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Comparisons of embryonic development in *Drosophila, Nasonia*, and *Tribolium*

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Abstract

Studying the embryogenesis of diverse insect species is crucial to understanding insect evolution. Here we review current advances in understanding the development of two emerging model organisms: the wasp *Nasonia vitripennis*, and the beetle *Tribolium castaneum* in comparison to the well-studied fruit fly *Drosophila melanogaster*. Although *Nasonia* represents the most basally branching order of holometabolous insects, it employs a derived long germband mode of embryogenesis, more like that of *Drosophila*, while *Tribolium* undergoes an intermediate germband mode of embryogenesis, which is more similar to the ancestral mechanism. Comparing the embryonic development and genetic regulation of early patterning events in these three insects has given invaluable insights into insect evolution. The similar mode of embryogenesis of *Drosophila* and *Nasonia* is reflected in their reliance on maternal morphogenetic gradients. However, they employ different genes as maternal factors, reflecting the evolutionary distance separating them. *Tribolium* on the other hand relies heavily on self-regulatory mechanisms other than maternal cues, reflecting its sequential nature of segmentation and the need for reiterated patterning.

Introduction

For the past three decades, the fruit fly *Drosophila melanogaster* has served as the premiere model for understanding the molecular basis of developmental patterning mechanisms. In particular, the array of powerful genetic tools available in this organism has allowed the thorough dissection of the events leading to the establishment of cell fates in the *Drosophila* embryo, and the regulatory networks underlying this process stand as the mostly thoroughly characterized among multi-cellular organisms. For those interested in the evolution of developmental mechanisms, the highly detailed description of *Drosophila* embryonic patterning is an appealing starting point for understanding the origin of complex gene regulatory networks, and how these networks can change in the course of evolution.

One of the appealing features of the *Drosophila* embryo as a developmental model system is that, until gastrulation, it can be thought of as a two dimensional Cartesian coordinate

system, with the two orthogonal axes (anterior-posterior (AP) and dorsal-ventral (DV)) being patterned independently, but more or less simultaneously. The ability to rapidly establish cell fates representing the entire future organism at this early stage is enabled by maternally localized mRNAs which provide high levels of positional information that can be interpreted and refined by downstream target genes.

The mode of embryogenesis employed by *Drosophila* is termed long germ embryogenesis, which for the purposes of this review is defined by the establishment of all segmental fates at the blastoderm stage prior to gastrulation [1, 2]. This is a derived mode of embryogenesis that is only found in scattered species among the Holometabola. Insects of more basally branching lineages use a conceptually different mode of embryogenesis, where only the most anterior segments are specified before gastrulation, while the more posterior segments are generated and patterned progressively from a posterior region called the growth zone. This is termed short, or intermediate germ embryogenesis, depending on the number of segments established at the blastoderm stage [1].

Different approaches have been combined to understand how major transitions in embryonic patterning strategies have occurred in the course of insect evolution. One approach has generated a highly detailed understanding of embryogenesis in an insect employing the ancestral, short/intermediate germ mode of embryogenesis. By comparing this to the derived mode found in *Drosophila*, many insights into how regulatory networks were rewired to pattern a long germ embryo have been revealed. In a complementary strategy, the embryo of an insect that independently derived a long germ mode of embryogenesis has been examined and then compared to *Drosophila* as well as the short/intermediate germ species. This approach has uncovered common strategies underlying the transition from short to long germ embryogenesis.

Two holometabolous insects, the intermediate germ beetle *Tribolium castaneum* and the long germ wasp *Nasonia vitripennis*, have emerged as powerful comparative model organisms with which these topics can be addressed. In this review, we will describe the contributions of these “non-model” models to understanding the evolution of development, and provide perspectives on the bright future such lines of research hold.

A tale of two models: *Nasonia* and *Tribolium*

To enable the characterization of developmental regulatory networks at high enough resolution to make evolutionary comparisons meaningful, tools for identifying, cloning and manipulating genes and their regulatory elements must be available and robust in each comparative model system. The availability of such tools is summarized in Table 1 and described in detail below. Access to the fully sequenced genomes of both *Nasonia* and *Tribolium* [3, 4] facilitates the identification of *Drosophila* gene orthologs, including descriptions of complete gene families, and identification of potential regulatory elements. The main method used to manipulate gene function in both of these organisms is parental RNA interference (pRNAi) [5, 6]. In this technique, *in vitro* synthesized dsRNA of the gene of interest is injected into the abdominal hemocoel of female pupae or adults, where it is taken up by the cells, and leads to the degradation of the target mRNA of interest. If developing oocytes take up the dsRNA the effect perdures in the fertilized eggs (unless the gene knock-

down causes adult lethality or sterility), and thus the developmental consequences of reducing the expression of any gene of interest can be examined in embryos or early larvae. This technique is so efficient in *Tribolium* that there is currently an effort underway to knock down and characterize every gene in the genome (Gregor Bucher, personal communication, see http://ibeetle.uni-goettingen.de/).

Another critical technique for characterizing developmental networks is the ability to engineer the genome to alter endogenous sequences or express exogenous ones through the use of germ-line transformation. These techniques are well established in *Tribolium* [7], and have been used to perform a genome-wide insertional mutagenesis and enhancer-trapping screen that has yielded many interesting and useful transgenic lines [8]. Furthermore, techniques for tissue and time specific misexpression have been developed by implementing the GAL4-UAS system in *Tribolium* [9]. The methodology in *Nasonia* is not as advanced, but some promising success has been gained in establishing germ-line transformation in *Nasonia* (Claude Desplan, personal communication).

Nasonia has an additional attractive feature, which is its haplo-diploid mode of sex determination. Unfertilized eggs develop as haploid males, allowing mutations affecting embryonic patterning to be rapidly identified by screening the progeny of the F1 generation [10]. A screen employing this method has already provided valuable insight into the mechanisms patterning the *Nasonia* embryo [11].

Nasonia and *Tribolium* represent the two most speciose orders of insects (the Hymenoptera and Coleoptera, respectively). Together with *Drosophila*, these three comparative models represent most of the diversity of holometabolous insects. Recent molecular phylogenies place the Hymenoptera at the base of the holometabolous radiation (Figure 1), with the Coleoptera and Diptera diverging a relatively short time later. These phylogenetic relationships allow hypotheses concerning the direction of evolutionary change in embryonic patterning as well as trends associated with the different modes of embryogenesis to be tested. For example, characters shared by *Tribolium* and *Drosophila*, but not *Nasonia*, may represent novelties that arose after the divergence of the Hymenoptera from the rest of the Holometabola (alternatively, the character could be ancestral but lost in *Nasonia*), while characters shared by *Nasonia* and *Drosophila*, but not *Tribolium*, might represent common strategies for dealing with the long germ mode of embryogenesis. Any such hypothesis can then be tested by sampling other insect lineages, including more basally branching hemimetabolous species, for the character of interest. The highly detailed understanding of embryonic patterning now obtainable in *Nasonia, Tribolium* and *Drosophila* can be used to generate hypotheses that will both broaden and deepen our understanding of how developmental strategies can evolve. Indeed, such studies have already contributed much to understanding how establishment and patterning of the AP and DV axes of the embryo, as well as the establishment of the germline, have evolved within holometabolous insects.

AP polarity and patterning

The AP axis of *Drosophila* is patterned by a hierarchy of regulatory gene functions that progressively increases the resolution and precision of positional information. This process starts with maternal coordinate genes that provide broad, graded information emanating

from both ends of the embryo. These gradients are interpreted by the gap genes (the first zygotically activated segmentation genes). The expression domains of these genes subdivide the embryo in large, partially overlapping yet precisely defined blocks, and also provide short-range graded information. The pair-rule genes interpret these short-range gradients and are expressed in a double segment periodicity to give the first hints of the metameric body plan of the fly. Pair-rule gene expression patterns are then interpreted by the segment polarity genes, which function to establish the borders between, and fates within each segment. Contributions to understanding the evolution of patterning mechanisms at each level of this hierarchy have been made by studying *Tribolium* and *Nasonia*.

Maternal inputs into AP patterning

In *Drosophila*, three maternally derived patterning systems provide spatial information that progressively subdivides the embryo along the anterior-posterior axis: the anterior, posterior, and terminal systems. Two of these systems, the anterior and posterior, rely on localized mRNAs responding to the internal polarity of the oocyte (*bicoid* and *nanos*, respectively), while the terminal system relies on modifications to the eggshell during oogenesis to provide its patterning function (see Figure 2). Work in *Nasonia* and *Tribolium* has provided insights into how strategies for providing patterning information may be correlated with the mode of embryogenesis employed.

The Anterior system—Establishment of cell fates along AP axis of the *Drosophila* embryo requires a symmetry-breaking event leading to AP polarity and subsequent patterning along the AP axis. In *Drosophila* both steps are provided by *bcd*: anterior localization of *bcd* mRNA provides a symmetry breaking event and its translation into a protein gradient along the AP axis provides positional information to the gap genes for patterning.

The Bcd gradient of *Drosophila* acts as a morphogen with both permissive and instructive functions that provides positional information along most of the length of the embryo. The permissive function of the Bcd gradient arises from the ability of this protein to bind and repress translation of *caudal* mRNA into protein where it would interfere with anterior development [12, 13]. The instructive function arises from the differential sensitivity of AP target genes to Bcd concentration, such that the expression of genes that respond to low levels of Bcd extends further towards the posterior, while the expression of those that are less sensitive is restricted to the anterior [14, 15]. Although essential to AP patterning in *Drosophila, bcd* is an evolutionary novelty of higher Diptera [16]. It is derived from the Hox cluster, but unlike most Hox proteins, possesses a Lysine (K) at position 50 of its homeodomain, giving it a distinct DNA binding affinity in comparison to typical Hox homeodomains which possess a glutamine (Q) at this position [17].

Localized mRNAs, in combination with permissive and instructive protein functions, are also critical in patterning the anterior half of the *Nasonia* embryo [18]. *Nv-orthodenticle1* (*Nv-otd1*) mRNA is tightly localized to the anterior (and posterior, see below) pole of the embryo [19], where a gradient of translated protein performs the instructive function (Figure 2). pRNAi against *Nv-otd1* significantly shifts the anterior fatemap, resulting in the loss of

all head segments, including some thoracic segments in the most extreme cases. *Nv-otd1* does not act alone, but rather cooperates with Nv-*hunchback* (*Nv-hb*) in activating target genes. This is similar to the cooperation of Bcd with Hb in activating anterior target genes in *Drosophila*, and indicates that cooperation between K50 homeodomain proteins and Hb is an ancient strategy for anterior patterning in holometabolous insects [19] (Figure 2).

