was further investigated. The second objective was to continue studying the existing signaling network, specifically potential interactions between the JNK cascade and ecdysone signaling, and how this may regulate communication between different tissues.

The overall goal at the end of my study was to have a better understanding of the signaling network between the AS and DME by analyzing how unknown/known players of multiple signaling pathways cooperate with each other during DC. As actomyosin regulation is at the core of the process, transcriptional modulation of the *zip* gene was particularly focused on, where its expression was used as a read-out for up-regulated or down-regulated JNK signaling. What is learned from this study will provide more information on how tissues coordinate their morphogenesis during development and tissue regeneration.

Chapter 2. Materials and Methods

2.1. Fly Stocks and Crosses

Fly stocks were maintained under standard conditions at room temperature (Ashburner and Roote 2007). w^{1118} was used as a wild-type control unless noted otherwise. The following lines were ordered from the Bloomington *Drosophila* Stock Center (Indiana University, USA): w^{1118} (3605), spo^{1} (3276), dib^{2} (2776), *UAS-rhoGEF2* (9386, 11369), *UAS-rho1* (28872, 58819), *UAS-rho1*^{V14} (7330, 8144, 58817), *UAS-rho1*^{N19} (7327, 8144), *UAS-EcR*^{W650A} (9449), *UAS-bsk*^{DN} (6409), *prd-Gal4* (1947), *c381-Gal4* (3734), *69B-Gal4* (1774) *Df(1)JA27/FM7c, kr-Gal4, UAS-GFP* (5193), *In(2LR)Gla/CyO, twi-Gal4, UAS-GFP* (6662) and *Dr*^{Mio}*/TM3, twi-Gal4, UAS-GFP* (6663). The following RNAi transgenic lines were ordered from the Vienna *Drosophila* RNAi Center (Campus Vienna Biocenter, Austria): *UAS-fog*^{RNAi} (101125). For other transgenic RNAi lines used, please see the appendix (**Table B1**). *UAS-fog* was a gift from Eric F. Wieschaus (Princeton University, USA). sqh-smogC::GFP was a gift from Dr. Thomas Lecuit (Aix Marseille Université, France). *UAS-cta*^{WT} and *UAS-cta*^{Q303L} were kindly provided by Dr. Fuse Naoyuki (Kyoto University, Japan).

For transgenic analysis, males homozygous for the *UAS*-transgene were crossed to virgin females homozygous for the tissue-specific *Gal4* to ensure that all progeny carried one copy of each. In these experiments, a separate wild-type control was used, which was treated under identical conditions but in a different reaction tube. For cases when either the transgene or Gal4 line was not homozygous viable, or for mutant stocks, the flies were re-balanced over GFP-tagged balancers so that non-balanced progeny can be distinguished from balanced progeny based on the absence of GFP signal when viewed under a fluorescent microscope. In these experiments, the balanced sibling progeny were used as an internal control, and were treated under identical conditions in the same reaction tube. Unless otherwise stated, all stocks and crosses used for experiments were incubated at 25°C, with the exception of crosses involving RNAi transgenes, which were instead incubated at 29°C.

2.2. cDNAs

The following cDNA clones were ordered from the *Drosophila* Genomics Research Center (Indiana University, USA): *zip* (LD21871), *dpp* (RE20611), *fog* (SD02223), *mist* (RE13854), *smog* (RE70685), *cta* (LD04530), *rhoGEF2* (SD04476), *rho1* (LD03419), *jar* (FI18104), *jupiter* (GH10365), *zasp52* (RH03424), *imp-e1* (RE39081), *imp-l1* (LP06390) and *medea* (LD22279). Clones arrived on FTA discs, and were processed and transformed as per the instructions supplied by DGRC. Resulting single colonies on the Lysogeny Broth (**LB**) agar plates were picked and individually cultured in liquid LB with the appropriate antibiotic for 16-18 hours at 37°C. The QIAGEN Plasmid Mini Preparation Kit was used to purify the plasmid DNA, and restriction digestion mapping was applied to verify the identity of the cDNA insert.

2.3. Antisense RNA Probe Synthesis

cDNA sequences, which were inserted into the multiple cloning site of vectors containing flanking T3, T7 or SP6 promoters, were first linearized at the 5' end of the coding strand with an appropriate restriction enzyme (see **Figure B1**) to prevent transcription of vector DNA. Digoxigenin (**DIG**)-labeled antisense RNA probes were then generated by *in vitro* transcription starting at the 3' end of the coding strand with the appropriate RNA polymerase (see **Table B2**), following the company's instructions (Roche Applied Science). Reactions were performed for 4 hours at 37°C. Synthesized RNA probes were purified using MicroSpin S-200 HR columns (GE Healthcare, 27-5120-01). The size of the probes was roughly estimated by non-denaturing agarose gel electrophoresis, and the quality and quantity were obtained by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The purified RNA probes were stored at -80°C until ready to use

2.4. Antibodies

The primary antibodies used for immunohistochemistry were as follows: 1:250 mouse anti-phosphotyrosine (Cell Signaling, 9411), 1:100 rabbit anti-GFP (Sigma-Aldrich,

G1544), 1:10 rabbit anti-Jun (Santa Cruz Biotechnology, sc-25763), and 1:5 mouse anti-EcR (Developmental Studies Hybridoma Bank, DDA2.7). The secondary antibodies used were as follows: Fluorescein-labeled horse anti-mouse (Vector Laboratories, FI-2000), Texas Red-labeled horse anti-mouse (Vector Laboratories, TI-2000), Fluorescein-labeled goat anti-rabbit (Vector Laboratories, FI-1000), and Texas Red-labeled goat anti-rabbit (Vector Laboratories, TI-1000). All secondary antibodies were used at a 1:200 dilution.

2.5. Embryo Fixation

Washing Solution:
0.01% Triton-X (Sigma-Aldrich, T8787)
Phosphate Buffered Saline (PBS):
3mM $NaH_2PO_4 \cdot H_2O$
7mM Na_2HPO_4
1.3M NaCl
Adjust to pH 7.0, and autoclave
4% Paraformaldehyde (PFA):
4% PFA
1mM NaOH
PBS (pH 7.0)
Dissolve PFA in water with NaOH at 56°C, then add buffer
Make fresh each time
Heptane (Caledon Laboratories, 5400-1)
Methanol (Caledon Laboratories, 6700-1)

Embryo fixations were performed according to Rothwell and Sullivan (Rothwell and Sullivan 2007; Rothwell and Sullivan 2007; Rothwell and Sullivan 2007) but some modifications were applied. Cages were incubated at 25°C unless otherwise stated. Embryos for experiments and their controls were collected at the same time under identical conditions.

Flies placed in cages were allowed to lay eggs on grape juice agar plates with yeast paste for 16-18 hours. Embryos ranging in age from 0 to 18 hours after egg laying

were then washsed off into a collecting basket using washing solution. The embryos were dechorionated in 50% bleach diluted in washing solution, followed by three 3.5-minute washes in washing solution. After final wash, embryos were transferred to 20mL glass scintillation vials containing a biphasic devitellinization mixture: a bottom aqueous layer consisting of 5mL of 4% PFA and a top organic layer consisting of 5mL of heptane. The embryos were then agitated vigorously for 25 minutes. After agitation, the bottom layer was removed and replaced with 5mL of methanol. The embryos were then manually shaken for 1 minute, which allowed the embryos to sink to the bottom methanol layer after being properly devitellinized and thus fixed. The top heptane layer, along with unfixed embryos, was discarded. After a few more washes with methanol, the fixed embryos were transferred into 1.5mL Eppendorf tubes and stored in methanol at -20°C until ready for experiments.

2.6. Fluorescent *in situ* Hybridization

1x Phosphate Buffer Saline with Tween (PBTween):

PBS (pH 7.0, see section 2.5)

0.1% tween 20 (Fisher Scientific, BP337-500)

4% Paraformaldehyde (PFA):

4% PFA

1mM NaOH

Dissolve PFA in water at 56°C, and add

PBS (pH 7.0)

0.1% tween 20

Make fresh each time

3mg/mL Proteinase K Stock:

Proteinase K (Sigma, P6556)

Dissolve Proteinase K in sterile water. Store at -20°C

3µg/mL Proteinase K Solution:

Freshly dilute from 3mg/mL proteinase K stock in PBTween (i.e. 1:1000)

20mg/mL Glycine Stock:

glycine (BioShop, GLN001.500)

Dissolve glycine in PBTween and filter sterilize. Store at -20°C

2mg/mL Glycine Solution:

Freshly dilute from 20mg/mL glycine stock in PBTween (i.e. 1:10)

20x Saline Sodium Citrate (SSC):

8.8% sodium citrate

17.5% sodium chloride

Dissolve in water and adjust pH 7.0 with HCl. Autoclave.

50x Denhardt's Reagent:

1% ficoll 400

1% polyvinylpyrrolidone

1% bovine serum albumin

Dissolve in water and filter sterilize. Store at -20°C.

Hybridization Solution:

50% formaldehyde

4x SSC

1x denhardt's reagent

10% tween 20

5% dextran sulfate (mol. wt. ≤10,000)

250µg/mL salmon sperm DNA

50µ/mL heparin

Dissolve in water and filter sterilize – store at -20°C

3% Bovine Serum Albumin (BSA):

3% bovine serum albumin

PBS (pH 7.0)

0.1% tween 20

Dissolve in water and filter sterilize – store at 4°C

Fluorescent *in situ* hybridization (FISH) was performed as previously described (Lecuyer, Parthasarathy et al. 2008) but with some modifications. Unless otherwise indicated, all incubations and washes were performed with shaking at room temperature, and wash volumes used were 400µL. Note that the reagents used for FISH were DEPC-treated, and where needed, RNAse-free water (Invitrogen) was used.

Fixed embryos in methanol (as described in section 2.5) were once rinsed in methanol, once in 1:1 mixture of methanol:PBTween, and twice in PBTween. The embryos were then post-fixed in 4% PFA for 20 minutes, followed by three washes in PBTween for 2 minutes each. After 3µg/mL proteinase K solution was added to each embryo sample and incubated for 2 minutes, the samples were transferred to ice and incubated for 1 hour on ice without shaking. Following 1-hour incubation, the proteinase K solution was removed, and proteinase digestion was terminated by washing the embryos twice in 2mg/mL glycine solution for 2 minutes each time. To remove the glycine, the embryos were washed two times in PBTween for 2 minutes each time. The embryos were post-fixed again for 20 minutes in 4% PFA, followed by five 2-minute washes in PBTween to remove any remaining traces of fixative. The embryos were then rinsed once in 1:1 mixture of PBTween:hybridization solution, and once in hybridization solution alone. In the meantime, a pre-hybridization solution was prepared in a separate tube by boiling the hybridization solution at 100°C for 5 minutes and subsequent cooling on ice at least for 5 minutes. After removing the hybridization solution, the prepared pre-hybridization solution was added to each embryo sample and incubated at 56°C for 2 hours. In the meantime, a RNA probe solution was prepared as following: 800ng of RNA probe (see section 2.6) was added to 200µL of hybridization solution in a separate tube, and then it was heated at 80°C for 3 minutes, which was subsequently cooled on ice at least for 5 minutes. After the pre-hybridization solution was removed, the embryos were incubated in RNA probe solution for 16-20 hours at 56°C without shaking. This step was generally performed overnight.

All wash solutions were pre-warmed to 56°C. The RNA probe solution was removed, and the embryos were first rinsed once in pre-warmed hybridization solution, and next washed in fresh pre-warmed hybridization solution for 20 minutes at 56°C. Afterwards, the embryos were washed in pre-warmed 3:1, 1:1, and 1:3 mixtures of hybridization solution:PBTween for 15 minutes each time at 56°C. The embryos were further washed in pre-warmed PBTween three times at 56°C for 15 minutes each time and one time at room temperature for additional 15 minutes. After washes, the embryos were blocked by incubating in 3% BSA for 10 minutes. The embryos were then incubated with 1uL of anti-DIG-POD (Roche Applied Science, 11 207 733 910) in 200µL of 3% BSA (i.e. 1:200) for 2 hours, followed by three 10-minute washes in PBTween and three additional

washes in PBS for 5 minutes each. A 1/50 dilution of the appropriate tyramide conjugate (i.e. Cyanine 3 Tyramide) in 200µL of amplification buffer provided in the tyramide kit (Perkin Elmer Life Sciences, SAT705A) was prepared. The tyramide solution was added to the embryos and incubated in the dark at 4°C overnight. Note that, for the remainder of the protocol, the samples were kept in the dark to reduce photobleaching.

After discarding the tyramide solution, embryos were washed in 3% BSA twice for 5 minutes each. The embryos were then incubated with other desired primary antibodies in 200µL of 3% BSA for 2 hours, followed by three 10-minute washes in 3% BSA. Appropriate secondary antibodies in 200µL of 3% BSA was then added to the embryos and incubated for 2 hours. Following final washes in PBS three times for 10 minutes each, PBS was removed, and then the embryos were gently resuspended in VECTASHIELD mounting medium (Vector Laboratories, H-1000). The samples were stored at 4°C until ready for imaging by a Nikon A1R laser scanning confocal microscope with NIS-Elements software.

2.7. Immunohistochemistry of *Drosophila* Embryos

1x Phosphate Buffer Saline with Triton (PBTriton):
PBS (7.0)
0.01% triton
1% Bovine Serum Albumin (BSA) with Triton:
1% bovine serum albumin
Dissolve in PBTriton. Store at 4°C

Immunostaining of embryos was conducted as previously described (Harden, Lee et al. 1996) but with some modifications. Unless otherwise indicated, all steps were performed at room temperature with shaking.

Fixed embryos in methanol (as described in section 2.5) were first washed in PBTriton three times for 10 minutes each time, and then blocked in 1% BSA for 1 hour. The embryos were next incubated overnight at 4°C with desired primary antibodies, appropriately diluted in 1% BSA. Following three washes in PBTriton for 10 minutes each,

the embryos were incubated with fluorescently-conjugated secondary antibodies for 2 hours. Note that, for the remainder of the protocol, the samples were kept in the dark to reduce photobleaching as the fluorophores are light sensitive. After incubation with the secondary antibodies, the samples were washed in PBTriton three times for 10 minutes each time, and subsequently resuspended in VECTASHIELD mounting medium. The embryos were stored at 4°C until ready for imaging. The embryos were imaged by a Nikon A1R laser scanning confocal microscope with NIS-Elements software.

2.8. Proximity Ligation Assay

Proximity ligation assay (**PLA**) was conducted as described in the manufacturer's instructions (Sigma-aldrich, Duolink *in situ* PLA kit) but with some modifications. Unless otherwise indicated, all steps were performed at room temperature with shaking.

Fixed embryos of the desired genotype were first incubated with primary antibodies against the two proteins of interest raised in two different species, i.e. mouse and rabbit, as described in section 2.9. After three washes with PBTriton for 10 minutes each, the embryos were incubated in Probe solution for 1.5 hours at 37°C. In this Probe solution, a pair of species-specific secondary antibodies, termed PLA probes, diluted 1/5 in 1X Diluent (provided in the kit) detects the primary antibodies. The secondary antibodies were then removed by washing twice with 1X Wash Buffer A for 5 minutes each. The embryos were next incubated with Ligation solution, prepared by diluting Ligase 1/40 in Ligation buffer, for 1 hour at 37°C. Followed by two washes with 1X Wash Buffer A for 2 minutes each to remove the Ligation solution, the embryos were incubated in Amplification solution, prepared by diluting Polymerase 1/80 in Amplification buffer, for 2 hours at 37°C. Note that, due to the use of light-sensitive reagents in Amplification solution, the samples were kept in the dark from this point onwards. After amplification step, any desired fluorophore-conjugated secondary antibodies (diluted in 1% BSA) were added for 2 hr at room temperature (or overnight at 4°C) to detect the marker. As the PLA signals were later visualized with a red fluorophore, another fluorophore was used to detect the marker, e.g. FITC-conjugated secondary antibody. After final washes with 1X Wash Buffer B twice and 0.01X Wash Buffer B once, the embryos were mounted in VECTASHIELD mounting

medium with DAPI, and stored at 4°C until ready for imaging. The embryos were imaged by a Nikon A1R laser scanning confocal microscope with NIS-Elements software.

2.9. Co-immunoprecipitation

Wild-type embryos ranging from 9-13 hours after egg laying were homogenized in lysis buffer on ice. The lysate was then centrifuged at 13000rpm for ten minutes at 4°C. The pellet was discarded and the extract was stored at -80°C until ready for immunoprecipitation.

All steps regarding protein immunoprecipitation were performed at 4°C. For each immunoprecipitation, 100µL of 50% Protein G Agarose slurry (Santa Cruz Biotechnology, sc-2002) was centrifuged at 6000rpm for five minutes, after which the supernatant was discarded. The beads were subsequently washed with PBS for five minutes, and then centrifuged at 6000rpm for five minutes. The supernatant was discarded and two more PBS washes were performed. After the final wash was removed, the beads were incubated with 5µL of antibody in 150µL of Lysis Buffer I overnight. The beads were next centrifuged at 6000rpm for five minutes, after which the supernatant was discarded. The beads were subsequently washed with Lysis Buffer I for ten minutes, and then centrifuged at 6000rpm for five minutes. The supernatant was discarded and two more Lysis Buffer I washes were performed. After the final wash was removed, the extract was incubated with the beads for two hours. The beads were next centrifuged at 6000rpm for five minutes, after which the supernatant was discarded. The beads were subsequently washed with lysis buffer with salt for ten minutes, and then centrifuged at 6000rpm for five minutes. The supernatant was discarded and two more washes were performed. After the final wash was removed, loading dye was added and the samples were stored at -20°C until ready for SDS-PAGE.

After boiling the samples for ten minutes, each sample was loaded onto an 10% discontinuous SDS-PAGE gel along with a prestained ladder (Bio-Rad, 161-0318). The samples were run at 100V in Running Buffer until the dye front migrated to the end of the gel, and then were transferred onto a nitrocellulose membrane (Bio-Rad, 162-0115) at

15V with Transfer Buffer for one hour using a semi-dry transfer apparatus. The membranes were stored in TBS at 4°C until ready for immunoblotting.

The membranes were first washed with TBST three times for ten minutes each, and then blocked with 5% BSA for one hour. The membranes were next incubated with a primary antibody overnight at 4°C (see a list of the primary antibodies in the next paragraph). Following two washes with TBST and two blocks with 2.5% BSA for ten minutes each, the membranes were incubated with an HRP-conjugated secondary antibody for two hours (see a list of the secondary antibodies in the next paragraph). The membranes were then washed with TBST four times for 15 minutes each. Detection was accomplished with the BM Chemiluminescence Western Blotting Peroxidase Substrate (Roche Applied Science, 11500694001).

Chapter 3. Results Part I: The diffusible Fog ligand is a potential regulator of *zip*-encoded myosin II during DC

For DC to proceed properly, the DME cells appear to communicate with the AS, and vice versa, to ensure coordinated morphogenesis of these two tissues during the process. This communication between the two tissues regulates morphogenesis, at least in part, by modulating transcription of *zip*, which encodes non-muscle myosin heavy chain required for cell shape changes in these tissues. Our lab believes that the regulation of *zip*-encoded myosin is a critical component of DC. As mentioned in the Introduction, two signaling pathways are major contributors to actomyosin contractility during DC: the JNK cascade, which induces *zip* expression, and the Rho1 pathway, which activates myosin (Harden 2002; Fernandez, Arias et al. 2007; Khoo, Allan et al. 2013). Rho1 activation of myosin has best been characterized in gastrulation, and is mediated by the Fog pathway, which is composed of the diffusible ligand Fog, the GPCRs Mist and Smog, the G_{α} subunit Cta, and the GEF RhoGEF2 (Barrett, Leptin et al. 1997; Nikolaidou and Barrett 2004; Dawes-Hoang, Parmar et al. 2005). The involvement of RhoGEF2 in the pulsation and constriction of AS cells during DC (Azevedo, Antunes et al. 2011) raises the possibility that the Rho1 pathway might also play a role in DC. Moreover, the $G_{\alpha 12/13}$ proteins are capable of activating the JNK cascade in mouse cells (Jho, Davis et al. 1997; Dermott, Ha et al. 2004). David Cheng, a former MSc student in the Harden lab, performed immunohistochemistry to see if Fog protein was present during DC. Strikingly, he found that Fog was found in both the AS and DME cells. Thus, Fog is a strong candidate that may contribute to the activation of Rho1 and/or JNK signaling during DC.

