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This is the submitted version of a paper published in *Gene Expression Patterns*.

Citation for the original published paper (version of record):

Janssen, R., Posnien, N. (2014)

Identification and embryonic expression of Wnt2, Wnt4, Wnt5 and Wnt9 in the millipede *Glomeris marginata* (Myriapoda: Diplopoda).

Gene Expression Patterns, 14(2): 55-61

<http://dx.doi.org/10.1016/j.gep.2013.12.003>

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Identification and embryonic expression of *Wnt2*, *Wnt4*, *Wnt5* and *Wnt9* in the millipede *Glomeris marginata* (Myriapoda: Diplopoda)

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Word count: 5545

Figures: 4

Tables: 1

Supplementary data: ---

Keywords: segmentation, limb development, tergite formation, evolution, arthropod development

Abstract

The Wnt genes encode secreted glycoprotein ligands that are key players during animal development. Previous studies revealed the presence of 12 classes of Wnt genes in protostomes, although lineage specific losses of Wnt genes are common. So far, the gene expression profile of only two complete sets of arthropod Wnt genes has been studied; these are the Wnt genes of the fly *Drosophila melanogaster* and the beetle *Tribolium castaneum*. Insects, however, do not represent good models for the understanding of Wnt gene evolution because several Wnt genes have been lost in the lineage leading to the insects, or within the different orders of insects. Comparative gene expression data from non-insect arthropods are rare and restricted to a subset of Wnt genes.

This study aims to fill this gap and describes four newly detected Wnt genes from the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Together with previous studies, now 11 *Glomeris* Wnt genes have been isolated and their expression has been studied. The only predicted but hitherto undetected Wnt gene is *Wnt10*. The new data provide a platform for the comparison of Wnt gene expression patterns in arthropods and reveal conserved as well as diverged aspects of Wnt gene expression in Arthropoda. Prominent expression of *Wnt4* in dorsal tissue implies a role in dorsal segmentation and suggests that *Wnt4* may be the predicted substitute for the previously reported missing expression of *wg/Wnt1* in dorsal tissue.

Introduction

The Wnt genes represent key regulator molecules in metazoan development. They encode secreted glycoprotein ligands that bind to a number of different transmembrane receptors, and thereby activate various intracellular signaling cascades that regulate target gene transcription ([Logan and Nusse 2004](#), [Murat et al. 2010](#), [Perrimon et al. 2012](#), [Swarup and Verheyen 2012](#)). The last common ancestor of the arthropods most likely possessed a set of 12 Wnt genes ([Janssen et al. 2010a](#)). At present, most knowledge about the expression and function of Wnt genes comes from studies in the fruit fly *Drosophila melanogaster*, which still is the main arthropod model organism. In *D. melanogaster*, however, as in many insects, the set of Wnt genes is reduced (summarized in [Janssen et al. 2010a](#)). Even in the conservatively developing beetle *Tribolium castaneum*, only nine Wnt genes are present ([Bolognesi et al. 2008a](#)). Data sets on non-insect arthropods are either incomplete or gene expression analysis has not been performed yet (summarized in [Janssen et al. 2010a](#)). To fully understand the importance of Wnt gene function during development and evolution it is imperative to investigate the expression patterns of all Wnt genes in various arthropod species representing all four (or five depending on the phylogenetic position of pycnogonids) main classes of arthropods: insects, crustaceans, myriapods and chelicerates. Today, however, exhaustive knowledge on Wnt gene expression is still restricted to insects ([Bolognesi et al. 2008a](#), reviewed in [Murat et al. 2010](#)).

This study aims to complete earlier work on the myriapod *Glomeris marginata* to provide comprehensive insight into the Wnt gene expression landscape of a non-insect arthropod. In earlier studies the expression of *wg/Wnt1*, *Wnt6*, *Wnt7*, *Wnt8*, *Wnt11*, *Wnt16* and *WntA* has been studied. Based on the distribution of Wnt genes

along the phylogenetic tree of arthropods the presence of further Wnt genes, such as *Wnt2*, *Wnt4*, *Wnt5*, *Wnt9* and *Wnt10* was predicted (Janssen et al. 2010a).

