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Evolution of Developmental Control Mechanisms

Evolution of the insect terminal patterning system—Insights from the milkweed bug, *Oncopeltus fasciatus*

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ABSTRACT

The anterior and posterior ends of the insect embryo are patterned through the terminal patterning system, which is best known from the fruitfly *Drosophila melanogaster*. In *Drosophila,* the RTK receptor Torso and its presumed co-activator Torso-like initiate a signaling cascade, which activates two terminal gap genes, *tailless* and *huckebein*. These in turn interact with various patterning genes to define terminal structures. Work on other insect species has shown that this system is poorly conserved, and not all of its components have been found in all cases studied. We place the variability of the system within a broader phylogenetic framework. We describe the expression and knock-down phenotypes of the homologues of terminal patterning genes in the hemimetabolous *Oncopeltus fasciatus*. We have examined the interactions among these genes and between them and other patterning genes. We demonstrate that all of these genes have different roles in *Oncopeltus* relative to *Drosophila*; *torso-like* is expressed in follicle cells during oogenesis and is involved in the invagination of the blastoderm to form the germ band, and possibly also in defining the growth zone; *tailless* is regulated by *orthodenticle* and has a role only in anterior determination; *huckebein* is expressed only in the middle of the blastoderm; finally, *torso* was not found in *Oncopeltus* and its role in terminal patterning seems novel within holometabolous insects. We then use our data, together with published data on other insects, to reconstruct the evolution of the terminal patterning gene network in insects. We suggest that the *Drosophila* terminal patterning network evolved recently in the lineage leading to the Diptera, and represents an example of evolutionary "tinkering", where pre-existing pathways are co-opted for a new function.

Introduction

Developmental patterning is largely governed by interactions of multiple genes in complex networks kno`wn as GRNs (gene regulatory networks). The past few years have seen a growing interest in the structure, function and conservation of such networks (Davidson, 2006, 2009; Jaeger et al., 2004; Jaeger, 2011; Peel, 2008; Wagner, 2007). However, there are relatively few studies addressing the question of how such networks evolve and how they are assembled.

A fairly simple and well-studied GRN is the terminal patterning system in insects. This network is responsible for defining the anterior and posterior of the embryo and for patterning the terminal structures, including the anterior of the head, and the posterior of the abdomen. It is best known in the fruitfly *Drosophila melanogaster*. In *Drosophila* terminal patterning is initiated through the broadly distributed tyrosine kinase receptor Torso (Klingler et al., 1988; Sprenger et al., 1989). Torso's localized activation in the poles of the embryo is done in interaction with the Torso-like protein, through a mechanism that remains unclear (Martin et al., 1994). Following this interaction, a tyrosine-kinase signaling cascade is initiated in the poles of the embryo (Cleghon et al., 1996; Furriols and Casanova, 2003). This localized cascade inhibits the repressor Capicua, and thus relieves the repression of two key transcription factors: the terminal gap genes, *tailless* and *huckebein* (Bronner and Jackle, 1991; Cinnamon et al., 2004; Jimenez et al., 2000). These in turn interact with various patterning genes to define terminal structures (Bronner and Jackle, 1996). Several components of this GRN have been studied in other holometabolous insects, and have generally been found to have divergent functions (McGregor, 2006). An obvious complication in comparative studies of this system lies in the fact that the development of *Drosophila* is far from representative for insect development (Peel et al., 2005). This is most notable in the development of its terminal structures. The posterior end of the *Drosophila* early embryo represents the posteriormost segment of the larva. In a majority of other insects, while the terminal tip of the embryo is probably determined very early (Minelli, 2001), most of the posterior portion of the early embryo represents the growth zone, from which additional segments will be formed

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throughout development. Likewise, the anterior end of the egg in *Drosophila* early development represents the anterior of the embryo, whereas in many insects a significant anterior portion of the egg is composed of extra-embryonic tissues.

The terminal patterning system has not been studied in its entirety in many cases, and has rarely been placed within a phylogenetic framework. Here we present results from our work on the system in the hemimetabolous milkweed bug *Oncopeltus fasciatus* (Hemiptera). We combine these data with previously published results from other species and analyze them within a phylogenetic framework. We use this analysis to reconstruct the evolutionary history of the terminal patterning network and to provide a hypothesis on the stages of its assembly throughout this history.

Material and methods

Animal husbandry and embryo collection

Cultures of *O. fasciatus* were kept as previously described (Ben-David and Chipman, 2010) at 25 °C with a 14/10 h light/dark cycle. Embryos were collected by placing individual cotton balls in the cages for a defined period of time (usually 2 h). Cotton balls with egg clutches were collected at the end of the period, giving us a timing error equal to half of the period. Variability in the precise stage of the embryo at egg laying gives us a maximum precision (minimum error) of $\pm$1 h. After collection we incubated the eggs at 25 °C until they reached the desired age, stated as hours after egg laying or hAEL (All hAELs in this paper are at 25 °C, and include an error of $\pm$1 h). Fixation and dechorionation were as described previously (Ben-David and Chipman, 2010).

