

# Stochastic WNT signaling between nonequivalent cells regulates adhesion but not fate in the two-cell leech embryo

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**Background:** In the leech *Helobdella robusta*, an annelid worm, the early pattern of cell divisions is stereotyped. The unequal first cleavage yields cells AB and CD, which differ in size, cytoplasmic inheritance, normal fate, and developmental potential.

**Results:** Here we report a dynamic and transcription-independent pattern of WNT signaling in the two-cell stage of *H. robusta*. Surprisingly, HRO-WNT-A is first expressed in a stochastic manner, such that either AB or CD secretes the protein in each embryo. This stochastic phase is followed by a deterministic phase during which first AB, then CD expresses HRO-WNT-A. When contact between the cells is reduced or eliminated, both AB and CD express HRO-WNT-A simultaneously. Finally, bathing embryos in anti-HRO-WNT-A antibody during first cleavage reduces the adhesion between cells AB and CD.

**Conclusions:** Our findings show that the stochastic phase of HRO-WNT-A signaling in the two-cell stage of *Helobdella* is negatively regulated by cell-cell contact and that this early signaling affects cell adhesion without affecting cell fate. We speculate that the primordial function of *wnt* class genes may have been to regulate cell-cell adhesion and that the nuclear signaling components of the *wnt* pathway arose later in association with the evolution of diverse cell types.

## Background

Studying development in species with fixed cell lineages (such as ascidians, leeches, nematodes, and sea urchin [1–4]) allows experimental analysis at the level of individually identified cells. An axiomatic assumption is that this stereotypy is based on invariant patterns of transcription, translation, secretion, etc, i.e., that homologous cells have the same molecular identity from embryo to embryo. Here we present a counter example to this assumption by showing a dramatic variability in the secretion of a WNT class protein in the two-cell stage in embryos of the glossiphoniid leech, *Helobdella robusta*.

Glossiphoniid leech eggs are fertilized internally and escape from meiotic arrest upon zygote deposition (0 hr after zygote deposition, or AZD). The first two divisions are roughly meridional but slightly unequal so that yolk-deficient cytoplasm is segregated into blastomere D, precursor of the segmental mesoderm and ectoderm (Figure 1). The third round of cell division is obliquely equatorial and highly unequal in all quadrants, and it yields quartets of animal micromeres (a'–d') and vegetal macromeres (A'–D'). Cell cycles are shorter in D lineage cells than in other cells; thus, cell CD divides before cell AB, and cell D divides before cells A, B, and C (Figure 1). Here we analyze the pattern of HRO-WNT-A secretion in embryos up to the division of the cell to form D' and d'.

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*Wnt* class genes encode secreted glycoproteins that act locally to regulate cell adhesion, transcription, and cell polarity [5–8]. *Wnt* class genes have been described in all three major bilaterian animal groups and exist as multi-gene families in many species [9]. Research on insects, nematodes, and vertebrates has revealed the existence of a well-conserved molecular pathway, which in *Drosophila* has been shown to act by affecting cytoplasmic levels of a  $\beta$ -CATENIN class protein [10, 11]. A *wnt* class gene designated *htr-wnt-A* was identified in the glossiphoniid leech *Helobdella triserialis*, and its expression was studied immunohistochemically. The polyclonal antibody used was shown to give similar staining patterns in embryos of other glossiphoniid species from the five-cell stages to the beginning of gastrulation [12]. Here we examine the pattern of secretion of leech WNT-A protein during earlier stages of development (zygote to five-cell stage, 0–7 hr AZD, Figure 1) in a closely related species, *H. robusta*.

## Results

In normal embryos, the first indication of HRO-WNT-A signaling is seen approximately 30 min after the appearance of the first cytokinetic furrow (approximately 270 min AZD). At this time, both cells stain faintly for HRO-WNT-A, but in only a small proportion of the embryos examined. These results suggest that this pattern persists for less than the approximately 5 min time window used

