

Arthropod-like Expression Patterns of *engrailed* and *wingless* in the Annelid *Platynereis dumerilii* Suggest a Role in Segment Formation

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Summary

The origin of animal segmentation, the periodic repetition of anatomical structures along the anteroposterior axis, is a long-standing issue [1] that has been recently revived by comparative developmental genetics [2–6]. In particular, a similar extensive morphological segmentation (or metamerism) is commonly recognized in annelids and arthropods. Mostly based on this supposedly homologous segmentation, these phyla have been united for a long time into the clade Articulata [7, 8]. However, recent phylogenetic analysis [9–10] dismissed the Articulata and thus challenged the segmentation homology hypothesis [11]. Here, we report the expression patterns of genes orthologous to the arthropod segmentation genes *engrailed* and *wingless* in the annelid *Platynereis dumerilii*. In *Platynereis*, *engrailed* and *wingless* are expressed in continuous ectodermal stripes on either side of the segmental boundary before, during, and after its formation; this expression pattern suggests that these genes are involved in segment formation. The striking similarities of *engrailed* and *wingless* expressions in *Platynereis* and arthropods may be due to evolutionary convergence or common heritage. In agreement with similarities in segment ontogeny and morphological organization in arthropods and annelids, we interpret our results as molecular evidence of a segmented ancestor of protostomes.

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Results and Discussion

Recent phylogenetic studies' results (see Figure 1) and comparative molecular analyses [12–15] have challenged the long-standing hypothesis of the homology of segmentation between arthropods and annelids. In order to address this contentious issue, we have undertaken a study of segment formation in an annelid representative, *Platynereis dumerilii*. Among the genes that are essential for segment formation in arthropods are the segment polarity genes, including *engrailed* and *wingless*. These genes define parasegments, which are primary metamer units upon which adult segments will later form [16–19]. For this study, we have cloned orthologs of *engrailed* (*Pdu-en*, see the Supplemental Data available with this article online) and *wingless* (*Pdu-wnt1* [20]) in the polychaete annelid *Platynereis dumerilii* and have examined their expression patterns.

We chose *Platynereis dumerilii* as a species for which segment formation mechanisms would hopefully be primitive among annelids. In *Platynereis*, as in most annelids, segment formation relies on the sequential addition of an indefinite number of new segments from a subterminal posterior growth zone during postlarval development. These segments are morphologically identical (homonomous segmentation), and this is in opposition to more-derived species in which various patterns of tagmatization exist [21]. As in most annelid species, the three anterior-most segments form simultaneously during larval ontogeny and exhibit developmental and morphological larval specificities. In addition, most annelids, including *Platynereis*, are capable of caudal regeneration. After a posterior truncation, the pygidium (the terminal-most structure) and the growth zone are rapidly regenerated from a blastema, and segment formation restarts similar to normal growth (see the Experimental Procedures). As segment formation in *Platynereis* proceeds through distinct cellular mechanisms during larval and postlarval development, we examined gene expression patterns during both phases.

Pdu-en and *Pdu-wnt1* Expression Patterns during Postlarval Development

During posterior growth, both during normal juvenile segment formation (Figure 2A) and after caudal regeneration (Figures 2B–2F), *Pdu-en* is expressed in ectodermal circular stripes in developing segments. This segmental expression appears in continuous rings of cells immediately after the growth zone has produced them (in younger, posterior-most segments) and persists in differentiating (more anterior) segments (Figures 2A–2D). The pattern is more complicated on the ventral face, as, in addition to the continuous segmental expression, *Pdu-en* is expressed in mesodermal groups of cells and in forming ganglia of the ventral nerve cord (Figures 2C and 2D, arrowheads). A longitudinal section shows that the segmental stripes of expression occur long before segmental coelomic cavities or segmental boundaries