Gene cloning

Cloning of *Of-tll* and *Of-hkb* was done using degenerate PCR, followed by RACE extension as described by Birkan et al. (2011). A list of primers used appears in Table S1. *Of-tsl* was found in the published *Oncopeltus* transcriptome (Ewen-Campen et al., 2011), and cloned using the primers detailed in Table S1 (GenBank accession number KC576905). This sequence is an unequivocal orthologue of *tsl*, based on reciprocal BLASTing and on sequence alignments to other published *tsl* orthologues (see Fig. S1).

In situ hybridization

Detection of mRNA through in situ hybridization was performed as previously described (Ben-David and Chipman, 2010). In situ hybridization on ovaries was done by dissecting complete ovaries out of mature anaesthetized females in cold PBS. Ovaries were fixed for 5 min in 4% formaldehyde and peeled of their outer sheath. They were then fixed again for 1 h and transferred to methanol for storage. The RNA hybridization was done in the same way as for embryos.

Parental RNAi

RNAi was performed by injection into virgin *Oncopeltus* females as described by Liu and Kaufman (2004). The templates for transcription of dsRNA were PCR products with T7 RNA recognition sites added to both ends (see Ben-David and Chipman, 2010 for detailed protocol). Injected females were reared individually with untreated males. Eggs were collected for in situ hybridization as soon as the females started laying (usually 3 to 6 days after injection). Eggs were collected normally and either kept until hatching or close to hatching to assess RNAi phenotypes or fixed for in situ hybridization at blastoderm or germ-band stages the same as wildtype embryos. Efficacy of knockdown was confirmed by in situ staining of the injected gene in knock-down embryos (see Table S2).

Microscopy and imaging

Prior to visualization, embryos were cleared by stepping them gradually into 70% glycerol via 25%/50% Glycerol/PBT each for at least 30 min. Images of blastoderm stage embryos and of hatched or pre-hatching larvae were captured using a Nikon 'digital sight' console connected to a DS-Fi1 digital camera mounted on either a Nikon SMZ1500 dissecting scope or a Nikon AZ100 Zoom Stereoscope. Germband stage embryos were dissected out of the yolk and flatmounted on microscope slides. Mounted embryos were viewed on a Nikon Eclipse 80i microscope and photographed as above. DAPI stained embryos were photographed using the Eclipse 80i microscope and a Nikon Intensilight C-HGFI fluorescent light source. Minimal image manipulation (brightness, color balance and contrast enhancement) was done using Adobe Photoshop.

Results

Terminal gap genes

We have looked at terminal patterning in the milkweed bug *O. fasciatus*. We have cloned (Table S1) and tested the expression of the *Oncopeltus* homologues of the orphan nuclear receptor encoding gene *tailless* (*Of-tll* GenBank accession number KC576907) and the Zn-finger transcription factor encoding gene *huckebein* (*Of-hkb* GenBank accession number KC576906), the two terminal gap genes. *Of-tll* is expressed in an anterior cap during blastoderm stages, starting at about 25 hAEL (hours after egg laying, $\pm$1 h at 25 °C). At 33 hAEL this cap begins to refine to a pair of large lateral patches. As the blastoderm invaginates to form the germ band, these patches move posteriorly, and ultimately represent expression in the lateral head lobes and the eye anlagen (Fig. 1A–E). Knocking down the expression of *Of-tll* through RNAi leads to lesions of the most anterior structures of the pre-hatching larva. In mild phenotypes the head is shorter than usual, and the eyes are narrower. In stronger phenotypes the head lobes, including the eyes, are missing (Fig. 2).

Due to the similarity in expression pattern and in knock-down phenotype between *Of-tll* and *Of-otd* (Birkan et al., 2011), we wanted to test if there is a functional relationship between them. We knocked down *Of-otd* through RNAi, and tested expression of *Of-tll*. Indeed, expression of *Of-tll* was disrupted, and in nearly half of the cases, entirely absent (Fig. 3A and B). This indicates that *Of-tll* is under the control of *Of-otd*.

The *Oncopeltus* homologue of the terminal gap gene *hkb* is expressed in a single dorsal stripe during blastoderm stages, starting from about 31 hAEL. When blastoderm invagination begins, a second expression focus appears at the invagination site. In the early germband, *Of-hkb* is expressed in small patches in the head lobes/eye anlage, and in narrow longitudinal stripes in the base of the antennae. In addition there is a segmental expression pattern in lateral spots in the anterior of each segment. In the later germband, the segmental pattern resolves to a series of spots in what we believe to be neuronal precursor cells, but we have not followed this pattern in detail (Fig.1 F–J). Attempts to knock down *Of-hkb* through RNAi proved unsuccessful. We tried two different dsRNA sequences, and in both cases recovered no more abnormal phenotypes than controls. When we stained RNAi treated embryos for *Of-hkb* as a positive control, we found almost normal expression, suggesting that in this case the dsRNA did not significantly

