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The roles of *wingless* and *decapentaplegic* in axis and appendage development in the red flour beetle, *Tribolium castaneum*

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Abstract

Axis patterning and appendage development have been well studied in *Drosophila melanogaster*, a species in which both limb and segment morphogenesis are derived. In *Drosophila*, positional information from genes important in anteroposterior and dorsoventral axis formation, including *wingless* (*wg*) and *decapentaplegic* (*dpp*), is required for allocating and patterning the appendage primordia. We used RNA interference to characterize the functions of *wg* and *dpp* in the red flour beetle, *Tribolium castaneum*, which retains more ancestral modes of limb and segment morphogenesis. We also characterized the expression of potential targets of the WG and DPP signaling pathways in these embryos. *Tribolium* embryos in which *dpp* had been downregulated had defects in the dorsalmost body wall, but did not appear to have been globally repatterned and had normal appendages. Downregulation of *wg* led to the loss of segment boundaries, gnathal and thoracic appendages, and lateral head lobes, and to changes in the expression of *dpp*, *Distal-less*, and Engrailed. The functions of *wg* varied along both the anteroposterior and dorsoventral axes of the embryo. Phylogenetic comparisons indicate that the role of WNT signaling in segment boundary formation is evolutionarily old, but that its role in appendage allocation originated in the common ancestor of holometabolous insects.

Keywords: *Tribolium castaneum*; *Wingless*; *Decapentaplegic*; Axis patterning; Appendage development; RNA interference

Introduction

The developmental mechanisms insects, and animals in general, use to set up the body axes early in development are paramount in directing the rest of morphogenesis. Morphogenesis in *Drosophila* is a result of progressive subdivision of the embryo along both the dorsoventral (DV) and anteroposterior (AP) axes. Development of each of these axes is initiated by maternally supplied factors, and development proceeds largely independently along the two axes (reviewed in Lall and Patel, 2001). Appendage development requires establishment of the third embryonic axis, the proximodistal (PD) axis. As generally described by Meinhardt (1983), intersecting signals from two perpendicular axes are sufficient to define a third axis or "distal organizer" of the appendage tip. As expected based on this model, PD axis development in *Drosophila* is initiated embryonically and requires positional information from both the AP and DV axes (Cohen et al., 1993; Goto and Hayashi, 1997; Raz and Shilo, 1993). The organization of the AP and DV axes at the blastoderm stage is derived in *Drosophila* and differs from that in many other lineages of insects. In *Drosophila*, all segmental primordia are already present in the blastoderm, and segmentation occurs nearly simultaneously throughout the entire germ band (Sander, 1976). This long germ mode of development contrasts with segmentation in short and intermediate germ band insects, like the beetle *Tribolium*. In these, primordia of anterior segments are present in the blastoderm, whereas primordia of more posterior segments are generated from a posterior growth zone during germ band extension (Sander, 1976). The DV axis of blastoderm stage embryos has also been reorganized in cyclorrhaphan flies in conjunction with reduction and modification of the extraembryonic membranes (Schmidt-Ott, 2000).

Although appendages are not visible morphologically in embryonic and larval *Drosophila*, appendage primordia are allocated early in embryogenesis. These cells form imaginal discs, which invaginate, undergo growth and patterning during larval development, and evert during the pupal stage to form the

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adult appendages (Cohen, 1993). Cohen and colleagues (1993) demonstrated that imaginal discs originate from cells at the intersections of segmentally reiterated stripes of the secreted signaling factor Wingless (WG), a WNT family member, and a horizontal stripe of the TGF-β family member Decapentaplegic (DPP). Signaling from both of these genes is necessary for proper allocation of the imaginal discs, as well as for later appendage patterning. A major question is to what extent these functions are conserved in species with different modes of appendage development and different blastoderm fate maps. For this reason, we explored the functions of *wg* and *dpp* in *Tribolium castaneum*, the red flour beetle, an intermediate germ band species that develops its legs directly rather than from imaginal discs.

The segmental polarity gene *wg* is the most downstream AP patterning gene for which null mutants lack all imaginal disc tissue in *Drosophila*, showing that it plays a key role in allocation of the appendage primordia (Simcox et al., 1989). Expression of *Distal-less* (*Dll*), one of the earliest molecular marker of imaginal disc allocation, is also dependent on *wg* in the blastoderm (Cohen, 1990). During embryogenesis, continued WG signaling is required for maintenance of leg imaginal disc fate (Kubota et al., 2003). Experiments using a temperature sensitive *wg* allele show that continued *wg* signaling is required for proximal imaginal disc fate in the embryo, while experiments using downstream components of the *wg* pathway show that both proximal and distal leg fates are WNT-dependent (Cohen et al., 1993; Kubota et al., 2003). The required WG signal comes from the segmental stripe of *wg* expression along the posterior edge of the anterior compartment of each segment (Cohen et al., 1993). *wg* mutants also fail to develop segment boundaries, in part because *wg* is required to maintain the expression of the segment polarity gene *engrailed* (*en*) (Baker, 1988; Bejsovec and Martinez Arias, 1991; Bejsovec and Wieschaus, 1993; Heemskerk et al., 1991; Perrimon and Mahowald, 1987).

Although *wg* is expressed along the entire DV axis of each segment when imaginal discs are allocated in *Drosophila*, imaginal disc allocation and *Dll* expression are restricted to a ventrolateral region of each segment by the activity of two DV patterning genes. Ventrally, imaginal disc identity is inhibited by the expression of the EGF ligand *spitz* (Goto and Hayashi, 1997; Kubota et al., 2000; Raz and Shilo, 1993). Dorsally, it is inhibited by *dpp* (Goto and Hayashi, 1997). Somewhat later, during germ band retraction, decreased DPP signaling leads to malformation of both the proximal and distal leg regions (Goto and Hayashi, 1997; Kubota et al., 2000, 2003). In the blastoderm, *dpp* is expressed throughout the dorsal region of the embryo; this expression resolves into two horizontal stripes along the length of the embryo, which run perpendicular to the stripes of *wg* expression; the more medial of these stripes is required for proper allocation of imaginal discs (Goto and Hayashi, 1997). In *dpp* mutants, dorsal cell fates are missing and embryos are completely ventralized, indicating that *dpp* plays a central role in patterning of the DV axis of the embryo (Arora and Nüsslein-Volhard, 1992; Ferguson and Anderson, 1992; Irish and Gelbart, 1987).

In larval leg discs, *wg* and *dpp* continue to play important roles in limb patterning. *wg* expression in the anterior ventral portion and *dpp* expression in the anterior dorsal portion of the disc are stabilized by inhibitory interactions between the genes (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Because *wg* and *dpp* act cooperatively to activate and repress target genes, discrete domains of gene expression form along the PD axis, which runs from the center to the edges of the disc. The distal region of the limb primordia is characterized by *Dll* expression, the intermediate region by expression of *dachshund* (*dac*), and the proximal region by co-expression of nuclearly-localized Homothorax (n-HTH) and Extradenticle (n-EXD) (Diaz-Benjumea et al., 1994; reviewed in Kojima, 2004; Lecuit and Cohen, 1997; Mardon et al., 1994; Wu and Cohen, 1999). Subsequently, these PD expression domains are maintained at least in part by autoregulation and by repressive interactions between *Dll*, *dac* and *hth* (Abu-Shaar and Mann, 1998; Castelli-Gair and Akam, 1995; Dong et al., 2001; Galindo et al., 2002; Lecuit and Cohen, 1997; Wu and Cohen, 1999).

Available comparative data from insects and other arthropods suggest that the subdivision of the PD axis by *Dll, dac,* n-Exd and n-Hth is evolutionarily ancient. DLL expression in the distal region of limbs characterizes the limbs of all arthropod species examined to date (Abzhanov and Kaufman, 2000; Grenier et al., 1997; Mittmann and Scholtz, 2001; Niwa et al., 1997; Palopoli and Patel, 1998; Panganiban et al., 1994, 1995; Popadić et al., 1998; Prpic and Tautz, 2003; Schoppmeier and Damen, 2001; Thomas and Telford, 1999; Williams, 1998; Williams et al., 2002), and its function in distal limb outgrowth has been confirmed by analysis of *Dll* mutants in *Tribolium* (Beermann et al., 2001) and by RNA interference (RNAi) in the milkweed bug *Oncopeltus fasciatus* (Angelini and Kaufman, 2004) and a spider, *Cupiennius salei* (Schoppmeier and Damen, 2001). RNAi also demonstrates the conservation of *dac* and *hth* functions in patterning intermediate and proximal regions of the limbs of *O. fasciatus* (Angelini and Kaufman, 2004). Although data on the function of *dac*, *hth* and *exd* are not yet available in other lineages, expression of n-EXD and n-HTH proximally and of *dac* in an intermediate domain are conserved across arthropods (reviewed in Angelini and Kaufman, 2005b). Thus, available data are consistent with the hypothesis that downstream leg (PD axis) patterning is functionally conserved across insects and crustaceans.

