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Hedgehog signaling regulates segment formation in the annelid *Platynereis*

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Abstract

Annelids and arthropods share a similar segmented organization of the body whose evolutionary origin remains unclear. The Hedgehog signaling pathway, prominent in arthropod embryonic segment patterning has not been shown to have a similar function outside arthropods. We show that the ligand *Hedgehog*, the receptor *Patched*, and the transcription factor *Gli* are all expressed in striped patterns prior to morphological segment appearance in the annelid *Platynereis dumerilii*. Treatments with small molecules antagonistic to Hedgehog signaling disrupt segment formation. *Platynereis Hedgehog* is not necessary to establish early segment patterns but is required to maintain them. The molecular similarity of segment patterning functions of the Hedgehog pathway in an annelid and in arthropods supports a common origin of segmentation in protostomes.

In the fruitfly, the axial patterning of each individual segment is controlled and maintained by two signaling pathways operating across the segment: Wnt/ β -catenin and Hedgehog (1, 2). The function of the Hedgehog (Hh) pathway in segment formation is conserved in other holometabolous insects (3) and probably also in non-insect arthropods (4, 5). By contrast, mesodermal somites in vertebrates are patterned by non-homologous segment polarity genes (6). Here, we investigated the role of the Hh pathway during segment formation in an annelid representative of the third great branch of Bilaterians, Spiralian (Fig S1). The nereid *Platynereis dumerilii* presents two phases of segment formation: larval metamorphosis and juvenile posterior growth (Fig S2). We cloned by degenerate PCR four genes of the Hh pathway in *Platynereis* coding respectively for orthologs of the ligand *Hedgehog* (*Pdu-hh*), its receptor *Patched* (*Pdu-ptc*), the transmembrane activator *Smoothened* (*Pdu-smo*) and the transcription factor *Cubitus-interruptus/Gli* (*Pdu-Gli*) (7, predicted primary structures in Fig S3, phylogenetic trees in Fig S4). *Pdu-hh*, *Pdu-ptc* and

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Pdu-Gli are expressed in segment polarity-like patterns strikingly reminiscent of their arthropod counterparts, i.e. in ectodermal segmental stripes visible before segment anlagen become apparent both during larval (Fig 1A, B, D, E, G, H, Fig S5A-F) and posterior growth (Fig 1C, F, I, Fig S5G-L) (detailed descriptions in Supporting Online Text). The thin stripes of *Pdu-hh* expression are located at the anterior border of each segment (Fig S5G, H), mostly in the same cells as two previously identified *Platynereis* segment polarity-like genes orthologous to *engrailed* (8) and *NK4/tinman* (9). The broader stripes of *Pdu-ptc* are centered on these *Pdu-hh/en/NK4* stripes spreading on each side of the segment border (Fig S5I, J) while *Pdu-Gli* shows a complementary pattern in the middle of each future segment (Fig S5K, L). *Pdu-smo* does not show distinctive patterns in whole mount *in situ* hybridization (WMISH) until the 3-segmented larva when it is expressed predominantly in the pharyngeal area (Fig S6). Real-time PCR measurements nevertheless show that *Pdu-smo* is expressed at low, presumably ubiquitous levels early on in trochophore development and that maternal mRNAs are present in fertilized eggs (Fig S6), similar to the early embryos of the fruitfly and vertebrates (10-12).

These expression patterns suggest that the Hh pathway is involved in segmental patterning in *Platynereis*. However, striped expressions of Hh pathway genes appears distinctly later during trochophore development (Fig S5A-F) than those of the previously identified *Platynereis* segment polarity-like genes *Pdu-en* (8), *Pdu-NK4* and *Pdu-Lbx* (9). Thus the Hh pathway might play a late role in segment formation, in shaping the individual segments after the specification of the segmental pattern by other genes. To test this hypothesis, we used two small molecules, cyclopamine (13) and SANT-1 (14), known for antagonizing Hh signaling in vertebrates by binding to Smo proteins (7). We defined three time windows for treatments (Fig S7): an early trochophore window before any component of the Hh pathway is distinctly expressed in stripes (20 to 30 hpf), a mid-trochophore window when *Pdu-hh*, *Pdu-Gli* and *Pdu-ptc* are all expressed in stripes (32 to 48 hpf) and a late trochophore window (54 hpf onward) when striped expression for *Pdu-ptc* and *Pdu-Gli* fade, but still a few hours before the larva starts to elongate and to form externally visible segments (15). Mid-trochophore treatments with increasing doses of cyclopamine or SANT-1 on mixed batches of trochophores coming from several females gave remarkably similar abnormal larvae (Fig S8.I and II). They fail to develop properly shaped segments at 3 days: They do not elongate properly, remaining ovoid in shape (Fig 2 A, B, Fig S8.II.A-C); Segmental grooves do not appear (Fig 2A, B, yellow arrows) and segmental trochae (circular ciliary belts borne by larval segments) are reduced (Fig S8.II.D-F); Appendage outgrowths and chaetae do not appear (Fig S8.II.A-C). In addition, the axonal scaffolds of the ventral nerve cord and segmental peripheral nervous system (Fig 2C, D, Fig S8.II.D-F) both appear underdeveloped and disorganized. In place of the metameric peripheral nerves (red asteriks), an asymmetric proliferation of axons projecting haphazardly to the periphery forms, indicating that the segmental pattern of the nervous system is lost. The effects of two unrelated drugs and of different treatment time windows on embryos suggest that the phenotypes obtained are caused by blocking Hh signaling (Fig S8, Supporting Online Text). In particular, the phenotypes cannot be due to a general toxicity of the drugs since late trochophore treatments at a time of massive proliferation and morphogenesis cause few defects (Fig S8.II.G-K). We also tested the role of *Pdu-smo* on posterior segment addition by treating individuals with cyclopamine after caudal regeneration. One half (12/24) of treated individuals show regenerated posterior parts with fewer, abnormally shaped segment anlagen and reduced segmental grooves (Fig S12).