No clear analog of the permissive function of Bcd has been identified for Nv-Otd1, which is not surprising, given the posterior localization of *Nv-cad* mRNA (see below). However, an anteriorly localized, permissive patterning factor with novel function has been found in the wasp. *Nv-giant (gt*) mRNA is localized to the anterior pole of the oocyte (Figure 2). After egg activation it disperses, forming a cap of *Nv-gt* mRNA at the anterior pole at the early blastoderm stage. Once translated into protein, this early source of Nv-Gt is critical to prevent the expansion of *Nv-Kr* and concomitant loss of *Nv-hb* expression in the anterior half of the embryo [20]. When *Nv-gt* and *Nv-Kr* are knocked down simultaneously, most of the anterior patterning defects seen in *Nv-gt* single pRNAi, are rescued, demonstrating that the primary function of maternal *Nv-gt* is to prevent the expression of *Nv-Kr* in the anterior half of the embryo, either by repressing it directly or indirectly through activation of *Nv-hb*.

The situation in *Tribolium* appears to differ significantly from what has been observed in *Nasonia* and *Drosophila*. This is likely due, at least in part, to differences in the blastodermal fate map of short/intermediate type embryos. Instead of many head segments that require high levels of patterning information to set their borders, most of the anterior end of the *Tribolium* embryo is devoted to extraembryonic cell fates, fates that are restricted to the dorsal side of the embryo in *Drosophila* and *Nasonia*. Thus, it is not clear whether *Tribolium* needs the high levels of patterning information provided by localized mRNAs as in *Nasonia* and *Drosophila*.

However, positional information is needed in *Tribolium* at least to specify the border between embryonic and extraembryonic tissues. It was proposed that *Tribolium orthodenticle1* (*Tc-otd1*), which is provided maternally and appeared to result in an anterior to posterior protein gradient due to translational repression [21] (Figure 2), along with hunchback act as bicoid-like morphogens in *Tribolium*. Recently, doubt has been cast on the putative role of *Tc-otd1* in providing positional information along the AP axis [22, 23]. However, anteriorly localized mRNAs have been found in *Tribolium* (Bucher, Farzana et al. 2005), and seem to have effects on early fatemap (S. J. Brown, manuscript under preparation).

The Posterior System—In *Drosophila*, posterior patterning relies on two major maternal factors: *caudal (cad)* and *nanos (nos)* (Figure 2). Together these two genes, acting in distinct ways, ensure proper specification of abdominal fates: *cad* encodes a transcription factor that activates posterior target genes, while *nos* encodes a translational factor that acts permissively for abdominal specification. Both of these genes are conserved in *Tribolium* and *Nasonia*, and analyses of their functions have revealed both conserved and divergent strategies for patterning the posterior segments in insects.

1. caudal function and regulation: In the early *Drosophila* embryo, ubiquitous maternal *cad* mRNA, translationally repressed by the Bicoid gradient, produces a reciprocal Cad gradient [12, 13]. The posterior patterning function of Cad provides activating input to pair-rule genes to form posterior stripes but only weakly activates posterior gap genes. It does not appear to act as a morphogen, and it seems that the repressive function of Bcd on *cad* mRNA is mainly to prevent ectopic Cad from disrupting head patterning [24].

Nv-cad mRNA is localized to the posterior pole of the oocyte, and upon egg laying it diffuses, forming a posterior to anterior gradient. This posterior localization of maternal *Nv-cad* obviates the need for translational repression at the anterior. *Nv-cad* has a more fundamental role in patterning the wasp embryo, as the strongest mutant and pRNAi phenotypes delete all abdominal and thoracic segments, while the strongest *Drosophila cad* mutants have variable defects restricted to the abdomen. [25] In addition, *Nv-cad* functions higher in the patterning hierarchy than does its fly counterpart, as it is absolutely required for the proper expression of posterior gap genes [25].

caudal also plays a critical role in patterning most of the segmented embryo in *Tribolium*. At the embryonic stage just prior to the onset of gastrulation (termed differentiated blastoderm stage), there are three developmentally distinct regions: the extraembryonic membrane primordia, the anterior head (pregnathal), and segmented germband (that part of the embryo patterned by pair-rule genes, including gnathal and trunk segments). *Tc-cad* is responsible for specifying this last region (Figure 2); its knock down leads to the complete loss of posterior head and all trunk segments. The phenotype, even more severe than that of *Nv-cad* RNAi, is also observed in most non-*Drosophila* insects [26, 27], indicating a larger ancestral role for Caudal in general body patterning. An important downstream target of Tc-Cad is likely to be *Tc-even-skipped (Tc-eve)*, a gene which is critical for the maintenance of the progressive segmentation process of *Tribolium* (see below the section on pair-rule genes).

As is the case for its *Drosophila* and *Nasonia* orthologs, Tc-Cad function must be repressed in the anterior regions of the embryo. However, *Tribolium* does not contain a *bcd* ortholog and *Tc-cad* mRNA is not posteriorly localized as it is in *Nasonia*. The beetle uses another strategy: two zygotically activated genes, *Tc-mex3*, expressed in the anterior head primordia, and *Tc-zen2*, expressed in the serosal anlage (the anteriormost extraembryonic region), are required to repress Tc-Cad protein production in these regions of the differentiated blastoderm embryo. Interestingly, the *C. elegans* ortholog of *Tc-mex3* is also involved in translational regulation of the worm *cad* ortholog, indicating that this mechanism for regulating cad expression along the AP axis was in place in at least the common ancestor of the Ecdysozoa [28, 29].

2. nanos regulation and function: In the fly, localization of *nanos* (*nos*) mRNA to the posterior pole of the embryo is critical for proper patterning of the abdominal segments. Only localized *nos* mRNA (which represents just 4% of total *nos* mRNA present in the embryo) is translated [30], giving rise to a gradient of Nos protein in the posterior half of the embryo that represses translation of maternal *hb* mRNA, which would, if translated, interfere with posterior gap gene regulation, and result in the disruption of posterior abdominal segments [31]. Localization of *nos* mRNA occurs late in oogenesis and depends on properly

assembled germ plasm [32]. Thus, *osk, vas,* and *tudor* mutants have abdominal defects identical to that of *nos*.

So far no expression or functional data have been presented for *nos* in *Tribolium*, so it is not clear what, if any, role this factor plays in patterning the beetle embryo. Intriugingly, Nanos Response Elements (NREs), the sequence motifs through which Nanos exerts its repressive influence, have been detected in the 3′ UTRs of both *Tc-otd1* and *Tc-hb*, and both of these genes show evidence of translational repression at the posterior pole [21, 33]. However, the functional significance of these NREs requires further validation.

In *Nasonia, Nv-otd1* and *Nv-hb* mRNAs both also possess NREs in their 3′ UTRs, and both also show posterior translational repression [19, 34]. *Nasonia nanos* (*Nv-nos*) mRNA is localized to the posterior pole, in the oosome, a structure that is the wasp equivalent of polar granules in the fruitfly. When *Nv-nos* is knocked down via pRNAi, the domain of maternal Nv-Hb protein expression expands to the posterior pole, revealing that the role of *Nv-nos* in repressing *Nv-hb* translation is conserved with its *Drosophila* ortholog. *Nv-nos* pRNAi also leads to the misregulation of posterior gap genes, and the eventual loss of posterior segments in the larva [35].

Regulation of the localization and function of *Nv-nos* shows some conservation with, as well as significant divergence from, the strategies employed in *Drosophila.* Like their *Drosophila* counterparts, *Nv-vas* and *Nv-osk* are required for the proper translation of *Nv-nos* mRNA at the posterior. Also as in the fly, this function of germ plasm genes seems to be in opposition to the activity of *smaug,* which represses translation of unlocalized *nos* mRNA [36]. A major difference between the wasp and fly is that while *nos* localization in *Drosophila* is completely abolished in germ plasm deficient mutants, *Nv-nos* shows significant posterior localization after *Nv-vasa* or *Nv-osk* pRNAi, despite the absence of the oosome. Another major difference in the function of *Nv-nos* is that its action is delayed due to maternal provision of Nv-Hb protein, which is absent in *Drosophila*, but which is also found in the holometabolous locust *Schistocerca* [37]. The lack of maternal Hb protein may have been important to the evolution of the rapid early syncytial cleavage divisions prior to blastoderm formation in *Drosophila*, which are considerably more rapid than in *Nasonia*.

The terminal system—In addition to the Bcd gradient, the terminal system is involved in patterning the termini of *Drosophila* embryo [38]. *torso*, encoding a receptor tyrosine kinase [39], and its ligand *trunk* are provided maternally and ubiquitously to the embryo [40]. Spatial specificity for the system is first provided by the expression of *torso-like* (*tsl*) in the anterior and posterior follicle cells during oogenesis [41], and later by incorporation of Tsl protein into the vitelline membrane [42]. *tsl* is required for production of the active form of *trunk*, by a still unknown mechanism, which defines the regions of terminal system activity at both embryonic poles [43, 44]. Targets of Torso signaling include the terminal gap genes *tailless* (*tll*) and *huckebein* (*hkb*) [45], which are initially expressed in caps covering both anterior and posterior poles. Both *tll* and *hkb* are important in patterning the unsegmented posterior region, the posterior abdominal segments, and the head [46] (however, it was shown that in case of increased *bcd* expression, the terminal system is dispensable for head development [47]).

In *Tribolium, Tc-tsl* is activated in the follicle cells located at both ends of the oocyte. Both *Tc-torso* and *Tc-tsl* knockdown result in the same defects at both embryo poles (no serosa is formed anteriorly, and no post-blastodermal segments emanate from posterior), indicating that Torso signaling plays similar roles in beetles and flies [48]. However, *Tc-tll* is activated only at the posterior terminus in the early blastoderm. Only later, in the germ rudiment, is it activated in the head [49] (note that the *Tribolium* head anlage is located mid-embryo and not at the anterior pole, as in *Drosophila*). This indicates that the terminal system must signal through a gene other than *Tc-tll*. Torso signaling at the posterior pole of the *Tribolium* embryo is required for both Wnt signaling and zygotic activation of *Tc-cad* (with the possibility that Torso signaling is regulating zygotic *Tc-cad* through Wnt signaling), and thereby affecting post-blastodermal segmentation [48]. However, it is not known whether this posterior action of Torso signaling is carried out through *Tc-tll*. Anterior Torso signaling is required for *Tc-zen1* activation, and hence its importance in serosa formation.

An ortholog of *tailless* is expressed at the posterior pole and in the head anlage at the anterior during the blastoderm stages of *Nasonia* [50]. However, these expression domains are not regulated by the *torso* signaling cascade, as no ortholog of *trunk* could be found in the *Nasonia* genome, and activated MAP kinase is not observed at the poles of the embryo (JAL, personal observation). Rather, both the anterior and posterior domains of *Nv-tll* depend on *Nv-otd1* for their activation [50]. The terminal system is also lacking in the honey bee *Apis mellifera* [51], indicating that either the terminal system originated after the divergence of Coleoptera and Diptera from the Hymenoptera, or that the terminal system was lost specifically in the Hymenoptera. Sampling from more basally branching hemimetabolous lineages will be required to resolve this question.

