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***Tribolium* Wnts: evidence for a larger repertoire in insects with overlapping expression patterns that suggest multiple redundant functions in embryogenesis**

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Abstract

wingless (wg)/Wnt family genes encode secreted glycoproteins that function as signaling molecules in the development of vertebrates as well as invertebrates. In a survey of *Wnt* family genes in the newly sequenced *Tribolium* genome, we found a total of nine *Wnt* genes. In addition to *wg* or *Wnt1*, *Tribolium* contains orthologs of the vertebrate *Wnt5–7* and *9–11* genes. As in *Drosophila*, *Wnt1*, *6* and *10* are clustered in the genome. Comparative genomics indicates that *Wnt9* is also a conserved member of this cluster in several insects for which genome sequence is available. One of the *Tribolium* *Wnt* genes appears to be a member of the *WntA* family, members of which have been identified in *Anopheles* and other invertebrates, but not in *Drosophila* or vertebrates. Careful phylogenetic examination suggests an *Apis* *Wnt* gene, previously identified as a *Wnt4* homolog, is also a member of the *WntA* family. The ninth *Tribolium* *Wnt* gene is related to the diverged *Drosophila* *WntD* gene, both of which phylogenetically group with *Wnt8* genes. Some of the *Tribolium* *Wnt* genes display multiple overlapping expression patterns, suggesting that they may be functionally redundant in segmentation, brain, appendage and hindgut development. In contrast, the unique expression patterns of *Wnt5*, *Wnt7* and *Wnt11* in developing appendages likely indicate novel functions.

Introduction

During embryogenesis, all insects reach a phylotypic stage when all the future segments are present, although there are often species-specific differences in the events leading to this stage. In the long-germ insect *Drosophila melanogaster*, segmentation of the embryo occurs almost simultaneously during the blastoderm stage. In contrast, in short-germ insects such as *Tribolium castaneum*, most segments are added sequentially from the posterior growth zone. These different mechanisms of segmentation might require diverse sets of genes, some of which are likely to be evolutionarily conserved among insects and possibly all arthropods.

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The *Wnt* gene family encodes secreted signalling molecules that control cell fate specification, proliferation, polarity and movements during animal development. Each *Wnt* protein has one or more sites for N-linked glycosylation and up to 24 cysteines, the positions of which are highly conserved. Most of the deduced proteins are about 350 to 380 amino acids in length, with over 100 conserved residues scattered across the entire sequence (Nusse and Varmus 1992).

Phylogenetic comparison between *Wnt* family genes from diverse animal phyla including chordates, echinoderms, insects, cnidarians and bilaterians have identified 12 phylogenetically conserved *Wnt* subfamilies (Prud'homme et al. 2002; Kusserow et al. 2005). In addition, orphan *Wnt* genes have been found in *Drosophila* (Ganguly et al. 2005), nematodes and humans (Kusserow et al. 2005). These studies, combined with the availability of the full sequence of the nematode *Caenorhabditis elegans* and human genomes, plus the insects genomes including that of the fly *Drosophila melanogaster*, the mosquito *Anopheles gambiae* and the honey bee *Apis mellifera*, allow comparison with the set of genes present in other organisms, including the newly sequenced *Tribolium* genome (*The Tribolium genome consortium*).

Within these genomes, *Wnt* genes are generally dispersed, however a cluster of three genes was found to be highly conserved when comparing *Wnt* genes from human and *Drosophila* (Nusse 2001). In *Drosophila*, *wingless* (*wg*), *Wnt6* and *Wnt10* are immediately adjacent to each other and are transcribed in the same orientation, with no recognizable genes between them. On human chromosome 2q35, *Wnt6* and the adjacent gene *Wnt10A*, are transcribed in the same direction, while *Wnt1* (the ortholog of *Drosophila wg*) and *Wnt10B*, immediately adjacent to each other on chromosome 12q13, are transcribed of opposite strands. Since *Wnt10A* and *Wnt10B* are thought to be the result of a recent duplication, these observations suggest that there was a common ancestral cluster of *Wnt* genes containing *Wnt1*, *Wnt6* and *Wnt10* (Nusse 2001, Sullivan et al. 2007).