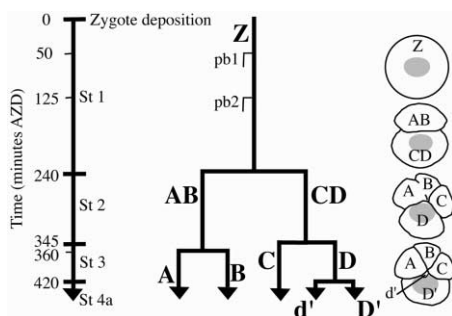
to synchronize embryos (see Materials and methods). Throughout most of the rest of the two-cell stage (275–345 min AZD), strong staining is found in just one cell, either AB or CD, in each embryo. This period of staining comprises an initial stochastic phase (275–295 min AZD), during which half the embryos show AB staining and half show CD staining (Figure 2a). The stochastic phase is followed by a deterministic phase (300–345 min AZD), during which AB stains in almost all embryos from 300–315 min AZD and CD stains in almost all embryos from 325–345 min AZD (Figure 2a). During the 3–4-cell stage, 345–420 min AZD (Figure 1), only weak HRO-WNT-A staining is observed, in cells C and D (Figure 2a). As cell D begins dividing to form macromere D' and micromere d', 420 min AZD (Figure 1), HRO-WNT-A is once again secreted in a stochastic pattern. Macromere D' and micromere d' never stain, but cells A, B, and C stain in various combinations (Figure 2a). Beginning approximately 10 min after it has been born (i.e., approximately 430 min AZD), micromere d' stains strongly in all embryos, as reported previously ([12]; not shown).

The inferred rapid changes in the pattern of immunostaining suggest that this early expression of HRO-WNT-A is not controlled at the level of transcription. Despite numerous attempts, we were unable to observe *Hro-wnt-A* transcripts by in situ hybridization (data not shown). This suggests that the transcripts were of low abundance. Thus, we were unable to determine the relative levels of *Hro-wnt-A* transcripts in cells AB and CD. However, semi-quantitative RT-PCR revealed that *Hro-wnt-A* transcripts

were present maternally and throughout stages 1–8, including stages where no immunostaining for HRO-WNT-A was detected (Figure 2b; in these experiments, we used *Hro-wnt-A* oligos that span an intron to avoid problems of genomic contamination, and we amplified 18S rRNA in parallel for each sample to control for variability in RNA yield between samples). This suggests that the early patterns of HRO-WNT-A expression can be regulated post-transcriptionally. Further support for this conclusion was obtained by the immunostaining of staged embryos that had been injected in  $\alpha$ -amanitin approximately 2 hr before the onset of the first cleavage. As described previously [13], such embryos cleaved normally through the end of stage 4. We found that the pattern of HRO-WNT-A staining during stage 2 in  $\alpha$ -amanitin-injected embryos was as in the controls except that cell CD stained less intensely than cell AB during the stochastic phase, and it did not stain at all during the deterministic phase (Figure 3). It has been shown previously that, while large-scale zygotic transcription is not activated until approximately 27 hr AZD in *Helobdella* [13], zygotic transcription of *wnt-A* begins at least as early as stage 5 [12]. Our results suggest that zygotic transcription of *Hro-wnt-A* may actually begin during stage 2 and that HRO-WNT-A expression during stage 2 reflects a mixture of maternal and zygotic transcripts.

The existence of the stochastic phase suggests that AB and CD participate as equals in the initial signaling despite the fact that they differ in volume, cytoplasmic inheritance, cell cycle duration, and developmental potential [2, 15]. The reciprocity of HRO-WNT-A secretion during the stochastic phase in the two-cell stage suggests that cell-cell communication is involved in coordinating HRO-WNT-A secretion between cells AB and CD. Either negative or positive regulation could be at work. To distinguish between these alternatives, we removed zygotes from their fertilization membranes (we devitellinized them) [14], and cells AB and CD were separated at first division by the application of gentle pressure along the cytokinetic furrow with a siliconized glass needle ([15], Materials and methods) as cytokinesis reached completion (approximately 275 min AZD). In these isolated pairs of blastomeres, both cells always stained for HRO-WNT-A (Figure 3), and these results suggest that the normal pattern results from inhibitory interactions between these two nonequivalent cells. Further support for this conclusion comes from the observation that both cells also stained in devitellinized but otherwise intact embryos (Figure 3). In such embryos, the extent of cell-cell contact between AB and CD is reduced during cell division relative to such contact in the controls, and the restoration of contact is delayed during the two-cell stage (Figure 4), [15]. In these devitellinized embryos, the simultaneous staining of AB and CD persisted until the last 20 min of stage 2 (325–345 min AZD), at which time staining ceased