In contrast to the evidence for similar patterning of the PD limb axis in species that develop their limbs directly and from imaginal discs, the evidence for conservation at earlier stages is limited; conservation of these earlier developmental interactions may be less likely for two reasons. First, limb imaginal discs are an evolutionary innovation of higher flies (Svacha, 1992; Truman and Riddiford, 1999). A straightforward model of imaginal disc evolution would suggest that discs originated by insertion of developmental events at the beginning of limb development or by modification of the earliest events in limb development. Second, the distribution of secreted signaling molecules is expected to be more sensitive than the distribution of transcription factors to the changes in tissue geometry that distinguish two-dimensional imaginal discs from three-dimensional limb buds (Jockusch et al., 2000; Prpic et al., 2003).

wg expression in segmentally reiterated stripes is highly conserved across insect species including *Tribolium*, as is *wg* expression along a ventral stripe in developing appendages (Abouheif and Wray, 2002; Angelini and Kaufman, 2005a; Dearden and Akam, 2001; Jockusch et al., 2000, 2004; Miyawaki et al., 2004; Nagy and Carroll, 1994); it is also relatively conserved in other arthropods (Damen, 2002; Hughes and Kaufman, 2002; Nulsen and Nagy, 1999; Prpic, 2004; Prpic et al., 2003). However, RNA interference targeting downstream components of the WNT signaling pathway of two hemimetabolous insect species with direct limb development did not lead to the complete loss of limbs, as would be predicted if the earliest function of *wg* in appendage development were similar in hemimetabolous species and in *Drosophila* (Angelini and Kaufman, 2005a; Miyawaki et al., 2004). *dpp* expression patterns show greater variation between *Drosophila* and other species of arthropods examined to date, both in early embryonic patterning and in limb development (Akiyama-Oda and Oda, 2003; Angelini and Kaufman, 2005a; Dearden and Akam, 2001; Giorgianni and Patel, 2004; Jockusch et al., 2000, 2004; Niwa et al., 2000; Prpic, 2004; Prpic et al., 2003). While the *dpp* expression pattern in *Tribolium* is divergent from that of *Drosophila*, Prpic et al. (2003) suggested that the function of *dpp* may nonetheless be similar in direct developing limbs and imaginal discs.

We used RNAi to examine the functions of *wg* and *dpp* in *Tribolium* embryogenesis, and particularly to assess whether their roles in axis patterning and limb allocation are conserved. *Tribolium* is more closely related to *Drosophila* than are hemimetabolous insects, but limb and segment morphogenesis are more similar between *Tribolium* and hemimetabolous species. Thus, functional data from *Tribolium* present an interesting comparison to the data from hemimetabolous species and from *Drosophila*. We find strong similarities between phenotypes observed in *Drosophila wg* null mutants and in *Tribolium* embryos in which *wg* has been downregulated, including complete loss of most appendages. These data also reveal variation in the developmental roles of WG signaling along the AP and DV axes in *Tribolium.* By contrast, *Tribolium* embryos in which *dpp* has been downregulated show much milder phenotypes than were observed in *Oncopeltus* using RNAi (Angelini and Kaufman, 2005a) and in *Drosophila dpp* mutants (Irish and Gelbart, 1987). While development of the dorsalmost row of body wall cells was disrupted in *Tribolium*, other aspects of the phenotype appeared largely normal. The phenotypes we observe are consistent with the changes in expression of several potential downstream targets of *wg* and *dpp*, including *wg, dpp, Dll* and *en*. Together, these data suggest that *wg* has an ancestral role in segment boundary formation, and that its role in limb allocation resulted from co-option of the segmental stripe of *wg* expression in the common ancestor of holometabolous insects.

Methods

Insect rearing

Tribolium castaneum embryos were reared at room temperature in white flour supplemented with brewer's yeast.

RNA interference

Injection of double-stranded (ds) RNA into a mother's hemocoel results in knockdown of zygotic genes in offspring embryos (Bucher et al., 2002). Double-stranded RNA was produced from an 801 bp fragment of the *T. castaneum wingless* gene (fragment positions 123–903; Nagy and Carroll, 1994) and 818 bp of the *T. castaneum dpp* gene (Sanchez-Salazar et al., 1996; bases 3596–4413 in GenBank accession U63132; this fragment includes 668 bp at the C terminal end of the coding region and 150 bp of 3′ UTR). PCR primers were designed with the T7 promoter site added to the 5′ end of both primers. T7 RNA polymerase (Invitrogen) was used to produce both strands of RNA simultaneously.

For injection, female *T. castaneum* pupae were affixed to microscope slides with double-stick tape at their posterior abdomen. Using a simple micromanipulator set-up, approximately 2 μl of dsRNA solution (0.076–0.210 μg/μl in 1:10 TE for *wg*, 0.496–0.596 μg/μl in 1:10 TE for *dpp*) were injected into each pupa, at a ventrolateral position between abdominal segments three and four (Bucher et al., 2002). Additional female pupae were injected with 1:10 TE only. After completion of metamorphosis, injected females were kept with untreated males at room temperature. Beginning 1 week after metamorphosis, eggs were harvested at 4-day intervals for 30 days. Because of the 4-day egg collection interval, embryos were scored for phenotypic defects at a range of developmental stages. To test whether early segmentation happened normally, one pool of embryos was collected at 30 h and subdivided. Half of the embryos were fixed immediately, and stained for EVE expression, while the other half were allowed to develop for 4 days before fixation.

Embryos from RNAi treatments and controls were bleached for 2 min in 50% bleach, fixed in 8% formaldehyde in phosphate-buffered saline, 50 mM EGTA, 0.1% Tween for 20–45 min, with heptane and rapid shaking to improve the penetration of the fixative, then sonicated or hand dissected to remove the membranes, and stored in 100% methanol at −20°C. Embryos were stained with 1 μg/ml DAPI, and mounted in 80% glycerol. Embryonic structures that express *wg* or *dpp* during normal development were examined for deformities, and embryos were scored as wild type (unaffected), or as deformed in one or more of the structures examined.

In situ hybridization and immunohistochemistry

In situ hybridization of embryo whole mounts using digoxigenin (DIG)-labeled riboprobes was carried using a protocol similar to that in Jockusch et al. (2000). Probes were made from the following templates: a 1.3 kb fragment of *T. castaneum dpp* containing primarily 3′UTR (Doctor et al., 1992), a 1.6 kb fragment of *T. castaneum wg* containing part of the coding region and the 3′UTR (Nagy and Carroll, 1994), and a 468-kb fragment of *T. castaneum Dll* containing coding region (Beermann et al., 2001). To confirm downregulation of *wg* and *dpp* expression in RNAi embryos, dsRNA injected and normal embryos were carried through the in situ protocol together, and the speed of signal development was compared. The Engrailed (EN) antibodies 4D9 and 4F11 (Patel et al., 1989) and Even-skipped (EVE) antibody 2B8 (Patel et al., 1994) were generously supplied by N. Patel and the Developmental Studies Hybridoma Bank. A 1:10 (EN 4D9) or 1:100 (EN 4F11, EVE 2B8) dilution of the primary antibody was used following the immunohistochemical protocol described in Panganiban et al. (1995) with an HRP- or Cy3-conjugated secondary antibody (Jackson Labs). Embryos were stained with 1 μg/ml DAPI to visualize nuclei and mounted in 80% glycerol. Photographs were taken on a Zeiss Axiophot microscope with an Optronics Magnafire digital camera.

Results

Phenotypes of Tcwg^{RNAi} embryos

Of the 173 *T. castaneum* embryos obtained from pupae injected with ds *wg* RNA and examined for morphological defects, 64% were scored as phenotypically abnormal (hereafter called *Tcwg*^{RNAi} embryos). This contrasts with the 100%

frequency of normal embryos obtained from control injections (zero deformed out of 84 observed, $P < 0.001$). The defects affected a wide range of morphological structures, including segments, limbs, spiracles, and lateral head lobes, all of which express *wg* during normal embryogenesis in *Tribolium*. To aid in interpreting these results, we also review relevant expression data, supplementing published results (Jockusch et al., 2004; Liu and Friedrich, 2004; Nagy and Carroll, 1994).

Segmental expression and defects: Normally, *Tribolium* embryos contain three anterior head (ocular, antennal, and intercalary), three gnathal, three thoracic, and ten abdominal segments. All segments except the ocular and antennal are separated by morphologically demarcated boundaries that extend along the entire DV ectodermal axis of the embryos. *wg* is expressed in a stripe in the anterior of each segment except the ocular from early in embryogenesis (expression of most stripes is initiated during germ band elongation) through the latest embryonic stages investigated (Figs. 1A, B). *wg* expression abuts *en* expression in the posterior compartment, and segmental boundaries form immediately posterior to the *en* domain (Nagy and Carroll, 1994). The *wg* stripes initially extend from the mesoderm boundary along the ventral midline to the epithelium bordering the dorsal edge of the embryo, leaving the dorsal most row of cells without *wg* expression; subsequently *wg* is downregulated laterally, leaving two discrete expression domains: a ventral region extending to the tip of the developing limb primordia in gnathal and thoracic segments and to the edge of the sternal region in abdominal segments, and a dorsal patch bordering the dorsal epithelium (Fig. 1A). The ventral expression persists throughout the stages we have examined, while *wg* expression in the dorsal and lateral regions is more dynamic (see below).

In 85% of *Tcwg*^{RNAi} embryos, segmental boundaries were disrupted (Figs. 2D–I). In most of these, segmentation was more strongly disrupted ventrally than dorsally, resulting in a lack of visible segmental boundaries ventrally. In less severely affected embryos, most segmental boundaries formed dorsally, but the full complement of segmental boundaries was usually absent and segmental boundaries that formed were not always symmetrical (Figs. 3A, D). In more extreme phenotypes, both ventral and dorsal segmental boundaries failed to form (Fig. 2E). The most severely affected embryos (Fig. 2F; 14% of *Tcwg*^{RNAi} embryos) were dwarfish with greatly shortened AP axes and few signs of segmentation externally.