Gap genes

In the fly, the gap genes function at the second level of the embryonic AP patterning hierarchy, and are generally the first zygotic genes to interpret the maternal gradients. The transcription factors encoded by the gap genes form short-range gradients that are generally mutually repressive, leading to precise overlap of opposed gradients. Loss of gap gene function leads to the misregulation of downstream pair-rule genes and the loss of one or more contiguous blocks of segment primordia. In addition to their role in regulating pair-rule genes, gap genes also regulate the domains of Hox gene expression [52, 53].

Although limited, the data on the function of gap genes in *Nasonia* indicate that their functions and interactions are generally similar to their fly counterparts. In the first functional study of *Nasonia* embryogenesis, a number of potential gap gene mutations were identified by their phenotypic similarity to fly mutants, [10]. One of these has been identified as a mutation in the *Nasonia hunchback* (*Nv-hb*) ortholog [34]. This mutation is of particular interest, since it affects only zygotic expression of the protein, yet results in a loss of segments (all of the head and most thorax) that is greater than the combined loss of maternal and zygotic contributions of *hb* in the fly. pRNAi against *Nv-Kr* and *Nv-gt* also showed that these genes possess canonical gap gene functions, and act at similar positions along the AP axis as their fly homologs [20, 35].

The mutually repressive interactions between gap genes also appear to be conserved in *Nasonia*, at least those between *Nv-Kr* and *Nv-hb*. In cases where the domain of either of these genes is altered by pRNAi or mutant backgrounds, the other expands or contracts to maintain a sharp border between the posterior edge of the *Nv-hb* domain and the anterior edge of the *Nv-Kr* domain[20].

Tribolium orthologs of *hunchback, giant* and *Krüppel* are required for proper segmentation in the beetle [22, 54, 55]. Surprisingly, *Tc-knirps* is important for head development but has only a minor effect on trunk segmentation [56]. In addition, a newly identified gap gene, *milles-pattes (mlpt)* [57] is unusual in that it encodes a series of small peptides, rather than a transcription factor.

In wildtype beetle embryos, the expression of *Tc-hb, Tc-gt, Tc-Kr* and *Tc-mlpt* changes dynamically between their initiation in the blastoderm stage and their resolution into domains covering one or more segments in the head and germband. *Tc-hb*, provided maternally, is found ubiquitously and then clears from the posterior end. This early expression domain resolves to the serosa, while a new posterior blastoderm domain arises and resolves to the anterior head through first thoracic segment. Finally, a domain arises in abdominal segments A7 through the posterior end of the germband as those segments are added [33]. *Tc-gt* is also expressed in a broad blastoderm domain that resolves to cover the anterior head as well as the mandibular and maxillary segments. An additional *Tc-gt* domain arises at the posterior pole of the embryo in the growth zone, the region from which the abdominal segments arise. As the germband elongates this domain broadens and splits into two stripes, one in third thoracic segment (T3) the other in the second abdominal segment (A2) [54]. *Tc-Kr* expression initiates in the posterior blastoderm, eventually resolving to cover the thorax [55]. *Tc-mlpt* expression initates in the blastoderm and resolves into domains covering the anterior head and mandibular segment, T2 – A4, and a small domain in A7 [57].

Although on the surface, gap gene functions in *Tribolium* appear similar to those of their *Drosophila* and *Nasonia* counterparts, in the end they are quite different. As in *Nasonia* and *Drosophila, Tribolium* gap genes are expressed in domains that overlap several segment primordia, show some evidence of cross-regulation (see Figure 3 for a comparison between expression domains and regulatory relationships between gap genes in both *Drosophila* and *Tribolium*), and regulate pair-rule and homeotic genes to some extent. Their loss of function through RNAi or mutation leads to large patterning gaps in the resulting cuticles. However, careful analysis of the segmentation process during embryogenesis revealed that these apparent deletion phenotypes are actually combinations of germband truncation (halted elongation) and homeotic transformations [22, 23, 55, 58] (see Figure 4 for a comparison between gap genes phenotypes of *Drosophila* and *Tribolium* and Figure 5 for Hox genes expression patterns for *Tribolium* in wildtype as well as gap genes mutants or RNAi embryos). Moreover, only very few pair-rule stripes appear to be specifically regulated by particular gap genes (S. J. Brown and M. Klingler, unpublished data), and most pair-rule pattern changes can be explained by halted elongation.

Pair-rule genes

Seven stripes of primary pair-rule gene expression (*even-skipped, runt* and *hairy*) are positioned in the *Drosophila* blastoderm by input from specific combinations of maternal and gap genes [59]. These primary pair-rule genes interact to regulate one another and the secondary pair-rule genes (*ftz, odd, odd-paired, paired* and *slp*) [60-62]. A complex network of interactions between primary and secondary pair-rule genes has been described [63] that positions stripes of the segment polarity genes *wingless* (*wg)* and *engrailed* (*en)* to define the borders between segments. Although the use of *wg* and *en* to define segmental boundaries is highly conserved, the expression patterns and functions of pair-rule gene orthologs differ greatly among insects and arthropods [64].

Similar to their *Drosophila* counterparts, *Tribolium* pair-rule genes are expressed in patterns of double segment periodicity [65-68]. However, in contrast to the simultaneous formation of pair-rule gene stripes in the *Drosophila* blastoderm, the *Tribolium* orthologs of *Drosophila* pair-rule genes are expressed in stripes sequentially from anterior to posterior, starting in the blastoderm and continuing during germband elongation. RNAi studies revealed that certain genes, *Tc-ftz, Tc-hairy* and *Tc-odd-paired,* are not required for proper segmentation, despite their striped expression patterns [67, 69]. Classical pair-rule phenotypes are observed in *Tc-prd* and *Tc-slp* RNAi or mutant embryos [68, 70], but depletion of *Tc-even-skipped (Tc-eve), Tc-runt (Tc-run)* or *Tc-odd-skipped (Tc-odd)* leads to loss of all (in the case of *Tc-eve*) or almost all (in the case of *Tc-run* and *Tc-odd*) gnathal and trunk segments [67]. Epistatis analysis revealed a negative feedback loop in which *Tc-eve* activates *Tc-run*, which activates *Tc-odd*, which in turn represses *Tc-eve* [67] (Figure 6) (however, this analysis was done using pupal injection; so while this verifies the circuit in the blastoderm phase, it is yet to be verified in the germband phase using embryonic injection). This negative feedback loop could serve as a self-regulatory segmentation mechanism, employing no or little cues from upstream maternal or gap genes. This is in contrast to the fly case, where pair-rule genes are under strict control of upstream maternal and gap genes.

Components of this negative feedback loop in *Tribolium* are categorized as primary pair-rule genes, since the depletion of any one of the three affects the expression of the other two, as well as the expression of *Tc-prd* and *Tc-slp*, which, hence, are categorized as secondary pair-rule genes. Although the functions of *prd* and *slp* are conserved in that they regulate segment polarity genes in both beetles and flies, the evolutionary flexibility of these modules is reflected in the register of *slp* function; it is necessary for odd-numbered *en* stripes in flies, but for even-numbered stripes in beetles [68]. In *Drosophila*, stripes of *en* expression are precisely positioned by *eve* in odd-numbered segments and by *ftz* in even-numbered segments through complex interactions with several secondary pair-rule genes [63]. As noted above, the beetle orthologs of many of these genes are not required for proper segmentation. However, *Tc-eve* may regulate segment polarity genes in every segment since it can, in combination with *Tc-prd* or *Tc-runt,* activate *Tc-en* [67, 68]. It is not known if other, as yet unidentified, genes function as secondary pair-rule genes in *Tribolium*. However, the different interactions between pair-rule genes that have evolved in beetles and flies still produce the same outcome; juxtaposed stripes of *wg* and *en* that define the segmental

boundaries. This underscores the evolutionary modularity of the segmentation gene network in insects.

So far the only pair-rule ortholog described from *Nasonia* is *Nv-paired (prd)*. Unlike its fly ortholog, *Nv-prd* expression initiates progressively from anterior to posterior, and shows no classical pair-rule expression, rather it appears in segmental stripes that alternate between strong and weak expression [71]. However, it is likely that genes with pair-rule functions exist in *Nasonia*, as several mutants with classic pair-rule phenotypes have been identified [10]. Preliminary evidence also indicates that the pattern of pair-rule expression is similar to that of the honey bee, where pair-rule stripes are initiated progressively from anterior to posterior [72]. In addition, the most posterior abdominal segments may not be patterned by the pair-rule paradigm, as only six pair-rule stripes are observed at the blastoderm stage; the later segmental stripes appear to progressively split from the most posterior pair-rule stripe [72] (Claude Desplan, personal communication). This may indicate that elements of the progressive growth and patterning that typify short germ embryogenesis are maintained in many “long-germ” embryos.

Segment polarity genes

Once segment polarity genes have been activated by pair-rule genes in *Drosophila*, their expression is maintained through a gene circuit including the transcription factor En, and two signaling pathways, Wnt and Hedgehog [73]. En and the components of the signaling pathways are highly conserved and have been identified in the both *Nasonia* and *Tribolium.* In *Tribolium*, RNAi analysis indicates both the Wnt and Hedgehog pathways play conserved roles in the segment polarity gene module [74-76].

In *Tribolium*, loss of canonical Wnt signaling via depletion of *Tc-arrow* (which encodes a co-receptor for canonical Wnt pathway) or *Tc-porcupine* (which encodes a Wnt ligand processing component), leads to germband truncation in the growth zone [77]. Detailed RNAi analysis revealed that multiple Wnts, specifically Wnt1 and WntD/8, are involved in posterior growth and patterning [78]. Additional studies will be required to determine the molecular mechanisms sensitive to Wnt signaling in the growth zone, but the need for a posterior source of Wnt signaling is conserved in other short germ insects [79, 80], other arthropods [81] and indeed most other metazoans [82]. Interestingly, although *wg* is expressed at the posterior pole of *Drosophila* eggs, it is not required for germband elongation or posterior patterning; mutations in genes encoding Wnt pathway components lead to segment polarity defects [83].

Homeotic genes

Homeotic genes were the basis of the first “evo-devo” studies in many organisms [84], and like most insects examined to date, *Drosophila, Nasonia* and *Tribolium* contain a single complement of homeotic genes, including labial, maxillopedia, Deformed, Sex combs reduced, Antennapedia, Ubx, abd-A and *Abd-B*. As in other organisms, they direct regional development along the AP axis. Their expression domains are similar in beetles and flies, and loss of function phenotypes affect similar body regions. In homeotic mutants, one developmental fate is replaced by another; thus, legs may appear where mouth parts should

be [85], depending on the resulting combination of homeotic genes expressed there (one exception to this is *Tc-labial* in *Tribolium*, which upon RNAi leads to deletion of the respective segment: intercalary [86]). In addition to specifying a specific regional identity along the AP axis, homeotic genes also repress anterior development; complete loss of homeotic gene function in a particular segment or region results in antennae developing on that segment or the segments in that region [87], and in the most extreme case, antennae develop on every segment [88].