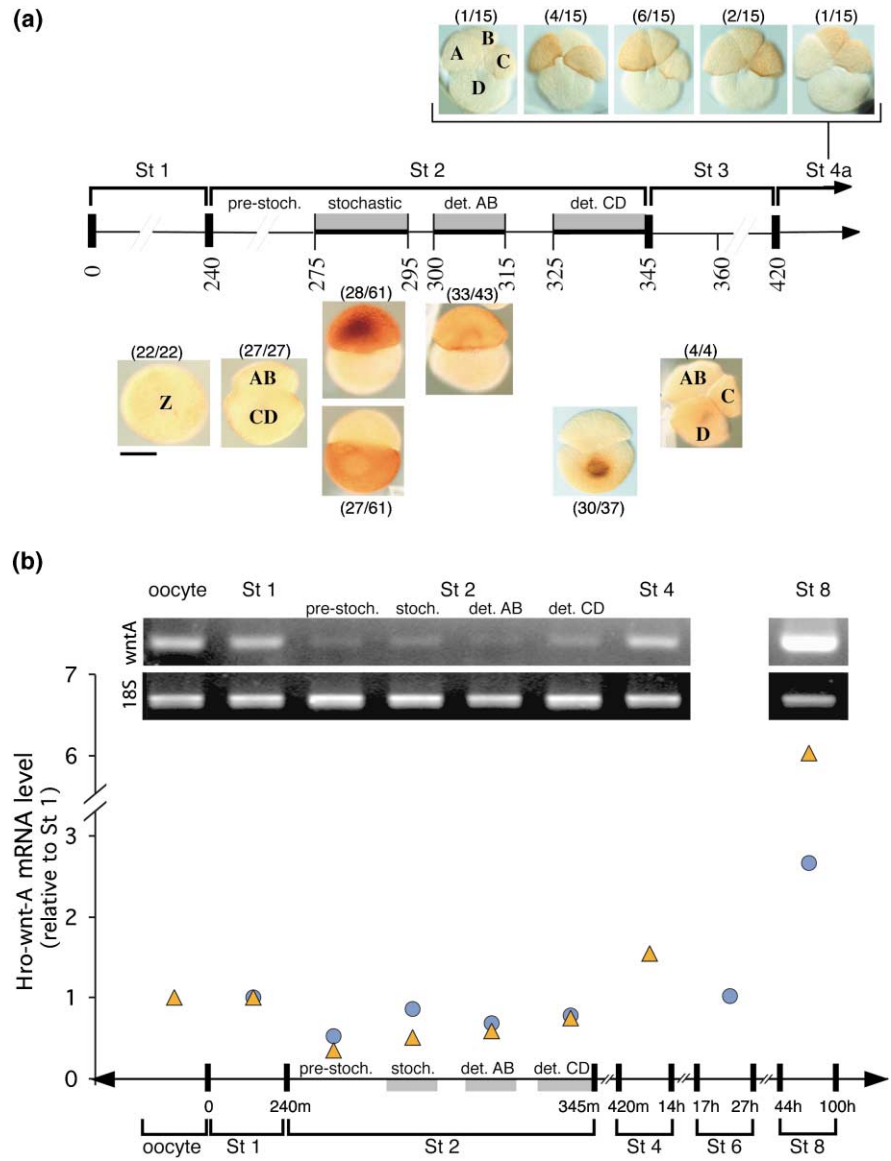
Figure 1



Lineage diagram for stages 1–4a of *Helobdella robusta*. In this hermaphroditic annelid, eggs are fertilized internally and arrest in metaphase I of meiosis until after zygote (Z) deposition. Cleavages (horizontal lines) are stereotyped, and this along with the fact that most are unequal gives rise to identified cells (AB, CD, A, B, C, D, D' and d'; denoted by vertical lines). Corresponding developmental stages (St) and time (minutes) after zygote deposition (AZD) are indicated on the timeline at left. Line drawings at right represent embryos viewed from the animal pole. Shading indicates yolk-deficient cytoplasm, which arises from cytoplasmic reorganization following polar body (pb) formation and is inherited by the D lineage precursor cell in the early divisions.