Appendage expression and defects: In *Tribolium*, *wg* is expressed in a stripe along the ventral side of the developing antennal, gnathal and thoracic limb primordia. In all appendages except the mandible, this expression in the main limb axis persists throughout the developmental stages examined (Jockusch et al., 2004; Nagy and Carroll, 1994). In the mandible, *wg* is downregulated distally. In both the antennae and the labrum, *wg* expression is initiated relatively late in development, and is not continuous with a segmental stripe in the ventral ectoderm (Nagy and Carroll, 1994). In the labrum, two wedges of *wg* are present, one on each side; they are located about midway along the PD axis, and are relatively lateral (Fig. 1A). There is no expression along the presumptive ventral sides of the labral primordia which are fused along most of their length.

In 76% of *Tcwg*^{RNAi} embryos, there was no visible outgrowth of gnathal or thoracic limbs (Figs. 2C–F). In 3% of *Tcwg*^{RNAi} embryos, gnathal and thoracic limbs were present but malformed (Fig. 2I). Abnormal limbs were stunted or thin. Surprisingly, however, the antennae and labrum were present in all embryos, and underwent extensive outgrowth. Limb defects do not appear to be merely a downstream effect of segmentation defects, since in 10% of *Tcwg*^{RNAi} embryos, all gnathal and thoracic segments were formed, but limbs were still absent (Fig. 2C).

Other expression domains and defects: *wg* is expressed dynamically in a wide range of other structures, including the

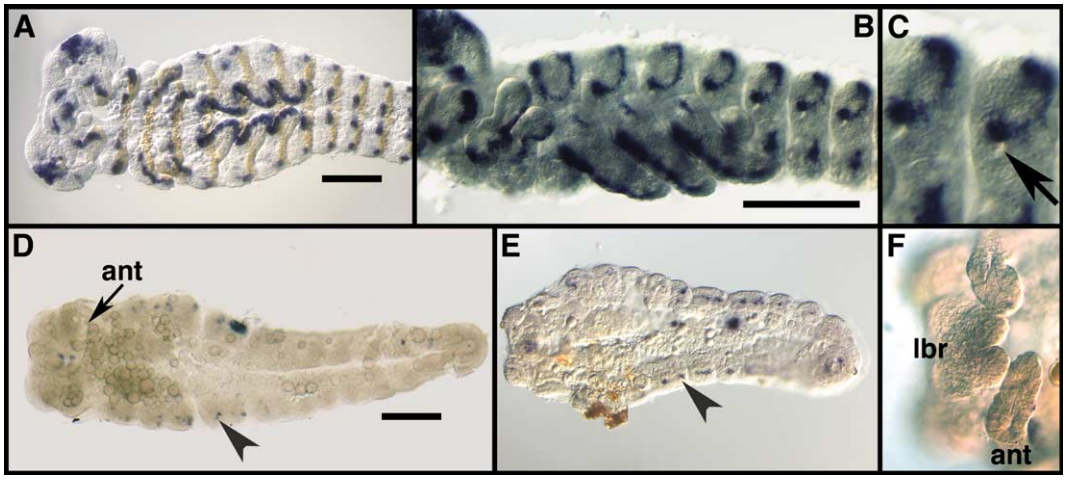

Fig. 1. *wg* expression in *T. castaneum* embryos. Unless indicated otherwise, embryos are oriented with anterior to the left and ventral side up in all figures. ant = antenna and lbr = labrum. Panels A–C show control embryos; panels D–F show *Tcwg*^{RNAi} embryos. (A) Embryo at 25% embryogenesis stained to show expression of *wg* (blue) and EN (brown). (B) Lateral view of control embryo at 40% embryogenesis. (C) Magnification of panel D showing curved stripes of *wg* expression in the dorsal region of abdominal segments 2–3, which ends at the spiracle (arrow). (D, E) *wg* expression in *Tcwg*^{RNAi} embryos showing downregulation of *wg*. The embryos are severely deformed; arrow indicates antenna in panel D and arrowheads indicate remnants of *wg* expression in dorsal region of embryo in panels D and E. (F) Close-up of head of panel E to show that labrum and antennae develop without *wg* expression. Scale bars indicate 100 μm.

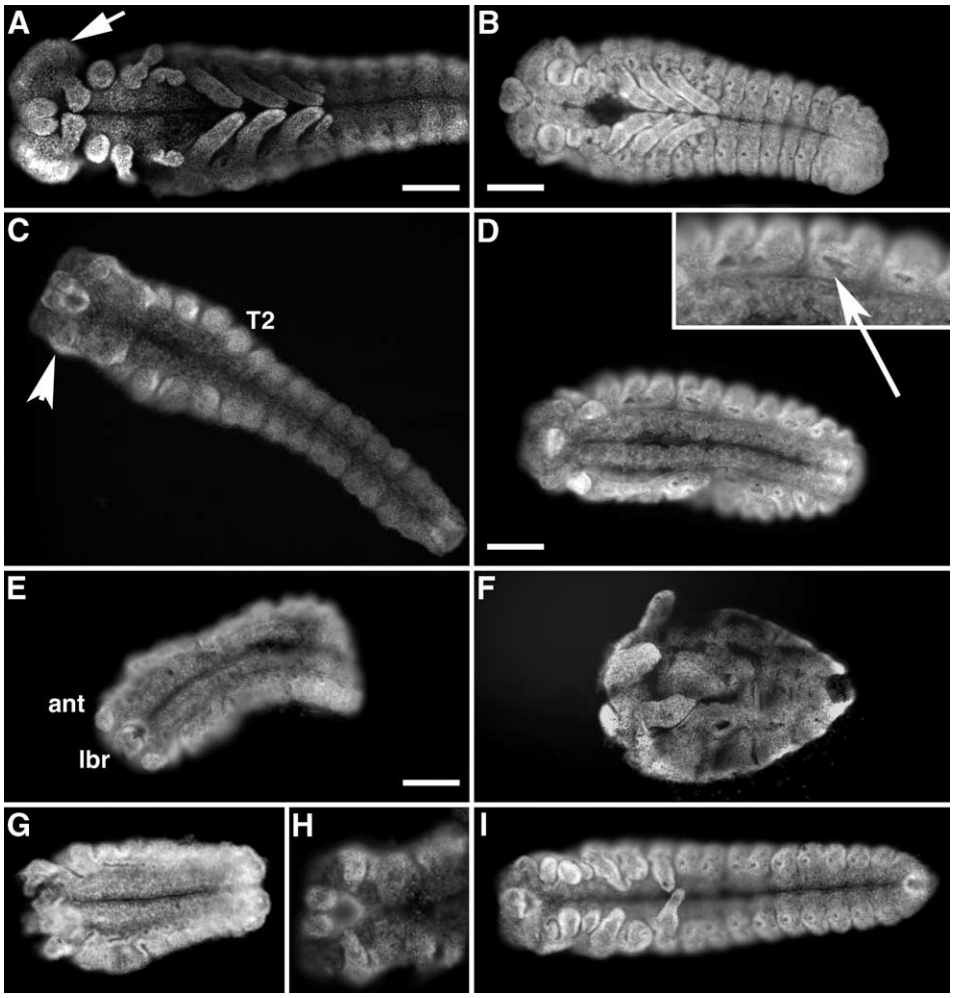

Fig. 2. *Tcwg*^{RNAi} phenotypes. All embryos are DAPI stained to show nuclei. Panels A and B show control embryos; panels C–I show *Tcwg*^{RNAi} embryos. ant = antenna; lbr = labrum; T2 = 2nd thoracic segment. (A) Control embryo at 40% development; arrow indicates the lateral head lobe. (B) Control embryo at 50% development; note the round morphology of the spiracles. (C) *Tcwg*^{RNAi} embryo at approximately 40% embryogenesis. The full complement of segments is present, as are the antennae and labrum, but gnathal and thoracic limbs are absent. Arrowhead indicates reduced lateral head lobes. (D) *Tcwg*^{RNAi} embryo at approximately 50% development. Segmentation is severely disrupted, especially in the ventral region, and some spiracles are fused (arrow in panel D inset). (E) *Tcwg*^{RNAi} embryo in which segment boundary development was severely disrupted dorsally as well as ventrally. (F) *Tcwg*^{RNAi} embryo showing dwarf phenotype. Note the well-developed antennae and labrum. Some *wg* expression from in situ hybridization is apparent. (G) *Tcwg*^{RNAi} embryo with severe disruption of segment boundaries and extensive fusion of spiracles in the presumptive thorax. (H) Close-up of head region of a severely deformed embryo showing development of labrum and antennae. (I) *Tcwg*^{RNAi} embryo in which all limbs were present but malformed. Some limbs appear truncated distally while others have reduced distal structures. Scale bars indicate 100 μm.

gut, lateral head lobes and spiracles; those we examined all showed phenotypic alterations in *Tcwg*^{RNAi} embryos. *wg* is strongly expressed in several regions of the lateral head lobes (Friedrich and Benzer, 2000; Liu and Friedrich, 2004), which were greatly reduced in all phenotypically abnormal embryos (Figs. 2C, E, I); a portion of this region ultimately gives rise to both larval and adult eyes, but we scored most of our embryos before eye formation would normally occur.

