GENETIC INTERACTIONS PATTERNING THE TRIBOLIUM FATE MAP

by

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B.S., Sichuan University, China, 2004
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Abstract

A segmented body plan is conserved in vertebrates and arthropods. Comparative studies implicate a conserved mode of A-P axis patterning and segmentation among vertebrates: Wnt signaling is involved in fate map patterning along the A-P axis and in segmentation in the posterior region of the embryo. On the other hand, comparative studies in arthropods have revealed distinct modes of development between long and short germ insects, which differ both morphologically and genetically. In the short germ insect Tribolium, a Wnt activity gradient contributes to A-P axis patterning and generates a posterior *Tc-cad* expression gradient that regulates segmentation through a pair-rule gene clock, forming segments sequentially as in vertebrates. In contrast, instead of Wnt activity, a hierarchy of regulatory genes regionalizes the blastoderm and defines segments simultaneously in the long germ insect Drosophila.

In Tribolium, *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* play key roles in patterning serosa, head and trunk regions, respectively, of the blastoderm fate map, which are impacted by changes in Wnt activity levels. However, interactions between these genetic factors have not been described. My work revealed that cross talk between the Wnt and Dpp signaling pathways regulates the expression of *Tc-zen-1* to determine the serosa. Furthermore, mutually repression between *Tc-otd-1* and *Tc-cad* defines the head and trunk regions while mutual repression between *Tc-zen-1* and cad determines the posterior extent of the dorsal serosa.

Analysis of target genes of the posterior *Tc-cad* gradient indicates that the *Tc-cad* gradient regulates the serial expression of gap genes, which are predominately regulators of Hox genes. Thus the posterior *Tc-cad* gradient determines segment formation through regulation of pair-rule genes in the insect segmentation clock, and defines segmental identity through regulation of gap genes. Given its effect on *Tc-zen-1* and *Tc-cad*, the Wnt activity gradient is a key organizer of the Tribolium blastoderm fate map.

Since homologs of these genes as well as the Wnt signaling pathway have also been identified in several other short germ band insects, and affect cell fates along the A-P body axis, this work provides a new paradigm for the short germ mode of development to guide studies in other arthropods.

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Major Professor
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Dedication

To my beloved grandfather and grandmother, you are in my heart, always.

To my beloved parents, brother and his family, thanks for all your support and understanding.

Love you and Miss you.

Chapter 1 - Introduction

Tribolium castaneum, the red flour beetle*,* is a short germ insect in which only the head and thorax are formed in the blastoderm, and the abdomen is formed later during the germ band elongation stage. In Tribolium, segments form sequentially and two extra-embryonic tissues, serosa and amnion form on both anterior and dorsal sides of blastoderm. This is quite different from long-germ insects, such as *Drosophila melanogaster*, in which all segments are formed simultaneously in the blastoderm (Driever and Nusslein-Volhard, 1988). The amnioserosa is localized on the dorsal-most side of the Drosophila egg. Therefore, the blastoderm fate maps are different in Tribolium and Drosophila. However, research on Tribolium embryonic development benefits from Drosophila studies. The homologs of many genes involved in Drosophila embryogenesis also regulate embryonic development in Tribolium, although the functions of these homologs are not always completely conserved. Furthermore, comparative studies between Drosophila and Tribolium, as well as other insects, provide insight into understanding embryogenesis in other insects and the evolution of the genetic regulation of embryonic development from short-germ to long-germ insects.

Embryogenesis in Drosophila and Tribolium

In Tribolium, after the first several rounds (approximately 8-10 cycles) of nuclear cleavage deep inside the yolk (Figure 1.1 a'), nuclei migrate to the cortex (Figure 1b'). Cell membranes start invaginating between nuclei, and, during interphase of the 13th nuclear cleavage, completely enclose the nuclei forming a uniform, undifferentiated blastoderm (Figure 1.1 b') (Benton et al., 2013). Until then, the blastoderm is at the syncytial stage. The 13th nuclear cleavage, also the first round of cell division, occurs asymmetrically in the blastoderm. Only cells in the prospective embryonic region divide and become more tightly packed (Figure 1.1 c'). In the prospective serosa, DNA replication occurs in the absence of division to form loosely scattered polyploid nuclei. Hence at this stage, the blastoderm can be regionalized by cell types and is known as the differentiated blastoderm. Several studies have shown no gross cell

movement in the blastoderm stage (Figure 1.1d', Figure 1.2 B) (Benton et al., 2013; El-Sherif et al., 2012; Handel et al., 2000).

Before the differentiated blastoderm stage, the prospective serosa and embryonic tissue, as well as amnion that separates them, are already genetically patterned. We considered a multi-step patterning process that occurs in the blastoderm before morphologic differences are observed. First is the patterning of extra embryonic serosa (Figure 1.1 A). Second is the patterning of the second extra embryonic tissue, the amnion, adjacent to the embryo and composed of a similar cell type (Figure 1.1 B) (Handel et al., 2000). Third is the distinction of the pre-gnathal head from the rest of the embryo that includes the gnathal region, thorax and posterior growth zone (Figure 1.1 C). These patterning steps that regionalize the blastoderm and produce the fate map are not strictly ordered and overlap temporally.

Similar early embryonic development is observed in Drosophila. The first nine rounds of nuclear cleavage take place in the middle of the yolk. Then, nuclei move to the surface of the embryo to form the blastoderm. Nuclear cleavage rounds 10-13 continue in the syncytial blastoderm. After round 13, cellularization occurs as cell membranes encompass the nuclei. Meanwhile, the blastoderm fate map is also determined. However, the Drosophila blastoderm fate map defines the entire body plan as well as the single extra embryonic tissue, amnioserosa (Campos-Ortega, 1985) (Figure 1.2 A).

Genetic factors in Drosophila blastoderm fate map

In Drosophila, a higher dipteran, the A-P and D-V axes are patterned by two largely independent gene networks, in which morphogen gradients are critical to the establishment of the body axis(Klingler, 1990; Winslow et al., 1988). A Dm-Bicoid (Dm-Bcd) protein gradient emanates from the anterior pole (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988). *Dm-Hunchback (Dm-hb)* and other anterior gap genes sense various Dm-Bcd concentrations and are activated along this gradient (Tautz, 1988). Meanwhile, Dm-Hb protein also forms an anterior morphogen gradient, which cooperates with Dm-Bcd to inhibit *Dm-caudal (Dm-cad)* and *Dm-nanos (Dm-nos)* expression anteriorly (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). In

response, Dm-Nos and Dm-Cad proteins form gradients emanating from the posterior end of the embryo to activate gap genes and repress *Dm-bcd* and *Dm-hb* expression posteriorly (Hulskamp et al., 1989; Irish et al., 1989; Schulz and Tautz, 1995). Inputs from these four protein gradients determine cell fates along the A-P axis via the segment gene hierarchy (Figure 1.2 C) (Gavis and Lehmann, 1992; Lehmann and Nusslein-Volhard, 1987). Similarly, along the D-V axis, a Dm-Dorsal (Dm-Dl) morphogen gradient emanating from the ventral midline of the embryo is established by the Dm-Toll pathway, which was activated during oogenesis (Rusch and Levine, 1994). Furthermore, Dm-Dl and its downstream Dm-Decapentaplegic (Dm-Dpp)-antagonists restrain and establish a Dm-Dpp gradient in the dorsal half of the embryos (Francois et al., 1994b)(Figure 1.5 A). Genes downstream of Dm-Dl and Dm-Dpp are activated by these two morphogen gradients and determine cell fates along the D-V axis.

This mechanism of gene regulation by morphogen gradients in Drosophila has been described in the 'French flag model', in which protein gradients position downstream gene expression patterns in a dose-dependent manner (Rogers and Schier, 2011; Wolpert, 1969). The French flag model is a perfect strategy for Drosophila embryogenesis, in that molecules freely diffuse in the long syncytial blastoderm stage to form gradients. Meanwhile, many genes can be activated within a short time in the blastoderm by sensing the different concentrations of proteins and all segments are determined in the blastoderm, enabling fast embryogenesis in Drosophila (Driever et al., 1989). However, this mechanism appears to be unsuitable for embryogenesis in short-germ insects. First, the syncytial blastoderm stage is relatively short and diffusion of proteins is impeded by cell membranes during the blastoderm stage; second, posterior segments are added sequentially during the elongation stage, rather than in the blastoderm; third, homologs of aforementioned morphogens are not all conserved in short-germ insects. However, homologs of several genes regulating Drosophila embryogenesis also play similar roles, more or less, in Tribolium and other insects during embryogenesis.

Genetic factors in Tribolium blastoderm fate map

In Drosophila, the blastoderm fate map is defined by gap gene expression, in that gap genes determine both identities of certain body regions as well as positions where these regions

form (Figure 1.2 B). In Tribolium, gap gene function is more limited to the determination of identity along the A-P axis. Instead, expression of three genes regionalizes the blastoderm. *Tc-zen-1* is expressed in the anterior region of the blastoderm. Knockdown of *Tc-zen-1* results in no serosa formation. *Tc-cad* is expressed in the posterior region of the blastoderm, where it regulates segmentation. Depletion of *Tc-cad* expression leads to no trunk. *Tc-otd-1* is expressed maternally and zygotically. Zygotic *Tc-otd-1* is expressed in the prospective pre-gnathal region, where it regulates head formation. The expression of these three genes determines cell fates the Tribolium blastoderm. To review the function of homologous genes in different insects, *Tc-zen-1* is discussed as part of D-V patterning; *Tc-otd-1* in defining the anterior fate map; and *Tc-cad* in defining the posterior fate map (Figure 1.2 D).

Defining the fate map in the anterior region of the embryo

Dm-Bcd protein forms an anterior to posterior gradient to activate anterior and repress posterior gap genes. Different concentrations of Dm-Bcd activate different gap genes at certain positions along the A-P axis. The affinity between Dm-Bcd and the cis-regulatory module (CRM) in gap genes that responds to it, appears to be providing positional information for gap gene expression (Hulskamp et al., 1989). However, flattening the Dm-Bcd gradient at different concentrations still activates several genes, rather than expanding the expression domain of specific genes expected to respond to certain Dm-Bcd concentrations as predicted by the French flag model (Chen et al., 2012). Instead, several studies have shown that the affinity between Dm-Bcd and the CRMs in its target genes is not sufficient to determine all anterior gap gene expression domains (Gregor et al., 2008). Precise gap gene expression domains along the A-P axis require some general repressors, such as *Dm-Krüppel* (*Dm-Kr*) and *Dm-runt* (Chen et al., 2012; Perry et al., 2012).

otd is an anterior cell fate organizer in non-dipteran insects

In non-dipteran insects, *otd* is likely to functionally substitute for *Dm-bcd*. *Dm-bcd* encodes a lysine residue at the 50th position (K50) of the homeodomain (Chahda et al.), which appears to be essential for binding site recognition in its DNA and RNA targets (Baird-Titus et al., 2006; Brown et al., 2001; Ma et al., 1996; Zhang et al., 2005). However, this K50 site also

appears in the HD of Otd. *otd* is an anterior gap gene, mutations in which cause development defects in antennae and eyes. In most insects, there are two homologs of *otd*. There are two *otd* homologs in Tribolium and Nasonia, one of which (*otd-2)* is expressed in late stages of embryogenesis, and does not contribute to early anterior patterning (Kotkamp et al., 2010; Li et al., 1996; Lynch et al., 2006). The other homolog (*otd-1*) impacts anterior patterning in several long and short-germ insects including Tribolium and Nasonia, as does the single homolog in Gryllus (Nakamura et al., 2010). In these insects, *otd-1* is expressed maternally and its mRNA ubiquitously distributed in the early blastoderm, and later expression is restricted to the head region. Similarly, Otd-1 protein is evenly distributed through the entire freshly laid egg. Later, Otd-1 protein recedes from the posterior to form a gradient at the anterior end. In Nasonia, *Nv-otd-1* also appears later in the posterior end of the embryo, where it also activates posterior gap genes (Lynch et al., 2006). Depletion of both *Tc-otd-1* and *Tc-hb* expression results in more severe phenotypes than the single knockdown of *Tc-otd-1* or *Tc-hb* in Tribolium. This double knockdown phenotype is similar to the *Dm-bcd* mutant phenotype in Drosophila, which causes deletion of head, thorax and part of the abdomen (Kotkamp et al., 2010). Similar impacts of *otd-1* and *hb* RNAi have also been seen in Nasonia. Considering the protein structure in combination with RNAi results suggests that *otd-1* and *hb* together functionally substitute for *bcd* in Tribolium and Nasonia.

Defining the fate map in the posterior region of insect embryos

Similar to the vital role Dm-Bcd plays anteriorly, Dm-Nos, another morphogen, is critical for patterning the posterior region of the Drosophila embryo (Lehmann and Nusslein-Volhard, 1991). Maternal *Dm-nos* mRNA is localized at the posterior pole of the embryo and protein translated from the mRNA diffuses into a gradient from posterior to anterior (Wang and Lehmann, 1991). *Dm-nos* and its helper *Dm-pumilio (Dm-pum)* repress translation of *Dm-hb* in the posterior half of the embryo to allow posterior gap genes such as *Dm-Kr* and *Dm-knirps* (*Dm-kni*) to be expressed and form the abdomen correctly (Murata and Wharton, 1995). Inhibition of *Dm-hb* expression can partially rescue the abdomen in *Dm-nos* mutants, which indicates that the function of *Dm-nos* in posterior pattering is through inhibition of Dm-HB (Irish et al., 1989). Unlike *Dm-bcd*, *Dm-nos* is more conserved among long and short -germ insects.

Orthologs of *nos* have been found in another long germ insect Nasonia and short germ insects, Gryllus and Tribolium (Lall et al., 2003; Lynch and Desplan, 2010; Schmitt-Engel et al., 2012).

In Nasonia, only a very small proportion of *Nv-nos* localizes at the end of embryo and a Nv-Nos gradient emanates from the posterior (Lynch and Desplan, 2010). *Nv-hb* translation in Nasonia is repressed by *Nv-nos* at the posterior end. This repression suggests a conserved function of *nos* in posterior embryonic fate patterning (Lynch and Desplan, 2010). Unexpectedly, in Tribolium, no *Tc-nos* mRNA or protein can be detected via *in situ* hybridization or immunocytochemistry, respectively. But, RT-PCR results demonstrate the presence of *Tc-nos* transcripts. Parental RNAi of *Tc-nos* leads to loss of abdominal segments as well as malformation of the head (Schmitt-Engel et al., 2012).

Role of the Wnt singling pathway in Tribolium fate map and segmentation

Noticeably, there is no Bcd homolog in Tribolium. Although morphogen coding genes *Tc-hb* and *Tc-nos* are both involved in segmentation, their influence on embryogenesis appears to be limited in Tribolium as compared to their roles in Drosophila. However, as in vertebrates but not Drosophila, the Wnt signaling pathway provides patterning input along the A-P axis in Tribolium (Beermann et al., 2011; Bolognesi et al., 2008a; Bolognesi et al., 2008b; Martin and Kimelman, 2009). Especially in vertebrates, canonical Wnt signaling is required for somitogenesis, which is the counterpart of insect segmentation (Bajard et al., 2014; Jensen et al., 2010). In the absence of Wnt signal, a protein complex phosphorylates Beta-catenin and degrades it. This Beta-catenin degradation complex is constructed of Axin, adenomatosis polyposis coli (APC) and other components. On the other hand when Wnt signal is present, Wnt ligands bind receptors to recruit the degradation complex to the cell membrane. Thus, Beta-catenin proteins accumulate in the cytoplasm. Eventually, Beta-catenin enters the nucleus and functions as a co-activator, binding to a TCF/LEF family transcription factor to activate Wnt target genes (Li et al., 2012) (Figure 1.3).

Although the underlying mechanism of somitogenesis regulated by Wnt and other signal regulators, is still not completely elucidated, disruption of Wnt signaling causes defects in

somitogenesis, resulting in fewer somites and a shortened body axis (Cooke and Zeeman, 1976; Greco et al., 1996). Therefore, canonical Wnt signaling is essential for formation of somites in vertebrates. The Wnt signaling pathway is also conserved in the long-germ insect, Drosophila. Even though the Drosophila homolog of Beta-catenin, Dm-Armadillo (Dm-Arm) and the homolog of TCF, Dm-Pangolin (Dm-Pan) are known, their mutation phenotypes exhibit defects in eyes, wings and segment polarity, but not in the proper enumeration of segments (Brunner et al., 1997). Loss-of-function of *Dm-arm* or *Dm-pan* mutants contain the normal number of segments, but lack the naked cuticle in each segment, similar to the defects observed in *Dm-wingless (Dm-wg)* mutants (Baker, 1987; Greaves et al., 1999) In contrast, expression of constitutively active Dm-Arm in Drosophila results in the replacement of denticles in the anterior region of each segment, at least partially, with naked cuticle (van de Wetering et al., 1997). Hence, *Dm-arm* and *Dm-pan* act as segment polarity genes similar to *Dm-wg* in Drosophila segmentation; the canonical Wnt signaling pathway does not regulate the process of segment formation as in vertebrates.

In addition to its function in segment polarity, which is conserved among insects, canonical Wnt signaling in Tribolium also regulates segmentation in a manner resembling, to some extent, its role in vertebrate somitogenesis. A homolog of *Dm-wg, Tc-Wnt1* RNAi embryos undergo elongation normally but do not maintain segmental integrity during retraction. Overly retracted germ bands lack segmental boundaries. Thus, *Wnt1* functions as a segment polarity gene in Tribolium. Although Wnt8, Wnt3, Wnt 5 are also expressed in the posterior growth zone, only knocking down the expression of both *Wnt1* and *Wnt8* produces severe germ band truncation (Bolognesi et al., 2008b). The posterior growth zone is also affected, which indicates that Wnt signaling is required for germ band elongation and segmentation in Tribolium. Knockdown of Wnt receptors, *Tc-frizzled1* and *Tc-frizzled2* results in formation of only head and some thoracic segments (Beermann et al., 2011). Disruption of the co-receptor of Wnt ligands, *Tc-arrow,* also impacts elongation. *Tc-porcupine* (*Tc-porc*) is involved in Wnt ligand formation. In *Tc-arrow* and *Tc-porc* RNAi embryos, expression of *Wnt1* in the posterior growth zone vanishes, the segmental expression of *Wnt1* fades, and *Wnt8* expression is slightly reduced (Bolognesi et al., 2009). In addition, depletion of *Gb-arm* in Gryllus and *Of-pan* in Oncopeltus also causes abdominal truncation, which demonstrates that the Wnt signal pathway plays a role

in segmentation, at least during the germ band elongation stage in other short-germ insects (Angelini and Kaufman, 2005; Shinmyo et al., 2005).

In vertebrates, the canonical Wnt signal plays more roles in addition to regulating somite formation along the A-P axis. Wnt signaling levels are critical to head formation. Dickkopf-1 (Dkk-1), a known antagonist of Wnt ligands, binds extracellularly to Frizzled, thus blocking Wnt signal activation in recipient cells. Over-expression of *Dkk-1* results in enlarged heads in zebrafish (Krupnik et al., 1999). In contrast, depletion of *Dkk-1* in mouse and frog removes the suppression of Wnt signaling in the head region, leading to deletion and malformation of head (Niehrs, 2006). In fish, up-regulated Wnt signaling levels stimulate the formation of posterior head structures more anteriorly (Hashimoto et al., 2000). Similar effects of Wnt signaling levels on A-P axis determination have also been described in Tribolium (Figure 1.4). *Tc-axn* RNAi embryos die before hatching, and produce cuticles with partial or no head structures (Fu et al., 2012). Thus, activating the Wnt signaling pathway in Tribolium by knocking down its repressors also causes head formation defects. Therefore, repression of Wnt signaling in the prospective head region could be a general criterion for head development in insects and vertebrates.

Furthermore, repressing Wnt signaling activity in the prospective head region and activating it in the posterior suggests that a gradient of Wnt signaling activity along the A-P axis is required for proper development in both vertebrates and Tribolium. This Wnt gradient is visible in several vertebrates, evident as a Beta-catenin gradient in mice and Xenopus (Aulehla et al., 2008; Kiecker and Niehrs, 2001), It has not been possible to detect a Wnt signaling gradient in Tribolium. However, expression of downstream targets is often used as readout of active signaling, and in Tribolium, *Tc-cad*, a target gene of Wnt signaling, is expressed in a gradient from the posterior end to the prospective gnathal region in embryos. This *Tc-cad* gradient is critical to segmentation and A-P patterning and indicates the presence of a Wnt activity gradient in Tribolium (El-Sherif et al., 2014; Sarrazin et al., 2012).

Caudal gradient contributes to posterior fate map of the embryo

Drosophila *Cad* is expressed both maternally and zygotically (Mlodzik and Gehring, 1987). Maternal Dm-Cad is sufficient to support normal abdomen formation. Maternal or zygotic *cad* expression can rescue formation of the abdomen in *Dm-cad* null mutants. However, only maternal *Dm-cad* mRNA is translated into a posterior gradient, which might play an instructive role for downstream genes (Schulz and Tautz, 1995). In contrast, activating zygotic *Dm-cad* alone in maternal *Dm-cad* null mutants does not produce the protein gradient as in wild type. Possibly, maternal *Dm-cad* produces the gradient and zygotic *Dm-cad* maintains this gradient at later stages (Mlodzik et al., 1985). This redundant *Dm-cad* expression strategy reflects the important role that *Dm-cad* plays in the determination of posterior embryonic fates, for instance, posterior activation of gap genes and pair-rule genes.

A gradient of *cad* mRNA is established at early blastoderm stages in other insects such as Tribolium, Gryllus and Nasonia, which also have both maternal and zygotic *cad* expression. In Tribolium and Gryllus, this gradient only exists at the blastoderm stage. Later, from the onset of gastrulation, *cad* is expressed only in the posterior growth zone (Schulz et al., 1998; Shinmyo et al., 2005; Wolff et al., 1998). In Nasonia, a similar *Nv-cad* mRNA gradient is positioned in the mid-posterior of the embryo, flanked by *Nv-otd-1* expression (Olesnicky et al., 2006). Although, *cad* expression differs between short germ insects (Tribolium and Gryllus) and long germ insect (Nasonia), lack of *cad* expression produces similar phenotypes. In Tribolium and Gryllus, only pre-gnathal head segments are formed in the strongest *cad* RNAi embryos. In Nasonia, *Nv-cad* mutants fail to develop almost all abdominal segments. This dramatic phenotype is more severe than that displayed in the *Dm-cad* null mutant. Moreover, in Drosophila, Dm-Hb is a repressor of abdomen cell fate, repressing *Dm-cad* in the anterior embryo to allow activation of anterior gap genes. But in Tribolium, Gryllus and Nasonia, *cad* activates *hb* instead (Olesnicky et al., 2006; Schoppmeier et al., 2009; Shinmyo et al., 2005). Hence, *cad* might function as a general activator of gap genes to participate in the determination of fate map of the entire embryo, except the pre-gnathal head region. This implies that *cad* plays a more restricted role in Drosophila, compared to other insects.

Furthermore, in Tribolium and Gryllus, *cad* is regulated by the Wnt signaling pathway (Shinmyo et al., 2005). Depletion of Wnt signaling by removing positive regulators of the pathway abolishes *cad* expression in these short-germ insects (Figure 1.4). Recently, the impact of Wnt signaling on *Tc-cad* expression has been described in Tribolium (El-Sherif et al., 2014).

Further studies on the impact of the *Tc-cad* gradient on segmentation indicate that the *Tc-cad* gradient is a key regulator of the primary pair-rule gene, *Tc-eve*, the expression of which is the first genetic sign of segment formation. The *Tc-cad* gradient induces *Tc-eve* expression, determining its position, tempo and stripe width in the blastoderm. Changes in Wnt activity that produce changes in the *Tc-cad* gradient also produce coordinated changes in *Tc-eve* expression (El-Sherif et al., 2014). *Tc-eve* expression during the blastoderm stage suggests that instead of relying on the French flag model of morphogen gradients, Tribolium may use a different mechanism, similar to the 'clock and wave front' model used to describe vertebrate segmentation (somitogenesis). In Tribolium, expression of *Tc-eve* is periodic during the blastoderm stage. Three *Tc-eve* primary stripes are formed by three cycles of *Tc-eve* expression oscillation, to pattern six segments in the blastoderm. In Tribolium, the *Tc-cad* gradient activates *Tc-eve* expression and the level of *Tc-cad* expression regulates the frequency of *Tc-eve* expression in cells. Higher *Tc-cad* levels induce higher oscillation frequencies of *Tc-eve* expression, evident as a kinematic wave sweeping from the posterior end of the embryo to the anterior border of the *Tc-cad* gradient. This regulation of *Tc-eve* expression by the *Tc-cad* gradient resembles the oscillating expression of several signaling pathway components in vertebrate somitogenesis (El-Sherif et al., 2014). Specifically, the Wnt signaling pathway functions on many levels in the clock and wavefront model. Involvement of Wnt signaling in Tribolium segmentation offers more evidence of the similarity between Tribolium and vertebrate segmentation (Figure 1.2 D).

In vertebrates, the periodic expression of several genes as in the presomitic mesoderm (PSM) is known as gene expression oscillation. These periodically expressed genes are considered to be the molecular clock of somitogenesis. One cycle of gene expression forms one somite. Several oscillating genes are components or target genes of the Notch, Wnt and FGF signaling pathways, for example, *hairy and enhancer of split 1* (*Hes1*) and *Hes7* (Notch pathway), as well as *Axin2* (Wnt pathway) (Henry et al., 2002; Klinck et al., 2011; Rodriguez-

Gonzalez et al., 2007). Notch, FGF and Wnt signaling gradients cover most of the PSM to regulate the frequency of the oscillation (Aulehla and Pourquie, 2008; Aulehla et al., 2003; Dubrulle et al., 2001; Greco et al., 1996). Up-regulated gradients result in a higher frequency of oscillation and somite formation, without changing the physical position of somites (Sanchez-Villagra, 2010). This indicates a closely correlated spatial-temporal regulation during somitogenesis. These gradients are anchored at the posterior end of the PSM, and move posteriorly together with the PSM during germ band elongation. Oscillation of gene expression is continual in cells within the in these signaling pathway gradients, but gene expression stabilizes in cells exiting these gradients, due in part to caudal growth of germ band. In addition, an opposite gradient of retinoic acid that functions to determine the position of the new somite formation also moves posteriorly in parallel with movement of the PSM (Diez del Corral et al., 2003).

Gap genes

In Drosophila and other insects, *hunchback* contributes to A-P axis patterning along with Bcd or Otd. Both expression and function of *hb* in these insects are conserved with minor variations. All the aforementioned insects have both maternal and zygotic *hunchback* gene expression. Although generally delivered as mRNA, in the grasshopper Schistocera, maternal Hb protein is delivered to the embryo (Patel et al., 2001). Zygotic *hb* expression is also conserved among these insects. In the blastoderm stage of short-germ insects, anterior zygotic *hb* expression partially covers the presumptive head as in Drosophila and posterior *hb* expression is required for proper abdominal segmentation (Marques-Souza et al., 2008; Mito et al., 2005). In another long-germ insect, Nasonia, *Nv-hb* has similar but not identical anterior and posterior expression domains, and is required for proper head patterning (Pultz et al., 2005).

Cuticles of mutant Drosophila embryos lacking both maternal and zygotic *Dm-hb* expression exhibit anterior defects, but in this case anterior segments are replaced by mirror images of abdominal segments. Zygotic and homozygotic mutants of *Dm-hb* lack thorax and posterior abdominal segments (Lehmann and Nusslein-Volhard, 1987). However, in *Dm-bcd* mutants, only the abdomen forms normally and is shifted anteriorly, while the head and thorax

are missing (Driever et al., 1989). This *Dm-bcd* mutant phenotype can be partially rescued to form normal thorax by the expression of maternal and zygotic *hb* (Wimmer, 2000). These results demonstrate the indispensable role of *hb* in A-P axis patterning in Drosophila and other insects.

Disruption of *hb* expression in Tribolium and Nasonia causes malformation of head and trunk. Parental *hb* RNAi in Tribolium, Gryllus and Oncopeltus produces an incomplete gap gene phenotype (Liu and Kaufman, 2004a). In weak knockdown phenotypes, thoracic segment identity is transformed to abdomen. In the strongest knockdown embryos, the gnathal segments are also transformed to abdomen, and the resulting embryos are much shorter than wild type. Segment transformation is also observed in some weak mutant alleles of *Dm-hb* in Drosophila (Lehmann and Nusslein-Volhard, 1987). This transformation appears to be due to regulation of Hox genes by *Dm-hb*. Therefore, regulation of Hox genes by *hb* is mostly conserved among short and long-germ insects. In Drosophila, the A-P axis is determined by the input of maternal morphogens. These inputs also activate downstream gap genes to subdivide the embryo into several regions. Amorphic gap gene mutants, such as, *Dm-Kr*, exhibit typical gap phenotypes that lack the body part corresponding to the region where they are expressed in the blastoderm (Preiss et al., 1985). Mutual repression between gap genes not only allows them to have stable expression profiles in varying maternal morphogen backgrounds in different individuals, but also provides a subtle regulation mechanism for their target pair-rule genes (Small et al., 1991; Sokolowski et al., 2012). Detailed studies of the expression of pair-rule gene in Drosophila reveal the need for several gap genes to regulate the proper expression of a single stripe (Wu et al., 1998). In addition, different stripes of a single pair-rule gene are regulated by various combinations of gap genes (Papatsenko and Levine, 2008). Therefore, gap genes are the upstream regulators of pair-rule genes in Drosophila.