The purpose of this project was to address possible interactions between Fog and the JNK cascade during DC, i.e. determine if Fog can promote JNK-mediated actomyosin contractility. The effects of Rho1 signaling on DC were also studied to assess whether it can act as an upstream regulator of the JNK cascade. More specifically, studies were done to determine whether Fog and known proteins downstream of the ligand can regulate JNK signaling using *zip* transcription as a read-out. To do this, *UAS*-transgenes that encode for each Fog-Rho1 pathway component were overexpressed in the embryo with various Gal4 drivers (**Figure 3.1**). *zip* transcription levels in the AS and DME cells during

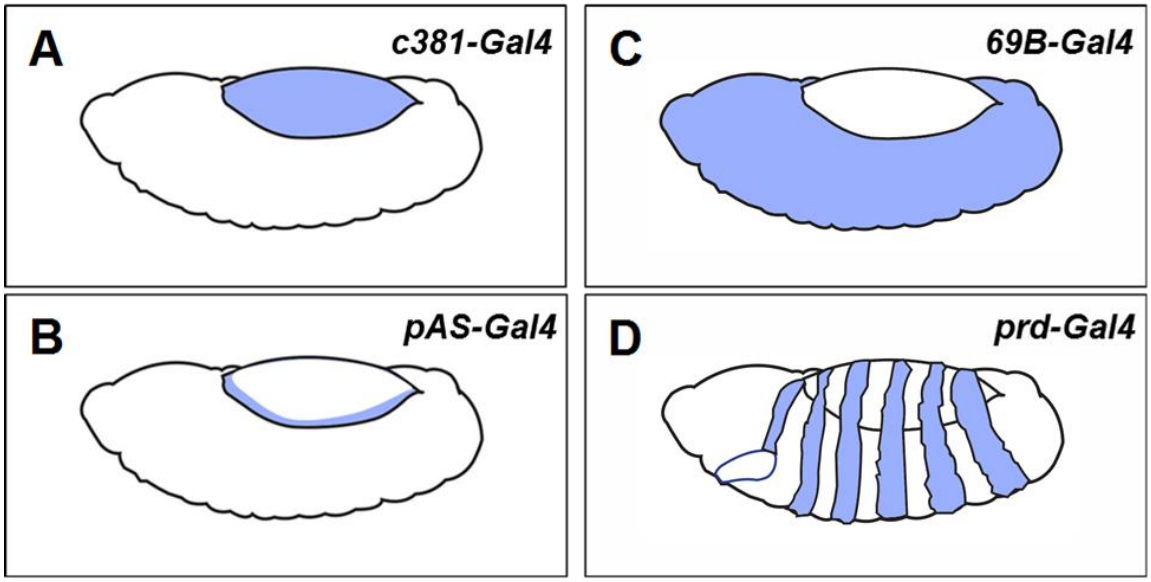

Figure 3.1. Gal4 drivers used in this project.

UAS-transgenes were either driven throughout the AS by *c381-Gal4* (**A**), in the peripheral-most AS cells, i.e. the outer row of AS cells abutting the leading edge epidermis, by *pAS-Gal4* (**B**), in the epidermis by *69B-Gal4* (**C**), and in paired-positive stripes extending from the epidermis into the AS by *prd-Gal4* (**D**). The yeast Gal4/UAS system was applied as previously described (Brand and Perrimon 1993), which allows the selective activation of cloned gene of interest in a various range of cell- and tissue-specific patterns. The gene encoding the yeast transcriptional activator Gal4 is inserted randomly into the *Drosophila* genome, generating transgenic lines expressing Gal4 in a wide variety of cell- and tissue-specific patterns. These lines induce Gal4 expression from one of various genomic enhancers. Next, subcloning any gene/sequence downstream of Gal4 binding sites within its promoter can construct a Gal4-dependent target gene *X* (*UAS-Gene X*). In order to activate the target gene *X* in particular patterns, flies carrying *UAS-Gene X* are crossed to flies expressing cell-/tissue-specific Gal4 (Enhancer Trap Gal4). In the progeny of this cross, *UAS-Gene X* in cells where Gal4 is expressed can then be activated, and the effect of this directed misexpression of *Gene X* on developmental processes can be studied. The target gene is silent in the absence of Gal4.

DC were then assessed by FISH. Parallel loss-of-function experiments was also performed by analyzing mutants, in addition to knockdowns using transgenic RNAi.

3.1. The diffusible Fog ligand promotes *zip* expression in both the AS and epidermis during DC

Fluorescent *in situ* hybridization (**FISH**) was first performed to assess whether the *fog* gene was actively expressed at this stage. Consistent with preliminary immunohistochemistry data from our lab that assessed Fog protein distribution in embryos (unpublished), *fog* expression was indeed observed throughout the AS and epidermis in wild-type embryos from germband retraction to DC (**Figure 3.2**). *fog* transcripts were localized to punctate structures that are scattered throughout the embryo. Interestingly, puncta in the AS appeared to accumulate especially at the canthi during DC (**Figure 3.2 B**), while puncta in the epidermis were predominately localized in a segmental pattern (**Figure 3.2 A,B,C'**). Future experiments will involve performing *fog* FISH in wild-type embryos co-immunostained with various nuclear and cytoplasmic markers to assess the exact subcellular location of the *fog* transcripts during DC.

The effects of Fog on *zip* transcription were next evaluated via FISH analysis on embryos overexpressing a wild-type *fog* transgene, i.e. *UAS-fog*, in the AS. AS-specific overexpression of *fog*, using either *c381-Gal4* or *pAS-Gal4*, caused an overall increase in *zip* transcript levels in both the AS and epidermis when compared to wild-type embryos (**Figure 3.3 A-C**). Similar results were also observed when Fog was overexpressed in the epidermis using *69B-Gal4* (**Figure 3.3 D**). Note that when making these conclusions, at least 5-10 embryos for each mutant and control genotype were imaged by confocal microscopy and evaluated.

In each experiment, males homozygous for the *UAS-fog* transgene were crossed to virgin females homozygous for the *Gal4* driver to ensure that all progeny carried one copy of each. Thus, the wild-type control, though incubated at the same time with master mixes during the FISH experiments, was in a different reaction tube. Therefore, it is possible that the control embryos stained less efficiently than the embryos overexpressing the *fog* transgene strictly by chance. To remedy this issue, *prd-Gal4*, which is homozygous

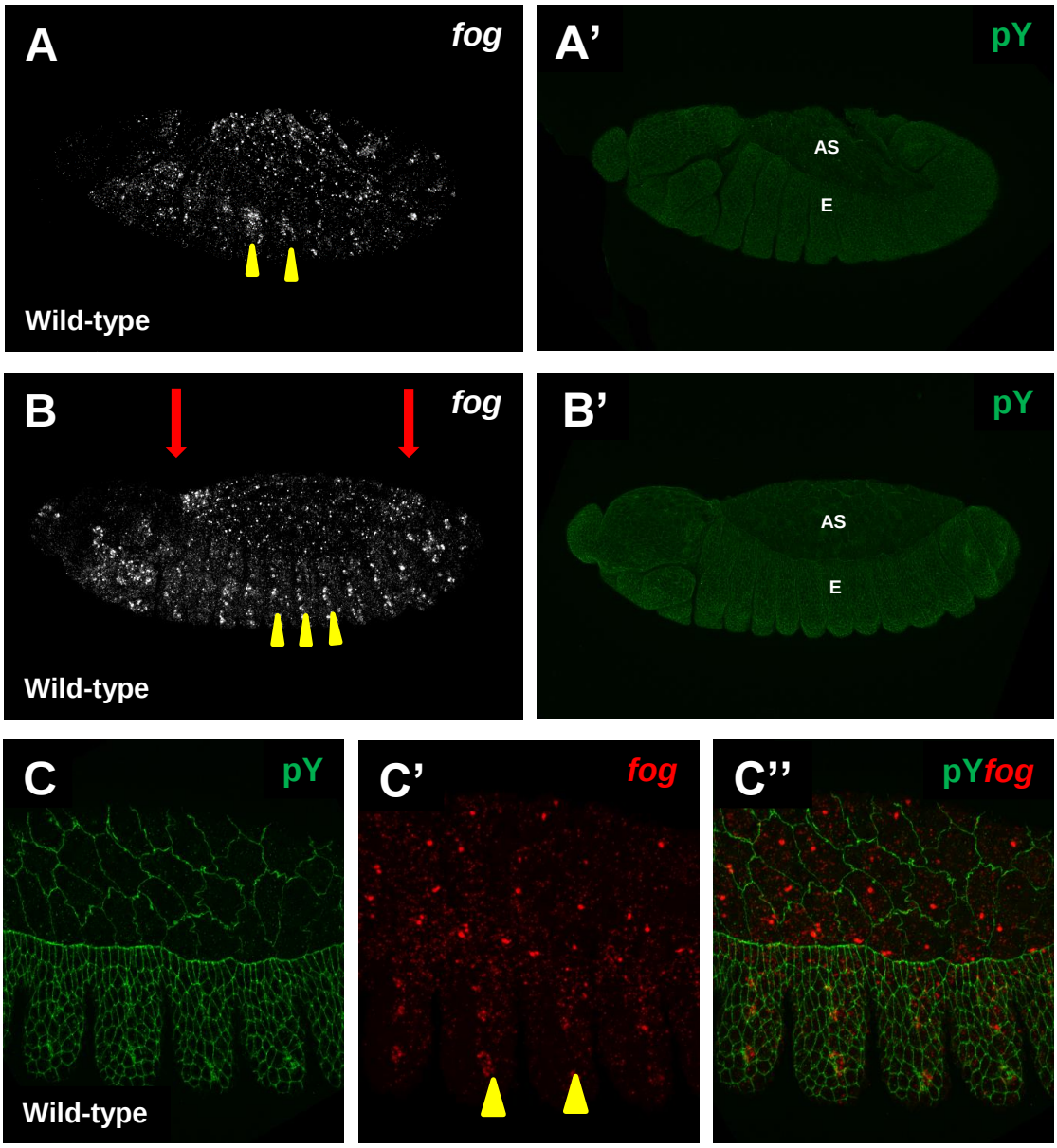

Figure 3.2. ***fog*** **is expressed in the AS and epidermis from germband retraction through to DC.**

fog FISH on wild-type embryos. Note the relatively high levels of *fog* in the AS and epidermis during germband retraction (**A**) and DC (**B**). Anti-phospho-tyrosine antibodies mark the cell membranes (**A**',**B**'). (**C-C''**) Magnified images of *fog* expression during DC indicate that the diffusible ligand Fog is efficiently expressed as puncta in both the AS and the epidermis. AS = amnioserosa, E = epidermis, pY = phospho-tyrosine. Puncta accumulation at the canthi in the AS is indicated by red arrows (**B**), and a number of puncta in the epidermis in a segmental pattern is indicated by yellow arrowheads (**A,B,C'**).

lethal, was balanced over a GFP balancer so that when outcrossed, progeny that received the *Gal4* can be distinguished from those that received the balancer via the lack of GFP expression. In other words, when crossed to *UAS-fog*, half the progeny will express the *fog* transgene via *prd-Gal4*, whereas the other half, which can be used as an internal control, will not due to the presence of the balancer instead of the *Gal4*. In this case, the mutant and internal control sibling embryos will be treated identically during FISH experiments as they are in the same reaction tube. Overexpression of fog with *prd-Gal4* consistently caused an increase in *zip* transcript levels in both the AS and epidermis when compared to the internal control (**Figure 3.3 E**). Due to the use of an internal control, the observed effects of Fog on *zip* expression can be considered more reliable. In the future, to further validate these results, quantification of expression from the *zip* gene can be determined using quantitative PCR (qPCR).

Notably, though Fog was overexpressed in a tissue-specific pattern, *zip* transcription was elevated throughout the AS and epidermis. This is likely due to the secretable nature of Fog, which affects not only cells expressing the *fog* transgene, but also neighbouring non-expressing cells, thereby causing a non-autonomous effect. All of the data above suggest that the diffusible Fog ligand, when expressed in either the AS or epidermis, can promote *zip* expression in both tissues during DC.

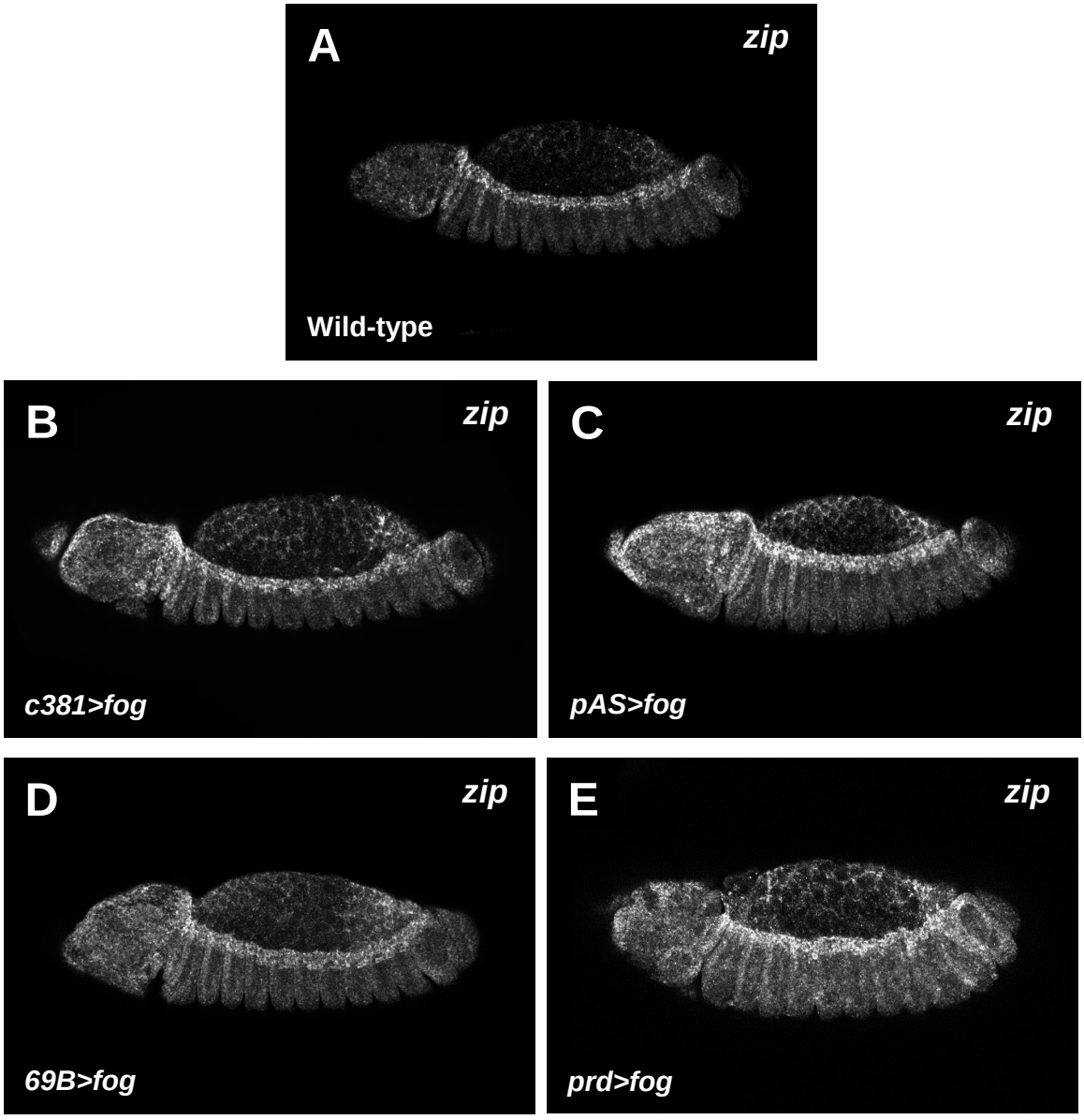

Figure 3.3. Overexpression of Fog in various tissues may activate *zip* expression during DC.

zip FISH performed in wild-type embryos (**A**) is used as a control. *zip* FISHs conducted in embryos overexpressing *fog* using AS-specific *c381-Gal4* (**B**), peripheral AS-specific *pAS-Gal4* (**C**), epidermis-specific *69B-Gal4* (**D**) and *prd-Gal4* (**E**) are shown. Compared to the wild-type control (**A**), embryos overexpressing *fog* shows the overall increase in *zip* levels. (Note that each FISH experiment was done separately with its own wild-type control. Only one image of wild-type embryo was used here as the same effects were observed in every FISH experiment.)

3.2. Overexpression of downstream components of Fog signaling also up-regulates *zip* expression

Having found that Fog might positively regulate *zip* expression, known downstream components of the Fog pathway, including the Fog receptors Mist and Smog, the G_{α} protein Cta, the GTPase Rho1, and the Rho1 GEF activator RhoGEF2, were also investigated. Individual FISH against these downstream components were first performed in wild-type embryos to determine whether each component of the pathway was expressed during DC. These experiments showed that all of the components were indeed present in both the AS and the epidermis during DC, with each component displaying a distinctive expression pattern (**Figure 3.4**). The distribution of the *mist* transcripts was more prominent in the epidermis than that in the AS, and they appeared to be accumulated in the head area (**Figure 3.4 A**). Similar to *mist*, the *cta* expression was largely found in the epidermis (**Figure 3.4 B**) rather than in the AS, and the accumulation of *cta* at the leading edge became more noticeable when less confocal images were merged (**Figure 3.4 B'**), suggesting its potential involvement in DC at the leading edge. Consistent with Rho1's critical function in the regulation of myosin and actin dynamics in embryogenesis (Abreu-Blanco, Verboon et al. 2014), the *rho1* transcripts were largely expressed throughout the epidermis (**Figure 3.4 C**). As briefly mentioned before, the role of RhoGEF2 in the regulation of AS cells during DC has been demonstrated (Azevedo, Antunes et al. 2011). Consistently, the transcription of *rhoGEF2* was not only high in the epidermis, but also strong in the AS (**Figure 3.4 D**). Unfortunately, where Smog, another receptor for Fog, is expressed in wild-type embryos was not determined due to the difficulty of generating a good antisense *smog* RNA probe. Nevertheless, with other known components of Rho1 signaling being expressed in the epidermis and the AS, possibly activated by its known ligand Fog, the Rho1 pathway might play an essential role in DC.

Next, transgenes encoding either the wild-type or constitutively active form of the G_{α} protein Cta, i.e. *UAS-cta*^{WT} and *UAS-cta*^{Q303L} respectively, were expressed using the segmental *prd-Gal4* driver, and *zip* levels were analyzed using FISH. Both the wild-type and constitutively active form of Cta increased *zip* transcription in *prd-positive* stripes (**Figure 3.5 A-C**) in the epidermis. The downstream Cta effector, Rho1, was next investigated by overexpressing wild-type Rho1 using *prd-Gal4*, which also caused

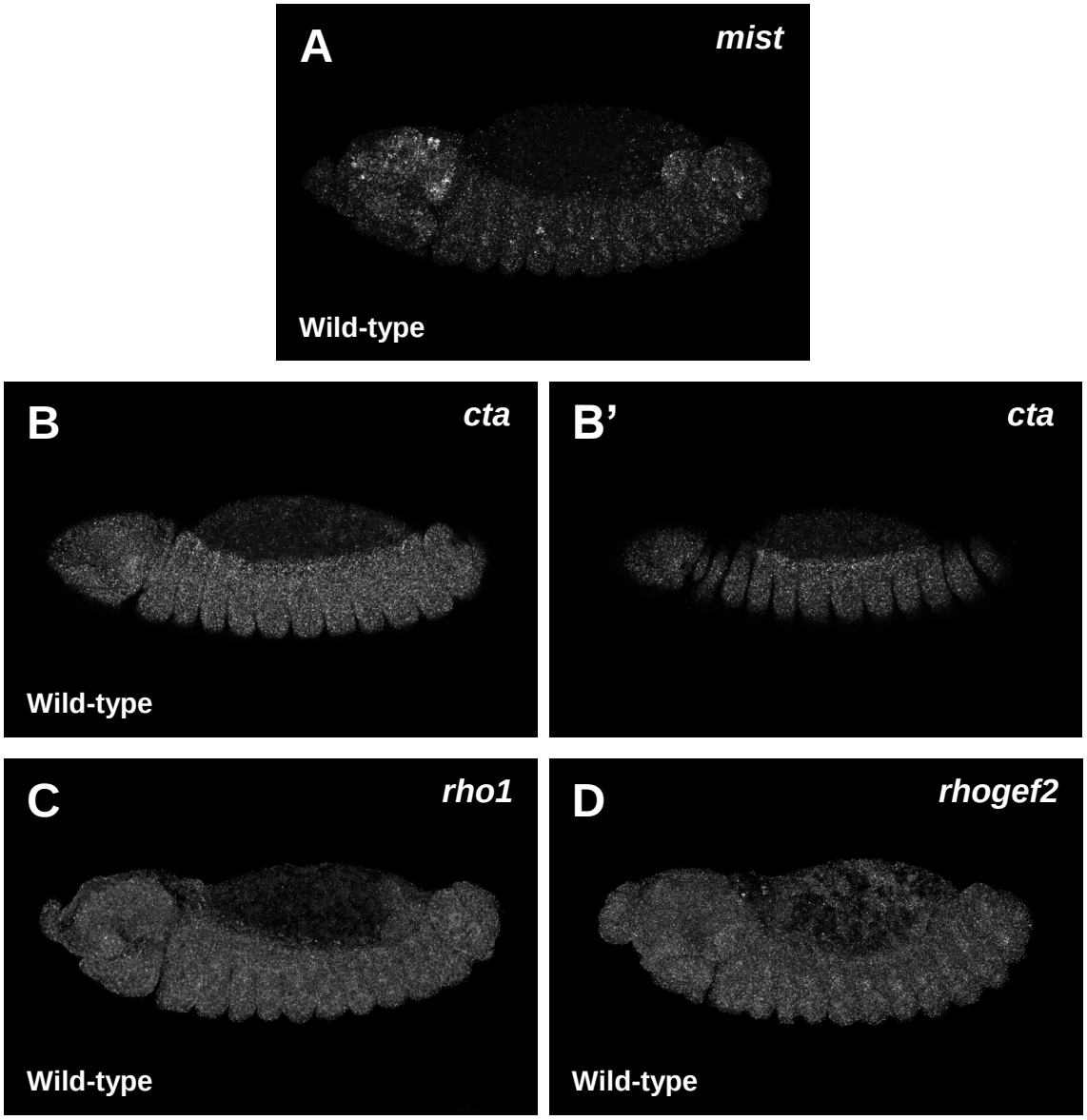

Figure 3.4. Downstream components of the Rho1 pathway are expressed during DC.