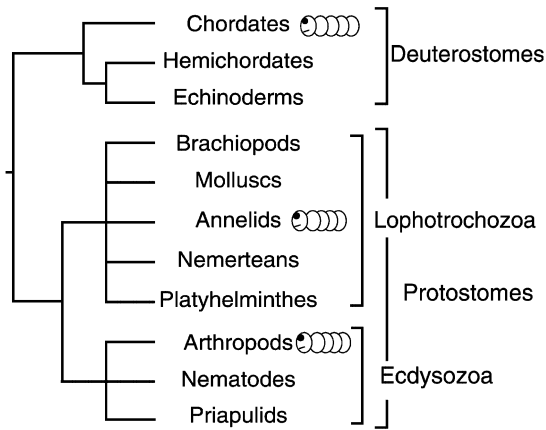


Figure 1. The Phylogeny of Bilaterian Animals

This consensus molecular phylogenetic tree (simplified from [10]) shows the distribution of overtly segmented phyla, namely, annelids, arthropods, and chordates, in the three main branches of the tree, lophotrochozoans, ecdysozoans, and deuterostomes, respectively. Each of these segmented groups is more closely related to unsegmented phyla than to each other. Of note in this tree, the Articulata, a group gathering annelids and arthropods, is dismissed, and so the hypothesis of the homology of the segmentation between these two phyla is challenged.

are visible (Figure 2E, arrowhead). As segments mature, it becomes apparent that continuous segmental stripes of *Pdu-en* expression are always restricted to the anterior-most row of epidermal cells within a segment immediately posterior to the forming segmental groove corresponding to the actual segmental boundary (Figure 2F, arrowheads). These segmental grooves are the only ones to form and do not seem to shift during segment differentiation, as indicated by the relative position of an appendage marker, *distal-less* (data not shown). Hence, this expression pattern suggests that during postlarval growth in *Platynereis*, *engrailed* is involved both in the establishment of the segmental boundaries in the ectoderm and in the specification of particular cell types in the mesoderm and the central nervous system.

Pdu-wnt1 is also expressed early in ectodermal stripes in each developing segment both during normal juvenile segment formation (Supplemental Data) and after caudal regeneration (Figures 2G–2I), although the signal level is much weaker compared to that in *Pdu-en*. *Pdu-wnt1* is expressed in the posterior-most ectodermal cells of each developing trunk segment, immediately anterior to the segmental boundary (Figures 2H and 2I, arrowheads). In contrast with *Pdu-en*, the thickness of *Pdu-wnt1* stripes increases in proportion with the segment length (Figure 2G). *Pdu-wnt1* is also expressed in the posterior part (Figures 2G and 2I, arrows) and in an anterior-proximal spot of the parapodia, as well as in the proctodaeum (Supplemental Data).

Based on morphological landmarks (i.e., segmental grooves), our results suggest that *Pdu-en* and *Pdu-wnt1* are expressed in adjacent domains on either side of the segmental boundary and play a role in the formation and maintenance of this boundary. According to our observations, *Pdu-en* and *Pdu-wnt1* are most likely expressed in directly neighboring cells. However, due to

technical difficulties with double in situ stainings, we have not succeeded yet in ascertaining this point.

Pdu-en and *Pdu-wnt1* Expression Patterns during Larval Development

Platynereis develops through a typical trochophore larval stage [21]. The trochophore rapidly metamorphoses and exhibits a head and three trunk segments that form almost simultaneously. In contrast with postlarval segment formation, no coelomic cavities form, and the first morphological manifestations of segment formation are the appearance of three sets of internal chaetal sacs that appear simultaneously from 24 hr postfertilization (p.f.) and will later evaginate to form the parapodia (Supplemental Data). Additional signs of morphological segmentation appear progressively from 48 hr p.f. In particular, epidermal differentiation proceeds in a posterior to anterior direction (opposite to the direction shown in juvenile growth), as indicated by the sequential formation of rings of ciliated cells (trochae) on larval segments (Figures 3A and 3B). These trochae that form in the posterior third part of the segment are specific to the larval segments, as they are not found in postlarval segments (not shown). Then, the body elongates and segmental grooves form.

The expression of *engrailed* appears very early during *Platynereis* embryogenesis. *Pdu-en* is expressed in the dorsal-posterior area of the postgastrula embryos in two bilateral territories of the presumptive larval ectoderm (Supplemental Data). At 18 hr p.f. (Figure 3C), *Pdu-en* is expressed in two transversal stripes, extending ventrally, and a third (Figure 3D) and fourth (Figure 3E) stripe are soon visible. Stripes of *Pdu-en* expression are restricted to superficial cells and correspond to the limit between the head and the anterior-most segment, the margins between larval segments, and the limit between the posterior-most segment and the pygidium (Figure 3E).

