Evolution of Developmental Control Mechanisms

Deciphering the onychophoran ‘segmentation gene cascade’: Gene expression reveals limited involvement of pair rule gene orthologs in segmentation, but a highly conserved segment polarity gene network

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A B S T R A C T

The hallmark of the arthropods is their segmented body, although origin of segmentation, however, is unresolved. In order to shed light on the origin of segmentation we investigated orthologs of pair rule genes (PRGs) and segment polarity genes (SPGs) in a member of the closest related sister-group to the arthropods, the onychophorans. Our gene expression data analysis suggests that most of the onychophoran PRGs do not play a role in segmentation. One possible exception is the even-skipped (eve) gene that is expressed in the posterior end of the onychophoran where new segments are likely patterned, and is also expressed in segmentation-gene typical transverse stripes in at least a number of newly formed segments. Other onychophoran PRGs such as runt (run), hairy/Hes (h/Hes) and odd-skipped (odd) do not appear to have a function in segmentation at all. Onychophoran PRGs that act low in the segmentation gene cascade in insects, however, are potentially involved in segment-patterning. Most obvious is that from the expression of the pairberry (pby) gene ortholog that is expressed in a typical SPG-pattern. Since this result suggested possible conservation of the SPG-network we further investigated SPGs (and associated factors) such as Notum in the onychophoran. We find that the expression patterns of SPGs in arthropods and the onychophorans are highly conserved, suggesting a conserved SPG-network in these two clades, and indeed also in an annelid. This may suggest that the common ancestor of lophotrochozoans and ecdysozoans was already segmented utilising the same SPG-network, or that the SPG-network was recruited independently in annelids and onychophorans/arthropods.

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Introduction

The arthropods are numerically by far the most diverse animal phyla on our planet. One reason why arthropods are so successful in conquering virtually every habitat since their appearance at around approximately 520 Mya in the lower Cambrian (e.g. Budd and Telford, 2009) is their ability to evolve new morphological features to adapt to changing ecological conditions. A likely reason for this ability is that the arthropods body is subdivided into segments, serially repeated homologous units along the anterior–posterior (AP) body axis.

Our current knowledge of the genetic and developmental mechanisms underlying arthropod segmentation are mainly based on the fruit fly Drosophila melanogaster where the body is subdivided into segments by a hierarchic segmentation gene cascade (reviewed in e.g. Damen, 2007): maternal-effect genes control the gap genes (GGs) in distinct domains along the AP axis of the developing embryo. The pair rule genes (PRGs) such as even-skipped (eve), runt (run) and paired (prd) then act downstream of the GGs, and are typically expressed in a double-segmental pattern in transversal stripes in every other segment. Primary PRGs such as eve regulate secondary PRGs such as opa. In a combinatorial mode the PRGs then activate the segment polarity genes (SPGs) such as engrailed (en), wingless (wg) and hedgehog (hh), that are needed to establish and maintain the parasegment boundaries and to establish the AP polarity of the segments (e.g. Hatini and DiNardo, 2001; Sanson, 2001; Damen, 2007). Orthologs of these segmentation genes have subsequently been studied in arthropods other than Drosophila. Although a great amount of data concerning SPG orthologs is available today, most data are restricted to the analysis of single genes or only a single component of the network. Only a few studies present comprehensive analyses of SPG expression in one single arthropod species. These data are mainly restricted to arthropod model species such as Drosophila (Ingham, 1991; DiNardo et al., 1994), the beetle Tribolium castaneum (Oppenheimer et al., 1999; Farzana and Brown, 2008), the spider Cupiennius salei (Damen, 2002), or the millipede Glomeris marginata (Janssen et al., 2004, 2008; Janssen, 2012). Data on crustacean SPG expression are restricted to the analysis of the SPG engrailed (en) (Manzanares et al., 1993; Scholtz et al., 1994; Abzhanov...
and Kaufman, 2000; Simonnet et al., 2004). The accumulated data suggest, however, a high degree of conservation on the level of SPGs (and associated factors) in arthropods. Comprehensive data on PRG expression are available from insects, myriapods and chelicerates (Pankratz and Jäckle, 1990; Chipman et al., 2004; Damen et al., 2000, 2005; Choe et al., 2006; Chipman and Akam, 2008; Choe and Brown, 2007, 2009; Janssen et al., 2011, 2012), but again data on crustaceans are scarce (Copf et al., 2003; Davis et al., 2005). These data suggest that at least some of the PRGs are involved in segmentation in these species, and thus that PRGs are generally involved in arthropod segmentation. The origin of pair rule patterning, however, is still unresolved (Chipman et al., 2004; Janssen et al., 2012; Sarrazin et al., 2012; El-Sherif et al., 2012).