This paper reports on the presence of all ‘missing’ *G. marginata* Wnt genes (except *Wnt10*) and describes their embryonic expression. The myriapod Wnt gene expression profiles are discussed in the light of possible redundant/combinatorial function during segmentation, brain regionalization and limb patterning. Beyond that, the results are compared with Wnt gene expression data from other arthropods in order to reconstruct the ancestral Wnt patterning landscape of Arthropoda. It is found that one of the Wnt genes, *Wnt4*, appears to be a good candidate to substitute for the classic segment polarity gene *wg/Wnt1* in dorsal tissue. This is an important finding since the existence of such a ‘factor X’, a *wg/Wnt1* substitute, has been predicted, but not as yet found (Janssen et al. 2004, 2008, Fusco 2009).

1. Results

1.1. *Glomeris marginata* Wnt genes

The embryonic transcriptome contains 11 Wnt genes. Seven of them, i.e. *wg/Wnt1*, *Wnt6*, *Wnt7*, *Wnt8*, *Wnt11*, *Wnt16* and *WntA* have previously been described and isolated by means of RT-PCR with degenerate primers (Janssen et al. 2004, 2010). At least four Wnt genes, however, remained undetected in these PCR screens, but have been detected in a sequenced embryonic transcriptome (see Experimental Procedures section). These new Wnt genes are *Wnt2*, *Wnt4*, *Wnt5* and *Wnt9*. In the conducted phylogenetic analysis these genes cluster with high reliability with their orthologs from other arthropods and an annelid (Fig. 1). The other previously studied Wnt genes also cluster with their orthologs supporting earlier phylogenetic analysis (Janssen et

al. 2010). Altogether, there is little doubt about Wnt gene orthology, and the newly isolated gene fragments were thus designated as *Glomeris Wnt2* (*Gm-Wnt2*), *Wnt4* (*Gm-Wnt4*), *Wnt5* (*Gm-Wnt5*) and *Wnt9* (*Gm-Wnt9*).

1.2. Expression patterns of *G. marginata* *Wnt2*, *Wnt4*, *Wnt5* and *Wnt9*

1.2.1. Expression of *G. marginata* *Wnt2*

Wnt2 is exclusively expressed in two domains in the developing brain throughout all investigated embryonic stages (Fig. 2A,B). Expression starts at stage 1.1 as faint patches in the ocular fields (not shown). This expression persists until at least stage 6.1 (Fig. 2B).

1.2.2. Expression of *G. marginata* *Wnt4*

From the early blastoderm stage onwards *Wnt4* is strongly expressed the complete limb-less premandibular segment (Fig. 2C); this is reminiscent of the early expression of the head gap gene ortholog *collier* (*col*) in the same segment (Janssen et al. 2011a,b). Later, *Wnt4* expression covers most of the segment in the form of two ventral patches (Fig. 2D-F). These patches likely represent expression in the ventral nervous system. At later stages similar expression appears in an anterior to posterior order in the trunk segments (Fig. 2E,F). *Wnt4* is expressed prominently in transverse stripes in the middle of the dorsal segmental units (Fig. 2E-G). One single stripe of *Wnt4* is located in the dorsal portion of each embryonic diplosegment (see Janssen 2011) in the posterior trunk. This pattern is reminiscent of that of the segment polarity genes *engrailed* (*en*) and *hedgehog* (*hh*) (Janssen et al. 2004, 2008). *Wnt4* is also expressed in the inside of the anal valves and the complete hindgut (Fig. 2C,D,H-J). Dichromatic double-hybridization experiments with *Wnt4* and either *en* or *hh* failed,

but monochromatic double-hybridization experiments with *Wnt4* and *en* revealed co-expression of these two genes in the dorsal segmental units (Fig. 2K; the combined expression domain is of the same width as the single ones). At later stages, it is obvious that *en*, *hh* and *Wnt4* are all restricted to the dorsal grooves that demarcate the tergite boundaries (Fig. 2G) (cf. Janssen et al. 2004, 2006). *Wnt4* is weakly expressed in the distal part of the antennae, strongly in a ring close to the distal end of the antennae, and ventral to the basis of the antennae (Fig. 4A).