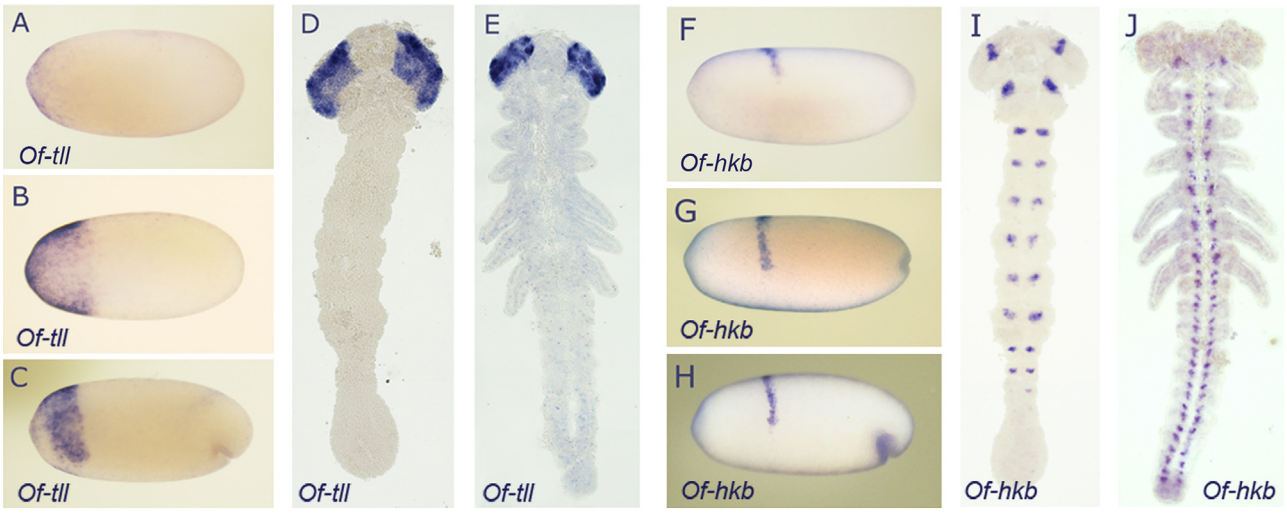

Fig. 1. Expression patterns of the terminal gap gene in *O. fasciatus*. (A–E) *Of-tll*. (F–J) *Of-hkb*. (A) Expression of *tll* is first seen at 25 h after egg laying (hAEL), as a weak anterior signal. (B) At 27 hAEl there is a clear cap. (C) This resolves to lateral patches at 35 hAEL. (D) In the germband these patches can be seen in the lateral head lobes at 49 hAEL. (E) At 73 hAEL. (F) Expression of *Of-hkb* is first seen as a dorsal stripe at 31 hAEL. (G) The stripe expands, and a posterior patch appears at 33 hAEL. (H) Strong expression in the invagination site at 35 hAEL. (I) In the germband there are paired patches at the base of the antennae and in the eye anlage at 47 hAEL, as well as paired segmental patches. (J) A complex pattern develops in the nervous system by 64 hAEL. In all blastoderm images anterior is to the left, and dorsal to the top. In all germband images, anterior is to the top.

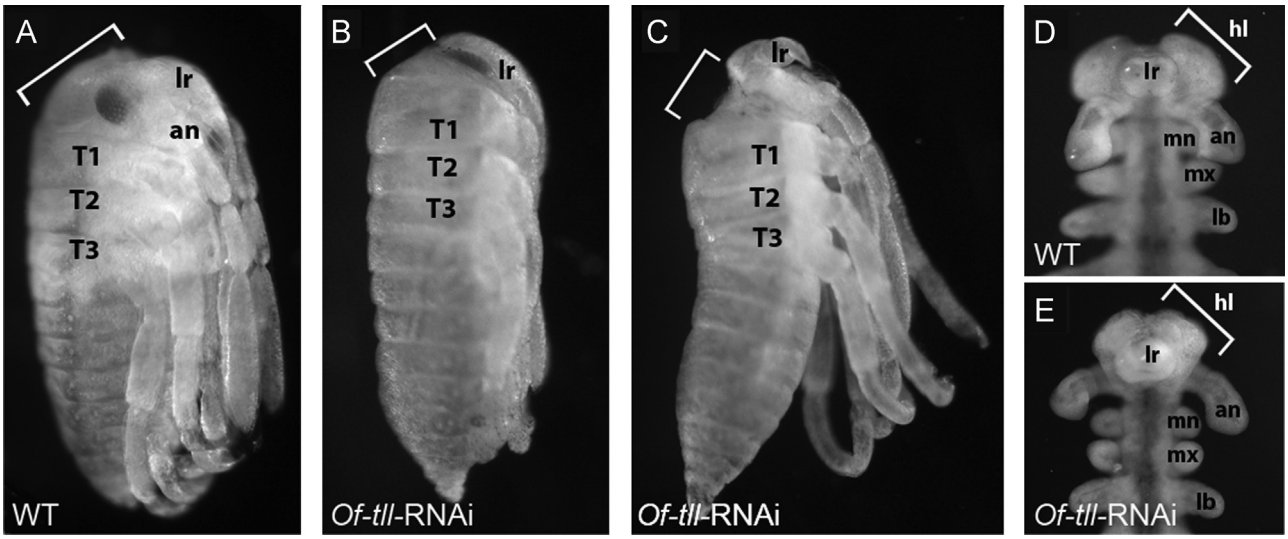

Fig. 2. Knockdown phenotypes following RNAi against *Of-tll*. (A) Wildtype pre-hatching larva. (B) Moderate knockdown phenotype where the posterior of the head is reduced. C) Severe phenotype where most head structures are missing entirely. The labrum and other median structures are not affected. (D) Wildtype germband embryo. (E) Knockdown phenotype with reduced headlobes. hl—head lobes, lr—labrum, an—antenna, mn—mandibula, mx—maxilla, and lb—labium. Bracket indicates the extent of the head.