In this paper we investigated the expression profiles of most of the canonical Drosophila PRG orthologs in the onychophorans Euperipatoides kanangrensis. The function and expression of fushi-tarazu, that belongs to the PRGs in Drosophila, but is still expressed as a typical Hox gene in arthropods (reviewed in Hughes and Kaufman, 2002a). The canonical Drosophila PRG tenascin-major (ten-m) represents an atypical PRG in Drosophila and does not encode a transcription factor, like all other PRGs. It has only been studied in Drosophila where it is expressed in a pair rule pattern on protein level, but not on mRNA level. Therefore ten-m has not been investigated in this study.

We found that PRGs that act first in the pair rule module in arthropods, except even-skipped (eve), play no role in onychophoran segmentation. The expression of PRGs, however, that act at lower levels in arthropods, and thus in close connection with the SPG network, suggests that these genes may also play a conserved function during segment formation and maintenance in onychophorans.

To further investigate this possibility we then investigated the expression patterns of SPGs and associated factors beyond previous studies on engrailed (en) and wingless (wg) expression (Eriksson et al., 2009). We found that these genes are expressed in highly conserved patterns in arthropods and the onychophoran suggesting that the interaction of these genes is also likely to be conserved. The conserved expression of SPGs and low-level PRGs relative to one another suggests that the interaction of these genes is a conserved feature of a clade Arthropoda+Onychophora, but that the function of PRGs early during segment formation represents an apomorphy of the Arthropoda.

Our data thus provide insight into the evolution of segmentation mechanisms in arthropods and the nature of the last common ancestor of the arthropods.

We also provide (in the supplementary data) a fine-scaled sequence of the most relevant embryonic stages in onychophoran development that are commonly investigated in situ hybridisation-based comparative analyses.

Material and methods

Animal husbandry and embryo preparation

Mature fertilised females of Euperipatoides kanangrensis were collected in Kanangra Boyd National Park, NSW, Australia. Embryos were dissected from the females from September to December to obtain all developmental stages. Each female carries around 30–60 embryos representing different stages often ranging from the 1-cell stage up to fully developed embryos that are close to birth. Embryonic membranes were removed manually with Dumont size 5 forceps and the embryos were fixed in 4% formaldehyde in 0.1 M phosphate buffered saline with 0.1% Tween-20 (PBST) (pH 7.4) for four to six hours at room temperature. The embryos were then dehydrated stepwise in 100% methanol and stored at −20 °C for at least three weeks prior to using them in hybridisation experiments.

RT-PCR and gene cloning

RNA isolation and cDNA synthesis were described in Janssen et al. (2004). Gene fragments of the pair rule gene orthologs even-skipped (eve), runt (runt), hairy/Hes (h/Hes), Hes2, Hes3, odd-skipped (odd), odd-paired (opa), sloppy-paired (slp) and pairbody (pby) have been isolated by means of PCR with gene specific primers based on the sequences found in a sequenced embryonic transcriptome. The transcriptome was made from embryonic stages 1 to 22 (Supplementary Fig. S1). These stages cover the full segmentation process. Total RNA was sent to Macrogen (South Korea). Library preparation was conducted according to the Illumina standard RNAseq protocol. Half of one Illumina HiSeq2000 lane resulted in 221,218,272 paired-end 101 bp reads. Low quality bases and Poly(A) stretches longer than ten bps were trimmed using custom perl scripts. The initial dataset was split randomly into two sub-datasets. The first dataset was 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. Assembled transcripts were then used as input (-long flag) for the second assembly. This resulted in 421,361 assembled sequences (470,016,133 bp). The N50 of the assembly is 2275 bp. The embryonic transcriptome contains sequences of all previously investigated Euperipatoides gene fragments that were isolated by means of RT-PCR with degenerate primers. We therefore believe that this transcriptome has a good coverage.