Individual FISH performed against each component of the Rho1 pathway in wild-type embryos. The receptor Mist (**A**), the G_{α} protein Cta (**B, B'**) and the small GTPase Rho1 (**C**) are expressed in the AS and the epidermis during DC, with most of expression enriched in the epidermis. The Rho1 activator RhoGEF2 is not only highly expressed in the epidermis but also in the AS (**D**), consistent with its role in AS cell constriction during DC (see Chapter 3). Note the slightly accumulated expression of Cta at the leading edge is seen when fewer confocal images are contained in the stack (**B'**).

elevated *zip* levels in *prd-positive* stripes (**Figure 3.5 D**). When expressing the constitutively active version of Rho1, i.e. *UAS-rho1^{V14}*, a similar *zip* transcriptional up-regulation effect was observed (**Figure 3.5 E**). Several different transgenic lines of Rho1 were investigated as well, all of which led to an increase in *zip* expression (**Figure 3.6**). Moreover, the effects of the GPCRs Mist and Smog on *zip* transcription were studied. Overexpression of wild-type Mist using *prd-Gal4* did not appear to have any effects on *zip* levels (Data not shown). Due to the lack of available *UAS*-transgenes of wild-type Smog to date, the UAS/Gal4 system could not be applied; However, a stock that overexpresses a functional GFP-fused Smog under the control of the natural *sqh* promoter was kindly provided by the Lecuit Lab (Kerridge, Munjal et al. 2016). The effects of the receptor Smog on *zip* levels, therefore, could be analyzed. In homozygous embryos overexpressing Smog proteins, an up-regulation of *zip* transcription was observed when compared to the wild-type *zip* levels (**Figure 3.7**), suggesting Smog's potential role in promoting *zip* levels as a receptor for Fog. Compared to wild-type controls (for example, **Figure 3.3 A**), an uneven morphology of the leading edge was displayed, possibly due do more *zip* transcripts expressed in the DME cells causing stronger constriction of cells.

Finally, another known component of the Rho1 pathway, RhoGEF2, might be expected to cause a similar *zip* transcriptional up-regulation effect when overexpressed. Nevertheless, when the wild-type RhoGEF2 was overexpressed using the segmental *prd-Gal4* driver, an effect on *zip* levels was not obvious. (**Figure 3.8**).

All of my data, thus far, indicated that Fog potentially acts as an upstream regulator of the Rho1 signaling pathway, which in turn contributes to *zip* transcription during DC. However, this hypothesis was based on overexpression, gain-of-function analysis which can be unreliable as non-physiological levels of signaling can occur. Loss-of-function data needed to be obtained to further confirm these results.

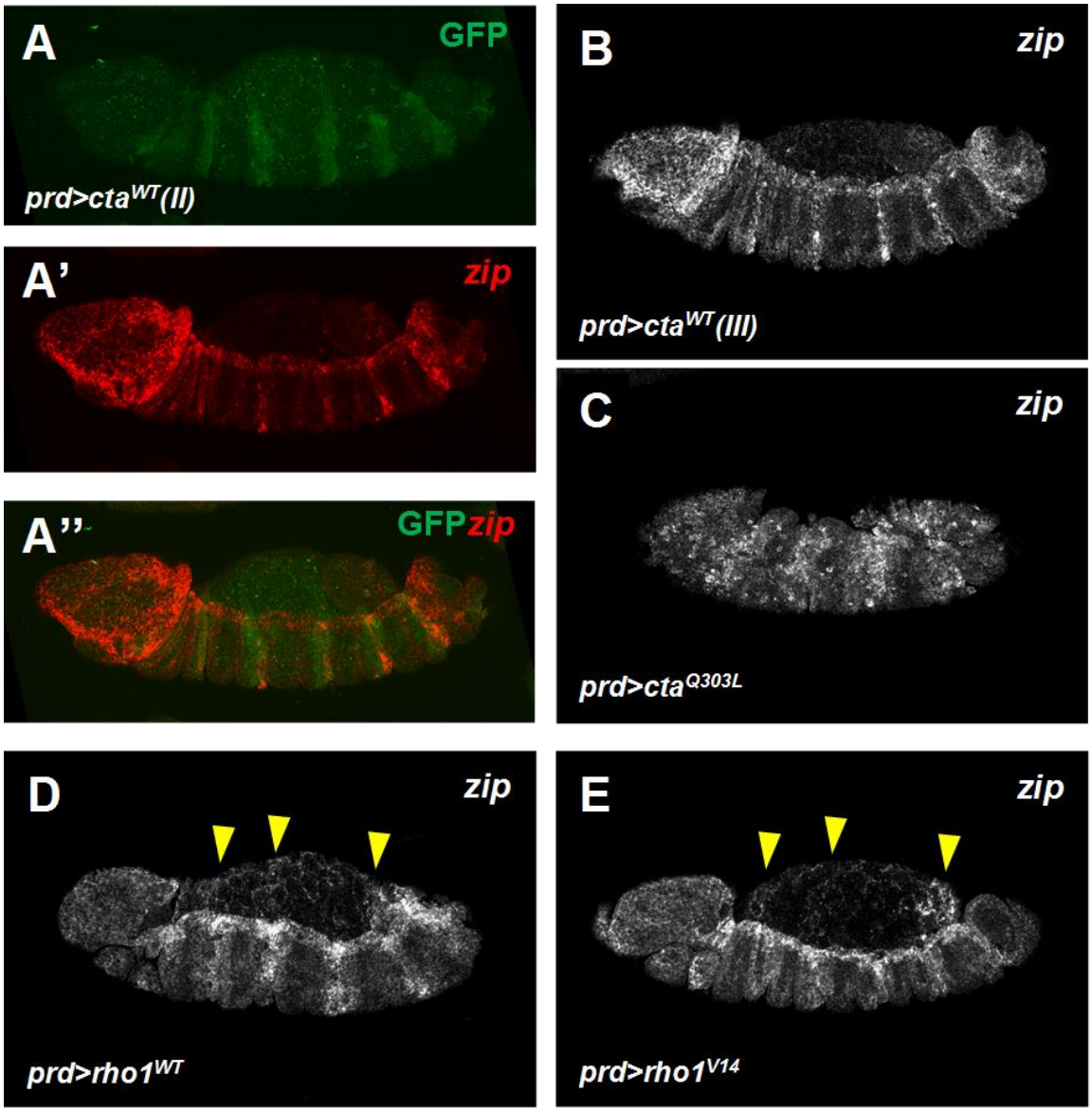

Figure 3.5. Overexpression of Cta and Rho1 up-regulates *zip* transcription.

zip FISH. (**A-A''**) The *UAS* transgene of Cta on chromosome II is expressed using the segmental *prd-Gal4* driver. (**A**) GFP staining marks the *prd* stripes that express the *UAS* transgenes. (**A'**) *zip* expression is elevated in *prd* stripes that are GFP-positive, as seen in a merged image of A and A' (**A''**). Similar *zip* up-regulation effects are also seen in embryos overexpressing the wild-type Cta on chromosome III (**B**), the wild-type Rho1 (**D**), and the constitutively active form of Cta and Rho1, cta^{Q303L} (**C**) and rho^{V14} (**E**), respectively. In some of embryos expressing more functional Rho1, the elevated *zip* transcripts in the AS were also seen in *prd* stripes, as indicated by yellow arrowheads (**D,E**).

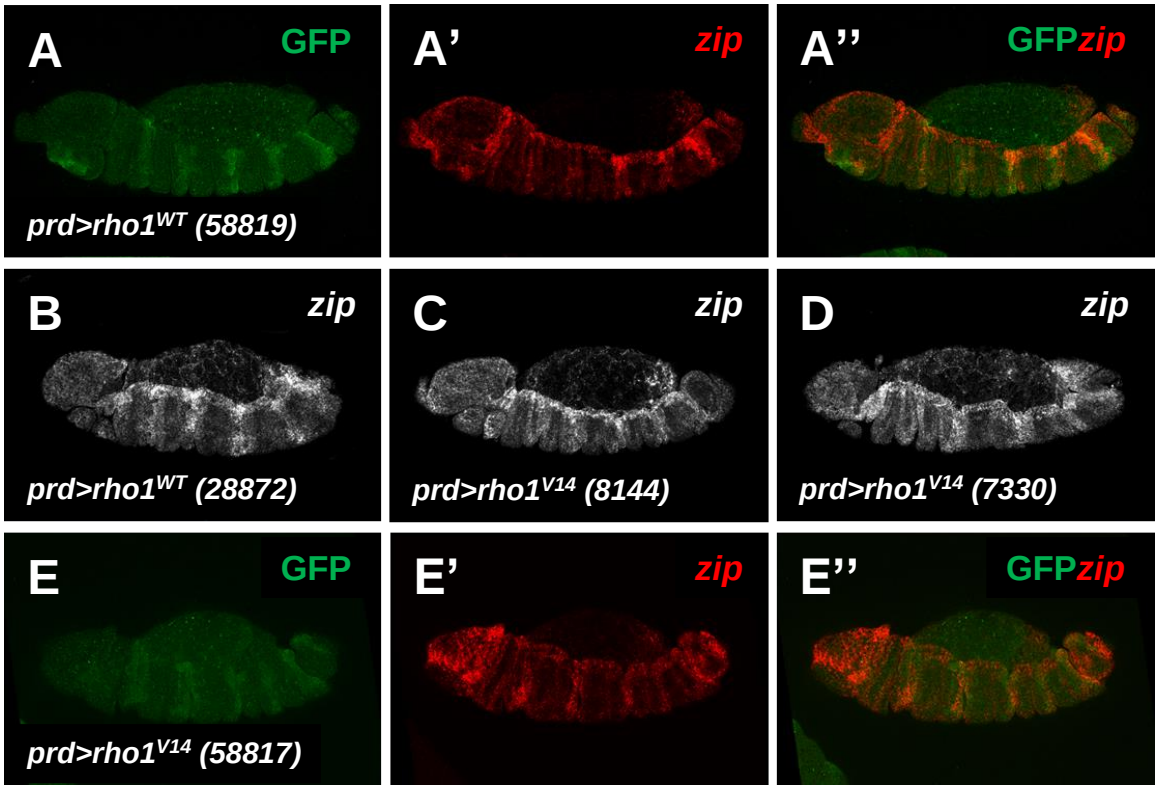

Figure 3.6. Overexpression of the wild-type Rho1 and the constitutively active Rho1 upregulates *zip* transcription.

zip FISH. Two different transgenic strains of the wild-type Rho1 are expressed using *prd-Gal4*: *rho1^{WT}-58819* (**A-A''**) and *rho1^{WT}-28872* (**B**). Three different transgenes of the constitutively active Rho1 are also expressed using *prd-Gal4*: *rho1^{V14}-8144* (**C**), *rho1^{V14}-7330* (**D**) and *rho1^{V14}-58817* (**E**). Embryos expressing these transgenes show *zip* upregulation. (**A, E**) GFP staining marks the *prd* stripes that express the *UAS* transgenes. (**A', E'**) *zip* expression is elevated in *prd* stripes that are GFP-positive, as seen in a merged image of A and A' (**A''**) or E and E' (**E''**). Note that each number represents a stock number from Bloomington *Drosophila* Stock Centre.

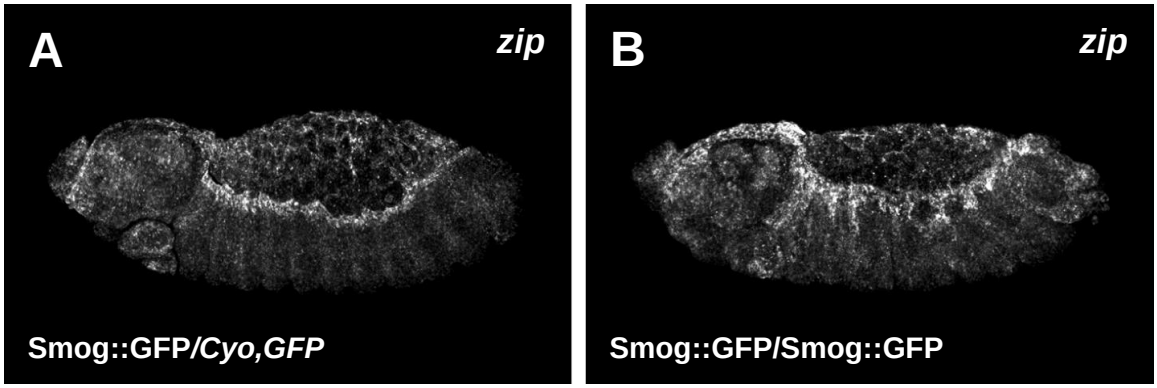

Figure 3.7. Overexpression of functional Smog protein promotes the transcription of *zip*.

zip FISH shown in embryos heterozygous (**A**) or homozygous (**B**) for overexpression of wild-type Smog protein. The up-regulated *zip* levels are apparent in homozygotes. Note that in B *zip* transcripts are elevated in a segmental pattern. Note the uneven morphology of the leading edge cells directly flanking the AS in both heterozygotes and homozygotes.

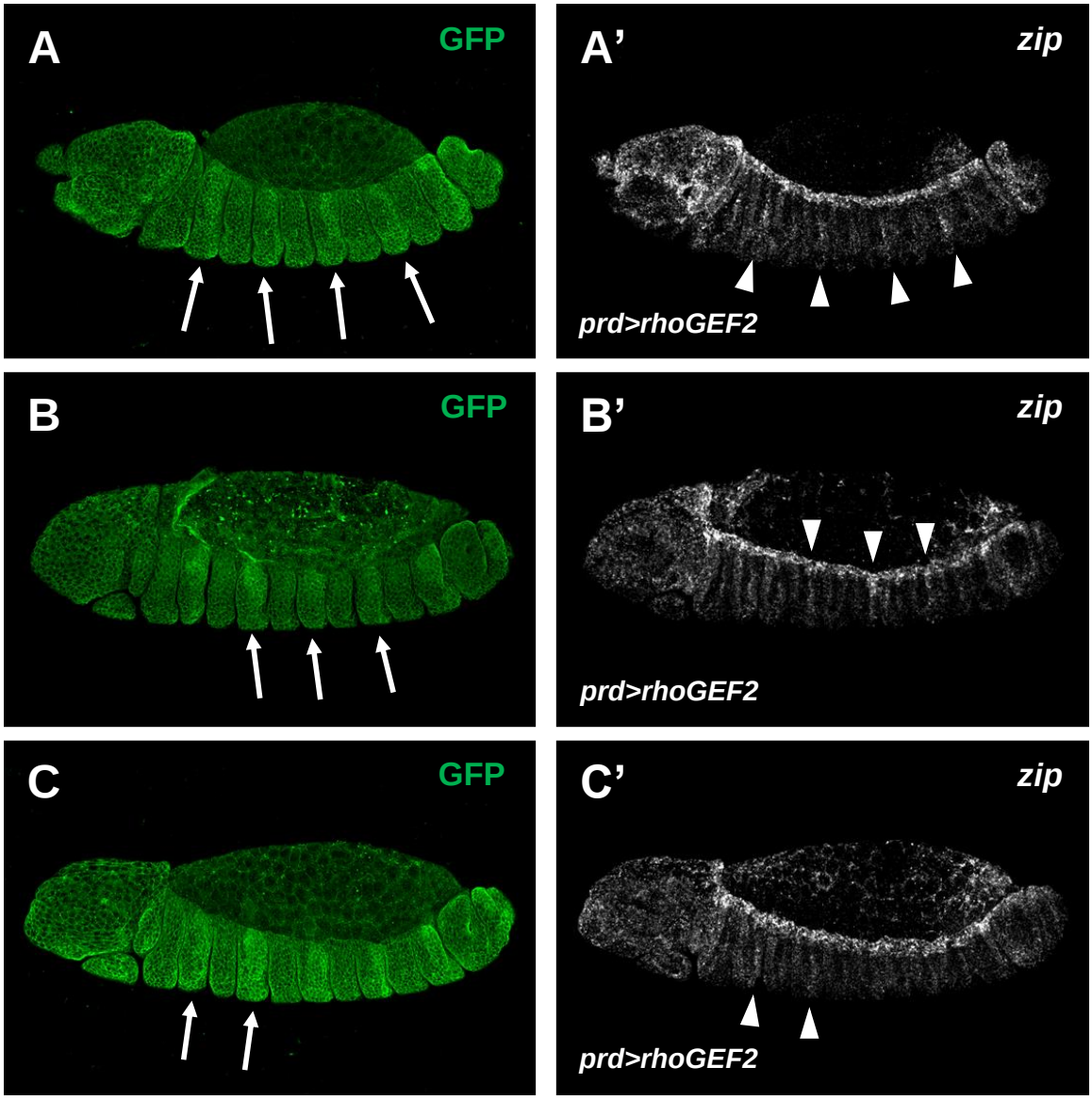

Figure 3.8. RhoGEF2 overexpression cannot robustly promote *zip* expression.

(**A'-C'**) *zip* FISH is shown in embryos overexpressing wild-type RhoGEF2 under the control of the *prd-Gal4* driver. (**A-C**) GFP staining marks the *prd* stripes that express the *UAS-rhoGEF2* transgene. A slight elevation of *zip* levels (white arrowheads) in GFP-positive stripes (white arrows) is observed in few embryos. Note that embryo cages were incubated at 29°C for this experiment.

3.3. Down-regulation of several Rho1 components causes a reduction in *zip* levels

To determine whether loss of Fog-Rho1 signaling would have the opposite effect on *zip* transcription, RNAi transgenes against multiple components of the pathway were expressed under the control of the segmental *prd-Gal4* driver. No obvious effects on *zip* levels were observed (See **Table B1 in Appendix**), except for one Fog RNAi transgene, i.e., *UAS-fog*^{RNAi}. Consistent with the gain-of-function data for Fog, embryos expressing RNAi against Fog using *prd-Gal4* caused a down-regulation of *zip* levels when compared to that of wild-type embryos (**Figure 3.9**). During early germband retraction, the *zip* transcripts in the AS expressed by the segmental *prd-Gal4* driver (**Figure 3.9 C**) were not as high as in its internal control embryos (**Figure 3.9 A**). The overall *zip* levels during DC appeared to be decreased, especially in the head area (Compare **Figure 3.9 D** with **Figure 3.9 B**). In order to obtain more loss-of-function data, instead of expressing RNAi transgenes, multiple mutations for each Fog-Rho1 signaling pathway component were re-balanced over GFP balancers so that homozygous mutant embryos could be identified. When *zip* transcription levels in *fog* homozygous mutant embryos were assessed, an overall decrease in *zip* levels was observed when compared to the heterozygous mutant control (**Figure 3.10 A, B**). The *zip* transcriptional down-regulation effect at the leading edge in homozygous *fog* mutant embryos was mild, but consistent with the observation seen in *prd>fog*^{RNAi} embryos (**Figure 3.9 D**), the decreased *zip* expression in the head area was apparent (Compare **Figure 3.10 B** with **Figure 3.10 A**). Similar results were also seen when *rhoGEF2* was mutated, i.e. the overall *zip* levels were decreased in the homozygous mutant embryos (**Figure 3.10 C, D**). These results further support the hypothesis that Fog and Rho1 signaling make a contribution to *zip* expression during DC.