Pdu-wnt1 expression is only observed from around 48 hr p.f. as segmental rings made of epidermal cells in each larval segment (Figure 3F). These rings of *Pdu-wnt1* expression are larger than the *Pdu-en* staining and appear progressively from posterior to anterior segments. This *Pdu-wnt1* larval expression occurs after *Pdu-en* segmental expression but before the formation of trochae and segmental grooves and correlates with the direction of segmental epidermal differentiation.

Expression Patterns of *engrailed* and *wingless* Suggest a Role in Segment Formation in *Platynereis*

During postlarval segment formation, *Pdu-en* and *Pdu-wnt1* are expressed in continuous and circular stripes of ectodermal cells that lie on either side of the forming segmental boundaries. These specific expression patterns are strikingly similar to those found in arthropods and, therefore, are highly suggestive that *engrailed* and *wingless* are involved in the segment formation in *Platynereis*. During larval segment formation, *Pdu-en* is segmentally expressed before any sign of morphological segmentation, while *Pdu-wnt1* expression appears later, but before segment epidermal differentiation. However,

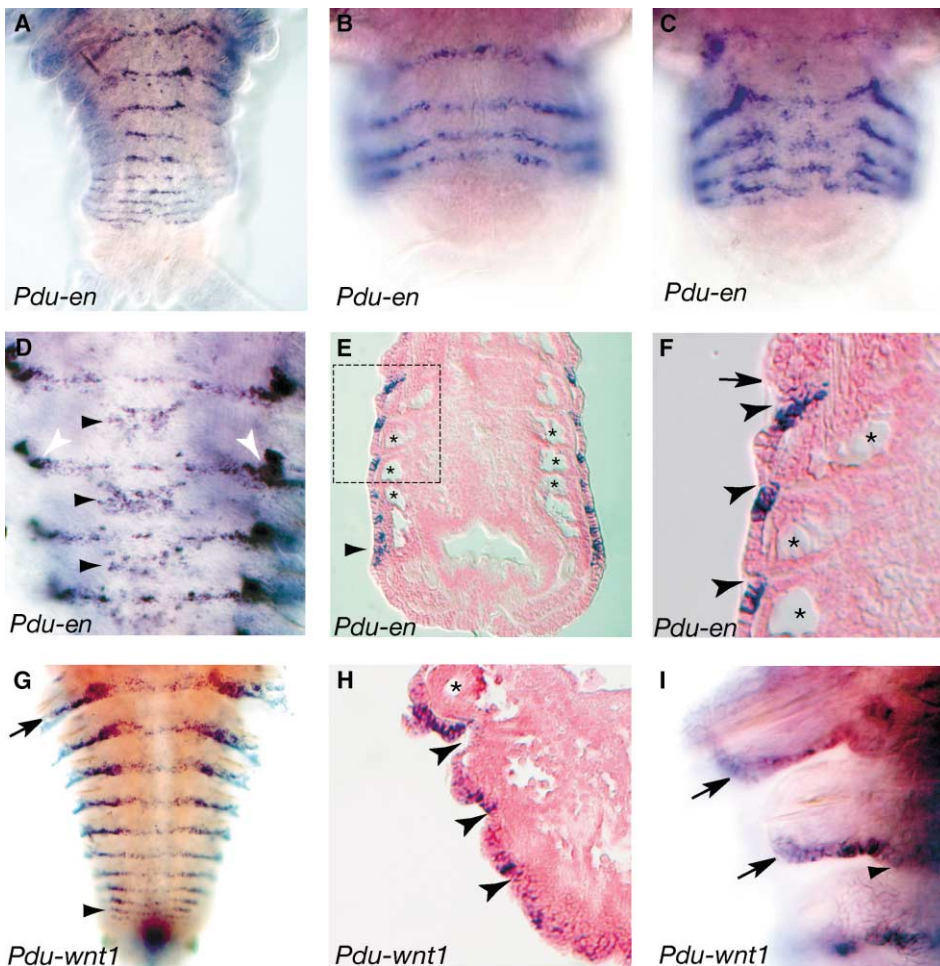


Figure 2. Expression Patterns of *Pdu-en* and *Pdu-wnt1* during Postlarval Development

(A) A ventral view of a worm during normal juvenile growth. Segments are produced and develop sequentially. Distinct stages of segment development (the posterior-most segment being the youngest) can thus be observed in a single individual. *Pdu-en* expression encircles each developing segment; as segments mature the space between two consecutive *Pdu-en* stripes becomes larger. The focus is on ventral stripes. (B and C) (B) Dorsal and (C) ventral views of a young regenerating worm showing that the circular expression of *Pdu-en* appears early during segment formation.