affect expression. In our experience with *Oncopeltus*, certain genes do not respond to RNAi (Ben-David and Chipman, 2010; Birkan et al., 2011). The level of response to RNAi is unpredictable, and we have no mechanistic explanation at this stage.

In order to test which genes regulate *Of-hkb* we tested its expression at blastoderm stages following RNAi against the three trunk gap genes for which RNAi is possible (*Of-hb*, *Of-gt*, and *Of-kr* —Ben-David and Chipman, 2010) (Table S2), as well as against *Of-tll*. Only *Of-gt* RNAi had any effect on *Of-hkb* expression, causing a posterior duplication of its expression domain (Fig. 3C and D).

The torso pathway

We could not detect localized expression of *torso-like* (*Of-tsl*) in the *Oncopeltus* blastoderm. However, when we carried out in situ hybridization experiments within the first hour after egg laying we obtained a strong uniform staining, that is clearly different from sense-strand controls (Fig. 4A and B). This suggests that *Of-tsl* mRNA is loaded maternally, and is rapidly degraded after egg laying. To test this we carried out in situ hybridization experiments on ovaries. We found strong specific expression in the follicle cells surrounding the oocytes, but no expression in nurse cells (Fig. 4C and C').

Knocking down the expression of *Of-tsl* through parental RNAi led to a range of severe larval phenotypes. Over 50% of the larvae exhibited significantly disrupted development or no development at all. Nearly half of the remainder exhibited a phenotype in which all of the appendages were absent, with the exception of the antennae, and the head was partially or entirely detached from the thorax and had an unusual spherical shape (Fig. 4D).

In order to understand the larval knock-down phenotypes, we collected RNAi blastoderm and germband stage embryos and

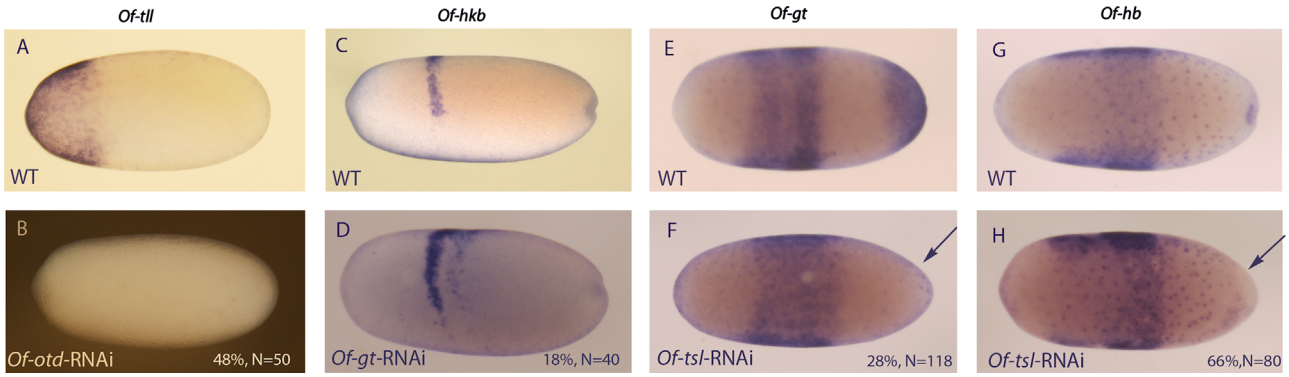

Fig. 3. Interactions between different patterning genes as evidenced by changes in the blastodermal expression pattern of one gene, following RNAi against another gene. Panels in the top row are normal expression patterns of (A) *Of-tll* (C) *Of-hkb* (E) *Of-gt* and (G) *Of-hb*. Panels in the bottom row are expression patterns of the corresponding gene in similarly staged embryos following RNAi against: (B) *Of-otd*. Expression of *Of-tll* is completely lost. (D) *Of-gt*. The expression of *Of-hkb* is duplicated in an ectopic posterior band. (F) *Of-tsl*. The posterior expression domain of *Of-gt* (arrow) is lost (this is found in a relatively low percentage of treated embryos). (H) *Of-tsl*. The posterior expression domain of *Of-hb* (arrow) is lost. All embryos shown with anterior to the left. (A–F) lateral view. (G–H) ventral view. See Table S2 for all of the interactions tested.

looked at their morphology. We found that knocking down *Of-tsl* disrupts the invagination process. Rather than the normal process in which the blastoderm invaginates into the yolk, and the germband forms embedded inside the yolk (Fig. 4E and F), in RNAi treated embryos the germband forms lying on top of the yolk, or only partially embedded in the yolk, depending on severity (Fig. 4G and H). In addition, the two halves of the head fail to fuse, leading to separated head lobes on the surface of the egg. Later in development the head lobes disconnect from the trunk at the level of the intercalary segment. An additional, though less common, phenotype is a splitting of the posterior growth zone into 2–3 lobes. A sample of the range of knock-down phenotypes is shown in Fig. S2.