The roles of gap and pair-rule gene homologs vary in other insects. In short-germ insects, fewer gap genes are expressed in the blastoderm; only anterior regions of the body plan are patterned during the blastoderm stage. In Tribolium, Oncopeltus, Gryllus and Nasonia, anterior expression of *giant* (*gt*) and *Kr* are observed in the blastoderm. During the germ band stage, *gt* is also expressed in a similar posterior region as in Drosophila, except that *gt* is expressed a third time, more posteriorly in the Oncopeltus germ band, which is not observed in other insects (Liu and Patel, 2010). Furthermore, parental RNAi against *Kr* and *gt* in Oncopeltus and Gryllus yields

canonical gap phenotypes as in the fly (Liu and Kaufman, 2004b; Mito et al., 2006). In these insects, mutual repression between these two gap genes is also conserved. The similarity of expression and function of gap genes among those non-dipteran insects and fly implies a fundamental gene regulation during embryogenesis from basal insects to the highly derived Drosophila model (Liu and Kaufman, 2004a).

A novel segmentation gene was discovered Tribolium, which highlights the difference between embryogenesis in Tribolium and Drosophila. *Tc-mille-pattes* (*Tc-mlpt)* is a polycistronic gene encoding four peptides (Savard et al., 2006). *Tc-mlpt* is expressed in the blastoderm in the head and at the posterior end. The most severe *Tc-mlpt* RNAi produces an extreme phenotype of head followed by 10 pairs of legs and only one or two abdominal segments in the posterior end of the embryo. The impact of *Tc-mlpt* on segmentation is similar to other gap genes, suggesting that *Tc-mlpt* functions as a gap gene in Tribolium. Moreover, interactions between *Tc-mlpt* and other gap genes confirm this conclusion. The discovery of *Tc-mlpt* provides a new type of candidate gene regulating segmentation, homologs of which may also function similarly in other insects. However, except for *Tc-mlpt*, no polycistronic genes have been yet reported to be involved in segmentation in other insects (Savard et al., 2006).

Hox genes

In Drosophila, the segment gene hierarchy defines the position of each segment in the blastoderm, which is observed morphologically in the germ band elongation stage by a groove formed between segments. However, as segments form, the identity of each segment is also determined along the A-P body axis by homeotic genes (Hox genes). Embryos homozygous for Hox gene mutations display a loss of certain morphologic characteristics or the replacement of one segmental identity by another identity. Homeotic genes encode transcription factors with a 60 amino acid homeodomain, which is critical for DNA binding site recognition upstream of target gene (McGinnis et al., 1984).

In Drosophila, Hox genes are in two clusters, the Antennapedia complex (ANT-C) (Diederich et al., 1989) and the bithorax complex (BX-C) (Duncan, 1987). The Antennapedia complex determines the identities of gnathal and thoracic segments, and includes the genes *labial*

(*lab*), *prosocipedia* (*pd*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*). The bithorax complex patterns posterior segmental identities, mainly in the abdomen, and includes the genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *abdominal-B* (*abd-B*). These genes are collinearly localized on the chromosome, such that the spatial order of these Hox genes corresponds to their expression and function in regions along the A-P body axis during embryogenesis. For example, Loss of Function *Dm-pb* mutations transform maxillary and labial segments into thorax (Diederich et al., 1989). *Dm-Scr* is required for normal development of posterior head and the first thoracic segment (Riley et al., 1987). *Dm-abd-A* is significant for the identity of anterior abdominal segments (A1-A4) and *Dm-abd-B* for posterior segments (A5-A9) (Karch et al., 1990).

Similar impacts on segmental identities are also observed in Tribolium mutants. A group of genes has been identified by morphologic mutants. These genes are located in a single homeotic gene complex (HOM-C) (Beeman et al., 1989), which is the juxtaposition of ANT-C and BX-C in Drosophila. Both expression patterns and mutation phenotypes of these genes indicate that they are orthologs of corresponding Drosophila homeotic genes, although expression and mutant phenotype of these genes are not identical to their Drosophila homologs. Analysis of Tribolium HOM-C genes also reveals a high similarity of homeobox sequence between Tribolium HOM-C genes and Drosophila Hox genes.

Homologs of Drosophila Hox genes also have been identified in other arthropods and even in vertebrates. Instead of ANT-C and BX-C clusters, homeotic gene homologs in other non-fly arthropods and vertebrates appear in one or several HOX gene complexes (Izpisua-Belmonte et al., 1991; Karch et al., 1990; Krumlauf, 1992). Expression and functional domains of these homologs of HOM-C genes also correspond to the collinear order of the genes in the complex. The homeobox sequences in these genes are also conserved. For example, expression of murine Hox genes in Drosophila produces a phenotype similar to that caused by over-expression of its Drosophila homolog, and a murine Hox gene can also activate the expression of the target genes of the Drosophila Hox genes. These results further demonstrate a conserved molecular function of Hox homologs from vertebrates to insects (Izpisua-Belmonte et al., 1991). Therefore, Hox

genes are general segment identity determinative factors in all three phyla with segmented body plans.

In addition to the homeotic genes, other developmentally important genes also contain homeobox sequences. Within ANT-C and HOM-C, three genes containing homeobox sequences are not homeotic genes: *Bicoid* and *zen,* which lie between *pd* and *Dfd*, as well as *fushi tarazu* (*ftz*) which lies 5' of *Scr* (Stauber et al., 2002). These genes are thought to have duplicated during evolution in insecta. Their homeobox sequences evolve much faster than the homeobox in Hox genes. Their expression does not follow the spatial, collinear order of other genes in the ANT-C or HOM-C, in addition that they do not determine segmental identity as Hox genes do (Lohr et al., 2001). Besides these three homeobox genes, several other genes containing homeoboxes do not function as homeotic genes, which might result from changes in amino acids in several key sites in the homeodomain (Gehring and Hiromi, 1986).

In Drosophila, Hox gene expression is regulated by genes at different levels in the segment gene hierarchy. Initiation of Hox gene expression occurs during cycle 14 of nuclear division in a cellularizing blastoderm and can be detected during the germ band elongation stage. In embryos with mutations in maternal morphogen coding genes or gap genes, several segments are affected or deleted, due to the regulation of pair-rule gene expression. Mutations of pair-rule genes, for example, *Dm-ftz* and *Dm-eve,* impact Hox gene expression (Ingham and Martinez-Arias, 1986; Tremml and Bienz, 1989). Therefore, the effect on Hox gene expression by either maternal morphogen coding genes or gap genes could be through pair-rule genes. However, careful examination of pair-rule and Hox gene expression patterns indicates that both gap and pair-rule genes are required for proper Hox gene expression (Harding and Levine, 1988; Reinitz and Levine, 1990). Furthermore, a segment polarity gene, *Dm-engrailed* (*Dm-en*) plays a repressive role in regulating Hox gene expression to define the identity of metathorax in Drosophila (Mann, 1994). Thus, expression of Hox genes in Drosophila requires various inputs from gap, pair-rule and segment polarity genes. The effects on Hox gene expression by maternal morphogens can be due to their impact on downstream gap genes.

The Drosophila segment gene hierarchy is unique and has not been described in detail non-drosophilid insects. Regulation of Hox gene expression in other insects varies in the absence of this hierarchy. Tribolium forms head and thorax in the blastoderm and abdomen in germ band elongation stage. The expression of Hox genes also occurs in different stages. *Tc-Dfd* is expressed in the blastoderm, while *Tc-abd-A* expression only can be detected after the blastoderm stage (Brown et al., 2000; Brown et al., 1999; Shippy et al., 1998; Stuart et al., 1993). In Tribolium, Hox genes are also regulated by gap and pair-rule gene homologs. As in Drosophila, homologs of pair-rule genes are expressed in pair-rule patterns, covering every two segments. However, disruption of pair-rule genes, such as *Tc-eve*, produces a more severe truncation phenotype with only the pre-gnathal region formed. Expression of Hox genes is affected by this deletion of segments in these embryos. Nevertheless, the main regulators of Hox gene expression in Tribolium are gap genes. Knockdown of gap gene expression affects Hox gene expression. In the weakest gap gene knockdown embryos, the normal number of segments is observed and the only effect is changing segmental identities (Bucher and Klingler, 2004). Similar regulation of Hox genes by gap genes is also detected in Oncopeltus *hb* RNAi embryos (Liu and Kaufman, 2004a). In Gryllus, the transformation of segmental identity by knocking down gap genes has not been observed yet, which could be due to deletion of segments overriding transformation in segments (Mito et al., 2006; Zhang et al., 2005).

Drosophila, Tribolium and Gryllus share some common features, but segmental identity varies among them. . All these insects contain a single Homeotic gene complex, which regulates metameric characteristics. Thus, the Hox gene expression pattern as well as the regulation of Hox target genes is important to determine and develop segment-specific and species-specific identities. *Distal-less (Dll*) expression is repressed by *Ubx* and *abd-A* in insects to suppress the formation of limbs in the abdomen. On the contrary, expression of *Dll* is not suppressed in centipede by *Ubx* and *abd-A* (Brena et al., 2006; Hughes and Kaufman, 2002; Palopoli and Patel, 1998). Interactions between a Hox gene and its target genes could explain morphological differences between insects.. Therefore, further understanding evolution at the genetic level requires comprehension of the input from other genes to regulate Hox gene expression as well as the regulation of Hox gene targets.

Determination of the fate map along the D-V axis of the embryo

In Drosophila, patterning the fate map along the D-V axis occurs in a similar dose-dependent manner as along the A-P axis, relying on two opposing gradients, a Dm-Dl gradient in the ventral half of the embryo and Dm-Dpp in the dorsal half. Drosophila Dm-Toll receptors in oocytes perceive ligands in the perivitelline space to activate the Dm-Toll signaling pathway during oogenesis (Belvin and Anderson, 1996). The activated Dm-Toll signaling pathway leads to phosphorylation and degradation of Dm-Cactus (Dm-Cact) that retains Dm-Dl proteins in cytoplasm. The degradation of Dm-Cact results in translocation of Dm-Dl into the nucleus, producing a gradient in the ventral half of the embryo, the region of prospective neural ectoderm and mesoderm (Bergmann et al., 1996; Rusch and Levine, 1994). Expression of the *Dm-Dl* target genes *Dm-sog* and *Dm-twist* is activated by low Dm-Dl levels found below the midline of D-V axis (Reeves and Stathopoulos, 2009).

Establishing the Dpp gradient in the dorsal side of the embryo also depends upon the Dm-Dl gradient. Dpp signaling is reduced by Dm-Dorsal target genes in the ventral embryo. Dm-Dpp forms a gradient in the dorsal half of the embryo (Ferguson and Anderson, 1992). The peak of Dm-Dpp is detected in the dorsal most cells. In Drosophila, two ligands of Dpp signaling pathway, Dm-Dpp and Dm-Screw (Dm-Scw), form a heterodimer, which further binds to Dm-Sog and Dm-Tsg (Ashe and Levine, 1999; Neul and Ferguson, 1998). As the complex of Dm-Dpp, Dm-Scw, Dm-Sog and Dm-Tsg is transported dorsally, the metalloprotease Dm-Tolloid (Dm-Tld) liberates Dm-Dpp from these antagonists, resulting in the activation of Dm-Dpp signaling pathway (Carneiro et al., 2006; Shimmi and O'Connor, 2003). Dm-Sog and Dm-Tsg exhibit a higher affinity with the heterodimers of Dm-Dpp and Dm-Scw, rather than the homodimers of either Dm-Dpp or Dm-Scw. Dm-Tsg contributes to the binding of Dm-Sog to Dm-Dpp ligands (Entchev et al., 2000).

In addition to *Dm-sog*, other ventral genes also repress Dpp signaling. *Dm-brinker* (*Dm-brk*) is also expressed in the ventral lateral side of the embryos and contributes to the reduction of Dpp signals. However, inhibition by *Dm-brk* exhibits strong preference for low-level Dpp signaling target genes. In *Dm-sog* and *Dm-brk* double mutant embryos, *Dm-dpp* is repressed by *Dm-twi* in the region of the ventral midline, causing the complete deletion of the neuroectoderm

(Jazwinska et al., 1999b). As in either *Dm-sog* or *Dm-brk* mutant embryos, dorsal expression of *Dm-dpp* and its target genes dorsalizes part of the ventral lateral region. Disrupting expression of *Dm-dpp*, *Dm-sog* or *Dm-scw* causes amnioserosa formation defects, which are not observed in *Dm-brk* mutant embryos (Jazwinska et al., 1999a; Rushlow et al., 2001).

Target genes of Dm-Dpp or Dm-Dl are activated by different levels of the Dm-Dpp or Dm-Dl gradients along the D-V axis (Francois et al., 1994a). In a Drosophila mutation screen, Mothers against Dpp (MAD) was found as an enhancer of a weak Dm-dpp mutant. Mad is phosphorylated via the Dpp pathway. Phosphorylated Mad (pMAD) is the transducer of Dpp signal and required to trigger Dpp target gene expression (Figure 1.5 A). From dorsal to ventral, these gradients direct formation of the amnioserosa, dorsal ectoderm, neuroectoderm and mesoderm (Ray et al., 1991). Formation of amnioserosa also requires *Dm-zen* expression. Early *Dm-zen* expression is weak and uniform in the dorsal half of the embryo. Later, *Dm-zen* is only expressed in the prospective amnioserosa region, which overlaps with the highest Dm-Dpp expression. Ectopic expression of either *Dm-zen* or *Dm-dpp* can produce ectopic amnioserosa. However, in *Dm-dpp* mutant embryos, early ubiquitous *Dm-zen* expression is present and quickly fades. In addition, *Dm-zen* is not expressed dorsally later (Rusch and Levine, 1997) (Decotto and Ferguson, 2001). Therefore, *Dm-zen* expression appears to be activated by some unknown input and maintained by Dpp signaling.

Homologs of most of the aforementioned D-V patterning genes have been identified in Tribolium, with the exception of *brk* and *scw*. The only Tribolium Toll receptor is detected in the ventral-most region of the embryos, and also regulates D-V polarity (Maxton KuchenmeisterMaxton et al., 1999). This Toll expression overlaps and activates Tc-Dl expression. Instead of forming a static Tc-Dl gradient as in Drosophila, the expression of Tribolium Tc-Dl is more dynamic and transient in the ventral side of the blastoderm, which vanishes before gastrulation. It is unknown whether Tc-Dl functions similarly in Tribolium. However, the function of Tribolium Toll pathway resembles that of Drosophila. *Tc-sog* and *Tc-twi* are both expressed in the ventral most side of the embryos. In Toll RNAi Tribolium blastoderm embryos, no *Tc-sog* expression is detectable; expression of *Tc-cact* and *Tc-twi* is only

observed in the primitive pit, which indicates diminishment of ventral fates in these embryos (Nunes da Fonseca et al., 2008).

In Drosophila, *Dm-cact* is the target gene of Toll signaling and an upstream antagonist of Dm-Dl. On the contrary, *Tc-cact* appears to be activated by *Tc-twi*, a potential target gene of *Tc-Dl* and/or *Tc-Toll*. No *Tc-Dl* knockdown embryos have been studied. According to the dynamic expression of *Tc-Dl* and *Tc-cact* in the Tribolium blastoderm, the transient expression of *Tc-Dl* can be explained by the presence of overlapping *Tc-cact* expression. As *Tc-Dl* expression fades in the wild type differentiated blastoderm, *Tc-cact* expression is up-regulated, resulting from activation by *Tc-twi*, which is demonstrated by *Tc-twi* RNAi. Therefore, in Drosophila, the genes from the Toll receptor to the *Dm-Dl* target genes function in a hierarchy through Dm-Cact and Dm-Dl. In contrast, in Tribolium, *Tc-cact, Tc-Dl* and the *Tc-Dl* target gene, *Tc-twi* compose a self-regulatory circuit to determine the ventral fate map of the blastoderm. In addition, it is hard to imagine *Tc-Dl* target genes can be activated in a dose-dependent manner by transient *Tc-Dl* expression (Nunes da Fonseca et al., 2008).

zen is involved in the extra-embryonic tissue formation

Tribolium eggs have two kinds of extra-embryonic cells, the serosa and amnion. Unlike the expression of Drosophila *Dm-zen* in dorsally located cells, Tribolium gene *Tc-zen-1* is expressed in presumptive serosa cells in a dorsally tilted anterior domain, and appears to be involved in both A-P and D-V axis patterning (van der Zee et al., 2005). In *Tc-Toll* knockdown embryos, *Tc-zen-1* is also ectopically expressed on the ventral side in more posterior regions, which reflects the dorsalization of these embryos (Nunes da Fonseca et al., 2008). The paralog of *Tc-zen-1, Tc-zen-2*, is first expressed in the same region as *Tc-zen-1* and functions downstream of *Tc-zen-1*. Later it is expressed in the dorsal amnion, and is likely to be involved in amnion movement during the gastrulation (van der Zee et al., 2005).

So far, in Tribolium, only one *dpp* homolog has been found. *Tc-dpp* is expressed throughout the entire early embryo, with a somewhat stronger short-range mRNA gradient emanating from the anterior pole. Soon after, *Tc-dpp* expression covers the anterior end of the

embryo with a tilted posterior border, which is the boundary between serosa and the embryonic tissue along the D-V axis. Later, before the blastoderm differentiates, *Tc-dpp* is expressed uniquely in the presumptive anterior amnion cells (Sharma et al., 2013). Although no direct protein interaction results have been reported, it appears that interactions between Tc-Dpp and Tc-Sog is conserved in the Tribolium blastoderm to patterning the D-V axis (van der Zee et al., 2006) (Figure 1.5 B). Disrupting the expression of *Tc-dpp*, *Tc-sog*, *Tc-tsg* or *Tc-tld* alters the tilted border, which is also the posterior boundary of serosa, as well as the expression of other dorsal and ventral fate marker genes (van der Zee et al., 2005). Hence, this alteration changes the D-V polarity of the blastoderm. Lack of *Tc-dpp* expression causes loss of posterior dorsal marker gene expression. Lack of *Tc-sog* expression transforms the dorsal amnion into ventral fated cells (Figure 1.5 C). Since knocking down *Tc-tsg* and *Tc-tld* expression produces ventralization effects on the blastoderm similar to knockdown of *Tc-sog* expression, D-V axis pattering in Tribolium can be explained by the dorsal transportation of Tc-Dpp by Tc-Sog and Tc-Tsg and liberation of Tc-Dpp by Tc-Tld in the dorsal side of the embryos (Nunes da Fonseca et al., 2010).

In other insects, the D-V patterning is not well studied. In the long germ insect, Nasonia, gene expression patterns in the blastoderm are almost identical to Drosophila. The significant difference appears to be cell movement during gastrulation (Buchta et al., 2013). In Oncopeltus, only the expression and function of a *zen* homolog has been studied. Although *Of-zen* is also expressed in the early blastoderm, the effect of *Of-zen* on the embryogenesis is more likely due to later *Of-zen* expression in the serosa during gastrulation, which is critical to serosal function in katatrepsis (Panfilio et al., 2006). Knock down of *Of-zen* expression produces inverted embryos, which is also observed in Tribolium embryos lacking *Tc-zen-2* expression. This similarity implicates a conservation of *zen* function in these short germ insects (Panfilio, 2009; Panfilio et al., 2006). It is possible that the regulatory interactions of other D-V patterning genes are also conserved among short germ insects.

In this chapter, the function and expression of genes involved in patterning both A-P and D-V axes of the blastoderm have been reviewed, mostly in Drosophila and Tribolium, as well as another long germ insect, Nasonia and two short germ insects, Gyllus and Oncopeltus. The patterning of body axes in these insects is conserved to some extent, reflected by expression

patterns and functions of many gene homologs. Although, compared to Tribolium, Nasonia is farther from Drosophila in the evolutionary tree, the regulation of gene expression in these two long germ insects are more similar. Gene regulation also appears more comparable among the short germ insects. Recently, studies of pair-rule and gap genes in Nasonia reveal a mode that combines elements of long and short germ development (Rosenberg et al., 2014). In Nasonia, gene regulation in the anterior region of the embryo resembles that of the Drosophila hierarchy, while in the posterior embryo, gene expression is more dynamic, similar to gene expression in short-germ insects. Hence, it is possible that the underlying mechanisms of either long or short germ insects co-exit. Referring to a specific insect, a mechanism has evolved to fit the timing or other requirements of embryogenesis. Further comparative studies of embryogenesis in these insects could yield more information to explicitly explain the mechanism applied in long and short insects and understand the difference between long and short insect embryonic development processes.

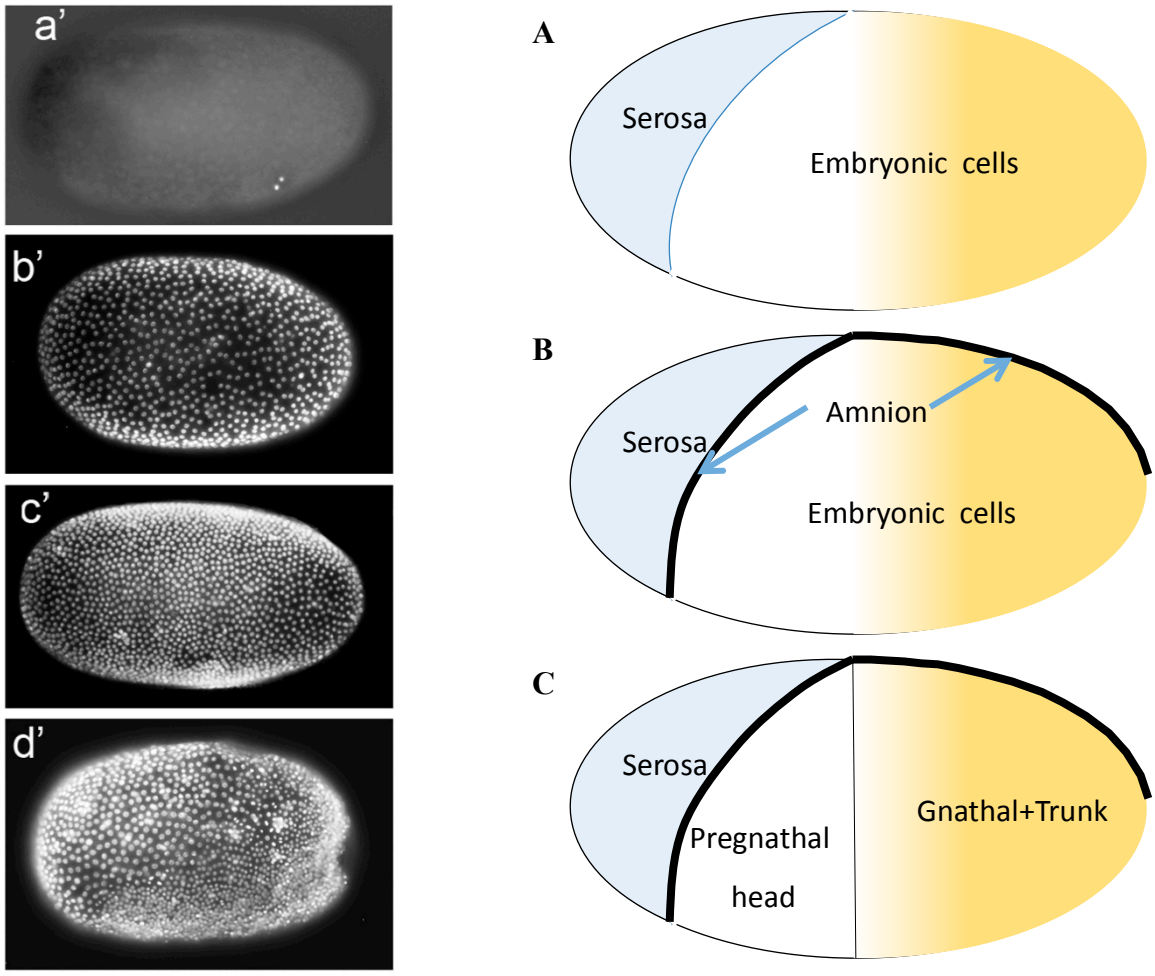
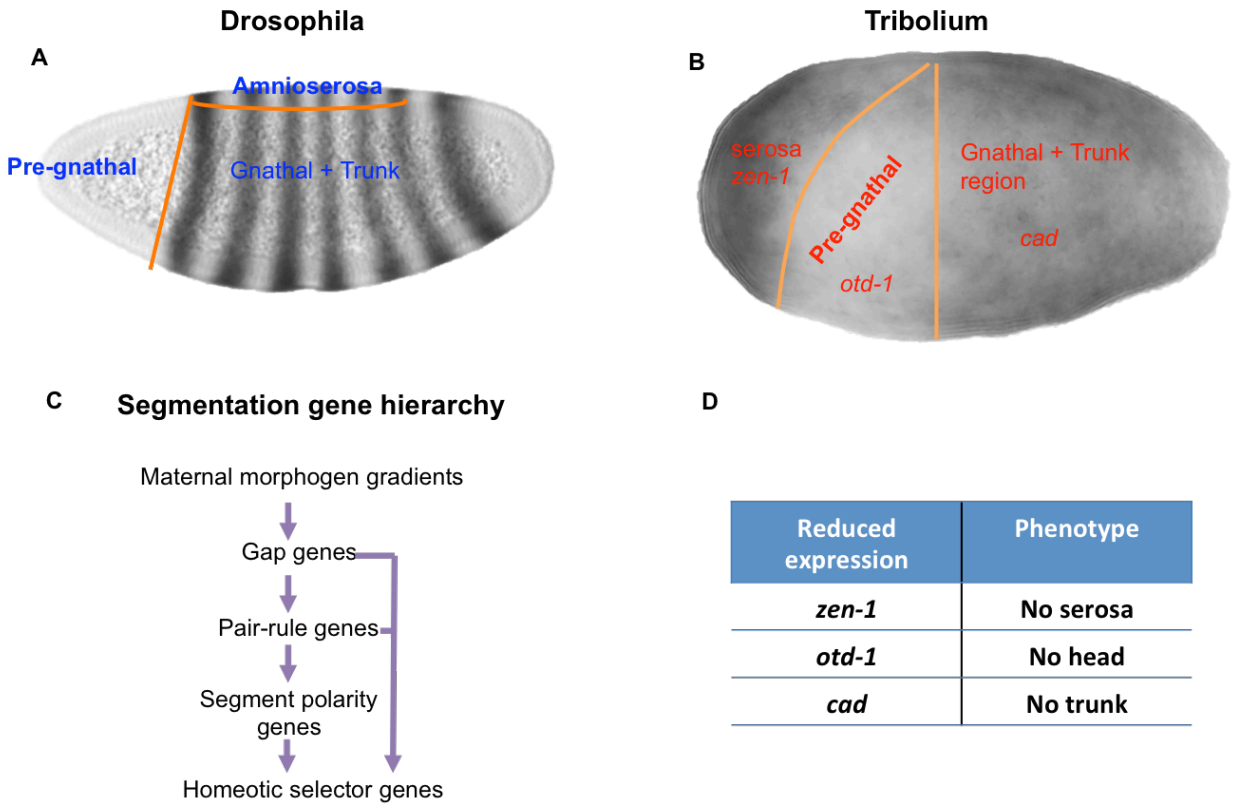


Figure 1-1 Embryogenesis in Tribolium blastoderm stage

(a'-d') Embryos stained with DAPI to visualize nuclei in the blastoderm. (a') Nuclei are in the middle of yolk. (b') Nuclei are reaching the surface of the embryo. (c') During encompassing the cell membrane of every nucleus, asymmetric cell division is undergoing to form differentiated blastoderm, in which, serosa is soon noticeable. (d') Differentiated blastoderm, gastrulation commenced.

(A-C) Steps to pattern fate map in Tribolium, which are ordered by the time of notable changes in morphology. (A) Serosa is first morphologically separated from other fated cells. (B) Another extra-embryonic tissue separates from the embryonic rudiment, although cell shapes in these two tissues are similar. (C) Cells in the embryonic rudiment also have different fates in different regions of the embryos.



Reduced expression	Phenotype
zen-1	**No serosa**
otd-1	**No head**
cad	**No trunk**

Figure 1-2 Genetic interactions underlying segmentation mechanisms

(A) In Drosophila, the entire body plan is determined in the blastoderm embryos, including the extra-embryonic tissue, the amnioserosa. (B) In Tribolium blastoderm, two extra-embryonic tissues, the serosa and amnion are formed. Only the head and thorax are formed. The rest is formed in the later germ band stage. (C) Segment gene hierarchy regulates the segmentation in Drosophila. (D) Phenotypes of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* RNAi embryos.

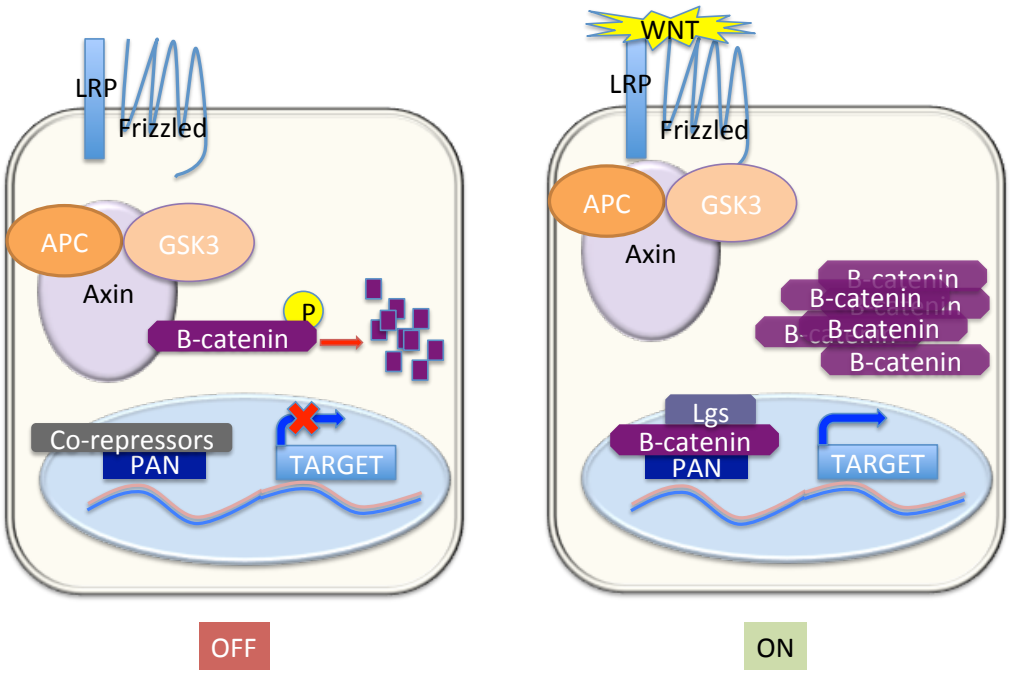


Figure 1-3 Wnt signaling pathway

Left: When Wnt ligand is absent, the destruction complex composed of APC, Axin, GSK3 and other proteins phosphorylates Beta-catenin, resulting in its degradation. In these cells, the co-repressor Groucho binds to the transcription factor TCF (or Pangolin in Drosophila) to repress expression of Wnt target genes. Therefore, Wnt signaling pathway is off.