Gene fragments of hedgehog (hh), patched (ptc) and cubitus-interruptus (ci) have been amplified using the primers described in Janssen et al. (2004, 2008). We used the primer pair fw (TTY YTN GAR GGN GGN TGG TAY TG) and bw1 (GNL CYT CRT CRA ANA RCC AYT G) in an initial and the pair fw and bw2 (TCT AAT ARC CAY TGR AAN ACR AA) in a nested PCR to amplify a fragment of the Notum gene. Longer fragments of hh and ptc were obtained by RACE (GeneRacer cDNA amplification Kit, Invitrogen). All fragments were cloned into the pCR II vector (Invitrogen). Sequences of all fragments were determined by means of Big Dye chemistry on an ABI3730XL analyser by a commercial sequencing service (Macrogen). The sequences are available from the EMBL nucleotide database under Accession Numbers HG004114 (hh), HG004115 (ptc), HG004116 (ci), HG004117 (Notum), HG004118 (eve), HG004119 (run), HG004120 (h/Hes), HG004121 (Hes2), HG004122 (Hes3), HG004123 (odd), HG004124 (opa), HG004125 (slp), and HG004126 (pby).

In-situ hybridisation, cell nuclei staining and data documentation

In-situ hybridisation experiments were performed as described previously (Janssen et al., 2010). Digoxigenin (DIG) labelled RNA probes were transcribed from the entire cloned gene fragments. Cell nuclei were stained with 1 µg/ml DAPI (4-6-Diamidino-2-phenylindole) in PBST for 20 min followed by several washing steps in PBST. Embryos were analysed under a Leica dissection microscope equipped with a Leica DC100 digital camera. Brightness, contrast and colour values were adjusted in all images using the image processing software Adobe Photoshop CS2 (Version 9.0.1 for Apple Macintosh).

Phylogenetic analysis

Sequences of SPGs were compared to published sequences via BLAST search. Sequences of PRGs were aligned in Clustal_X (Thompson et al., 1997) (BLOSUM matrix (Henikoff and Henikoff, 1992)). Maximum likelihood analysis was performed with the Quartet Puzzling method (Strimmer and von Haeseler, 1996) as implemented in PAUP 4.0b10 (Swofford, 2002).
Results

Onychophoran pair rule gene and segment polarity gene orthologs

We isolated one single copy of each potential pair rule gene ortholog, i.e. even-skipped (eve), runt (run), hairy/Hes (h/Hes), odd-skipped (odd), odd-paired (opa), sloppy-paired (slp) and pairberry (pby), from a sequenced embryonic transcriptome. To determine orthology of the isolated fragments we conducted a simple phylogenetic analysis for eve, odd, opa and slp. It shows that the onychophoran genes cluster with their arthropod and mouse orthologs (Supplementary Fig. S2). For cases where this analysis failed to produce convincing results (unresolved trees), we provide alignments of conserved regions of onychophoran, arthropod and mouse genes (Supplementary Fig. S3). Onychophoran run and pby show strong similarity to their orthologs in other animals.

Three genes with similarity to hairy and enhancer of split homologues have been found in the transcriptome. Of these, the gene we designated as potential hairy/Hes ortholog, Ek-h/Hes, is similar to other arthropod hairy genes (Supplementary Fig. S3). It is, however, most similar to mouse Hes genes, indicating that it may have a diverged function from that of arthropod hairy genes. We isolated gene fragments with high sequence similarity to the signalling molecule Hedgehog (Hh), the Hh-receptor Patched (Ptc), the C2H2 zinc finger containing transcription factor Cubitus-interruptus (Ci) and the hydrolase Notum. Orthology of all gene fragments recovered in this study is unambiguous. These genes have unique conserved domains such as a DNA binding domain or other motifs that make them unlikely to be mixed up with other distantly related genes. For the SPGs we therefore only conducted a simple sequence similarity analysis searching GenBank using the alignment programme Gapped BLAST (Benson et al., 2003; Altschul et al., 1997).

Fig. 1. Expression of Euperipatoides even-skipped (eve) and runt (run). In all panels anterior is to the left. (A–D) Expression of eve, (E–G) Expression of run. (A) lateral view. Arrow marks the posterior pit that is free from expression. Arrowheads point to segmental stripes. Note that staining in the head is background, (B) ventral view. Close-up on the posterior region. Arrowheads as in (A), (C) ventral view, (D) close-up on the posterior end of the embryo shown in (C), (E) lateral view. Arrowhead points to expression in the optical region, (F) ventral view. Arrow marks segmental expression between the limb buds, (G) ventral view. Arrowheads point to segmental expression ventral to the limb buds. (A’) shows DAPI counter-staining of the same embryo as shown in (A). Abbreviations: a, anus; fap, frontal appendage; hl, head lobe; j, jaw; L, walking limb; pp, posterior pit; sp, slime papilla; st, stage.
Expression of onychophoran pair rule gene and segment polarity gene orthologs