To gain a better understanding of the early role of *Of-tsl*, we checked whether it controls any of the trunk gap genes in *Oncopeltus* (Ben-David and Chipman, 2010). We tested the expression of the four gap genes in the early blastoderm following *Of-tsl* RNAi, and found that the posterior domains of *Of-hb* and (more rarely) *Of-gt* are disrupted, to the extent that they are frequently entirely absent (Fig. 3E and H). Similar experiments were carried out to test whether *Of-tsl* controls the terminal gap genes. The results of these experiments indicate that there is no noticeable effect of *Of-tsl* RNAi on *Of-hkb* or on *Of-tll* expression (not shown).

The receptor tyrosine kinase encoding gene *torso* (*tor*) was not recovered in our attempts to clone it using degenerate PCR, nor could we find it in the published *Oncopeltus* transcriptome (Ewen-Campen et al., 2011). We found several other RTK genes in the transcriptome, but they were all very distant from tor and were not tested further. A *tor* homologue is found in the sequenced genome of the related pea aphid *Acyrthosiphon pisum* (Shigenobu et al., 2010), but does not have an apparent role in terminal patterning (Duncan et al. 2013; G. Davis—pers. comm).

Discussion

The expression pattern of the two terminal gap gene homologues in *Oncopeltus* is very different from that seen in *Drosophila*. The expression of *tll* and *hkb* in *Drosophila* is in both poles during blastoderm stages (Bronner and Jackle, 1996; Pignoni et al., 1990; Steingrimsson et al., 1991), with the expression of *hkb* restricted to the extreme termini of the blastoderm. The anterior expression domain of *hkb* is under control of *tor* and *bicoid* and the posterior domain is under control of *tor* (Bronner and Jackle, 1996). Mutations of *hkb* lead to loss of endodermal structures. *Drosophila tll* is expressed in somewhat larger anterior and posterior caps. The anterior cap resolves to a patch, similar in position to that found in late blastoderm stages in *Oncopeltus*. Both the anterior and the posterior domains are under the control of *tor* (Steingrimsson et al., 1991). Loss of *tll* in *Drosophila* leads to a loss of the posteriormost segments, and to a disruption of anterior structures (Mahoney and Lengyel, 1987; Strecker et al., 1986).

Although we have no functional data for this gene, the expression pattern of *Of-hkb* strongly suggests that its role is very different from the role of its *Drosophila* homologue, as there is no expression in the termini either at blastoderm or at germband stages. The only other species outside of *Drosophila* where *hkb* has been studied are two dipterans: the brachyceran moth midge *Clogmia albipunctata* (García-Solache et al., 2010), where blastodermal expression was not found, and the cyclorrhaphan hoverfly *Episyrphus balteatus* (Lemke et al., 2010), where the expression pattern is very similar to *Drosophila*. The germband expression described for *Clogmia* is very similar to what we have found in *Oncopeltus*. This suggests that the terminal gap role seen in *Drosophila* and its close relative *Episyrphus* is a cyclorrhaphan novelty, while the neural pattern seen in the more basal *Clogmia* and in *Oncopeltus* represents the ancestral role of the gene. Recent partial results suggest that *Tribolium castaneum hkb* is also not involved in anterior patterning, and has a late neural expression, as we show here (Kittelmann et al. 2013).

On the other hand, the expression pattern and the function of *Of-tll* are reminiscent of the anterior *tll* domain in *Drosophila*, suggesting that the role of *tll* in the anterior is conserved. However, there is nothing in the expression or function in *Oncopeltus* that is similar to the posterior patterning role of *tll* in *Drosophila*. Unlike *hkb*, *tll* has been studied in a number of insects. In the beetle *T. castaneum, tll* is found in a posterior domain only during early blastoderm stages, with an anterior domain appearing later (Schoppmeier and Schröder, 2005). There is no functional data on the role of *tll* in *Tribolium*. In the wasp *Nasonia vitripennis* (Lynch et al., 2006), and in the honeybee *Apis mellifera* (Wilson and Dearden, 2009) *tll* is expressed in anterior and posterior domains, as it is in *Drosophila*, though the regulation of these domains is very different (Lynch et al., 2006; Wilson and Dearden, 2009). In both cases, the anterior domain regulates the formation of anterior brain structures, and the posterior domain regulates the posteriormost abdominal segments.