**Figure 2**

Normal patterns of *Hro-wnt-A* protein and mRNA expression in early development. **(a)** Photographs of embryos immunostained for HRO-WNT-A at various times and viewed from the animal pole. The timeline is as in Figure 1. Cell identities are indicated for one specimen of each stage. Preliminary experiments revealed little or no HRO-WNT-A secretion prior to 275 min AZD or during stage 3 (see text for details). Thickenings of the timeline indicate time periods during which all batches of embryos gave the same distribution of staining patterns. Numbers in parentheses indicate the distribution of different staining patterns for each period. At various time points, a minority of embryos (not shown) either failed to stain or showed faint and hybrid patterns consistent with transitions in the staining pattern. In particular, 9/9 embryos fixed at 320 min AZD gave faint staining of both AB and CD (not shown). The scale bar represents 200  $\mu$ m. **(b)** Semiquantitative RT-PCR analysis reveals maternal and zygotic expression of *Hro-wnt-A*. Top: ethidium bromide-stained gel showing *Hro-wnt-A* and 18S rRNA bands from the stages indicated. This is the raw data for experiment 1. Bottom: graph showing the relative levels of *Hro-wnt-A* at the stages indicated, with stage 1 defined as 1. Triangles (orange) and circles (blue) represent data from experiments 1 and 2, respectively (see Materials and methods for details).



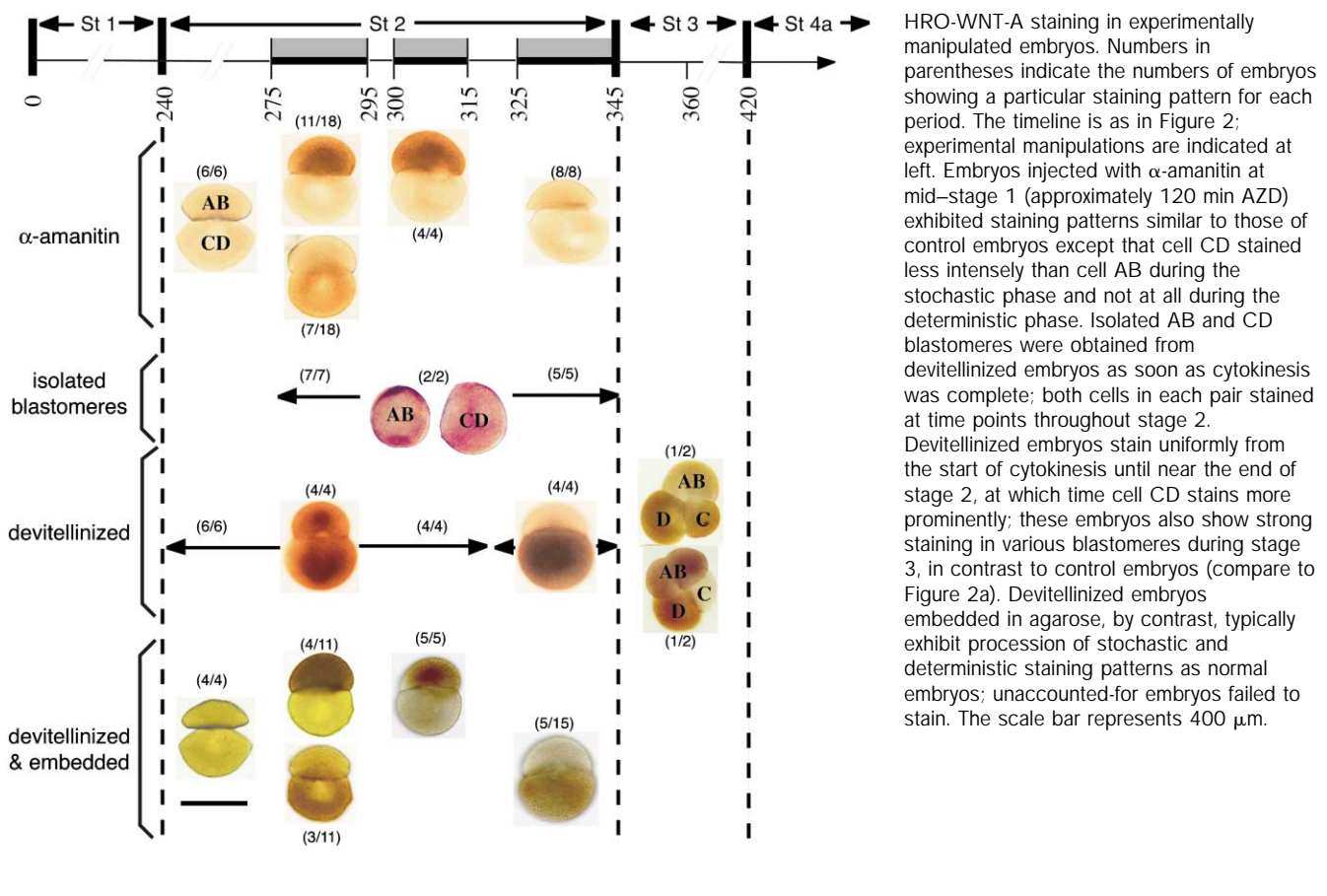
in AB but persisted in CD, as in control embryos of an equivalent age (Figure 3).