The single onychophoran eve gene is exclusively expressed in the rear end of the developing embryos (Fig. 1A–D). While the posterior pit itself remains free from expression, eve is strongly expressed on either side of the posterior pit where the new segments are formed, and is ubiquitously expressed in the nascent segment(s). Faint stripes of expression are visible in the posterior region of the last few newly formed segments in early stages (Fig. 1A, and not shown) (until approximately stage 13 (Fig. 1B)). As the segments develop further (become older and thus located more anteriorly), the segmental expression of eve disappears. In later stage embryos eve is only present in a small domain to either side of the posterior pit (Fig. 1C/D). This expression remains throughout the investigated developmental stages (until stage 22 (Supplementary Fig. S1)). Notably, this expression is thus present after the last segment (L15) has formed.

The earliest expression of run is detectable in stage 10 embryos (Fig. 1E and Supplementary Fig. S4). At this stage it is expressed as single patches below the eyes. This remains the only detectable expression until at least stage 16. At stage 17 run is expressed in patches between and slightly ventral to the bases of the appendages until inclusively the fourth walking limb (Fig. 2F). At this point run is also strongly expressed in the anterior of the head bulbs (Fig. 2F). At subsequent stages the segmental expression progresses towards the posterior (not shown), and then successively disappears from older (i.e. more anterior segments). In stage 20 embryos, for example, this expression is only present between the posterior walking limbs of segments 11–14; expression between 14 and 15 has not yet appeared (Fig. 2G). At these later stages de novo expression appears in a complex pattern in the ventral nervous tissue (Fig. 2G). This pattern is present in all trunk segments in which the previously described segmental pattern has disappeared. The broad domain of expression in the head lobes is now refined to a position anterior to and between the bases of the frontal appendages. Weaker expression is in the posterior rim of the head lobes (Fig. 2G). The posterior end of the embryo, i.e. the posterior pit region, where new segments are patterned does not express run.

The onychophoran Hes-like gene with most similarity to arthropod hairy genes, h/Hes is expressed ubiquitously in early stage embryos. Expression in the mouth appears to be enhanced (Fig. 2A). Later expression is in the limbs and the head, except for the centre of the head lobes (Fig. 2B–D). Enhanced expression is at the posterior rim of the head lobes and at the anterior rim of the head lobes at the bases of the frontal appendages (Fig. 2D). Dorsal and ventral tissue (compared to the position of the limbs) does not, or only weakly expresses h/Hes. From approximately stage 16 on, two spots of expression appear in each limb, except the frontal appendages and the jaws (Fig. 2D/E). A second gene with similarity to arthropod Hes genes, Euperipatoides Hes2, is strongly expressed in the developing brain and the ventral nervous system, as well as in a dot-like pattern in the limbs and the tissue dorsal to the outgrowing limbs (Supplementary Fig. S5). At no stage is it expressed in a pattern that would suggest a role during segmentation. Expression of a third gene, Euperipatoides Hes3, could not be obtained with our in situ hybridisation technique. It is either active at stages earlier than stage 1, or at stages older than stage 22, or it is expressed at extremely low levels that remain undetected by our protocols.

Expression of odd first appears in the jaw-bearing segment and faintly in the slime papillae and anterior limb buds (Fig. 3A). This latter expression is inside the limb buds (except the jaws and the frontal appendages) (Fig. 3B/C). It is mesodermal and is likely located in the onychophoran nephridia. This assumption is

Fig. 2. Expression of Euperipatoides hairy/Hes (h/Hes). In panels (A–D) anterior is to the left. (A) Ventral view. Ubiquitous expression, except in the posterior pit. (B) lateral view. (C) lateral view. Arrowhead marks expression in the posterior rim of the head lobes. Asterisk marks expression anterior to the base of the frontal appendages. (D) Ventral view of the same embryo as shown in (C). Arrowheads and asterisk as in (C). Ventral view. Anterior is up. Close-up on a walking limb. Arrowheads point to dot-like expression. (B) and (C) show DAPI counter-staining of the same embryos as shown in (B) and (C). Abbreviations as in Fig. 1; m, mouth.
Expression of *pby* is reminiscent of that of a typical SPG ortholog in transverse segmental stripes in every segment, except the head segments anterior to the jaws (Fig. 5A). The intrasegmental position of *pby* is in the middle of the segment (Fig. 5B/C). The posterior end of the embryo does not express *pby*.