Intriguingly, the vertebrate orthologue of *tll* is involved in the formation of the eye and anterior structures, as part of a network that also includes *Otx* (vertebrate *otd*) and *Pax6* (Zuber et al., 2003). It is thus possible, that the anterior patterning function of *Of-tll* represents a role within an ancient and conserved network

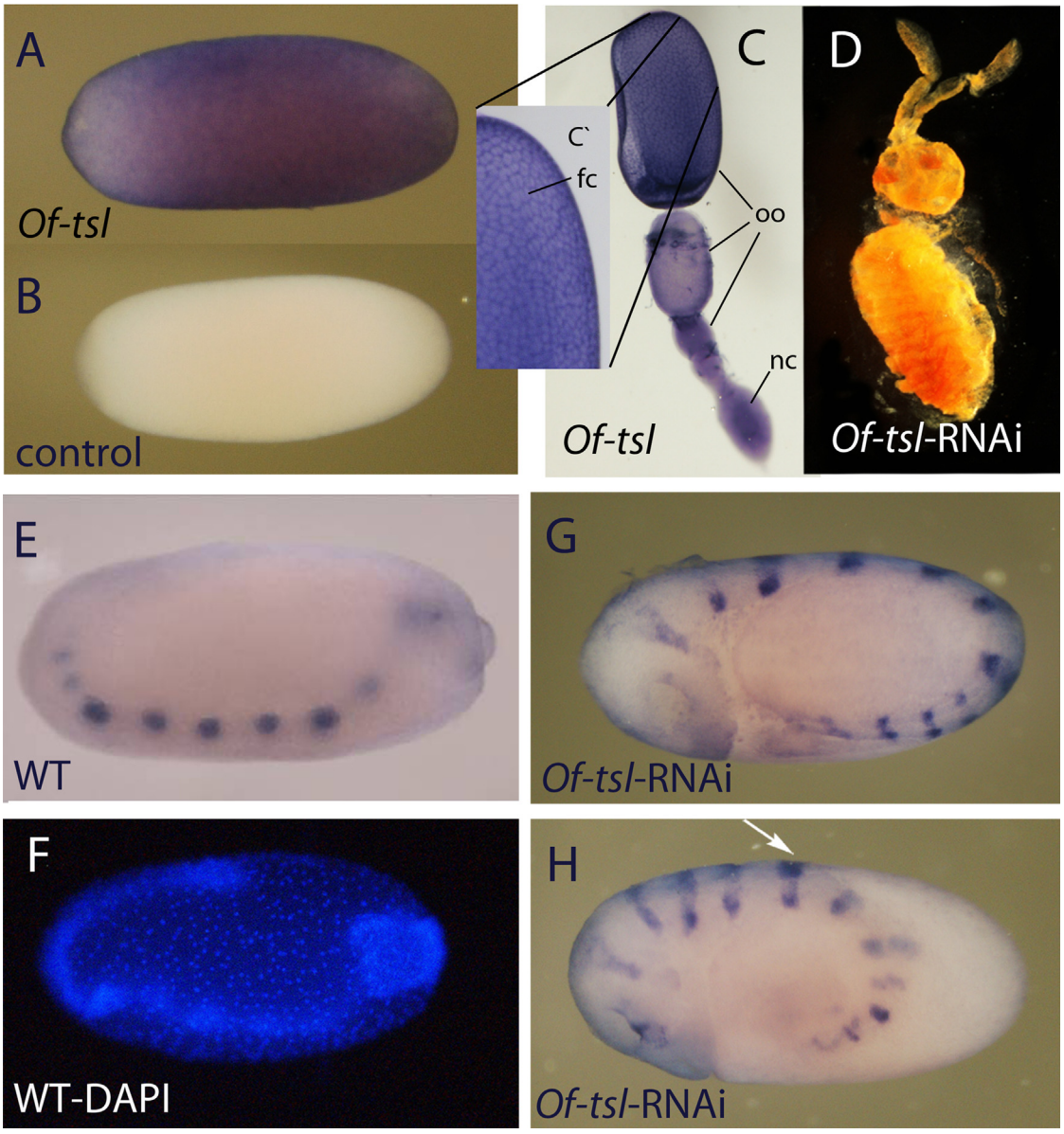

Fig. 4. Expression and RNAi knockdown phenotypes of *Of-tsl*. (A) Ubiquitous expression of *Of-tsl* 1 hAEL. (B) Control embryo at same age as (A) detected with a sense-strand probe. (C) Ovary stained for *Of-tsl*. The posterior-most, most developed oocyte (top of image) shows expression in the follicle cells, which form a thin layer surrounding the oocyte. Younger oocytes in this specific ovary have lost the follicle cells during their preparation. There is no staining in the nurse cells (anterior of ovary, out of the focal plane in this image). oo—oocytes, nc—nurse cells, and fc—follicle cells. Posterior is to the top. (C') Higher magnification of part of the most developed oocyte, showing clear cytoplasmic staining in the flat follicle cells around the oocyte. (D) Larva following RNAi against *Of-tsl*. (E) Wildtype early germband embryo, stained for the segmental marker *engrailed*. (F) Similar stage embryo stained with the fluorescent nuclear marker DAPI. G) Germband stage RNAi embryo. Invagination has failed, and the germband surrounds the yolk, rather than being embedded in it as seen in (E–F). (H) Similar stage RNAi embryo, where invagination has occurred partially. The white arrow marks the ectopic invagination site. See Fig. S1 for more examples.

in bilaterians. Parts of this network are also conserved in *Drosophila* (Daniel et al., 1999), although the link between *tll* and *otd* seems to have been lost there.