1.2.3. Expression of *G. marginata* *Wnt5*

Wnt5 is expressed in the posterior segment addition zone (SAZ) and in transverse stripes in nascent segments (Fig. 3A,B). This expression is restricted to the ventral segmental units, while the expression in the SAZ extends into dorsal tissue (Fig. 3B). The ventral segmental pattern transforms into a refined pattern in older (i.e. more anterior) segments (Fig. 3B,C). *Wnt5* is also expressed in all appendages, except the antennae (Fig. 4B-E). The labrum expresses *Wnt5* as two dorsal patches (Figs 3C and 4B). The complete mandibles express *Wnt5*, but in the maxillae the expression is mainly restricted to a sub-anterior region (Fig. 4C,D). From there, two thin offshoots of expression run to the distal tip of each maxilla leaving three distal *Wnt5*-negative regions in each tip. Expression in the walking limbs is restricted to the dorsal side (Fig. 4E). Two patches of weak expression are located in the brain lobes, possibly associated with the developing eyes (Fig. 3B-D).

1.2.4. Expression of *G. marginata* *Wnt9*

Wnt9 is expressed in the tips of the limbs, including the labrum (Fig. 4J-M). Expression in the maxillae and antennae is stronger than expression in the walking

limbs and the mandibles (Fig. 4J-M). In the antennae and the walking limbs the spot-like expression of *Wnt9* is not in the extreme distal end, but is slightly shifted ventrally (Fig. 4J,M). In contrast, expression in the labrum is shifted dorsally (Fig. 4J). Expression in all limb buds first appears shortly after they become morphologically visible, and in a strict anterior to posterior order (Fig. 3E-H). Expression in the labrum, however, appears later when the labrum begins to grow out from a position anterior to the mouth (Fig. 3E,F). From approximately stage 4 onwards, *Wnt9* is strongly expressed in short segmental stripes at the interface between the dorsal edge of the embryo proper and the extraembryonic tissue (Fig. 3H,I). Weaker expression is found in the continuation of these stripes in the dorsal extraembryonic tissue. These latter stripes are broader than the “embryonic” stripes and span the complete dorsal extraembryonic tissue (Fig. 3H-K). Thus, they “connect” the dorsal edges of the germ band. At later stages, this dorsal tissue will grow over and finally fuse in a process called “dorsal closure”. *Wnt9* is expressed in the inside of the anal valves (Fig. 3E, G-I).

2. Discussion

2.1. *Wingless-independent, but not Wnt-independent tergite boundary formation?*

Previous studies revealed that the segment polarity gene network as known from *D. melanogaster* is highly conserved in ventral segmental tissue in *G. marginata* (Janssen et al. 2004, 2008), other arthropods (e.g. Damen 2002, Hughes and Kaufman 2002, Farzana and Brown 2008) and even in an annelid (Dray et al. 2010). There are, however, differences in dorsal segment-polarity gene signaling in *G. marginata*, such as the lack of dorsal *wg/Wnt1* expression (Janssen et al. 2004, 2008, Fusco 2009).

Therefore, the idea was brought forward that an as yet unknown ‘factor X’, likely another Wnt gene, could possibly substitute for the function of *wg/Wnt1* in this tissue (Janssen et al. 2004).

The dominant expression in a transversal stripe in each dorsal segmental unit makes *Wnt4* a good candidate for being the predicted ‘factor X’ in dorsal segmentation. *Wnt4* is co-expressed with *engrailed* (*en*) and *hedgehog* (*hh*), and not, as in ventral tissue, expressed anterior adjacent to it. Thus, the interaction of the segment polarity genes (SPGs) in dorsal tissue and *Wnt4* must be different from that in ventral segmental tissue beyond the replacement of *wg/Wnt1* by *Wnt4*.