(D) A ventral view of more differentiated segments. *Pdu-en* is expressed in cells forming the ganglia of the central nervous system (black arrowheads) and in bilateral mesodermal derivatives, probably a subpart of the nephrostome (white arrowheads).

(E and F) Longitudinal sections of a regenerating worm. (F) Higher magnification of the framed area in (E) showing forming segments in which segment boundaries are starting to appear. *Pdu-en* is segmentally expressed in stripes before morphological segmentation (arrowhead in [E]). In each forming segment, only the anterior-most row of ectodermal cells adjacent to the segmental boundary (arrowheads in [F]) expresses *Pdu-en*. The posterior part of a given parapodium corresponds to the posterior of the trunk segment (arrow in [F]).

(G) The *Pdu-wnt1* expression pattern after caudal regeneration (ventral view). The expression appears very early during segment formation (arrowhead) as segmental stripes both in the trunk and in the posterior part of the parapodia in more mature segments (arrow).

(H) A longitudinal section showing that *Pdu-wnt1* is expressed in the posterior-most rows of ectodermal cells in the trunk, just anterior to the forming segmental boundaries (arrowheads).

(I) *Pdu-wnt1* is expressed in the ectoderm in the posterior part of parapodia and in the posterior-most row of cells in trunk segments. The arrowhead indicates the segmental boundary.

Black stars indicate coelomic cavities in regenerating animals. Anterior is oriented toward the top in all panels.

it must be stressed that postlarval segmentation mechanism by sequential addition of new segments from a posterior growth zone is highly conserved in annelids and is certainly ancestral. In contrast, larval segmentation displays tremendous morphological diversity and relies on derived cellular mechanisms that result from an acceleration of normal development [21].

Are These Arthropod-like Expression Patterns of *engrailed* and *wingless* in *Platynereis* Due to Evolutionary Convergence?

There are two ways to interpret the similarities of *engrailed* and *wingless* expression patterns in *Platynereis* and arthropods. Either these similarities are due to the recruitment of these two genes in segment formation