The role of the *Oncopeltus* homologue of *tsl* is very different from *Drosophila*. It apparently involves determination of the invagination site and/or coordination of the invagination process. When invagination is disrupted following the knock-down of the gene, germband extension and segmentation occur almost normally, but on the outside of the egg yolk, rather than embedded in it. The signals that trigger limb outgrowth are lacking in this conformation, for reasons that we do not understand. Surprisingly, dorsal closure still manages to take place, generating a segmented, intact larva, but with no appendages. The detached head is likely due to improper fusion of the head capsule, because of the dissociation of the head lobes.

Of-tsl also regulates the posterior domain of two of the trunk gap genes, *Of-gt* and *Of-hb*. We identify these posterior domains as being part of the network that defines the posterior growth zone (Ben-David and Chipman, 2010). The fact that *Of-tsl* lies upstream of these gap gene posterior domains strengthens our interpretation that one of the main early roles of this gene is in coordinating or regulating the invagination process, which seems to be tightly linked with the generation of the growth zone. It is important to note in this context that the effect of *Of-tsl* on posterior patterning must be indirect, as its expression is neither spatially nor temporally connected with invagination or growth zone formation.

As in *Drosophila* and *Tribolium* *Of-tsl* is expressed in somatic follicle cells (Martin et al., 1994; Schoppmeier and Schröder, 2005). *Oncopeltus* follicle cells cover the entire oocyte throughout oogenesis, and not just the termini as in the other two species. We could

not see expression in the oocytes themselves during oogenesis, but this could be due to the sensitivity of the protocol during these stages. Expression is found in the early embryo just after laying, but not later. We hesitate to suggest that RNA or protein is deposited by follicle cells into the oocyte, as there is no previously recorded example of this happening. However, we cannot rule out such a possibility.

The posterior role of *Of-tsl* is surprising, given its lack of localized expression during oogenesis and early blastoderm stages. Nonetheless, based on the effects of the RNAi experiments, we suggest that either the gene product remains active well into the blastoderm invagination phase and there is an additional, as yet unknown, signal that localizes the *tsl* gene product in the posterior, or else that *Of-tsl* is interacting with an unknown localized posterior factor that conveys a signal that is maintained for a long time before being manifested. Either way, this signal functions in defining the posterior growth zone and the invagination site.

Evolution of the terminal patterning system

Our results from the hemimetabolous milkweed bug add a significant phylogenetic data point, as it belongs to the closest outgroup to the holometabolous insect radiation. The addition of these results make it possible to offer a possible reconstruction of the evolutionary history of the terminal patterning system leading up to *Drosophila*. We can see how the pathway was assembled piecemeal, as the various components changed their roles throughout evolution (Fig. 5).

According to this reconstruction, the original role of *tll* was in anterior patterning and eye patterning, and it is part of an ancient network involving *orthodenticle* and *Pax6* (Zuber et al., 2003). It maintains this role in most insect lineages, although the link with *Pax6* and *otd* has been lost in *Drosophila* (Daniel et al., 1999), where it has come under the control of *Bicoid* (*bcd*)—the major anterior determinant in cyclorrhaphan flies (Brown et al., 2001; Stauber et al., 1999). The addition of *bcd* is linked with several significant changes in anterior patterning in *Drosophila* (Dearden and Akam, 1999). At some point within holometabolous insects, *tll* acquired a secondary role in the posterior as a terminal gap gene. In Hymenoptera, at least in *Nasonia*, this posterior domain is under control of *otd*, but in *Drosophila* it comes under the control of the recently recruited *tor* pathway. The second terminal gap gene *hkb* is apparently a very recent recruitment to the terminal patterning network, as it only seems to have such a role in *Drosophila* and probably closely related species. The original role of *hkb* may have been in regulating neural development, as might be deduced from its expression pattern in *Oncopeltus*, in *Tribolium* and in *Clogmia*. A possible vestige of this original role is still seen in *Drosophila* where *hkb* has a role in the differentiation of serotonin neurons (Lundell et al., 1996).