To analyze further the effects of cyclopamine on the segmental pattern, we looked at the expression patterns of the previously known segment polarity-like genes in cyclopamine treated larvae (7). To take into account a strong maternal effect polymorphism in the response to cyclopamine (Supporting Online Text and Fig S11A), we carefully selected only

those batches of embryos that presented medium and strong morphological phenotypes (scored in 3-day larvae) for the gene expression study. Expression patterns for medium strength morphological phenotypes are presented in Fig 2E-L and Fig S10. Complete results including 1 or 2 repeat experiments for each gene indicate that the effects of cyclopamine on gene expression correlate well with the strength of the phenotype in each same-female batch studied (Fig S11B). Early-stage cyclopamine treatment has no effect on the striped expressions of *Pdu-en*, *Pdu-NK4* and *Pdu-Lbx* (Fig S10 and Fig S11B) seen in 30 hpf larvae, showing that *Pdu-Smo* does not act on the segmental pattern independently of the morphogen *Pdu-Hh* and that the Hh pathway does not intervene in the initial laying of the segmental pattern. By contrast, mid-trochophore cyclopamine treatment at high dose leads to a complete or almost complete loss of striped expression for *Pdu-hh*, *Pdu-en*, *Pdu-Wnt1* (Fig 2E, F, G, respectively), showing that the segmental pattern is largely affected well before metamorphosis. *Pdu-ptc* is also downregulated (Fig S9) as it is observed in *hh*-mutant flies (16) and vertebrates lacking *shh* expression (17). *Pdu-Gli* (Fig 2I) stripes are mostly persistent even at high doses of cyclopamine. The specificity of the effects of mid-trochophore cyclopamine treatment on the patterning of the segmental ectoderm is demonstrated by the persistence of gene expression in tissues other than segmental ectoderm. Hence the expression of *Pdu-en* and *Pdu-Lbx* in segmental mesoderm (Fig 2F and 2K, red arrows), the stomodeal expression of *Pdu-hh* and *Pdu-Wnt11* (Fig 2E and 2H, blue asterisks) and the pygidial expression of *Pdu-Wnt11* and *Pdu-Cdx* (Fig 2H and 2L, green asterisks) are maintained whereas the proctodeal expression of *Pdu-Wnt1* is slightly enlarged (Fig 2G, green asterisks).

The effect of cyclopamine on *Pdu-hh* stripes must be indirect if we suppose that the direct targets of Hh signaling are only those cells expressing *Pdu-Gli*, away from segment borders. In *Drosophila* (Fig 3), *wingless* (*wg*) expression is maintained by Hh signaling in the anterior compartment just anterior to the *en* stripe whereas *wg* signaling is necessary to maintain *en* and *hh* striped expression in the posterior compartment (18). In *Platynereis*, *Pdu-Wnt1*, the orthologue of *Drosophila wingless* (8), is expressed just anterior to *Pdu-en*/*Pdu-hh* stripes on the other side of segmental grooves. Its strong downregulation by cyclopamine (Fig 2G) is consistent with a loop of regulation similar to the one known in *Drosophila*. *Ladybird*, the fly gene orthologous to *Pdu-Lbx* is expressed in epidermal stripes overlapping *wingless* stripes in the anterior compartment of the epidermis and is positively regulated by *wingless* (19). By contrast, cyclopamine treatment in the annelid does not abolish completely *Pdu-Lbx* stripes in trochophores (Fig 2K), suggesting that other factors are involved in its maintenance. Another difference between the annelid and the fly is that *Pdu-Wnt1* is not expressed in complete circular stripes in the trochophore (it does during posterior growth) but only in the lateral parapodial field (Fig 2G). It was thus interesting to look at *Pdu-Smo* regulation of other *Wnt* genes potentially involved in segment formation. *Pdu-Wnt11* is expressed in thick stripes in the posterior halves of segments and is strongly downregulated by cyclopamine (Fig 2H). *Pdu-Wnt5* is expressed in the anterior halves of segments and is by contrast much more resistant to cyclopamine (Fig 2J).