However, when the dominant negative version of Rho1, i.e. *UAS-rho1*^{N19}, was expressed under the control of the *prd-Gal4* driver, *zip* transcript levels were slightly increased in *prd*-positive stripes (**Figure 3.11**). These results were unexpected as loss of Rho1 was hypothesized to cause a decrease in *zip* levels based on the previous results. Nevertheless, the up-regulated transcriptional effect of *zip* in embryos expressing dominant negative Rho1 was milder than that in embryos expressing either the wild-type Rho1 or the constitutively active Rho1 (compare with **Figure 3.6**). There are many

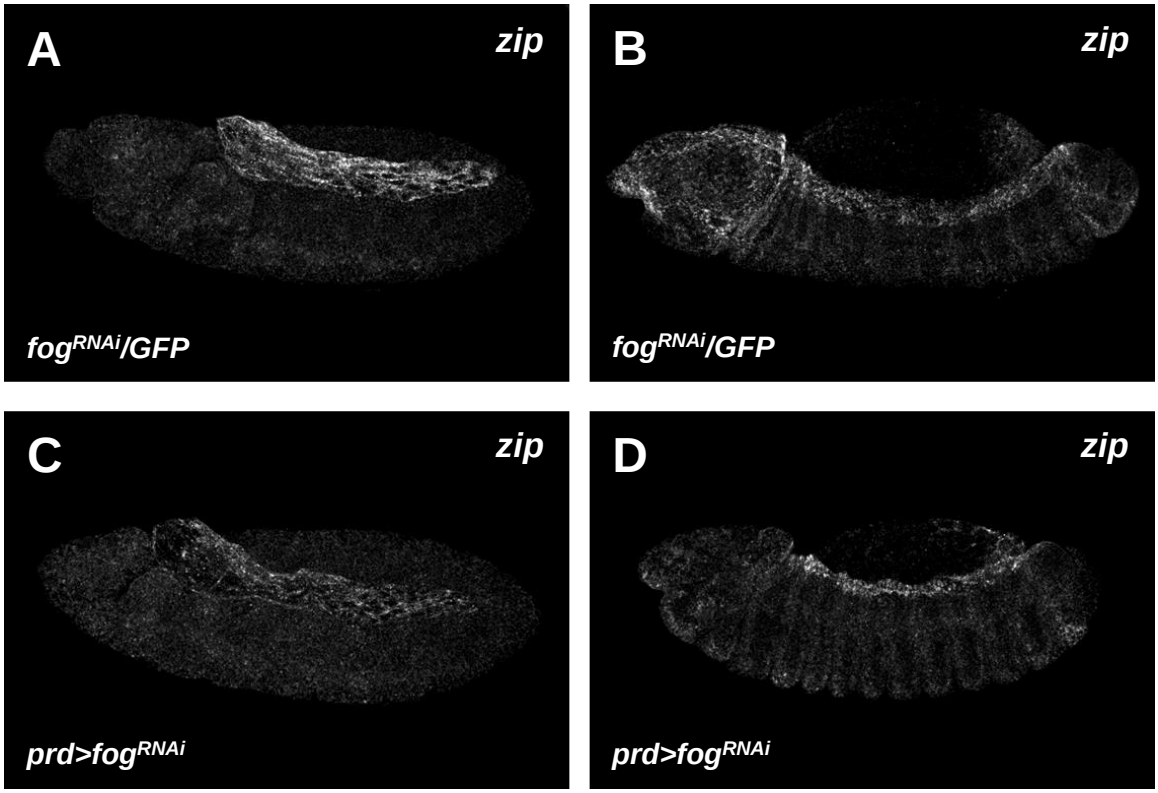

Figure 3.9. Expression of transgenic RNAi against Fog might cause a decrease in *zip* levels.

zip FISH during germband retraction (**A,C**) and DC (**B,D**) are performed in embryos overexpressing *fogRNAi* using *prd-Gal4*. Overall transcriptional levels of *zip* in embryos expressing *fogRNAi* (**C,D**) is reduced when compared to that in *fogRNAi* non-expressing embryos, which serve as a control (**A,B**), both before and during DC. Note that embryo cages were incubated at 29°C for this experiment.

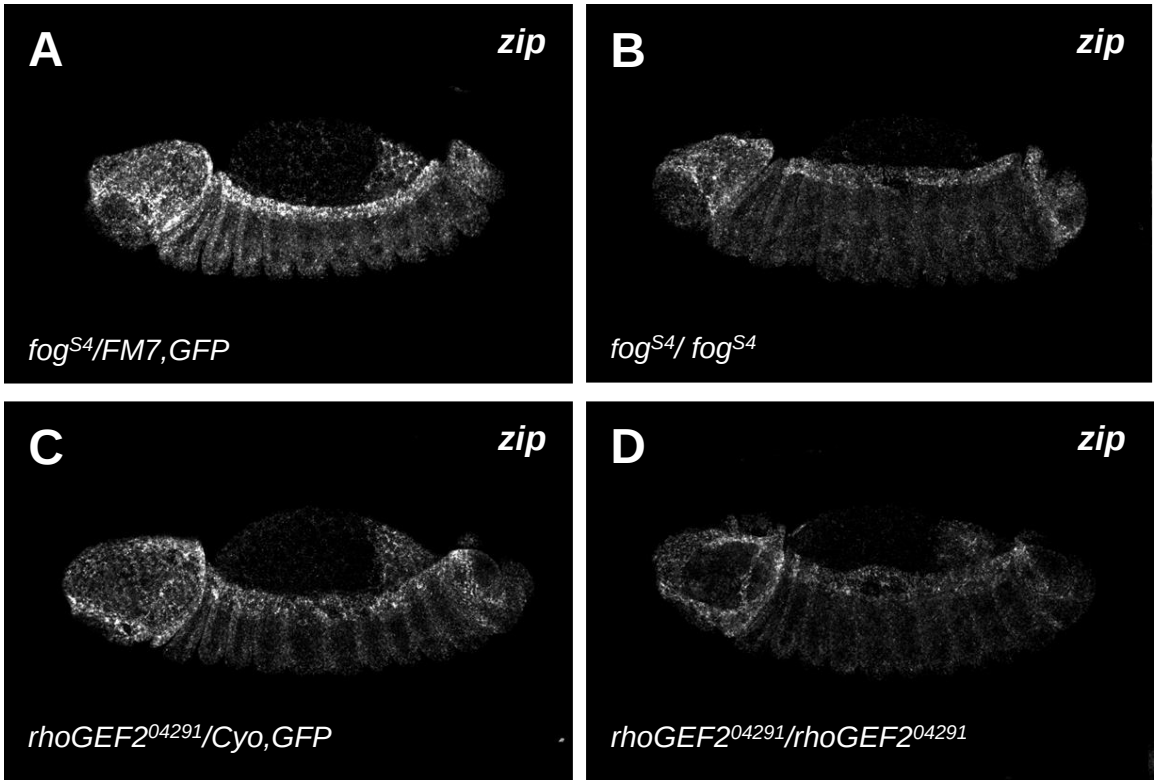

Figure 3.10. Down-regulation of either functional Fog or RhoGEF2 reduces *zip* expression.

(**A-D**) *zip* FISH during DC. In homozygous embryos mutant for Fog, i.e. fog^{S4}/fog^{S4} (**B**), an overall transcriptional level of *zip* is decreased when compared to that in corresponding heterozygous control embryos, i.e. $fog^{S4}/FM7,GFP$ (**A**). A similar down-regulation effect on *zip* is also observed in homozygous RhoGEF2 mutant embryos, i.e. $rhoGEF2^{04291}/rhoGEF2^{04291}$(**D**) compared to the control heterozygotes, $rhoGEF2^{04291}/Cyo,GFP$ (**C**).

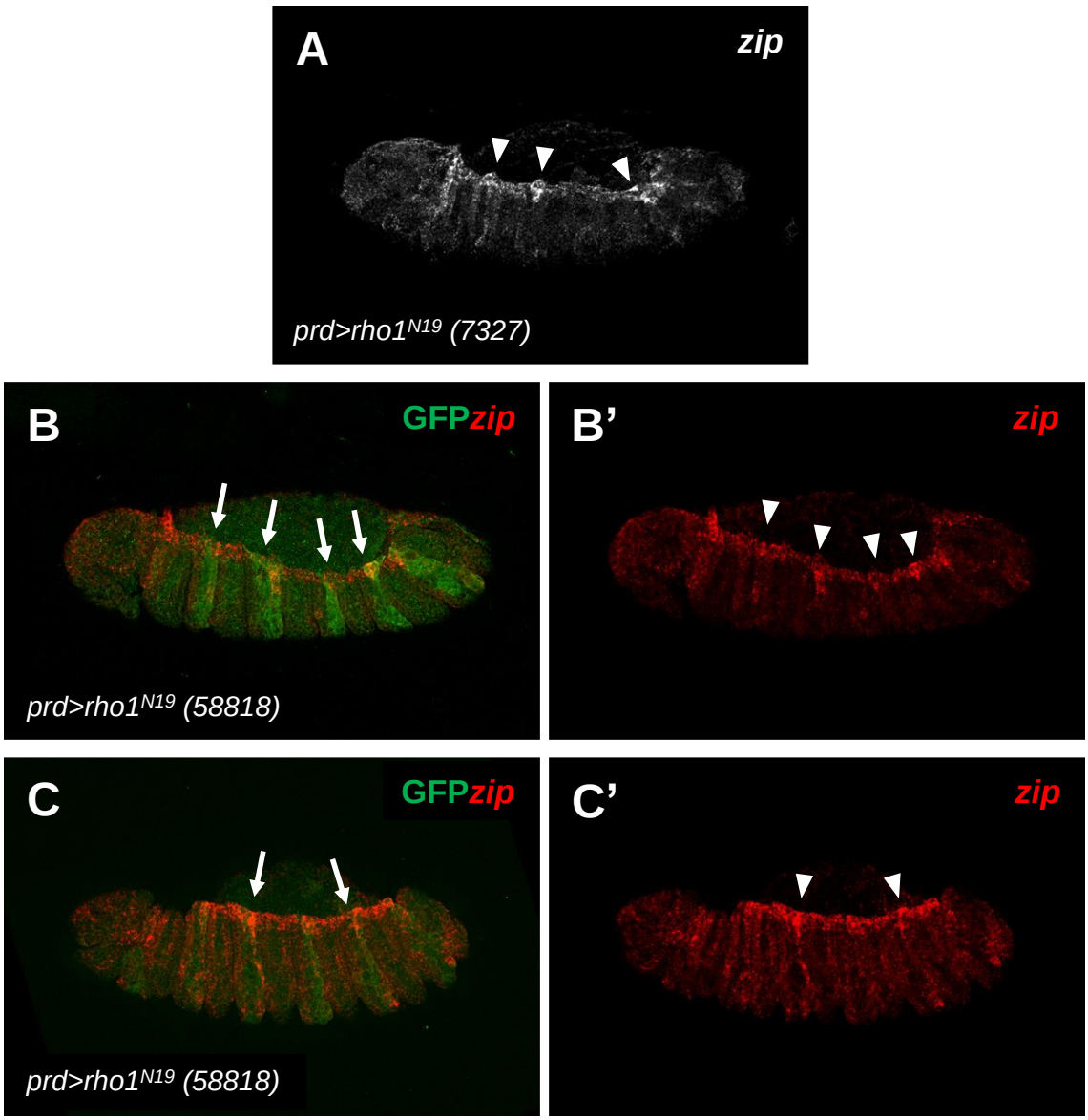

Figure 3.11. Unexpectedly, dominant negative version of Rho1, *UAS-rho1^{N19}*, causes a slight increase in *zip* levels in *prd*-positive stripes.

Two different transgenic strains of the dominant negative form of Rho1 are expressed using *prd-Gal4*: *rho1^{N19}*-7327 (**A**) and *rho1^{N19}*-58818 (**B,B',C,C'**). (**B,C**) GFP staining marks the *prd* stripes that express these UAS transgenes. Embryos expressing the transgenes result in the up-regulation of *zip*, indicated by white arrowheads (**A,B'C'**), in *prd*-positive stripes, indicated by white arrows (**B,C**).

examples in the literature of dominant negative and constitutively active versions of a small GTPase having the same phenotypic effect, one interpretation of such results is that cycling of the small GTPase between on and off states is a critical part of its signaling. For example, gains and losses of Rap1 have the same effect on epithelial folding in the *Drosophila* embryo (Wang, Khan et al. 2013).

3.4. Evidence that regulation of *zip* expression by Cta and Rho1 is JNK-dependent

Both the gain- and loss-of-function data suggested that Fog-Rho1 signaling may positively regulate *zip* transcription in DC. In order to address if the observed *zip* transcriptional effects were JNK-dependent, i.e. if the Fog, acting upstream of the Rho1 pathway, acts as an upstream regulator of JNK-mediated *zip* expression, *dpp* FISH was next considered. As discussed in the Introduction, the JNK cascade controls the transcription of *dpp* at the leading edge (**Figure 3.12 A**), whereas *dpp* transcriptional regulation in the ventral epidermis is not affected by the JNK cascade. As the wild-type Cta and Rho1 transgenes, *UAS-cta*^{WT} and *UAS-rho1*^{WT} respectively, showed an increased *zip* levels clearly in *prd-positive* stripes when overexpressed under the control of the *prd-Gal4* driver (as seen in **Figure 3.5 B, D**), these two transgenes were selected for *dpp* FISH. If the up-regulation of *zip* transcription observed in these embryos was JNK-dependent, a similar *dpp* transcriptional up-regulation effect at the leading edge, not in the ventral epidermis, would result. Overexpression of wild-type Cta and Rho1 using *prd-Gal4* caused an increase in *dpp* levels more apparently at the leading edge in *prd-positive* stripes (**Figure 3.12 B, C**) than *dpp* expression in the ventral epidermis, which remained almost unaffected. In embryos overexpressing wild-type Rho1, a slight up-regulation in *dpp* transcripts in the ventral epidermis was observed (**Figure 3.12 C'**). This might be due to ectopic activation of the JNK cascade in the ventral epidermis. These results suggested that Fog-Rho1 signaling likely regulates *zip* transcription through a JNK-mediated pathway.

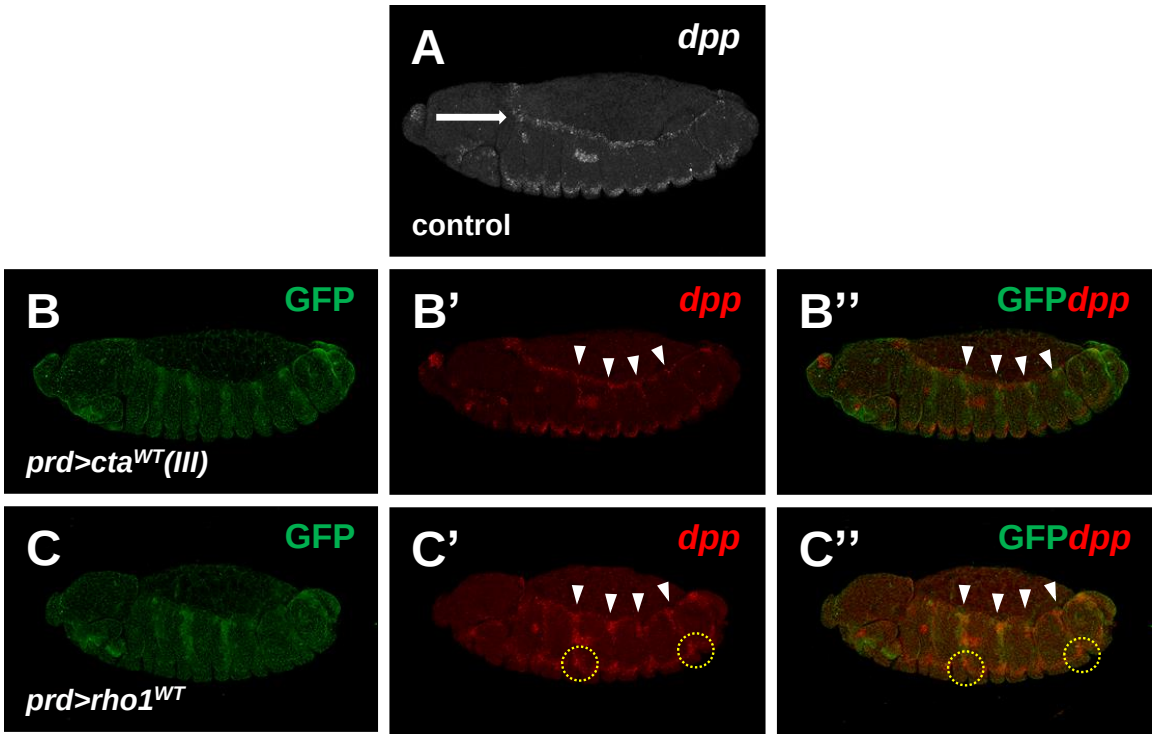

Figure 3.12. Overexpression of Cta and Rho1 up-regulates *dpp* transcription at the leading edge while *dpp* levels in the ventral epidermis remain almost unaffected.

dpp FISH. (**A**) Two regions expressing *dpp* in a control embryo are shown: one at the leading edge and the other in the ventral epidermis. *dpp* expression indicated by a white arrow is the region where its transcription is regulated by the JNK cascade. (**B, C**) GFP staining marks the *prd* stripes that express the UAS transgenes. In embryos expressing either wild-type Cta or Rho1 (**B', C'**), *dpp* expression at the leading edge is elevated in *prd* stripes that are GFP-positive, as seen in a merged image of B and B' (**B''**) or C and C' (**C''**). The elevated *dpp* expressions are indicated by arrowheads. Slightly affected *dpp* expression in the ventral epidermis is indicated by yellow dotted circle.

Chapter 4. Results Part II: A novel mechanism of ecdysone signaling in cooperation with Jun, a component of the AP-1 transcription factor, to regulate gene expression during DC

As described in the Introduction, ecdysone signaling is involved in DC as mutations in members of the Halloween group of genes, which are involved in ecdysone biosynthesis, lead to DC defects (Chavez, Marques et al. 2000; Giesen, Lammel et al. 2003; Kozlova and Thummel 2003; Ono, Rewitz et al. 2006; Niwa, Namiki et al. 2010). In the canonical pathway, a heterodimer consisting of EcR and Usp translocates into the nucleus in the presence of ecdysone. There, EcR/Usp binds to EcRE sequences to promote the expression of downstream target genes (Henrich, Sliter et al. 1990; Cherbas, Lee et al. 1991; Koelle, Talbot et al. 1991; Yao, Segraves et al. 1992; Thomas, Stunnenberg et al. 1993; Yao, Forman et al. 1993; Riddiford, Cherbas et al. 2000; Bonneton, Zelus et al. 2003). Previous work in our lab by Weiping Shen and Xi Chen has uncovered a signaling network involving ecdysone and the TGF-β ligand, Dpp, that mediates communication between the AS and DME cells during DC (unpublished data). Ecdysone, which is produced in the AS, can promote *zip* expression during DC, as demonstrated by observed increases in *zip* transcript levels throughout the AS and epidermis when bathing wild-type embryos in exogenous ecdysone. Conversely, in embryos mutant for *spo* or *dib*, where the production of ecdysone is inhibited, *zip* expression in the DME cells is abolished. It has also been shown that Dpp, which is expressed at the leading edge epidermis during DC, is required for the up-regulation of *spo* transcription in the AS, thus triggering the production of ecdysone, and consequently promoting *zip* expression. These results have provided evidence of a signaling network between the AS and epidermis mediated by diffusible molecules. In this model, Dpp produced in the DME cells prior to and during DC diffuses into the AS, where it promotes the transcription of *spo*. Spo, along with the other Halloween members, then enables the production of ecdysone, which can diffuse to neighbouring AS and DME cells. This activates its receptor, EcR, which in turn regulates expression of the *zip* gene in both the AS cells and possibly the DME cells (summarized in **Figure 4.1**).