Two additional aspects of this last experiment are of particular interest. First is the fact that strong HRO-WNT-A staining in devitellinized embryos is observed as soon as cytokinesis begins, i.e., more than 30 min earlier than in control embryos; these devitellinized embryos also exhibit abnormal staining during stage 3 (Figure 3). This cannot be a fixation artifact because no staining is observed in devitellinized embryos before the onset of cytokinesis, and no such precocious staining is seen in devitellinized embryos that have been embedded in agarose (see below). Second, these pronounced changes in the HRO-WNT-A expression pattern have no noticeable effect on cell fate,

as judged from the orientation or timing of subsequent cell divisions and from the phenotypes of the definitive progeny of cells AB and CD. We show here, as has been shown previously [14, 15], that devitellinized embryos can develop normally at least through stage 10, by which time the entire body plan is well established and segments are differentiated.

To further test the hypothesis that both cells stained in devitellinized embryos because of the reduction in (inhibitory) cell contacts, we embedded devitellinized zygotes in low-melt agarose ([16], see Materials and methods). When these embedded embryos underwent the first cleavage, AB and CD were constrained to remain in close contact despite the absence of the fertilization membrane.

Figure 3

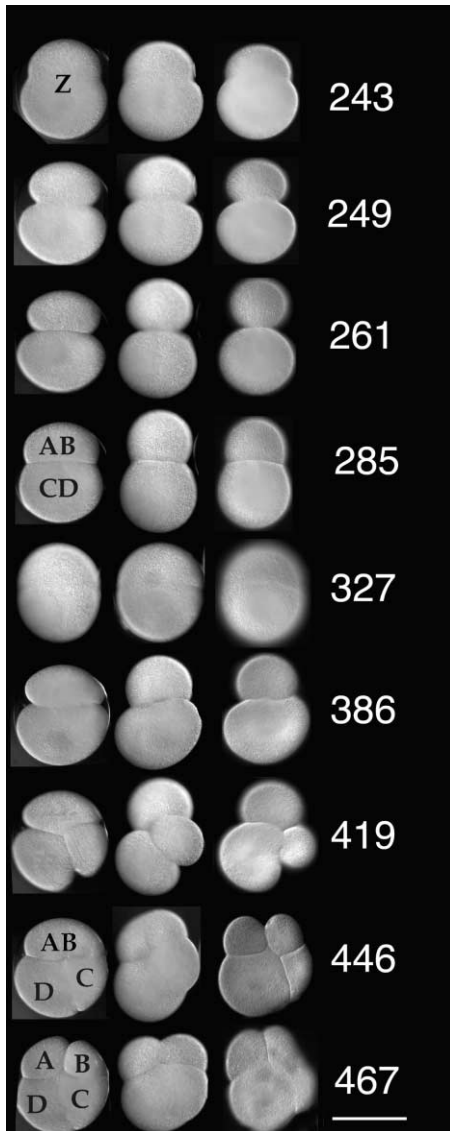


In these embryos, both the normal staining levels and the normal complementary patterns of HRO-WNT-A secretion were observed (Figure 3).