Our work demonstrates the involvement of the Hh pathway in segment formation outside of arthropods. It comes in contrast with earlier studies of *hh* orthologues in annelid species (20, 21). The comparison of the effect of Hh signaling inhibition in *Platynereis* and in insects, i.e. the fruitfly and the coleopteran *Tribolium* (3), reveals extensive similarities. As in insects, *Platynereis* Hh presumably diffuses anteriorly to maintain *Wnt* signaling anterior to the *hh* expressing cells (Fig 3), which may in turn be crucial to maintain segment boundary gene expressions, including *Pdu-hh*. As in insects, Hh is not necessary in the initial setting of the segmental pattern but is required to maintain this pattern before the morphological appearance of segments. The comparison of segment polarity gene patterns between *Platynereis* and arthropods (Fig 3) reveals four independent players with remarkably similar

expressions: *engrailed*, *ladybird/Lbx*, the Hh pathway and *Wnt1/wingless* signaling. The most likely explanation of these similarities is that these genes were already playing similar roles in a metameric protostome ancestor. The alternative explanation would be a parallel recruitment of these genes for similar functions in annelids and arthropods. Since they are not known to be part of a conserved core regulatory network or “kernel” (22) that might have been co-opted *en bloc*, each gene would have been recruited independently, which seems unlikely. Altogether, these four players are expressed in the same spatial relationship across the annelid segment boundary as they are across the parasegmental boundary in arthropods (Fig 3). This suggests that these two boundaries are homologous as was proposed earlier (8). Therefore, the segmented exoskeleton of the arthropods must have evolved out of frame with the ancestral protostome segmentation (Fig S13). This ancestral protostome segmentation is nowadays “recapitulated” as parasegmental patterning in the embryos of extant arthropods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression patterns of *hh* (A-C), *ptc* (D-F) and *Gli* (G-I) orthologues in *Platynereis* late trochophores (48 hpf), early three-segmented larvae (72 hpf) and during posterior growth. All views are ventral except I, lateral right (Lat). Anterior or apical is up. Black dashed line: prototroch of larvae. The stomodeum is delimited by a red dashed circle. Larval segment anlagen are numbered 1-3. Posterior growth panels (post. gr., C, F, I) show patterns in posterior regenerates one week after caudal amputation, in front of the thin segment addition zone (yellow dashed line). Scale bars represent 50 μ m. py: pygidium.

Figure 2.

Effects of mid-trochophore stage cyclopamine treatments on the development of *Platynereis* larvae. (A-D) 3-day larvae stained with an anti-acetylated tubulin antibody revealing the ciliary belts (trochae) (A, B) and the axonal scaffold of the nervous system (C, D). (A, B) are mid-body frontal confocal sections of Hoechst 33342 stained larvae whereas (C, D) are ventral frontal confocal sections. (E-L) Effects of cyclopamine treatment on the expressions of segment polarity-like genes and *Pdu-Cdx*, a pygidial ectoderm marker. The first and second columns show expression in untreated larvae and in the majority of treated larvae, respectively. Pie graphs show the frequencies of abnormal expressions with normal in blue, abnormal in red and the maternal batch variability in violet where available. Small fractions of abnormal expressions in the untreated control reflect both a small proportion of spontaneous abnormal development and the probe-dependent imperfect sensitiveness of the WMISH protocol. Prototrochs: dashed black line. Stomodeum: white dashed circle and "s". p: prototroch. 1, 2, 3: larval segments in A, B, segmental trochae in C, D. Yellow arrows: segmental grooves. Red asteriks: main lateral nerve roots. n: protonephridium. h: head lobes. Red arrows, blue asterisks and green asterisks indicate persistent mesodermal, stomodeal or pygidial/proctodeal expressions, respectively.

Figure 3.

(A) Comparison of the antero-posterior extension of striped expression patterns of proven and presumed segment polarity orthologous genes in *Platynereis* and *Drosophila*. Segment boundaries and limb anlagen positions are represented with a schematic body wall section in black. Orthologous genes are color-coded whereas segment polarity-like genes specific to *Platynereis* are represented by gray rectangles. The morphogenetic actions of the diffusible Hh proteins are represented with blue arrows. A white arrow with question mark indicates that Hh action on the anterior parts of annelid segments is not proven in this study. Unlike in fly, *Pdu-ptc* is expressed in *en/hh* positive cells and *Pdu-Gli* expression does not abut *en/hh* positive cells in *Platynereis*. (B) Comparison of expression patterns of some of these segment polarity-like genes in the early stages of appendage formation. The homology of annelid segments with arthropod parasegments implies that annelid parapodia do not correspond in embryonic position with the arthropod appendages, suggesting that these appendages do not share a common origin in the protostome ancestor. PSB: parasegmental boundary.