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Functional analyses in the milkweed bug *Oncopeltus fasciatus* (Hemiptera) support a role for Wnt signaling in body segmentation but not appendage development

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Abstract

Specification of the proximal–distal (PD) axis of insect appendages is best understood in *Drosophila melanogaster*, where conserved signaling molecules encoded by the genes *decapentaplegic* (*dpp*) and *wingless* (*wg*) play key roles. However, the development of appendages from imaginal discs as in *Drosophila* is a derived state, while more basal insects produce appendages from embryonic limb buds. Therefore, the universality of the *Drosophila* limb PD axis specification mechanism has been debated since *dpp* expression in more basal insect species differs dramatically from *Drosophila*. Here, we test the function of Wnt signaling in the development of the milkweed bug *Oncopeltus fasciatus*, a species with the basal state of appendage development from limb buds. RNA interference of *wg* and *pangolin* (*pan*) produce defects in the germband and eyes, but not in the appendages. *Distal-less* and *dachshund*, two genes regulated by Wg signaling in *Drosophila* and expressed in specific PD domains along the limbs of both species, are expressed normally in the limbs of *pan*-depleted *Oncopeltus* embryos. Despite these apparently paradoxical results, Armadillo protein, the transducer of Wnt signaling, does not accumulate properly in the nuclei of cells in the legs of *pan*-depleted embryos. In contrast, *engrailed* RNAi in *Oncopeltus* produces cuticular and appendage defects similar to *Drosophila*. Therefore, our data suggest that Wg signaling is functionally conserved in the development of the germband, while it is not essential in the specification of the limb PD axis in *Oncopeltus* and perhaps basal insects.

Keywords: Oncopeltus; Milkweed bug; Hemiptera; *Wingless*; *Pangolin*; *Decapentaplegic*; *Engrailed*; RNA interference; Appendage patterning

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Introduction

Appendages are present in several of the most successful animal groups, and they are a defining feature of the arthropods. Much of our understanding of appendage development has been taken from the foremost model arthropod, the fruit fly *Drosophila melanogaster* (Diptera). However, the universality of *Drosophila* appendage-patterning mechanisms is questionable, particularly given the derived nature of limb development from imaginal discs in *Drosophila*. Imaginal discs are epithelial sheets of cells set-aside during embryogenesis but patterned during larval development. These structures are unique to the Holometabola, but appendage development from imaginal discs is not universal among this group. In Coleoptera, Trichoptera, Neuroptera, and Lepidoptera, only some adult appendage types develop from imaginal discs (Svacha, 1992), while the phenomenon is most pronounced in the cyclorhaphous Diptera, where all adult appendages are produced from imaginal discs. In contrast, limb development in most insect orders proceeds directly from three-dimensional embryonic limb buds. Given these differences of topology, potential differences in the specification of the limb proximal–distal (PD) axis are possible.

In *Drosophila*, the adult appendages develop from imaginal discs during the larval stages. The discs are sheets

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of epithelia, in which the central and presumptively distal region of the limb telescopes out during pupal development to yield the mature appendages. However, larval patterning of the disc occurs in an essentially two-dimensional sheet of cells.

The specification of the limb PD axis is best understood in the leg disc, where it is defined by the overlap of signaling molecules encoded by the genes *decapentaplegic* (*dpp*) and *wingless* (*wg*). In the embryo, *wg* is required for the formation of the imaginal disc primordia (Kubota et al., 2003; Simcox et al., 1989). Removal of *wg* activity during this period eliminates the appendages and other imaginal disc derivatives (Cohen et al., 1993). In the imaginal leg disc, *dpp* and *wg* are expressed in stripes along the anterior–posterior (AP) compartment boundary on the dorsal and ventral sides, respectively (Baker, 1988a; Masucci et al., 1990), in response to activation by *hedgehog* signaling from the posterior compartment (Diaz-Benjumea et al., 1994). *wg* encodes a secreted Wnt signaling molecule (Rijsewijk et al., 1987) that also acts as a segment polarity gene in the germband (Ingham and Martinez-Arías, 1992). Similarly, *dpp* encodes a signaling molecule of the TGF-β protein family (Padgett et al., 1987), which also acts to establish the dorsal–ventral (DV) body axis (Irish and Gelbart, 1987). The imaginal leg disc is an essentially two-dimensional structure in which these signaling pathways interact. In this context, Dpp and Wg signaling mutually inhibit one another's expression to define dorsal and ventral territories of the disc, respectively (Theisen et al., 1996). Because of the two-dimensional character of the imaginal disc, Wg and Dpp ligands overlap in a graded manner only at its center. There, they cooperatively activate distal appendage-patterning genes, such as *Distal-less* (*Dll*) and *dachshund* (*dac*), while repressing proximal genes such as *homothorax* (*hth*) (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). In this way, *wg* and *dpp* cooperate to specify the first distinct domains along the limb PD axis.