The onychophoran *hedgehog* (*hh*) ortholog is expressed in the posterior compartment of each trunk segment (Fig. 6A,C,E). In the head, *hh* is expressed at the posterior rim of the brain buds and anterior to the mouth between the two brain buds. The expression of *hh* is thus different from that of *en* in the head (cf. Eriksson et al., 2009). The segmental expression in the trunk (jaw to last leg bearing segment) lies in the very posterior of the appendages and reaches into the ventral tissue (Fig. 6A,C,E). Compared to the expression of *en*, *hh* is expressed in fewer cells and does not extend as far posterior as *en* in the ventral area of its region of expression (cf. Eriksson et al., 2009). Another difference is that *hh* appears considerably later in the segments compared to the very early appearance of *en* (cf. Supplementary Fig. S6 and Eriksson et al., 2009). The earliest detectable expression of *hh* is in the future mouth region (Supplementary Fig. S6). This latter expression is also known from arthropods, but there it appears relatively later (Janssen, 2012).

*patched* (*ptc*) is expressed in segmental stripes in the trunk (Fig. 6B,D,F). Its intra-segmental position is anterior to that of *hh* as it is obvious from the lack of expression in the posterior of the limb buds ((Fig. 6F)). The segmental expression is prominent in the appendages and the ventral tissue. In contrast to *hh*, expression of *ptc* extends into the ventralmost tissue of the developing embryo; the ventral extraembryonic ectoderm does not express *ptc*. In the head *ptc* is expressed along the posterior and ventral, but not the anterior and dorsal rim of the brain buds (Fig. 6D).

*Notum* is expressed in iterated stripes in each trunk segment, and likely anterior to *en* and *hh* expressing cell, as it is expressed in the middle of the limb buds (Fig. 8). It is expressed dominantly in the ventral ectoderm and faint expression is even seen in the ventral extraembryonic ectoderm (Fig. 8C). Expression is weaker ventral to the limb buds, and in the developing limbs *Notum* is only faintly expressed in the ventral mesoderm and the mesoderm of the tips of the limbs (but not of the frontal appendage) (Fig. 8D). This weak mesodermal expression in the limbs is reminiscent of the expression of *Notum* in the limbs of the spider *Cupiennius salei* (Pricic and Damen, 2005) and the myriapod *Glomeris marginata* (Janssen et al., 2008). A spot of *Notum* expression is visible at the ventral base of the appendages (Fig. 8D). In the head *Notum* is expressed in a domain in the oral lips, a lateral ectodermal spot in each of the two ocular regions, a mesodermal patch-like domain inside of each of the brain buds at the base of the frontal appendages, and a dorsal ectodermal half-ring in the fourth annulus of the frontal appendages (Fig. 8C and Supplementary Fig. S4). *Notum* is also expressed in the developing dorsal tube (heart) (Supplementary Fig. S4). The anterior border of expression in the heart lies in dorsal tissue aligned with the slime papilla segment.

**Discussion**

The potential role of onychophoran PRGs in segmentation

In *Drosophila* the expression of pair rule genes in transverse stripes in alternating segments is the first indication of metameric body patterning, and the knock-out of PRGs leads to defects in
alternating segments (Nüsslein-Volhard and Wieschaus, 1980). Although the expression patterns and most probably also the function of PRGs in *Drosophila* are different from those in most arthropods, many of these genes likely play prominent roles during segment formation even in basally-branching arthropods. The function of PRGs in non-insect arthropods has yet to be investigated, but gene expression studies indicate that most of the PRG orthologs are likely to have a function during segmentation (Damen et al., 2000, 2005; Hughes and Kaufman, 2002b; Chipman et al., 2004; Chipman and Akam, 2008; Janssen et al., 2011, 2012). Typically these genes are expressed in transverse stripes early during segment-patterning, either in the segment addition zone (the posterior pit area of onychophorans), or in the newly-formed segments. The so-called primary PRGs in *Drosophila* are *eve*, *run* and *h* (newer studies also include *odd* and *ftz* (Schroeder et al., 2011)). In arthropods with a short-germ developmental mode they are expressed in the segment addition zone and control the expression of the secondary (*odd*, *opa*, *slp*, *prd*) PRGs. The latter genes are expressed later, i.e. in the nascent segment or anterior in the segment addition zone, but not in the posterior of this zone. If the onychophoran PRGs were involved in segmentation they would most likely be expressed in patterns that are reminiscent of those in arthropods, i.e. in transverse segmental stripes in the posterior pit region and the nascent posterior segment(s).