The *tsl* gene product is an ancient member of a family of pore-forming proteins (Rosado et al., 2008). We suggest that its original role may have been in initiating the coordination of posterior growth in sequentially segmenting insects. Posterior growth is tightly coupled with invagination in *Oncopeltus*, explaining the severe invagination phenotypes we see. The fact that *Of-tsl* regulates the posterior domains of the trunk gap genes, and is involved in the invagination process, provides further support to this suggestion. The recruitment of the *tor* signaling pathway to terminal patterning seems to have occurred within the holometabolous insects. Even within holometabolous insects its exact role is variable (Duncan et al., 2013). In the hoverfly *Episyrphus tor* not only controls the terminal gap genes, but also activates *otd* and transiently represses anterior *cad* (Lemke et al., 2010). Schoppmeier and Schröder (2005) suggest that the original role of *tor* signaling was in coordinating posterior growth. Adding our

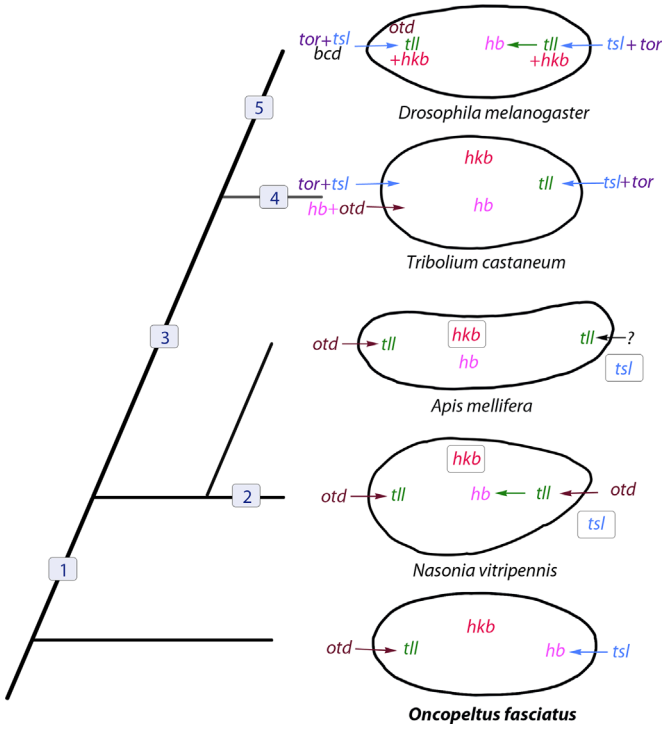

Fig. 5. A schematic summary of the interactions of the key genes in the terminal patterning system during blastoderm stages, mapped on a phylogenetic tree to show their changing roles throughout insect evolution. Genes listed outside the embryo indicate a general role in determining anterior and/or posterior structures. Genes listed in bounding boxes are assumed to be present, but there are no data on their expression or interactions. Expression of *tsl* is in somatic follicle cells where known. We suggest it is expressed in follicle cells in other species as well. The scheme includes only the genes discussed explicitly in the paper and omits e.g. anterior targets of *otd+hb* in *Tribolium*, and others. The pattern in *O. fasciatus* is taken to be the plesiomorphic pattern. Rectangles marked 1–5 on the tree indicate key events in the evolution of the terminal patterning system. (1) At the base of Holometabola *tll* acquires a posterior patterning role (in addition to its ancestral anterior role). (2) Posterior expression of *otd* is apparently an autapomorphy for *N. vitripennis*. (3) The gene *tor* is recruited to the terminal system, and interacts with *tsl* as an initiator of the network. The two genes assume a role in the anterior. (4) In *T. castaneum* anterior function of *tll* is lost in the blastoderm. Anterior patterning involves *otd+hb* (though this may be plesiomorphic). (5) Within Diptera *hkb* is recruited as a terminal gap gene. The role of *otd* as an anterior determinant is taken over by *bcd*, and *otd* is expressed in the anterior as a head gap gene. Posterior *hb* is under the control of *tll* (this may be plesiomorphic). Embryo sketches are not to scale.

results, we can modify their suggestion to say that *tsl* was the original coordinator of posterior growth, and *tor* signaling was added later in evolution. Our previous work on the development of *Oncopeltus* indicates that it often has a development pattern that represents the plesiomorphic pattern prior to the radiation of holometabolous insects (Ben-David and Chipman, 2010; Birkan et al., 2011). If this is indeed the case with terminal patterning as well, then we can hypothesize that a gene that already had posterior roles in hemimetabolous insects – *tsl* – was co-opted for the developmentally earlier process of defining the posterior pole of the embryo, together with a novel signaling pathway—the *tor* pathway, and later still was recruited to defining both termini.

Our data on terminal patterning genes in *Oncopeltus* provide a crucial piece of a puzzle, which together with previous reports on parts of the terminal patterning system in other insects, allows us to reconstruct the early evolutionary history of this well-studied system. The reconstruction shows that the *Drosophila* terminal patterning system is not the result of gradual evolutionary change, nor is it an evolutionary novelty. The evolution of this system along different lineages from hemimetabolous insects to *Drosophila* is a classic example of what has been called evolutionary tinkering

(Duboule and Wilkins, 1998; Jacob, 1977). The idea of co-option of genes from an ancestral role to a novel role is well known in evolutionary developmental biology (Arthur, 2002), but there are few clear examples of the assembly of a full developmental network from individual unrelated elements. The *Drosophila* terminal patterning system includes elements from networks originally involved in neural development (*hkb*), eye development (*tll*), and growth zone determination (*tsl*) linked together and activated by a newly recruited receptor tyrosine kinase (*tor*). We believe that as our knowledge of non-model species increases, we will find more and more such examples of tinkering.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.04.030.

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