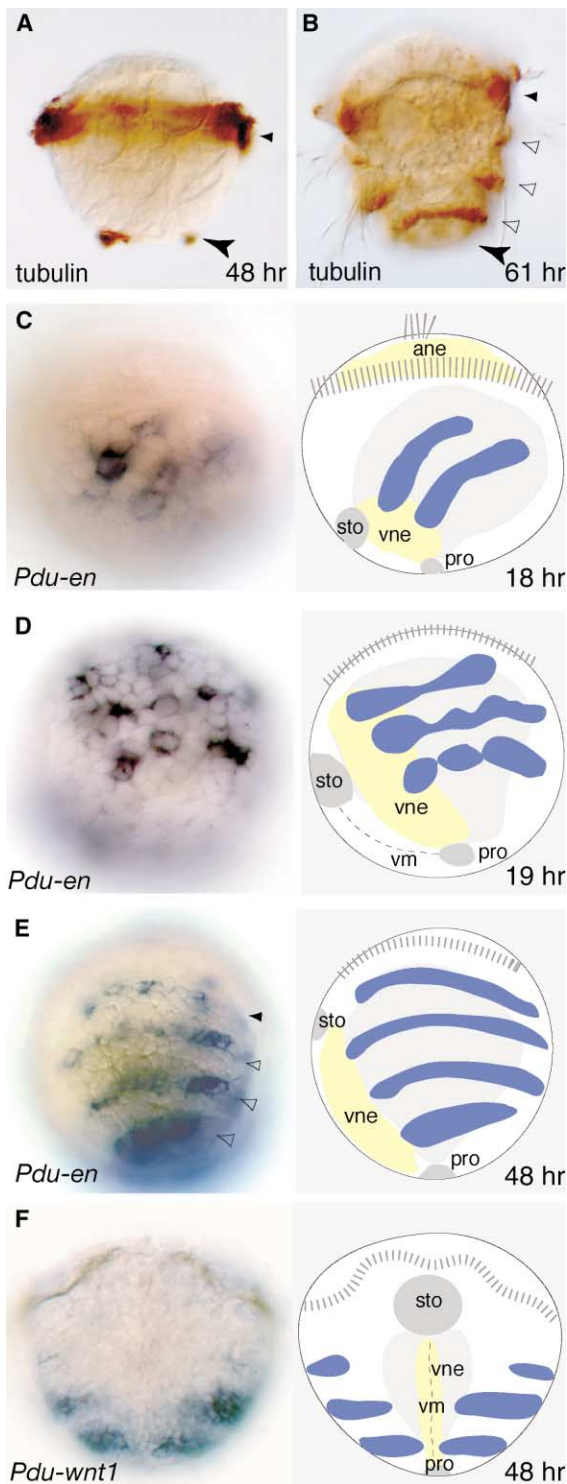


Figure 3. Expression Patterns of *Pdu-en* and *Pdu-wnt1* during Larval Development

(A and B) α -tubulin stainings of a (A) 48 hr p.f. and a (B) 61 hr p.f. larva showing the position of rings of ciliated cells. (A) At 48 hr p.f., only the prototroch (small arrowhead) and the telotroch (large arrowhead) are visible on the nonsegmented anterior and posterior part of the larva, respectively. At this stage, segmental grooves have not yet developed. (B) At 61 hr p.f., segmental grooves are visible

independently in arthropods and *Platynereis*, or these specific expression patterns were already established in the common ancestor of arthropods and *Platynereis* (i.e., the common ancestor of all protostomes) and have been conserved in both groups.

The *engrailed* expression pattern has been described in a few other annelid species. In all of them, *engrailed* is only expressed in subsets of specific precursor cell types that are themselves distributed in a segmentally iterated pattern, notably in the nerve cord or chaetoblasts, and thus does not play a general role in segment formation [12–15]. This argues for an independent recruitment of *engrailed* in segment formation in arthropods and *Platynereis*. However, it should be noted that species for which data are available, two clitellates and a chaetopterid [12–14], are highly derived with respect to segment formation, so it would be possible that in these species, *engrailed* has lost an ancestral segmentation function. A similar loss of segmentation function scenario has been demonstrated for some key arthropod segmentation genes, for instance, *even-skipped*, which is not involved in segmentation in some insects [11]. As evolutionary relationships among the distantly related annelid families are poorly resolved, it is not currently possible to determine when segmentation function of *engrailed* has been gained or lost during annelid evolution.

Based on their role in parasegmental boundary formation in *Drosophila*, it has been proposed that *engrailed* and *wingless* have been recruited for similar function in various developmental systems [22]. However, these genes have been very rarely reported as being directly involved in morphological boundary formation other than segments. A well-known example of recruitment of *engrailed* and *wingless* is for the formation of the midbrain-hindbrain boundary in the vertebrate nervous system [23]. However, *engrailed* is expressed on both sides of the vertebrate midbrain-hindbrain boundary, so the spatial relationships of *engrailed* and *wingless* are not the same as in *Drosophila*. Gene expression similarities reported in this study concern comparable morphological structures (segments). So, if these similarities were indeed due to independent recruitments of *en-*

and segmental rings of ciliated cells differentiate in a posterior to anterior direction (open arrowheads). The small arrowhead indicates the prototroch; the telotroch is out of focus (large arrowhead).

(C and D) Lateral views of a (C) 18 hr p.f. and a (D) 19 hr p.f. larva. Ectodermal stripes of *Pdu-en* expression are one cell row wide and extend ventrally as morphogenesis of the larva proceeds.

(E) A lateral view of a 48 hr p.f. larva. *Pdu-en* expression persists and is restricted to epidermal cells of the larval trunk segments. *Pdu-en* expression outlines segment boundaries. The black arrowhead marks the head-trunk boundary; open arrowheads mark the trunk segment boundaries

(F) A ventral view of a 48 hr p.f. larva. *Pdu-wnt1* is expressed in epidermal cells of larval segments before segmental grooves are visible.

Stomodaeum (sto) and proctodaeum (pro) anlage (dark gray), yolky midgut anlage (light gray), neurectoderm (vne, ventral neurectoderm and ane, anterior neurectoderm; yellow), and gene expression patterns (blue) are shown; the stippled line represents the ventral midline (vm). Anterior is oriented toward the top.