Our data suggest that of the onychophoran PRGs only *eve* may have a function in segmentation, as it is the only gene that is expressed in the posterior pit area. Additionally, at least at earlier developmental stages, it is expressed in the last few nascent segments, in form of segmentation-gene typical transverse stripes. Notably, *eve*, the PRG that acts at the highest level in insects with both a long-germ developmental mode (e.g. *Drosophila melanogaster* (e.g. Pankratz and Jäckle, 1990)) and short-germ developmental mode (e.g. *Tribolium castaneum* (Choe et al., 2006; Choe and Brown, 2007)), and most likely also in basally-branching arthropods, is the only gene that is expressed in a segmentation-gene like pattern in

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**Fig. 4.** Expression of *Euperipatoides* *odd-paired* (*opa*) and *sloppy-paired* (*slp*). In all panels anterior is to the left. (A–D) Expression of *opa*. (E–G) Expression of *slp*. (A) Lateral view. Arrow points to expression in the head lobes. (B) same embryo as in (A). Ventral view. (C) Lateral view. Arrowheads point to the limb buds that do not express *opa*. (D) Ventral view. Arrowheads as in (C). Asterisks mark complex segmental expression along the trunk. (E) Lateral view. Arrow points to segmental expression ventral to the limb buds. (F) Same embryo as in (E). Ventral view. Arrowheads point to three domains of expression in the head lobes. Asterisk marks expression in the base of the frontal appendages. (G) Lateral view. Arrow as in (E) Note that the expression is now in more posterior segments. (H) Same embryo as in (G). Ventral view. (A) and (E) show DAPI counter-stainings of the same embryos as shown in (A) and (E). Abbreviations as in Fig. 1.
onychophorans. This suggests that eve may have been a key gene in the evolution of arthropod segmentation.

Onychophoran segmentation shows some peculiarities that set it apart from that of the arthropods, in particular in the reduced or absent segmentation of the ectoderm (Budd, 2001; Eriksson et al., 2009). It is thus possible that, if derived relative to the last common ancestor of onychophorans and arthropods, the apparent employment only of eve in onychophorans is a remnant of the arthropodan system. However, it seems at least morphologically from the fossil record that it is arthropodan segmentation that is derived relative to that of the onychophorans, which seem to exhibit the plesiomorphic state (Budd, 2001), and it thus seems reasonable in the absence of other information to consider the genetic basis for this also to be plesiomorphic. Whatever the evolutionary pattern is however, all other investigated onychophoran PRGs apart from eve are not expressed in a pattern that would suggest an important role in segment formation, since none of these genes are expressed in the posterior pit area. Other PRGs that act high in the PRG-network of either Drosophila or Tribolium, i.e. run, h/Hes and odd, do not appear to have a function during onychophoran segmentation at all.

The arthropod PRG orthologs that act low in the hierarchy, i.e. opa, slp and pby, however, are expressed in a segmental pattern that is reminiscent (for opa and slp) of that of segmentation genes such as the segment polarity genes (SPGs). The expression of opa resembles that of its ortholog in Glomeris, where it is expressed in tissue at the base of the limbs and in stripes between the limbs, but not in the limbs or ventral tissue aligned with the limbs (Janssen et al., 2011). Since this feature of opa-expression is conserved in an onychophoran and a millipede, it is likely that one of the ancestral functions of opa is the repression of limb development. Notably, in Glomeris opa is expressed in the position where the limb buds would grow in otherwise limbless segments (Janssen et al., 2011). It remains, however, unclear whether this late segmental pattern in Euperipatoides has any function during segment formation. The same is true for slp. In both the onychophoran and the myriapod slp is expressed at the base of the limbs, but not the limbs themselves, suggesting a (non-segmentation) function in the control of limb-development. Both genes opa and slp lack the typical expression in transverse ventral stripes that are reminiscent of the expression of SPGs in onychophorans and arthropods (Janssen et al., 2004, 2008; Eriksson et al., 2009; this study). The last of the onychophoran PRGs, pby, is expressed in a pattern that is typical for SPGs. pby is expressed in transverse stripes in the middle of each trunk segment, and thus anterior to en and likely co-expressed with wg (Eriksson et al., 2009; this study). The intra-segmental position of pby is thus conserved in onychophorans and arthropods (cf. Janssen et al., 2011). Notably, pby acts directly on the expression of SPGs in at...
least insects (Baumgartner and Noll, 1990; Choe and Brown, 2007), and is thus one of the genes that articulates PRG- and SPG-function. This strongly suggests that, despite the limited involvement of the PRGs in onychophoran segmentation, the SPGs network may be conserved to a much greater degree.