DV patterning

A key player in patterning the DV axis in *Drosophila* is the NFκB transcription factor Dorsal. Dorsal protein is expressed in all cells of the early *Drosophila* embryo. However, its nuclear uptake occurs in the form of a gradient with maximal levels on the ventral side of the embryo [89]. The differential expression of Dorsal target genes define various cell fates (broadly, the mesoderm, ectoderm and extraembryonic fates) along the DV axis (Figure 7) [90, 91]. Cross-regulatory relationships between these target genes further refine DV gene expression domains, in a manner reminiscent of Bicoid and gap gene action in AP axis patterning. For example, while *twist* (*twi*) and *snail* (*sna*) respond to high levels of nuclear Dorsal ventrally to specify the mesoderm, they also repress expression of many genes that are activated by intermediate and low levels of nuclear Dorsal (e.g., *single-minded* (*sim*), *rhomboid* (*rho*) and *short gastrulation* (*sog*)) [92, 93].

Dorsal target genes of particular interest are those that encode components of other signaling pathways. For example, the BMP ligand *dpp* and the protease *tolloid* (*tld*) are both strongly repressed by Dorsal, and are thus restricted to the dorsal side of the embryo [94, 95], while *sog* (an extracellular BMP binding protein), activated by low Dorsal levels [96](and repressed by *snail*), is expressed in broad lateral stripes. The arrangement of these factors inhibits BMP signaling in the ectoderm, and produces peak levels of BMP signaling at the dorsal midline that are critical to specifying the amnioserosa. This latter phenomenon results from the binding of Sog to Dpp, which prevents signaling in ventral regions and enhances diffusion. The following cleavage of Sog by Tld on the dorsal side of the embryo releases transported Dpp from Sog mediated inhibition [97]. In addition, a local gradient of the *rho* protease in mirror image ventrolateral stripes, regulated by intermediate levels of Dorsal activation and Snail repression, provides EFG signaling activity [98, 99]. The nuclear Dorsal gradient, in combination with BMP and EGF signaling further refines patterning of neuroectoderm into three domains defined by the columnar genes: *vnd, ind*, and *msh* [100].

There are similarities as well as differences in DV patterning between *Tribolium* and *Drosophila*. As in *Drosophila*, the ventral-most expression of *Tc-twist* and *Tc-snail* defines the mesoderm, and adjacent stripes of *Tc-sim* demarcate the mesectoderm [101, 102]. As in *Drosophila*, a ventral domain of *Tc-sog* (somewhat wider than the ventrolateral domain of *sog* in *Drosophila*) is needed to transport Tc-Dpp to the dorsal side of the embryo where it is released from Tc-Sog by Tc-Tld [103, 104]. Active EGF signaling in the ventral neuroectoderm in *Tribolium* presumably regulates columnar gene expression [105]. Major blastoderm fate map differences between *Tribolium* and *Drosophila* are visible in the regulation of *dpp*. While *dpp* is restricted to the dorsal side of the embryo of *Drosophila, Tc-dpp* is initially

expressed in a weak AP gradient, and then in an oblique stripe along the AP axis that defines the boundary between embryonic and extraembryonic cell fates. Despite these differences, the gradient of activated BMP signaling (as detected by phosphorylated-MAD) centered on the dorsal midline, is conceptually similar in the fly and beetle [103].

While nuclearized Dorsal is essential to activate most DV patterning genes in *Drosophila*, it plays a less prominent role in *Tribolium*. The Tc-Dorsal gradient fades at the end of the blastoderm stage (see below), completely disappearing prior to gastrulation [106]. This transient Tc-Dorsal expression initiates (but does not maintain) the most ventral fates. *Tc-twist* expression is activated at the ventral midline of the blastoderm and continues to be expressed at the posterior end of the germband after the Tc-Dorsal gradient disappears. As a result, abdominal segments emanating from the caudal end of the embyro continue to be correctly patterned with DV fates. *Tc-twist* expression is never initiated after *Tc-dorsal* depletion by RNAi, producing embryos that are symmetrical along the DV axis [107]. However, additional sources of patterning capacity exist, as demonstrated by the self-regulatory features of BMP signaling. The initial AP-asymmetric, DV-symmetric expression of *Tc-dpp* during early blastoderm phase is transformed into both AP and DV asymmetric BMP signalling activity in the late blastoderm by Tc-Sog mediated transportation towards the dorsal side[103]. In *Tc-dorsal* RNAi, probably due to the depletion of *Tc-sog*, the late blastodermal BMP signalling loses its DV asymmetry and becomes purly AP asymmetric. As a result, during germband elongation, additional DV symmetric stripes of BMP form along the AP axis in cells distant from the initial peak of BMP activity, indicating that a BMP-mediated self-regulatory mechanism is at work [107] (Figure 8). Interestingly, this same phenotype is not observed in *Tc-sog* RNAi embryos where DV fate specification is mostly normal, but with an additional peak of BMP activity replacing *Tc-sog* expression at the ventral midline [103]. This indicates that another factor downstream of Tc-Dorsal is capable of orienting BMP activity, and emphasizes the self-regulatory properties of BMP signaling in *Tribolium*.

The source of nuclear Dorsal gradient

In contrast to the Bcd protein gradient that forms by diffusion of newly translated protein from a localized mRNA source at the anterior pole, there is no localized point-like source from which to initiate a Dl nuclear uptake gradient [108]. Formation of the gradient can be traced back to the dorsal localization of *gurken* (*grk*) mRNA in the late oocyte, which results in dorsal activation of EGF signaling in the adjacent follicle epithelium. EGF signaling represses *pipe*, restricting its expression to the ventral side of the follicle epithelium [109]. Pipe, a sulfotransferase, modifies vitelline membrane proteins [110] to define the region in which a protease cascade is initiated that ultimately activates the Toll ligand Spätzle (Spz). In the embryo, binding of the Spz to the Toll receptor leads to the phosphorylation and degradation of the Dorsal inhibitor Cactus (Cact), permitting translocation of Dorsal to the nucleus [111]. A still unanswered question is how the homogenous, sharp-bordered *pipe* domain is translated into the graded distribution of Toll activation through Spz. Some clues come from mutants in which Pipe expression is wider than in wildtype, leading to two ventrolateral peaks of Dorsal nuclear uptake instead of one ventral peak [112]. This suggests that behind the Spätzle gradient is a self-regulatory mechanism with a domain of action that

is restricted ventrally by the maternal factor Pipe. Extending the Pipe domain makes it possible to adopt more than one peak of nuclear Dorsal [113].

In contrast to the stable gradient of nuclear Dorsal in *Drosophila, Tribolium* nuclear Dorsal is highly dynamic. Starting as a very broad domain of nuclear localization along the DV axis, Tc-Dorsal clears from the dorsal side in a gradually shrinking gradient with its peak at the ventral side, and finally vanishes at the differentiated blastoderm stage [106]. While the formation of the *Drosophila* Dorsal is to a great extent set by maternal factors, the dynamics of Tc-Dorsal gradient is generated mainly through its positive regulation of zygotic *Tc-Toll* as well as its own negative regulator, *Tc-cact* [107]. The negative component of this self-regulatory system is stabilized by the inclusion of the autoactivation of Tc-Twist as a late positive regulator of *Tc-cact* transcription, which is required to keep Tc-Dorsal out of the nuclei during the germband extension stages.

The exact molecular interactions among Tc-Dorsal, Tc-Toll, and Tc-Cact that give rise to the dynamic nature of the Tc-Dorsal gradient are as yet unknown. The regulatory relationships between these factors indicate both negative and positive feedback loops, which in principle could generate self-regulated patterns by local activation and long-range inhibition in the mode proposed by Meinhardt and Gierer [114]. The positive feedback loop between Tc-Toll and Tc-Dorsal satisfies the local activation requirement, while, in principle, Tc-Cact could satisfy the lateral inhibition requirement; however, it not yet know whether Tc-Cact has a long range effect, which is an important requirement of the model [107].

Self-regulatory mechanisms of the type found for Tc-Dorsal gradient formation are capable in principle of generating patterns by intensifying random early fluctuations without any maternal bias. However, in order to be useful in establishing axial polarity, such a system needs a mechanism to consistently orient itself perpendicula to the AP axis. This function is carried out by asymmetric activation of EGF signaling in the follicular epithelium overlying the oocyte nucleus during oogenesis. When EGF signaling is disrupted by pRNAi, the affects seen in the resulting embryos are highly variable, some show chaotically-positioned, duplicate peaks of nuclear Tc-Dorsal, while others produce Tc-Dorsal gradients oriented perpendicular to the DV axis [115]. Yet to be discovered is the sequence of events connecting EGF signaling in the follicle epithelium of the *Tribolium* oocyte to the point at which the symmetry of early nuclear Tc-Dorsal expression is lost.

Nasonia is an attractive model in which to further explore the evolution of DV patterning mechanisms, for much the same reasons it is attractive for studying the AP axis: at gastrulation, DV genes homologs are expressed in very similar patterns in *Nasonia* and *Drosophila* [115]. What is known so far is that, as in *Tribolium* and *Drosophila*, proper establishment of the DV axis depends on maternal EGF signaling [115]. In an interesting parallel, mRNA for the *Nasonia* EGF ligand is localized to the dorsal cortex, much like *grk* is localized in *Drosophila*, which is in sharp contrast to the lack of localized factors in *Tribolium.* Thus, heavier reliance on localized mRNAs also extends to patterning the DV axis in long germ insects.

Head development

The embryonic head anlage of *Drosophila* is partitioned by means of segment-polarity genes into six segments (listed in a posterior to anterior order): the labium, maxillary and mandible (composing the gnathocephalon), and intercalary, antenna, and ocular (composing the procephalon). Molecular arguments for the existence of a labral segment have been found in *Drosophila* [116] but not in other insects [86, 117]. Segmentation in the gnathocephalon is under the control of the segmentation hierarchy that defines the trunk segments, while procephalon segmentation is controlled by a different set of genes, the so-called "head gap-like genes" (*orthodenticle* (*otd*), *buttonhead* (*btd*), *empty-spiracles* (*ems*) and sloppy-paired) [118, 119]. These genes are expressed in overlapping domains, and mutations in them result in segmental deletions similar to the effects of gap gene mutations in the trunk (hence the name "gap-like"). Therefore, it was thought that they regulate the patterning of segment-polarity genes in the procephalon in a fashion similar to that of the trunk gap genes (directly or with the help of second order regulators instead of pair rule genes [120]). However, later studies indicate that head gap-like genes play only a permissive role for segment polarity genes expression, and do not regulate their metameric postions [121], leaving the problem of head segmentation unresolved [122].