After approximately 35% development, additional modulation of the ectodermal *wg* expression occurs. Expression is initiated in an anterolateral domain; following formation of the spiracles, this domain lies on the anterodorsal side of each spiracle (Fig. 1C). The dorsal patch also expands, arcing around through the EN domain to join the anterolateral domain (Figs. 1C, 3C). Spiracles were either absent or laterally fused (Figs. 2D, G) in 80% of older *Tcwg*^{RNAi} embryos.

Gene expression in Tcwg^{RNAi} *embryos*

The effectiveness of *wg* RNAi at downregulating *wg* expression was confirmed by the near complete loss of *wg* expression in *Tcwg*^{RNAi} shown using in situ hybridization; small patches of low level *wg* expression were detected in some embryos after very long development times (Figs. 1D, E). To investigate the conservation of regulatory relationships between *wg* and some of its targets in *Drosophila*, we examined the expression of EN, *dpp* and *Dll* in *Tcwg*^{RNAi} embryos. To investigate the ontogeny of segment defects, we also examined expression of the pair-rule protein EVE.

Because of the segmentation defects, we characterized EN expression in a subset of *Tcwg*^{RNAi} embryos. Normally in *Tribolium*, EN is expressed in a stripe at the posterior of each segment that extends along the complete DV axis of the embryo,

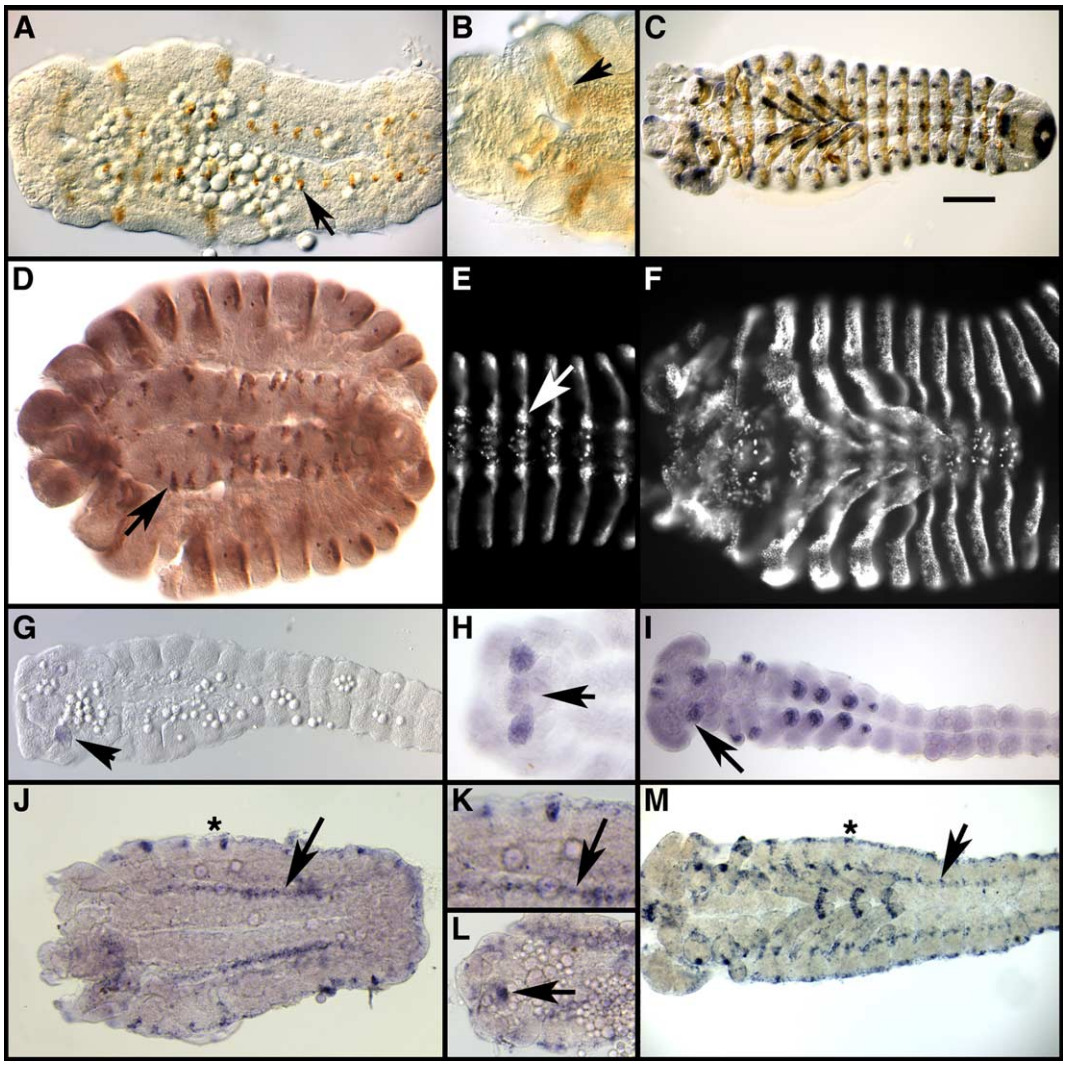

Fig. 3. Comparison of gene expression in normal and *Tcwg*^{RNAi} embryos. (A) Severely affected *Tcwg*^{RNAi} embryo stained with an antibody for EN. EN expression is greatly reduced or absent at the segment boundaries. EN is still expressed in a segmentally repeated pattern in the central nervous system (arrow). (B) *Tcwg*^{RNAi} embryo showing normal EN expression in the posterior region of the antennae (arrow). (C) Normal embryo (45% development) double-labeled for *wg* RNA (blue) and EN protein (brown). At this stage, *wg* and EN are co-expressed dorsally in part of the posterior of the segment. (D) Severely affected *Tcwg*^{RNAi} embryo stained for EN. Note the close correlation between EN expression and segment boundary formation dorsally. (E) Normal embryo stained for EN, focused on nervous system expression (arrow), which largely underlies the segmental stripe expression at this stage. (F) Older normal embryo stained for EN. (G) *Dll* expression in a *Tcwg*^{RNAi} embryo is absent in the gnathal region and thorax, but persists in the anterior head (arrow). (H) *Tcwg*^{RNAi} embryo (without DIC imaging) showing *Dll* expression in the antennae and labrum (arrow). (I) Normal *Dll* expression at ca. 30% embryogenesis. An arrow points to the antenna. (J) *dpp* expression in a *Tcwg*^{RNAi} embryo. Note the absence of a gut ring. The asterisk indicates the dorsal stripe of *dpp* expression, which appears patchier in *Tcwg*^{RNAi} embryos, while the arrow indicates the more medial stripe, which is more robust in *Tcwg*^{RNAi} embryos. (K) Close-up of J showing the medial stripe of *dpp* (arrow). (L) *Tcwg*^{RNAi} embryo showing *dpp* expression in the labrum (without DIC imaging). (M) *dpp* expression in a control embryo, labeled as in panel J. Scale bar indicates 100 μm.

and in underlying patches of cells in the nervous system (Brown et al., 1994; Nagy and Carroll, 1994; Figs. 3C, E, F). In *Drosophila*, *wg* is required for maintenance of EN expression in most segments (Bejsovec and Martinez Arias, 1991). Alterations in EN expression in *Tcwg*^{RNAi} embryos showed a close correlation with the disruptions in segment boundary formation. In the dorsal ectoderm, where some segment boundaries persisted, EN expression was found at the posterior of many morphologically visible segments, and in these segments, there was a good correlation between the DV extent of morphological segment boundaries and the DV extent of EN expression (Figs. 3A, D). Furthermore, in ventral ectodermal regions where segment boundaries were absent, EN expression was absent or highly disrupted at the developmental stages examined. However, even in embryos in which segment boundaries were severely disrupted, EN expression persists in a segmentally repeating pattern in a cluster of cells in the central nervous system (Fig. 3A). In mid-stage embryos, we observed 15–16 of these clusters, suggesting that all segments had been generated. In addition, EN was expressed in the posterior region of the antennae as in normal embryos (Fig. 3B), indicating that *wg* is not needed for EN expression in this region. Unlike in *Drosophila wg* mutants, gnathal expression of EN resembled its expression in the trunk of *Tcwg*^{RNAi} embryos.

Normally in *Tribolium*, EVE is expressed in stripes during segmentation but in a more spatially dynamic pattern than in

Drosophila. Pair-rule stripes are delineated sequentially from the posterior domain of EVE expression; subsequently, each broad pair-rule stripe is transformed into two segmental stripes by downregulation of EVE in the center of the stripe (Patel et al., 1994). In *Tcwg*^{RNAi} embryos, EVE is expressed in the same pattern (Fig. 4) as in control embryos, suggesting that downregulation of *wg* does not affect the generation of segments. Although the ds RNA injected embryos stained for EVE appeared normal, 80% of their siblings that were allowed to continue developing had defects in segment boundary, limb and head lobe development, indicating that the embryos examined are unlikely to have remained phenotypically normal later in ontogeny.

In *Tribolium*, as in *Drosophila*, *Dll* is normally expressed in the distal tip of all of the developing appendages except the mandibles (Beermann et al., 2001; Fig. 3I). In embryos that lacked gnathal and thoracic appendages, no *Dll* expression was detected in these segments (Fig. 3G). *Dll* expression was, however, detected at the distal tip of the antennae and labrum, in an expression pattern identical to that in control embryos (Fig. 3H).