These results demonstrate that HRO-WNT-A secretion is regulated in a negative manner during the stochastic phase of stage 2 by the extent of contact between AB and CD. Moreover, these results suggest that HRO-WNT-A signaling may function in part to restore cell contacts after cytokinesis by modulating cell-cell adhesion, presumably by the regulation of  $\beta$ -catenin and thus  $\alpha$ -catenin and cadherin function [10]. To begin to test this possibility, we assayed the adhesivity of AB and CD in two-cell embryos derived from devitellinized zygotes bathed continuously in either normal HL medium [17], anti-HRO-NOS (a polyclonal antibody to the leech NANOS homolog [16]; 1/100 in HL medium), or anti-HRO-WNT-A (1/300 in HL medium). We gauged adhesivity by attempting to separate the two cells with a siliconized glass needle as described above. Immediately after cytokinesis was complete (approximately 275 min AZD; see Figure 4), AB and CD could be separated with equal success in all three groups of embryos; separating the cells required only a few seconds for each embryo. But by approximately 300

min AZD, the cells in embryos bathed in HL medium with or without anti-HRO-NOS (six embryos in each condition) were very difficult to separate. More pressure was required, and the embryos rolled back and forth with the motion of the needle. It took approximately 2 min to successfully separate the cells in each embryo, and even at this rate, cells lysed in one–two embryos of each type. In contrast, when embryos were bathed in anti-HRO-WNT-A, the probe slid between the two blastomeres as in control embryos immediately after cytokinesis, and in two of nine embryos, AB and CD separated spontaneously. Thus, we conclude that in the two-cell *Helobdella* embryo, one effect of HRO-WNT-A signaling is to increase cell-cell adhesion. All else being equal, increases in adhesion between two cells should lead to an increased area of contact between them that is analogous to the zippering together of two velcro strips. Having also shown that increasing cell-cell contact downregulates HRO-WNT-A secretion, we propose that HRO-WNT-A signaling in the two-cell stage of *Helobdella* serves in part as a homeostatic mechanism for reestablishing and/or maintaining embryonic integrity after the first cleavage. Note that the identity of the signaling pathway by which cell-cell contact downregulates HRO-WNT-A signaling remains to be de-

Figure 4



Selected images from a time lapse video comparing first and second cell divisions in synchronized sibling embryos that were either intact (left), devitellinized (center), or devitellinized and bathed in anti-HRO-WNT-A. In this experiment, images were taken every 3 min for approximately 4 hr, starting at the onset of first cleavage (243 min AZD). For each row of images, the number at the far right indicates developmental time (minutes AZD). The images in each row were aligned digitally. Cell labels in the lefthand column indicate the completion of each mitosis; thus, the zygote (Z) cleaved at approximately 273 min AZD, cell CD cleaved at approximately 431 min AZD, and cell AB cleaved at approximately 452 min AZD. The scale bar represents 400 microns.

terminated. Moreover, since we cannot demonstrate any effects on cell fate when HRO-WNT-A signaling is perturbed, another formal possibility is that WNT-A signaling in the early leech embryo has no real function in *Helobdella*.

## Discussion

The WNT signaling described here in the two-cell *Helobdella* embryo is by definition the earliest possible point at which intercellular embryonic signaling can occur. Genetic and embryological evidence suggests that, in the nematode *Caenorhabditis elegans*, a *wnt* class gene (*mom-2*) is expressed in cell P2 at the four-cell stage. This expression affects cell polarity and fate in the adjacent EMS cell [7, 8]. With our present results, this suggests the possibility that WNT signaling among early blastomeres was a feature of the last common ancestor of ecdysozoans and lophotrochozoans. In this case, one might predict that the leech *wnt-A* and nematode *mom-2* genes should be orthologs. Constructing phylograms for the *wnt*-gene family is problematic due to the limited amount of useful sequence, the large numbers of duplications in this gene family, and the absence of a close outgroup. Nonetheless, some support for such a relationship comes from preliminary phylogenetic analyses (A. E. B., unpublished data).

Garcia-Bellido applied the term “syntagma” to the analysis of development to designate a group of genes or gene products involved in a given developmental operation and linked by direct interactions [17]. For studies of comparative development at the molecular or genetic level, this concept leads to the notion that the conservation, modification, and/or new use of such interacting groups of genes, rather than individual genes, are essential components of the evolution of developmental mechanisms [18]. The WNT signaling pathway is one example of a syntagma that is widely conserved and used in numerous developmental processes across diverse species [9–11]. Our results strongly suggest that the WNT signaling pathway is also operating in leeches.