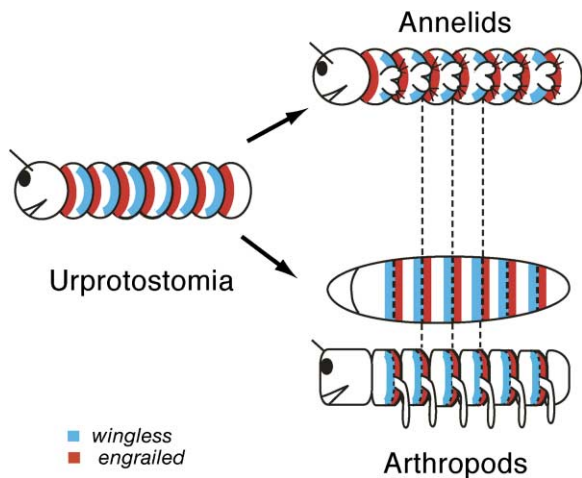


Figure 4. A Hypothetical Scenario of the Evolution of Segmentation in Protostomes

engrailed and *wingless* expressions would define the segmental unit of the body plan in the common ancestor of arthropods and annelids ("Urprotostomia"). This ancestral segmental unit corresponds to parasegments in arthropods and to adult segments in annelids. Arthropods' transient embryonic parasegments would be the only trace of these ancestral segmental units. The black dotted lines indicate parasegmental boundaries. *Urprotostomia* is arbitrarily represented limb-less.

grailed and *wingless* in *Platynereis* and arthropods, then this example would constitute an extreme case of convergence.

A Scenario for the Evolution of Segmentation in Protostomes

An alternative explanation to these striking similarities in gene expression patterns between *Platynereis* and arthropods is that these similarities reflect an evolutionary conservation. In arthropods, *engrailed* and *wingless*, which are essential for segment formation, are expressed on either side of the transient parasegmental boundary. Our results suggest that in *Platynereis*, *engrailed* and *wingless* are expressed in similar spatial relationship but across the segmental boundary. These data raise the interesting hypothesis that annelid segments may be homologous with arthropod parasegments (Figure 4). In arthropods, definitive adult segments form later through a specific process of resegmentation. A similar process occurs in vertebrates, in which vertebrae are formed out of phase with the mesodermal somites. So there are several examples in which embryonic segmented structures are not in register with the morphological definitive segmented structures they form.

Conclusions

Although the hypothesis of evolutionary convergence cannot be ruled out at this stage of analysis, we propose that our data are molecular and developmental evidence that extensive segmentation (i.e., metamerism) is ancestral to annelids and arthropods and thus to all protostomes. Studying the expression of *engrailed* and *wingless* and additional arthropod segmentation genes in some other annelid species may solve this debate.

Our evolutionary scenario of ancestral metamerism in protostomes implies that extended segmentation has been secondarily lost or reduced during evolution of various protostome phyla. Such a scenario would explain why many seriated organs or structures are still seen in organisms that belong to nonmetameric phyla such as molluscs. Indeed, this scenario is consistent with the segmental expression of *engrailed* in stripes in a chiton [24] and with the recent description of a fully segmented fossil mollusc [25]. Our results suggesting ancestral segmentation in protostomes are in agreement with the hypothesis of ancestral segmentation in Bilateria that so far has only been supported by data from a limited number of taxa [3, 4, 6] and certainly requires the comparative analysis of mesodermal segmentation between chordates and annelids [26].

Experimental Procedures

Animal Culture

Larval stages and adults were obtained from established breeding cultures in Gif, Heidelberg, and Mainz.

Regenerating Worms

The rate of juvenile segment formation is quite slow and variable among individuals, and in situ hybridizations on juvenile worms often yield high background. Because of these practical difficulties, we preferred to analyze gene expression patterns in regenerating worms. After a posterior amputation of a few segments, worms rapidly form a blastema that regenerates the pygidium (the terminal posterior structure that bears the anus) and the growth zone. The regenerated growth zone starts the sequential production of new segments, but at a much higher rate compared to normal growth. As we have always observed similar gene expression patterns in nonregenerating and in regenerating worms (after 7 days), we conclude that segmentation mechanisms are fundamentally similar during normal growth and after regeneration (this study and unpublished data).

Supplemental Data

Supplemental Data including additional data and detailed Experimental Procedures and an *Engrailed* sequences alignment are available at <http://www.current-biology.com/cgi/content/full/13/21/1876/DC1/>.

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Accession Numbers

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