2.2. Conserved and diverged aspects of Wnt expression in arthropods

Relatively little is known about the expression of *Wnt2*, *Wnt4*, *Wnt5*, and *Wnt9* orthologs during arthropod embryogenesis. *Wnt2* and *Wnt4* are absent from the genome of insects. Expression data from crustaceans are lacking, although the presence of these Wnt genes has been revealed for the water flea *Daphnia pulex* (summarized in Janssen et al. 2010a).

Expression of *Wnt2* has been studied in the spider *Achaearanea tepidariorum* (synonym *Parasteatoda tepidariorum*) (Janssen et al. 2010a) and the myriapod *G. marginata* (this study). In at least these two species the expression is virtually identical and suggests a conserved function during brain development.

Data on *Wnt4* expression are available for *A. tepidariorum* (Janssen et al. 2010a) and *G. marginata* (this study). The expression profiles are fundamentally different in these species suggesting that *Wnt4* has changed its function during arthropod evolution.

Expression of *Wnt5* is very similar in *D. melanogaster* (Eisenberg et al. 1992, Fradkin et al. 1995, Russell et al. 1992), *T. castaneum* (Bolognesi et al. 2008a), *A.*

tepidariorum (Janssen et al. 2010a), *Cupiennius salei* (Damen 2002, Janssen and Damen 2008), and *G. marginata*, suggesting partially conserved functions in arthropods.

Wnt9 has previously been studied in *D. melanogaster* (Graba et al. 1995, Gieseler et al. 1995) and *T. castaneum* (Bolognesi et al. 2008a). In many respects, the complex expression patterns observed in *G. marginata* are different from those in *D. melanogaster*. In the latter, *Wnt9* is expressed in a segment-polarity gene like pattern. Expression of *D. melanogaster Wnt9* in the visceral mesoderm, the ventral nerve cord, and the developing gut, is not conserved in *G. marginata*. In contrast, *G. marginata Wnt9* is expressed in the tips of the appendages. This expression has not been reported for the fly, but it is unclear if expression in the larval imaginal discs has been investigated at all. Exclusive expression of *Wnt9* in the interface of hindgut and midgut in *T. castaneum* is different from the expression patterns in the fly and the millipede. This suggests that the expression and function of *Wnt9* gene orthologs has undergone dramatic changes during evolution in at least two of the investigated arthropod lineages.

In order to possibly reveal the ancestral expression patterns of arthropod *Wnt9*, and also *Wnt4*, data on further arthropods including crustaceans, and arthropod sister groups such as onychophorans and tardigrades (e.g. Meusemann et al. 2010, Campbell et al. 2011) are needed.

2.3. The *G. marginata Wnt* gene-expression landscape

Brain. Several Wnt genes (*wg/Wnt1*, *Wnt2*, *Wnt5*, *Wnt6*, *Wnt7*, *Wnt8*, *Wnt16* and *WntA*) are expressed in the developing brain of the millipede (Janssen et al. 2004,

2010a, this study). Expression in the brain, however, differs considerably for each gene suggesting a possible role of Wnt signaling in brain-regionalization.

SPG-like-patterns. The segment polarity gene function of *wg/Wnt1* is most likely conserved in *G. marginata* (Janssen et al. 2004, 2008). Some other Wnt genes (*Wnt5*, *Wnt6*, *Wnt16*) have comparable expression patterns in the form of transverse segmental stripes in the middle of each ventral segment. It is possible, therefore, that these genes fulfill partially redundant, or combinatorial, functions during the process of segment formation and differentiation.