Right: Interaction between Wnt ligand and receptor triggers the destruction complex to move to the cell membrane and prevents phosphorylation of Beta-catenin. In these cells, Beta-catenin accumulates in the cytoplasm and translocates into the nucleus, where it replaces Groucho and binds TCF (Pan) and another co-activator Lgs to activate Wnt target genes. Therefore, Wnt signaling pathway is on.

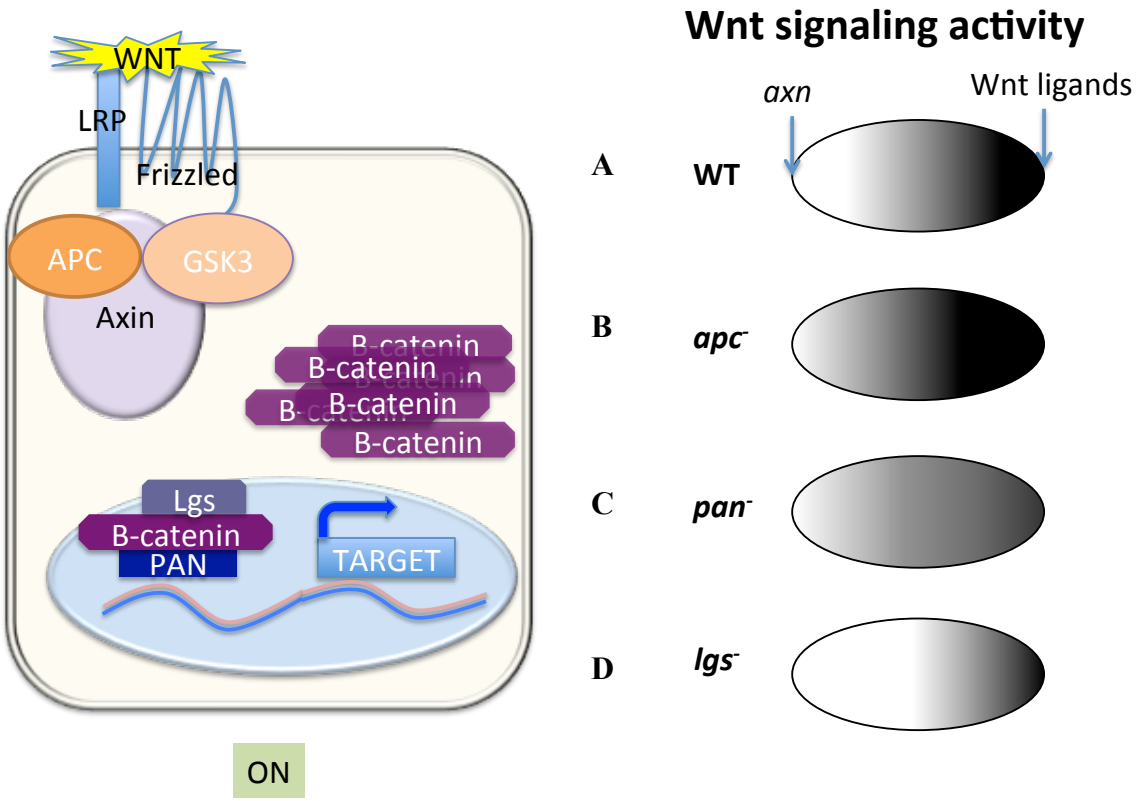


Figure 1-4 Wnt signaling activity in the Tribolium blastoderm

Wnt signaling activity can be regulated with Wnt ligand, but also cell-autonomously.
(A) In Tribolium, *Tc-axn* is maternally expressed in the anterior pole of the embryo, which represses Wnt activity in this region. In the posterior region of the embryo, no expression of *Tc-axn* has been detected, which suggests that in this region Wnt activity is not repressed. Moreover, Wnt ligands are expressed in the posterior end of the embryo to activate Wnt activity at later blastoderm stages.

(B) In *Tc-apc-1* or *Tc-axn* RNAi embryos, the destruction complex fails to form and repression of Wnt signaling activity is reduced in these cells. In other words, Wnt signaling activity is up-regulated in the anterior region of the embryo.

(C) *Tc-pan* is the homolog of Drosophila *Dm-pan* encoding the transcription factor in Wnt signaling pathway. Tc-Pan protein can bind to both co-repressor and co-activator. Therefore, knocking down *Tc-pan* expression in cells where Wnt signaling is repressed, Wnt activity is de-

repressed. In other words, Wnt target genes are not fully repressed. In cells Wnt targets should be activated, Wnt target genes are not fully activated.

(D) *Tc-lgs* is a co-activator of the Wnt signaling pathway. In *Tc-lgs* RNAi embryos, even if Wnt ligand binds with Wnt receptor, Wnt target genes cannot be fully activated.

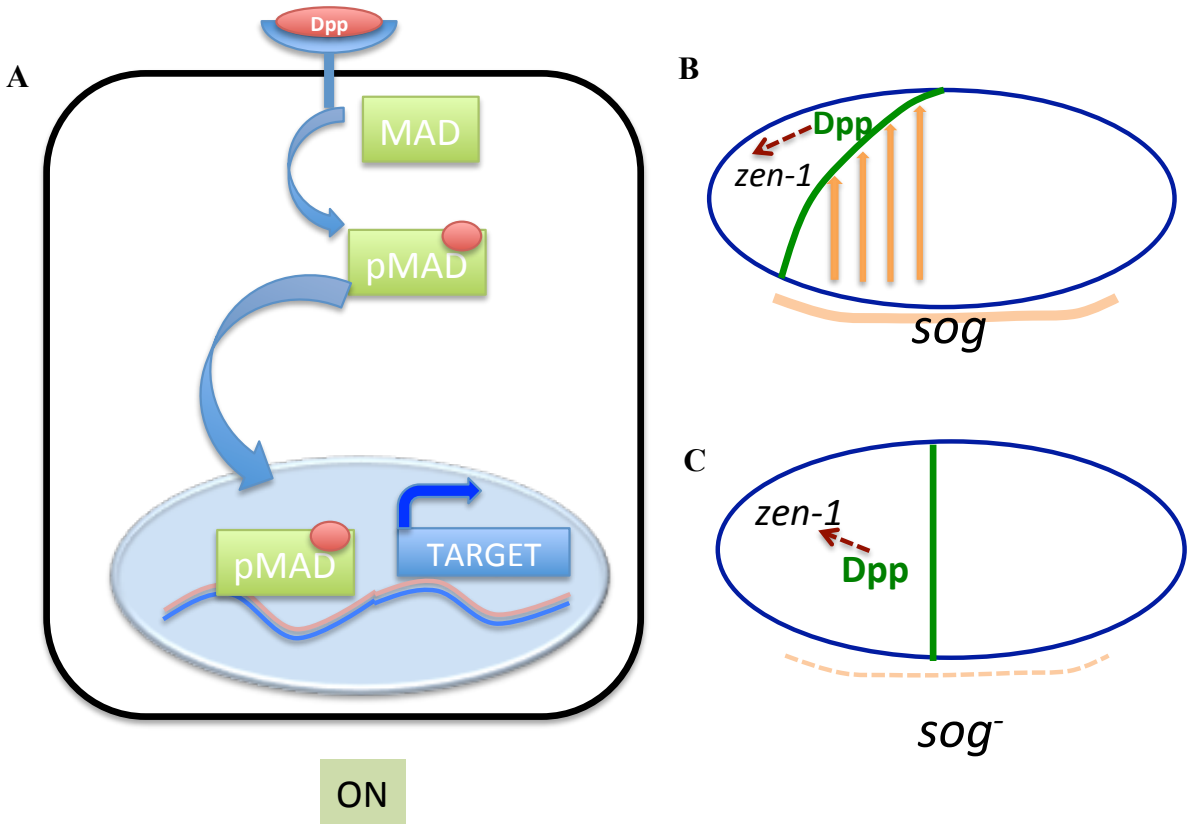


Figure 1-5 Potential regulation of Dpp signaling activity in the Tribolium blastoderm

Left: (A) In Tribolium, only one Tc-Dpp ligand and one receptor Tc-Tvk have been identified. Presumably, Tc-Dpp ligand binds to the receptor to phosphorylate the protein Mother against Dpp (pMAD). Then pMAD translocates into the nucleus to activate Dpp signaling target genes.

Right: In Drosophila, Dm-Sog is an antagonist of Dm-Dpp ligand that binds to Dm-Dpp and Dm-Screw heterodimers or Dm-Dpp homodimers to reduce Dpp activity in the ventral half of the embryo and also build a steep gradient in the dorsal-most cells of the embryo. (B) In Tribolium, the Tc-Dpp expression pattern can be similarly explained by interactions between Tc-Dpp and Tc-Sog. (C) In *Tc-sog* RNAi embryos, the posterior border of *Tc-dpp* expression domain is vertical. *Tc-zen-1* expression in the prospective serosal cells is also positively regulated by Dpp signaling.

Materials and methods

Strains and maintenance

Tribolium castaneum Ga-1 strain is a pest of stored grain. It has been used as a genetic model organism for developmental genetics and evolution of development studies. *Tribolium castaneum* was stored at room temperature in whole-wheat flour with 5% dried yeast.

RNA interference (RNAi)

Total RNA was extracted from five larvae using RNeasy Mini Kit (Qiagen). Then Tribolium cDNA was produced by reverse transcription PCR from larval RNA (SuperScript III, Invitrogen). The template of double-stranded RNA (dsRNA) was amplified by using gene-specific primers flanked by T7 primer sequence at the 5'-end, and was from 300pb to 1200pb base-pair long. This template was inserted into a Topo 4 vector. Constructed vector was transformed into *E.coli*. *E.coli* containing constructed vector was examined by primer pair of T7 and Sp6, or mix pairs of T7 or Sp6 with specific primers. dsRNA was then synthesized using the T7 megascript kit (Ambion) and purified using the Megaclear kit (Ambion). Concentration of dsRNA was determined by Nanodrop 1000 using the measure constant 45 for dsRNA (Bucher, 2005). dsRNA was mixed with injection buffer (5 mM KCl, 0.1 mM KPO_4, pH 6.8) in a 1:1 ratio of volume before injection.

For some genes, the template of their dsRNA was amplified by using gene-specific primers that were not flanked by T7. TOPO RNAi primers were used to produce dsRNA of them.

To examine specificity of RNAi effects, cuticles of RNAi embryos were observed and compared to known phenotypes reported in previous publications. For *Tc-hb*, *Tc-gt*, *Tc-Kr* and *Tc-mlpt*, different fragments within each gene coding region were used to make dsRNA. Only one region of each gene was chosen for later studies. RNAi

phenotypes of different regions of each gene were identical. To assess weak *Tc-cad* RNAi, *Tc-cad* expression was detected in *Tc-cad* RNAi embryos with different concentrations of *Tc-cad* dsRNA.

Parental RNAi and egg collection

Females were injected with dsRNA as previously described (Bucher et al., 2002). Different concentrations of dsRNA (5ng/ul - 2ug/ul) were used to generate different gene knockdown effects. According to previous studies in our lab as well as published paper, for most genes, 2ug/ul is sufficient to produce strong RNAi impacts. To analyze effects of weak *Tc-cad* expression levels, 5-10 ng/ul were used in *Tc-cad* RNAi experiments. After knocking down *Tc-axn* expression, egg laying was dramatically impacted. To produce enough embryos for experimental analysis, the concentration of *Tc-axn* dsRNA was reduced to 10ng/ul.

After injection, females were put on whole-wheat flour with 5% dried yeast at 30°C for 5 days to recover and to release eggs that developed before RNAi effects could occur. Then, females were transferred onto triple-sifted flour with 5% dried yeast at 23-24°C. To examine eggs at particular developmental stages after injection of dsRNA, eggs were collected after either one- or three-hours egg-laying time and then incubated at 23-24°C for a certain length of time (Incubation Time, IT). For general egg collection, not targeting a particular stage, eggs were collected every 72 hours to allow complete embryonic development and divided into two groups, one for cuticle preparation and one for prompt embryo fixation.

For timing gap gene expression, eggs were collected after 3hrs and put at 23-24°C to develop. Eggs were fixed after 14, 17, 21 and 23 hours incubation. Then eggs were fixed as described below

To compare cad gradients in embryos with different Wnt activity levels and wild type, one wild type control was set up for each gene dsRNA injection. Wild type and RNAi embryos were collected, fixed and stained in parallel.

Cuticle preparation

Eggs were collected and put at 30°C for 4-5 days, which is the time required for them to complete embryogenesis and form cuticles, if they could. Then, eggs were put into 75% lactic acid for at least 6 hours at 60°C to digest cell contents, leaving naked cuticle.

Egg fixation

Eggs were harvested and put into a fixation basket. Eggs were briefly washed with water to remove flour. Eggs were washed with 50% bleach for 1-2 min in a beaker to remove the chorion and then rinsed 1-2 min in running water. Briefly, dried eggs were fixed in 2 dram scintillation vials containing a biphasic solution of 3ml 1× PBS, 1ml paraformaldehyde solution (PFA) and 5ml heptane. Fixation vials were shaken at 200 rpm for between 30 min to 2 hrs. After shaking, eggs were at the interface. The bottom layer (PBS and PFA) was replaced with 5ml methanol and eggs were devitellinized by shaking the bottle vigorously for at least 30sec. Once the vitelline membrane is removed, the embryos sink to the bottom. If the vitelline membrane was difficult to remove, they were pulled through a 20G needle to mechanically devitelline them. Devitellinized eggs were collected in a new tube and washed 3 times with methanol for 5min on a rotatory platform at 35rpm. After washing, eggs were stored in methanol at -20°C for in situ hybridization and immunocytochemistry.

In situ hybridization and immunocytochemistry

Target sequence of genes were amplified and cloned into Topo 4 vector. Antisense of target sequences were obtained by using T7 with SP6 or T3 primers to preform PCR and used as the template for digoxigenin (DIG)-labeled antisense RNA

probes. Reaction between anti-DIG:: alkaline phosphatase (AP) antibody (Roche) and NBT/BCIP (BM purple, Roche) substrate produced a blue signal.

Mouse antibody to pMAD (a gift from Gines Morata) was recognized by secondary antibody anti-mouse::POD (ABC Kit, Vector), which reacts with the substrate diaminobenzidine (DAB) to produce a golden brown signal.

To examine the specificity of gene expression, sense strand ribo-probes were also produced following the same steps as for antisense probes. And embryos were stained in parallel with sense and antisense probes. Staining results were also compared to results in previous studies to ensure that probes produced expected gene-specific patterns.

Microscopy and imaging

Cuticles and embryos were observed by using Olympus BX50 microscope. Pictures were taken with Nikon Digital Camera DXM 1200F camera attached to Olympus BX 50 microscope using Nikon ACT-1 Version 2.62 software. Samples were optically sectioned and these layered images were assembled using MONTAGE software. For *Tc-cad* gradient analysis, embryos were flattened under a cover slide. The staining signal was converted into grey scale using ImageJ and then the staining intensity was detected and analyzed with ImageJ. Pictures were adjusted by Adobe Photoshop CS5.1.

Table 1-1 Gene names, abbreviation of gene names and primers used

Gene	Abbreviation of gene name	Abbreviation of protein	Primers
abdominal-A	*abd-A*	Abd-A	Not used
abdominal-B	*abd-B*	Abd-B	Not used
Adenomatous polyposis coli	*apc*	Apc	5'-TCC CTA ATT TGG TGG CTT CA 5'-CGA GTG TTG TTC GAT GCT GT
Antennapedia	*Antp*		Not used
armadillo	*arm*	Arm	Not used
arrow	*arrow*		Not used
Axin	*Axn*	Axn	(Fu et al., 2012)
bicoid	*bcd*	Bcd/Bicoid	Not used
brinker	*brinker*	Brk	Not used
cactus	*cact*	Cact/Cactus	Not used
caudal	*cad*	Cad	5'-TAA TAC GAC TCA CTA TAG GGT GAC AAG TGC GTG TGA CC 5'-TAA TAC GAC TCA CTA TAG GGT CTT GCC GAT TGC GTC C
caudal-related homeobox	*cdx*	Cdx	Not used
decapentaplegic	*dpp*	Dpp	5'-TCG CGA TGC GTT TGA ACA TG 5'-TGC CCA GAT ACA GCA TGG ACA T
deformed	*dfd*	Dfd	Not used
Dickkopf-1	*Dkk-1*	Dkk-1	Not used
dorsal	*dl*	Dl/Dorsal	Not used
dorsocross	*doc*	Doc	5'-TAA TAC GAC TCA CTA TAG GGG CAC TCG ATT TCC CAT TTT C 5'-TAA TAC GAC TCA CTA TAG GGC CTG CAG ACG GAG ATG ATA A

Gene	Abbreviation of gene name	Abbreviation of protein	Primers
frizzled	*frizzled*		Not used
fushi tarazu	*ftz*		Not used
giant	*gt*	Gt	5’-TAA TAC GAC TCA CTA TAG GGA CAC GCG AGA CTA CGA ATC C 5’-TAA TAC GAC TCA CTA TAG GGA ACT CGG AAG TCT CCA GCA A
hairy and enhancer of split	*Hes*		Not used
hunchback	*hb*	HB/ Hunchback	5'-TAA TAC GAC TCA CTA TAG GGA CGA CAA CCA GGA CCA CCT 5’-TAA TAC GAC TCA CTA TAG GGT TAG CAT GGA CTT GTC ACA CA
knirps	*kni*	Kni	Not used
Krüppel	*Kr*	Kr	5’-AGG TGT GCA GGA AGA TGG CG 5’-CAC GTC CTG CAG AAC CAC GA
labial	*lab*		Not used
legless	*lgs*	Lgs	5’-TAA TAC GAC TCA CTA TAG GGA TCA GAA GAA CAA CCA TCA TCT C 5’-TAA TAC GAC TCA CTA TAG GGG GTT CGG TGT TAC TGC TAT TTA C
Mex-3	Mex-3	Mex-3	Not used
mille-pattes	*mlpt*	Mlpt	5’-TAA TAC GAC TCA CTA TAG GGC CGA TAA AAT CGC CTC TTT G 5’-TAA TAC GAC TCA CTA TAG GGC AAC AAA TTA AGA AAA ACT GAG TGT CA
mother against dpp	*mad*	MAD/ pMAD	Not used
nanos	*nos*	Nos	Not used
orthodenticle	*otd*	OTD	5’-TAA TAC GAC TCA CTA TAG GGG AAC ATG CAA GGA TTT GTT AAG C 5’-TAA TAC GAC TCA CTA TAG GGT AGT TGT GTG AGG AGG TGT TGT G

Gene	Abbreviation of gene name	Abbreviation of protein	Primers
pangolin	*pan*	Pan	5'-TAA TAC GAC TCA CTA TAG GGA GAT GCG AGC G 5'-TAA TAC GAC TCA CTA TAG GGA GAT GGA CTG G
porcupine	*porc*		Not used
prosocipedia	*pb*		Not used
sex combs reduced	*scr*	Scr	Not used
Short gastrulation	*sog*	Sog	(van der Zee et al., 2006)
T-cell factor		TCF	Not used
toll	*toll*	Toll	Not used
tolloid	*tld*	Tld	Not used
twist	*twi*	Twi	Not used
Twisted gastrulation	*tsg*	Tsg	Not used
Ultraithorax	*ubx*	Ubx	Not used
Wingless	*wg*	Wg	Not used
wnt1	*Wnt1*	Wnt1	Not used
wnt8	*Wnt8*	Wnt8	Not used
zerknüllt	*zen*	Zen	5'-TAA TAC GAC TCA CTA TAG CAA CTT ACG AGT ATT ACG AG 5'- TAA TAC GAC TCA CTA TAG TTT GGC CGT TCC ACC CTT CC
			TOPO RNAi primers: 5'-TAA TAC GAC TCA CTA TAG GGC GAA TTC GCC CTT

Table 1-2 Insects names

Insects	Specie name	Abbreviation of insect name
Drosophila	***Drosophila melanogaster***	Dm
Gryllus	***Gryllus bimaculatus***	Gb
Nasonia	***Nasonia vitripennis***	Nv
Oncopeltus	***Oncopeltus fasciatus***	Of
Tribolium	***Tribolium castaneum***	Tc

Chapter 2 - The Tribolium blastoderm fate map is patterned by a mutual repression network regulated by Wnt and Dpp signaling

Abstract

In Drosophila through the segment gene hierarchy, expression of gap genes along the A-P axis regionalizes the blastoderm and determines the position and identity of different body tissues (regions). Therefore, expression of gap genes defines the Drosophila blastoderm fate map. Although Tribolium homologs of gap genes are also expressed along the blastoderm A-P axis, the function of gap genes is limited and more involved in the regulation of Hox genes to define segment identity. Instead, expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* labels cell fates in the blastoderm, which will develop into the serosa, head and trunk, respectively. Wnt signaling also affects posterior trunk formation through *Tc-cad* and anterior serosa through *Tc-zen-1*. Here, we closely examine the role of Wnt signaling in defining the Tribolium blastoderm fate map and found that Wnt signaling appears to be an activator of a Dpp signaling antagonist, *Tc-sog* in the anterior region of the blastoderm. To form serosa, Wnt signaling activity must be repressed in this region to allow activation of *Tc-zen-1*. We also discovered interactions between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* that are critical to patterning the Tribolium blastoderm fate map, and also appear to be regulated by the Wnt signaling activity gradient.

Introduction

A segmented body plan is a defining feature of arthropods. Segmentation is the critical criterion by which long germ and short germ insects are categorized. Long germ insects form all segments simultaneously in the blastoderm. In contrast, short germ insects add segments sequentially. Prior to segmentation, cell fates are already determined in the blastoderm of both long and short germ insects. In Drosophila, this blastoderm fate map is determined by the expression of gap genes, which are activated by maternal morphogen gradients. Mutual repression between gap genes regionalizes the blastoderm to pattern the head, thorax and abdomen along the A-P axis, while the extra embryonic tissue, the amnioserosa is restricted to the dorsal side of the egg. In Tribolium, extra-embryonic tissue, specifically the serosa, forms

anteriorly, followed by the germ band rudiment, containing the head and thorax, along the A-P axis. Comparative studies have shown that disruption of gap gene homolog expression does not always produce a corresponding gap in the Tribolium body plan (Bucher and Klingler, 2004; Cerny et al., 2005; Cerny et al., 2008). Moreover, maternal morphogen gradients similar to those functioning in Drosophila have yet to be reported in any non-dipteran insects (Brown et al., 2001). Expression of genes that mark the serosa, head and trunk respectively have been detected in the early Tribolium blastoderm, but the genetic interactions regulating this fate map have yet to be revealed. Expression of *Tc-zerknüllt-1* (*Tc-zen-1*) marks the prospective serosa, expression of *Tc-orthodenticle-1* (*Tc-otd-1*) marks the prospective pre-head and expression of *Tc-caudal (Tc-cad)* identifies the prospective thorax and posterior end of the embryo (Schroder, 2003; van der Zee et al., 2005; Wolff et al., 1998). Knockdown of these genes in the embryo results in the deletion of the corresponding serosa, head and trunk in the larvae.

Tc-cad is a downstream target of the Wnt signal in Tribolium, but not in Drosophila. Knockdown of Wnt ligands or a co-activator of the Wnt signaling pathway, *legless* (*lgs*) causes the reduction of *Tc-cad* expression in the blastoderm and truncation of germ band, suggesting similar regulation of *Tc-cad* genes by the Wnt signaling pathway during segmentation in Tribolium and vertebrates (El-Sherif et al., 2014). Homologs of *cad* in Tribolium, Oncopeltus, and Gryllus form a long-range gradient from the posterior, activating gap and pair rule gene expression in the thorax and abdomen (Liu and Kaufman, 2004b; Shinmyo et al., 2005). Depletion of *cad* in these insects produces severe truncation; only the head forms in the most severely affected embryos. In contrast, maternally expressed Drosophila *Dm-cad* encodes a long-range morphogen that represses posterior Hb expression, but its zygotic expression is limited to a narrow stripe in the posterior region of the embryo, where it regulates some pair-rule genes and hindgut formation. Thus *Dm-cad* plays a comparatively minor role in Drosophila segmentation (Mlodzik et al., 1985; Schulz and Tautz, 1995).

On the other hand, similar roles for the Wnt signaling pathway are also observed in the anterior regions of Tribolium and vertebrate embryos. In mouse, *dkk1* is expressed in the prospective head region to repress Wnt activity (Hashimoto et al., 2000; Lewis et al., 2008). In fish, Wnt signaling is also repressed anteriorly. Altering the Wnt signal in the future head region

causes the transformation between forebrain and midbrain (Hashimoto et al., 2000). In Tribolium, removal of negative regulators of the Wnt signaling pathway, *Tc-axin* (*Tc-axn*) causes reduction of *Tc-zen-1* expression and loss of anterior structures in a dose dependent manner (El-Sherif et al., 2014). Therefore, Wnt signaling must be repressed for proper head formation..

Tc-zen-1 is expressed in the extra embryonic tissue in both Drosophila and Tribolium. In Drosophila, the amnioserosa is the dorsal-most tissue and is regulated by the D-V patterning genes of the Dpp pathway (Entchev et al., 2000). Dm-Dpp proteins are localized in the dorsal half of the embryo to activate the dorsal fate determining genes (Nunes da Fonseca et al., 2010). Disruption of Dpp pathway components only impacts the D-V axis. In Drosophila, *Dm-zen-1* expression and the amnioserosa are missing in embryos lacking Dpp signaling (Rushlow et al., 2001). Both Tribolium and Drosophila require Dpp signaling to regulate gene expression along the D-V axis and form extra embryonic tissue. In Tribolium, perturbation of the Dpp signal produces abnormalities in both A-P and D-V axes. Furthermore, either up-regulating the Wnt signal or down-regulating the Dpp signal reduces the serosa region near the anterior pole of the egg (Fu et al., 2012; Nunes da Fonseca et al., 2008). In leg and wing development in Drosophila, *Dm-dpp* is a target of the Wnt signal, thus it is possible that Wnt signaling regulates Tribolium serosa formation through the Dpp signaling pathway (Campbell and Tomlinson, 1999; Takaesu et al., 2008). Alternatively, Dpp and Wnt signals may contribute to the formation of the serosa and define the blastoderm fate map independently.

We performed RNAi with *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* and found the expression of each of the other genes was affected, suggesting a network of predominantly mutual repressive interactions. In addition we performed RNAi against both positive and negative regulators of the Wnt signaling pathway and again found effects on the expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad*. Furthermore, various double RNAi combinations allowed us to determine the extent to which Wnt activity functions through *Tc-cad* to determine the blastoderm fate and the extent to which it influences other genes independently.

Results

In Drosophila, gap genes are activated and their expression domains positioned by a concentration gradient of Bicoid protein. However, refining boundaries depends on mutual repression between gap genes (Lynch et al., 2012). In Tribolium, previous results suggest *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* determine the blastoderm fate map (Copf et al., 2004; Schroder, 2003; van der Zee et al., 2005). However, interactions between them are still unclear, which may be also primarily via mutual repression. To test this hypothesis, we examined the expression of these genes in different RNAi backgrounds that altered the fate map.

First we examined the expression of *Tc-otd-1* in *Tc-zen-1* and *Tc-cad* RNAi embryos. In the differentiated blastoderm, *Tc-otd-1* is expressed in the pre-gnathal head region (Figure 2.1 B). We hypothesized that the anterior boundary of *Tc-otd-1* expression is positioned by *Tc-zen-1* expression while the posterior boundary is positioned by *Tc-cad* expression. According to this hypothesis, reduction of *Tc-cad* or *Tc-zen-1* expression should cause expansion of the *Tc-otd-1* expression domain. In *Tc-cad* RNAi embryos, the *Tc-otd-1* expression domain expanded all the way to the posterior pole (Figure 2.1 H). While in *Tc-zen-1* RNAi embryos, which lack extra embryonic serosa, *Tc-otd-1* expression extended to the anterior pole (Figure 2.1 E). These shifts in *Tc-otd-1* expression imply that is it repressed anteriorly by *Tc-zen*-1 and posteriorly by *Tc-cad*, consistent with the hypothesis.

Next we examined the expression of *Tc-zen-1* in *Tc-otd-1* and *Tc-cad* RNAi embryos. If the domain of *Tc-zen-1* expression is regulated by *Tc-otd-1,* we might expect it to expand posteriorly when *Tc-otd-1* expression is reduced. However, previous studies indicated that the *Tc-zen-1* expression domain was reduced in *Tc-otd-1* RNAi embryos, suggesting that *Tc-otd-1* actually activates *Tc-zen-1* (Figure 2.1 D). We observed even stronger effects, suggesting *Tc-otd-1* activates *Tc-zen-1* dorsally as well as anteriorly. On the dorsal side of wild type embryos, *Tc-zen-1* expression almost abuts the *Tc-cad* expression domain. The serosa is greatly expanded in *Tc-cad* RNAi embryos. Dorsally, *Tc-zen-1* expression in the differentiated blastoderm expanded to the posterior pole of the egg, while there is little or no change on the ventral side, suggesting *Tc-cad* represses *Tc-zen-1* mainly on the dorsal side of the egg (Figure 2.1 G). Since knockdown of *Tc-cad* also impacted *Tc-otd-1* expression, which may cause changes in *Tc-zen-1* expression,

double knockdown of *Tc-otd-1* and *Tc-cad* was applied to examine whether interaction between *Tc-cad* and *Tc-zen-1* is *Tc-otd-1* independent. In *Tc-otd-1* RNAi embryos, *Tc-zen-1* expression was dramatically reduced. In *Tc-otd-1*;*Tc-cad* RNAi embryos, expression of *Tc-zen-1* on the dorsal side of the embryos was rescued (Figure 2.1 J), which indicates that *Tc-cad* repressed *Tc-zen-1* on the dorsal side of the embryos.