In insects, the expression pattern and segment polarity function of the canonical *wg* gene are widely conserved, but little is known about other *Wnt* molecules. In *Drosophila*, *wg* gene expression in the presumptive head region and in a caudal ring is detected in the early blastoderm (Baker, 1988). The expression of the *wg* ortholog in *Tribolium* (*Tcwg/Wnt1*) is first seen during the blastoderm stage at the posterior pole of the egg. Shortly thereafter stripes appear in the presumptive head lobes (Nagy and Carroll 1994). Later, *wg* is expressed in both insects in segmental stripes. In *Drosophila*, the stripes are one cell wide and appear simultaneously in response to the activity of pair-rule genes (Baker 1988; Ingham & Hidalgo 1993). In *Tribolium*, these stripes develop in a sequential order, as the germ band elongates (Nagy and Carroll 1994). The first *Tcwg* stripe appears in the presumptive mandibular segment, followed sequentially by stripes in the other gnathal segments, three thoracic segments and ten abdominal segments. Additional expression domains in the developing head appear on an independent schedule (Nagy and Carroll 1994). Although the expression dynamics of *Tcwg* expression differ from *Drosophila*, the timing and position of segmental *wg* stripes are consistent with their regulation by pair-rule genes (Choe et al. 2006). In contrast to this detailed analysis of *Tcwg*, there are no data concerning the extent to which other *Wnt* family genes are conserved in *Tribolium* or how they might be expressed. However, in *Drosophila*, studies on *Wnt* genes have implicated *Wg* signalling in several developmental processes in different life stages (*Wnt5*: Fradkin et al. 1995; *Wnt6*, *10*: Janson et al, 2001; *Wnt7*: Kozopas et al. 1998; *Wnt9*: Cohen et al. 2002; *WntD*: Gordon et al. 2005, Ganguly et al. 2005).

Here we describe nine *Wnt* family genes in the *Tribolium* genome, four of which are located in a highly conserved cluster. We also analyse the expression pattern of the newly identified

Wnt genes in *Tribolium* and discuss possible differences and/or redundancies in gene function among the *Wnt* gene family members.

Materials and Methods

Isolation of *Wnt* genes from *Tribolium castaneum* and *in situ* hybridization

Tribolium Wnt genes were identified by BLAST analysis of the genome. Candidate genes were amplified from cDNA, cloned into TOPO4 vector (Invitrogen) and sequenced to confirm their identity. 400–600-bp fragments were used as template for digoxigenin-labeled RNA probes. *Tribolium* eggs were collected and whole-mount *in situ* hybridization was performed as previously described (Klingler and Gergen 1993; Tautz and Pfeifle 1989; Brown et al. 1997).

Nomenclature

The insect *Wnt* genes are named to match previous nomenclature for vertebrates and other metazoans.

Alignment and phylogenetic analysis of *Wnt* gene sequences

Wnt protein sequences were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>). ClustalW within the MEGA 3.0 software was used to generate a multiple alignment of the protein sequences, which was subsequently manually improved by considering only the conserved regions of the proteins. Neighbour joining analysis in MEGA 3.0 was performed using a bootstrap value of 1000.

Results

Wnt family genes in *Tribolium*

We found a total of nine *Wnt* genes in the *Tribolium* genome. Alignment of the predicted *Tribolium* proteins with other insect and human *Wnt* proteins was used to identify conserved blocks of amino acid sequence for phylogenetic analysis. The neighbour-joining algorithm in the MEGA phylogenetic software package identified *Tribolium* homologs of most of the human *Wnts* (Fig. 1a). In addition to an ortholog encoding *wg* or *Wnt1*, *Tribolium* contains obvious orthologs of vertebrate *Wnt5–7* and *9–11*. Similar to other sequenced insect genomes (Prud'homme et al. 2002), orthologs of *Wnt2*, *3* or *4* were not found in the *Tribolium* genome (Fig. 1).

Among insects with completed genomes examined to date, *Tribolium* appears to contain the largest complement of *Wnt* genes (Fig. 1). Compared to the nine *Wnt* genes in *Tribolium*, seven have been identified in honeybee (*Apis*), seven in *Drosophila* and six in *Anopheles* (Fig. 1b). However, the quality of each genome project may influence the number of *Wnt* genes identified. Orthologs of *wg/Wnt1*, *Wnt5*, *6* and *10* have been identified in all of these insects. A *Wnt11* ortholog is found in *Apis* and *Tribolium*, but not in *Anopheles* or *Drosophila*. Of these insects, *Anopheles* is the only one that appears to be lacking a *Wnt7* ortholog, while *Apis* is the only one that appears to be lacking a *Wnt9* gene.