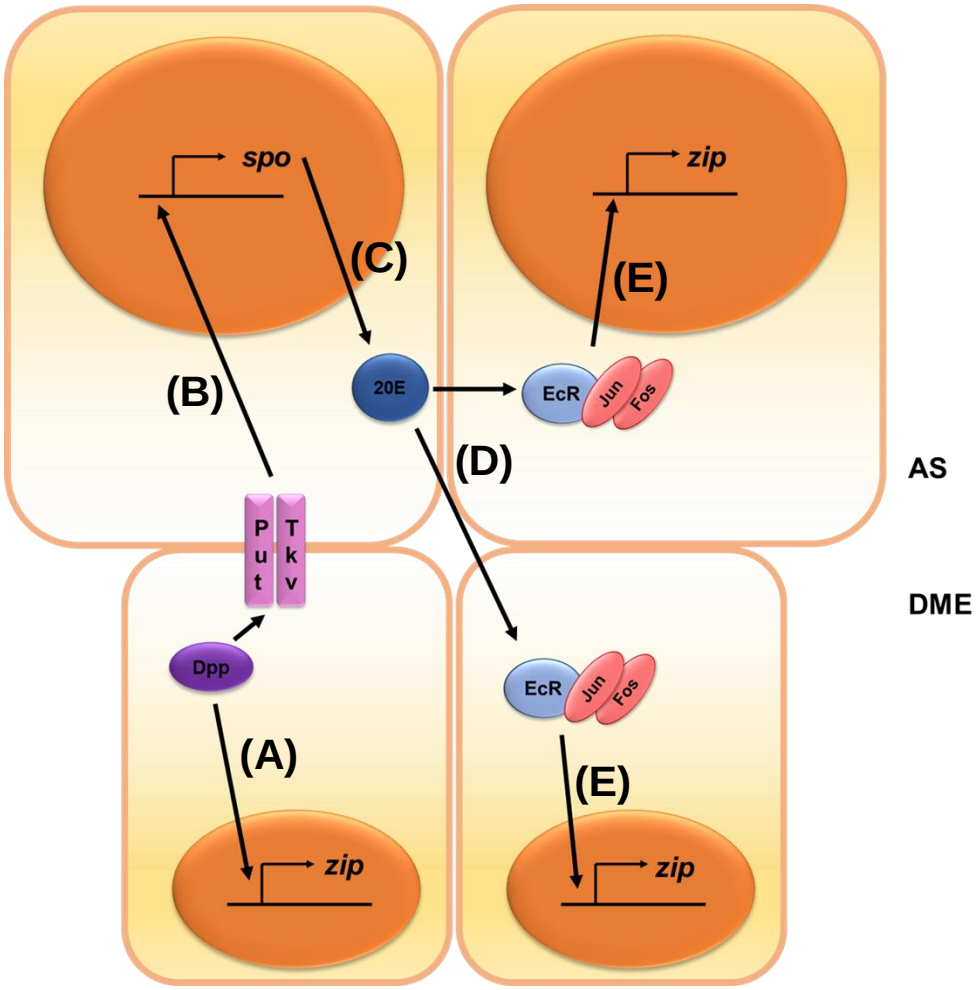

Figure 4.1. A schematic representation of hypothesized bidirectional signaling that mediates *zip* expression in the AS and DME cells during DC.

(**A**) Dpp produced in the dorsal epidermis regulates JNK-mediated *zip* expression along the leading edge. (**B**) It can also diffuse into neighbouring AS cells, where it promotes *spo* transcription. (**C**) Spo positively regulates the synthesis of ecdysone, (**D**) which then spreads to other AS and DME cells activate its receptor EcR. (**E**) *zip* expression in both the AS cells and DME cells is subsequently regulated through EcR complexing with the AP-1 transcription factor component, Jun.

Interestingly in this previous study, a novel mechanism of ecdysone signaling has been uncovered where EcR forms a complex with Jun to control the expression of the *zip* gene during DC (**Figure 4.1**). Xi Chen, a former PhD student in the Harden lab, performed proximity ligation assay (**PLA**) in an attempt to look for protein-protein complexes *in situ* between EcR and Jun. PLA is a technique that can detect interactions between two endogenous proteins that are in close proximity to each other (i.e. within 40nm), and has successfully been used to identify protein-protein complexes in the nuclei of *Drosophila* embryos (Soderberg, Gullberg et al. 2006; Petruk, Sedkov et al. 2012). These PLA experiments demonstrate that complex formation between EcR and Jun, marked as fluorescent puncta, occurs in AS and DME cell nuclei during germband retraction through to DC (**Figure 4.2 A-A''**). If EcR/Jun complexes bind to the *zip* gene in order to promote its expression, then it is predicated that the amount of PLA signal should be reduced in embryos lacking *zip*. Indeed, in embryos homozygous for the deficiency, *Df(2R)BSC608*, which deletes the entire *zip* locus, the number of PLA-positive puncta is significantly reduced by roughly half (**Figure 4.2 B-D**). The AS cells are polyploid (Buchenau, Saumweber et al. 1997), which explains why so many puncta could be localizing to one gene, i.e. *zip*. As PLA signal is still present even in the complete absence of the *zip* locus, this suggests that EcR not only acts in a complex with Jun to bind and potentially regulate *zip*, but also at least one other gene during DC.

The objective of this research project was to further investigate non-canonical ecdysone signaling involved in the regulation of gene expression via the EcR/Jun complex during DC. More specifically, candidate genes, other than *zip*, that are potentially regulated by this complex were identified and characterized. In addition, providing biochemical evidence of EcR and Jun binding was attempted.

4.1. *jar*, *jupiter*, and *zasp52* are expressed similarly to *zip*

In a search for candidate genes that could possibly be bound and regulated by the EcR/Jun complex, an interesting published paper came to our attention. The authors showed that three genes, named *jaguar* (***jar***), *jupiter*, and *Z-band alternatively spliced*

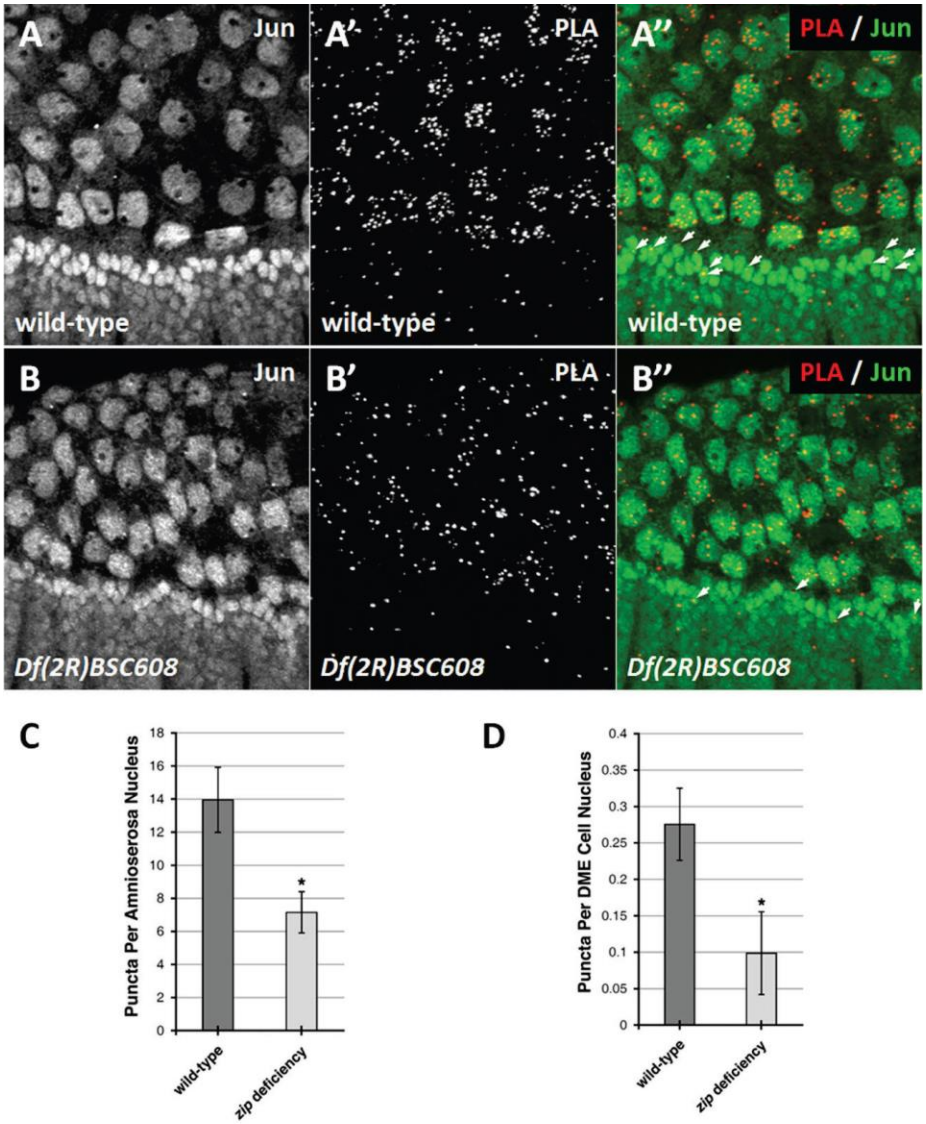

Figure 4.2. The number of PLA-positive complexes both in the AS and DME cells is reduced in embryos deficient for the *zip* locus.

(**A-A''**) Wild-type embryo showing Jun immunostaining in nuclei (**A**), PLA-positive puncta between EcR and Jun in the AS and epidermis (**A'**), and a merged image of the two (**A''**). (**B-B**'') Embryo homozygous for *Df(2R)BSC608*, which is deficient for the *zip* locus, showing Jun immunostaining (**B**), overall reduced PLA-positive puncta compared to the wild-type control (**B'**), and a merged image of the two (**B**''). White arrows mark PLA signal in DME cells. Scale bar: 50μm. (**C**) Quantification analysis of PLA-positive puncta in the AS observed in wild-type and *zip* deficient embryos. Puncta are counted at the beginning of DC when the AS cells are flat and unfolded. 43 nuclei in six wild-type embryos and 46 nuclei in six *zip* deficient embryos are counted. * $p<0.0001$. (**D**) Quantification analysis of PLA-positive puncta in DME cells observed in the wild-type and *zip* deficient embryos. 285 nuclei in six wild-type embryos and 311 nuclei in six *zip* deficient embryos are counted. * $p<0.0002$. These experiments were done by Xi Chen.

PDZ-motif protein 52 (***zasp52***), are expressed in the DME cells in a JNK and Dpp-dependent manner (Ducuing, Keeley et al. 2015). To confirm their results, the expression patterns of each gene were first analyzed via FISH. Strikingly, the distribution of their transcripts was very similar to that of *zip* (**Figure 4.3**). In wild-type embryos, both *zip*, *jar*, *jupiter*, and *zasp52* transcript levels in the DME cells all remained high from mid germband retraction through to DC (**Figure 4.3 B,E,H,K**). Also similar to *zip*, *jar* and *zasp52* transcript levels in the AS began to progressively diminish from mid germband retraction through to DC (**Figure 4.3 A-C,G-L**). Interestingly, *jupiter* transcripts in the AS contrastingly persisted much longer than the others, localizing into unidentified puncta-like structures (compare **Figure 4.3 F** with **Figure 4.3 C,I,L**).

4.2. Ecdysone promotes the expression of *zip* and *jupiter*, but suppresses *zasp52 and jar* transcription

Due to their comparable expression patterns to the *zip* gene, it is highly possible that *zip*, *jar*, *jupiter* and *zasp52* are regulated via similar signaling mechanisms. Thus, work was done to see if these three genes, like *zip*, could also be regulated by ecdysone. To do so, the expression of each gene in embryos mutant for one of the Halloween group of genes, i.e. either for *spo* or *dib*, was evaluated. Consistent with previous data in the lab generated by Xi (Chen, 2014), *zip* expression in the AS during germband retraction was considerably reduced in embryos homozygous mutant for *dib* when compared to *dib* mutant heterozygotes, which served as a control (**Figure 4.4 A,C**). Effects in the AS during DC could not be ascertained as *zip* transcript levels are naturally downregulated in wild-type embryos (**Figure 4.4 B,D**). These results confirmed that ecdysone is necessary for the regulation of *zip*. Next, FISH against *jar*, *jupiter and zasp52* transcripts was performed *dib* mutants to determine whether ecdysone also had an effect on the expression of these genes. Though effects during germband retraction were not obvious, a decrease in *jupiter* transcript levels in the AS during DC was observed, with a possible slight reduction of expression in the DME cells (**Figure 4.5 A-D**). Unlike with *zip*, the persistence of *jupiter* expression in the AS during DC in wild-type embryos made it possible to observe effects of the *dib* mutation at this stage. In contrast, the expression of *zasp52* in *dib* homozygous mutant embryos was increased in the AS (**Figure 4.6 A-D**). In confirmation of all of these

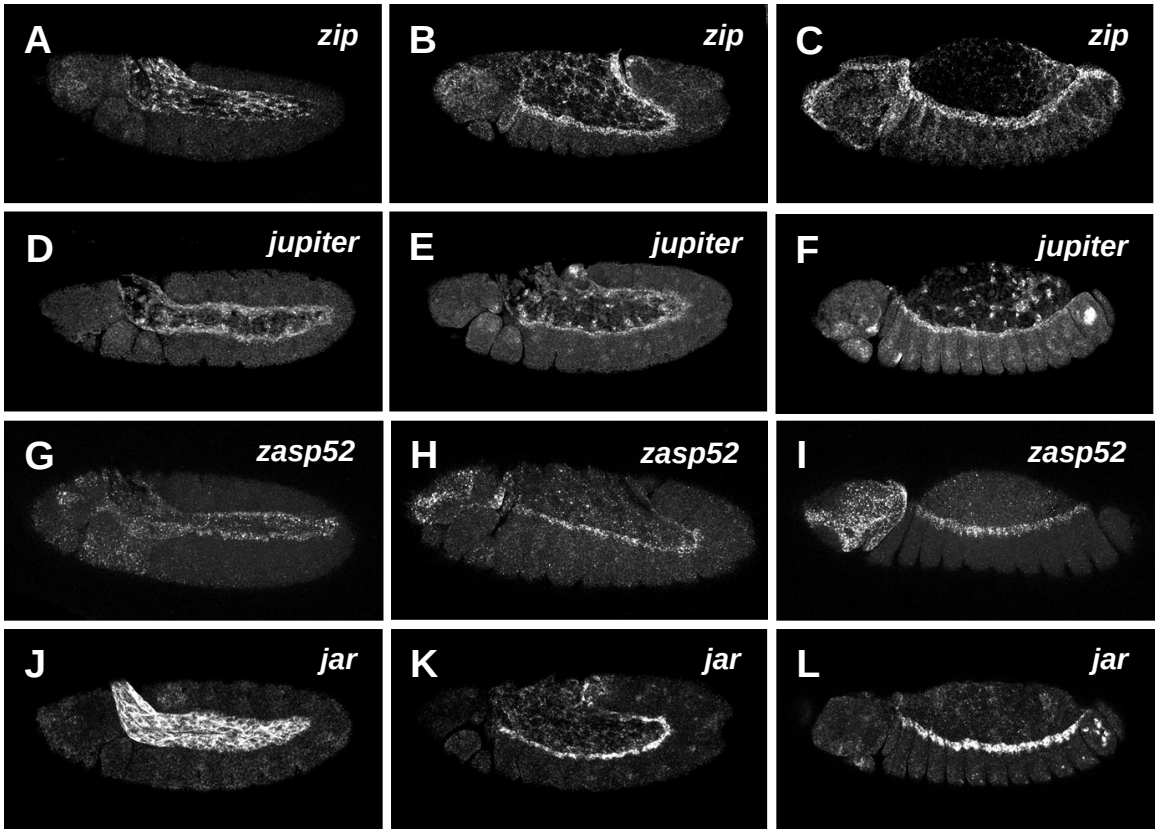

Figure 4.3. Expression of the *zip*, *jupiter*, *zasp52* and *jar* genes during germband retraction and DC.

Embryos are progressively older from left to right, from the onset of germband retraction to mid DC. All four genes showed sustained expression in the DME cells from mid germband retraction (**B,E,H,K**) to DC (**C,F,I,L**), which eventually fades as DC progresses to the completion (data not shown). *zip*, *zasp52* and *jar* transcript levels in the AS begin to progressively diminish from mid germband retraction through to DC (**A-C,G-L**). On the other hand, the expression of *jupiter* in the AS persists longer than the other genes, localizing into puncta-like structures (**D-F**).

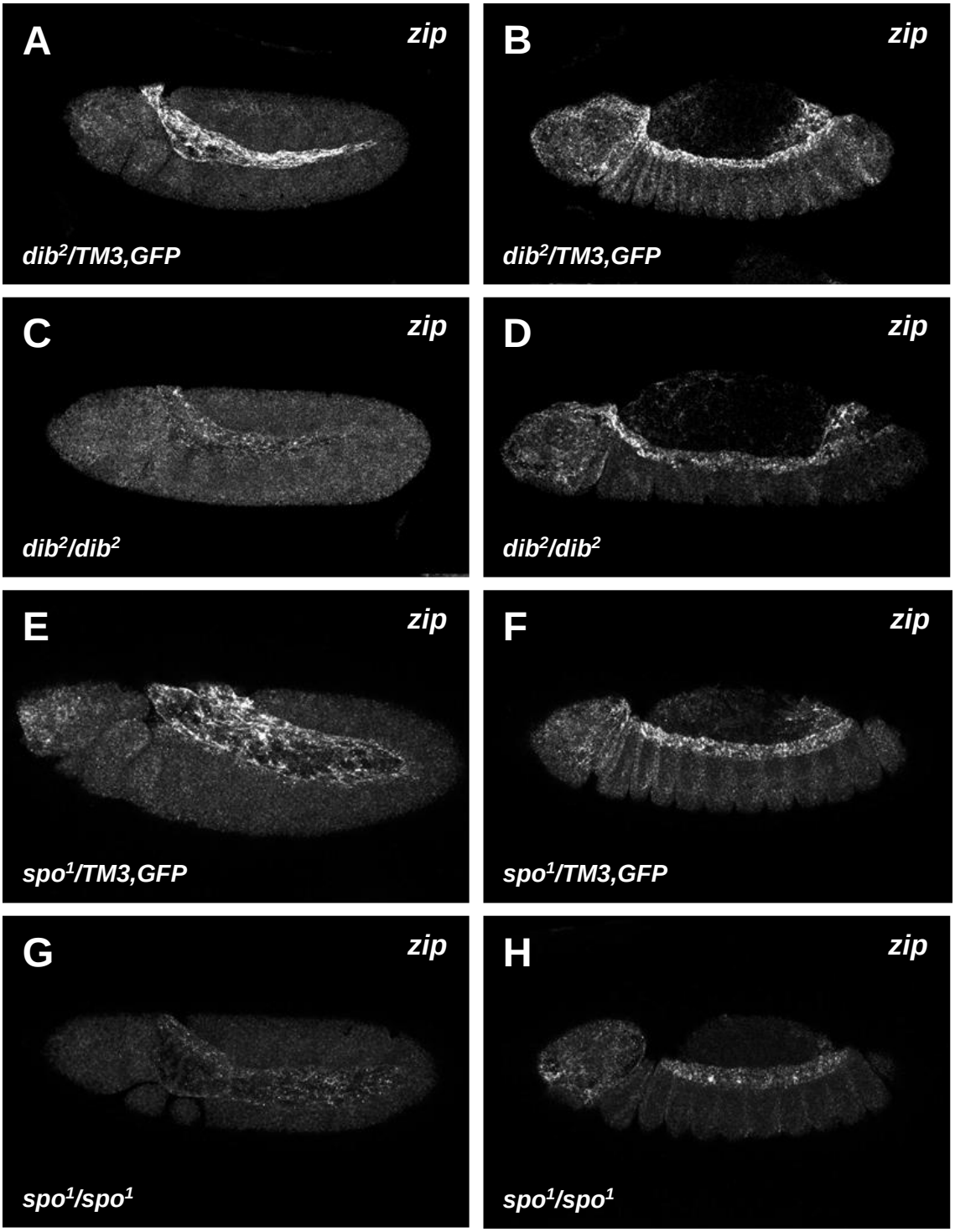

Figure 4.4. ***zip*** **transcription in the AS is lost but retained in the DME cells the genes encoding enzymes in the ecdysone biosynthetic pathway are mutated.**

zip FISH during germband retraction (**A,C,E,G**) and DC (**B,D,F,H**). *zip* levels in the AS are decreased in *dib* homozygous mutant embryos (**C**) compared to those in heterozygous embryos, which serve as controls (**A**). Similar down-regulated *zip* transcription in the AS is also observed in homozygous embryos mutant for *spo* (**G**) compared to their corresponding heterozygotes (**E**). Note robust *zip* expression in DME cells in all genotypes.