Finally we examined the expression of *Tc-cad* in *Tc-otd-1* and *Tc-zen-1* RNAi embryos. When *Tc-otd-1* was reduced, the *Tc-cad* expression domain expanded anteriorly, suggesting *Tc-otd-1* is required to properly position the *Tc-cad* gradient in the blastoderm fate map (Figure 2.1 I). In *Tc-zen-1* RNAi embryos, *Tc-cad* expression also expanded anteriorly (Figure 2.1 F). Thus, interactions between the zygote expression of these three genes contributed to the fate map of the differentiated blastoderm. *Tc-otd-1* expression is restricted to the presumptive head lobes by zygotic expression of *Tc-zen-1* in the serosa and *Tc-cad* in the posterior half of the blastoderm. The *Tc-cad* gradient is positioned by *Tc-otd-1* and *Tc-zen-1*. *Tc-zen-1* expression and the extent of the serosa are primarily determined by *Tc-otd-1* with a dorsal contribution by *Tc-cad*. The regulatory interactions appear to be predominantly mutual repression with the exception of the fact that early *Tc-otd-1* expression activates *Tc-zen-1*(Figure 2.1 K).

Input of Wnt activity into the blastoderm fate map gene network

Since *Tc-cad* is a target of Wnt signaling, and a Wnt activity gradient was recently found to be essential for proper A-P development in Tribolium, we examined the effects of knocking down several key components of the Wnt signaling pathway on the formation of the blastoderm fate map. *Tc-axn mRNA,* which encodes a repressor of Wnt signaling, is localized to the anterior pole of Tribolium egg (Fu et al., 2012). *Tc-pan* mRNA is also localized to the anterior pole of newly laid eggs, but is ubiquitously expressed later (Bucher et al., 2005). As a transcription factor, Pan binds to Beta-catenin and other co-activators to trigger the expression of downstream target genes, such as *cad*. Without Beta-catenin, Pan interacts with repressors to repress Wnt target genes. *legless* (*lgs),* one of the co-activators, is required for activation of Wnt target genes (El-Sherif et al., 2014). Depleting *Tc-axn* or *Tc-apc-1* relieves repression of Wnt target genes increasing their expression; while depletion of *Tc-lgs* reduces activation of Wnt target genes,

lowering their expression. On the other hand, in *Tc-pan* RNAi embryos, anterior Wnt target genes are not fully repressed, while posterior Wnt target genes are not fully activated.

In Tribolium, knocking down these three key Wnt pathway components altered the expression of *Tc-cad* as expected for a Wnt target gene. In *Tc-axn* RNAi embryos, *Tc-cad* mRNA expression expends anteriorly (El-Sherif et al., 2014). The same result was also observed in *Tc-apc-1* RNAi embryos (Figure 2.2 F). In contrast, in *Tc-lgs* RNAi embryos, *Tc-cad* expression was much weaker than in wild type and restricted to the posterior pole (Figure 2.2 L). Knockdown of *Tc-pan* expression produced an anterior expansion in the *Tc-cad* expression gradient, due to de-repression of the Wnt pathway in the anterior embryo, as in *Tc-apc-1* RNAi embryos (Figure 2.2 I).

Since interactions between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* affected their expression, changes in Wnt signaling that alter the *Tc-cad* expression gradient, should also affect the blastoderm fate map, impacting the *Tc-zen-1* and *Tc-otd-1* expression domains. Therefore, we also examined the expression of *Tc-zen-1* and *Tc-otd-1* in *Tc-apc-1*, *Tc-pan* and *Tc-lgs* RNAi embryos. *Tc-otd-1* expression domain shifted anteriorly in *Tc-apc-1* (Figure 2.2 E) and *Tc-pan* (Figure 2.2 H) RNAi embryos. *Tc-zen-1* expression was restricted in the anterior pole of *Tc-apc-1* RNAi embryos (Figure 2.2 D). In *Tc-pan* RNAi embryos, *Tc-zen-1* expression was detected in two separate domains (Figure 2.2 G): One was in the anterior pole of the embryos as in *Tc-apc-1* RNAi embryos; the other was on the dorsal side of *Tc-pan* RNAi embryos. In *Tc-lgs* RNAi embryos, *Tc-otd-1* expression domain shifted posteriorly similar to that in *Tc-cad* RNAi embryos (Figure 2.2 K). The expression of *Tc-zen-1* expanded posteriorly on both the ventral and dorsal sides of the embryo (Figure 2.2 J), compared to dorsal only expansion in *Tc-cad* RNAi embryos (Figure 2.1 G). In both cases, reduction of *Tc-cad* expression is associated with a posterior extension of *Tc-zen-1* expression on the dorsal side of the embryo. However, on the ventral side, the posterior extension of *Tc-zen-1* expression was observed in *Tc-lgs* RNAi embryos, but not in *Tc-cad* RNAi embryos, suggesting that the Wnt pathway regulates *Tc-zen-1* expression independent of *Tc-cad* here.

In addition to the Wnt signaling pathway, the Dpp signaling pathway also regulates *Tc-*

zen-1 expression and serosa formation in the Tribolium blastoderm. The alteration of *Tc-zen-1* expression can be caused by changes in Dpp signaling activity in *Tc-apc-1*, *Tc-pan* and *Tc-lgs* RNAi embryos. Therefore, we further examined the expression of several components of Dpp signaling pathway in these RNAi embryos to analyze the interaction between Wnt and Dpp signaling.

Changes in Wnt activity impact the Dpp signaling pathway

In Tribolium, knockdown of *Tc-dpp* expression produces a smaller serosa, which is indicated by the ectopic expression of *Tc-dorscross* (*Tc-doc*), a downstream target gene of Dpp signaling (Nunes da Fonseca et al., 2010). In Drosophila wings and legs, *Dm-dpp* expression is regulated by both Wnt and Dpp signaling pathways, and interactions between them provide determinative information to establish polarity in wings and legs (Morel and Arias, 2004). In vertebrates, the crosstalk between them is also important during embryogenesis (Eivers et al., 2009; Henriquez et al., 2011; Tzahor et al., 2003). Therefore, we considered the hypothesis that the Wnt signaling pathway regulates serosa formation through the Dpp signaling pathway. To examine whether the Wnt pathway impacts the Dpp signaling pathway, we checked the expression of *Tc-doc,* which initiates in the dorsal serosa in wild type (Figure 2.3 A). In *Tc-axn* RNAi embryos with up-regulated Wnt signaling in the anterior, the expression of *Tc-doc* mRNA was dramatically weakened, and hardly detectable on the dorsal side of the blastoderm (Figure 2.3 B). The difference between *Tc-doc* expression in *Tc-axn* RNAi and wild type embryos suggests that the Wnt signaling pathway impacts the Dpp signaling pathway and not only *Tc-zen-1*.

To further study the impact of altered Wnt signaling activity on the Dpp signaling pathway, we studied distribution of phosphorylated Mother-against-Dpp (pMAD) in different Wnt activity backgrounds. pMAD is the transducer of Dpp signal and required to trigger Dpp target gene expression (Fujise et al., 2003). Although, the regulation of pMAD distribution by Dpp signaling is still not fully elucidated in Tribolium, Dpp has been shown to activate pMAD in Tribolium (Kotkamp et al., 2010). We used pMAD antibody to visualize the target sites of the active Dpp signaling in Tribolium. In wild type, pMAD protein is distributed broadly on the dorsal side and in posterior end of the undifferentiated blastoderm (Figure 2.4 A). After

increasing Wnt activity by knockdown *Tc-axn* expression, pMAD was detected on the dorsal side of the embryo as well as in the posterior end of the embryo. However, the pMAD antibody signal was extremely weak in the anterior-dorsal region of the egg (Figure 2.4 B). This reduced pMAD protein signal suggests that the Dpp signal was weaker in the anterior end of blastoderm, which might also explain the reduced expression of *Tc-doc* in these embryos. Distribution of pMAD should be also affected in *Tc-pan* RNAi embryos if the Wnt signal represses pMAD expression anteriorly. Indeed, pMAD was dramatically reduced in the anterior end of the egg, but distributed normally along the dorsal side of embryo as in wild type (Figure 2.4 C). In contrast, in *Tc-lgs* RNAi embryos, pMAD protein expression resembles that in wild type (Figure 2.4 D). Therefore, changes in pMAD distribution in these RNAi embryos are correlated to changes in Wnt signaling activity. Since pMAD distribution reflects the active sites of Dpp signaling, the alteration of pMAD distribution may result from the change of Dpp expression.

Tc-dpp expression is very dynamic in wild type Tribolium embryos. In the syncytial blastoderm, the expression of *Tc-dpp* is nearly ubiquitous (Figure 2.5 A). Soon thereafter, enhanced expression appears at the anterior pole and expands over the dorsal side of the egg (Figure 2.5 B). Later, *Tc-dpp* is only expressed in the presumptive anterior amnion (Figure 2.5 C) (Sharma et al., 2013). In *Tc-apc-1, Tc-pan* and *Tc-lgs* single RNAi embryos, the initial expression of *Tc-dpp* was still ubiquitously with strong short-range gradient in the anterior pole of the eggs (Figure 2.5 D, G, J). However, *Tc-dpp* expression was affected at later stages by changes in Wnt activity. In *Tc-apc-1* RNAi embryos, the dorsal extension of *Tc-dpp* expression was greatly restricted relative to wild type (Figure 2.5 E) In most *Tc-apc-1* RNAi embryos, no extension was detected. Then, *Tc-dpp* expression faded from the anterior end and formed a vertical ring on the D-V axis abutting the embryonic anlage that was shifted anteriorly (Figure 2.5 F). In *Tc-pan* RNAi embryos, the initial expression of *Tc-dpp* was similar to both wild type and *Tc-apc-1* RNAi embryos. But later the expression of *Tc-dpp* was stronger only at the anterior pole (Figure 2.5 H). Finally as the blastoderm differentiated, the expression of *Tc-dpp* was detected as a ring around the anterior pole of the egg separating the anterior serosa from the embryo rudiment (Figure 2.5 I). In *Tc-lgs* RNAi embryos, the expression of *Tc-dpp* initiated as in wild type but then expanded more dramatically both dorsally and laterally, resulting in a larger region of *Tc-dpp* expression than in wild type (Figure 2.5 K). Later, the expression of *Tc-dpp*

was limited to a narrow region separating the embryo from the serosa (Figure 2.5 L).

A notable change in these embryos was in the width of the *Tc-dpp* expression domain in the ventral-lateral regions of the egg. In wild type, the *Tc-dpp* expression domain is wider ventrally than laterally. But in *Tc-apc-1* and *Tc-pan* RNAi embryos, no difference appeared between the ventral and lateral expression domains, which were both narrower than in wild type (Figure 2.5 F, I). On the other hand, the ventral *Tc-dpp* expression domain was wider in *Tc-lgs* RNAi embryos than in wild type. As a final comparison, the expression of *Tc-dpp* in *Tc-cad* RNAi embryos resembled the expression of *Tc-dpp* in wild type (data not shown). These results showed that the expression of *Tc-dpp* was altered by different levels of Wnt activity, mostly likely independent of the level of *Tc-cad* expression.

In Drosophila, Dm-Sog is an antagonist of Dpp signaling pathway, which transports Dpp ligands dorsally to form a Dpp gradient in the dorsal side of the embryo to determine the dorsal fate map. Although, there is no direct no protein binding evidence in Tribolium, interactions between Sog and Dpp appears to be conserved. However, the expression pattern of *Tc-sog* is quite different in these two holometabolous insects. In Drosophila, *Dm-sog* is activated by Dorsal (Dl) in ventrolateral regions of the embryo, between the dorsal expression of *Dm-dpp* and ventral expression of *Dm-twist* (*Dm-twi*). In Tribolium, *Tc-sog* is expressed in a wide domain along the ventral midline, excluding the anterior region where *Tc-zen-1* is expressed (Figure 2.6 A). At the later blastoderm stages, *Tc-sog* expression fades in the anterior region of its expression domain and is relatively stronger expressed on the ventral side of the head region in the embryos (Figure 2.6 B) (Nunes da Fonseca et al., 2008).

Changes in the level of Wnt activity affected the expression of *Tc-sog*. Depletion of *Tc-apc-1 or Tc-pan* expression resulted in the ectopic anterior expression of *Tc-sog.* In addition to expression on the ventral side of the embryo, *Tc-sog* was anteriorly expressed in the lateral and dorsal regions of *Tc-apc-1* RNAi embryos, forming a vertical ring around the anterior pole (Figure 2.6 E). This ectopic anterior expression of *Tc-sog* did not fade at the later blastoderm stages (Figure 2.6 F). In *Tc-pan* RNAi embryos, *Tc-sog* expression was expanded anteriorly, approaching the anterior pole of the embryo, consistent with the anterior shift of the germ

rudiment (Figure 2.6 G). As in *Tc-apc-1* RNAi embryos, anterior fade of *Tc-sog* expression was not observed in *Tc-pan* RNAi embryos (Figure 2.6 H). While depletion of *Tc-lgs* shifted the *Tc-sog* expression domain posteriorly, correlated with the posterior shift of the head (Figure 2.6 C). Later, *Tc-sog* expression was reduced anteriorly in *Tc-lgs* RNAi embryos as that in wild type (Figure 2.6 D).

Changes in *Tc-sog* expression in *Tc-lgs* RNAi embryos can explain the different impacts of *Tc-lgs* and *Tc-cad* on *Tc-zen-1* expression. In *Tc-lgs* RNAi embryos, shifts in *Tc-sog* expression resulted in the expansion of *Tc-dpp* expression domain, which led to the ectopic posterior expression of *Tc-zen-1* on the ventral side of the embryos. On the contrary, knockdown of *Tc-cad*, *Tc-sog* expression was not affected. Hence, *Tc-zen-1* expression on the ventral side of *Tc-cad* RNAi embryos was not impacted. Therefore, Wnt activity was up-regulated in the *Tc-apc-1* or *Tc-pan* RNAi embryos and activated ectopic *Tc-sog* expression in the anterior region, where Tc-Sog limited Dpp signaling. The reduced Dpp signaling in this region resulted in reduced *Tc-zen-1* expression and serosa formation. And the anterior border of *Tc-sog* expression domain defines the boundary between extra-embryonic and embryonic tissues.

Serosa formation in the Tribolium blastoderm

In the Tribolium blastoderm, serosal cells can be indicated by *Tc-zen-1* expression, which is regulated by the Wnt and Dpp signaling pathways as well as *Tc-cad* and *Tc-otd-1.* In *Tc-pan* RNAi embryos, the serosa was split into two domains, anterior and dorsal (Figure 2.7 B). To further understand the formation of two separate serosa regions in *Tc-pan* RNAi embryos, *Tc-zen-1* expression was examined in *Tc-pan;Tc-otd-1* double RNAi embryos. Previous studies have shown that *Tc-zen-1* expression in the anterior serosa is activated by *Tc-otd-1* (Kotkamp et al., 2010). Thus, depleting *Tc-otd-1* in addition to *Tc-pan* should reduce or eliminate anterior *Tc-zen-1* expression. As expected, only a dorsal domain of *Tc-zen-1* expressing cells was found in *Tc-pan;Tc-otd-1* double RNAi embryos (Figure 2.7 C). Furthermore, we knocked down *Tc-apc-1* and *Tc-otd-1* to analyze the source of the remaining serosa in *Tc-apc-1* RNAi embryos. If the serosa in *Tc-apc-1* RNAi embryos is also dependent upon *Tc-otd-1,* as in *Tc-pan* RNAi embryos, then in *Tc-apc-1;Tc-otd-1* double RNAi embryos, this region should be greatly reduced or

absent. Indeed, in *Tc-apc-1;Tc-otd-1* double RNAi embryos, the serosa was greatly reduced (Figure 2.7 F). Hence, although in *Tc-pan*, *Tc-axn* or *Tc-apc-1* RNAi embryos, *Tc-zen-1* expression was observed in the anterior region, where pMAD activity was dramatically reduced, This *Tc-zen-1* expression was activated by *Tc-otd-1* not through the Dpp signaling pathway.

On the other hand, serosa on the dorsal side of *Tc-pan* RNAi embryos is not due to *Tc-otd-1*, but may be regulated by *Tc-cad*. Although the *Tc-cad* expression domain shifted anteriorly in embryos lacking either *Tc-apc-1* or *Tc-pan*, the expression level of *Tc-cad* was not same as wild type in *Tc-pan* RNAi embryos. Comparison of *Tc-apc-1* and *Tc-pan* RNAi embryos, each stained in parallel with wild type embryos, revealed that *Tc-cad* mRNA expression, although expanded toward the anterior, is greatly reduced in *Tc-pan* RNAi embryos (El-Sherif et al., 2014). Lower *Tc-cad* levels in *Tc-pan* RNAi embryos might explain the observed differences in serosa formation. Similar reduction of the anterior serosa in *Tc-pan* and *Tc-apc-1* RNAi embryo was most likely due to the anterior expansion of the *Tc-cad* gradient, while absence of dorsal serosa in *Tc-apc-1* RNAi is likely due to the higher levels of *Tc-cad* expression there than in *Tc-pan* RNAi embryos. Thus, we knocked down both *Tc-axn* and *Tc-cad* to produce the *Tc-cad* expression as that in *Tc-pan* RNAi embryo. In these double RNAi embryos, *Tc-zen-1* was expressed in two separate domains as in *Tc-pan* RNAi embryos. Therefore, the higher *Tc-cad* expression in *Tc-apc-1* appears to repress the dorsal expression of *Tc-zen-1*, which is consistent with previous results. Therefore, a moderate level of *Tc-cad* expression is critical to the serosa formation and *Tc-zen-1* expression on the dorsal side of the Tribolium blastoderm embryos, which is also regulated by Wnt signaling activity.

Discussion

Most comparative studies have highlighted the differences in segmentation between Drosophila and non-dipteran insects. Before the onset of segmentation, regions of the blastoderm fated to segment are already genetically patterned. Our examination of the Tribolium fate map revealed additional differences between the long germ mode of development displayed by Drosophila and the short germ mode of development displayed by Tribolium.

A mutual repression gene network and Wnt activity determine the Tribolium blastoderm fate map

In Drosophila, a network of mutual inhibition between gap genes regionalizes the blastoderm. In Tribolium, instead of gap genes, the expression of three genes, *Tc-zen-1, Tc-otd-1* and *Tc-cad* regionalize the blastoderm. Maintaining the expression of these genes is important to determining the boundary between serosa and embryonic rudiment, as well as between pre-gnathal head and the rest of the body. In this study, we found that although gap genes are not responsible for the regionalization in Tribolium blastoderm, boundary formation between gene expression domains is still essential to determine the aforementioned boundaries in the Tribolium blastoderm, through a network of mutual repressive interactions. This mutual repression network senses the gradient of Wnt activity. *Tc-cad* is the target gene of Wnt signaling, which is activated in the posterior half of the egg (Wolff et al., 1998). To form serosa, marked *by Tc-zen-1* expression, requires the abolishment of the Wnt activity in the anterior region of the egg (Fu et al., 2012). Increasing Wnt activity in the anterior or decreasing Wnt activity in the posterior of the egg shifts the fate map along the A-P body axis through the mutual repression network. Moreover, this mutual repression interaction between *Tc-otd-1, Tc-zen-1* and *Tc-cad*, restricts the expression of *Tc-otd-1* to the pre-gnathal head region. Although regulation of *Tc-otd-1* by Wnt activity was not detected in our study, the possibility cannot be ruled out. Furthermore, Wnt activity also impacts the expression of the ventral marker gene, *Tc-sog*. When Wnt activity is increased, ectopic expression of *Tc-sog* near the anterior pole ultimately redefines the domain of Dpp activity and affects the size and orientation of the serosa. Together, these results indicate that the Wnt signaling activity gradient from posterior to anterior is important to patterning the fate map in Tribolium blastoderm (Figure 2.8).

Tc-otd-1 functions as a general activator in the early blastoderm

Tc-otd-1 appears to contribute to the blastoderm fate map in Tribolium at different levels. Drosophila Bcd is a maternal morphogen that patterns the anterior region of the embryo. Bcd possesses a K50 homeodomain, which is thought to be essential proper binding to DNA or RNA (Baird-Titus et al. 2006; Ma et al., 1996; Zhang et al., 2005)). This K50 site is also present in the homeodomain of Otd-1, which implies a potential role similar to Drosophila Bcd. In Nasonia,

Nv-otd-1 and *Nv-hb* together function as Bcd does in Drosophila, patterning most regions from the anterior to posterior of the body (Lynch et al., 2006). Tribolium *Tc-otd-1* regulates several anterior gap genes. Parental *Tc-otd-1* RNAi produces more severe impacts than embryonic RNAi; cuticles lack all head and the first thoracic segment in the most severe *Tc-otd-1* RNAi phenotype (Schroder, 2003). Therefore, maternal *Tc-otd-1* may also provide input to fate map determination in Tribolium as an early general activator of several genes.

Maternal *Tc-otd-1* is expressed uniformly throughout the egg. Soon thereafter, expression is observed only in the anterior region of the egg, covering the presumptive serosa and pre-gnathal head. Somewhat later, in the blastoderm, the expression of *Tc-otd-1* fades at the anterior pole and is restricted to the pre-gnathal head (Li et al., 1996). This dynamic expression of *Tc-otd-1* can be explained by the repressive interactions between *Tc-otd-1*, *Tc-zen-1* and *Tc-cad*, in that the *Tc-otd-1* expression domain shifted in both *Tc-zen-1* (anteriorly) and *Tc-cad* (posteriorly) RNAi embryos. However, closer examination suggests that the situation is more complicated since *Tc-zen-1* and *Tc-cad* expression domains do not expand exactly as expected in *Tc-otd-1* RNAi embryos. In fact, the *Tc-zen-1* domain is greatly reduced, and *Tc-cad* expression though expanded is reduced in intensity. These results suggest that an earlier function of *Tc-otd-1* is to activate both *Tc-zen-1* and *Tc-cad*. Once activated, *Tc-cad* and *Tc-zen-1* in turn, repress *Tc-otd-1* in the posterior and the anterior, respectively to generate the blastoderm fate map.

Conserved functions of cad in short-germ insects, which are reduced in Drosophila

The function of *Dm-cad* in Drosophila is restricted posteriorly compared to its homologs in vertebrates and other insects. Maternal Cad contributes to the establishment of the zygotic HB gradient from the anterior to posterior (Schulz and Tautz, 1995). *Dm-cad* zygotic expression is limited to a narrow stripe near to the posterior end of the embryo to regulate several posterior stripes of pair-rule genes. In contrast, in vertebrates, *cdx* genes are expressed in the posterior end of germ band and contribute to germ band elongation (Chawengsaksophak et al., 2004; Gaunt et al., 2008; Lohnes, 2003; Pilon et al., 2006; Prinos et al., 2001). Meanwhile, these *cdx* genes regulate downstream Hox genes to pattern cell fates along the A-P body axis (Young et al., 2009). In several other insects including Tribolium, Gryllus and Oncopeltus, *cad* is expressed in

a posterior to anterior gradient in the posterior half of the embryo (Shinmyo et al., 2005; Wolff et al., 1998). The *cad* gradients in these non-dipteran insects function as the posterior core determinator to regulate gap and pair-rule gene expression, and therefore, control the posterior fate of the embryos. Disruption of the *cad* gradients in these insects results in the absence of posterior gap and pair-rule genes expression leading to truncation. The extent of truncation corresponds to the extent of disruption of *cad* expression. In another basally branched long-germ insect Nasonia, the *cad* gradient is located in the middle of the blastoderm. In the Nasonia *Nv-cad* mutation and RNAi embryos, the expression of gap genes in this region is abolished, which also suggests activation of gap genes by *Nv-cad*. Moreover, the segment polarity genes are also affected in these *Nv-cad* minus embryos (Olesnicky et al., 2006). Therefore, in non-dipteran insects, *cad* functions upstream of gap genes and appears to act as the organizer of the mid-to-posterior embryo.

In our study, we found evidence that *Tc-cad* contributes to dorsal patterning as well as posterior patterning. Both extra embryonic tissues form on the dorsal side of the blastoderm embryo, serosa anteriorly and amnion posteriorly. *Tc-zen-1* is expressed in the serosa and when *Tc-cad* is reduced, as in *Tc-cad* RNAi embryos, *Tc-zen-1* expression extends posteriorly, indicating that the fate of these cells is converted from amnion to serosa. Reduced *Tc-cad* expression in *Tc-lgs* RNAi embryos also leads to additional dorsal serosa in the posterior egg. In contrast, increasing *Tc-cad* expression anteriorly reduces the amount of serosa formed. In both *Tc-apc-1* and *Tc-axn* single RNAi embryos, anterior Wnt activity is increased, the *Tc-cad* expression domain expands anteriorly and the serosa forms only at the anterior pole. However, in *Tc-pan* RNAi embryos, *Tc-zen-1* expressing serosa forms on the dorsal side as well as at the anterior pole. In these embryos, Wnt activity is increased anteriorly and reduced posteriorly, leading to ectopic anterior *Tc-cad* expression, but at reduced intensity compared to *Tc-axn* or *Tc-apc-1*. Reducing *Tc-cad* in the *Tc-axn* RNAi background produced similar results. Thus, lower or no *Tc-cad* expression appears to be permissive for dorsal serosa formation, at least when anterior Wnt activity is up-regulated. In addition, the repressive interaction between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* helps restrict *Tc-cad* expression to the posterior half of the egg, where no serosa is formed. Therefore, in Tribolium, *Tc-cad* function is required for proper determination of both embryonic and extra-embryonic tissues in the posterior blastoderm, while elimination of *Tc-cad*

in the anterior embryo is crucial for serosa formation there.

Dpp signaling pathway contributes to the blastoderm fate map

In Drosophila extra embryonic tissues are reduced to the amnioserosa, which is located dorsally and determined by the D-V patterning system. In Tribolium, formation of extra embryonic tissues, serosa anteriorly and amnion posteriorly, requires input from both A-P and D-V patterning systems.

The function of Dpp signaling in D-V patterning in Tribolium is different than in Drosophila

In Tribolium, Dpp signaling is essential to the developmental of anterior as well as dorsal cell fates. The expression of *Tc-dpp* is quite dynamic and somewhat different than in Drosophila. In Tribolium *Tc-dpp* is first expressed ubiquitously throughout the entire embryo with slightly stronger expression anteriorly. Then, it is expressed anteriorly in the presumptive serosa (Sharma et al., 2013; van der Zee et al., 2006). Although there is only one known ligand, Dpp, in Tribolium, the interaction between Tc-Dpp and its antagonists Tc-Sog and Tc-Tsg seems to be conserved. Reduction of *Tc-dpp*, *Tc-sog* or *Tc-tsg* expression results in the loss of D-V polarity (Nunes da Fonseca et al., 2010). As *Tc-dpp* expression is depleted, the dorsal-most serosa and posterior amnion take on embryonic fates. A larger head forms due to transformation of additional serosal cells to embryonic fate. In contrast, knockdown of *Tc-sog* or *Tc-tsg* results in a larger serosa in addition to embryonic transformation of the posterior amnion (Nunes da Fonseca et al., 2010).

That early ubiquitous expression of *Tc-dpp* presumably determines the Dpp activity gradient, evident as pMAD expression on the dorsal side of the embryo, to pattern the extra-embryonic tissues: serosa and posterior amnion. The later slightly stronger anterior expression of *Tc-dpp* generates an oblique boundary between serosa and embryonic rudiment.

In Drosophila, both distribution and intensity of pMAD correlate closely with the expression of *Dm-dpp*. In Tribolium, as in Drosophila, Dpp signaling results in the phosphorylation of MAD, and higher expression of pMAD indicates higher Dpp activity.

However, in the Tribolium blastoderm fate map, expression of *Tc-dpp* does not totally overlap the distribution of pMAD (van der Zee et al., 2006). The dorsal pMAD gradient extends into the presumptive poster amnion where *Tc-dpp* is no longer expressed. It is likely that formation of this pMAD gradient relies on the earlier ubiquitous expression of *Tc-dpp*. When *Tc-dpp* is expressed throughout the entire blastoderm, *Tc-sog* is expressed on the ventral side of the embryonic rudiment (van der Zee et al., 2006). Theoretically, Tc-Sog binds Tc-Dpp dimers and transports them to the dorsal-most side of the egg. Tc-Dpp accumulates dorsally are released from Tc-Sog by Tc-Tld. Then, Tc-Dpp interacts with receptors to activate Dpp pathway and phosphorylate MAD, forming a dorsal gradient of pMAD that determines dorsal cell fates there.

However, proper formation of the serosa embryo boundary in Tribolium requires Dpp activity. The early ubiquitous Dpp expression is slightly higher anteriorly, and changes in the ratio of Dpp dimers to Sog along the A-P axis may determine the orientation of the boundary between serosa and embryonic rudiment. Higher Dpp expression might provide more Dpp dimers that readily saturate Sog, limiting the ability of Sog to clear Dpp only from the ventral side of the egg. Lower Dpp expression might lead to more gradual saturation of Sog, allowing Dpp dimers farther from the ventral midline to be transported dorsally. In more posterior regions, Sog levels are sufficient to transport Dpp dimers even farther dorsally. Therefore, more anteriorly, Dpp signaling is activated more ventrally, and posteriorly, Dpp signaling is activated more dorsally, forming an anterior Dpp activity domain with a tilted boundary between the serosa and the embryo.