Drosophila and *Tribolium* each contain an additional *Wnt* gene, that appear to be related to one another (Fig. 1a). The *Drosophila* gene, *WntD* is required for proper development of the mesectoderm (Ganguly et al. 2005; Gordon et al. 2005). In phylogenetic analysis, the length of the *Drosophila WntD* branch suggests that this gene is diverging more rapidly than its *Tribolium* counterpart. In addition, these insect *WntD* genes phylogenetically group in a larger clade with *Wnt8* subfamily genes, consistent with the previous analysis of *Drosophila*

WntD (Ganguly et al. 2005; Gordon et al. 2005) and supporting the hypothesis that these are divergent members of the *Wnt8* subfamily.

The ninth *Tribolium* *Wnt* gene has no obvious *Drosophila* ortholog. Surprisingly, it groups with both *Anopheles WntA* and *Apis Wnt4* (Fig. 1a); two *Wnt* genes that have not been previously grouped together. This group also includes *WntA* genes from polychaete and cnideria (Fig. 1a), suggesting that these three insect *Wnt* genes are most likely *WntA* subfamily members. The presence of *WntA* genes in *Anopheles*, *Apis* and *Tribolium* suggests that this *Wnt* gene family, although not represented in *Drosophila*, is conserved in insects. and perhaps among arthropods. Indeed, a spider *Wnt* gene has been grouped with *WntA* family genes in a previous analysis (Prud'homme et al. 2002). Similar results were obtained using the same multiple sequence alignment in tree puzzle (Strimmer and von Haeseler, 1996), which uses a maximum likelihood method to derive the phylogenetic tree (data not shown).

A conserved *Wnt* gene cluster

Most of the *Tribolium* *Wnt* genes are dispersed throughout the genome. However, four genes, including *wg/Wnt1*, *Wnt6*, *Wnt9* and *Wnt10*, are closely linked on LG 5 (Fig. 2). They are all clustered within a 52 kb region that contains only one other unrelated gene, which encodes CDP diglyceride synthetase. *Wnt9* is located 5' of *Wnt1*, while *Wnt6* and *Wnt10* are located 3' of *Wnt1*. *Wg* and *Wnt10* are transcribed off one strand while *Wnt6* and *Wnt9* are transcribed off the opposite strand (Fig. 2).

A similar *Wnt* gene cluster exists in other insects (Nusse 2001; Dearden et al. 2006). In *Drosophila*, *Wnt9* (initially identified as *DWnt4*, which groups with *Wnt9* family members in phylogenetic analysis (Prud'homme et al. 2002; Fig. 1a), is located 5' of *wg* with a single gene (CG13785) between them. This *Wnt* cluster is contained within 120kb (Fig. 2) on chromosome 2L. The non-*Wnt* genes in the *Drosophila* and *Tribolium* clusters are not related to one another. In *Apis*, *Wnt1*, *Wnt6* and *Wnt10* are also clustered (Dearden et al. 2006) within an 80kb interval (Fig. 2). Here the cluster is composed of only three *Wnt* genes, since *Wnt9* appears to be absent from the *Apis* genome. This *Wnt* gene cluster seems to be highly conserved (Sullivan et al. 2007), thus loss of *Wnt9* may reflect the draft status of the genome sequence rather than a true gene loss. In *Anopheles*, *Wnt1*, 6 and 10 are clustered in a genomic interval spanning 100kb. *Wnt9* is located more distantly 5' of *Wnt1*, such that all four genes are located within 150kb and no other genes have been predicted within this interval (Fig. 2). In *Aedes aegypti*, the gene order is conserved (*Wnt1-Wnt6-Wnt10*), but they do not seem to be clustered as they span an interval of 420 kb (NCBI, *Aedes aegypti* mapviewer).

The orientation of genes within these insect *Wnt* clusters differs (Fig. 2), suggesting that lineage specific reorganisation of the *Wnt* cluster has occurred during evolution. The orientation of *Wnt* genes 1, 6 and 10 are the same in *Tribolium*, *Anopheles* and *Apis*. In *Drosophila*, the orientation of *Wnt6* is reversed relative to the other two genes. The orientation of *Wnt9* relative to the other three genes is identical in all the insects for which *Wnt9* orthologs have been identified. These results suggest an ancestral insect cluster existed in which *Wnt9* and *Wnt6* are transcribed off one strand while *wg/Wnt1* and *Wnt10* are transcribed off the other. Despite some degree of rearrangement, the overall structure of this cluster has been conserved arguing for a functional constraint on *Wnt*-cluster maintenance (Nusse, 2001; Sullivan et al. 2007).