In contrast to *Drosophila*, most other arthropods produce appendages directly from embryonic limb buds. A consistent and interesting theme has emerged from studies reporting the expression patterns of appendage-patterning orthologues in non-model species. Generally, the expression of PD domain genes, such as *Dll* and *dac*, is well conserved, in discrete regions of the legs (Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2004; Prpic and Tautz, 2003; Prpic et al., 2003). Similarly, the expression of *wg* orthologues appears conserved. In the red flour beetle *Triboliumcastaneum* (Nagy and Carroll, 1994), the cricket *Gryllus bimaculatus* (Miyawaki et al., 2004), and the spider *Cupiennius salei* (Prpic et al., 2003), *wg* expression extends in stripes along the parasegmental compartment boundaries into the limb buds to their distal tips. However, *dpp* orthologues examined in other arthropods show a pattern that is unlike *Drosophila* but fairly consistent among the diverse species examined. In species, such as *Tribolium* (Sanchez-Salazar et al., 1996), the grasshopper *Schistocerca americana* (Jockusch et al., 2000), and *Cupiennius* (Prpic et al., 2003), early *dpp* expression appears throughout the limb buds. As the limb buds elongate, rings of expression are formed at or just proximal of the distal tip. Later, additional weaker rings of expression appear at different PD levels along the legs of *Schistocerca* and *Cupiennius*.

The differences in *dpp* expression between *Drosophila* and other arthropods are striking and imply perhaps different modes of action in the specification of the limb PD axis for *Drosophila* as compared to more basal insects. However, a model has recently been proposed by Prpic et al. (2003), based on comparative data and mathematical models of the *Drosophila* imaginal leg disc (Almirantis and Papageorgiou, 1999). These authors have noted that because Wg and Dpp cooperate to active distal targets, it is crucial that these ligands remain spatially separated in areas fated to become proximal, where Wg and Dpp proteins should not co-occur. Since *dpp* and *wg* are expressed in separate DV territories of the anterior compartment of the *Drosophila* imaginal disc, the ligands cannot diffuse to all proximal areas of the disc, only those in their respective territory. Therefore, stripes of *wg* and *dpp* expression along the compartment boundary in different DV territories allow them to cooperatively activate distal target genes only in the central region of the disc. However, in a three-dimensional limb bud, the dorsal and ventral sides of the proximal limb bud are close enough spatially that the same pattern of *wg* and *dpp* stripes would activate distal target genes over too great a length of the limb bud. This model rationalizes the pattern of distal *dpp* rings in basal insects based on topology. If *dpp* is expressed distally, Wg and Dpp should only overlap in a distal area, and their combined concentration is thought to diminish proximally. Therefore, it is assumed that primitive insects share the same regulatory network architecture as *Drosophila*, in which overlap of Wg and Dpp ligands activates target genes, while inhibiting genes responsible for proximal limb fate.

The topology model, as we shall refer to it, is based on two assumptions: 1 The expression of *wg* and *dpp* in the limb buds of basal insects should be critical to the proper development of appendages in these species. 2. The genetic pathway of the *Drosophila* limb PD axis specification mechanism is conserved. That is, in basal insects, as in *Drosophila*, Wg and Dpp signaling should cooperate to activate distal targets, such as *Dll* and *dac*, while repressing proximal domain genes. Therefore, the topology model leads to at least two testable hypotheses: 1. Perturbations of Wg signaling in basal insects should produce appendage phenotypes similar to those seen in *Drosophila*. 2. The proper expression of genes, such as *Dll* and *dac*, along the limb PD axis of basal insects should require Wg and Dpp signaling.

Here, we test these hypotheses through functional analysis in a hemimetabolous insect, *Oncopeltus fasciatus* (Hemiptera). *Oncopeltus* is a member of the sister taxon to the Holometabola and therefore provides an important