Interestingly, Wnt component RNAi embryos, in which the serosa is greatly impacted, still display normal D-V polarity posteriorly. This can be explained by the fact that the early ubiquitous expression of *Tc-dpp* was not significantly altered in these embryos. The pMAD gradient formed normally for the most part, leading to the normal formation of the posterior amnion and other dorsal features.

Thus Dpp activity and D-V patterning contribute to the Tribolium blastoderm fate map at several levels. First, the initial ubiquitous expression of *Tc-dpp* appears to be essential to determine the fate of posterior amnion in the blastoderm stage. The stronger anterior expression

of *Tc-dpp* could be prerequisite to the formation of the oblique border between serosa and embryonic tissue. The expression of *Tc-dpp* in the presumptive serosa region is likely to contribute to activation *Tc-zen-1* expression. However, the final pattern of *Tc-zen-1* expression in the fate map appears to be impacted by additional genes, including *Tc-otd-1* and *Tc-cad* through the mutual repression network.

The role of Wnt activity in Tribolium blastoderm fate map

In Drosophila, no signaling pathway influences the blastoderm fate map. Gap genes interpret inputs from several opposing morphogen gradients to define the fate of cells along the A-P axis. Anterior morphogens activate anterior gap genes to form head, as well as repress posterior morphogens and gap genes to exclude posterior fates. The posterior morphogens determine posterior fates in a similar manner. Homologs of maternal morphogens are evenly distributed in the embryos of non-dipteran insects. Hence, A-P patterning may require input from regulators other than the maternal morphogens. In Tribolium, this input is likely to be provided by the Wnt signaling pathway. The maternal *Tc-axn* mRNA is localized at the anterior pole. Axn is the key component of the repressive complex of Wnt signaling. This asymmetric location of *Tc-axn* mRNA results in repression of Wnt signaling in anterior regions of blastoderm from very early stages. Wnt ligands are expressed at the posterior pole of the egg to activate Wnt signaling in the blastoderm. Anterior localization of a repressor and posterior expression of ligands suggests a Wnt activity gradient in the Tribolium blastoderm. However, the best evidence of a gradient, the expression of the target gene caudal, only demonstrates gradient readout in the posterior half of the embryo.

In our studies, this Wnt signaling gradient was shown to play an important role in patterning the blastoderm fate map along the entire A-P axis. In vertebrates, low or no Wnt activity is required to form head. In Tribolium, activating Wnt signaling in the presumptive head region also caused a headless phenotype. In addition, low Wnt signaling is a precondition for proper serosa formation in Tribolium. The impact of Wnt activity on serosa formation appears to be, at least in part, through regulation of *Tc-zen-1* expression. These results suggest the fate map can be largely determined by the activation of *Tc-cad* and repression of *Tc-zen-1* by Wnt

signaling, in combination with mutual repression between *Tc-zen-1* and *Tc-cad*. Increasing Wnt activity shifts the fate map anteriorly, resulting in less serosa and expanded domain of *Tc-cad* expression. In contrast, decreasing Wnt activity, shifts the fate map posteriorly, leading to larger serosa and a reduced, or even no, *Tc-cad* expression domain. Thus the mutual repression network is highly influenced by levels of Wnt activity. Although we did not observe any interaction between Wnt activity and *Tc-otd-1* in determination of the blastoderm fate map, the regulation of *Tc-otd-1* by Wnt signaling may contribute to head patterning later. In vertebrates, the moderate Wnt signaling is required for facial development and Wnt signaling regulates the expression of *otx*, the vertebrate homolog of *otd*. In Tribolium *Tc-apc-1* or *Tc-axn* RNAi embryos, *Tc-otd-1* is expressed in the blastoderm, but in these embryos head formation ultimately fails. Somehow, high levels of Wnt activity in the head antagonized the function of *Tc-otd-1*. However, we cannot rule out the possibility that effects of Wnt signaling on *Tc-otd-1* are simply masked by stronger interactions between Wnt signaling and *Tc-zen-1* and/or *Tc-cad*.

Wnt signaling regulation of *Tc-zen-1* expression appears to be, at least in part, through the Dpp signaling pathway. Up-regulation of Wnt activity leads to the ectopic expression of *Tc-sog* anteriorly, where *Tc-dpp* is strongly expressed in wild type embryos. The presence of Sog transports Dpp dimers to dorsal regions, abolishing Dpp signaling in this region. Reduced Dpp signaling causes defects in *Tc-zen-1* expression. In contrast, lower Wnt activity produces retraction of *Tc-sog* expression from the anterior border to produce a larger serosa correlated with increased Dpp activity and ectopic *Tc-zen-1* expression. However, changes in Wnt activity levels do not impact D-V polarity posteriorly (Fu et al., 2012). *Tc-axn* and *Tc-lgs* RNAi embryos still maintain D-V patterning during elongation. In contrast, cell fate is changed along the D-V axis in *Tc-sog* or *Tc-dpp* RNAi embryos. Hence, the repression of Wnt activity is more crucial for patterning the anterior blastoderm fate map, specifically serosa formation.

A-P and D-V axis patterning in the Tribolium blastoderm

In Drosophila, the fate map of the entire body is completely determined by the blastoderm stage. Cell fate along both A-P and D-V axes is simultaneously and independently determined through different gene networks. On the other hand, in short-germ insects, such as

Tribolium, embryogenesis is a relative long process. Abdominal segments form gradually during elongation, and A-P and D-V features need to be determined in newly added segments. In Tribolium, A-P and D-V fate map patterning are related to some extent, in that changing the identity of serosa or head affects both A-P and D-V patterning. Therefore, in Tribolium, at least some genetic factors are involved in patterning both axes.

In this study, the Wnt signaling pathway impacted D-V polarity in certain regions along the A-P axis of the blastoderm through Dpp signaling, the major contributor to D-V pattering in both blastoderm and germ band. In Drosophila, Toll signaling stimulates accumulation of Dorsal in the nuclei of cells on the ventral half of the embryo to activate the D-V patterning genes. *sog* is a target of the Dorsal morphogen to antagonize Dpp on the dorsal side of the embryo to establish the Dpp gradient and on the ventral-lateral side to pattern cell fates. In Tribolium, the Toll pathway also determines D-V polarity by inducing a transient Dorsal signal on the ventral-most side. This Dorsal gradient activates *sog* expression, which functions as a Dpp antagonist. Disruption of *Toll* or *sog* results in loss of D-V polarity. In Tribolium, formation of serosa requires Dpp signaling. Higher levels of Wnt activity induce expression of *Tc-sog* more anteriorly, resulting in less serosa. In contrast, lower levels of Wnt activity shift the *Tc-sog* expression domain posteriorly, producing more serosa. Therefore, the D-V patterning functions of the Toll and Dpp pathways are conserved in Tribolium. Additional regulation of *Tc-sog* expression along the A-P axis by Wnt signaling, which causes the loss of D-V polarity, integrates both pathways in patterning the anterior fate map in Tribolium.

In addition to the above-mentioned pathways, the mutual repression network of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* is also responsible for maintaining D-V polarity. Changes affecting *Tc-zen-1* or *Tc-otd-1* often cause reciprocal transformation of serosa and embryonic tissues, impacting the D-V polarity in this region. Changes affecting *Tc-cad* alter D-V cell fate, but not D-V polarity in general. Thus, in Tribolium A-P and D-V patterning requires the input of several signaling pathways, as well as interactions between the zygotic gene*s Tc-zen-1, Tc-otd-1* and *Tc-cad.*

Tc-Cad is also regulated by *Tc-Mex-3* and *Tc-zen-2* in head and serosa, respectively. *Tc-*

zen-2 is expressed the same domain as *Tc-zen-1* but its expression initiates somewhat later. In *Tc-zen-2* RNAi embryos, Cad protein accumulates in serosal cells (Schoppmeier et al., 2009). However, the serosa forms normally and does not change in size or position, suggesting *Tc-zen-2* does not appear to contribute substantially to the blastoderm fate map. In *Tc-Mex-3* RNAi embryos, Cad protein accumulates in the pre-gnathal head region, overlapping the *Tc-otd-1* domain and the resulting embryos produce cuticles lacking head structures (Schoppmeier et al., 2009). However, the boundary between serosa and embryonic tissue is not affected. Knocking down *Tc-Mex-3* expression does not produce defects as severe as knocking down *Tc-otd-1*. Thus Tc-Mex-3 appears to be required in addition Tc-Otd-1 to repress *Tc-cad* expression in the head lobes and may explain the remaining embryonic tissue that does not express *Tc-cad* in *Tc-otd-1* RNAi embryos. However, *Tc-Mex-3* RNAi produced milder effects at much lower penetrance in our hands, than previously reported. Thus, we conclude that Tc-Mex-3 may contribute to the fate map, but not as substantially as *Tc-otd-1*. In addition, in *Tc-torso* RNAi embryos, the expression of *Tc-cad* and *Tc-wg* are severely impacted and embryos develop into truncated cuticles (Schoppmeier et al., 2009; Schoppmeier and Schroder, 2005; Schroder et al., 2000). These results implicate a role of *Tc-torso* in the regulation of segmentation through the Wnt signaling pathway and *Tc-cad* expression.

Conclusion

Genetic patterning in the Tribolium blastoderm occurs before morphological differentiation appears. According to our results, before cells exhibit the difference between serosa and embryonic rudiment, an incomplete mutual repression network between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* mapped cell fate and established the boundary between serosa and embryonic tissue, as well as the boundary between the pre-gnathal head region the rest of the body. Moreover, our studies suggest that this mutual repression network is regulated by the Wnt signaling pathway, incorporating the Dpp signaling pathway, and maybe even earlier *Tc-otd-1* expression as well. Changes in Wnt signaling resulted in the alteration of *Tc-zen-1* and *Tc-cad* expression. *Tc-cad* is directly regulated by Wnt signaling as a target gene. Regulation of *Tc-zen-1* expression by Wnt signaling might be through regulation of Tc-Sog expression, an antagonist of Dpp ligands. Then, *Tc-zen-1* expression is affected by the ultimate shift in Dpp activity region,

eventually impacting the fate map. Furthermore, shifts in the mutual repression network in various Wnt signaling activity backgrounds also suggest that a Wnt activity gradient functions along the A-P axis in the Tribolium blastoderm. Meanwhile, the early expression of *Tc-otd-1* functions as a general activator to activate *Tc-zen-1* and *Tc-cad* expression. Inputs from Wnt and Dpp signaling, in combinations with early *Tc-otd-1* expression, regulate the zygotic expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* to define the Tribolium blastoderm fate. In addition to the aforementioned factors, several other genetic factors may contribute to the blastoderm fate map as shown in previous studies, such as *Tc-torso* and *Tc-Mex-3*. Therefore, other factors may influence the blastoderm fate map through pathways and genes we focused on in this study.

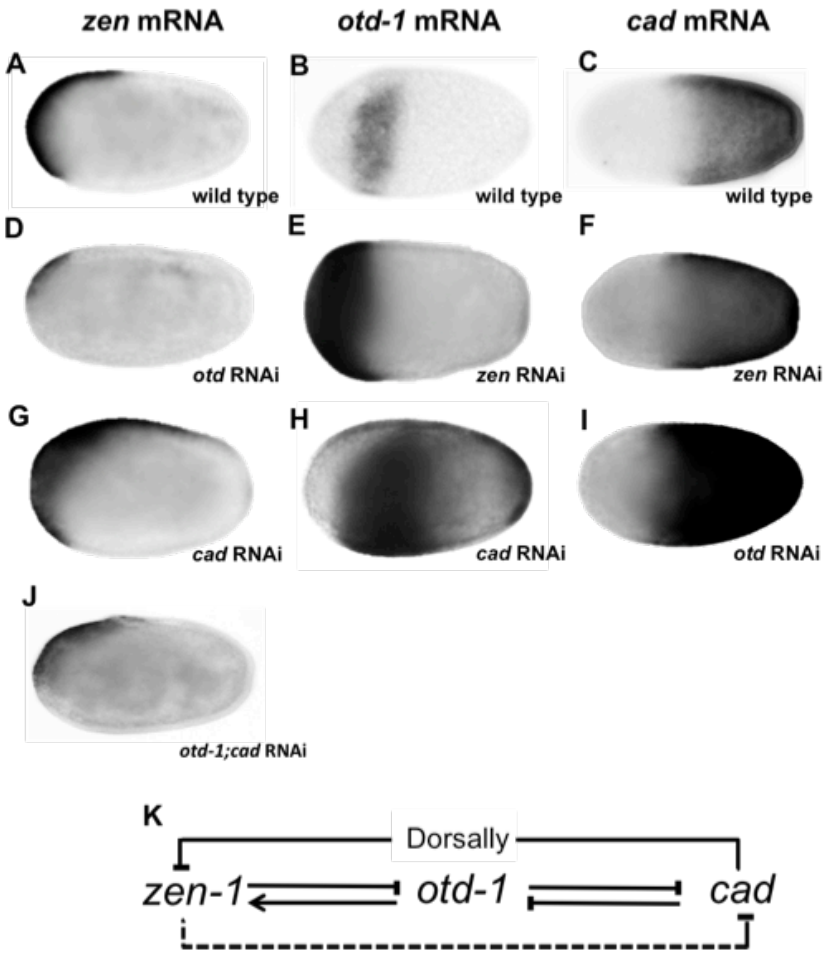


Figure 2-1 Interactions between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad*

(A-C) *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* expression patterns, respectively, in wild type blastoderm embryos before the onset of gastrulation. Knocking down *Tc-otd-1* expression reduced the expression of *Tc-zen-1* (D) and caused anteriorly expansion of *Tc-cad* expression (I). Knocking down *Tc-zen-1* expression shifted *Tc-otd-1* anteriorly (E) and *Tc-cad* expression expanded anteriorly (F). After knocking down *Tc-cad* expression, *Tc-otd-1* was ectopically expressed in the posterior region of the embryo (H), and *Tc-zen-1* expression expanded posteriorly on the

dorsal side of the embryo (G). In *Tc-lgs* and *Tc-otd-1* double RNAi embryos, dorsal *Tc-zen-1* expression expanded even more posteriorly (J). Mutual repression network developed based on the interaction between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* in the Tribolium blastoderm (K).

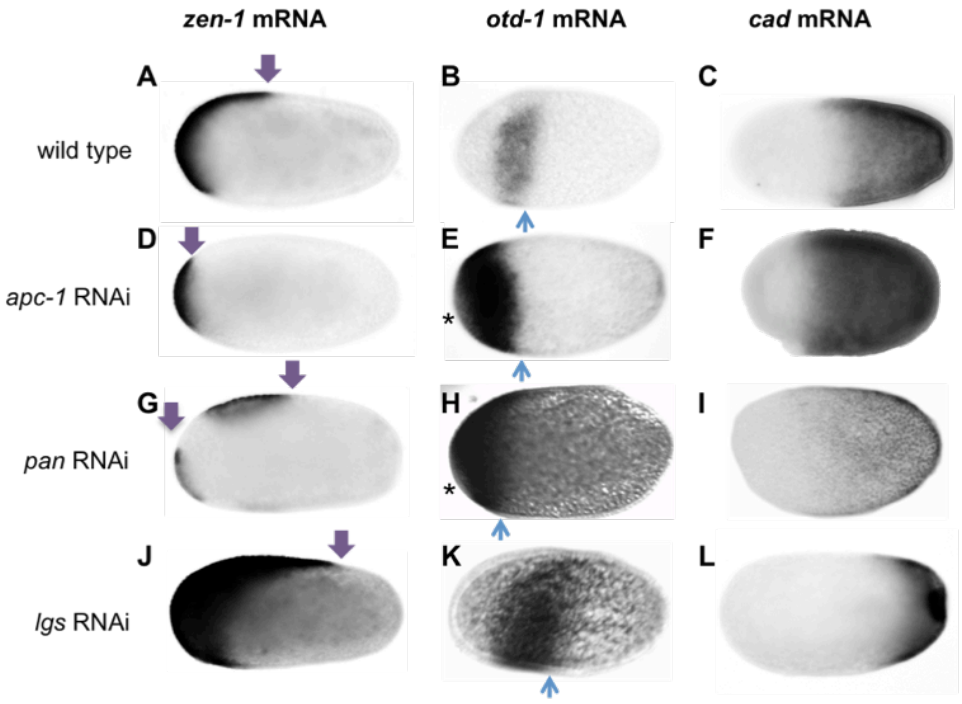


Figure 2-2 Wnt signaling impacts *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* expression

(A-C) Expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* in wild type blastoderm embryos.
(D-F) Expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* in *Tc-apc-1* RNAi embryos. (D) *Tc-zen-1* expression was reduced to a small patch at the anterior pole of the embryo. (E) *Tc-otd-1* also shifted anteriorly with no expression in the anterior pole where *Tc-zen-1* is expressed. (B) *Tc-cad* expression was anteriorly expanded.
(G-I) Expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* in *Tc-pan-1* RNAi embryos. (G) Two separate expression domains of *Tc-zen-1*, one at the anterior pole and another on the dorsal side of the embryo. (H) *Tc-otd-1* shifted anteriorly with no expression in the anterior pole where *Tc-zen-1* is expressed anteriorly. (I) *Tc-cad* expression was anteriorly expanded but weaker.
(J-L) Expression of *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* in *Tc-lgs* RNAi embryos. (J) *Tc-zen-1* expression expanded posteriorly in both dorsal and ventral sides. (K) *Tc-otd-1* also shifted posteriorly in a broader domain, which may due to the weak expression of *Tc-cad*. (L) *Tc-cad* expression was significantly reduced, and restricted to the posterior end of the embryo.

Purple and blue arrows indicate the posterior border of *Tc-zen-1* and *Tc-otd-1*, expression, respectively, in different embryos. Asterisks denote the anterior pole of embryos lacking *Tc-otd-1* expression.

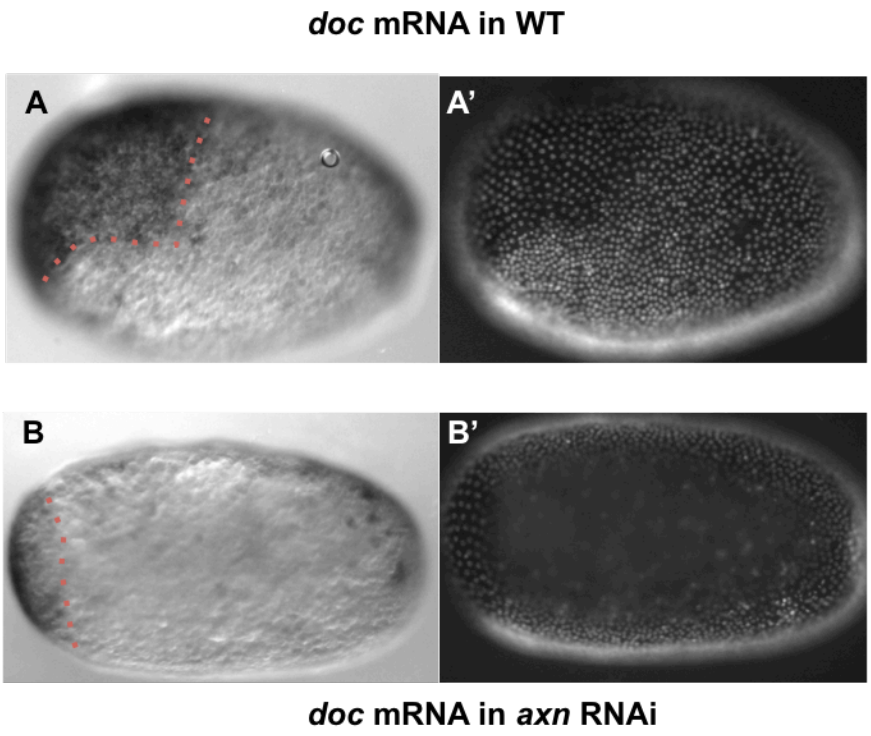


Figure 2-3 *Tc-doc* expression is affected by changing Wnt signaling activity

Tc-doc expression is reduced in *Tc-axn* RNAi embryos. (A) Dorsal view of *Tc-doc* expression in wild type blastoderms. (B) *Tc-doc* expression was dramatically reduced in the blastoderm of *Tc-axn* RNAi embryos.

(A', B') Embryos were stained with DAPI to check the distribution of nuclei in the blastoderm under UV light to stage the embryos.

Red dotted lines label the border of the *Tc-doc* expression domain.

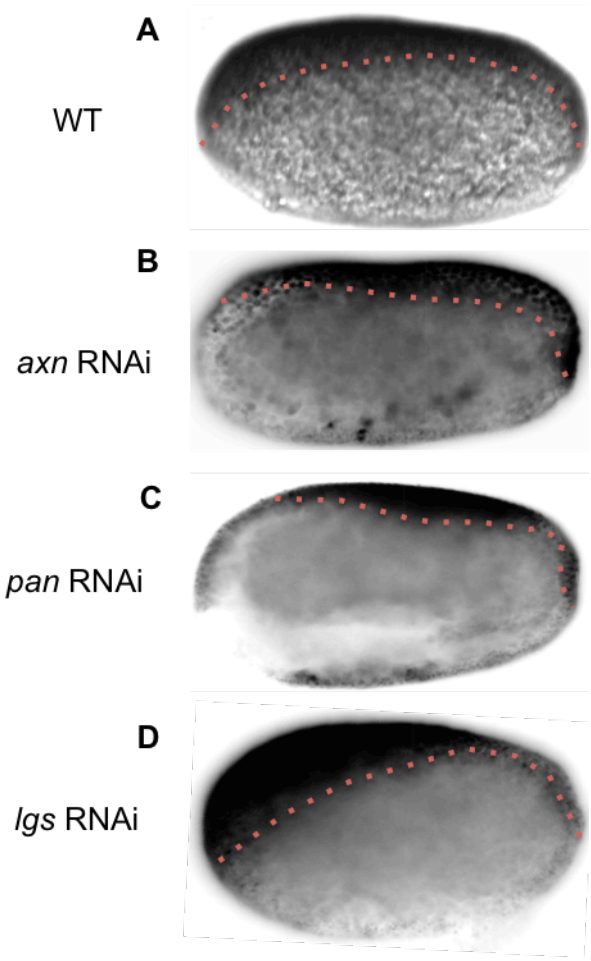


Figure 2-4 pMAD distribution is affected by changing Wnt signaling activity

(A, A') Distribution of pMAD in wild type blastoderm embryos.
(B, B') Distribution of pMAD in *Tc-axn* RNAi embryos, anterior pMAD was dramatically reduced, which is similar to the effects on pMAD expression in *Tc-pan* RNAi embryos (C, C').
(D, D') Distribution of pMAD in *Tc-lgs* RNAi embryos, the most anterior pMAD expression does not appear to be effected, while in the middle region of the blastoderm, pMAD expression expanded ventrally.
(A', B', C', D') Embryos were stained with DAPI, to check the distribution of nuclei in the blastoderm under UV light to stage the embryos.

Red dotted lines label the border of pMAD distribution domain.

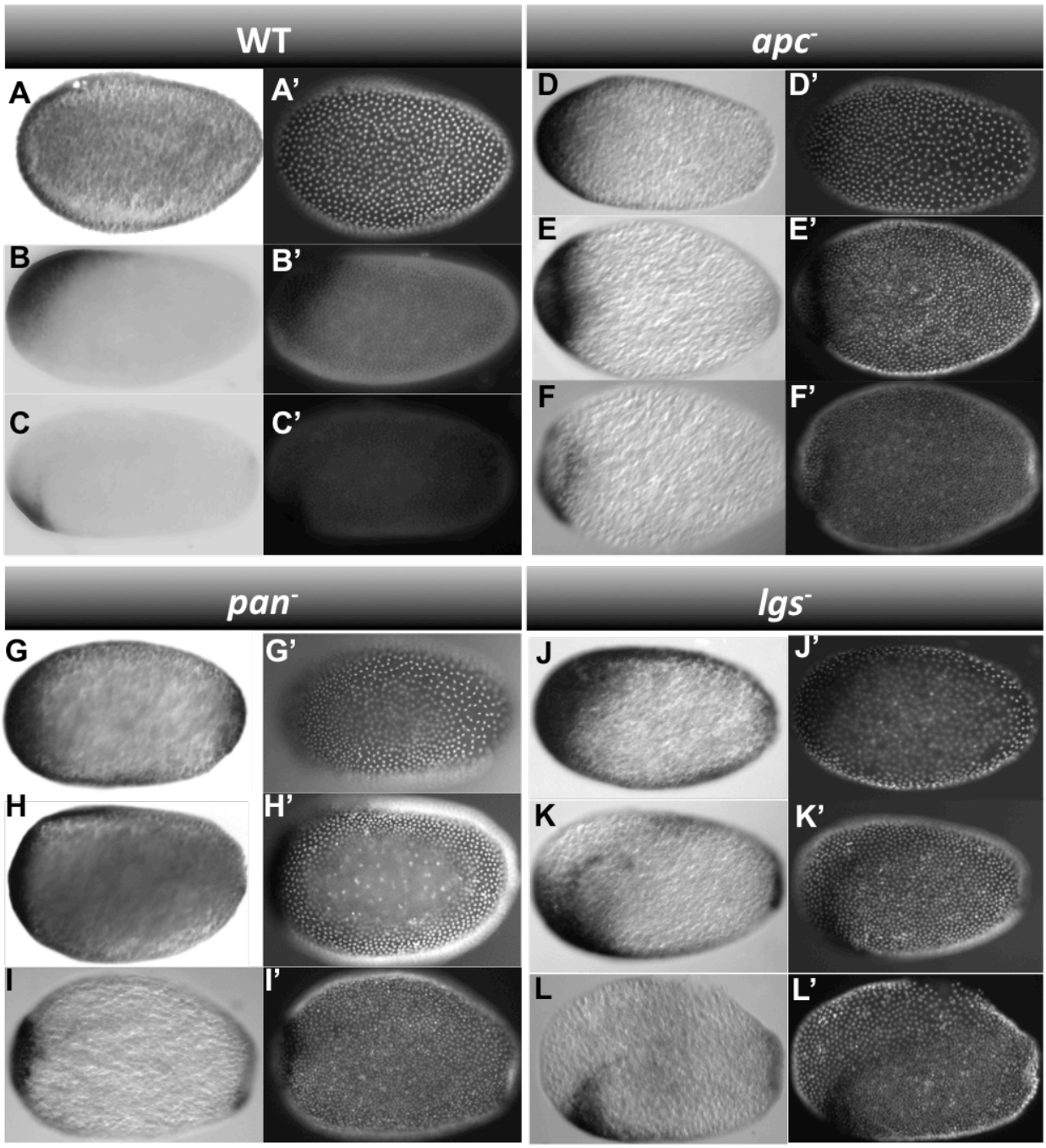


Figure 2-5 Zygotic expression of *Tc-dpp* is affected by levels of Wnt signaling activity

Tc-dpp is expressed ubiquitously in the early embryo with a strong anterior gradient in wild type (A) and in *Tc-apc-1* (D), *Tc-pan* (G) and *Tc-lgs* (J) RNAi embryos.

In wild type embryos, *Tc-dpp* expression expanded into the anterior-dorsal region (B). In *Tc-apc-1* (E) and *Tc-pan* (H) RNAi embryos, *Tc-dpp* failed to expand dorsally, and is reduced to a small domain at the anterior pole. In *Tc-lgs* RNAi embryos, *Tc-dpp* expression expanded posteriorly (K).
Later *Tc-dpp* was expressed along the dorsolateral edge of the presumptive embryo in wild type embryos (C). In *Tc-apc-1* (F) and *Tc-pan* (I) RNAi embryos, *Tc-dpp* was expressed in a ring at the anterior pole of the egg. *Tc-dpp* is expressed along the dorsal lateral edge of *Tc-lgs* RNAi embryos, which are located more posteriorly in egg (L). *Tc-lgs* RNAi embryos also display a larger anterior ventral expression domain of *Tc-dpp* than in wild type embryos.

(A', B', C', D', E', F', G', H', I', J', K', L') Embryos were stained with DAPI to check the distribution of nuclei in the blastoderm under UV light to stage the embryos.