Wnt expression patterns

To analyze the possible function of different *Wnt* genes during embryogenesis, we assayed the expression patterns of the nine *Tribolium* *Wnt* genes by *in situ* hybridization during embryogenesis (Fig. 3 and 4). The early blastoderm stage embryos contain head and tail primordia, as well as the presumptive gnathal and thoracic segments, while the late germband embryos also contain nine abdominal segments, sequentially added from the posterior growth zone. We followed the expression of the newly identified *Wnt* genes through these embryonic stages, comparing them with the expression of *wg* (Figs. 3 and 4).

wg/Wnt1 is expressed in *Tribolium* during embryogenesis at the blastoderm and germband stages (Nagy and Carroll 1994 and Fig. 3a, 4a–c). Initially, *wg* is expressed in a domain at the posterior end of the embryo (Fig. 3a), and then laterally in each head lobe (Nagy and Carroll, 1994). In the germ rudiment, *wg* is expressed at the anterior dorsal rim of the head lobes and posteriorly in a subterminal position that marks the developing hindgut and leaves the posterior pole free of expression (Fig. 4a). During axis elongation, segmental stripes of *wg* expression appear one at a time, anterior to the growth zone (Fig. 4b), and extend ventrally into the appendages as they develop (Fig. 4c). Later, *wg* expression appears in patches along the lateral edge of the embryo in each segment (Fig. 4c), as well as in the labrum and faintly in the stomodeum while the expression domains expand in the developing brain (Fig. 4c).

Similar to *wg*, *Wnt5* is expressed during germband elongation in the lateral head lobes, in segmental stripes and in the posterior growth zone (Fig. 4d, e). *Wnt5* expression also extends ventrally into the developing limbs (Fig. 4f). In contrast to *wg*, the domains of *Wnt5* expression in the head lobes are broader (compare Fig. 4b and e) and the segmental stripes cross the midline (compare Fig. 4e, f with b, c). The domain of *Wnt5* expression in the posterior growth zone is also broader than that of *wg* and includes the posterior tip of the growth zone (compare Fig. 4e with b), but fades after germband elongation (Fig. 4f). Interestingly, *Wnt5* is expressed in the tips of the developing limbs. Both *wg* and *Wnt5* are expressed in the labrum of older embryos (Fig. 4c, f).

As the germband begins to elongate, *Wnt6* is expressed similar to *wg* (albeit much fainter) in lateral domains of the head lobes, segmental stripes, and a subterminal region of the posterior growth zone (Fig. 4g). *Wnt6* is also expressed along the ventral edge of the developing limbs and in segmental patches along the lateral edges of the elongating germband similar to *wg* (Fig. 4g–h). In the abdomen, faint spots of expression in the CNS appear to overlap with *wg* expression (compare 4h with c). Unlike *wg*, *Wnt6* is expressed in the anlagen of the Malpighian tubules (arrowhead in Fig. 4h). During germband retraction, *Wnt6* expression appears in the anlagen of the fore- and hindgut, as well as in the precursors of the tracheal openings (Fig. 4i).

Wnt7 expression is first visible during germband elongation in segmental stripes that resemble *wg* stripes (Fig. 4j). As they develop, these stripes do not cross the ventral midline, similar to *wg*. However, later they extend into the dorsal mesoderm of the developing limbs, where *wg* is not expressed (Fig. 4l). *Wnt7* is expressed in only a few cells of the developing brain (Fig. 4k, l), some of which may be outside the *wg* domain.

Wnt8/D is one of the two other *Wnt* genes expressed in the blastoderm embryo (Fig. 3b), where it is expressed in a small domain at the posterior pole. As the germ rudiment condenses and elongates, *Wnt8/D* expression resolves into spots on either side of the ventral mesoderm in the posterior growth zone that are connected by a faint subterminal u-shaped line of cells (Fig. 4m). During germband elongation, the posterior domains of *wg* and *Wnt8/*

D appear to partially overlap. Unlike *wg*, expression of *Wnt8/D* fades completely after germband elongation (data not shown).

Unlike *wg*, *Wnt9* is not expressed during early stages of embryonic development. In the retracted germband, *Wnt9* marks cells in the fore- and hindgut at the border between the ectodermal and endodermal derivatives of the gut (Fig. 4n–p).

Wnt10 expression also resembles that of *wg* in many aspects. It first appears in a single stripe that marks the mandibular segment (Fig. 4q). During germband elongation, the segmental stripes of *Wnt10* do not cross the ventral midline, but ventrally extend into the developing appendages (Fig. 4r, s). Unlike *wg*, these stripes are not uniform but appear as dots. Three prominent neurons in each hemisphere of the brain that express *Wnt10* may also fall within the *wg* domains (Fig. 4r).