The situation is similar in *Tribolium*, where the head gap-like genes (*Tc-otd, Tc-btd*, and *Tc-ems*) are not even extensively overlapping [123]. *Tc-otd* is the only one of these genes to have a gap-like phenotype (antennal and intercalary segments are missing in late embryonic knockdown). *Tc-ems* knockdown results in a partial deletion of the antennal segment, while *Tc-btd* knockdown has no effect at all, ruling out possible gap-like functions in *Tribolium* [123]. However, RNAi analysis of the *knirps* homolog in *Tribolium* produces a gap in the head pattern [56], which is in contrast to the normal early metameric patterning and late head defects of *knirps* mutants in *Drosophila* [124]. Thus, *Tc-knirps* joins *Tc-otd* as a head gap-like gene in *Tribolium* [122].

In *Drosophila*, head gap-like genes receive input from three maternal systems: the anterior, terminal and DV systems [46]. In *Tribolium*, the terminal and DV systems are likely to be responsible for the high-level partitioning of the embryo into extraembryonic, head and trunk, mediated by the cross-regulation of *Tc-zen1, Tc-mex3*, and *Tc-cad* (see AP section). So far, *Tc-ems, Tc-knirps, Tc-collier* and *Tc-labial* have been shown to regulate segment polarity expression in the head [125]. Their exact mode of action and more genetic factors regulating their fine-tuning and ultimately positioning the metameric expression of segment-polarity genes are yet to be discovered.

Mapping embryonic head segments to their larval counterparts in *Drosophila* and insects in general was and still is subject to much debate [117]. This is mainly because the larval head of the best-studied insect, *Drosophila*, undergoes a complex process of involution that introduces technical difficulties to mutant screens and expression assays. In addition, the derived nature of the *Drosophila* is not representative of insect head development in general. In *Tribolium*, head segments are initially collinear with the AP axis during embryonic development (the later bend and zipper morphogenetic movements, however, change the situation such that the eye becomes located posterior to the antenna – see below), making this beetle, with its available genetic tools, a good model system for studying typical insect

head development [122]. To support this line of inquiry, many larval morphological markers as well as many embryonic genetic markers have been characterized in the *Tribolium* head. Comparison of gene expression domains in *Tribolium* and *Drosophila* has helped reassess the assignment of morphological structures to individual segments [126]. These markers have also been used to compare embryonic and larval phenotypes in a variety of mutants, providing new insight into the fate map of the insect head [127]. Recently the "bend and zipper" model was formulated to describe the morphogenetic movements the insect head undergoes during development [127]. In addition, evidence supporting the hypothesis of the non-segmental, appendage-bearing nature of labrum was presented [86]. However, more technical advances in *Tribolium* are required to give unequivocal answers to the problem of *Tribolium* and insect head development in general.

In contrast to the previously mentioned processes, head patterning is not well understood in *Drosophila*. Hence, it will be the task of future *Tribolium* research to establish the paradigm in this species while *Drosophila* and *Nasonia* provide interesting cases of parallel evolutionary adaptations to long germ embryogenesis. Additionally, in *Drosophila* the head involution is an intriguing evolutionary novelty the evolution of which remains to be understood.

Germline specification

In *Drosophila*, the germline is specified by cytoplasmic inclusions called polar granules, which are produced specifically at the posterior pole of the oocyte and the embryo (Figure 10, A1-A2). When the syncytial nuclei arriving at the plasma membrane during blastoderm formation become associated with these structures, they take on the germ line fate and become pole cells (Figure 10, A3-A4). The gene *oskar (osk)* is both necessary and sufficient to induce the polar granules [128], and thus the activity of this gene must be tightly regulated.

Neither maternally synthesized polar granules, nor early specification of the primordial germ cells in the form of pole cells, are ancestral features of insect development. These features are missing from all described hemimetabolous species, as well as from some holoemetabolous taxa, such as *Tribolium* and *Apis* [4, 129]. These species use zygotic induction mechanisms to specify germline fate at varying times later in embryonic development [130]. On the other hand, *Drosophila*-like, maternal inheritance modes of germline determination are found in most hymenopterans (including *Nasonia*) and dipterans, and in many beetles [131]. *osk* had, until recently, only been found in the genomes of Drosophilids and other Diptera, leading to the idea that *osk* was a novelty of the Diptera [132].

Recently, an *osk* ortholog was discovered in the genome of *Nasonia*. It is a component of the oosome (the functional equivalent of the posterior pole plasm in *Drosophila*), and is taken up into the pole cells as they form, until it is degraded during the cellular blastoderm stage in a pattern quite similar to *Drosophila osk* (Fig 10, B1-B4). pRNAi against *Nv-osk* leads to the loss of the oosome and pole cells, as well as posterior patterning defects [131].

The discovery of an *oskar* ortholog performing a conserved function in generating maternal germ plasm in *Nasonia* has shed light on the evolutionary history of germline determination among insects. First, the latest time for the origin of the *oskar* gene is pushed back to the last

common ancestor of the Holmetabola, indicating that it was lost independently multiple times (at least in the lineages leading *Apis, Tribolium* and *Bombyx*). Second, the general conservation of the regulatory networks both upstream and downstream of *Nv-osk* in the formation of germ plasm and pole cells, indicates that there was a single origin of the maternal inheritance mode of germline determination that coincided with *oskar*, and that the strong correlation of losses of *oskar* with losses of maternal inheritance are not coincidental [131].

The fact that the lack of pole cells is likely secondarily derived in *Tribolium* makes understanding how the germline is established in this species very interesting. So far little data exist. Tc-Vasa protein is not maternally localized, unlike the Vasa orthologs in *Drosophila* and *Nasonia* [131]. However, its initially ubiquitous mRNA (Figure 10, C1-C2) appears to be selectively degraded, so that it is only found at the posterior pole at the onset of gastrulation (Figure 10, C3), after which it marks the primordial germ cells (Figure 10, C4) [133]. It will be interesting to see how this degradation is regulated and what factors are responsible for the induction of the rest of the germline program.

Morphogenesis

During the blastoderm stages when cell fates are being determined, the *Drosophila* embryo is a relatively simple two dimensional structure. Once cellularization has been completed and cell fates have been determined, numerous cell movements and rearrangements including gastrulation, dorsal closure, and germ band extension occur which transform the embryo into a dynamic, three-dimensional entity [134]. Equivalent movements also occur in *Tribolium* and *Nasonia*, however the means by which they are carried out vary significantly due to the combination of the different embryonic architectures, and independent evolution of strategies to solve morphological problems in each species' respective lineage.

Gastrulation

In *Drosophila* gastrulation begins with the formation of the ventral furrow, which forms more or less simultaneously along the entire AP axis of the embryo. In this process, cells on the ventral surface of the embryo expressing *twist* and *snail* contract at their apical sides, which leads to the internalization of the presumptive mesoderm, and formation of the ventral midline when the formerly lateral, *single-minded* (*sim*) expressing cells of the mesectoderm meet and fuse over the internalized mesoderm (Figure 11) [134].

The mesoderm is also ventrally located in *Tribolium*, it internalizes after cellularization of the blastoderm, which, during early stages, resembles invagination of the ventral furrow in *Drosophila* [101]. In the fly, gastrulation is among the first morphogenetic movements, and is completed before the major movements of germ band extension and dorsal closure have been initiated. In contrast, gastrulation in *Tribolium* occurs first in the context of germ rudiment condensation, and continues during convergent extension of the germ band. In addition, extraembryonic membranes are enveloping the embryo [135]. Furthermore, both specification of new mesodermal cell populations and ventral closure leading to the formation of the ectodermal ventral midline occur progressively from anterior to posterior [101], contrasting sharply to the rapid, coordinated invagination along the AP axis in

the fly. Finally, while a ventral furrow similar to that of *Drosophila* is observed at certain positions along the axis, the morphogenetic movements involved in internalizing the mesoderm and closing the ventral ectoderm vary along the axis during *Tribolium* gastrulation. In segments derived from the growth zone, the presumptive mesodermal cells, which unlike *Drosophila* and more anterior *Tribolium* segments, do not express *twist*, lose their epithelial character to form a mass of multilayered tissue, and appear to be internalized chaotically as the ectoderm closes over them [101] (Figure 11 B-B"). Interestingly, after internalization at least a subset of the presumptive mesoderm cells reinitiate *Tc-twi* expression (Figure 11 B"). In addition, the mesoderm remains relatively flat in the head region, with ectodermal cells crawling over this substrate to meet at the midline (similar to what is seen in hymenopteran gastrulation, see below, Figure 11 C-C"). It will be interesting to see if these different morphogenetic movements are the result of different cell signaling interactions among the different cell types involved, or whether they result simply from different forces impinging on the mesoderm at different AP axis positions.

The cellular and molecular underpinnings of gastrulation have been relatively unexplored in *Nasonia*. Observations on living embryos have shown, however, that the general features of *Nasonia* [136] are similar to the unique strategies that are used in many other Hymenoptera [137]. Here, no ventral furrow forms, rather the prospective mesoderm remains as a stiff sheet of cells while the ectodermal cells at the border of mesoderm break their lateral contact with their mesodermal neighbors (Figure 11 C-C'). The resulting free edges move across the rigid mesoderm cells until they meet and fuse, forming the ventral midline (Figure C"). This process also occurs in an anterior to posterior progression, again in contrast to *Drosophila* [137](JAL personal observation). These observations indicate that the molecular and cellular basis of *Nasonia* gastrulation also differ from that of the fly.

Extraembryonic membranes

In *Drosophila*, the vast majority of cells present at the end of the blastoderm stage will give rise to the future tissues of the embryo. However there is a small population of cells restricted to the dorsal midline fated to become the amnioserosa. This single extraembryonic membrane in the fly is important for proper germband retraction [138] and dorsal closure of the embryo [139], and disappears in the course of the latter process. While the amnioserosa is critical for successful completion of embryogenesis, it is extremely reduced in comparison to the extraembryonic membranes found in most other insects [140].

Tribolium possesses a more typical insect complement of and investment in extraembryonic membranes [141]. There are distinct serosal and amnion tissues, the former occupying a large anterior-dorsal cap, while the latter is a relatively restricted domain located at the anterior and dorsal margins of the embryonic anlage [29]. At the onset of gastrulation, these membranes undergo radical rearrangements. The serosal cells flatten and expand, and eventually move to envelope the embryo proper, causing the latter to sink slightly into the yolk. The amnion remains closely associated with the embryo during these movements. After the completion of germband extension and retraction, it is thought that the amnion and serosa fuse by cell intercalation of the two tissues into one, which eventually weakens and ruptures, thus uncovering the embryo. This membrane contracts to the dorsal side of the

embryo, and eventually disappears as the embryonic flanks expand and meet at the dorsal midline [29].