Cell adhesion and cell fate specification were two key innovations in the evolution of metazoans from unicellular organisms. In most studied cases, WNT signaling affects developmental cell fates through modulation of cell-cell adhesion, cell polarity, and/or transcription. A priori, it seems unlikely that this complex functionality arose as a full-blown evolutionary novelty. Instead, we propose that the primordial role of WNT signaling was in just one of these processes. In the four-cell stage of *C. elegans*, for example, MOM-2 signaling by cell P2 affects spindle orientation and transcription in cell EMS with no obvious effects on cell adhesion [19]; from this, one might entertain the possibility that the primordial WNT syntagma affected just transcription and/or cell polarity. Here, in contrast, we have found an example in which WNT signaling regulates cell adhesion without affecting cell fate at all. This raises another possibility, namely that the primordial WNT syntagma comprised only those genes necessary for the regulation of cell adhesion. This primordial syntagma might have functioned primarily to regulate the integrity of primitive (cellularly homogeneous) multicellular ani-

mals. In this view, nuclear signaling components of the WNT syntagma would have been linked to it later in evolution as a mechanism for generating cell diversity.

## Materials and methods

### Isolation of *Hro-wnt-A*

From the sequence of *wnt-A* from *H. triserialis* (Kostriken and Weisblat, 1992), we designed primers *wntA-A*<sup>+</sup> (AGCCAGCAAGGAGTCAGCG TTIGT, in the single-letter nucleic acid code) and *wntA-B*<sup>-</sup> (GGC GTCTAAAAAGTTTTTCGCAIAA), and we amplified a 196 bp region of the *wnt-A* gene from *H. robusta*. We sequenced this fragment and designed new primers *wntA-C*<sup>+</sup> (CGTCTCTAAGAGTACTG) and *wntA-F*<sup>-</sup> (TTGACGTTTCGCACTACATC) to obtain additional sequence with 3'RACE and 5'RACE by using an *H. robusta* cDNA (stage 7–10) library in pBluescript SK (Lambda ZAP II, Stratagene) as a template. For 3'RACE, the first round of PCR was performed with the gene-specific primer *wntA-A*<sup>+</sup> and the vector primer M13(–20), and the second round of PCR was performed with the gene-specific primer *wntA-C*<sup>+</sup> and the vector primer KS. For 5'RACE, the first round of PCR was performed with *wntA-B*<sup>-</sup> and M13(–20), and the second round of PCR was performed with *wntA-F*<sup>-</sup> and KS. Both first-round and second-round PCRs were performed with 1X PCR buffer (Perkin-Elmer Cetus), 2.0 mM MgCl<sub>2</sub>, 200 μM dNTP, 0.6 μM each primer, and 1 U AmpliTaq Gold polymerase. Thermocycling conditions for the first round PCR were an initial incubation for 10 min at 94°C followed by 30 cycles of 30 s at 92°C, 30 s at 50°C, and 90 s at 72°C and a final extension for 10 min at 72°C. For second-round PCRs, parameters were the same except that the number of cycles was increased to 35 and the extension time (during the 35 cycles) was decreased to 45 s. PCR fragments were ligated into pGEM-T (Promega), cloned into DH5 cells (Gibco), and sequenced with an ABI automated sequencer.

### Developmental RT-PCR

Total RNA samples were prepared from *H. robusta* embryos at selected stages with RNAwiz (Ambion) according to the manufacturer's instructions. Either 30 (experiment 1) or 20 (experiment 2) embryos were used for each sample. Fresh embryos for experiment 1 were collected before RNA isolation, whereas embryos for experiment 2 were first stored in RNAlater (Ambion) for less than one month before processing. The RNA samples were reverse transcribed in 1X reverse transcription buffer (Gibco), 3.33 mM DTT, 0.33 mM dNTP, and 3.33 μM random decamer (Ambion) with 200 U reverse transcriptase (Gibco). The mixture was incubated at 42°C for 1 hr, and the cDNA was then purified with spin columns (QIAquick PCR Purification, Qiagen).