Wnt11 expression domains have little in common with those of *wg*. During early germband extension, *Wnt11* is expressed in cells of the serosa (Fig. 4t). A little later, *Wnt11* appears in the distal tips of newly formed gnathal and thoracic appendages, with exception of the mandibles (Fig. 4u). At this stage, *Wnt11* expression is also observed faintly at the border of the dorsal ectoderm (Fig. 4u). As the limbs develop, *Wnt11* is expressed in a ring at midlength and in a ventral patch slightly proximal to the distal tip, whereas in the maxilla and labium no such refinement is seen, and the mandibles remain free of expression (Fig. 4v). *Wnt11* is also expressed in a few cells of the antennae and strongly in the anal plate (Fig. 4v).

Similar to *wg* and *Wnt8/D* (although fainter), expression of *WntA* first appears at the posterior end of the embryo during the blastoderm stage (Fig. 3c). As the germ rudiment condenses, additional *WntA* expression appears in a mandibular stripe and in lateral head lobe domains (Fig. 4w). Soon after the germband begins to elongate, *WntA* is expressed in segmental stripes that initially resemble *wg* stripes, but quickly resolve into small domains on either side of the ventral midline (Fig. 4x). Head- and appendage specific *WntA* expression is also observed during later stages (Fig. 4x, y), however expression in the posterior growth zone fades during germband elongation (compare Fig. 4w and 4x) expression in the hindgut anlagen appears after the germband is fully extended (Fig. 4y). In the limbs, *WntA* expression extends proximally from the distal tips (Fig. 4y). *WntA* is also expressed in segmentally reiterated patches along the lateral edge of the embryo and in the labrum.

Discussion

Phylogenetic characterization of insect *Wnt* genes

Tribolium has the largest complement of *Wnt* genes among insects for which genome sequence data is available (Fig. 1b). We identified nine *Wnt* genes in *Tribolium* while seven have been identified in *Apis* and *Drosophila*, and six in *Anopheles*. The higher complexity of this important signalling gene family in the beetle *Tribolium* compared to the more derived dipteran insect *Drosophila* reflects specific gene losses in the lineage leading to *Drosophila* since the divergence of these groups appr. 250 million years ago (xx). Eleven *Wnt* family members have been found in phylogenetically basal cnidarians (Kusserow et al. 2005). *Drosophila* has representatives of seven *Wnt* gene subfamilies (*Wnt1*, 5–7, 9, and 10) including a diverged *Wnt8* gene (*WntD*). Although the complement of *Wnt* genes reported for *Anopheles* is smaller, (*Wnt7*, 11 and *D* appear to be missing) this dipteran possesses a *WntA* family member, suggesting that the common ancestor of diptera contained at least eight *Wnt* genes. When compared to the other insects (Fig. 1b), *Apis* and *Tribolium* share the presence of *Wnt11*, which is absent from the dipteran species. However, *Wnt9*, which is

present in the other three species, appears to be missing in *Apis*. All together, these data indicate that at least nine of the 12 different *Wnt* genes known so far from other studies (Kusserow et al. 2005) are represented in insects in different combinations. Thus, with its large repertoire of *Wnt* genes, *Tribolium* is the only insect to date to include representatives of all the known insect *Wnt* genes.

An insect *Wnt* gene cluster—Despite species-specific *Wnt* gene losses, all insects examined to date, including *Tribolium*, contain the evolutionarily conserved cluster of *Wnt1*, *Wnt6* and *Wnt10*. In addition, most insects for which genome data are available have a fourth gene (*Wnt9*), which also clusters with *Wnt1-Wnt6-Wnt10*. The *Wnt9* gene is located 5' of *Wnt1* and the four-gene cluster spans 70–150 kb, depending on the insect species. We also found evidence for a cluster of *Wnt* genes in the genome of *Bombyx mori*. In this Lepidopteran insect, *Wnt1*, *Wnt6* and *Wnt10* are located within 60 kb of one another in the same order as in other insects (NCBI BLAST results, data not shown). The *Wnt9* ortholog is also located 5' of *Wnt1*, resulting in a four-gene cluster of 99 kb, supporting our suggestion that a four-gene *Wnt* cluster may be ancestral to insects. It is interesting to note that in the current assembly version of the *Bombyx* genome, all four genes are oriented in the same direction. The conservation of this *Wnt* cluster during evolution implies that these genes may be co-ordinately regulated.