As in *Drosophila,* specification of extraembryonic membranes in *Tribolium* depends on transcription factors encoded by *zerknuellt* (*zen*) genes [29]. There are two *zen* paralogs in *Tribolium* (*Tc-zen1* and *Tc-zen2*); both of which are expressed in an anterior cap at the blastoderm stage corresponding to the presumptive serosa. *Tc-zen2* has an additional later domain in the amnion. Knockdown of *Tc-zen1* has dramatic effects on blastodermal patterning. The serosa is completely absent (and thus serosal *Tc-zen2* expression is also lacking), the amnion expands dorsally and anteriorly, and the anterior head anlage expands to the anterior pole of the egg. In addition, the embryo never sinks into the yolk, but remains on the surface of the egg throughout development. Despite these major early patterning defects, the majority of larvae look largely normal, indicating that there are mechanisms for compensating for the dramatic expansion of the head, and that the expanded amnion has some capacity to rescue the loss of the serosal fate or that the insect can develop in the absence of the serosa [29].

In contrast to *Tc-zen1*, the defects seen after *Tc-zen2* pRNAi are initially mild. No defects are seen at the blastoderm stage. However, after germband extension, the amnion fails to fuse with the serosa, and does not degenerate on the ventral side, thus preventing ventral eversion of the embryo. The persistent amnion contracts ventrally, dragging the dorsal flanks with it, leading to the inversion of the embryo trapped within the yolk [29].

The complicated movements of covering and uncovering the embryo in the course of extraembryonic development in *Tribolium* might seem superfluous, or at least unnecessarily baroque, given the ability of apparently wild type embryos to hatch in the absence of these movements and the entire serosal tissue after *Tc-zen1* pRNAi. However, the serosa likely has important roles beyond embryogenesis in, for example, providing protection to the embryo from a variety of environmental insults [141]. These insults are likely to be more extreme for embryos laid outside of laboratory conditions, thus the proper formation of the extraembryonic membranes is likely to be critical for the success of the organism in the wild. It is interesting to note that reduction in extrambryonic membranes is observed in many long germ species, which may indicate that deposition of the egg in a less harsh environment may be an ecological factor that contributes to the evolution of this type of embryogenesis.

The extraembryonic membranes of the long germ embryo of *Nasonia* are again comparatively less well described, but the data that do exist indicate that they possess both *Drosophila* and *Tribolium*-like features. Observations of living embryos indicate that unlike *Drosophila*, both a distinct serosa and an amnion are present in the wasp [136]. However, like the fly these tissues are defined in a very narrow domain restricted to the dorsal midline of the embryo [34] (JAL, personal observation). At the onset of gastrulation, the presumptive serosa expands from its dorsal domain, and crawls over the embryo proper until it completely envelops the embryo on the ventral side, while the amnion appears to expand from the dorsal flank of the embryo to cover the yolk until the completion of dorsal

closure [34, 136]. Further experiments are required to determine whether the specification of these two fates bears any similarity to either *Drosophila* or *Tribolium* mechanisms.

Conclusions and Perspectives

The tripartite comparative system of *Drosophila, Nasonia,* and *Tribolium* has allowed a number of significant insights into how early embryogenesis has evolved among holometabolous insects, especially in relation to their changing embryonic patterning environments. One striking observation was that, although the specific molecules and some of the details differ, both of the long germ species use very similar strategies based on localized mRNAs to rapidly specify cell fates along the entire AP axis of the early embryo. This strategy includes an instructive anterior factor (*bcd* in *Drosophila, Nv-otd1* in *Nasonia*), factors permissive for anterior patterning (also *bcd* in *Drosophila*, maternal anteriorly localized *Nv-gt* and the posterior localization of *Nv-cad* in *Nasonia*), and a permissive posterior factor (*nanos* orthologs in both species). *Nasonia* employs an additional posterior instructive factor, the posterior aspect of *Nv-otd1* localization. The dependence of long germ species on localized mRNAs extends to the DV axis, as both *Nasonia* and *Drosophila* use localized mRNA encoding EGF ligands to establish a stable axis. The nature of the long germ patterning system is likely a major factor driving the acquisition of precisely localized sources of positional information, as embryos of this type must specify a large number of distinct cell fates in a short period of time, and thus require precise and robust patterning information at a very early stage. Although localized factors have been found in *Tribolium* [142] that are functionally important (S. J. Brown, manuscript under preparation), it seems that they give guidance for self-regulatory mechanisms, other than giving extensive positional information as in the *Drosophila* case (E. El-Sherif, S. J. Brown, manuscript under preparation).

The reduced dependence of *Tribolium* on localized mRNAs is likely due in part to its mode of embryogenesis, where significantly fewer cell fates are established at a time accessible to maternally provided mRNAs. In place of maternally provisioned patterning information, *Tribolium* seems to rely on another powerful strategy for establishing pattern, namely emergent patterning based on self-regulatory systems. Examples of these include the zygotic self regulatory and feed-forward loops of the DV axis, the negative feedback pair-rule loop required for patterning and progressive growth of the germband, and perhaps the spatial and temporal cross regulation of the gap genes in the germband stage.

The ability to break with the *Drosophila* paradigm is another major development made possible by the establishment of *Nasonia* and *Tribolium* as comparative model systems. The discovery of the novel peptide encoding gene *mlpt* as a gap gene in *Tribolium*, the discovery that Wnt signaling is required for posterior growth zone and patterning, and the discovery of *mex-3* as an anciently conserved regulator of *cad* among the ecdysozoa are illustrative of this power. These types of discoveries should only increase as more genome wide and unbiased analyses of gene function in both species start bearing fruit.

Additional advances in understanding the developmental systems of *Tribolium* and *Nasonia* will come from taking advantage of the rapidly increasing set of transgenic and genomic

tools and techniques available in these organisms. For example, germline transformation allows the assessment of putative enhancer regions that can be identified by genomic techniques (such as those discussed below). In addition, the development of techniques for conditionally inducible expression, introduction of fluorescent hybrid proteins, and live imaging will allow for a much more detailed description and genetic analysis of developmental events. This will be especially valuable for dynamic processes, such as the operation of the growth zone or the movements of the extraembryonic membranes, where static observations on fixed material are not sufficient for rigorous interpretation.

The availability of fully sequenced genomes allows for the application of powerful techniques for comprehensively unraveling the regulatory interactions underlying development. These include genome-wide analysis of expression level perturbation (e.g. microarrays) and genome-wide discovery of transcription factor binding sites (e.g. ChIP-seq). The combination of such techniques should provide robust descriptions of gene regulatory networks robust enough to compare meaningfully with those already described for *Drosophila*.

Finally, the evolutionary significance of the similarities and differences in embryonic patterning among *Nasonia, Tribolium*, and *Drosophila* will only be fully clarified by placing them in a more robust phylogenetic and ecological context. For example, sampling of other short germ species both within the beetles, and among hemimetabolous insects will allow the differentiation of characteristics that are typical for short germ embryos, and those which arose uniquely in *Tribolium*. Reciprocally, additional long germ species, particularly those among the beetles, will be particularly informative in identifying potential constraints or common strategies used in the transition from short germ embryogenesis. Such broad sampling of insect developmental data is increasingly feasible, given the wide applicability of pRNAi, and the increasing power of next generation sequencing techniques to rapidly generate candidate gene sequence data.

Understanding the evolutionary meaning of such expanded developmental data will require a more detailed understanding of the ecological contexts under which these embryos develop and hatch. These conditions could result in selection on the speed of embryogenesis, the relative need for protective extraembryonic membranes, or the relative complexity of the hatching larva. All of these potential selective pressures could have major impacts on the strategies used for early patterning and embryogenesis. For these experiments, the highly developed techniques and resources available in *Tribolium* and *Nasonia* will confer on them a status as rich sources of hypothesis generation in relation to the numerous emerging satellite models roughly equivalent to that which *Drosophila* held in the early days of the wasp and beetle systems. Hopefully the process of reciprocal illumination will continue whereby the results obtained in satellite systems spur further experiments in core model species and vice versa, leading to both a broad and deep understanding of the evolution of developmental mechanisms throughout the insects.

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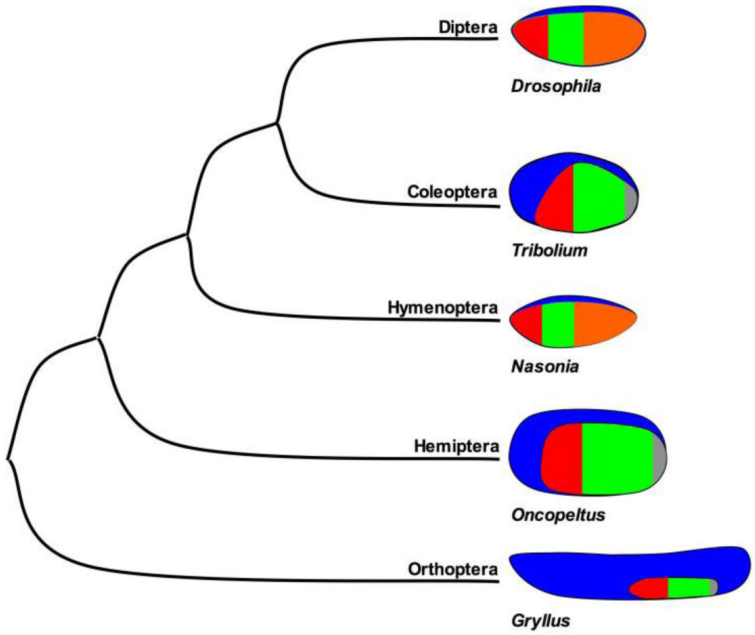

Figure 1. Phylogenetic relationships of selected insect models, and schematic representations of their early embryonic fate maps

Blue = extraembryonic tissue, Red = segments of the head, Green = thoracic segments, Orange = abdominal segments, and Gray = growth zone primordium.