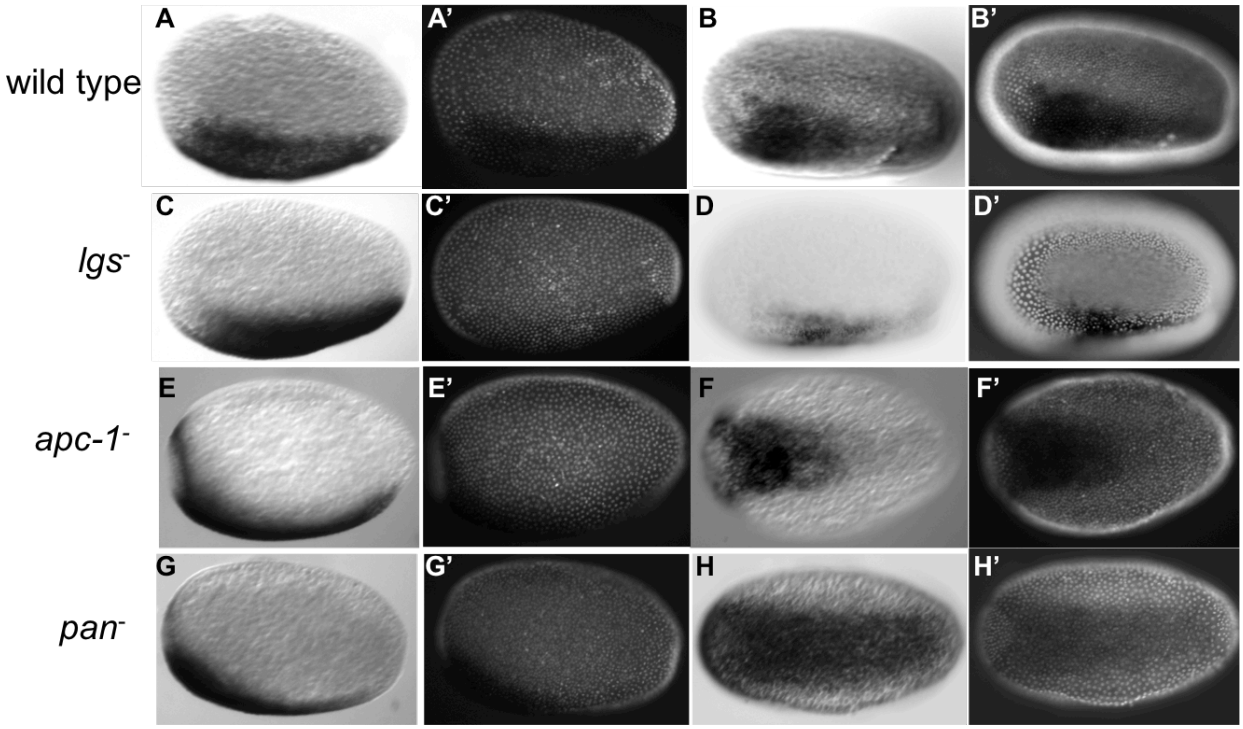


Figure 2-6 Dpp signaling is regulated by Wnt activity via *Tc-sog* in the anterior blastoderm

(A, A') Lateral view of *Tc-sog* expression in the undifferentiated blastoderm of wild type embryos. *Tc-sog* is strongly expressed along the ventral middle line.
(B, B') Lateral view of *Tc-sog* expression in the differentiated blastoderm of wild type embryos. *Tc-sog* is strongly expressed in the head region and weakly in the posterior region along the ventral middle of the embryos. The anterior expression border has retreated slightly posteriorly.
(C, C') Lateral view of *Tc-sog* expression in the undifferentiated blastoderm of *Tc-lgs* RNAi embryos. The anterior border of the *Tc-sog* expression domain was posterior shifted.
(D, D') Lateral view of *Tc-sog* expression in the differentiated blastoderm of *Tc-lgs* RNAi embryos. expression of *Tc-sog* was weaker than in wild type embryos, but relatively stronger expression remains in the head region. In these embryos, the anterior expression border has retreated more posteriorly than in wild type embryos.
(E, E') Lateral view of *Tc-sog* expression in the undifferentiated blastoderm of *in Tc-apc-1* RNAi embryos. Expression has surrounded the anterior pole of the egg.

(F, F') Ventral view of *Tc-sog* expression in the differentiated blastoderm of *Tc-apc-1* RNAi embryos. Expression remains around the anterior pole, and strong expression of *Tc-sog* has shifted more anteriorly compared to that in wild type embryos.
(G, G') Lateral view of *Tc-sog* expression in the undifferentiated blastoderm *of Tc-pan* RNAi embryos. *Tc-sog* expression has expanded anteriorly to the pole of the egg.
(H, H') Ventral view of *Tc-sog* expression in the differentiated blastoderm of *Tc-pan* RNAi embryos. *Tc-sog* expression remained stronger in a more anterior region that reaches the anterior pole of the egg.

(A', B', C', D', E', F', G') Embryos were stained with DAPI to check the distribution of nuclei in the blastoderm under UV light to stage the embryos.

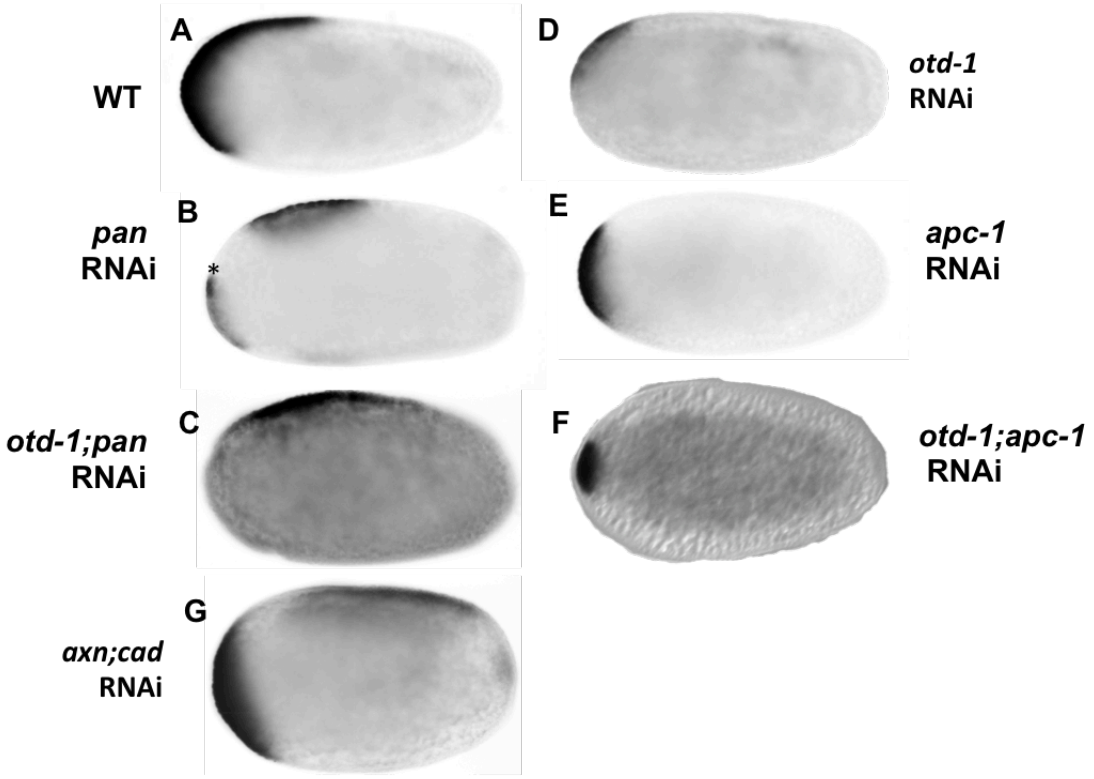


Figure 2-7 Analyzing determinative factors for *Tc-zen-1* expression in *Tc-pan* RNAi and *Tc-apc-1* RNAi embryos

(A) *Tc-zen-1* expression in wild type embryos.

(B) Two split *Tc-zen-1* expression domains in *Tc-pan* RNAi embryos.

(C) No *Tc-zen-1* expression in the anterior pole of the *Tc-otd-1and Tc-cad* double RNAi embryos.

(D) Reduced expression of *Tc-zen-1* in *Tc-otd-1* RNAi embryos.

(E) Reduced expression of *Tc-zen-1* in *Tc-apc-1* RNAi embryos.

(F) *Tc-zen-1* expression domain was more reduced in *Tc-otd-1*;*Tc-apc-1* double RNAi embryos than in *Tc-apc-1* RNAi embryos.

(G) Knocking down both *Tc-axn* and *Tc-cad* produced split *Tc-zen-1* expression domains, similar to that in *Tc-pan* RNAi embryos.

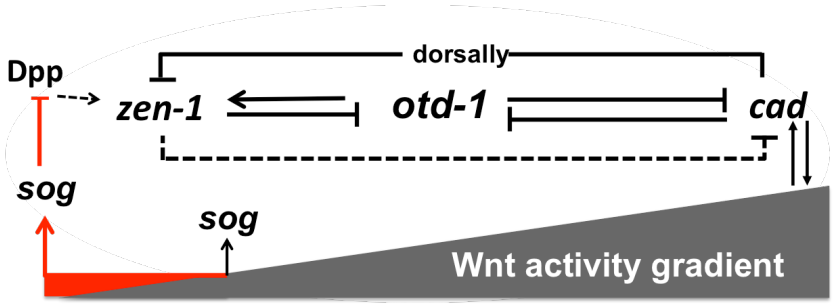


Figure 2-8 Fate map gene regulatory network

In wild type and these *Tc-pan*, *Tc-apc-1* and *Tc-axn* RNAi embryos, interactions between *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* define the boundaries between serosa and embryonic cells, as well as pre-gnathal region and the rest embryonic tissues. *Tc-otd-1* activates *Tc-zen-1* expression, instead of repressing *Tc-zen-1* expression. *Tc-cad* appears to only repress *Tc-zen-1* expression dorsally. The serosa formation and *Tc-zen-1* expression are affected by *Tc-dpp*. Since it has not been clearly demonstrated that *Tc-dpp* activates *Tc-zen-1*, dotted line is used to indicate this speculation.

In the anterior region of these RNAi embryos, up-regulated Wnt signaling activity (shown as red gradient) causes ectopic expression of *Tc-sog*. Then *Tc-sog*, as an antagonist of Dpp ligand, reduced Dpp signal activity in this region, resulting in the defects of serosa formation, via *Tc-zen-1*.

Therefore, to properly pattern fate map requires the repression of Wnt signaling activity in the anterior region of the embryos to form the serosa and normal expression domain of *Tc-zen-1,* and the activation of Wnt signaling activity in the posterior region to activate *Tc-cad* expression, which further regulates segmentation.

Chapter 3 - *Tc-cad* gradient regulates serial expression of gap genes in Tribolium blastoderm

Abstract

Segmentation in Drosophila is regulated by a gene hierarchy, in which the first genetic segment markers, pair-rule genes, as well as the genetic segment identity markers, Hox genes are regulated by up-stream gap genes, in addition Hox genes are also regulated by pair-rule and pair-rule targets, the segment polarity genes. Therefore, in Drosophila, segment formation and segmental identities are coordinately regulated by gap genes, the expression domains of which are determined through morphogen gradients and interactions between gap genes. In Tribolium, segments are also defined by pair-rule genes. However, expression of pair-rule genes is regulated by the posterior *Tc-cad* gradient. Gap genes are involved, to some extent, but they do not function as core determinators. In contrast, gap genes impact segment identities, via Hox genes. In this study, we demonstrate that the *Tc-cad* gradient also regulates serial expression of gap genes in the Tribolium blastoderm in a manner similar to the regulation of pair-rule gene expression. Hence, In Tribolium, the *Tc-cad* expression gradient regulates segmentation and segment identity coordinately.

Introduction

In Drosophila, a large portion of the fate map dedicated to the segmented portion of the body plan (gnathal and trunk regions) is regionalized by gap gene expression. Gap genes function early in the segmentation gene hierarchy; they are activated by maternally expressed genes, including a posterior gradient of *Dm-cad*, and regulate the expression of downstream pair-rule and homeotic genes to generate segments and define segmental identities (Dearolf et al., 1989; Hader et al., 1998; Lynch et al., 2012; Rivera-Pomar et al., 1995). Although vertebrates do not contain clear functional homologs of Drosophila gap genes, posterior gradients of *Dm-cad* homologs (*cdx* genes) and also Hox genes are required for proper axial elongation and segment identity (Chawengsaksophak et al., 2004; Ikeya and Takada, 2001; Lohnes, 2003; Pilon et al., 2006; Pilon et al., 2007).

However, the mechanisms of segment formation and segmental identity determination are quite different between them. A 'French Flag model' has been used to describe segmentation in the long-germ insect, Drosophila, in which maternal morphogen gradients emanate from both anterior and posterior poles of the blastoderm. These two opposing gradients not only activate but also provide position signals to downstream gap genes, which determine segment formation and regulate Hox gene expression via pair-rule and segment polarity genes. Maternal *Dm-cad* mRNA is expressed uniformly in the early embryo, but is only translated and forms a protein gradient in the posterior region of the embryo. (Niessing et al., 2002; Rivera-Pomar et al., 1996). This maternal Cad gradient patterns the posterior fate map. Zygotic *Dm-cad* expression is limited to a narrow region near the posterior pole and also regulates pair-rule gene expression in this region (Dearolf et al., 1989). Although, zygotic *cad* is not thought to be involved in posterior fate map patterning, knockdown of maternal and zygotic *Dm-cad* causes more severe phenotypes, which lack all segments after the second abdominal segment (A2), compared to a maternal *Dm-cad* null mutant (impacting segments posterior of A7) (Olesnicky et al., 2006).

In contrast, vertebrate segmentation is best described by a 'clock and wavefront' model. Expression of several genes oscillating in the presomitic mesoderm (PSM) forms the molecular clock of somite formation. Each cycle of gene expression forms one somite. This gene expression oscillation is regulated by several signaling gradients, which form in the posterior PSM, including Notch, Wnt and FGF signaling pathways (Jensen et al., 2010; Rodriguez-Gonzalez et al., 2007; Saga, 2012). *cdx1*, *cdx2* and *cdx4* are expressed in overlapping gradients in the posterior of the PSM during elongation in mouse. (Beland et al., 2004; Chawengsaksophak et al., 2004; Gaunt et al., 2005; Gaunt et al., 2008). Mutation of any one of these genes causes a shorter tail and a posterior shift of segment identities, which indicates key roles of *cdx* genes that impact axial elongation and segmental identity. Hox genes are also expressed along the A-P axis in vertebrates, patterning segmental identity. Although, disruption of *cdx* gene expression alters Hox gene expression, the impact of *cdx* genes on Hox genes indicates that the regulation of segment formation and segmental identity in vertebrates is not as closely coordinated by *cdx* genes (Chawengsaksophak et al., 2004; Gaunt et al., 2008; Young et al., 2009).

The expression and function of *cad/cdx* homologs are, to some extent, conserved in Tribolium, as well as other insects. In Tribolium, Gryllus and Nasonia, both maternal *cad* mRNA are found ubiquitously in the egg. Zygotic *cad* is expressed in a gradient emanating from the posterior end of Tribolium and Gryllus embryos and in the mid-posterior region of Nasonia embryos (Schulz et al., 1998; Wolff et al., 1998); (Olesnicky et al., 2006; Shinmyo et al., 2005). In Tribolium and Gryllus, knockdown of *cad* expression disrupts segmentation and truncates elongation. In a null *cad* mutation of Nasonia, only the head forms, similar to the phenotype produce by *cad* RNAi in Tribolium and Gryllus (Mito et al., 2005; Shinmyo et al., 2005; Wolff et al., 1998). In these embryos, gap and pair-rule gene expression is also affected by knocking down *cad* expression, which causes lack of posterior gap gene expression, indicating *cad* is required to activate gap gene expression there.

In Tribolium gap gene RNAi embryos and in the only mutant described to date, no clear patterning gaps along the A-P axis have been described. Instead, segmental transformations and posterior truncations are produced by *Tc-hb*, *Tc-gt*, *Tc-Kr* and *Tc-mlpt* RNAi (Bucher and Klingler, 2004; Cerny et al., 2005; Cerny et al., 2008; Savard et al., 2006). Therefore, in Tribolium gap genes play less important roles in segment formation, but are crucial for segment identity.

Mixed results have been reported in Oncopeltus and Gryllus , RNAi against *Of-gt*, *Of-Kr or Gb-Kr* produces typical gap phenotype, while RNAi against *Of-hb or Gb-hb* produces transformation (Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Liu and Patel, 2010; Mito et al., 2006; Mito et al., 2005). In general, the gap phenotypes suggest that gap genes regulate pair-rule genes in these two short germ insects while transformations indicate a more direct regulatory link between gap and homeotic genes. Moreover, gap phenotypes may camouflage transformation phenotypes as the case in Drosophila null mutants.

Recently, the mechanism by which *Tc-cad* regulates the pair-rule gene, *Tc-eve* in Tribolium has been described (El-Sherif et al., 2014). *Tc-eve* is a primary pair-rule gene as well as the first genetic marker of segments in the blastoderm. Disruption of *Tc-eve* eliminates segment formation in the trunk. The *Tc-cad* gradient is regulated by Wnt signaling activity along

the A-P axis in the Tribolium blastoderm. Disruption of the Wnt signaling pathway impacts the *cad* gradient and also *Tc-eve* expression (El-Sherif et al., 2014). Hence, Wnt signaling activity regulates the expression of *Tc-eve*, presumably through the *Tc-cad* gradient, and ultimately induces segmentation in Tribolium blastoderm. Moreover, knockdown of components of the Wnt signaling pathway or *Tc-cad* itself not only affects segment formation but also segmental identity coordinately. Since Tribolium gap genes regulate Hox gene expression to define segmental identities. Therefore, Hox genes are regulated by the *Tc-cad* gradient or Wnt signaling activity, maybe through gap genes.

In Tribolium, gap genes are expressed in multiple tissues at multiple stages. Here we focus on the expression of the gap genes *Tc-hb*, *Tc-Kr*, and *Tc-mlpt* in the region where the *Tc-cad* gradient forms in the blastoderm. Expression of each of these genes initiates at the posterior end of the blastoderm and resolves to a specific region along the A-P axis. *Tc-hb* appears to be an activator of *Tc-Kr*, and *Tc-mlpt* expression is affected by altering *Tc-Kr* expression. Knocking down *Tc-hb* expression, *Tc-Kr* expression is restricted to the posterior pole of the blastoderm embryo, almost no *Tc-Kr* expression is detected in the germ band, and *Tc-mlpt* is impacted (Marques-Souza et al., 2008).

In this study, we analyzed the expression of three gap genes, *Tc-hb*, *Tc-Kr*, and *Tc-mlpt*, both spatially and temporally in the blastoderm in various *Tc-cad* expression gradients produced by changing the level of Wnt activity. We showed that gap gene expression is regulated by *Tc-cad* gradient in the manner comparable to the regulation of *Tc-eve*. In addition, interactions between gap genes that contributed to defining their expression boundaries in the blastoderm were conserved as in Drosophila. Therefore, in Tribolium, *Tc-cad* regulates gap and pair-rule genes coordinately to define segments and segmental identities.

Results

Previous studies of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* have focused on their expression domains and not temporal order, since they were compared to their Drosophila homologs, which are expressed concurrently during the blastoderm stage. However, in Tribolium, these three genes

are expressed in temporal order with a slight overlap in their expression borders. Therefore, more accurate documentation of their temporal expression will provide more detailed understanding of the interactions between these genes as well as their possible regulation by the *Tc-cad* gradient. In this analysis, we focused on the expression domains of these genes that initiated in the posterior blastoderm and categorized embryos based on gene expression there: class Ø included embryos not yet expressing the gene under analysis at the posterior end; Class I included embryos with zygotic expression at the posterior end; Class II included embryos no longer expressing the gene under analysis at the posterior end.

Serial expression of Tc-hb, Tc-Kr and Tc-mlpt in the blastoderm

In wild type, prior to zygotic expression (Class-Ø), *Tc-hb* is maternally expressed and uniformly distributed throughout the early embryos (Figure 3.1 A) (Wolff et al., 1998). Zygotic *Tc-hb* expression is observed in the presumptive serosa and the posterior half of embryo (Tc-*hb* Class-I, Figure 3.1 A). *Tc-hb* expression in serosa is relatively stable. However, posterior *Tc-hb* expression was more dynamic (Figure 3.1 A). Expression faded at the posterior end first and continued to fade more anteriorly (Class *hb*-II), until *Tc-hb* expression covered only the presumptive gnathal region (Figure 3.1 A). *Tc-Kr* is not expressed maternally and no *Tc-Kr* expression was detected in the early blastoderm (*Kr* Class-Ø, Figure 3.1 B) (Cerny et al., 2005). Later, expression initiated at the posterior pole and gradually expanded anteriorly to cover about 30% of the blastoderm (*Kr* Class-I, Figure 3.1 B). Clearing of *Tc-Kr* expression from the posterior end is detected soon thereafter, at the beginning of the germ-band elongation stage (*Kr* Class-II, Figure 3.1 B). *Tc-mlpt* is also not expressed maternally, but is expressed anteriorly in the head region as well as later in the thorax and abdomen. Anterior expression of *Tc-mlpt* in the head region starts weakly in a wide region in the early blastoderm (Class *mlpt*-Ø, Figure 3.1 C) (Savard et al., 2006). Then, expression appears at the posterior end of the embryo (*mlpt* Class-I, Figure 3.1 C). *Tc-mlpt* expression expands anteriorly covering less than 20% of the blastoderm (*mlpt* Class-I, Figure 3.1 C). During gastrulation, *Tc-mlpt* expression in the invaginating tissue cannot be monitored. Similar to *Tc-Kr* expression, *Tc-mlpt* fades at the posterior end until it covered the thorax in the elongating germ-band (Class *mlpt*-II, Figure 3.1 C). There are additional expression domains of *Tc-hb* and *Tc-mlpt* that initiate later during germ band

elongation. In this study, we focused on expression of these three gap gene homologs during the blastoderm stage, specifically at the posterior end where the *cad* gradient form is formed.

If we consider the dynamics of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression in the posterior blastoderm, they resemble the dynamics of *Tc-eve* expression (El-Sherif et al., 2012). Posterior *Tc-hb* expression overlaps the initial *Tc-eve* expression covering about the half of the embryo. Thereafter, expression of both genes clears from the posterior end of the embryo, and this clearing border moved anteriorly gradually to form stable expression domains. The dynamics of *Tc-Kr* and *Tc-mlpt* expression are similar to the second oscillation of *Tc-eve* expression, which emanates from the posterior pole of the blastoderm and expands anteriorly. In addition, the clearing of their expression also starts from the most posterior end of this expression domain and continues in anterior direction. However, clearing of *Tc-Kr* and *Tc-mlpt* expression occur during gastrulation, which was not detected, but fade of second *Tc-eve* expression takes place in the blastoderm. Although, the expression of both *Tc-Kr* and *Tc-mlpt* initiates at the posterior end of the blastoderm, *Tc-mlpt* posterior expression appears later than *Tc-Kr* expression and expands less anteriorly. Thus, *Tc-mplt* expression is more comparable to the third oscillation of *Tc-eve* expression. This sequential expression of the gap genes *Tc-hb*, *Tc-Kr* and *Tc-mlpt*, in combination with their dynamic expression at the posterior end of the embryo, suggests they determines the tempo at which segmental identity is defined via regulation of Hox gene expression.

To further understand the temporal connection between *Tc-hb*, *Tc-Kr* and *Tc-mlpt*, we measured the expression intervals of these genes. Previous work shows that *Tc-eve* is activated at about 14 hours after egg laying (AEL). We also observed early posterior *Tc-hb* expression at this time. Meanwhile, both *Tc-Kr* and *Tc-mlpt* expression were not detected. Hence, we monitored the expression of these three gap genes in the same time intervals as in the previous study of pair-rule gene expression. Moreover, alteration of gap gene expression starts from the posterior end of the embryo, suggesting that the highest frequency of their expression is in this region, as for *Tc-eve* expression oscillation. Therefore, we examined expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* at the posterior pole of the blastoderm at 14-17 hours, 17-20 hours and 20-23 hours AEL.

First, posterior expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* were examined in the eggs in 14-

17 hour AEL by *in situ* hybridization (Figure 3.1 A, B, C). All these embryos were in Class-Ø of *Tc-Kr* and *Tc-mlpt* expression. Most of them were also in Class-Ø of *Tc-hb* expression, with a small proportion in Class-I of *Tc-hb* expression. Thus, in 14-17 hour AEL interval, the serial expression of gap genes initialed the expression of *Tc-hb*, not *Tc-Kr* or *Tc-mlpt*. Later, eggs from 17 to 20 hours AEL showed that the majority of embryos were in Class-I of *Tc-hb* and *Tc-Kr* expression. Few of eggs were still in Class-Ø of *Tc-Kr* expression. Meanwhile, some embryos had entered Class-II of *Tc-hb* expression. For *Tc-mlpt* expression, almost all embryos were in Class-Ø and very few proceeded to Class-I. Thus, during 17 to 20 hours AEL, the posterior gap gene expression is transferring from *Tc-hb* to *Tc-Kr*, and *Tc-mlpt* expression was about to start. Eggs from 20 to 23 hours AEL are all in Class-I of *Tc-Kr* expression and almost all eggs are in Class-II of *Tc-hb* expression, which indicates that in the posterior end, serial expression of gap genes is has switched from *Tc-hb* to *Tc-Kr* and *Tc-mlpt* expression has initiated. From 23 to 26 hours AEL, embryos are in the germ-band elongation stage. In this time window, expression of all three genes entered Class-II, which means none of them were expressed in the posterior end of the germ band. This dynamic serial gene expression is the first indication that the blastoderm is regionalized by gap genes.

Serial expression of gap genes is regulated by the Tc-cad gradient

Expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* are all impacted in *Tc-cad* RNAi embryos. Moreover, expression of the gap genes resembles that of *Tc-eve* in the blastoderm, which is controlled by the levels of *Tc-cad* expression gradient. Therefore, we hypothesized that this serial expression of gap genes is also regulated by the *Tc-cad* expression gradient in a dose-dependent manner. The *Tc-cad* expression gradient in the wild type blastoderm is well described with three aspects: the position of anterior border, the slope of the gradient and the maximum value of the gradient. The position of anterior border of *Tc-cad* mRNA gradient is the distance from the anterior pole to the anterior border of the gradient. *Tc-cad* mRNA gradient has a platform in posterior end of the blastoderm with the highest *Tc-cad* expression. The maximum value of the gradient is in the posterior most end of the blastoderm (El-Sherif et al., 2014). In Tribolium, *Tc-cad* is a target gene of the Wnt signaling pathway. Formation of this *Tc-cad* mRNA gradient is regulated by Wnt signaling activity. Knocking down the transcription factor

of Wnt signaling pathway, *Tc-pan*, results in anterior shift of the position of the anterior border of the gradient and reduces the maximum value, which produces a much shallower slope of this gradient. This shallower slope explains wider stripes of *Tc-eve* in *Tc-pan* RNAi embryos (El-Sherif et al., 2014). Knocking down a co-activator of Wnt signaling pathway, *Tc-lgs* decreases the maximum value and causes posterior shift of the position of anterior border. The slope of this *Tc-cad* mRNA gradient is also less steep than that in wild type embryo, but dose not lead to a notable result.

To investigate regulation of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression by *Tc-cad*, we used *Tc-lgs* RNAi and *Tc-pan* RNAi to change the cad expression levels. We focused on the expression of these three genes both spatially and temporally correlated to the *Tc-cad* gradient. In wild type embryos, the anterior border of posterior *Tc-hb* expression is in the anterior region of the embryo, passing the midline of the blastoderm A-P axis. Majority of embryos expressing *Tc-hb* at the posterior end were during 17-20 hours AEL; some of them during 14-20 hours AEL, but seldom in 20-23 hours AEL periods (Figure 3.1 A; 3.2 A blue). In *Tc-lgs* RNAi embryos, this border was posterior to the A-P axis midline, closer to the posterior end. Similar percentage of embryos was expressing *Tc-hb* during 14-20 hours AEL. But majority embryos expressed *Tc-hb* from 17-23 hours AEL. The clearance of *Tc-hb* expression was detected after 23 hours AEL during germ band elongation stage. The posterior shift in the anterior border is in accordance with the limited expression of *Tc-cad* in the posterior region of *Tc-lgs* RNAi embryos, which is reflected by the relative position of serosa and *Tc-cad* expression (Figure 3.1 D; 3.2 B Blue). In *Tc-pan* RNAi embryos, posterior *Tc-hb* expression is expanded more anteriorly than in wild type embryos. Similar to *Tc-lgs* RNAi embryos, *Tc-hb* expression in the posterior end of embryos was detected in the embryos during 17-23 hours AEL; and clearing of this expression was also observed during the germ band stage after 23 hours AEL (Figure 3.1 G; 3.2 C Blue).

As mentioned, posterior *Tc-hb* expression overlaps *Tc-cad* mRNA gradient in wild type embryos and *Tc-cad* expression is adjacent to serosa on the dorsal-most side of the wild type and knockdowns embryo. The relative position between the serosa and *Tc-cad* expression indicates that the shift of *Tc-hb* expression is in correlation with alteration of *Tc-cad* expression gradient in these knockdown embryos. Moreover, to complete posterior *Tc-hb* expression pulse in wild type

needs 3 hours (Figure 3.2 A' Blue). In contrast, this process in *Tc-lgs* or *Tc-pan* RNAi embryos lasted for 6 hours (Figure 3.2 B', C' Blue). Therefore, decrease of *Tc-cad* levels slows the speed of *Tc-hb* expression switch. In addition, in *Tc-pan* RNAi embryos the initial posterior expression region of *Tc-hb* at the blastoderm stage and its stable expression domain at germ band stage are wider than those in wild type and *Tc-lgs* RNAi embryos (Figure 3.1 A, D, G *hb*-I during 17-20 hours AEL and *hb*-II). This is due to the effect of a shallower slope of *Tc-cad* expression gradient, which also produced a wilder stripe of *Tc-eve* expression.

Correspondingly, initial expression of *Tc-Kr* and *Tc-mlpt* were delayed in *Tc-lgs* and *Tc-pan* RNAi embryos. *Tc-Kr* expression in the posterior was detected from 17-23 hours AEL. Its expression faded before germ band elongation stage (Figure 3.1 B; 3.2 A Red). In either *Tc-lgs* or *pan* RNAi embryos, *Tc-Kr* was mostly detected in the primitive pit (Figure 3.1 E; 3.2 B Red; 3.1 G; 3.2 C Red). Its expression detail was hard to be observed due to gastrulation. During the germ band stage from 23 to 26 hours AEL. *Tc-Kr* expression was faded at the posterior end of the germ band. The anterior expansion of *Tc-Kr* was not observed in *Tc-pan* RNAi embryos in that postponed initiation of expression results in that expansion occurs during gastrulation, which is problematic to be detected (Figure 3.1 G). Posterior *Tc-mlpt* expression was only observed in germ band elongation stage in *Tc-lgs* or *Tc-pan* RNAi embryos in 23-26 hour AEL (Figure 3.1 F; 3.2 B; 3.1 I Green; 3.2 C Green). In contrast, posterior *Tc-mlpt* expression started before the onset of the gastrulation 17-20 hours AEL. In *Tc-lgs* RNAi blastoderm, the anterior *Tc-mlpt* expression shifted posteriorly, suggesting the posterior shift in anterior border of the posterior *Tc-mplt* expression (Figure 3.1 F). In *Tc-pan* RNAi blastoderm, the anterior *Tc-mlpt* expression shifted anteriorly, correlating to anterior expansion of *Tc-cad* expression gradient (Figure 3.1 I). Moreover, the anterior shift of anterior expression and delay of posterior expression implicated the increased distance between these two expression domains, which could be explained by the shallower slope of the *Tc-cad* expression gradient.