Based on the sequence obtained for *Hro-wntA*, we designed the primers *wntA-R*<sup>+</sup> (TGCCAGCAAAGAGTCAGCGTTCGT) and *wntA-P*<sup>-</sup> (TGA AACACCGGAGCACTTGCAG), which amplified an approximately 300 bp fragment of *Hro-wnt-A* from cDNA. These primers span an intron of about 300 bp, which permitted us to distinguish bands that had been amplified from cDNA from those that had been amplified from contaminating genomic DNA. To amplify the *Hro-wnt-A* fragment, we used cDNA equivalent to three embryos in a reaction containing 1X PCR buffer (Perkin-Elmer Cetus), 1.5 mM MgCl<sub>2</sub>, 200 μM dNTP, 0.6 μM each primer, and 1 U AmpliTaq Gold polymerase. Thermocycling conditions were an initial incubation for 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C.

As an internal standard to adjust for differences in efficiency of RNA extraction between samples, an approximately 450 bp fragment of 18S rRNA was amplified in parallel to each *Hro-wnt-A* sample. For this purpose, and to attenuate the signal obtained from the abundant rRNAs, we used bonafide and nonextending 18S primers (competimers; Ambion) in a 3:7 ratio, respectively. PCR conditions for amplifying the 18S rRNA fragment were as described for *Hro-wnt-A* except that cDNA equivalent to one embryo was used and 30 amplification cycles were performed.

To quantitate PCR products, we ran out each sample in a 2% agarose

gel containing ethidium bromide. We measured band intensity with an Alphaimager (Alpha Innotech Corp.) by using the AlphaEase (v3.3b) program. To correct for differences in RNA extraction efficiency between samples, we normalized each *Hro-wnt-A* band intensity with the corresponding 18S band intensity. The RT-PCR bands analyzed clearly result from amplification of cDNA rather than contaminating genomic DNA since a sample processed in a manner identical to the others except that no enzyme was added to the reverse transcription reaction yielded no PCR product. Moreover, the only *Hro-wnt-A* bands produced from PCR were the size expected from cDNA (approximately 300 bp) and not genomic DNA (approximately 600 bp). All negative PCR controls, in which water rather than cDNA was added to the PCR, were blank.

### Staging embryos

A total of 225 embryos from 8 clutches was used to establish the pattern of *Hro-wnt-A* secretion from stages 1–4a shown in Figure 2. Of these, 4 clutches (152 embryos) were used to examine the dynamics of the pattern during the period from 275 to 345 min AZD. For this purpose, each clutch was divided into small groups (2–9 embryos each) of synchronous embryos (within 5 min of one another), as judged from the appearance of the first cytokinetic furrow, and maintained in HL medium (4.8 mM NaCl, 1.2 mM KCl, 2.0 mM MgCl<sub>2</sub>, 8.0 mM CaCl<sub>2</sub>, and 1.0 mM maleic acid [pH 6.6]). Synchronous groups were fixed at 5 min intervals.

### Immunostaining

Immunostaining for HRO-WNT-A was carried out essentially as previously described [12] except that embryos were fixed for 1 hr at room temperature, then 4° C for 4–12 hr. Evidence that the antibody was reacting with leech WNT-A selectively, and not any general cell-adhesion factor(s), was provided by the fact that it stained a single band on western blots [12] and also by the cell-specific staining patterns obtained here.

### Agarose embedding

For agarose embedding, molten low-melting temperature agarose (Nu-Sieve GTG; FMC BioProducts; 1% in HL medium) was cooled to approximately 25°C and poured over a precast bed of regular agarose (ultrapure, Gibco BRL; 0.75% in HL medium). Devitellinized and rinsed zygotes were then pipetted into the upper layer while it was still liquid.

### Cell separation

Glass capillary tubes (FHC; 1.0 mm OD × 0.75 mm ID/fiber) were pulled with a micropipette puller (Sutter Instrument model P80/PC). Each electrode was treated with silicone (Rain-X) prior to its use as a probe for separation of AB and CD cells. The embryos were bathed in zero-divalent medium (19.8 mM NaCl, 1.2 mM KCl) at the first sign of cytokinesis.

### Video time lapse

Samples were bathed in a petri dish with HL medium. They were viewed with a Dage-MTI 3CCD camera mounted on a Zeiss Axiophot. The camera was operated with Scion NIH image software.

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