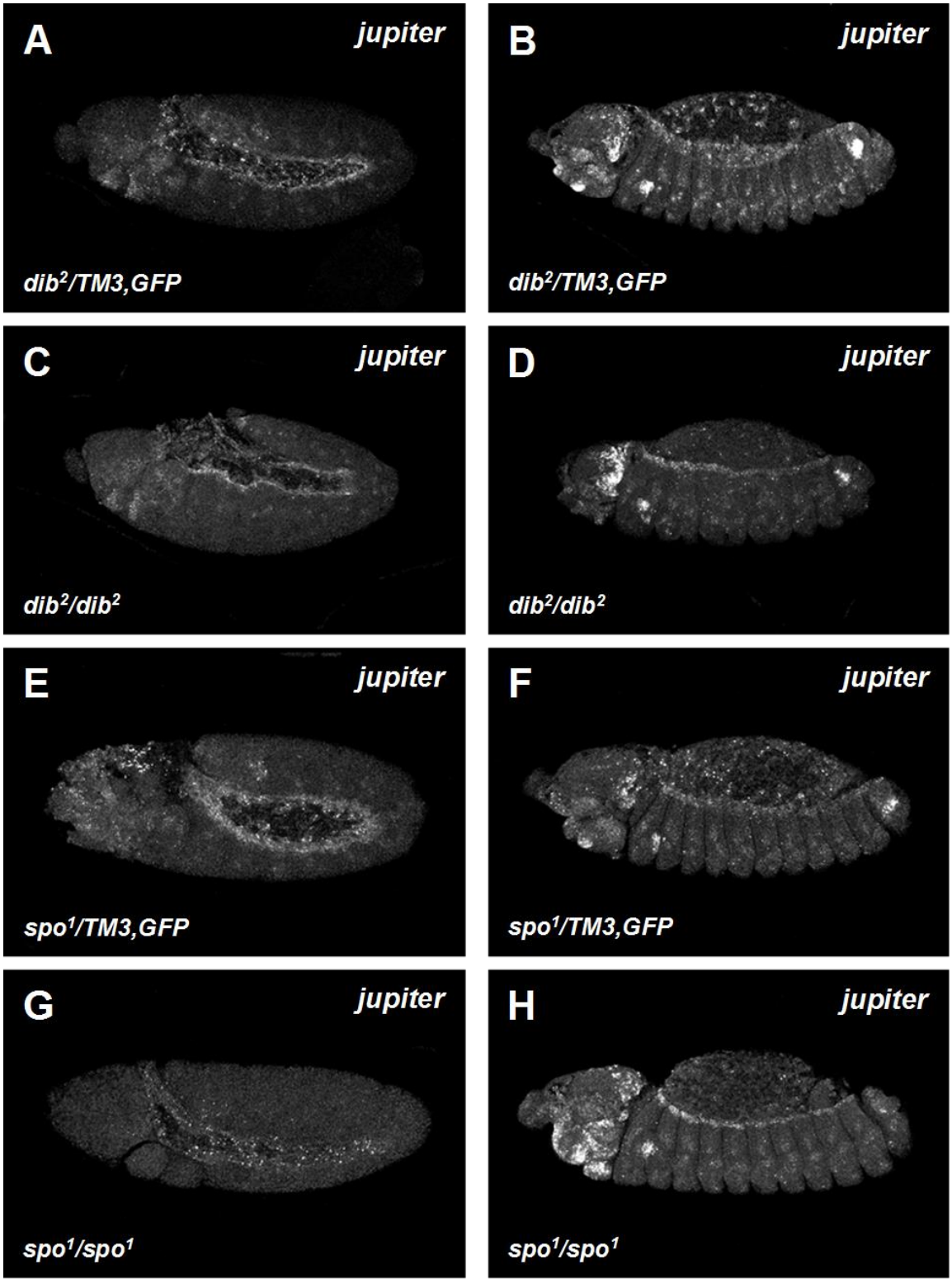

Figure 4.5. ***jupiter*** **transcription in the AS is reduced when the genes encoding enzymes in the ecdysone biosynthetic pathway are mutated.**

jupiter FISH during germband retraction (**A,C,E,G**) and DC (**B,D,F,H**). *jupiter* levels in the AS are decreased in homozygous embryos mutant for *dib* (**C,D**) compared to those in heterozygous embryos, which serve as controls (**A,B**). Similar *jupiter* down-regulation is also observed in homozygous embryos mutant for *spo* (**G,H**) compared to their corresponding heterozygotes (**E,F**).

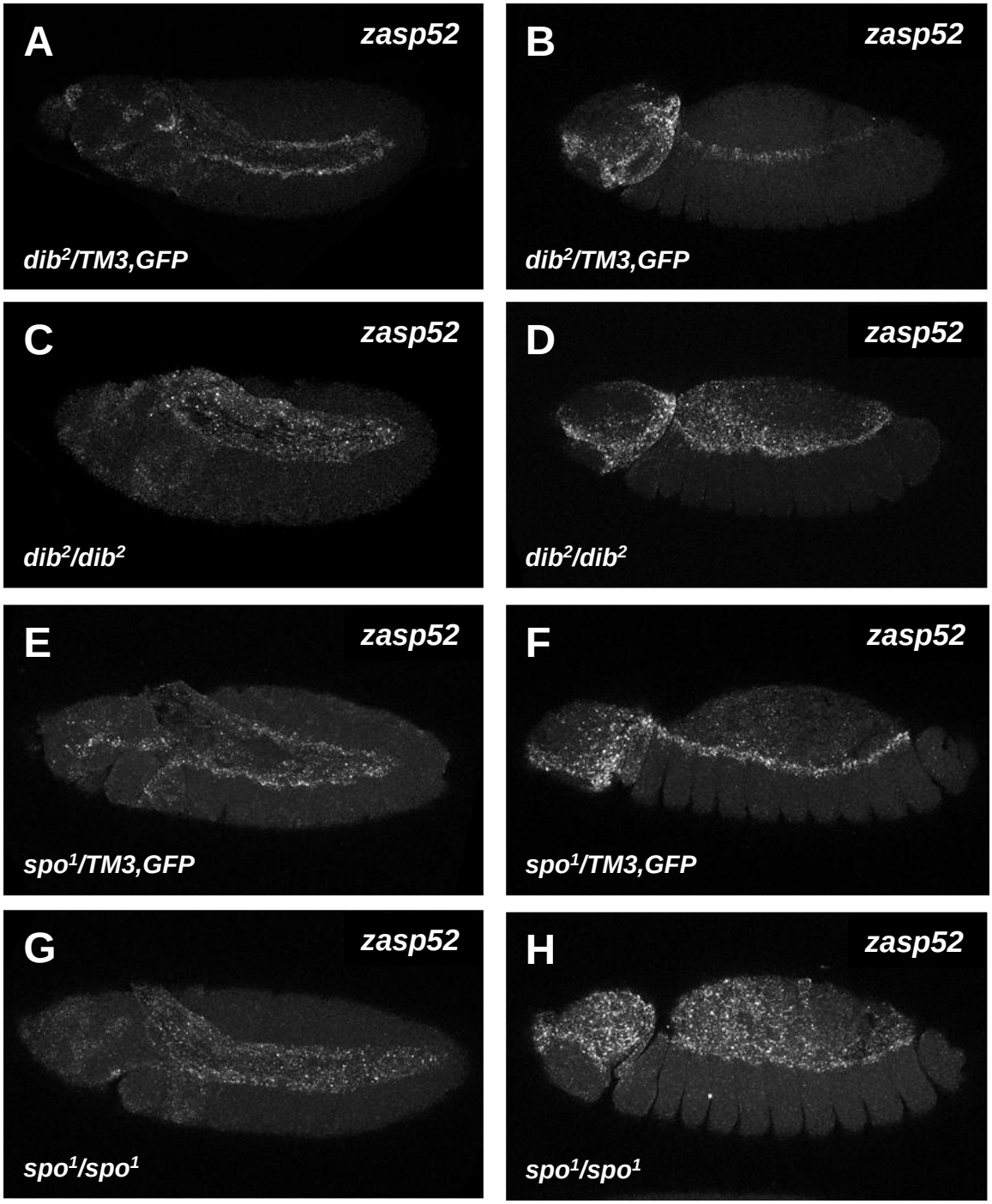

Figure 4.6. ***zasp52*** **expression in the AS is up-regulated when the genes encoding enzymes in the ecdysone biosynthetic pathway are mutated.**

zasp52 FISH during germband retraction (**A,C,E,G**) and DC (**B,D,F,H**). *zasp52* levels in the AS during DC are increased in homozygous embryos mutant for *dib* (**C,D**) compared to those in heterozygous embryos, which serve as controls (**A,B**). Similar *zasp52* up-regulation is also observed in homozygous embryos mutant for *spo* (**G,H**) compared to their corresponding heterozygotes (**E,F**).

results, similar observations were made in *spo* homozygous mutant embryos (**Figures 4.4 E-H, 4.5 E-H, 4.6 E-H**). The transcription of *jar* in the AS appeared to be increased in *spo* mutant homozygotes during DC (**Figure 4.7**), which was similar to the *zasp52*. Note that in all of these experiments, the homozygous and heterozygous mutant siblings were treated identically in the same reaction tube. These results indicate that ecdysone plays a role in the regulation of these genes in the AS during DC. If this steroid hormone is ultimately found to modulate the transcription of these four genes, then ecdysone may have a dual role: it is required to promote *zip* and *jupiter* transcription, but repress *jar* and *zasp52* expression, through unknown mechanisms.

4.3. Preliminary evidence that *zip* and *jupiter* expression is regulated by a putative EcR/Jun transcription complex during DC

As mentioned before, previous experiments indicated the involvement of a novel form of ecdysone signaling, where EcR acts in a complex with Jun, to regulate the transcription of the *zip* gene. As PLA signal between EcR and Jun in *zip* deficient embryos is only reduced by half (Chen, thesis), there should be at least one other gene that can be bound by this putative transcription factor complex. Due to the observed decreases in both *zip* and *jupiter* expression in the AS when ecdysone signaling is downregulated, this other gene may likely be *jupiter*. Addressing this hypothesis was done by performing PLA between EcR and Jun in embryos homozygous for deficiencies that delete the entire *jupiter* locus. If some of the PLA signal observed in the AS nuclei of wild-type embryos is attributed to EcR/Jun complexes binding to the *jupiter* gene, then the amount of PLA signal should be reduced in embryos lacking the *jupiter* locus. Although the experiments need to be repeated and quantified, preliminary data showed that the number of PLA puncta was reduced in embryos homozygous for the *jupiter* deficiency, *Df(3R)Exel6276*, compared to wild-type embryos (**Figure 4.8**). Future experiments will involve performing similar PLA experiments in embryos deficient for both *zip* and *jupiter* to see if the PLA signal observed in wild-type AS tissue can be completely eliminated.

To biochemically confirm the presence of the EcR/Jun complex shown via PLA, co-immunoprecipitations were also performed using lysates prepared from whole, wild-

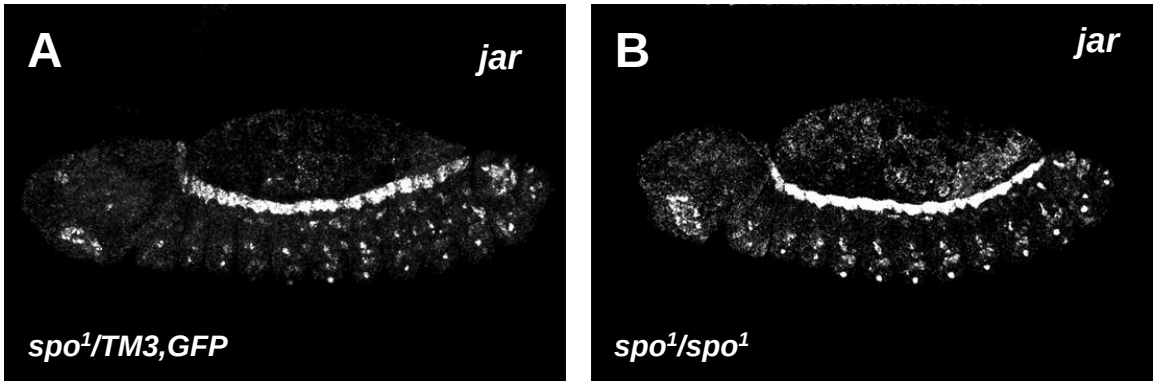

Figure 4.7. *jar* expression in the AS is increased when the *spo* gene encoding an enzyme in the ecdysone biosynthetic pathway is mutated.

jar FISH during DC (**A-B**). *jar* levels in the AS appear to be up-regulated in homozygous embryos mutant for *spo* (**B**) in comparison to *jar* expression in heterozygous control embryos (**A**).

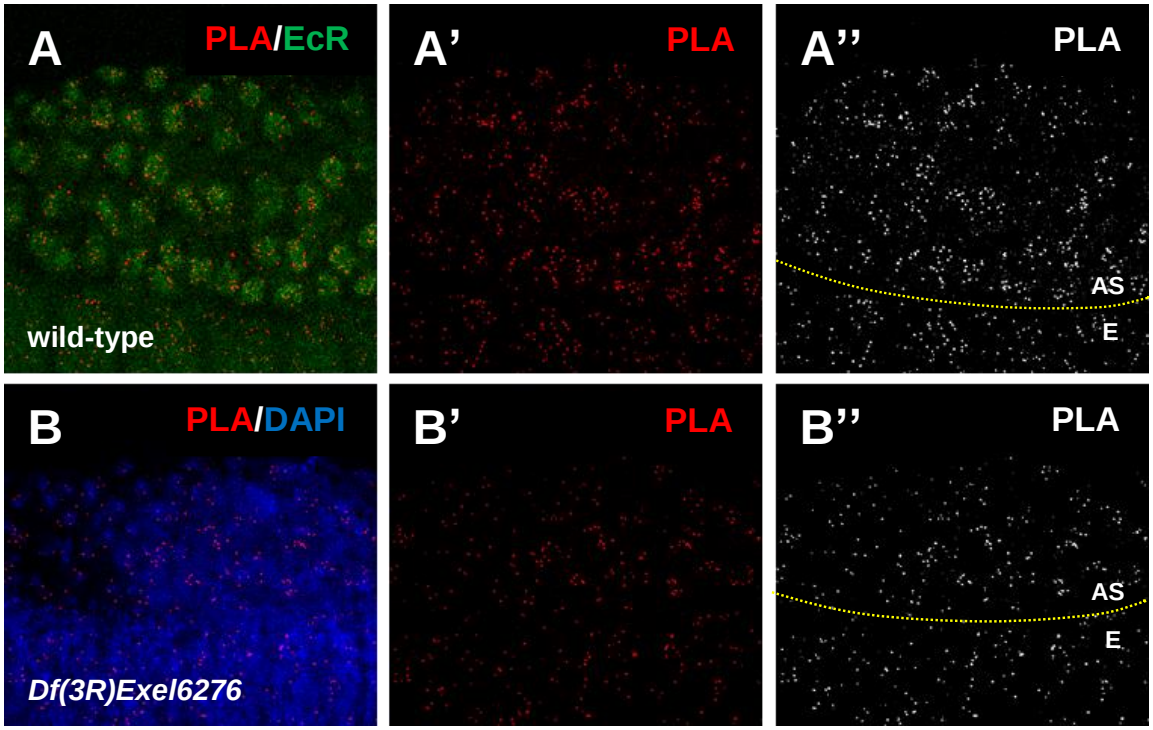

Figure 4.8. The number of PLA-positive puncta in AS nuclei appears to be reduced in embryos deficient for *jupiter* locus.

(**A-A''**) Wild-type embryo showing EcR immunostaining (**A**) and PLA signals in the AS and epidermis (**A',A''**). Homozygous *jupiter* deficient embryo, *Df(3R)Exel6276* (**B-B''**), showing DAPI staining (**B**) and PLA signals in the AS and epidermis (**B',B''**). Note that the yellow dotted line marks the boundary between the AS and Epidermis. AS: amnioserosa. E: epidermis. Due to poor nuclear staining by DAP, result could not be quantified.

type embryos staged 8 to 16 hours after egg laying. Reciprocal co-immunoprecipitations were accomplished with the use of two commercially available antibodies against EcR and Jun. Despite repeated attempts, neither EcR nor Jun was observed to immunoprecipitate the other protein (**Figure 4.9**). One possible issue is that only a fraction of the EcR and Jun proteins may actually be in a complex with each other, which might be below the detection limit for this assay – note that PLA involves amplification of the signal. This problem is exacerbated by the fact that the lysate is also made up of embryos that are not undergoing DC, i.e. either too young or too old, in addition to the presence of other "contaminating" embryonic tissues other than the AS.

4.4. Evidence that the EcR/Jun complex drives gene expression in a JNK- and EcRE-independent manner

The PLA data shown in previous sections have helped identify the presence of an EcR/Jun complex that is potentially involved in the regulation of target genes, such as *zip* and *jupiter*, during DC. Due to the involvement of Jun, an AP-1 transcription factor component in this complex, it was next determined whether the function of this complex relies on the JNK pathway. To investigate this question, *zip* and *jupiter* expression in the AS was assessed in embryos with inhibited JNK signaling. Surprisingly, in embryos overexpressing the dominant negative form of Bsk, i.e. *UAS-bsk*^{DN}, using the AS-specific *c381-Gal4* driver, no effects on the transcriptional levels of either *zip* or *jupiter* (**Figure 4.10**). This suggests that the EcR/Jun complex regulates gene expression independently of the JNK cascade.

To determine whether canonical ecdysone signaling is required for the function of the EcR/Jun complex, *zip* and *jupiter* FISH were performed in embryos overexpressing the dominant negative version of EcR, i.e. *UAS-EcR*^{W650A}, in the AS via *c381xGal4*. EcR^{W650A} is believed to block endogenous EcR by competitively binding to Usp and thereby repressing transcription of genes next to EcRE sequences (Cherbas, Hu et al. 2003). Suppression of the canonical ecdysone pathway affected neither *zip* nor *jupiter* transcript levels in the AS during DC (**Figure 4.11**). This suggests that ecdysone does not promote the expression of these genes through EcRE sequences, but through another mechanism.

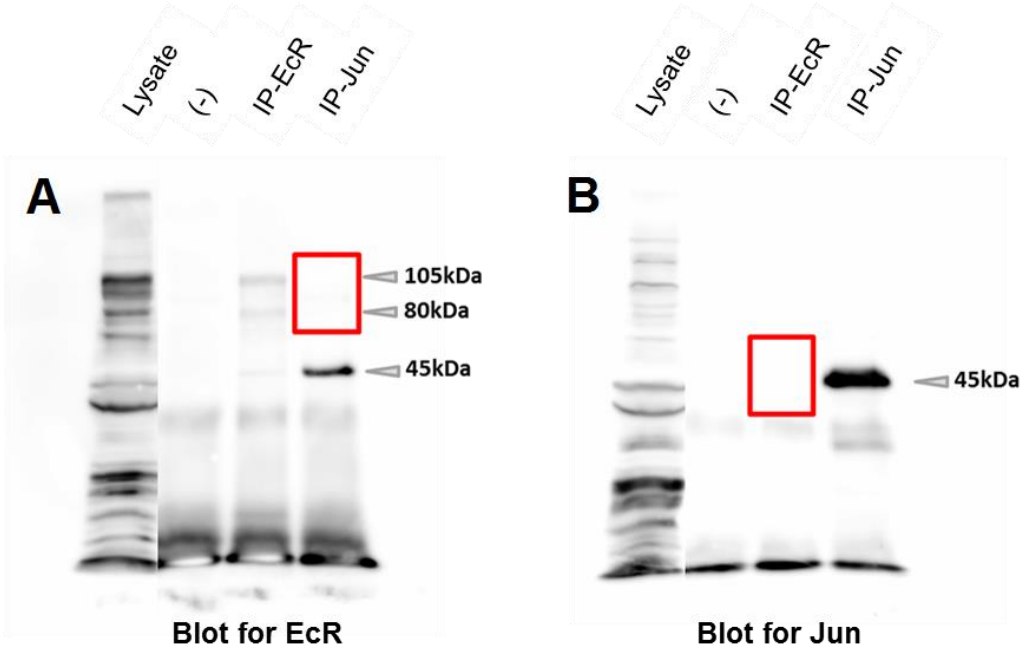

Figure 4.9. Neither EcR nor Jun is observed to immunoprecipitate the other protein in reciprocal immunoprecipitations.

From left to right, the loading scheme in each membrane is as follows: Lysate, Negative control (Lysate without any antibody), Lysate with mouse-anti-EcR (IP-EcR), and Lysate with rabbit-anti-Jun (IP-Jun). (**A**) The membrane is blotted for EcR. In lane 4, an antibody against Jun is used to pulldown itself and its potential interacting protein EcR; However, no bands corresponding EcR isoforms, 105kDa and 80kDa, are observed, indicated by a red box. (**B**) The membrane is blotted for Jun. In lane 3, an antibody against EcR is used to pulldown itself and its potential interacting molecule Jun, but no band corresponding Jun, 45kDa, is observed, indicated by a red box.