SAZ. The posterior segment addition zone (SAZ) is the origin of all posterior segments, including the second walking limb-bearing segment. The only Wnt gene that is strongly expressed in the complete SAZ is *Wnt5*. *Wnt6*, *Wnt7* and *Wnt11* are only expressed in the posterior part of the SAZ. *wg/Wnt1*, *Wnt4*, *Wnt9* and *Wnt16* are only expressed in the inside of the anal valves (av), and thus rather in the developing proctodaeum than in the SAZ. Although the function of Wnt genes in the SAZ/av is unclear, the expression suggests a function in posterior segment addition. Such a function has been shown for Wnt signaling in a spider (McGregor et al. 2008), a beetle (Bolognesi et al. 2008b), a true bug (Angelini and Kaufman 2005a), and a cricket (Miyawaki et al. 2004).

Appendages. Seven of the eleven reported Wnt genes (i.e. *wg/Wnt1*, *Wnt5*, *Wnt6*, *Wnt9*, *Wnt11*, *Wnt16* and *WntA* (summarized in Fig. 4)) are expressed in at least one type of appendage. A detailed analysis of *wg/Wnt1* expression in the appendages of *G. marginata* has been published previously (Prpic 2004). In this study, it was suggested that *wg/Wnt1* (as part of WG/DPP signaling) is involved in dorso-ventral (DV) and proximo-distal (PD) limb patterning/development (Prpic 2004, Prpic et al. 2005). The walking limbs most likely represent the most ancestral type of limbs, since their

function is mainly restricted to locomotion and, unlike the head-appendages, has not evolved multiple specialized functions. This assumption is supported by conserved expression profiles in arthropod and onychophoran walking limbs (e.g. [Abzhanov and Kaufman 2000](#), [Prpic et al. 2001, 2003](#), [Angelini and Kaufman 2005b](#), [Prpic and Damen 2009](#), [Janssen et al. 2010b](#)). *wg/Wnt1*, *Wnt6*, *Wnt9*, *Wnt11* and *Wnt16* are expressed in almost identical patterns along the ventral side of the walking limbs. This suggests either that these genes function redundantly, or more likely, that they act in a combinatorial fashion, as shown for Wnt-signaling in other arthropods ([Llimargas and Lawrence 2001](#), [Bolognesi et al. 2008b](#), [van Amerongen and Nusse 2009](#), [Beermann et al. 2011](#)). They are likely involved in ventral patterning of the limbs, as shown for *D. melanogaster* (e.g. [Brook and Cohen 1996](#), [Penton and Hoffmann 1996](#), [Prpic et al. 2003](#), [Grossmann et al. 2009](#), but [Angelini and Kaufman 2005a](#)). Remarkably, *Wnt5* is expressed along the dorsal surface of the legs. This expression is reminiscent of that of *decapentaplegic (dpp)* and *optomotor-blind* type genes, which most likely have a conserved Wnt-antagonistic function in dorsal patterning ([Prpic et al. 2003](#), [Prpic 2004](#), [Penton and Hoffmann 1996](#), [Brook and Cohen 1996](#), reviewed in [Brook 2010](#)). Thus, *Wnt5* may act as an antagonist of the ventrally expressed Wnt genes.

The nature and homology of the labrum, the upper lip, is controversial ([Eriksson and Budd 2000](#), [Budd 2002](#), [Scholtz and Edgecombe 2006](#), [Kimm and Prpic 2006](#), [Brenneis et al. 2008](#), [Posnien et al. 2009](#); [Liu et al. 2009](#), [Pechmann et al. 2010](#)). *G. marginata* Wnt genes are expressed exclusively as two dots in or along the dorsal side of the labrum. This is in line with the fact that the arthropod labrum evolved by the fusion of two initially separate anlagen ([Kimm and Prpic 2006](#), [Liu et al. 2009](#)). Remarkably, although the expression of *G. marginata* Wnt genes is predominantly in

ventral tissue in the walking limbs, the labrum always expresses Wnt genes in dorsal tissue. This can be explained by the hypothesis that the labrum evolved through a 180° rotation ([Kimm and Prpic 2006](#)).

2.4. *G. marginata* *Wnt10*: lost or simply not found yet?