Analysis of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression in posterior end of the blastoderm demonstrated that *Tc-cad* expression gradient regulates gap gene expression including the position, speed of progression and width of expression domain. With lower level of *Tc-cad* gradient, initiation of gene expression was delayed, which is reflected by less percentage of

Class-I embryos at same time window such as *Tc-hb* expression at 14-17 hours AEL or similar percentage of Class-I in a later time window such as *Tc-Kr* expression at 17-20 hours AEL in wild type embryos and 20-24 hours AEL in knockdown embryos. In *Tc-lgs* or *Tc-pan* RNAi embryos, posterior *Tc-mlpt* that is a blastoderm gap gene was only expressed at the germ band stage. However, these three genes were expressed in order, as in wild type embryos but at a slower pace.

Interactions between Tc-hb, Tc-Kr and Tc-mlpt suggest an activation and repression network

This sequential expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression is regulated by the *Tc-cad* gradient. However, determination of their expression boundaries is unclear. By careful examining the expression of these gene expression patterns, we noticed a small overlap between two contiguous expression domains. We hypothesized that expression of the posterior gap gene strongly represses expression of the anterior gap gene to determine the posterior boundary of anterior gene expression domain.

We first examined interactions between *Tc-hb* and *Tc-Kr*. *Tc-hb* is known to activate *Tc-Kr* expression in the blastoderm (Marques-Souza et al., 2008). In wild type germ band, *Tc-hb* is expressed weakly in the pre-gnathal head region and strongly in the gnathal segment (Figure 3.3 A). Knockdown of *Tc-Kr* expression, *Tc-hb* expression is still weak in the pre-gnathal region, but strong in a broader region that covers gnathal segments and contiguous several more posterior segments at the germ band stage (Marques-Souza et al., 2008). Since *Tc-Kr* RNAi embryos develop into a much shorter body, this strong expression of *Tc-hb* could be due to either posterior expansion of labial segment expression, or earlier and more anterior abdominal *Tc-hb* expression. Hence, we analyzed *Tc-hb* expression in *Tc-Kr* RNAi blastoderm embryos. In these embryos, clearance of *Tc-hb* expression at the posterior pole was not observed (Figure 3.3 B), indicating that *Tc-Kr* is required to repress *Tc-hb* expression from the posterior end.

Furthermore, knockdown of *Tc-Kr* also results in weaker expression of *Tc-mlpt* in the germ band (Savard et al., 2006). However, initiation of *Tc-mlpt* expression in the *Tc-Kr* RNAi

blastoderm has not been reported. Therefore, we examined the expression of *Tc-mlpt* in *Tc-Kr* RNAi embryos. In these embryos, initiation of *Tc-mlpt* was not affected; it was still expressed in the posterior end of the embryos as the primitive pit forms (Figure 3.3 D). Therefore, *Tc-Kr* might play a role in activating *Tc-mlpt* expression, but is more likely to cooperate with other genes. One candidate is *Tc-cad*. In *Tc-hb* RNAi embryos, expression of *Tc-Kr* is dramatically reduced, but still expressed at the posterior end of the embryo (Figure 3.3 F). On the other hand, in severe *Tc-cad* RNAi embryos, posterior expression of *Tc-hb* and *Tc-Kr* was not detected (Figure 3.3 G, H). Hence, *Tc-cad* appears to regulate *Tc-Kr* expression as does *Tc-hb*. Similarly, the loss of *Tc-mlpt* expression in *Tc-cad* RNAi embryos suggests that its expression is also regulated by *Tc-cad.* Furthermore, in *Tc-mlpt* RNAi embryos, *Tc-Kr* expression expends posteriorly (Savard et al., 2006). This result indicates that *Tc-mlpt* is involved in patterning, if does not determine, the posterior boundary of *Tc-Kr* expression.

In summary, in the Tribolium blastoderm, *Tc-hb* activates *Tc-Kr* expression and *Tc-Kr* contributes to the initiation of *Tc-mlpt* expression while *Tc-cad* regulates the activation of all three genes. In addition, the *Tc-cad* expression gradient determines the tempo of the gap gene expression composed of these three genes. Furthermore, *Tc-Kr* represses *Tc-hb* expression posteriorly; *Tc-mlpt* also negatively regulates *Tc-Kr* posterior expression. Therefore, a network of activation and repression among them can be described (Figure 3.4). The *Tc-cad* gradient is upstream of this network. To further study the regulation between these genes, we analyzed double knockdowns of these genes. In *Tc-hb*;*Tc-mlpt* double RNAi embryos, *Tc-Kr* expression is restricted to the posterior end of the blastoderm (Figure 3.5 B), which resembles the impact on *Tc-Kr* expression by *Tc-hb* (Figure 3.3 F). In *Tc-mlpt*;*Tc-Kr* double RNAi embryos, *Tc-hb* expression did not fade from the posterior end (Figure 3.5 D) as in *Tc-Kr* RNAi embryos (Figure 3.3 B). If the anterior border of *Tc-mlpt* is determined by repression from both *Tc-Kr* and *Tc-hb* as hypothesized, one might expect *Tc-mlpt* expression to shift anteriorly into the region where *Tc-hb* and *Tc-Kr* are expressed in wild type. However, it did not expand to completely encompass the region where *Tc-hb* and *Tc-Kr* are expressed (Figure 3.5 F).

The cause of this unexpected result may be due to additional regulation by other genes. In the Tribolium blastoderm, another gap gene, *Tc-gt*, is expressed maternally and zygotically. In

new laid eggs, *Tc-gt* is expressed ubiquitously. Then it is expressed weakly in the pre-gnathal region, overlapping *Tc-hb* expression and more strongly in the maxillary segment (Bucher and Klingler, 2004). In *Tc-gt* RNAi embryos, expression of *Tc-Kr* expands anteriorly and posteriorly. *Tc-mlpt* expression also expands in both directions (Bucher and Klingler, 2004; Savard et al., 2006). If *Tc-gt* inhibits the expansion of *Tc-mlpt* expression in *Tc-hb*;*Tc-Kr* double RNAi embryos, then expression of *Tc-mlpt* should be expanded anteriorly significantly in *Tc-hb*;*Tc-gt* double RNAi embryos, since knocking down *Tc-hb* and *Tc-gt* not only reduces the expression of *Tc-hb* and *Tc-gt* directly, but also reduces the expression of *Tc-Kr* indirectly, through *Tc-hb*. However, in *Tc-hb*;*Tc-gt* RNAi embryos, the initiation of *Tc-mlpt* expression appears to be normal in the primitive pit of the embryos (Figure 3.5 G). Thus, the anterior border of *Tc-mlpt* expression domain in the blastoderm is also not determined by the repression from *Tc-gt.*

Discussion

In this study, we demonstrated that expression of certain gap genes, *Tc-hb*, *Tc-Kr* and *Tc-mlpt*, are regulated by the *Tc-cad* expression gradient in the Tribolium blastoderm. We examined the spatiotemporal dynamics of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression in wild type and in RNAi embryos with different *Tc-cad* expression backgrounds produced by disruption of Wnt signaling pathway components. Sequential expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* is regulated by the *Tc-cad* gradient, which resembles that of *Tc-eve* expression, suggesting both gap gene expression and the insect segmentation clock are regulated by *Tc-cad*. We also studied the interaction between gap genes. Genetic interactions between *Tc-hb* and *Tc-Kr* and *Tc-mlpt* largely determined their expression patterns, with additional repressive input from *Tc-gt* and activation input from upstream *Tc-cad*. Combining all these results suggests that the *Tc-cad* gradient regulates the gap genes within this regulatory network, rather than individually. The level of *Tc-cad* expression is correlated with the activation of gene expression at the posterior end of the embryo, to guide transitions within this serial expression of gap genes. The posterior expression boundaries of these three gene appear to be determined through interactions between them, which are mostly conserved in Tribolium and Drosophila. Since in Tribolium, gap genes are important regulators of Hox genes, which determine the identity of segments, it is likely that *Tc-cad* functions through the regulation of gap gene expression to determine segmental identity.

Regulation of the gap gene expression by the Tc-cad gradient

Analysis of *Tc-cad* RNAi embryos indicates an activation role for *Tc-cad* in the regulation of gap gene expression. Here, serial gap gene expression within a specific developmental window during the blastoderm stage in the embryos of wild type and RNAi against Wnt pathway components was studied. The gap gene timer appears to be regulated by the gradient of *Tc-cad* in a dose-dependent manner. Lower levels of *Tc-cad* expression in the posterior end of the embryos lead to slower transition of gap gene expression from one to the next. Furthermore, the slope of the *Tc-cad* expression gradient impacted the region of gap gene expression; less steep gradients led to broader expression domains. Therefore, *Tc-cad* is not only an activator of gap gene, but its expression gradient also determines the expression status of the serial expression of three gap genes. Interaction between gap genes is not only required to maintain expression boundaries between them, but is also important to determine downstream Hox gene expression, which eventually defines the identity of segments.

The *Tc-pan* RNAi embryo cuticles are consistent with the hypothesis that *Tc-cad* expression gradient regulates pair-rule and gap gene expression coordinately to regulate segmentation and segmental identity. Knocking down *Tc-pan* produces a *Tc-cad* gradient with a less steep slope, which results in wider *Tc-eve* stripes and ultimately wider than normal segments in *Tc-pan* RNAi cuticles. Appendages fail to develop due to the lack of Wnt signaling; only the most proximal segments, the coxa, form in these cuticles (Fu et al., 2012). However, segmental identities are normally fated, reflecting the wider gap gene and concomitant Hox gene expression domains observed in these embryos.

Both gap and pair rule genes are similarly regulated by gradients of Wnt signaling activity and *Tc-cad* in overlapping domains in Tribolium, which may represent a more ancestral form of insect segmentation. Such regulatory paradigms may prove conducive to the evolution of interactions between gap and pair rule genes, leading to the segmentation hierarchy found in Drosophila. In fact, we described secondary, minor interactions between gap and pair rule genes in Tribolium that support such speculation.

Interactions between gap genes are similar to those in Drosophila

In Drosophila, gap genes are expressed simultaneously in the early blastoderm. Expression of each gap gene in a certain region along the A-P axis requires positional input from maternal morphogen gradients. However, expression boundaries between gap genes are defined through mutual repression between them.

In developing a *Tc-hb*, *Tc-Kr* and *Tc-mlpt* regulatory network, we briefly described the interaction between *Tc-hb* and *Tc-Kr*, which is similar to that of their Drosophila homologs. In Drosophila, higher *Dm-hb* protein concentrations repress *Dm-Kr* expression at the anterior border of the *Dm-Kr* expression domain; lower *Dm-hb* concentrations activate *Dm-Kr* expression (Struhl et al., 1992; Schulz and Tautz, 1994). On the other hand, *Dm-Kr* is a transcriptional repressor, which can also repress *Dm-hb* expression (Zuo et al., 1991). In Tribolium, knocking down *Tc-hb* expression causes reduced *Tc-Kr* expression, only weak *Tc-Kr* expression remains at the posterior end of the blastoderm (Marques-Souza et al., 2008). On the contrary, *Tc-hb* expression was not repressed at the posterior end of *Tc-Kr* RNAi embryos. In Gryllus, similar interaction between the homologs of *Gb-hb* and *Gb-Kr* has also been observed (Mito et al., 2006). Therefore, the interaction between these two genes is conserved in most if not all insects.

We also examined the interaction between *Tc-gt* and *Tc-Kr*. In Tribolium, *Tc-Kr* expression covers the thoracic segment (Cerny et al., 2005). Maternal expression of *Tc-gt* appears throughout the entire early blastoderm (Bucher and Klingler, 2004). Then zygotic *Tc-gt* is expressed strongly in the maxillary segments and weakly in the head region. Later *Tc-gt* is expressed from T3 to A2 and refined to two stripes in these segments. In *Tc-gt* RNAi blastoderm embryos, *Tc-Kr* expression is expanded anteriorly compared to wild type (Figure 3.6 B). At the germ band stage, expression of *Tc-Kr* is not only more anterior, but also contiguous in several more posterior segments (Bucher and Klingler, 2004). It appears that *Tc-gt* is important to set both anterior and posterior boundaries of *Tc-Kr* expression. In contrast, knocking down *Tc-Kr*, posterior expansion of *Tc-gt* expression was detected in both the blastoderm (Figure 3.6 D) and germ band (Cerny et al., 2005). In summary, in Tribolium, mutual repression defines the

posterior expression border of gnathal *Tc-gt* expression as well as anterior border of *Tc-Kr* expression, the posterior border of which is determined by repression from the trunk *Tc-gt* expression domain. Similarly, in Drosophila, interaction between *Dm-gt* and *Dm-Kr* expression also contributes to the establishment of the expression boundary between these two genes (Kraut and Levine, 1991). However, in Tribolium, the second expression domain of *Tc-gt* in the trunk is likely to be activated by *Tc-Kr*. Knocking down *Tc-hb*, *Tc-Kr* or *Tc-mplt* results in the loss of *Tc-gt* expression in T3 and T4 (Savard et al., 2006). Hence, the expansion of *Tc-Kr* expression in *Tc-mlpt* RNAi embryos may be through the interaction between *Tc-Kr* and *Tc-gt.*

In Drosophila, interaction between gap genes is critical to activate pair-rule genes. For example, interaction between anterior *Dm-gt* and *Dm-Kr* determines the expression domain of the second *Dm-eve* stripe (Small et al., 1992; Small et al., 1996). However, in Tribolium, pair-rule gene expression is not primarily regulated by gap genes. In contrast, effects on segmentation from gap genes are mostly observed as changes in segmental identity. Therefore, interactions between gap genes predominantly pattern the expression of Hox genes, instead of pair-rule genes, to assign the identity of segment. Analysis of *Tc-cad* RNAi embryos indicates an activation role for *Tc-cad* in the regulation of gap gene expression. Here, serial gap gene expression within a specific developmental window during the blastoderm stage in the embryos of wild type and RNAi against Wnt pathway components was studied. The serial expression of gap genes appears to be regulated by the gradient of *Tc-cad* in a dose-dependent manner. Lower levels of *Tc-cad* expression in the posterior end of the embryos led to slower transition of gap gene expression from one to the next. Furthermore, the slope of the *Tc-cad* expression gradient impacted the region of gap gene expression; less steep gradients led to broader expression domains. Therefore, *Tc-cad* is not only an activator of gap genes, but its expression gradient also determines the expression status of the gap gene network. Meanwhile, the interaction between gap genes is not only required to maintain expression boundaries between them, but is also important to determine downstream Hox gene expression, which eventually defines the identity of segments.

Tc-cad determines segmental identities through the regulation of gap genes

In Drosophila, expression of Hox genes is regulated by gap, pair-rule and segmental polarity genes. In Tribolium, homologs Hox genes are mainly regulated by gap genes, as determined by examining the expression of Hox genes in gap gene RNAi embryos. In *Tc-hb* RNAi embryos, expression of *Tc-Antp*, *Tc-Ubx* and *Tc-abd-A* (which are expressed in thorax and abdomen in wild type) expand anteriorly to abut *Tc-Dfd* expression in the maxillary segment. Changes of expression of these three genes are correlated to the transformation of labium and thorax into abdominal segments (Marques-Souza et al., 2008)..

In *Tc-Kr* RNAi cuticles, instead of forming thorax and abdomen, the labial segment is followed by repeated segments of maxillary and labial identity (Cerny et al., 2005). Corresponding repetitive expression of *Tc-Dfd* and *Tc-Scr* are detected in these embryos (Cerny et al., 2005). On the contrary, knocking down *Tc-gt* expression causes maxillary and labial segments to transform into thoracic segments (Bucher and Klingler, 2004). *Tc-Dfd* expression is limited to one segment and no *Tc-Scr* expression has been detected in these embryos. Instead, *Tc-Antp* expression is anteriorly expanded. Thus, *Tc-Kr* positively regulates *Tc-Antp* and *Tc-Ubx* expression while *Tc-gt* and *Tc-hb* activate *Tc-Scr* and *Tc-Dfd* expression. In Drosophila, *Dm-gt* is responsible for establishing the expression border of *Dm-Antp* and activating *Dm-Scr* (Reinitz and Levine, 1990). The regulation of *Antp* and *Scr* homologs expression by the gap gene *gt* is likely to be conserved between these two insects.

Although, Hox gene expression has been analyzed in the gap gene RNAi embryos described above, the regulation of Hox gene by gap genes is still not completely understood. A more complicated case has been reported for *Tc-gt* RNAi performed in a *jaws* mutant background (*jaws* is a mutant in *Tc-Kr*) embryos. These embryos resemble *Tc-gt*;*Tc-Kr* double RNAi embryos in that they both display one extra maxillary and labial segmental repeat, but the identity of the following segment is indiscernible (Cerny et al., 2005). We also performed *Tc-gt* and *Tc-Kr* double RNAi and also could not determine the identity of the posterior segments. Therefore, further comparative studies of Hox gene expression in gap gene single and double RNAi embryos may provide a more detailed understanding of the interactions between gap genes as well as between gap and Hox genes, which may also contribute to our understanding of the

morphological difference between Drosophila and Tribolium.

Crosstalk between pair-rule gene clock and gap gene timer under the control of Tc-cad gradient

In Drosophila, gap genes are upstream of pair-rule genes. Indeed, pair-rule gene expression is regulated by various combinations of gap genes. However, similar interactions between gap and pair-rule genes have only been observed in certain regions of Nasonia embryos (Rosenberg et al., 2014), and not in any short germ insects. In Tribolium, three pair-rule genes are considered to be primary pair rule genes, *Tc-eve*, *Tc-odd* and *Tc-runt*. These three genes have been examined in different single gap gene RNAi embryos. In the blastoderm of these embryos, expression of these pair-rule genes appears to be normal. However, we did notice the ectopic expression of *Tc-eve* in the *Tc-gt* RNAi germ band. Instead of forming stripes, *Tc-eve* was expressed in a wide region in the trunk in the early germ band stage, which implicates a secondary impact on pair-rule gene expression by gap genes. We focused on *Tc-eve* expression in the blastoderm. While the primitive pit forms, the third stripe of *Tc-eve* expression starts to be noticeable at the posterior end of the wild type embryos. *Tc-eve* expression was examined in the blastoderm stage of different double gap gene knockdown embryos. *Tc-eve* expression oscillation was not to be disrupted in the blastoderm, and initiation of the third stripe was observed at the posterior end of different double knockdown embryos. However, since knockdown of gap genes affected germ band elongation causing truncation, the *Tc-eve* expression domain was also misshaped in these embryos, most likely due to the abnormal shape of the embryos.

On the other hand, after disruption of pair-rule genes, gap genes were still expressed in the blastoderm and germ band. However, in these embryos, segments are not well defined. It is impossible to correlate gap expression to a specific region of the body. In addition, embryos with severely reduced pair-rule gene expression still developed into the germ band stage. But the shape of these embryos is quite different from wild type, which also presents a challenge when analyzing gap expression in pair-rule gene RNAi embryos.

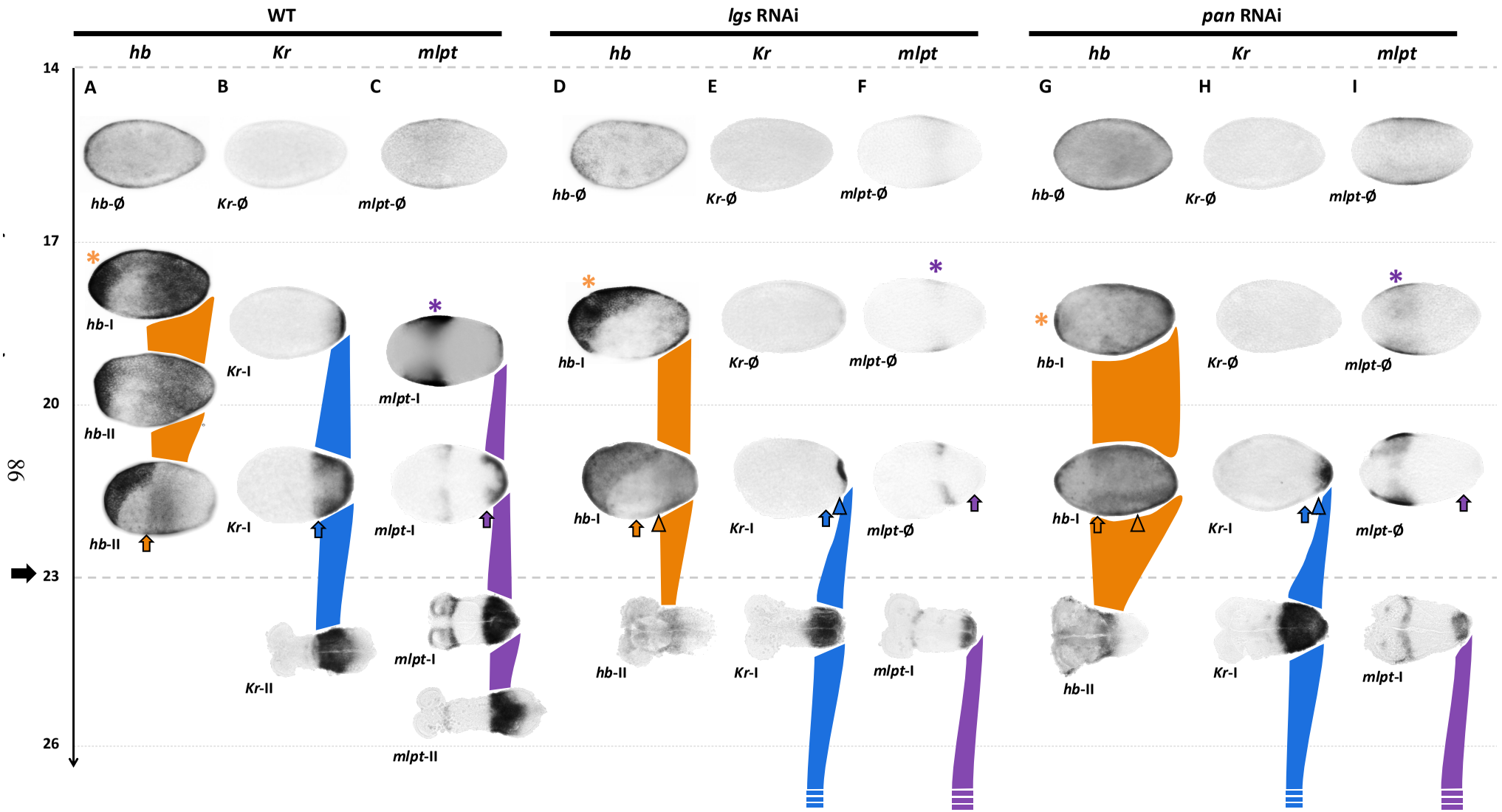


Figure 3.1 Expression of Tc-hb, Tc-Kr and Tc-mlpt in wild type embryos and Tc-lgs and Tc-pan RNAi embryos

Figure 3-1 Expression of *Tc-hb, Tc-Kr* and *Tc-mlpt* in wild type embryos and *Tc-lgs* and *Tc-pan* RNAi embryos

(A) *Tc-hb* expression in wild type embryos. (*hb*-Ø) Ubiquitous maternal expression before 14 hr AEL; (*hb*-I) Orange asterisk denotes expression in the anterior presumptive serosa region. Orange blocks track dynamic expression in the posterior region where gnathal and trunk segments will form. (*hb*-II) Expression fades from the posterior end of the embryo between 17-20 hr AEL. Eventually, posterior expression only covers gnathal region after 20 hr AEL. Orange arrow denotes the anterior border of the expression domain.
(B) *Tc-Kr* expression in wild type embryos. (*Kr*-Ø) No expression before 17 hr AEL. (*Kr*-I) Expression appears at the posterior end of the embryo within 17-20 hr AEL. Within 20-23 hr AEL, expression expands anteriorly. A bright blue arrow labels the anterior border of the posterior expression domain of *Tc-K*r and Bright blue colored blocks are used to track the dynamic expression of posterior *Tc-Kr*. (*Kr*-II) After 23 hr AEL expression clears from the posterior and is restricted to a domain covering the thoracic region.
(C) *Tc-mlpt* expression in wild type embryos. (*mlpt*-Ø) No expression prior to 17 hr AEL. (*mlpt*-I) Expression is observed at the posterior pole of the embryo between 17-20 hr AEL and expands anteriorly between 20-23 hr AEL. During the germ band stage after 23 hr AEL, *Tc-mlpt* is expressed in the posterior growth zone for a short time and then (*mlpt*-II) in a more anterior domain. A purple arrow labels the anterior border of the posterior expression domain of *Tc-mlpt* and purple colored blocks are used to track the dynamic expression of posterior *Tc-mplt*.
(D) *Tc-hb* expression in *Tc-lgs* RNAi embryos. (*hb*-Ø) Maternal expression is not affected. (*hb*-I) Initiation of posterior expression is not affected between 17-20 hr AEL. But the anterior border of the posterior expression domain (labeled by an orange triangle), is shifted posteriorly compared to wild type embryos, (orange arrow).
(E) *Tc-Kr* expression in *Tc-lgs* RNAi embryos. (*Kr*-Ø) no expression between 14-20 AEL (*Kr*-I) Initial expression is delayed until 20-23 hr AEL and retained at the posterior end of the embryo, as denoted by a blue triangle. (*Kr*-II) Anterior expansion of expression occurs in the germ band (tracked by bright blue blocks).
(F) *Tc-mlpt* expression in *Tc-lgs* RNAi embryos. (*mlpt*-Ø) no posterior expression, as in wild type. (*mlpt*-I) Posterior expression is delayed until the germ band stage after 23 hr AEL. A purple arrow denotes the anterior expression border in the wild type embryo (C *mlpt*-I) and where it

would be expected in the *Tc-lgs* RNAi embryo (F *mlpt-Ø)*

(G) *Tc-hb* expression in *Tc-pan* RNAi embryos. (*hb*-Ø) Ubiquitous maternal expression is normal. (*hb*-I) posterior expression initiates as in wild type, but in a broader domain due to anterior expansion past the normal anterior border (orange arrow). Dynamic expression is tracked by the orange blocks. Orange asterisk denotes expression in the anterior presumptive serosa region.

(H) *Tc-Kr* expression in *Tc-pan* RNAi embryos. (*Kr*-Ø) No expression from 14-20 hr AEL. (*Kr*-I) Initiation of expression is delayed to between 20-23 hr AEL as in *Tc-lgs* RNAi embryos. But its expression expands more anteriorly. Blue triangles mark the anterior border in *Tc-lgs* RNAi embryos and the equivalent position in *Tc-pan* embryos. (*Kr*-II) Expression domain in the germ band is wider than in wild type or *Tc-lgs* RNAi embryos. Dynamic expression is traced by bright blue blocks.

(I) *Tc-mlpt* expression in *Tc-pan* RNAi embryos. (*mlpt*-Ø) No posterior expression until after, 23 hr AEL. Head expression is shifted more anterior. (*mlpt*-I) Posterior expression initiates in the posterior end of the germ band.