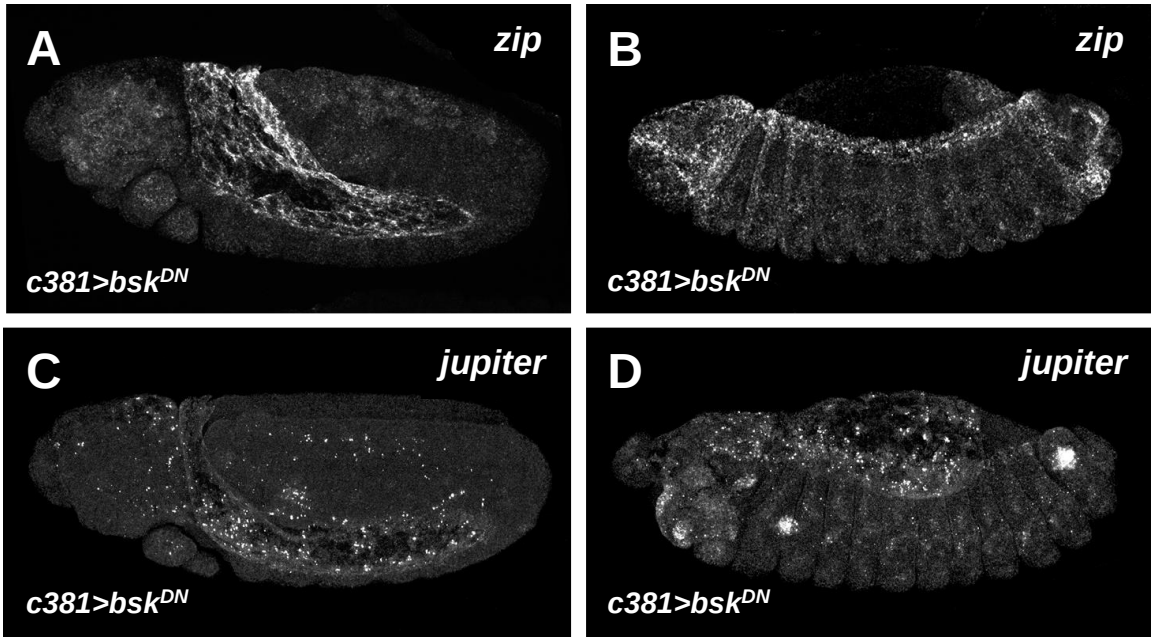

Figure 4.10. The dominant negative version of Bsk does not affect the transcriptions of *zip* and *jupiter*.

zip FISH (**A,B**) and *jupiter* FISH (**C,D**) in embryos overexpressing the dominant negative Bsk, *UAS-bsk^{DN}*, using *c381-Gal4* during germband retraction (**A,C**) and DC (**B,D**).

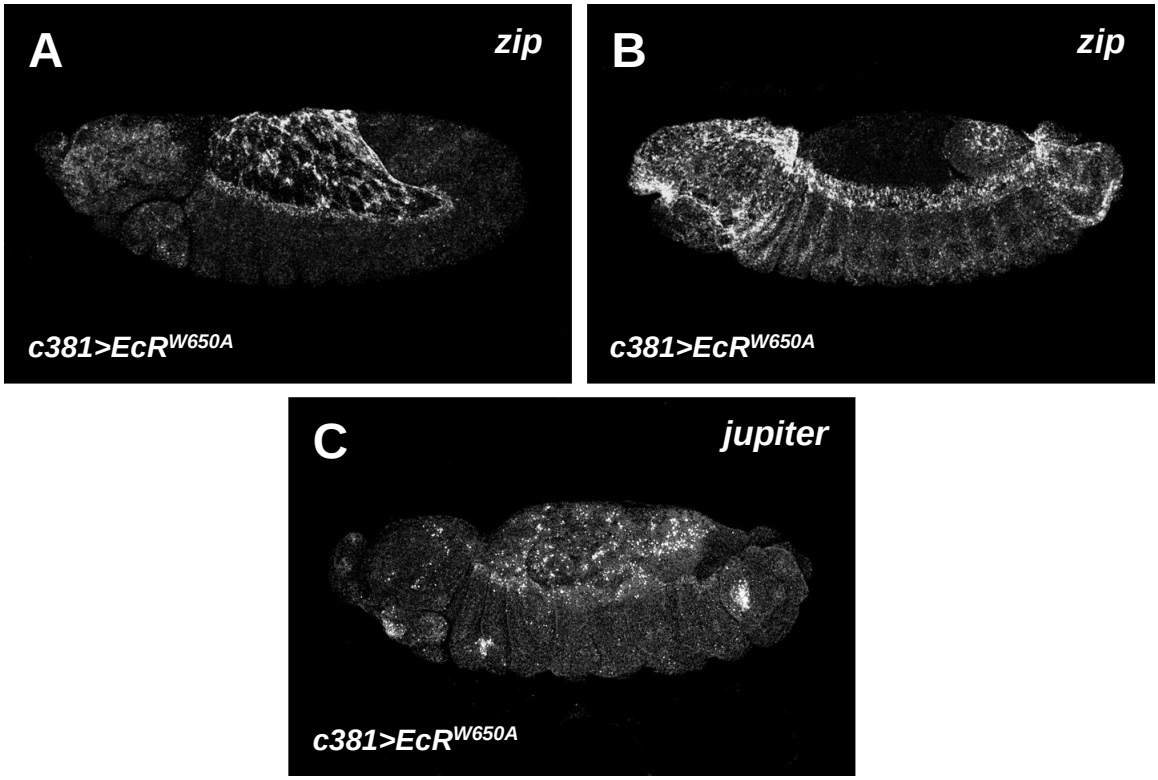

Figure 4.11. The dominant negative version of EcR does not affect the transcription of *zip* and *jupiter* in the AS.

zip FISH (**A,B**) and *jupiter* FISH (**C,D**) in embryos overexpressing the dominant negative EcR, *UAS-ecr^{DN}*, using the AS-specific *c381-Gal4* driver during germband retraction (**A**) and DC (**B,C**).

Chapter 5. Discussions

5.1. The diffusible Fog ligand and its known downstream pathway, Rho1 signaling, are potential regulators of the JNK-mediated *zip* expression in the epidermis during DC

The JNK signaling pathway has long been identified as a central regulator of DC, in which its components are up-regulated in the DME cells, which interface with the AS, prior to the commencement of DC. Previous studies on both *Drosophila* DC and vertebrate wound healing have identified numerous upstream activators and downstream effectors of the pathway (e.g. (Zhang, Zhang et al. 2010; Belacortu and Paricio 2011; Rios-Barrera, Gutierrez-Perez et al. 2015)). Despite the fact that JNK function has extensively been investigated, when the JNK cascade is turned on is not known, and the identities of a single signal or signals that trigger the cascade have remained a mystery. Several inputs, instead of only one trigger, may be integrated to regulate the JNK pathway. Growth factors, polarity cues, mechanical stress and many other triggers have been identified to trigger JNK signaling (Kushida, Kabuyama et al. 2001; Ramet, Lanot et al. 2002; Igaki, Pagliarini et al. 2006).

In a search for new regulators of DC, the diffusible ligand Fog was considered, as it has been well-known to activate the Rho1 actomyosin contractility pathway during gastrulation (Costa, Wilson et al. 1994; Barrett, Leptin et al. 1997; Dawes-Hoang, Parmar et al. 2005). Coordinated actomyosin contractility, as a vital component for proper cell morphogenesis in *Drosophila* and other eukaryotes, is promoted by the Rho1 signaling pathway through activation of the ROK kinase. Activated ROK subsequently phosphorylates myosin regulatory light chain and, at the same time, inhibits myosin light chain phosphatase, thereby leading to myosin activation (Settleman 2001). As evidenced in several previous studies, the Rho1 actomyosin contractility pathway is involved in DC by driving cell shape changes both in the AS and the epidermis (e.g. (Harden, Ricos et al. 1999; Jacinto, Woolner et al. 2002; Mizuno, Tsutsui et al. 2002; Tan, Stronach et al. 2003)). Here I addressed whether the Fog ligand can also act as a regulator of myosin-encoding *zip* expression through activating the JNK pathway.

I started by looking at the transcriptional levels of Fog before and during DC using FISH analysis. Strikingly, Fog expression was found at high levels in the AS and the DME cells, where a DC-triggering ligand would be predicted to be expressed. Given the enrichment of Fog in these two regions, further investigation was carried out by looking at the expression of the key DC gene, *zip*. As the *zip* gene is a critical component of DC for myosin regulation, it was worthy to study the effect of Fog on *zip* expression. In embryos overexpressing Fog using various drivers, *zip* levels were elevated compared to wild-type controls, and loss of Fog had the opposite effect on *zip* transcription. Having established the involvement of Fog in the regulation of *zip*, the effects of the downstream components of Fog, including Cta, Rho1 and RhoGEF2, on *zip* transcription were also investigated either by overexpressing each component or knocking it down by RNAi. Gain of Cta and Rho1 clearly resulted in the up-regulation of *zip*; However, despite repeated experiments, no obvious effects on *zip* levels were observed in embryos in which each component was knocked down by RNAi transgenes. These negative data may be due to the result of weak knockdown of target transcripts by the RNAi transgenes. When mutations for Fog pathway components were studied instead, only RhoGEF2 mutant embryos showed a decrease in *zip* levels.

Recent studies have identified that Fog acts via the GPCRs Mist and/or Smog during gastrulation (Manning, Peters et al. 2013; Kerridge, Munjal et al. 2016). To assess if the GPCRs also affect *zip* levels, *zip* FISHs were performed in embryos in which Mist was either knocked down in the epidermis or overexpressed, but no transcriptional effect was observed. When similar experiments were done to study the role of Smog, embryos expressing excessive endogenous Smog were found to exhibit an overall increase in *zip* levels; Nevertheless, loss of Smog did not affect *zip* expression (Table B1).

Interestingly, when *dpp* expression patterns were looked at in embryos overexpressing Cta or Rho1, *dpp* expression, which is JNK-dependent was up-regulated. This is an important indication that the regulation of *zip* by Fog and its downstream Rho1 pathway may be JNK-dependent. With various results above demonstrating that the Fog and Rho1 actomyosin contractility pathway has a similar effect on *zip* expression as the JNK pathway, the diffusible ligand Fog and Rho1 signaling may be operating upstream of the JNK signaling to regulate *zip*-encoded myosin levels during DC. Further thorough

studies on the relationship between these signaling pathways are yet needed to confirm my hypothesis.

Moreover, the effects of the two known receptors for Fog, Mist and Smog, on *zip* transcription remain largely elusive. A key experiment is to analyze *zip* transcription levels in embryos with both GCPRs knocked down. If Fog signaling is ultimately determined to regulate *zip* transcription, then follow-up genetic interaction studies can be done to determine if it crosstalks with other signaling pathways as well. Given the mild effects on zip expression seen in my experiments to date, Fog is unlikely to be a major regulator of the JNK pathway, but may represent a minor regulatory input.

5.2. Ecdysone, in cooperation with the AP-1 transcription factor component, Jun, regulates gene transcription during DC

Four biomechanical forces described in the Introduction contribute to native closure of the dorsal hole: 1) an actomyosin-rich pulse string surrounding the hole; 2) zippering events at the canthi, coordinated with filopodia/lamellipodia; 3) pulsed contraction of the actomyosin cytoskeleton in the AS, coupled with apoptosis of the cells within the tissue; 4) elastic forces in the lateral epidermis opposing DC, accounting for ventral-ward relaxation of the epidermis. Even though each force generated in the AS and the epidermis is required for native DC, it does not necessarily mean that all the forces must be present. Certain combinations of forces and tissues are essential for DC to complete. However, numerous laser ablation and genetic experiments have demonstrated that DC does not always fail when any key forces and/or tissues are perturbed (e.g.(Hutson, Tokutake et al. 2003; Peralta, Toyama et al. 2007; Toyama, Peralta et al. 2008; Wells, Zou et al. 2014; Ducuing and Vincent 2016; Duque and Gorfinkiel 2016; Pasakarnis, Frei et al. 2016). Likewise, when one tissue is either physically or genetically compromised, the other functional tissue has shown to up-regulate the force to overcome the perturbations (Kiehart, Galbraith et al. 2000; Reed, Wilk et al. 2001; Fernandez, Arias et al. 2007; Zahedi, Shen et al. 2008). Therefore, communication between the AS and epidermal cells likely plays a critical role in DC.

The purpose of my research project was to further elaborate on the signaling network that mediates coordinated morphogenesis of the AS and epidermis during DC, and our lab has made a considerable progress in elucidating such a complex signaling network. Previous data has shown that prior to the onset of DC, Dpp produced in the leading edge epidermis diffuses into the neighboring AS cells, where the transcription of the *spo* gene is promoted. As a consequence of *spo* expression, the steroid hormone ecdysone is produced in the AS. Ecdysone then signals to the AS, and possibly the DME cells, where it activates *zip* expression. What is exciting is the finding that ecdysone might act in a novel way that is independent of EcREs. Instead of the use of canonical ecdysone signaling, EcR may interact with Jun, a component of the AP-1 transcription factor, when in the presence of ecdysone to modulate the transcription of the *zip* gene. A genome wide study of EcR binding demonstrated a binding site at the *zip* locus in a region with no EcREs (Gauhar, Sun et al. 2009). Interestingly, Xi Chen used bioinformatic analysis to identify AP-1 binding sites in this region instead, which might be bound by the EcR/Jun complex as revealed by PLA. This may be the first example of non-canonical ecdysone hormone signaling in the regulation of gene transcription in *Drosophila*. However, whether the other component of the AP-1 transcription factor, Fos, is involved in this EcR/Jun complex remains unknown. Due to the lack of a good antibody against Fos in our hands, PLA between EcR and Fos could not be performed. Instead, other method can be used to address this issue in the future. For example, the effects of loss of Fos on *zip* levels by overexpressing a RNAi transgene against Fos using the AS-specific *c381-Gal4* driver may provide information about the non-canonical EcR complex. If *zip* expression is found to be affected by the FosRNAi transgene, that would suggest that the regulation of *zip* gene is mediated by EcR forming a complex with both Jun and Fos. If *zip* transcript levels remain the same, then EcR/Jun complex may operate independently of Fos.

Previous PLA experiments between EcR and Jun in embryos deficient for *zip* have indicated that there is at least one other gene bound and regulated by this EcR/Jun complex. While looking for other candidate genes, I have demonstrated through FISH analyses that ecdysone signaling regulates the expression of three genes in the AS during DC in addition to *zip*: *jar*, *jupiter,* and *zasp52*. However, non-canonical ecdysone-mediated expression appears to have opposing effects, i.e. ecdysone is required to enhance *zip* and *jupiter* expression, but repress *zasp52* and *jar* expression (through an unknown

mechanism). To investigate whether *jupiter* expression was regulated by the EcR/Jun complex similarly to *zip*, a PLA experiment between EcR and Jun was performed on *jupiter* deficient embryos. Although PLAs need to be repeated, my preliminary PLA data suggested that the transcription of *jupiter* may be also modulated by the non-canonical form of ecdysone. If EcR/Jun complexes detected via PLA were ultimately confirmed to be reduced in *jupiter* deficient embryos, more direct evidence showing EcR/Jun complex binding at the *jupiter* locus, as well as at the *zip* locus, needs to be accomplished in the future. First, are AS PLA signals completely abolished in embryos bearing deficiencies for both *zip* and *jupiter*? Second, can direct binding of EcR/Jun to these genes by demonstrated by chromatin immunoprecipitation? It might also be worthwhile doing PLA experiments between EcR and Jun on salivary gland chromosomes, to see if complexes bind to cytological locations of *zip* and *jupiter*.

The "negative result" observed in the immunoprecipitation in **Figure 4.2** may be due to a lack of sensitivity in detecting the endogenous complexes. Of note, signal from PLA is enhanced via an amplification process. To address this issue, co-immunoprecipitations between overexpressed transgenes may be attempted. Additionally, the experiments could be repeated on embryos genetically made to have more AS cells (Stronach and Perrimon 2001).

This newly discovered finding of non-canonical steroid hormone signaling in *Drosophila*, in which EcR forms a complex with Jun and acts independently of EcREs, has been described for steroid hormones in other animals as well. The estrogen receptor, a steroid nuclear receptor similar to EcR, has been reported to promote gene transcription at AP-1 sites in a complex with Jun/Fos along with other proteins. The estrogen receptor has demonstrated to be linked to AP-1 via p160 proteins and CBP/p300 (Kushner, Agard et al. 2000), and the formation of EcR/AP-1 complex in *Drosophila* could be mediated in a similar fashion. In support of this idea, the *Drosophila* homolog of p160 proteins called Tai has been determined to bind EcR directly (Bai, Uehara et al. 2000), further supporting the parallels present between mammalian and *Drosophila* non-canonical steroid hormone signaling.

5.3. Complex signaling networks mediate communication between the AS and DME cells

For cells or tissues to communicate with each other, paracrine signaling via secreted molecules can be used. My study focused on the diffusible signals present during DC: 1) Fog, which potentially acts as a weak upstream activator of the JNK pathway in the epidermis; 2) Dpp, which regulates production of the steroid hormone ecdysone in the AS; 3) ecdysone, which interacts with the transcription factor AP-1 to regulate gene transcription in the AS. Signaling via these molecules ultimately regulates myosin contractility expressed from the *zip* locus necessary for morphogenesis of both tissues during DC (Summarized in **Figure 5.1**). I suspect that this signaling network must tightly controlled in order to achieve smooth cell shape changes and epithelial migration. In a journey to build up a big picture of the signaling networks regulating this morphogenetic event, studying the relationships between Dpp, Fog and ecdysone will need to be further investigated using molecular methods in the future.

Numerous ablation and genetic studies that were previously done in our lab and many others have determined that *Drosophila* DC is a collective system that is robust. As seen in my data, tissue-specific genetic perturbations of any one of the key processes that promote closure, i.e. Fog-Rho1 pathway in the epidermis or ecdysone signaling in the AS, does not result in a complete loss of *zip*, suggesting multiple inputs into its expression which may enable DC to proceed under adverse conditions. Similarly, even when the forces from either one of the two tissues are genetically disrupted, the completion of DC is not always compromised. For example, closure still completes when functional myosin is either removed from the purse string or its activity is blocked (Franke, Montague et al. 2005; Duque and Gorfinkiel 2016; Pasakarnis, Frei et al. 2016). Likewise, several laser perturbation experiments designed either to dissect each tissue or to prevent zipping at both canthi have shown that such experiments initially result in qualitative morphologic changes in the dorsal hole with delayed closure rate, but eventually, DC resumes at near native rates (Peralta, Toyama et al. 2007; Rodriguez-Diaz, Toyama et al. 2008; Wells, Zou et al. 2014). These observations indicate that DC is indeed a robust system that continues to work regardless of physical or genetic perturbations by adapting to changes. How do cells during DC adapt to challenges? One property of the system can explain this:

redundancy. At the cellular level, when zippering events are compromised or function of the actomyosin cable is eliminated, up-regulation of the forces generated by the AS cells maintains DC to proceed (Hutson, Tokutake et al. 2003; Peralta, Toyama et al. 2007). How an embryo “knows” that one or another force for DC is perturbed remains still unknown. Nevertheless, crosstalk between the tissues is certainly one of the important molecular mechanisms that potentially mediate the up-regulation in the force produced by one tissue when the other is compromised.

Striking parallels exist between *Drosophila* DC and developmental epithelial closure and wound healing in other systems (Martin and Parkhurst 2004). Similar to DC, the involvement of the diffusible signals and complex signaling between tissues has been identified and described in vertebrate systems. For example, in vertebrate wound healing, a crosstalk link between keratinocytes and fibroblasts occupying the wound promotes gene regulation necessary for the healing process (Werner, Krieg et al. 2007). This crosstalk involves the TGF-β signaling from keratinocytes to fibroblasts, which subsequently triggers differentiation of fibroblasts into contractile myofibroblasts. Meanwhile, growth factors are secreted from fibroblasts back to keratinocytes, where they promote proliferation and differentiation of keratinocytes. Even though numerous genes have been identified that play important roles in vertebrate developmental epithelial migration and fusion events, as well as vertebrate wound healing, the complex signaling networks mediating these processes are poorly understood. Studies on signaling networks present in DC, on the other hand, have shown great progress. Therefore, the pieces of the signaling network I have discovered, along with extensive work done by others, can be used as a guideline to aid in the study of the vertebrate morphogenetic processes.