***Wnt* gene synexpression groups**—The nine *Tribolium* *Wnt* genes display distinct expression patterns. However, overlapping tissue-specific expression patterns suggests that *Wnt* genes may be functionally redundant in some aspects of embryonic development. In the discussion below we classify these genes into synexpression groups that share partially overlapping expression domains in the head, segments, growth zone and/or limbs. However, the intensity and developmental profile of each gene exhibits differences that are often subtle, but in some cases more dramatic, suggesting they are also involved in gene-specific functions.

Expression in the embryonic head /brain—*wg* expression in the developing head lobes of the differentiated blastoderm, is soon followed by expression of *Wnt5*, *Wnt6* and *WntA* as the germband begins to elongate. The wedge-shaped domains of *Wnt5* are broader and more intense than those of the other genes, while the domains of *Wnt6* and *WntA* are fainter. *Wnt7* and *Wnt10* are also expressed in the head, although they appear later and are restricted to a few neurons in the developing brain. *wg* and *WntA* are both expressed in the labrum as it develops.

Segmental aspects of *Wnt* gene expression—In addition to *wg/Wnt1*, *Wnt5*, 6, 7, 10 and *A* are expressed segmentally during germband elongation. While the segmental stripes of *Wnt6* are very faint and fade early during axis elongation, *wg/Wnt1*, *Wnt5* and *Wnt10* show much stronger expression that lasts until axis elongation has ended. On the other hand, *Wnt7* is not strongly expressed in every abdominal segment until late in germband extension.

***Wnt* gene expression in the growth zone**—Various *Wnt* genes are expressed in the posterior region of the embryo, in the anlagen of the hindgut or more broadly in the growth zone including *wg/Wnt1*, *Wnt5*, *Wnt6*, *Wnt8/D*, *Wnt9* and *WntA*. Although very faintly expressed, *Wnt6* resembles *wg/Wnt1* in that its early expression occupies a subterminal position. In contrast, early germband expression of *Wnt5* and *WntA* covers the posterior tip of the embryo. While posterior expression of *Wnt5* and *Wnt8/D* fade during germband retraction, continued expression of *wg* and *Wnt6* in the anlagen of the hindgut is joined by expression of *Wnt9*. Posterior *WntA* expression is the most dynamic, fading from the growth

zone during germband elongation, but reappearing in the hindgut during retraction. These expression patterns suggest these genes may function in germband extension as well as growth and differentiation of the integument.

Limb specific expression of Wnt genes—*wg/Wnt1* is strongly expressed ventrally along the proximo-distal axis of the growing limb buds in a domain shared by *Wnt5*, *6*, and *10*. In contrast, expression of *Wnt7* in the leg dorsal mesoderm suggests a distinct function. In young stages *Wnt5*, *11* and *A* are each expressed in the limb buds and resolve into distinct patterns as the limbs mature. *Wnt5* expression continues in a small cap at the tip of the limb in addition to expression along the ventral edge. *Wnt11* expression fades at the tip but develops into a ring at midlength and slightly more distal as a ventral patch. *WntA* expression extends more proximal than *Wnt5*, but is excluded from the distal tip. A more intense ring of *WntA* expression appears at midlength, similar to *Wnt11*. These complex dynamics suggest Wnt signalling may be required in the development of several different limb features.

Wnt gene expression in the blastoderm

In addition to the genes expressed in overlapping domains during germband elongation and retraction, we can consider another group of *Wnt* genes that are expressed at the blastoderm stage. This group includes *wg/Wnt1*, *Wnt8/D* and *WntA*. They are all expressed in the posterior region of the embryo. The expression of *Wnt8/D* is largely complementary to that of *wg/Wnt1*, suggesting they may be only partially redundant in function. Expression of *wg/Wnt1* and *Wnt8/D* quickly resolves into specific domains in the germ rudiment suggesting more specialized functions, while expression of *WntA* remains broad.

Genes in the *Wnt* cluster belong to similar synexpression groups, supporting the hypothesis that these genes may be co-regulated. *Wnt1*, *6* and *10* display overlapping expression patterns in the head, limbs and segments, while *Wnt1*, *6* and *9* are expressed in the developing gut. In *Drosophila*, *Wnt6* and *10* are not expressed segmentally, but are expressed in the gut (Janson et al. 2001). *DWnt9* has been implicated in the regulation of cell movement in the developing ovaries, but is not required for embryonic development (Cohen et al. 2002). Most *Drosophila* larvae lacking *Wnt9* activity appear outwardly normal, but die as first or second instars. Our analysis of *Wnt9* in *Tribolium* suggests a role in gut development, which, if conserved, might help explain the larval lethal phenotype observed in *Drosophila*.