It has been shown that in Tribolium, eve acts as a primary PRG and that it regulates en and wg expression through prd and slp (Choe and Brown, 2009). The expression patterns of eve, prd and slp suggest that this regulatory network may be conserved even in onychophorans, and thus may indeed represent an ancestral component of the arthropod (and onychophoran) segmentation gene cascade.

**Gene expression suggests a highly conserved SPG-network in arthropods and onychophorans**

In all hitherto studied arthropods the SPG network appears to be widely conserved (e.g. Akam, 1987; Patel, 1994; Hughes and Kaufman, 2002b; Janssen et al., 2004, 2008; Damen, 2002). In Drosophila the SPGs are activated by the pair rule genes (PRGs) as transverse segmental stripes (e.g. Lawrence et al., 1987; DiNardo and O’Farrell, 1987; Ingham et al., 1988). The transcription factor en then activates the expression of the secreted signalling protein hedgehog (hh); en and hh are thus co-expressed, and this co-expression is conserved in all hitherto examined arthropods (e.g. Lee et al., 1992; Manzanares et al., 1993; Simonnet et al., 2004; Farzana and Brown, 2008; Janssen et al., 2004). In Euperipatoides this expression is similarly conserved, suggesting that the interaction and function of en and hh in segmentation are conserved in onychophorans. Notably, however, hh appears much later in the segments after they have formed, suggesting that the very early interaction of en and hh in defining the segment border is not conserved in onychophorans. In Drosophila and other arthropods Hh protein binds to the Patched (Ptc) receptor on neighbouring cells (Hidalgo and Ingham, 1990). In the onychophoran, ptc is expressed in cells anterior abutting en/hh expressing cells. The direction of Hh-signalling is therefore also conserved: en/hh expressing cells communicate with anterior abutting cells. Hh signalling towards posterior is impossible since posterior cells do not express ptc. As a result of Hh binding to Ptc, in Drosophila, the transcription factor cubitus-interruptus (ci), which is expressed in non-engrailed expressing cells, is activated and transferred into the nucleus where it positively regulates the transcription of wg (reviewed in Aza-Blanc and Kornberg, 1999). In Euperipatoides this interaction is also likely conserved since ci is expressed in virtually all cells that do not express en (e.g. Eaton and Kornberg, 1990; Orenic et al., 1990; Damen, 2002; Janssen et al., 2004, 2008; Farzana and Brown, 2008). Released Wg protein interacts with heparin sulphate proteoglycans that are anchored to the cell membrane (Reichsman et al., 1996; Tsuda et al., 1999; Baeg et al., 2001) and is then transported to neighbouring en-positive cells (Franch-Marro et al., 2005; Han et al., 2005). There it causes these cells to maintain en-expression (reviewed in Sanson, 2001; Damen, 2007). Notum (aka Wingful) is a negative regulator of Wg-signalling that suppresses the activation of Wg target genes, and is expressed in cells that receive high levels of Wg-signalling (Gerlitz

**Fig. 7. Expression of Euperipatoides cubitus-interruptus (ci).** (A) Lateral view. Anterior is to the left, (B) ventral view. Anterior is to the left, (C) ventral view. Anterior is up. Shown is the anterior part of the embryo, (D) close-up on the slime papilla. Same embryo and position as in (C), (E) anterior is to the left. Dorsal view. Arrow marks anterior border of segmental expression. (C’) Shows DAPI counter-staining of the same embryos as shown in (C). Abbreviations as in Fig. 1.
and Basler, 2002; Giraldez et al., 2002; Traister et al., 2008; Piddini and Vincent, 2009). Notum is co-expressed with segmental wg in Drosophila, other arthropods, and Euperipatoides (Giraldez et al., 2002; Prpic and Damen, 2005; Janssen et al., 2008; this study). The spatial expression of all investigated components of the SPG network is thus conserved among arthropods and onychophorans, and we therefore suggest that also the function of these segmentation genes is highly conserved in arthropods and onychophorans.