In *Drosophila*, *wg* and *dpp* interact in a wide variety of contexts, including in limb, gut and eye development (Brook and Cohen, 1996; Domínguez and Hafen, 1997; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Szüts et al., 1998; Takashima and Murakami, 2001; Theisen et al., 1996). We therefore examined the expression of *dpp* in *Tcwg*^{RNAi} embryos. Normally, *dpp* is expressed in a dynamic fashion in the developing limbs (Jockusch et al., 2004; Sanchez-Salazar et al., 1996). This limb domain of *dpp* expression was absent in segments in which limb outgrowth failed. By contrast, *dpp* expression was normal in the developing antennae and labrum.

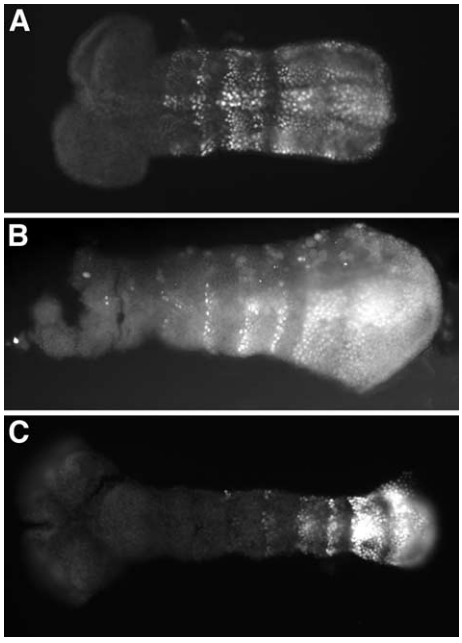

Fig. 4. EVE expression in *Tcwg*^{RNAi} embryos. (A) *Tcwg*^{RNAi} embryo in which EVE pair-rule stripes 1 and 2 are split into secondary segmental stripes. (B) A slightly older *Tcwg*^{RNAi} embryo showing downregulation of EVE anteriorly. (C) An older *Tcwg*^{RNAi} embryo showing continued delineation of EVE stripes from the posterior growth zone during germ band extension.

The ring of *dpp* expression in the hindgut was missing in *Tcwg*^{RNAi} embryos (Fig. 3J). We also noted differences in the body wall expression of *dpp* in *Tcwg*^{RNAi} embryos. Normally *dpp* is expressed in a longitudinal stripe in the dorsalmost cells of the body (Fig. 3M). Later in *Tribolium* embryogenesis, patchy expression of *dpp* occurs more medially (see below for a more complete description of normal *dpp* expression) (Fig. 3M). In *Tcwg*^{RNAi} embryos, the dorsalmost *dpp* stripe appeared more patchy than normal whereas the lateral stripe appeared more robust and less patchy, especially in the abdomen (Figs. 3J, K).

Phenotypes of Tcdpp^{RNAi} embryos

In addition to the dorsal edge and lateral longitudinal stripes of *dpp* described above, *dpp* is expressed dynamically in the limbs, palps and antennae, first at the appendage tip, and then in a subterminal ring and in a proximodorsal domain (Giorgianni and Patel, 2004; Jockusch and Ober, 2004; Jockusch et al., 2004). In the labrum, *dpp* expression is initiated in two crescent-shaped domains as the labrum begins to grow out from the body wall. Expression persists in a distal region of the developing labrum throughout the developmental stages we have examined here. The lateral longitudinal stripe of *dpp* includes the intersegmental patches described by Giorgianni and Patel (2004).

The phenotypes of *dpp* injected embryos were less penetrant and more subtle than those of *Tcwg*^{RNAi} embryos. 56% of embryos (84 of 151) appeared phenotypically normal. The remaining 44% (referred to as *Tcdpp*^{RNAi} embryos) were more slender than control embryos (a significant difference; $P < 0.001$), particularly in the thorax, which is substantially broader than the abdomen in control germ-band extending embryos but only slightly broader in *Tcdpp*^{RNAi} embryos (Fig. 5). These embryos appear to be lacking the dorsalmost row of cells. In almost all of these embryos, development of the antennae, gnathal and thoracic limbs is morphologically normal (Fig. 5). In 2% of embryos, which we scored at very late stages of development, legs seemed to be less robust than in normal embryos with possible defects in leg segmentation (Fig. 5E). The labrum also appeared less flared laterally in a small percentage of older embryos (Fig. 5G). The frequency of embryos scored as abnormal was much greater at early developmental stages (68% of embryos between 30 and 50% embryogenesis) than at later stages (32% of embryos examined around 70% embryogenesis). This may reflect an ability of the embryo to regulate development in the absence of the dorsalmost cell row, or our inability to detect a relatively small phenotypic difference at later stages. At the latest stages, we examined, 85% of embryos did not complete dorsal closure (compare Figs. 5F and H). The dorsalmost edge cells appeared ragged and did not meet to form a continuous dorsal epidermis.

Gene expression in Tcdpp^{RNAi} embryos

The effectiveness of ds *dpp* RNA injections in downregulating *dpp* mRNA expression was confirmed by examining expression of *dpp* using in situ hybridization. In phenotypically

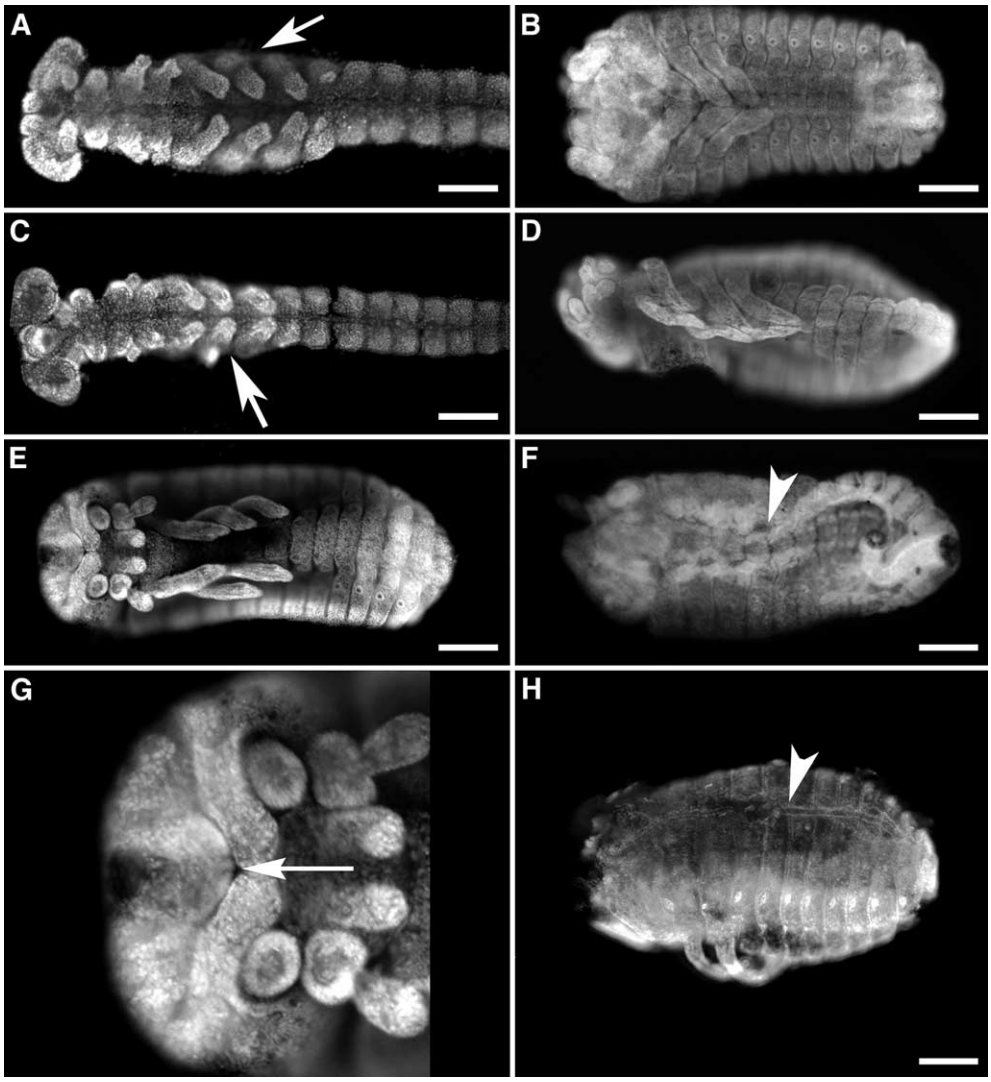

Fig. 5. *Tcdpp*^{RNAi} phenotypes. Panels A, B and H show control embryos; panels C–G show *Tcdpp*^{RNAi} embryos. All embryos are DAPI-stained to show nuclei. (A) Control embryo at 25% embryogenesis, with arrow indicating the dorsal edge. (B) Older control embryo. (C) *Tcdpp*^{RNAi} embryo at ca. 25% embryogenesis. Arrow points to underdeveloped dorsal edge (compare to panel A). (D) older *Tcdpp*^{RNAi} embryo with flimsy legs and abnormal dorsal closure. (E) *Tcdpp*^{RNAi} embryo with abnormal labrum and thoracic limbs. (F) Dorsal view of *Tcdpp*^{RNAi} embryo showing defects in dorsal closure (arrowhead). (G) Close-up of E showing abnormal labrum (arrow). (H) Dorsal view of control older embryo to show completion of dorsal closure (arrowhead).

abnormal embryos, *dpp* expression was never detectable along the dorsal edge of the body or in the lateral longitudinal stripe (Figs. 6F, G) and was substantially downregulated in the limbs, but a low level of expression in the limb tips was detected in some (56% of $N = 27$) embryos we examined.