The expression patterns of other *Wnt* genes do not appear to be largely conserved between *Drosophila* and *Tribolium*, suggesting they may be required for different functions in each insect. *Wnt5* is expressed in the labrum and the primordial of the thoracic limbs of both insects. Later it is expressed strongly in the CNS in *Drosophila* (Fradkin et al. 1995), but not *Tribolium*. During embryogenesis, *Wnt7* is expressed segmentally in both insects, but in flies it is expressed in dorsolateral and ventral patches (Russell et al. 1992), while in beetles it is expressed in segmental stripes. In *Drosophila*, *Wnt8/D* is expressed at both poles of the egg at the blastoderm stage, while in *Tribolium* it is only expressed at the posterior end. In *Drosophila* it is expressed continuously in the ventral mesoderm, and is required for proper mesoderm formation (Ganguly et al. 2005). In *Tribolium* it continues to be expressed at the posterior end of the growth zone during germ-band elongation.

Wg signalling has been implicated in germband extension from the posterior growth zone in *Gryllus bimaculatus*, and *Oncopeltus fasciatus*, but *wg/Wnt1* itself does not appear to be the ligand (Miyawaki et al. 2004; Angelini and Kaufman 2005). As described above, the expression of several *Wnt* genes in *Tribolium* appears to overlap in the posterior growth zone (*Wnt1*, *5*, *6*, *A* and *D*), providing several ligand candidates to activate the *wg* signalling

pathway involved in germband extension. Careful investigation of single and multiple RNAi experiments will be required to sort out the functions of the *Tribolium Wnt* genes in this domain.

The overlapping expression patterns of *Tribolium Wnt* genes suggest they participate in similar functions, perhaps redundantly. On the other hand, minor differences observed in the temporal and spatial expression of each gene within a synexpression group (eg. varied expression in the growth zone) might imply functional discrepancies. Functional analysis of each *Tribolium Wnt* gene is required to determine the relationship between expression patterns and functions. Furthermore, it may be necessary to analyze multiple genes simultaneously to elucidate genes that function redundantly.

By accessing the genome sequence we have completed a comprehensive analysis of the *Wnt* gene family in *Tribolium*. We found a larger complement of *Wnt* genes in *Trioblium*, which was used to provide a framework for a comparative genomic analysis of *Wnt* genes in insects. Our results suggest that the insect repertoire of *Wnt* genes includes nine of the more broadly conserved *Wnt* gene subfamilies: *wg/Wnt1*, *5–7*, *9–11*, *A* and a highly diverged *Wnt8/D*.

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Fig. 1.

a Neighbour-joining phylogenetic tree of *Wnt* gene family. Analysis in MEGA 3.0 was performed using a bootstrap value of 1000. *Tribolium Wnt8/D* is clustered with *Drosophila WntD* and other *Wnt8* family members. *WntA* from *Tribolium*, *Apis* and *Anopheles* also clustered together. Species abbreviations: Tc, *Tribolium castaneum*; Am, *Apis mellifera* (insect); Dm, *Drosophila melanogaster* Ag, *Anopheles gambiae* (insect); Dr, *Danio rerio* (vertebrate); Hs, *Homo sapiens*; Bf, *Branchiostoma floridae* (amphioxus); Nv, *Nematostella vectensis* (sea anemone); Pd *Platynereis dumerilii* (annelid). **b** *Wnt* gene families in insects. The hymenopteran *Apis* stands basal to *Tribolium*, *Anopheles* and *Drosophila* (Savard et al. 2006). *WntD* is placed according to its phylogenetic position close to *Wnt8*.

Fig. 2. Insect *Wnt* gene clusters. The relative size, order and orientation of *Wnt* genes, including *Wnt9*, *Wnt1/wg*, *Wnt6* and *Wnt10* are compared. Non-*Wnt* genes found in the *Tribolium* and *Drosophila* clusters are also shown. The *Drosophila* non-*Wnt* gene is not annotated, but is not orthologous to the *Tribolium* CDP-diglyceride synthetase. Orthologs are color-coded.

Fig. 3. Only three *Tribolium* *Wnt* genes show expression in blastoderm embryos (whole mount *in situ* hybridization). Anterior pole points to the left. **a** *wingless* / *Wnt 1*. **b** *Wnt 8/D*. **c** *Wnt A*.