Implications for the origin of segmentation

Segmentation in short-germ arthropods, annelids, and vertebrates shares some morphological similarities. Single body units are added one by one (or in pairs of two) from a posterior region in the embryo. This is the segment addition zone in arthropods, the posterior growth zone in annelids, and the presomitic mesoderm in vertebrates. The cyclic expression of genes that is involved in the formation of new somites in vertebrates resembles dynamic, and as shown for Tribolium, even cyclic gene expression of for example some of the PRGs in the arthropod segment addition zone (Schoppmeier and Damen, 2005a; Chipman et al., 2004; Chipman and Akam, 2008; Janssen et al., 2011; Sarrazin et al., 2012; El-Sherif et al., 2012). The cyclic gene expression in vertebrates is under control of a so-called ‘segmentation clock’ including Notch/Delta signalling (Pourquie, 2003). It has been shown that Notch/Delta signalling is also involved during segmentation in the relatively basally branching arthropod Cupiennius salei (Stollewerk et al., 2003; Schoppmeier and Damen, 2005b). That, and the involvement of Notch/Delta signalling in basally branching insects suggest that the lack of Notch/Delta signalling in Drosophila segmentation is derived (Pueyo et al., 2008, Mito et al., 2011; but see Kainz et al. (2011) for contradicting results). Similarities to segment formation in arthropods, annelids, and vertebrates, however, suggest that segmentation in these (or two of these) phyla may trace from a common ancestor, the ur-protostomian or even the ur-bilaterian.

Recent work has provided good evidence that the SPG network may be conserved in annelids and arthropods, revitalizing the idea of a segmented common ancestor (= ur-protostomian) (Prud’homme et al., 2003; Dray et al., 2010; see Seaver and Kaneshige (2006) for an alternative interpretation). Our finding that the SPG network is also conserved in onychophorans strengthens this idea as it allows us to reconstruct the state at a node (last common ancestor (LCA) of arthropods and onychophorans) in the tree closer to the LCA of bilaterians compared to that of the LCA of only arthropods. If the onychophorans had not shown conservation of this feature, it would have cast doubt (without being conclusive) on the homology between the SPG network of arthropods and annelids.
On the level of PRGs in onychophorans, only eve appears to be involved in segment addition. Notably the same role has been suggested for eve in the annelid Platynereis dumerilii (DeRosa et al., 2005; see Seaver et al. (2012) for an alternative interpretation). A second P. dumerilii gene that is according to DeRosa et al. (2005) likely to be involved in segment formation is the para-Hox gene caudal (cad). We have investigated the expression of Euperipatoides cad and found that its expression is indeed very similar to that of eve (Supplementary Fig. S4) suggesting involvement in segment formation. Altogether this implies, that annelids and onychophorans at least share the same (or very similar) interaction of SPGs, and the possible involvement of eve and cad during segmentation. Again, whether the recruitment of these genes is a matter of conservation or convergence remains unclear.

Divergent aspects of onychophoran SPG expression

Despite the overall conservation of the expression patterns of the SPGs (and associated factors), some aspects of expression are divergent. In Drosophila and other arthropods, ptc is initially expressed in broad segmental domains or in the complete blastoderm (in Drosophila) (Hooper and Scott, 1989; Nakano et al., 1989). Later, ptc is down-regulated in en-positive cells. As a consequence ptc is expressed in two stripes per segment, one anterior to en and one posterior to en (Hidalgo and Ingham, 1990). The conserved expression pattern of ptc in a myriapod and a chelicere suggest that regulation and function of the ptc receptor is conserved in all arthropods (Janssen et al., 2008; Akiyama-Oda and Oda, 2010). In contrast, in Euperipatoides, ptc is only expressed anterior to the en/hh expressing cells, but not posterior to them. In arthropods, the T-box transcription factor paralogous midline and H15 are expressed in the ptc-expressing cells posterior to the en/hh domain (Buescher et al., 2004; Pppic et al., 2005; Janssen et al., 2008). For at least Drosophila it has been shown that it acts as a repressor of Hh-signalling (Buescher et al., 2004). We have isolated a single H15 gene from the onychophorans Euperipatoides and found that it is not expressed posterior to the en/hh cells (unpublished data). This observation is congruent with the finding that ptc is not expressed in these cells in onychophorans either: since ptc is not expressed posterior to en/hh, Hh signalling towards posterior is not possible, and thus the repressing action of H15 on Hh-signalling is not required.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.07.010.

References