The expression of *wg* and *Dll* was also examined in *Tcdpp*^{RNAi} embryos. If *Drosophila* interactions are conserved, we would predict that loss of *dpp* signaling during limb allocation would lead to the expansion of the *Dll* domain, while loss of *dpp* later in limb development would lead to the loss of *Dll* expression. *Dll* expression was indistinguishable in control and *Tcdpp*^{RNAi} embryos (Fig. 6D), suggesting that *dpp* is not needed for *Dll* expression in *T. castaneum*. Because *wg* and *dpp* repress each other during leg imaginal disc patterning, loss of *dpp* would be predicted to lead to expansion of the *wg* domain in the limb if gene interactions are conserved between *Drosophila* and *Tribolium*. However, the expression of *wg* in the appendages of *Tcdpp*^{RNAi} embryos was indistinguishable from its expression in appendages of control embryos (Fig. 6E). We did observe one difference in *wg* expression between *Tcdpp*^{RNAi} and control embryos. In control embryos, the dorsal patch of *wg* in each segment does not reach the dorsal edge of the body wall (Fig. 6J), whereas in the *Tcdpp*^{RNAi} embryos, it does (Fig. 6I). Comparison of the dorsal *wg* patch in control and *Tcdpp*^{RNAi} embryos suggests that the patch size is the same, and thus that the extension of *wg* to the dorsal edge does not result from the expansion of the *wg* domain in the absence of *dpp* (Fig. 6). The difference in expression is, however, consistent with the absence of the dorsalmost row of cells.

Discussion

The RNAi data support hypotheses based on comparative analyses of gene expression in showing that the embryonic

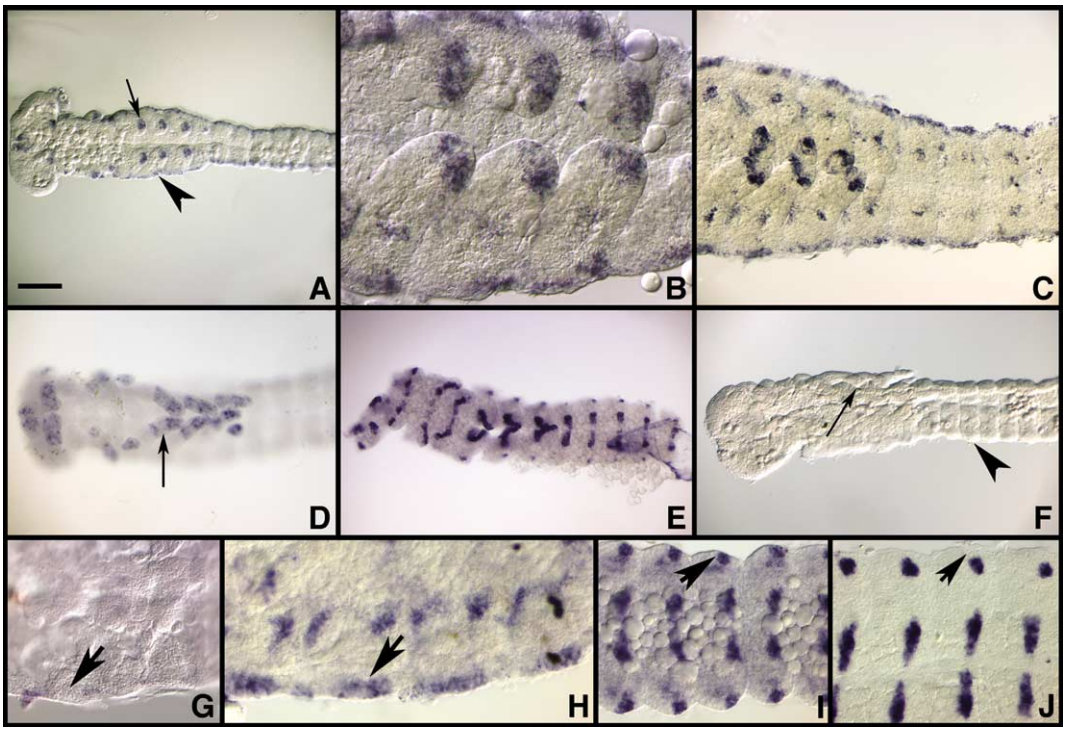

Fig. 6. Comparison of gene expression in normal and *Tcdpp*^{RNAi} embryos. Panels A–C, H, and J show normal embryos. Panels D–G and I show *Tcdpp*^{RNAi} embryos. (A) Normal *dpp* expression at ca. 25% embryogenesis. Arrowhead indicates *dpp* expression in dorsal most cells; arrow shows *dpp* expression in limb tips. (B) Close-up of embryo at ca. 25% embryogenesis showing limb tip and dorsal stripe expression. (C) Normal *dpp* expression at ca. 45% embryogenesis. *dpp* forms a distal ring in legs, and a patchy lateral stripe has appeared. (D) *Dll* is expressed normally in distal limbs in *Tcdpp*^{RNAi} embryos. An arrow separates the typical "sock and ring" *Dll* domains in *Tribolium* legs. (E) *wg* expression domains in a *Tcdpp*^{RNAi} embryo. Expression appears normal (compare to Fig. 1A) except that the dorsal patches of *wg* extend to the very dorsal edge of the embryo. (F) *Tcdpp*^{RNAi} embryo showing that *dpp* expression is not detected in the dorsal cells (arrowhead) or limbs (arrow). (G) Close-up of dorsal edge cells in a *Tcdpp*^{RNAi} embryo stained for *dpp* RNA showing reduced *dpp* expression and lack of shape differentiation between dorsal most cells and more lateral ones (arrow; compare to panel H). (H) Close-up of normal embryo to show dorsal most row of cells stained for *dpp* (arrow). (I) Abdomen of *Tcdpp*^{RNAi} embryo stained for *wg* expression. The dorsal patch of *wg* extends to the dorsal edge of the embryo (arrow). (J) Abdomen of normal embryo at a similar stage to panel I. The dorsal most cell row lacks *wg* expression (arrow).

functions of *dpp* are relatively divergent while the embryonic functions of *wg* are relatively conserved in *T. castaneum* and *D. melanogaster*, two species of holometabolous insects with contrasting modes of segment formation and limb development (Jockusch and Ober, 2004; Jockusch et al., 2000; Nagy and Carroll, 1994). The data point to a requirement for *dpp* in patterning the dorsalmost body wall, rather than a more global role in DV patterning in *Tribolium*. Furthermore, the RNAi data do not identify any essential role for *dpp* in appendage development in *Tribolium*, and suggest that interactions between *dpp*, *wg* and *Dll* differ between the two species. By contrast, the phenotypes of the *Tcwg*^{RNAi} embryos show a close correspondence to both the expression domains of *wg* during normal embryogenesis in *Tribolium* and the phenotypes observed in *Drosophila* hypomorphs and null mutants, suggesting that a wide array of *wg* functions are conserved between these two holometabolous insects, including segment boundary formation, regulation of *en*, limb allocation, head development, and spiracle development. After briefly discussing the implications of the *dpp* data below, we focus on comparative analysis of two major conserved functions of *wg*: appendage allocation and segment boundary formation. While the latter function is an evolutionarily ancient function of WNT signaling (Angelini and Kaufman, 2005a; Miyawaki et al., 2004), a role for *wg* in appendage allocation is inferred to have originated in the common ancestor of holometabolous insects.

Functions of dpp in Tribolium embryogenesis

Two lines of evidence suggest that *dpp* is required for development of the dorsalmost body wall epithelium in *T. castaneum*, where *dpp* is expressed in a longitudinal stripe one cell wide from before germ band extension through dorsal closure. First, *Tcdpp*^{RNAi} embryos are narrower than control embryos and the cells present at the dorsal edge lack the distinctive morphology of dorsal edge cells in control embryos. Second, in *Tcdpp*^{RNAi} embryos, the dorsal *wg* patch extends to the dorsalmost edge of the embryo rather than stopping one cell width in from the dorsal edge. These data suggest that *dpp* is required for one specific aspect of DV axis patterning in *T. castaneum*. By contrast, in *Drosophila*, *dpp* plays a role in organizing broad domains along the DV axis (Ashe et al., 2000; Ferguson and Anderson, 1992; Irish and Gelbart, 1987; Wharton et al., 1993) and induces other dorsal tissues (Frasch, 1995). The divergence in *dpp* function parallels the divergence in blastoderm stage expression in these two species. In *Tribolium*, blastoderm stage expression of *dpp* is restricted to an anterior domain that will form extra-embryonic membranes;

the earliest embryonic expression is restricted to the dorsalmost row of cells (Chen et al., 2000; Sanchez-Salazar et al., 1996). In the *Drosophila* blastoderm, the broad dorsal domain of *dpp* encompasses both the presumptive extraembryonic membranes and a more lateral region that will give rise to all ectoderm dorsal to the neurogenic ectoderm (Ashe et al., 2000; Ferguson and Anderson, 1992; Irish and Gelbart, 1987; Wharton et al., 1993). Other investigations of homologues of *Drosophila* DV patterning components in *Tribolium* have suggested that DV patterning uses some of the same genes as in *Drosophila*, but in different ways (Chen et al., 2000; Maxton-Küchenmeister et al., 1999). Together, these data suggest that a more global reorganization of DV patterning may have occurred in the lineage leading to *Tribolium* or *Drosophila*. Further work is needed to determine whether any other BMP family member, such as *glass bottom boat* (*gbb*), is more broadly involved in DV patterning in *Tribolium*, as functional shifts between paralogues are relatively common in evolution.