Fig. 4. Expression of *Tribolium* Wnt genes in germband embryos (whole mount *in situ* hybridization). Ventral views, anterior to the left

a–c wingless (*wg*)/*Wnt1*. **a** In the germ rudiment, *wg* is expressed in the head lobes and posteriorly in a subterminal position that marks the developing hindgut and leaves the posterior pole free of expression (*arrow*). **b** In the extending germband embryo, stripes of *wg* are visible in the antennal, gnathal and thoracic segments anterior to the growth zone. **c** *wg* expression extends ventrally into the appendages of the fully extended germband embryo. In the head *wg* expression appears in the labrum and the developing brain lobes. *wg* is also expressed in patches along the lateral edge of each segment and in the developing hindgut.

d–f *Wnt5*. **d** *Wnt5* is expressed faintly in the head lobes (*arrow*) and in segmental stripes in an embryo beginning germband elongation. More intense expression is visible at the posterior end of the growth zone. **e** Stripes of *Wnt5* in the gnathal and thoracic segments cross the ventral midline. *Wnt5* expression is more intense at the posterior end and in broad wedge shaped domains in the head lobes. **f** In the fully extended germband embryo, *Wnt5* is no longer expressed at the posterior end of the growth zone, but extends ventrally into the developing limbs and is also visible at their tips.

g–i *Wnt6*. **g** In an embryo beginning germband elongation, *Wnt6* is expressed faintly in the lateral head lobes (*arrow*), in segmental stripes and in a subterminal domain in the growth zone (*arrowhead*). **h** After elongation, a single segmental stripe remains, marking the eleventh abdominal segment, while expression is observed along the ventral edge of the limbs, along the dorsolateral edge of the embryo and in spots to the left and right of the midline. Expression near the posterior end of the germband marks the anlagen of the Malpighian tubules (*arrowhead*). **i** In the retracted germband, fore- and hindgut anlagen express *Wnt6*, as do precursors of the tracheal openings. Several spots of *Wnt6* expression are also evident in the brain. Non-specific expression in the pleuropodia is marked by an asterisk.

j–l *Wnt7*. **j** Faint segmental stripes of *Wnt7* are apparent in the elongating embryo. **k** In a slightly older embryo, gnathal expression of *Wnt7* is restricted to specific cells in the developing CNS, while in the thorax, expression encompasses the leg anlagen. **l** In the thorax and abdomen of the germband extended embryo, segmental stripes of *Wnt7*, which extend laterally into the dorsal mesoderm of the appendages, do not cross the midline. **m** *Wnt8/D*. During elongation, *Wnt8/D* remains expressed at the posterior end of the growth zone in two small patches, one at each side of the ventral mesoderm, that are connected by a narrow row of cells at a subterminal position.

n–p *Wnt9* **n** In late stage embryos *Wnt9* expression is visible in cells of the fore- and hindgut that appear to be positioned at the border of the ectodermal and endodermal derivatives of the gut. **o** Closeup view of the embryo in **n** focusing on the foregut (*arrow*). **p** Closeup view of *Wnt9* expression at the anterior end of the hindgut, where it connects to the midgut (*arrow*). Nonspecific staining of the pleuropodia in abdominal segment 1 is marked by an asterisk.

q–s *Wnt10*. **q** *Wnt10* expression is first visible in a single stripe in the mandibular segment. **r** In elongated embryos segmental stripes have resolved into dots of expression that extend ventrally into the developing limbs. Three prominent cells in each hemisphere of the developing brain also begin to express *Wnt10*. **s** During retraction, segmental expression of *Wnt10* remains in the ventral limbs and in a few cells on either side of the ventral midline while expression in the brain has expanded.

t–v *Wnt11*. **t** *Wnt11* expression is visible in cells of the serosa, remnants of which are evident along the edge of the embryo and covering part of the posterior end. **u** In the germ-band extended embryo, *Wnt11* expression is visible in the distal tips of the developing legs. The distal regions of the maxillary and labial appendages express *Wnt11* strongly but it is seen only weakly in the antennal lobes and there is no expression in the mandibles (*arrowhead*). *Wnt11* is also expressed at the border of the dorsal ectoderm (*arrow*). **v** As the legs mature during germband retraction, expression of *Wnt11* there resolves proximally into rings and distally into a ventral patch. Only a few antennal and labral cells express *Wnt11*, while expression is strong in the anal plate.

w–y *WntA*. **w** In a germband embryo beginning elongation, *WntA* is expressed faintly in lateral domains of the head lobes, segmentally and in the growth zone. **x** Segmental expression appears as stripes only in the most posterior abdominal segments where it resolves into spots on either side of the ventral midline (*arrows*). As elongation nears completion, expression in the growth zone fades, while head specific expression develops. **y** During germband retraction, *WntA* expression appears in the developing hindgut and in the labrum. Expression also appears in the limbs in a ring at midlength and in patches along the lateral edge of the embryo.