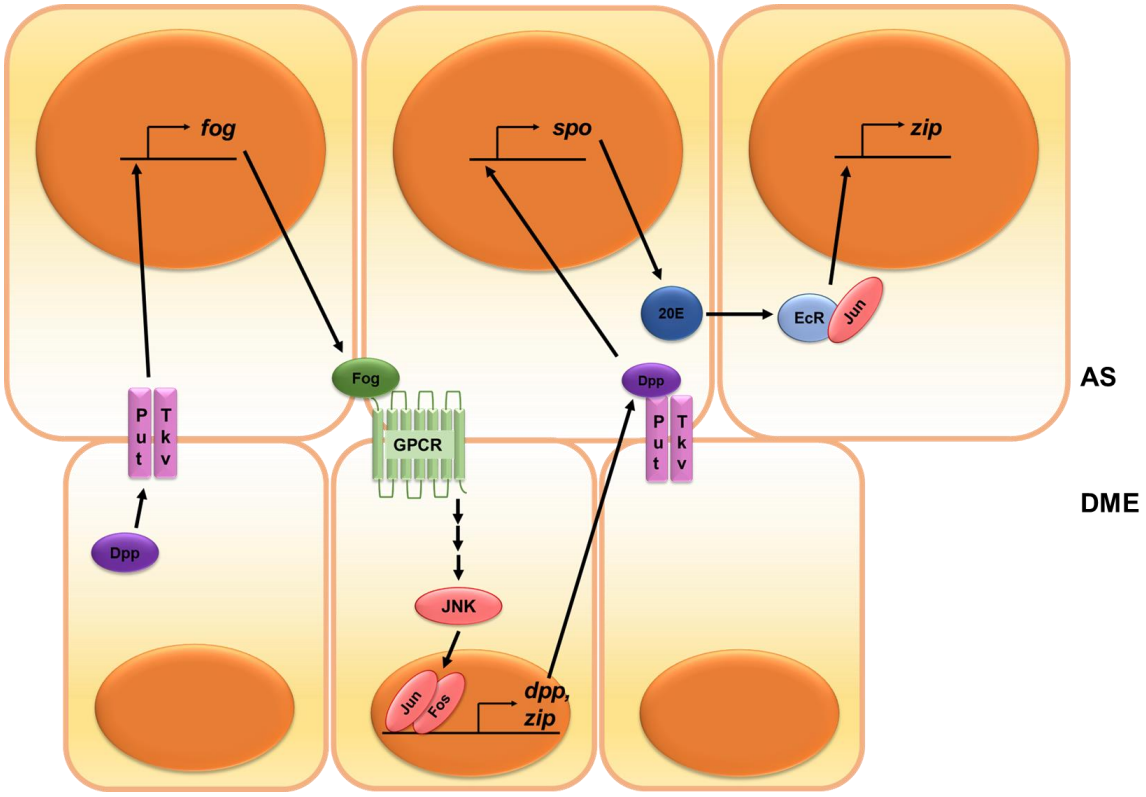

Figure 5.1. Simplified schematic representation of hypothesized signaling networks that mediate communication between the AS and the DME cells during DC.

The diffusible ligand Dpp, through its receptors Put and Tkv, regulates the transcription of *fog* in the AS (see Appendix A). Fog subsequently binds to GPCR, up-regulating the Rho1 pathway, which acts as a minor regulator of the JNK cascade. JNK-mediated Dpp turns on *zip* expression in the DME cells, and in the meantime, it is secreted into the neighboring AS cells to regulate the transcription of *spo*. The steroid hormone ecdysone is then produced in the AS. In the presence of ecdysone, EcR forms a complex with Jun to regulate *zip* (and possibly *jupiter*) expression in the AS.

Chapter 6. Conclusion

In this thesis, I have identified a new player that may act upstream of the JNK cascade: Fog. The diffusible Fog ligand, through activation of the Rho1 pathway, may promote actomyosin contractility during DC by regulating the *zip* and *dpp* genes. As to what exactly triggers the JNK signaling has for a long time remained a mystery, if Fog is ultimately found to be one signal that triggers the JNK cascade, we have stepped a little closer to better understanding of the process.

Communication between the AS and the epidermis has been identified, which modulates the coordinated morphogenesis of tissues during DC. Central to this communication is the involvement of the diffusible molecules, such as Dpp, Fog and ecdysone. Of interest, the non-canonical form of ecdysone signaling, in which EcR forms a complex with AP-1 to regulate the transcriptions of *zip* and possibly *jupiter* as well, has been evidenced. This is the first example of crosstalk link between the steroid hormone signaling and the AP-1 transcription factor in *Drosophila*. These experimental observations have revealed further parallels between *Drosophila* DC and vertebrate wound healing, and therefore, our recent findings may be of particular interest for vertebrate researchers to look for similar signaling networks mediating the extent of actomyosin contractility.

More complicated feedback loops by multiple signaling pathways have been evidenced, and further investigations need to be done in the future. Although there are still many unknowns to be discovered, further studies on DC certainly promise to provide researchers with richer knowledge not only about general epithelial fusion and migration events but also about developmental defects such as spina bifida.

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Appendix A.

Future studies

A preliminary search for new crosstalk links between signaling pathways

As discussed in previous sections, Dpp activates a signaling pathway in the AS that is critical for morphogenesis, and evidence has indicated that it likely plays a role in the communication between the AS and epidermis, which is essential for achieving coordinated morphogenesis of the tissues during DC. To further elaborate this communication, I considered crosstalk links between Dpp and other signaling networks known to be active in the AS and/or DME cells during DC, with focus on diffusible signals, such as Fog and ecdysone. Interestingly, two proteins that are known ecdysone-responsive genes can be turned on by JNK signaling in the epidermis during DC: IMP-E1 and IMP-L1 (Jasper, Benes et al. 2001). Furthermore, the Halloween group of genes have reported to regulate the expression of both genes in the embryonic epidermis during DC (Chavez, Marques et al. 2000).

Evidence of the crosstalk link between Dpp and ecdysone signaling pathways

To study possible Dpp-dependent effects on IMP-E1 and IMP-L1, FISH against *imp-e1* and *imp-l1* transcripts in embryos homozygous mutant for the Dpp receptor, *tkv*, were performed. In comparison to the heterozygous mutant siblings, overall *imp-e1* levels were consistently decreased in the epidermis (**Figure A1**). On the other hand, multiple effects on *imp-l1* expression were observed, which ranged from significant reductions to robust elevations in *imp-l1* levels in the epidermis (**Figure A2**). Interestingly, embryos exhibiting a more normal morphology showed reductions in *imp-l1* transcript levels, whereas embryos with more severe morphological defects showed increases in *imp-l1* levels. Why such a diverse range of effects on *imp-l1* expression was observed in *tkv* mutants is unclear. Unfortunately, further insights gained by transgenic expression of a constitutively active version of *tkv*, i.e. *UAS-tkv*^{CA}, during DC could not be assessed as there were no observed effects on *imp-e1* and *imp-l1* transcript levels (data not shown).

As mentioned above, IMP-E1 and IMP-L1 are expressed in the epidermis via the JNK pathway. However, they are not expressed in the AS (see figure panels **A1 A** and **A2 A**), thus indicating the presence of an unknown mechanism that actively suppresses their transcription. Dpp signaling involves complex formation between Mad and Med, which translocates into the nucleus to regulate the transcription of target genes (Affolter, Marty et al. 2001). In humans, it has been reported that Smad4, the homologue to *Drosophila* Med, has additional roles in estrogen signaling. Multiple target genes of estrogen signaling are repressed by Smad4 when in a complex with the estrogen receptor. In addition, evidence of a direct interaction between the two proteins has been confirmed by coimmunoprecipitation and ChiP assays (Wu et al., 2003). These results demonstrate cross-talk between Dpp and estrogen signaling pathway components that can act as corepressors of gene regulation, and this complex may be conserved during DC. In order to show such an interaction, PLA was performed between EcR and Med. The presence of PLA-positive complexes in the AS (**Figure A3**) provides evidence that the EcR/Med complex also is present in *Drosophila*, and may be the mechanism involved in the down-regulation of *imp-e1* and *imp-l1* expression in the AS during DC. If this were found to be true, further parallels between *Drosophila* and vertebrate processes would be uncovered, thus making further investigations on this hypothesis worth trying.

Evidence of the crosstalk link between Dpp and Fog

In a search for possible interactions between the two diffusible proteins present in DC, Dpp and Fog, the effects of Dpp on Fog were evaluated by performing *fog* FISH in embryos homozygous mutant for *tkv*. The level of *fog* transcription in the AS was largely reduced compared to the heterozygous control (**Figure A4**). This experimental observation suggested that Dpp, through its receptor Tkv, promotes the transcription of the *fog* gene in the AS. The *fog* transcripts in the epidermis, on the other hand, appeared to be increased in the homozygous *tkv* mutants (through an unknown mechanism). How these transcript levels of *fog* in the epidermis are regulated remained largely elusive.

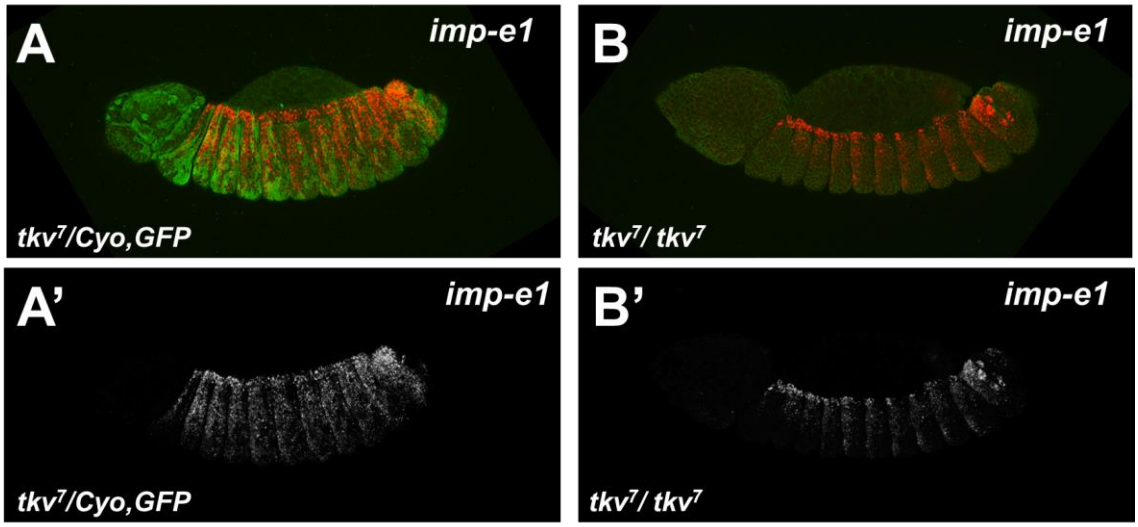

Figure A1. Overall *imp-e1* levels are reduced in homozygous *tkv* mutant embryos compared to controls.

imp-e1 FISHs in embryos heterozygous for *tkv*, which serves as a control (**A,A'**), and in corresponding homozygous embryos (**B,B'**) show that the transcription of *imp-e1* is dependent on Dpp in the epidermis during DC.

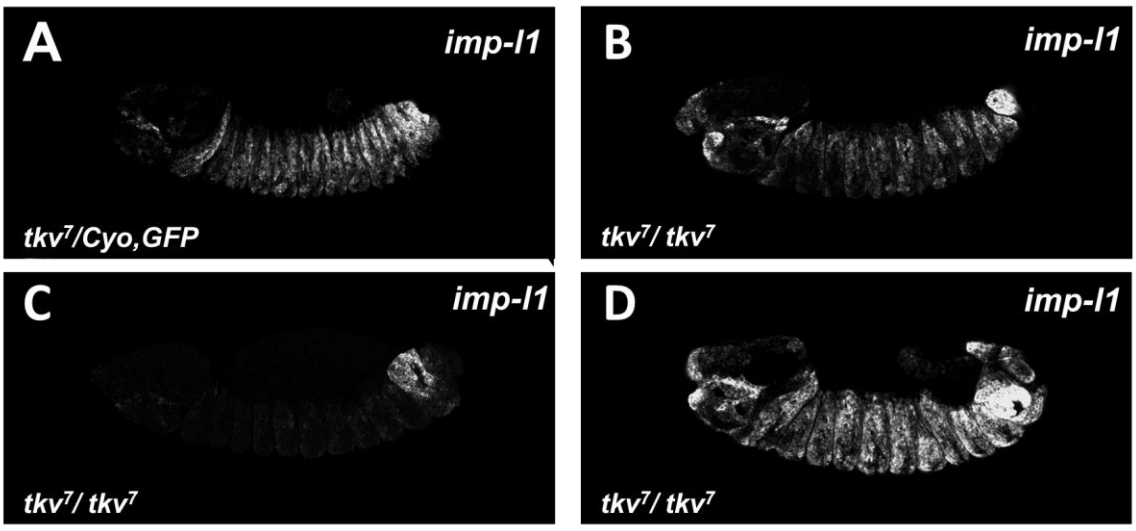

Figure A2. A diverse range of effects on *imp-l1* levels are observed in homozygous *tkv* mutant embryos compared to controls.

imp-l1 FISHs in embryos heterozygous for *tkv*, which serves as a control (**A**) and in corresponding homozygous siblings (**B-D**) demonstrate multiple effects of Dpp on *imp-l1* expression, ranged from significant reductions to robust elevations in the epidermis during DC.

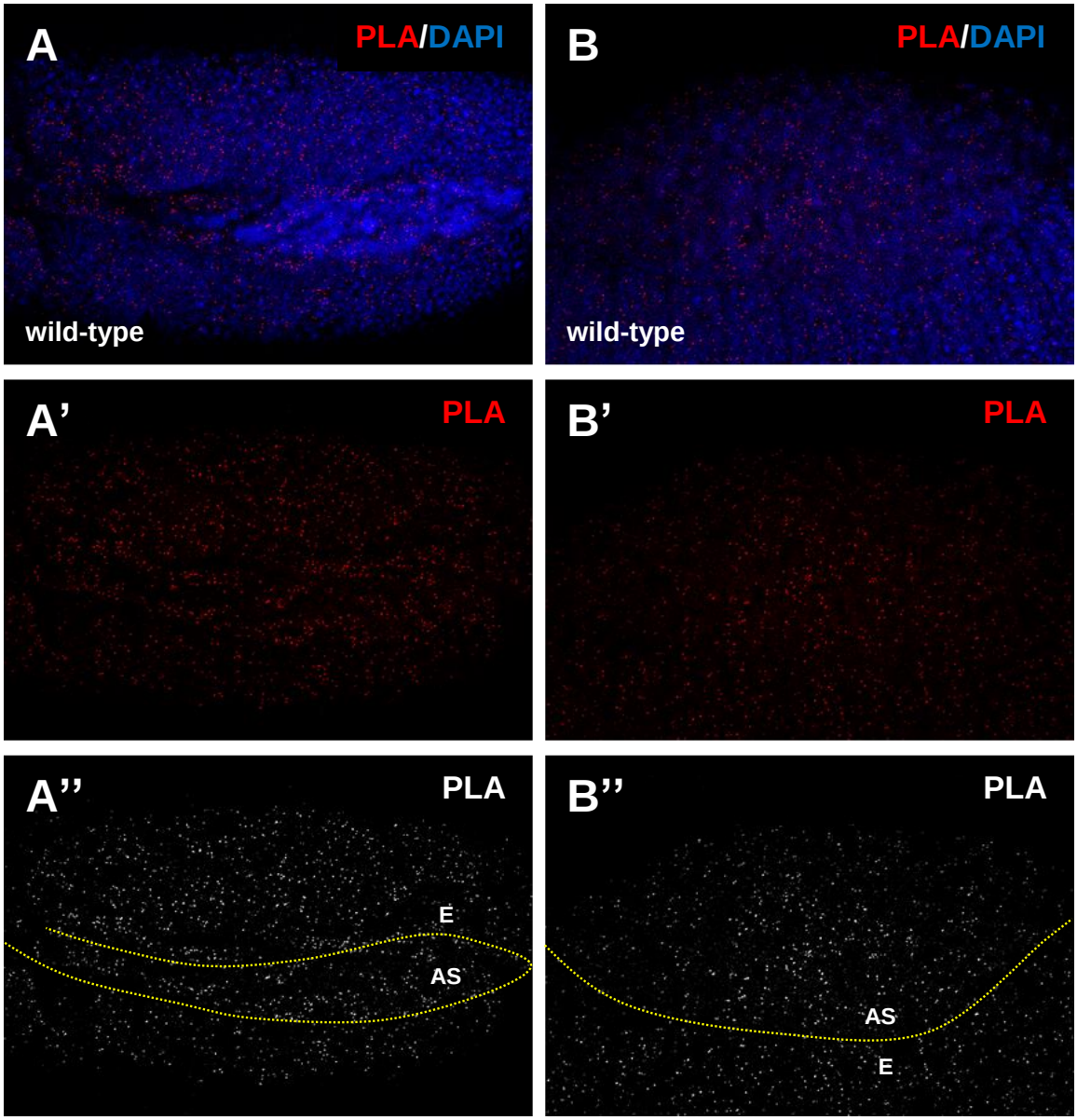

Figure A3. Preliminary PLA data demonstrates evidence for the presence of EcR-Med complex, possibly involved in the regulating of genes.

Wild-type embryo showing DAPI staining (**A,B**) and PLA signals in the AS and epidermis (**A-B''**), during germband retraction (**A,A'A''**) and DC (**B,B',B''**). AS: amnioserosa. E: epidermis.

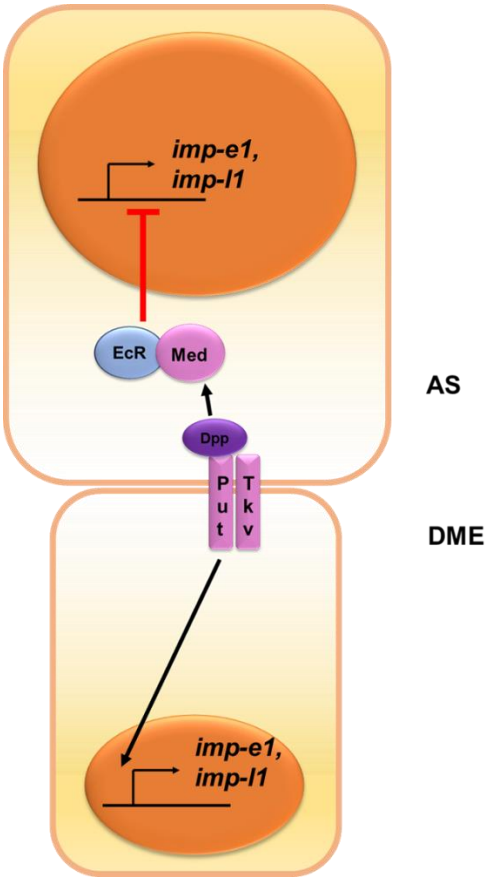

Figure A4. Simplified illustration of the hypothesis that crosstalk between Dpp pathway and ecdysone signaling may repress *imp-e1* and *imp-l1* transcriptions in the AS during DC.

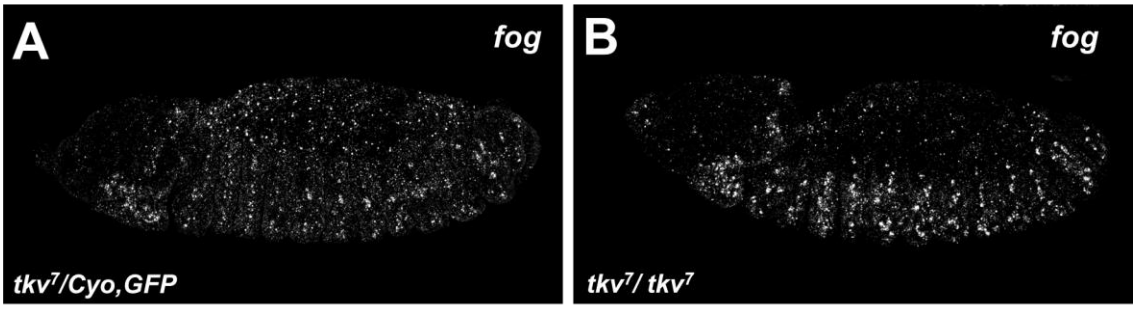

Figure A5. *fog* levels are decreased in the AS when the receptor Tkv is perturbed, while those in the epidermis appear to be up-regulated.

fog FISH in embryos homozygous for *tkv* (**B**) and in corresponding heterozygous siblings (**A**) suggests that Dpp is involved in the regulation of the transcription of *fog* in the AS through its receptor Tkv.

Appendix B.

Table B1. *UAS* transgenes and mutant stocks that caused no effect on *zip* expression when investigated to study the role of Rho1 signaling in the regulation of *zip* levels.

Gene	Stock	Stock Number
mist	UAS-mist	B: 21192
	UAS-mist[RNAi]	V: 33135
		V: 33136
	Mthl1/FM7,GFP	B: 51320
smog	UAS-smog[RNAi]	V: 110641
	Smog[-]/Cyo,GFP	Lecuit Lab
cta	UAS-cta[RNAi]	B: 31132
		B: 41964
		B: 51848
RhoGEF2	UAS-RhoGEF2[RNAi]	V: 110577
Rho1	UAS-Rho1[N19]	B: 7328
	UAS-Rho1[RNAi]	V: 12734
ROK	UAS-Rok[CAT]	B: 6667
		B: 6668
		B: 6669
	UAS-Rok[RNAi]	V: 104675
	UAS-Rok[RBD]-GFP	B: 52290
LIMK1	UAS-LIMK1	B: 9116
	UAS-LIMK1[RNAi]	V: 25343
		V: 25344

V: Vienna RNAi stocks; B: Bloomington stocks

Figure B1. Probe Synthesis.