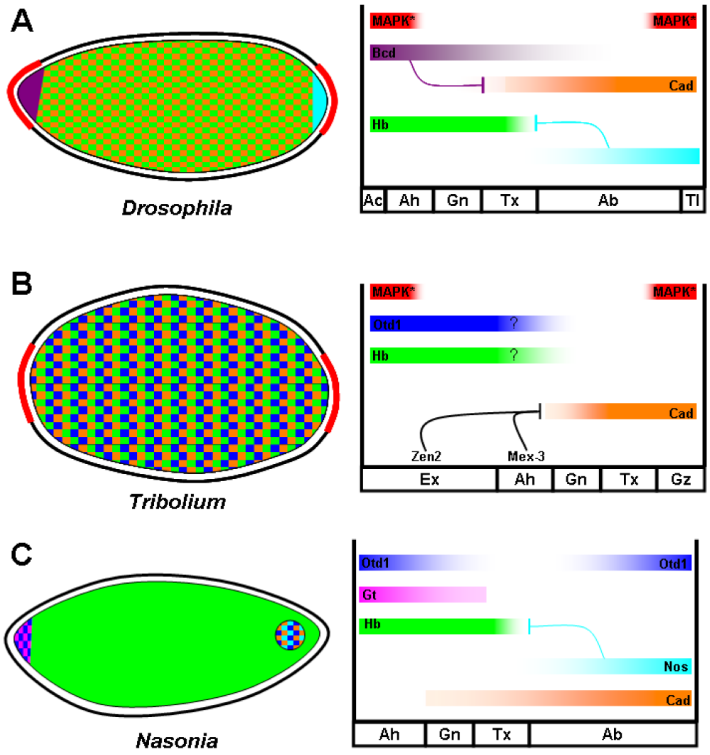

Figure 2. Maternal provision of AP patterning information in *Drosophila, Tribolium,* and *Nasonia*

Left: Schematic representation of *Drosophila* (A), *Tribolium* (B), and *Nasonia* (C) embryos showing distribution of maternally provided mRNAs. Right: representation of resulting protein gradients. Red curves in left panel on A and B represent the distribution of *tsl* expression, and correspond with the red bars at right representing the resulting activation of MAP kinase in the embryo. Question marks for Otd and Hb in panel B indicate their now doubted role in providing positional information to the early embryo. Ah=anterior head, Gn=gnathal, Tx=thorax, Ab=abdomen, Tl=telson, Gz=growth zone.

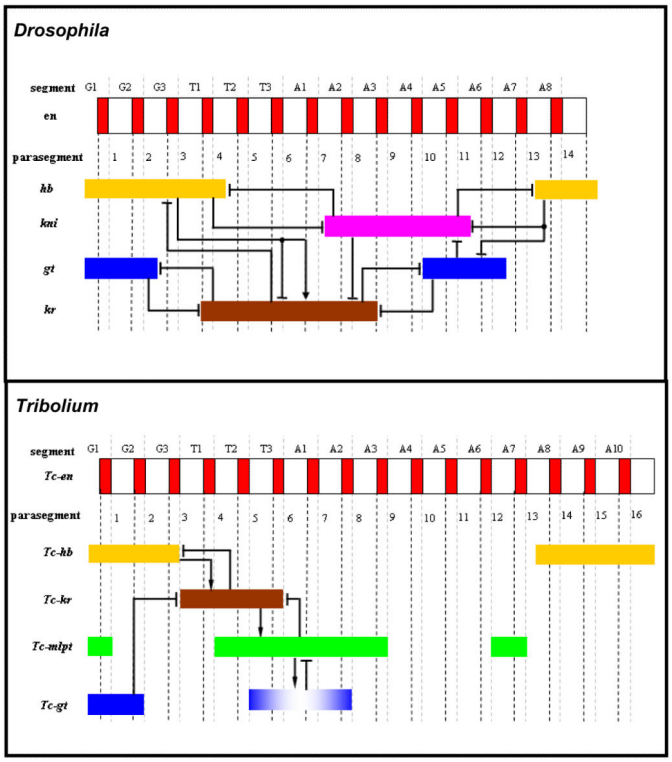

Figure 3. Comparison of gap gene expression patterns and their regulatory relationships in *Drosophila* and *Tribolium*

Top panel: Late blastodermal expression patterns of gap genes (non-terminal, non-head) in *Drosophila* and their regulatory relationships. Bottom panel: post-blastodermal expression patterns of gap genes (non-terminal, non-head) in *Tribolium* after they emanate from the growth zone and before they fade, and a parsimonious interpretation of their regulatory relationships. An arrow represents positive regulation and a blunt line represents negative regulation. G= gnathal segment, T= thoracic segment, A= abdominal segment.

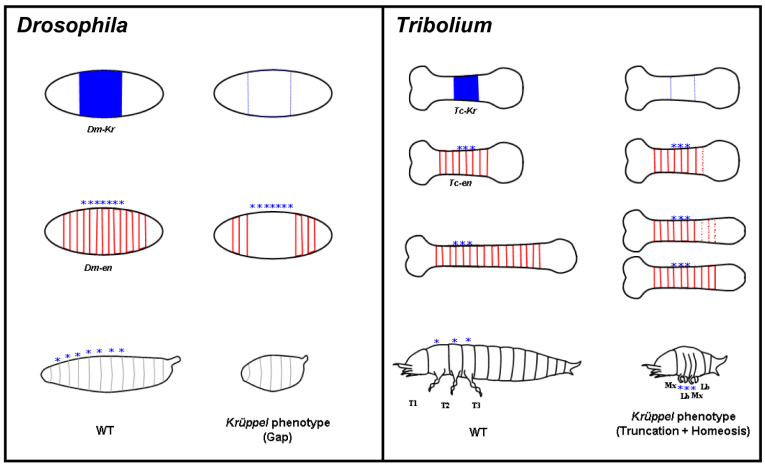

Figure 4. Comparison of gap genes phenotypes in *Drosophila* and *Tribolium*

Expression of *Krüppel* (in blue) and *engrailed* (in red) and the resulting larval cuticles (bottom of each panel) in WT (left of each panels) and *Krüppel* mutants (right of each panel). Left panel (*Drosophila*): The strongest *Krüppel* phenotype in *Drosophila* results in loss of seven segments (marked by stars) within the expression domain of the gene *Krüppel*, which is a typical "gap" phenotype. Right panel (*Tribolium*): In the *Krüppel* mutant (*jaws*), the segments within the *Tc-Krüppel* expression domain (marked by stars) form properly, and only a few more posterior segments develop, which are disorganized at first but later regulated to form intact segments. In addition to this "truncation" phenotype, a homeotic transformation is observed in the larval cuticle, in which the three thoracic segments and the first abdominal segment are transformed to gnathal identity (maxillary-labial-maxillary-labial). This phenotype is best described as "truncation plus homeosis", rather than "gap". The yet unexplained "truncation after expression domain" of *Tribolium* gap genes in contrast to "gap within expression domain" of *Drosophila* is proved valuable here to show clearly the homeotic effect of gap genes phenotypes in *Tribolium*, whereas it is precluded by the loss of segments in *Drosophila* (except for some hypomorphic mutants).

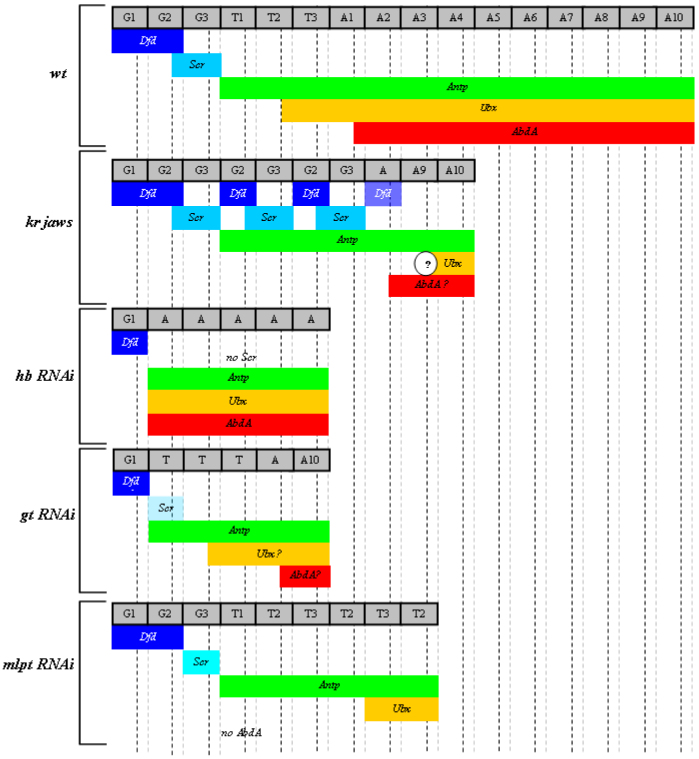

Figure 5. Hox gene expression in wt and gap gene RNAi and mutant embryos

The identity of segments that are formed is described in the gray boxes at the top of each panel. Diluted color in an expression domain indicates reduced expression. A question mark inside an expression domain indicates that this expression domain is not reported in literature. A question mark in a circle at the border of an expression domain indicates that the exact location of this border is not reported precisely in literature. G= gnathal segments, T= thoracic segments, A= abdominal segments.

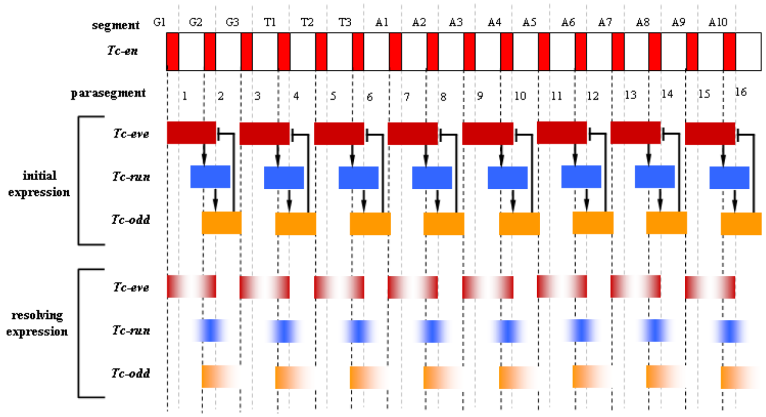

Figure 6. Initial and resolving expressions of primary pair-rule genes in *Tribolium*

A negative feedback loop between the three primary pair-rule genes *Tc-eve, Tc-run*, and *Tc-odd* are responsible for their initial double-segmental periodic expression. Later, they resolve into segmental periodic expression through a yet to be identified genetic mechanism. This later segmental primary pair-rule expression regulates downstream genes to ultimately position segment polarity genes. An arrow represents positive regulation and a blunt line represents negative regulation. G= gnathal segment, T= thoracic segment, A= abdominal segment. It is wothnoting that these regulatory relationships were verified for the anteriormost segments. Further expriments are needed to verify it for the following segments (using embryonic RNAi).