We also find no evidence for a conserved role of *dpp* in early limb development. In *Tcdpp*^{RNAi} embryos, appendages were present, underwent normal outgrowth and expressed *Dll* distally in 100% of embryos examined. In *Drosophila*, at least four distinct roles for *dpp* in limb development have been identified. Its earliest known role is in suppressing *Dll* expression dorsally, thereby helping to restrict imaginal disc identity to a ventrolateral region (Goto and Hayashi, 1997). Subsequently in embryogenesis, it is required for the specification of proximal limb fate (Kubota et al., 2000). During imaginal disc patterning, *dpp* is required for both PD outgrowth and DV patterning (Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit and Cohen, 1997; Theisen et al., 1996). Although many *Tcdpp*^{RNAi} embryos had no evidence of *dpp* expression, but normal appendages, one explanation for the lack of phenotypic effect is that even a very low level is sufficient to activate target genes along the PD appendage axis. However, given that in *Drosophila* imaginal discs *dpp* functions as a morphogen, with differential regulation of target genes depending on graded changes in expression levels (Lecuit and Cohen, 1997, 1998; Lecuit et al., 1996; Nellen et al., 1996), a lack of sensitivity in *Tribolium* would also indicate that some divergence in *dpp* function has occurred.

Lack of conservation of *dpp* function in *Dll* activation had previously been proposed based on the divergence of expression patterns between *Drosophila* and other arthropods in which *dpp* expression has been characterized (Akiyama-Oda and Oda, 2003; Angelini and Kaufman, 2005a; Dearden and Akam, 2001; Jockusch et al., 2000, 2004; Niwa et al., 2000; Prpic, 2004; Prpic et al., 2003). However, Prpic et al. (2003) argued that *dpp* could still be playing a conserved role of cooperative activation of genes along the PD axis because of the differences in tissue morphology between direct developing appendages and imaginal discs. Our data reject this model, as appendage outgrowth and *Dll* expression are normal in *Tcdpp*^{RNAi} embryos.

One possible explanation for a lack of a detectable role for *dpp* in limb development in *Tribolium* is that this function is redundantly served by the *dpp* paralogue *gbb*, which is broadly expressed in the limb primordia in a domain that appears to include the *dpp* domain (Giorgianni and Patel, 2004). The pattern of *gbb* expression closely parallels the expression of pSMAD, a downstream transducer of BMP signaling (Giorgianni and Patel, 2004). In *Drosophila*, *gbb* is also broadly expressed in leg imaginal discs, in a domain largely complementary to the *dpp* domain (Khalsa et al., 1998). It interacts with *dpp* during imaginal disc patterning, and in some contexts, these genes are functionally redundant (Khalsa et al., 1998; Ray and Wharton, 2001). Further work is needed to determine whether any BMP signaling is required for appendage development in species with direct limb development such as *T. castaneum*.

Data on the embryonic functions of *dpp* are available from one other insect species, the hemimetabolous bug *O. fasciatus*. In this species, *dpp* RNAi leads to severe developmental defects brought about by the failure of the early germ band to invaginate (Angelini and Kaufman, 2005a). Because the earliest functions of *dpp* appear to be different in the three species of insects studied to date, additional functional analyses in distantly related species are needed to draw inferences about ancestral *dpp* functions. Functional analyses of *dpp* are also needed in species that are more closely related to *Drosophila* than to *Tribolium* in order to determine whether the changes in *dpp* function in appendage development occurred concomitantly with the evolution of imaginal discs or reorganization of the extraembryonic membranes in higher flies.

The role of wg in appendage development in T. castaneum

The most striking phenotypic abnormality of *Tcwg*^{RNAi} embryos was the complete absence of gnathal and thoracic limbs. The loss of limbs is unlikely to be a downstream effect of altered segmentation as limbs were absent in some embryos in which the proper number of segment boundaries formed. The loss also cannot be attributed to a downstream effect of the loss of *Dll* expression, since strong *Tribolium Dll* mutants retain proximal limbs (Beermann et al., 2001), whereas *Tcwg*^{RNAi} embryos do not. Thus, these RNAi data indicate that *wg* is required for the initiation of limb outgrowth in *Tribolium*.

In *Drosophila*, interference with WG signaling affects appendage development during at least three different stages, leading to questions about which of these stages are conserved in *T. castaneum*. Initially in *Drosophila*, *wg* is required for allocation of appendage primordia (Simcox et al., 1989) and for initiation of *Dll* transcription (Cohen, 1990; Cohen et al., 1993). Because limbs are entirely absent and we do not detect any *Dll* expression in the gnathal and thoracic segments of mutant *Tcwg*^{RNAi} embryos, we conclude that the earliest functions of *wg* in *Tribolium* limb development are identical to its earliest functions in *Drosophila*. Thus, the earliest steps in limb development appear to be the same in two holometabolous insects with contrasting modes of limb development. Because of the complete loss of limbs in *Tcwg*^{RNAi} embryos, we are unable to evaluate the degree of conservation of later roles of *wg* in limb development; the occurrence of a small number of

embryos with truncated limbs suggests that *wg* is also required for distal appendage development in *T. castaneum*.

One interesting aspect of the *Tcwg*^{RNAi} appendage phenotypes is that the antennae and labrum developed in all embryos, including those that lacked the gnathal and thoracic limbs. Antennae are serially homologous to limbs (Snodgrass, 1935; Struhl, 1981). Whether the labrum is also appendicular in origin is debated, but this hypothesis has been suggested (e.g., Haas et al., 2001; Popadić et al., 1998; Prpic et al., 2001). Normal outgrowth and *Dll* expression in anterior appendages of *Tcwg*^{RNAi} embryos likely reflects a lack of requirement for *wg* signaling to initiate appendage outgrowth and *Dll* expression rather than an artifact of RNAi. In *T. castaneum*, *wg* expression generally proceeds along an AP gradient, but expression in the antennae, labrum, and intercalary segment is delayed (Nagy and Carroll, 1994). Expression of *Dll* also proceeds in an AP gradient (Beermann et al., 2001), with the earliest appendage expression in the antennae. Comparison of the stages at which *wg* and *Dll* expression are initiated make it clear that detectable levels of *Dll* expression precede detectable levels of *wg* expression in the labrum. Thus, *wg* is unlikely to be an upstream regulator of *Dll* in the labrum. Delayed expression of *wg* in the labrum is also characteristic of other insects with short and intermediate germ development (e.g., Angelini and Kaufman, 2005a; Dearden and Akam, 2001, Miyawaki et al., 2004). The timing of *wg* expression in the antennae varies greatly across insect species. In *Tribolium* and the orthopteran *Schistocerca gregaria*, the antennal stripe appears after five gnathal and thoracic stripes have appeared (mandible through T2) (Dearden and Akam, 2001; Nagy and Carroll, 1994). By contrast, in both the hemipteran *Oncopeltus* and the orthopteran *Gryllus* the *wg* antennal stripes are the first segmental stripes to form (D. Angelini, personal communication; Miyawaki et al., 2004). While our data support the hypothesis that the development of serially homologous appendages diverges very early in development, further work is needed to characterize the nature of these developmental differences. One other interesting implication of our data is that even though *Dll* is an extremely conserved marker of appendage outgrowth (Abzhanov and Kaufman, 2000; Beermann et al., 2001; Panganiban et al., 1994, 1995; Prpic and Tautz, 2003; Schoppmeier and Damen, 2001; Williams et al., 2002), the regulation of *Dll* differs among appendages within a single species.

In *Drosophila*, the role of *wg* in appendage development varies between larval and adult appendages. Although *Drosophila* larvae are apodous, they have sensory structures that are homologous to the appendages. Development of all larval appendage sense organs requires *Dll* (Cohen et al., 1990). *Dll* expression in and development of posterior, but not anterior (labral, antennal and maxillary), larval sense organs is dependent on *wg*, as shown by the phenotype of *wg* null mutants (Cohen, 1990). By contrast, all imaginal disc primordia are absent in *wg* null mutants, including the eye-antennal discs (Simcox et al., 1989). Thus, the presence of appendage outgrowths anteriorly *in Tcwg*^{RNAi} embryos suggests that anterior appendage development may occur by mechanisms used in the development of larval, rather than adult, appendages of *Drosophila*. To test this hypothesis, the function of homologues of head gap genes, which regulate *Dll* anteriorly in *Drosophila* (Cohen and Jürgens, 1990), should be investigated in *T. castaneum*.

The role of wg in development along the AP axis of T. castaneum

The *Tcwg*^{RNAi} embryos demonstrate a key role for *wg* in AP axis development in *Tribolium*. In the most severely affected embryos, the AP axis was greatly shortened, producing embryos reminiscent of the dwarfish phenotype of *Drosophila wg* null mutants (Baker, 1988; Perrimon and Mahowald, 1987). In most embryos, axis elongation appeared relatively normal, but the development of segment boundaries was disrupted. Examination of EVE expression showed that the early stages of segmentation were normal in *Tcwg*^{RNAi} embryos. Additional evidence that all segments are formed comes from the segmentally repeated expression of EN in the nervous system of *Tcwg*^{RNAi} embryos, an expression pattern that persists in the absence of *wg*. The changes in segment boundary formation were tightly correlated with alterations in EN expression (Fig. 3). In *Drosophila*, segment boundaries form immediately posterior to the *en* expression domain. Expression of both *wg* and *en* is required for boundary formation (Martinez-Arias et al., 1988; Nüsslein-Vollhard and Wieschaus, 1980) and *wg* and *en* maintain each other's expression in the trunk (Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991). Our data suggest that these embryonic functions of *wg* are also found in *Tribolium*. A conserved pattern of interactions between *wg* and *en* was previously suggested based on data from viral-mediated misexpression of *Drosophila wg* in *Tribolium* (Oppenheimer et al., 1999).