All predicted Wnt orthologs, except *Wnt10*, have been identified in *G. marginata*. The seven previously described Wnt gene orthologs (*wg/Wnt1*, *Wnt6*, *Wnt7*, *Wnt8*, *Wnt11*, *Wnt16* and *WntA*) have been isolated by means of extensive PCR screens. The Wnt genes described in this paper have been found in a sequenced embryonic transcriptome. It appears that *Wnt10* was lost in *G. marginata*, or that it has escaped from detection. The latter is possible because the sequenced transcriptome only represents embryonic stages. If lost, this loss was not in the lineage leading to the arthropods, since *Wnt10* orthologs have been identified in insects (except in the sequenced genome of the pea aphid *Acyrtosiphon pisum* ([Shigenobu et al. 2010](#))), and a crustacean ([Janssen et al. 2010a](#)). The situation in the spider *A. tepidariorum* (sequenced embryonic transcriptome) and the tick *Ixodes scapularis* (at the time not fully annotated genome) is unclear since *Wnt10* has not yet been identified in these species although it may be present. It could, however, be that *Wnt10* was lost in the lineage leading to myriapods and chelicerates (supporting the Paradoxopoda or Myriochelata hypothesis) (e.g. [Hwang et al. 2001](#), [Mayer and Whittington 2009](#)).

3. Experimental procedures

3.1. Species husbandry, transcriptome analysis, gene cloning, in situ hybridization, nuclei staining and documentation techniques

The general handling of *G. marginata* adults and embryos is described in [Janssen et al. \(2004\)](#). Embryos developed at room temperature. Staging follows [Dohle \(1964\)](#) and [Janssen et al. \(2004\)](#). Developmental stages were determined by using 4'-6-diamidino-2-phenylindole (DAPI) for all embryos. New Wnt genes were identified from a sequenced embryonic transcriptome. The total RNA of embryos (stages 0 to 6.1 ([Janssen et al. 2004](#))) was sent to Macrogen (Korea) for sequencing. The library preparation was done according to Illumina standard RNAseq protocol. Sequencing on half of one Illumina HiSeq2000 lane resulted in 200,241,866 paired-end 101 bp reads. Low quality bases and PolyA/T stretches longer than 10 bases were trimmed using custom perl scripts resulting in reads with 99 bp length on average. Due to computational limitations the dataset was randomly split into four sub-datasets. First, all four sub-datasets were independently assembled using Velvet ([Zerbino et al., 2008](#); version 1.2.08) and Oases ([Schulz et al., 2012](#); version 0.2.08) with a k-mer size of 27. The resulting assembled transcripts were used as input (-long flag) for a final merged assembly. This assembly strategy resulted in 246,076 assembled sequences composed of 457,149,108 bp. The N50 of the assembly is 3,614 bp which means that 50% of all sequences are 3,614 bp or longer. The assembly has a CG-content of 42.55%.

A local BLAST search comparison was used with previously isolated Wnt genes from *G. marginata*, and other arthropods. The fragments were isolated by means of gene specific primers (Table 1) and cloned into a plasmid vector (pCRII-TOPO, Invitrogen). Sequences of the complete coding region of the genes are available under accession numbers HG529218 (*Gm-Wnt2*), HG529219 (*Gm-Wnt4*), HG529220 (*Gm-Wnt5*), and HG529221 (*Gm-Wnt9*). Single whole mount in situ hybridization was performed essentially as described by [Prpic and Tautz \(2003\)](#). Double whole mount in

situ hybridization was performed as described by [Janssen et al. \(2008\)](#). Embryos were analyzed under a Leica dissection microscope equipped with either an Axiocam (Zeiss) or a Leica DC100 digital camera. Brightness, contrast and color values were corrected (linear transformations only) in all images using the image processing software Adobe Photoshop CS2 (version 9.0.1 for Apple Macintosh).