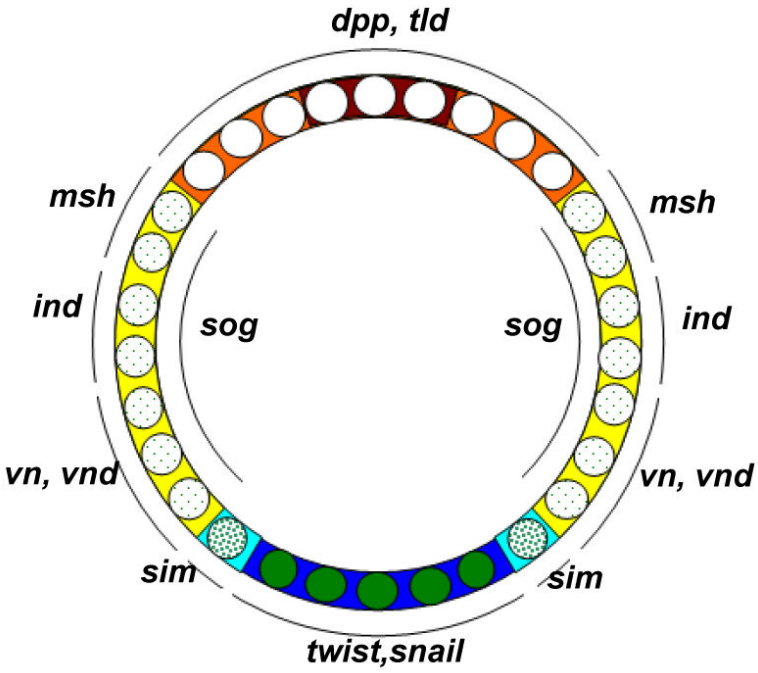

Figure 7. DV fate map and expression domains of genes involved in DV patterning in *Drosophila*

Different concentrations of nuclear Dorsal drive different sets of downstream genes and ultimately (with the help BMP and EGF signaling) define DV fates. Blue: mesoderm, cyan: mesectoderm, yellow: neurogenic ectoderm, orange: dorsal ectoderm, red: amnioserosa, opaque green circles: high nuclear Dorsal concentration, heavily dotted green circles: intermediate nuclear Dorsal concentration, lightly dotted green circles: low nuclear Dorsal concentration, white nuclei: no nuclear Dorsal.

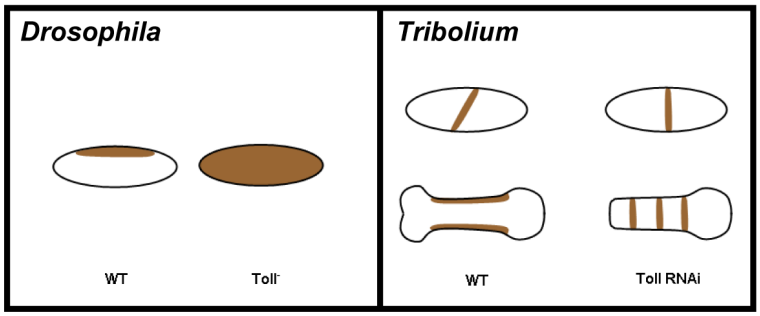

Figure 8. Comparison between *Toll* phenotype in *Drosophila* and *Tribolium*

Left panel (*Drosophila*): *dpp* (brown) is expressed dorsally in WT (left) and along the entire DV axis in a Toll mutant (right). Right panel (*Tribolium*): Early blastodermal *Tc-dpp* is expressed as an oblique stripe in WT (left upper embryo), and loses this obliqueness in *Toll* RNAi (right upper embryo). In *Tribolium*, the initial orientation of balstodermal *Tc-dpp* dictates the orientation of later reiterated germband expression of *Tc-dpp*. Consequently, *Tc-dpp* reiterates only twice in the DV direction in WT probably due to space constraints (left lower embryo), and many times in the AP direction in Toll RNAi (right lower embryo).

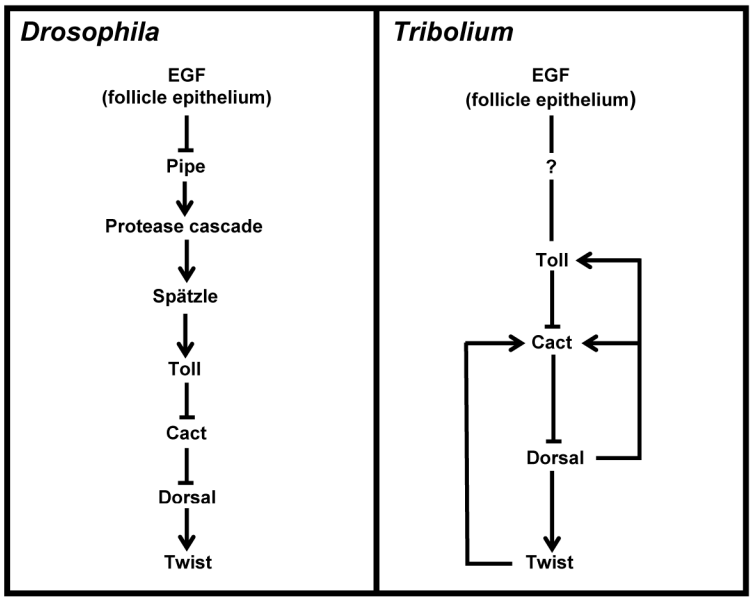

Figure 9. Regulatory relationships between major components in DV axis patterning

Left panel (*Drosophila*): the DV axis patterning network in *Drosophila* is mostly a linear cascade with the exception of multiple positive and negative feedback loops within the protease cascade components. Right panel (*Tribolium*): nothing is known about the involvement of a protease cascade in *Tribolium* similar to that involved in *Drosophila*. However, *Tribolium* posses multiple feedback loops at levels more downstream.

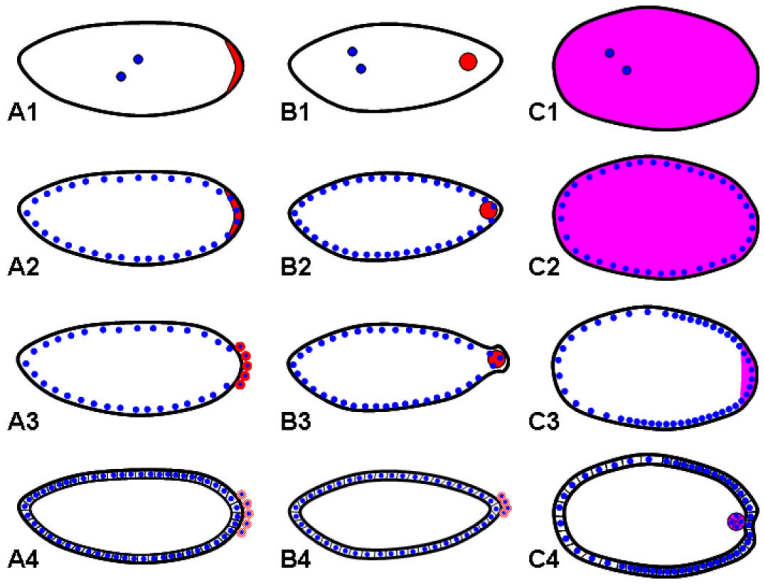

Figure 10. Germ cell formation in *Drosophila, Nasonia,* and *Tribolium*

Red=*osk* mRNA, magenta=*Tc-vasa* mRNA, blue=nuclei.

A1-C1: In the early cleavage stages of embryogenesis, *osk* mRNA is localized in the posterior pole plasm of *Drosophila* (A1) and oosome of *Nasonia* (B1), while *Tc-vasa* is ubiquitous in *Tribolium* (C1).

A2-C2: As the syncytial blastoderm forms, posterior nuclei interact with the pole plasm (A2), or the oosome (B2) in fly and wasp, respectively, while nuclei enter an apparently homogenous environment in *Tribolium* (C2).

A3-C3: Individual nuclei that enter the posterior pole plasm bud singly, forming the pole cells in *Drosophila* (A3). In contrast, the oosome along with multiple nuclei bud simultaneously from the posterior in *Nasonia* (B3). In the later blastoderm stages of *Tribolium, Tc-vasa* mRNA is cleared from the embryo, save for the most posterior pole.

A4-C4: Production of pole cells is completed, and *osk* mRNA begins to be degraded in the cellular blastoderm stage in both *Drosophila* (A4) and *Nasonia* (B4). Posterior cells that retained *Tc-vasa* expression delaminate from the blastoderm into the interior of the embryo (C4) and will become the primordial germ cells of the beetle.

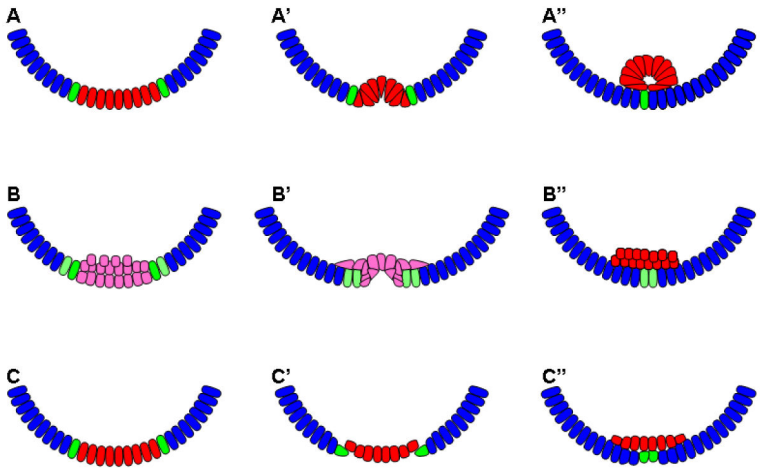

Figure 11. Mechanisms of gastrulation in *Drosophila, Tribolium*, and *Nasonia*

A-A": Gastrulation by furrow formation employed by *Drosophila*, and *Tribolium* in the anterior segments. B-B": Gastrulation mode found in the segments deriving from the growth zone of *Tribolium*. A multilayered mass of cells loses its epithelial character and is internalized by the migration of the lateral ectodermal cells. C-C": Hymenopteran mode of gastrulation, where the mesoderm forms a stiff plate, the ectoderm breaks contact with mesoderm and migrates ventrally until the free edges meet and fuse at the ventral midline. Blue= lateral ectoderm, Green=mesectoderm, Red= mesoderm precursors expressing twist, Pink= mesoderm precursors not expressing twist. Other cell fates have been omitted for clarity.

Table 1

Techniques and tools available in *Drosophila*, *Tribolium,* and *Nasonia*.

	Drosophila	Tribolium	Nasonia
in situ hybridization, immunohistochemistry	Y	Y	Y
Parental RNAi	N	Y	Y
Embryonic RNAi	Y	Y	Y
Germline Transformation	Y	Y	y
Enhancer Trapping	Y	Y	n
Misexpression constructs	Y	Y	n
Amenable to Forward genetics	Y	Y	Y
Ability to screen in F1 generation	N	N	Y
Sequenced Genome	Y	Y	Y
Detailed Transcriptome Data	Y	Y	Y
Availibility of Custom Transcriptome Microarray	Y	y	Y

Y = indicates availability is well established, N = technique is not, and is unlikely to become available, y = indicates the availability is preliminary and/or unpublished, n = technique not available, but could conceivably be developed in the near future.