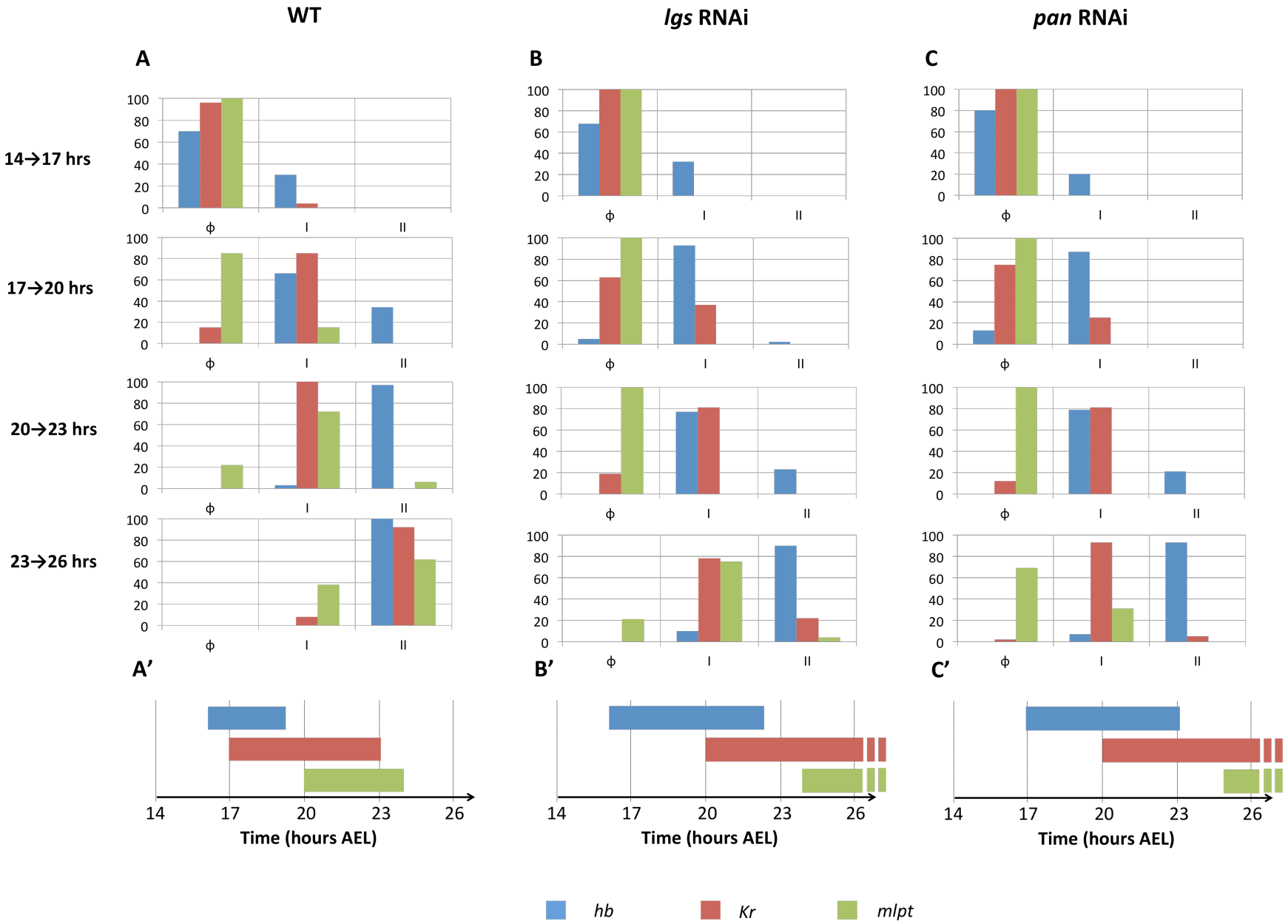

WT
lgs RNAi
pan RNAi
A
B
C
14→17 hrs
17→20 hrs
20→23 hrs
23→26 hrs
100
80
60
40
20
0
ϕ
I
II
A'
B'
C'
14
17
20
23
26
Time (hours AEL)
hb
Kr
mlpt

Figure 3-2 Quantitative analysis of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression at the posterior end of the wild type and *Tc-lgs* and *Tc-pan* RNAi embryos

Embryos prior to the expression of a gap gene at the posterior end of the blastoderm are considered to be in Phase Ø. Embryos with gap gene expression at the posterior end are considered to be in Phase I. Phase II is defined as the time at which gap gene expression has cleared from the posterior end of the embryo. Blue for *Tc-hb,* Red for *Tc-Kr,* Green for *Tc-mlpt.*

(A) In wild type embryos, between 14-17 hr AEL, several embryos are in *Tc-hb* Phase I, only a few are in *Tc-Kr* phase I and all embryos are in *Tc-mlpt* Phase Ø. Between 17-20 hr AEL, a majority of the embryos are in *Tc-hb* Phase I and *Tc-Kr* Phase I, but only a few embryos have entered *Tc-mlpt* Phase I at this time. Between 20-23 hr AEL, almost all embryos are in *Tc-hb* Phase II and *Tc-Kr* Phase I, and majority of the embryos were in *Tc-mlpt* Phase I. Between 20-23 hr AEL, only a few embryos are still in *Tc-Kr* Phase I and the majority of embryos are *in Tc-mlpt* Phase I.
(A') The duration of gap gene expression in Phase I in wild type embryos. Proportions of embryos in gap gene Phase I is converted into the length of expression time.
(B) In *Tc-lgs* RNAi embryos, between 14-17 hr AEL, several embryos are in *Tc-hb* Phase I and all embryos are in *Tc-Kr* Phase Ø, and *Tc-mlpt* Phase Ø. Between 17-20 hr AEL, almost all embryos are in *Tc-hb* Phase I and *Tc-mlpt* Phase Ø, and several embryos are in *Tc-Kr* Phase I. Between 20-23 hr AEL, a majority of embryos were in *Tc-hb* Phase I and *Tc-Kr* Phase I, and all embryos are still in *Tc-mlpt* Phase Ø.
(B') The duration of gap gene expression in Phase I in *Tc-lgs* RNAi embryos. The expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* at the posterior end of the embryos lasts longer than in wild type embryos. Although initiation of *Tc-Kr* and *Tc-mlpt* expression is delayed, these three genes are still expressed in the same order as in wild type embryos. Longer expression of an early, anterior gene is correlated with the delayed expression of a next, posterior gene.
(C) In *Tc-pan* RNAi embryos, *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression in all time widows are similar to that in *Tc-lgs* RNAi embryos, except the initiation of gene expression is somewhat delayed in *Tc-pan* RNAi embryos.

(C') The duration of gap gene expression in Phase I in *Tc-pan* RNAi embryos. The expression of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* at the posterior end of the embryos still occurrs in the same order as in *Tc-lgs* RNAi and wild type embryos.

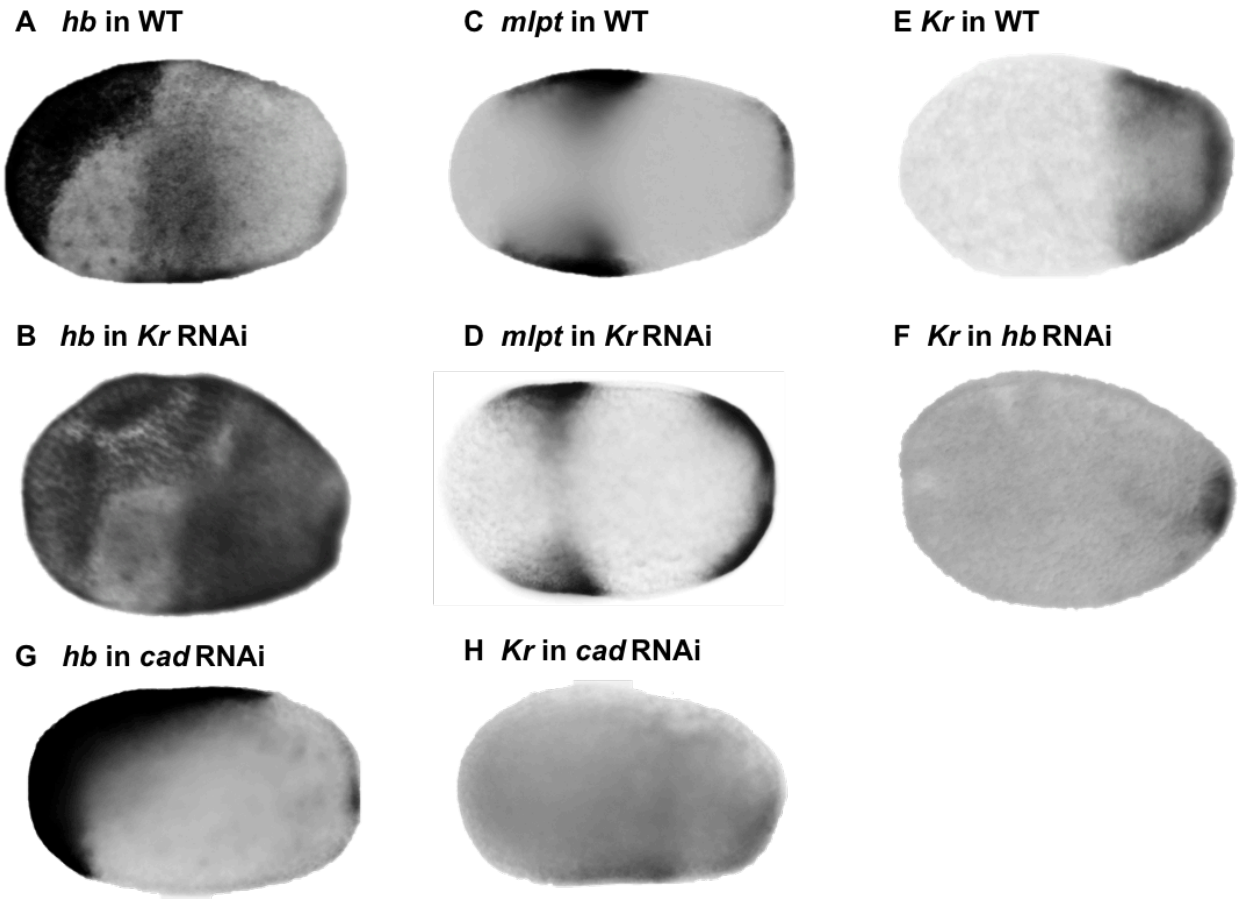


Figure 3-3 Interactions between *Tc-hb*, *Tc-Kr* and *Tc-mlpt* in the Tribolium blastoderm

(A) *Tc-hb* is expressed in the serosa and gnathal segments of the differentiated wild type embryo.

(B) *Tc-hb* expression is expressed in the posterior region of *Tc-Kr* RNAi embryos.

(C) *Tc-mlpt* is expressed in the head lobes and at the posterior end of the differentiated wild type embryo.

(D) *Tc-mlpt* is still expressed at the posterior end of *Tc-Kr* RNAi embryos.

(E) *Tc-Kr* is expressed in the posterior third of the differentiated wild type blastoderm.

(F) *Tc-Kr* expression is reduced to a small domain at the posterior pole of *Tc-hb* and *Tc-hb*; *Tc-mlpt* RNAi embryos.

(G, H) Expression of *Tc-hb* and *Tc-Kr* is not detected in the posterior region of the strong *Tc-cad* RNAi embryos.

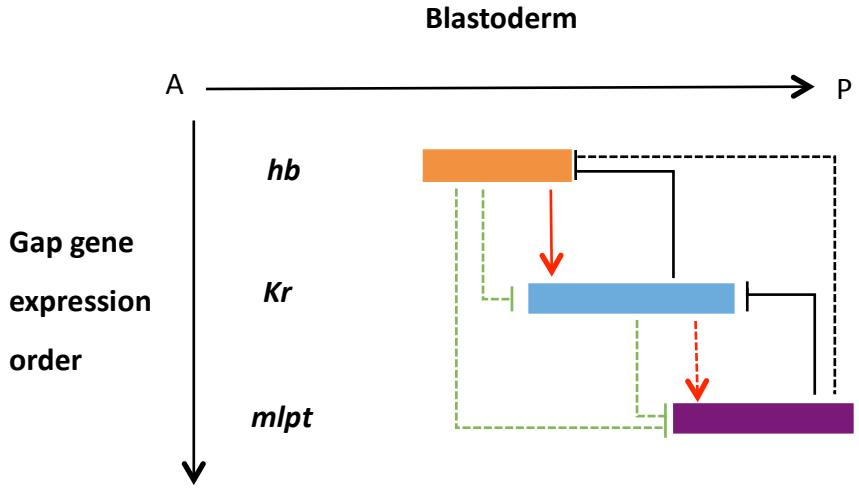


Figure 3-4 Expression boundary formation between *Tc-hb*, *Tc-Kr* and *Tc-mlpt*

In addition to activation in the posterior end of the embryo by *Tc-cad*, interactions between gap genes refine their expression domains. *Tc-hb* (orange) activates (denoted by red arrow) *Tc-Kr* (blue). *Tc-Kr* then represses posterior *Tc-hb* expression (denoted by solid black line). Some *Tc-Kr* input is required to activate and maintain *Tc-mlpt* expression (purple) (broken red arrow), which then represses posterior *Tc-Kr* expression. *Tc-mlpt* appears to repress posterior *Tc-hb* expression as faint ectopic *Tc-hb* expression appears at the posterior end of *Tc-mlpt* RNAi embryos (dashed black line).

Factors regulating the anterior border of *Tc-mlpt* were not revealed in these studies, *since Tc-mlpt* is still expressed in *Tc-Kr* single knockdown, and in *Tc-hb;Tc-Kr* or *Tc-hb;Tc-gt* double knockdown embryos (see text for details). Since *Tc-Kr* is not expressed in *Tc-hb* RNAi embryos, it is formally possible that high levels of *Tc-hb* repress *Tc-Kr* anteriorly as in Drosophila (green dashed line)

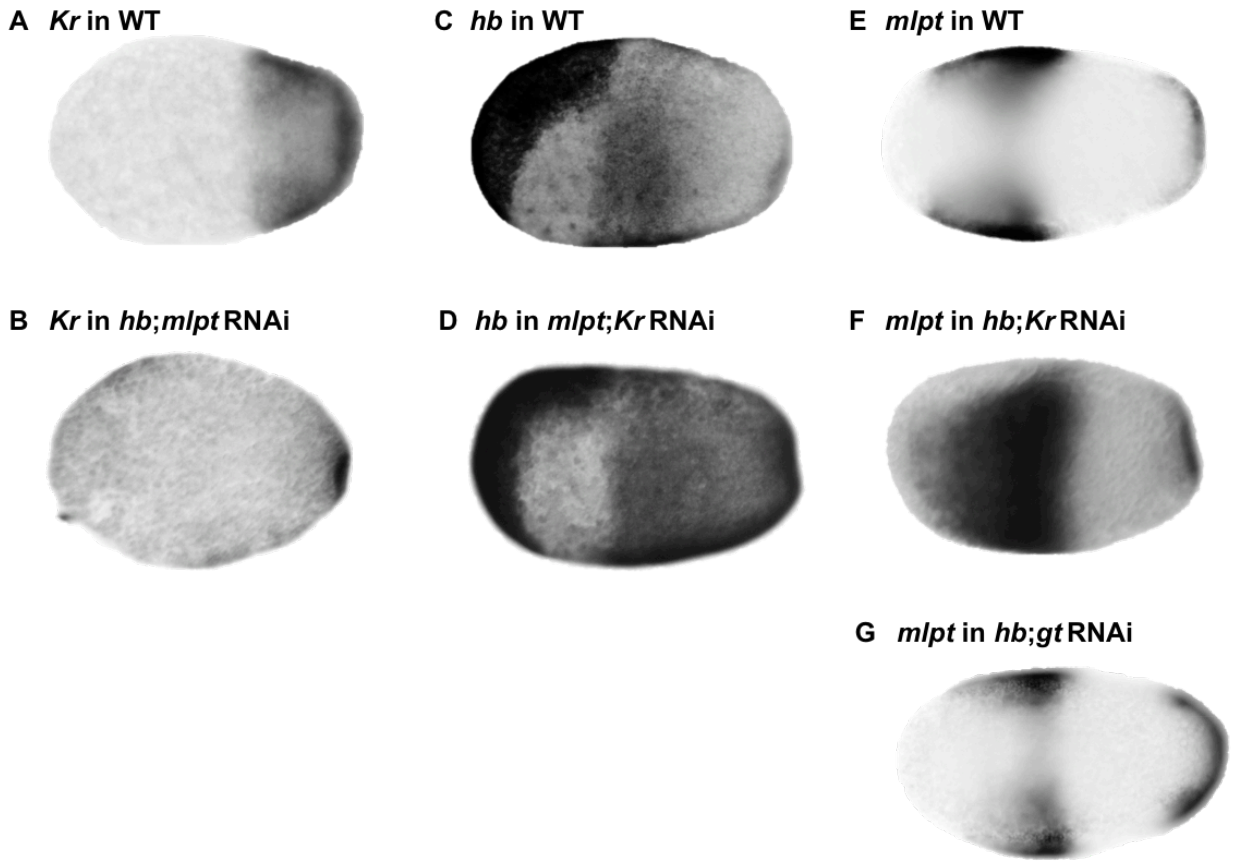


Figure 3-5 Analysis of *Tc-hb*, *Tc-Kr* and *Tc-mlpt* expression in RNAi and wild type embryos

(A) *Tc-Kr* is expressed in the posterior third of the differentiated wild type blastoderm.

(B) *Tc-Kr* expression is reduced to a small domain at the posterior pole of *Tc-hb*; *Tc-mlpt* RNAi embryos.

(C) *Tc-hb* is expressed in the serosa and gnathal segments of the differentiated wild type embryo.

(D) *Tc-hb* expression does not fade from the posterior end of *Tc-Kr*; *Tc-mlpt* RNAi embryos.

(E) *Tc-mlpt* is expressed in the head lobes and at the posterior end of the differentiated wild type embryo.

(F) *Tc-mlpt* is still expressed at the posterior end of *Tc-hb*;*Tc-Kr* RNAi embryos.

(G) *Tc-mlpt* expression in the posterior end of the *Tc-hb*; *Tc-gt* RNAi embryos appears to be normal in the blastoderm stage.

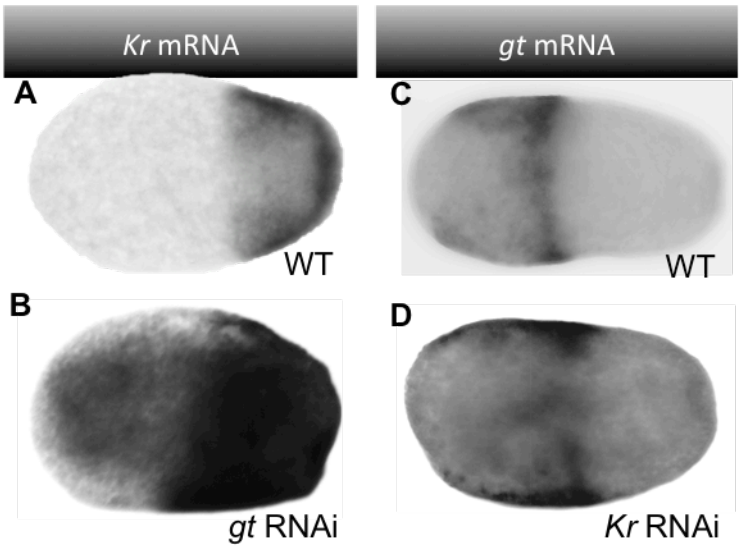


Figure 3-6 Mutual repression between *Tc-gt* and *Tc-Kr* in Tribolium blastoderm embryos

(A) *Tc-Kr* expression in wild type embryos covers approximately 30% of the embryo length from the posterior end.

(B) *Tc-Kr* is expressed in the posterior half of *Tc-gt* RNAi embryos.

(C) The posterior border of the *Tc-gt* expression domain is anterior to the mid-line on the A-P axis in wild type embryos.

(D) The posterior border of the *Tc-gt* expression domain expands posteriorly in *Tc-Kr* RNAi embryos.

Table 3-1 Number of embryos documented for examining the expression of each gene at different stages in the embryos with different Wnt activities

Expression stage	Number of embryos for *Tc-hb* expression	Number of embryos for *Tc-Kr* expression	Number of embryos for *Tc-mlpt* expression
EL3 IT14 in WT	27	24	25
EL3 IT17 in WT	29	26	26
EL3 IT20 in WT	29	26	32
EL3 IT23 in WT	22	25	22
EL3 IT14 in *lgs* RNAi	32	25	30
EL3 IT17 in *lgs* RNAi	42	24	30
EL3 IT20 in *lgs* RNAi	12	21	30
EL3 IT23 in *lgs* RNAi	10	35	28
EL3 IT14 in *pan* RNAi	25	35	30
EL3 IT17 in *pan* RNAi	23	20	30
EL3 IT20 in *pan* RNAi	28	26	22
EL3 IT23 in *pan* RNAi	15	43	29

Chapter 4 - Summary

Animals with segmented body plans are found in only three phyla, the annelida, arthropoda and chordata. However, these three phyla are distantly related in the phylogenetic tree, belonging to different superphyla. Their neighboring phyla within the same superphylum are all unsegmented. To understand the origin of metamerization, comparative studies have been conducted within and between these three metameric phyla.

Somitogenesis in several vertebrates, such as fish, chicken and mouse, appear to implement a general mechanism among them, described by the 'clock and wavefront' model. However, in arthropods, at least two different mechanisms appear to function in long germ and short germ insects.

Drosophila, a long germ insect, segments as well as the fate map of the embryos are determined by the segmentation gene hierarchy initiated by inverted morphogen gradients emanating from opposite ends of the embryo. However, the anterior morphogen is highly derived and absent in non-dipteran insects. Instead, an anterior localized *otd* gradient has been described as a functional substitute of this anterior morphogen gradient in Tribolium (a short germ insect) and Nasonia (a long germ insect). Results of analyzing the expression of two blastoderm fate map patterning genes, *Tc-zen-1* and *Tc-cad* implicates *Tc-otd-1* as a general activator of these genes in the anterior and posterior regions of the embryo, respectively. These two genes are also positively regulated by other genetic factors: Dpp signaling impacts *Tc-zen-1* expression and Wnt signaling for *Tc-cad* expression. *Tc-zen-1* and *Tc-cad* repress *Tc-otd-1* expression restricting it to the presumptive pre-gnathal region. Meanwhile, anterior expression of *Tc-zen-1* determines serial formation, while *Tc-cad* expression in the posterior region of the embryos regulates formation of all gnathal and trunk segments. Although Wnt signaling activity is required to form the *Tc-cad* gradient in the posterior region of the embryos, Wnt signaling activity also impacts the expression of *Tc-zen-1*, which ultimately impacts the formation of the serosa. Therefore, in Tribolium, Wnt activity is important to the fate of cells along the A-P axis, at least in the blastoderm.

Previously, studies conducted in our lab described the details of a repressive component of Wnt signaling pathway, *Tc-axn*. mRNA of this gene is localized at the anterior pole of the wild type embryos, repressing Wnt signaling in this region, which is important to serosa formation. When Wnt activity is up-regulated in *Tc-axn* RNAi embryos, *Tc-zen-1* expression is dramatically reduced and the serosa in these embryos is much smaller than in wild type. Furthermore, studies of *Tc-cad* expression in embryos with different levels of Wnt signaling activity indicate that the *Tc-cad* expression gradient is mostly, if not completely, determined by the Wnt signaling pathway. Moreover, several Wnt ligands are expressed at the posterior end of embryos. Since Wnt signaling is repressed in the anterior region of the embryo and activated in the posterior, a posterior to anterior Wnt signaling activity gradient is likely to be formed in the Tribolium blastoderm, which is interpreted as a gradient of *Tc-cad* expression.

The posterior *Tc-cad* gradient is required for the formation of gnathal and trunk segments. In embryos lacking the *Tc-cad* gradient, only pre-gnathal cuticle is formed. However, it was unclear how *Tc-cad* regulates other genes to induce segmentation. In recent work, we demonstrated that *Tc-cad* gradient regulates the oscillating expression of a pair-rule gene in Tribolium blastoderm, and the expression of pair-rule genes decides where and when segments form. The levels of *Tc-cad* expression determine the tempo of pair-rule expression oscillation. The position of the *Tc-cad* gradient sets the anterior boundary of the initial pair-rule expression. The slope of the *Tc-cad* gradient defines the width of pair-rule gene stripes. Therefore, the *Tc-cad* expression gradient regulates segment formation both temporally and spatially.

Similar regulation of gap genes by the *Tc-cad* expression gradient is described in this work. Unlike pair-rule genes, which are expressed reiteratively in the blastoderm and germ band, gap genes are expressed in more regionalized domains. However, the serial expression of the gap genes, *Tc-hb*, *Tc-Kr* and *Tc-mlpt* is also regulated by the *Tc-cad* expression gradient similar to the regulation of pair-rule gene oscillation. The position and maximum level of the *Tc-cad* expression gradient together determine the anterior border and timing of gap gene expression. The slope of the *Tc-cad* gradient decides the width of each gap gene expression domain. Since segments are gradually added in Tribolium, the width of gap gene expression also reflects the

duration of expression. In addition, these gap genes are expressed in a specific order, and changes in the width of gap gene expression domains also reflect the timing of transitioning from one gap gene to another. Hence, the slope of the *Tc-cad* gradient also regulates the duration and transition of gap gene expression. Furthermore, we demonstrated that conserved mutual repression between gap genes is also crucial to refine gap gene expression. Therefore, the *Tc-cad* expression gradient does more than serially activate gap gene expression; it regulates a gap gene timer network.

Our studies on the regulation of this gap gene timer network by *Tc-cad*, demonstrate the sequential activation of gap genes at the posterior end of the embryo, which provides a clue to the regulation of the pair-rule gene circuit. Work published from our lab describes a pair-rule gene circuit composed in which *Tc-eve* activates *Tc-runt* which activates *Tc-odd* which in turn represses *Tc-eve* (Choe et al., 2006). The sequential expression of these genes in a negative feedback generates pair-rule stripes. Studies to date have only focused on how the *Tc-cad* expression gradient regulates one of the primary pair-rule genes, *Tc-eve*, not the others. However, just as *Tc-cad* serially activates expression in the gap gene timer, it may also serially activate *Tc-runt* and *Tc-odd* in the pair-rule circuit.

Furthermore, Tribolium gap genes not only impact pair-rule gene expression, but also pattern the identity of segments through regulation of Hox gene expression. Therefore, the *Tc-cad* expression gradient can be seen to be at the core of segmentation in Tribolium, where *Tc-cad* regulates segment formation via the pair-rule genes and segmental identity via gap genes. Since the *Tc-cad* expression gradient is determined by the Wnt signaling pathway, segmentation is also regulated by Wnt signaling.

Considering anterior serosa formation and posterior segmentation, the Wnt signaling pathway in corporation with *Tc-otd-1* seems to be sufficient to pattern cell fates along the A-P axis in the Tribolium blastoderm. However, another signaling pathway determining the D-V axis also affects the A-P axis, specifically the Dpp signaling pathway. Depletion of *Tc-dpp* expression results in a larger head, and depletion of its antagonist, *Tc-sog* causes lack of head. We analyzed expression of several components in the Dpp signaling pathway and revealed that *Tc-sog*

expression was activated by higher levels of Wnt signaling activity in the anterior region of *Tc-axn* or *Tc-apc-1* RNAi embryos, whereas Wnt signaling activity is repressed in wild type. Therefore, the Dpp signaling pathway also appears to be regulated by the Wnt signaling pathway. Impacts on the A-P axis can be explained by changes in Tc-Dpp distribution regulated by Tc-Sog. However, the role of the Dpp signaling pathway in the regulation of serosa formation appears to be limited, since the serosa is almost eliminated in *Tc-otd-1*;*Tc-apc-1* RNAi embryos.

Furthermore, we also analyzed serosa formation in embryos with different levels of Wnt signaling activity. *Tc-pan* is the transcription factor of the Wnt signaling pathway, which can activate and repress Wnt signaling with co-activators posteriorly or co-repressors anteriorly in Tribolium embryos. Thus, knocking down *Tc-pan* leads to a flatter gradient of Wnt signaling activity, due to anterior up-regulation and posterior down-regulation. In these *Tc-pan* RNAi embryos, the serosa splits into two separate regions, one at the anterior pole and the other on the dorsal side of the embryo. In double *Tc-otd-1*;*Tc-axn* or *Tc-otd-1*; *Tc-apc-1* knock down embryos the anterior serosa was eliminated. For the serosa residue in the dorsal side, we hypothesized that serosa formation here is due to lower levels of *Tc-cad* expression in *Tc-pan* RNAi embryos. Unfortunately, we could not over-express of *Tc-cad* in *Tc-pan* RNAi embryos. However, when we directly reduced the level of *Tc-cad* by RNAi together with *Tc-axn* or *Tc-apc-1*, *Tc-zen-1* expression also split into two separate regions, as in Tc-pan RNAi embryos. In addition, in *Tc-cad* RNAi embryos, *Tc-zen-1* expression expanded dorsally, which also implicates the repression of *Tc-zen-1* by *Tc-cad*. Thus, serosa formation in Tribolium is regulated, at least in part, by Wnt signaling through its target gene *Tc-cad*.

In summary, the Wnt signaling pathway appears to be a core determinative factor to regulate Tribolium embryogenesis. Before the onset of segmentation in early blastoderm stages, lower Wnt signaling activity is required in the anterior region of the embryo to allow the formation of serosa. While high levels of Wnt signaling in the posterior region of the embryo activate *Tc-cad* expression to induce segmentation. By resolving the serosa anteriorly and the segmented regions of the embryo posteriorly, the intervening pre-gnathal region is determined. On the other hand, input from *Tc-otd-1* and the Dpp signaling pathway is also required to coordinate Wnt signaling activity in serosa formation. Further studying the interaction between

the Wnt signaling pathway, *Tc-otd-1* and the Dpp signaling pathway has led to a more comprehensive understanding of pattering both A-P and D-V axes in Tribolium. Since these genes are also involved in the fate map patterning in other insects, studying in Tribolium may provide general regulatory mechanisms to promote our understanding of embryogenesis in other insects.

Studies in Tribolium described in this dissertation have uncovered significant roles for specific genes in patterning the blastoderm fate map and generating segments sequentially, and provide insight into general principles of short germ development, that are likely to apply to other insects in which these genes are conserved and perhaps other animals that produce segments sequentially.

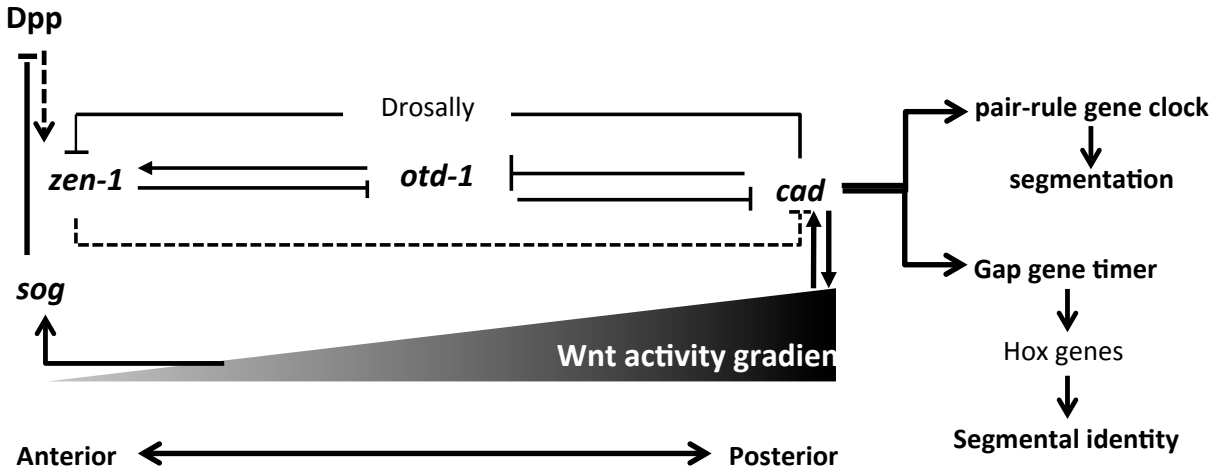


Figure 4-1 Genetic interactions pattern the fate map and regulate segmentation in the Tribolium blastoderm embryo. Wnt and Dpp signaling activity regulate a *Tc-zen-1*, *Tc-otd-1* and *Tc-cad* interaction network to define the blastoderm fate map. In the posterior region of the embryos, *Tc-cad* expression gradient regulates the pair-rule gene clock to generate segments, concordantly with the regulation of the gap gene expression to define the segmental identities.

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