3.2. Phylogenetic analysis

For the phylogenetic analysis the sequences were aligned using Clustal_X ([Thompson et al. 1997](#)) on the basis of the GONNET residue comparison matrix ([Gonnet et al. 1992](#)); a gap introduction penalty of 10 and a gap extension penalty of 0.1. Maximum likelihood analysis was then carried out using the Quartet Puzzling method ([Strimmer and von Haeseler 1996](#)) as implemented in PAUP 4.0b10 ([Swofford 2002](#)).

Acknowledgements

Graham E. Budd is thanked for financial help and ongoing discussion. The work of NP was funded by the Volkswagen Foundation. Alistair McGregor is thanked for drawing my attention to the importance of Wnt genes in development and evolution. Native English speaker John Peel is thanked for checking grammar and spelling.

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Figure Legends

Fig. 1 Phylogenetic analysis of *Glomeris* Wnt genes

Unrooted majority rule consensus tree calculated from 1000 intermediate trees produced with the Quartet Puzzling method. Numbers represent reliability values. Newly discovered *Glomeris* Wnt genes are in bold. Subfamilies are color-marked. At, *Achaearanea tepidariorum* (aka *Parasteatoda tepidariorum*) (Chelicerata); Cs,

Cupiennius salei (Chelicerata); Dp, *Daphnia pulex* (Crustacea); Gm, *Glomeris marginata* (Myriapoda); Pd, *Platynereis dumerilii* (Annelida); Tc, *Tribolium castaneum* (Insecta)

Fig. 2 Expression of *Wnt2* and *Wnt4*

Anterior is to the left in all panels, except panel (J) where anterior is up. (A), (C-F), (H) and (K) represent ventral views. (B), (G) and (J) represent lateral views. (I) represents a dorsal view. Yolk has been partially removed from embryos shown in (H-J). One hemisphere of embryo shown in (J) has been removed and the picture shows the “inside” of the embryo. (A-B) Expression of *Wnt2*. Arrows point to expression in the brain. (C-J) Expression of *Wnt4*. Asterisks mark expression in the ventral nervous system. Numbers indicate segmental dorsal stripes of expression (1 = dorsal tissue corresponding to the postmaxillary segment; 2-5 = corresponding to dorsal haplosegments; 6-7 = corresponding to dorsal diplosegments). Arrows in (H-J) point to the anterior border of expression in the gut; this is the hindgut-midgut boundary. (K) Expression of *Wnt4* + *engrailed* (*en*). Note *Wnt4*-specific expression in the pmd (green arrow), and *en*-specific expression in the ventral segments (red arrow) and the last-formed dorsal segmental unit (indicated by red number ‘7’). Numbers as in (E-G). Panels (A’-D’) and (G’) show the same embryos as in (A-D) and (G) with DAPI staining. Abbreviations: av, anal valves; hg, hindgut; pmd, premandibular segment; SAZ, segment addition zone; st, stage.

Fig. 3 Expression of *Wnt5* and *Wnt9*

Anterior is to the left in all panels. (A-C) and (E-G) represent ventral views; (D) and (I-J) represent lateral views; (K) represents a dorsal view. (A-D) Expression of *Wnt5*.

Arrows in (A) point to segmental stripes. Arrows in (D) point to patches of expression dorsal to the basis of the limbs. (E-K) Expression of *Wnt9*. Arrows in (E) point to segmental expression in the anlagen of the trunk limbs. Numbers indicate dorsal segmental stripes (cf. figure legend Fig. 2). Panels (A'-D'), (F') and (I') show the same embryos as in A, C and I with DAPI staining. Abbreviations: an, antenna; av, anal, valves; md, mandibular segment; mx, maxillary segment; (lr) anlagen of the labrum; lr, labrum; oc, ocular field; S, stomodaeum; SAZ, segment addition zone; T1, first walking limb bearing segment.

Fig. 4 Summary of Wnt gene expression in the limbs

(A) Expression of *Wnt4* in the antennae. (B-E) Expression of *Wnt5*. (F-I) Expression of *Wnt6*. (J-M) Expression of *Wnt9*. (N-Q) Expression of *Wnt11*. (R-U) Expression of *Wnt16*. (V-Y) Expression of *WntA*.