In *Drosophila*, the interactions between *wg* and *en* vary in the anterior head and gnathal segments (Gallitano-Mendel and Finkelstein, 1997), with loss of *wg* leading to expansion of *en* in the antennal and intercalary segments, to downregulation dorsally and ventrally, but not ventrolaterally, in the mandibular segment, and to persistence of *en* stripes (with reduced expression ventrally) in the maxillary and labial segments. Our data point to a difference between the antennal segment, where EN expression was always detected despite the downregulation of *wg*, and other segments. EN expression in the gnathal region of *Tcwg*^{RNAi} embryos paralleled its expression in the trunk and underwent extensive downregulation, suggesting that the interactions between these segment polarity genes vary less along the AP axis of the body in *Tribolium* than they do in *Drosophila*.

Our data also suggest that the role of *wg* in segment boundary formation varies along the DV axis of the embryo. Most *Tcwg*^{RNAi} embryos showed no morphological signs of segment boundaries ventrally, but many had developed some segment boundaries more dorsally (although usually a complete complement of segment boundaries was not present; Fig. 2D).

In more severe phenotypes, segment boundaries were disrupted along the entire DV axis (Fig. 2F). These data indicate that ventral regions are more sensitive than dorsal regions to downregulation of *wg*. This difference in sensitivity corresponds with DV differences in *wg* expression. *wg* expression initially extends along almost the entire DV axis of each segment (Nagy and Carroll, 1994). It persists ventrally throughout embryogenesis, but is downregulated in a dorsolateral domain after the completion of germ band expression, leaving only a small patch of *wg* dorsally (Fig. 1A). The differential sensitivity of segment boundaries also corresponds with DV differences in the effects of *wg* downregulation on EN expression. In control embryos, EN stripes are similar along the DV axis of each segment, indicating that WG signaling can only be required transiently in the dorsolateral region. In *Tcwg*^{RNAi} embryos, EN expression is highly disrupted or absent in the ventral ectoderm, where segment boundaries are lost, but generally present in partial stripes dorsally where segment boundaries form.

Differential interactions between *en* and *wg* along the DV axis also occur in the embryonic ectoderm of *Drosophila*. Using a temperature sensitive allele that produces phenocopies of null mutants at restrictive temperatures, Bejsovec and Martinez Arias (1991) showed that laterally in the trunk, *en* expression is only dependent on *wg* expression during a brief early phase of development, dorsal *en* expression retains dependence on *wg* expression slightly longer, and ventral *en* expression remains dependent on *wg* expression the longest.

Interactions between wg and dpp in T. castaneum

The alterations in *dpp* expression in *Tcwg*^{RNAi} embryos indicate that WG signaling regulates some aspects of the expression of *dpp* either directly or indirectly in *T. castaneum*. The increased patchiness of the dorsal *dpp* stripe suggests that *wg* normally activates *dpp* in a portion of this domain. By contrast, decreased patchiness of the more lateral expression domains when *wg* expression is reduced suggests that *wg* downregulates *dpp* in lateral portions of each segment during normal development. Because *wg* expression appeared normal in *Tcdpp*^{RNAi} embryos, these relationships do not appear to involve mutual interactions.

Neither of these body wall interactions would have been predicted based on data from *Drosophila*, in which no direct interactions between the WG and DPP signaling pathways have been documented during embryonic body wall development. *dpp* expression in *Drosophila* embryos mutant for the WNT signal transducer *arm* is indistinguishable from wildtype expression (Kubota et al., 2003). Alterations in *dpp* signaling are, however, expected to have an indirect effect on *wg* expression in the embryonic body wall of *Drosophila*. Downregulation of *wg* in a lateral domain depends on expression of the T-box gene *Dorsocross* (*Doc*) in segmentally reiterated lateral patches, which appear shortly before the downregulation of *wg* in this region. This segmental expression of *Doc* in turn depends on the expression of *dpp* (Hamaguchi et al., 2004; Reim et al., 2003). Thus, in *Drosophila*, the dorsal extent of the ventral *wg* stripes is indirectly determined by the activity of *dpp* (Reim et al., 2003). If similar interactions were present in *Tribolium*, we would predict that loss of *dpp* signaling would lead to persistence of continuous *wg* stripes; however, downregulation of *wg* in the lateral portion of the segmental stripes occurs normally in *Tcdpp*^{RNAi} embryos. *wg* and *dpp* also interact in leg imaginal disc patterning in *Drosophila. wg* and *dpp* mutually repress each other in the leg disc, predicting that a loss of *dpp* would lead to an increase of *wg* in the legs. Our data from *Tcdpp*^{RNAi} embryos do not support the presence of such an interaction between *wg* and *dpp* during limb development in *T. castaneum*.

Our data also suggest that *wg* is required for *dpp* expression in the hindgut in *T. castaneum*. *dpp* expression in the hindgut ring was abolished in *Tcwg*^{RNAi} embryos. In *Drosophila wg* mutants, *dpp* expression in the hindgut is also absent (Takashima and Murakami, 2001). Because the *wg* and *dpp* domains are not adjacent, this interaction is thought to be indirect (Takashima and Murakami, 2001). Together, these data suggest that the interaction between *wg* and *dpp* during gut development is evolutionarily conserved.

Evolution of wg functions

Data on embryonic functions of *wg* are now available from representatives of three distantly related insect orders: *O. fasciatus* in the Hemiptera, *T. castaneum* in the Coleoptera and *D. melanogaster* in the Diptera (Angelini and Kaufman, 2005a; Baker, 1988; Cohen, 1990; Cohen et al., 1993; Oppenheimer et al., 1999; Simcox et al., 1989). Additionally, the effects of downregulation of downstream components of the WNT signaling pathway have been studied using *pangolin* (*pan*) RNAi in *O. fasciatus* (Angelini and Kaufman, 2005a) and *armadillo* (*arm*) RNAi in the orthopteran *Gryllus bimaculatus* (Miyawaki et al., 2004). Because Coleoptera and Diptera span the basal split in holometabolous insects (Kristensen, 1991), *wg* functions shared by these two lineages are inferred to be ancestral for the group. Hemiptera are a member of the sister group of holometabolous insects, while the Orthoptera are equally distantly related to the other three orders. Thus, this taxon sampling, although limited, allows us to begin to make inferences about ancestral functions of *wg* and WNT signaling and to identify subsequent evolutionary changes in those functions.

The comparative data identify two major functions of *wg* that are shared by at least two lineages: formation of segmental boundaries and outgrowth of appendages. Decreased expression of *wg* leads to loss of segmental boundaries in all three lineages for which data are available, suggesting that this is an ancestral function of *wg*. In addition, segmental boundaries are disrupted by *arm* RNAi in *G. bimaculatus*, indicating that it is also a function of WNT signaling in this species. Generation of posterior segments requires WNT signaling in *G. bimaculatus* (Miyawaki et al., 2004) and *O. fasciatus* (Angelini and Kaufman, 2005a), but is not disrupted by *wg* downregulation in *O. fasciatus*, *T. castaneum* or *D. melanogaster*. Because of the relationships of these species, we conclude that generation

of segments posterior to T2, but not of more anterior segments, is also an ancestral function of WNT-signaling, but does not require *wg*. These data thus suggest that ancestrally, regulation of segmentation differed in the anterior, where segments are already present in the embryonic germ Anlage, and in the posterior, where segments form from a posterior growth zone. Additional work is required to determine whether the role of WNT signaling in generating posterior segments is retained in *T. castaneum*. There is other evidence that formation of segments differs in the anterior and posterior regions of *Tribolium*; the *T. castaneum jaws* mutant leads to a failure of segmentation in the posterior, but not in the anterior (Sulston and Anderson, 1996, 1998).

The effects of WNT signaling on limb development have also been examined in *G. bimaculatus* and *O. fasciatus*. The data from *Tribolium* make a good comparison because both *Gryllus* and *Oncopeltus* develop their limbs directly, like *Tribolium* and in contrast to *Drosophila*, but *Tribolium* is more closely related to *Drosophila*. In both *Gryllus* and *Oncopeltus*, appendages developed on all anterior segments that were present, indicating that WNT signaling, and thus also *wg*, are not required for the initiation of appendage outgrowth in these species. By contrast, limbs do not develop in the absence of *wg* in *Tribolium* or *Drosophila* (Cohen et al., 1993; Simcox et al., 1989). These data therefore point to a significant change in the earliest stages of appendage development in the common ancestor of holometabolous insects, a change that is not accompanied by any apparent change in the earliest stages of limb morphogenesis. One implication of these data is that the evolution of imaginal discs does not appear to have involved alterations in the earliest identified stages of appendage development. Rather, downstream changes must have occurred to arrest appendage development following allocation, and then promote invagination rather than outgrowth of the appendage primordia and delay of proliferation until the larval stage in *Drosophila*. Because the segment boundary formation and appendage allocation functions of *wg* use the same expression domain, and the segment boundary formation function is evolutionarily older, we conclude that the segmental stripes of *wg* were co-opted for a role in appendage allocation in the common ancestor of holometabolous insects.

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