

Expression of the *wnt* gene complement in a spiral-cleaving embryo and trochophore larva

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ABSTRACT The highly conserved *wnt* gene family has roles in developmental processes ranging from axis formation to cell fate determination. The polychaete *Platynereis dumerilii* has retained 12 of the 13 ancient *wnt* subfamilies and is a good model system to study the roles of the *wnt* ligands in spiralian development. While it has been shown that *Platynereis* uses a global β -catenin-mediated binary cell fate specification module in development, the early roles of the 12 *wnt* genes present in *Platynereis* are unknown. Transcriptional profiling by RNA-Seq during early development and whole-mount *in situ* hybridization of embryo and larval stages were used to determine the temporal and spatial regulation of the *wnt* complement in *Platynereis*. None of the 12 *wnt* transcripts were maternally provided at significant levels. In pregastrula embryos, zygotic *wnt4*, *wnt4*, and *wnt5* transcripts exhibited distinctive patterns of differential gene expression. In contrast, in trochophore larvae, all 12 *wnt* ligands were expressed and each had a distinct expression pattern. While three *wnt* ligands were expressed in early development, none were expressed in the right place for a widespread role in β -catenin-mediated binary specification in early *Platynereis* development. However, the expression patterns of the *wnt* ligands suggest the presence of numerous *wnt* signaling centers, with the most prominent being a bias for staggered posterior *wnt* expression in trochophore larvae. The similarity to *wnt* expression domains in cnidarians around the blastopore and the tail organizer in chordates supports a hypothesis of a common evolutionary origin of posterior organizing centers.

KEY WORDS: *wnt*, spiral cleaving, signaling center, trochophore, polychaete

Wnts are secreted, lipid-modified proteins that are thought to act as morphogens and travel to a receiving cell. There are a number of Wnt receptors, and the binding of a Wnt ligand to a receptor will trigger a variety of intracellular responses. The consequence of a Wnt signal is an activated transcriptional program that will give rise to a biological response, such as cell proliferation, cell fate determination, or axis formation (Logan and Nusse, 2004). The response to a Wnt signal and activated transcription is dependent on the cellular context, as Wnt signaling is involved in many processes from development to adult tissue homeostasis. There are three main pathways that are activated by secreted Wnt proteins: the canonical Wnt/ β -catenin pathway, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/ Ca^{2+} pathway. The Wnt/ β -catenin signaling pathway is highly conserved in metazoans and has two states;

OFF that causes the degradation of cytoplasmic β -catenin, and ON that occurs when Wnt ligands bind to the receptors Frizzled and LRP5/6, and triggers an intracellular signaling cascade ending with the transcription of Wnt target genes. The interaction of the Wnt ligand with its receptors will block the antagonistic activity of GSK3 β that normally targets cytoplasmic β -catenin for degradation (Logan and Nusse, 2004).

Wnt gene diversity is ancient, as phylogenomic analyses suggest the presence of thirteen *wnt* genes present in the last common ancestor between the diploblastic cnidarians and bilaterally symmetric animals (Cho *et al.*, 2010; Janssen *et al.*, 2010; Kusserow

Abbreviations used in this paper: hpf, hours post fertilization; WMISH, whole-mount *in situ* hybridization.

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et al., 2005). Significantly, this ancestral gene set has been largely retained by some animals, such as the cnidarian sea anemone *Nematostella vectensis* (Kusserow et al., 2005; Lee et al., 2006), several protostomes like the crustacean *Daphnia pulex* (Janssen et al., 2010), the mollusk *Lottia gigantea* (Cho et al., 2010), the annelids *Platynereis dumerilii* (Janssen et al., 2010) and *Capitella teleta* (Cho et al., 2010), and the deuterostome chordate *Branchiostoma floridae* (Croce et al., 2006; Holland, 2002) and echinoderm *Strongylocentrotus purpuratus* (Croce et al., 2006). In other animal lineages, the ancestral *wnt* gene complement has been modified with gene loss, dramatically seen in the ecdysozoan nematodes like *Caenorhabditis elegans* and the arthropod *Drosophila melanogaster* (Janssen et al., 2010; Ruvkun and Hobert, 1998), or gene duplication, like in *Xenopus tropicalis*, *Danio rerio*, and *Homo sapiens* (Garriock et al., 2007).

Platynereis dumerilii has retained 12 of the 13 ancient *wnt* genes lacking *wnt3* only. Thus, the *wnt* complement in *Platynereis* is well conserved and provides an opportunity to study individual *wnt* gene function in a species untainted by compensatory mechanisms occurring after *wnt* gene loss or gain as observed in other animal lineages. *Platynereis dumerilii* is a marine polychaete annelid and a member of the superphylum Lophotrochozoa. In early development, *Platynereis* embryos first exhibit a spiral cleavage pattern before transitioning into a pattern of bilaterally symmetric cell divisions (Fig. 1). The spiral cleavage pattern is invariant and unequal in *Platynereis* (Fig. 1) (Dorresteyn, 1990; Fischer and Dorresteyn, 2004; Fischer et al., 2010), with the first two cell divisions producing four cells of unequal size, named A, B, C, and D, that define the quadrants of the embryonic cell lineage. The third round of cell divisions creates the micromeres (1a, 1b, 1c, 1d) that are located at the animal pole, and macromeres (1A, 1B, 1C, 1D) located at the vegetal pole. The first micromeres (1a-1d) are located slightly to the left of the vegetal sister macromeres

(1A-1D). The orientation of the sister cells with respect to one another alternates with the next round of cell divisions (1a¹ is located slightly to the right of 1a²), and continue to alternate with each division, creating a spiral pattern. This stereotyped, unequal cleavage pattern enables individual cells to be identified by size and position, and has allowed the complete cell lineage of early *Platynereis* embryos to be determined up to the ~220 cell stage (Schneider and Bowerman, 2007). *Platynereis* embryos transition from a spiral cleavage pattern to a bilaterally symmetric cleavage pattern when two cells, 4d and 2d¹¹², progenitor cells for trunk mesoderm and ectoderm, respectively, divide equally (Fig. 1). These two cells divide perpendicular to the animal-vegetal axis and are important to establish the bilateral symmetry in the trunk of larvae and adult worms.

In later development, *Platynereis* has a larval phase with a few notable features (Fig. 1). Early (24hpf) and late (48hpf) trochophore larvae have three ciliated regions in common: the apical organ at the anterior pole, the equatorial prototroch, and the telotroch near the posterior. Also common between 24hpf and 48hpf larvae is the presence of the stomodeum on the ventral side and developing chaetal sacs (primordial appendages) laterally (Fischer and Dorresteyn, 2004; Fischer et al., 2010). The posterior end at 24hpf is the proctodeal region that will later give rise to the proctodeum and pygidium by 48hpf. In the 48hpf larva, three appendage bearing larval segments (1, 2, and 3) have formed in the trunk region. Also shown in the 48hpf larva (Fig. 1C) is a territory called the peristomium, a larval region described here as posterior to the prototroch, but anterior to the first segment, and contains the stomodeum.

Previous studies have revealed potential *wnt*-related mechanisms in embryos and larvae of annelids. During early *Platynereis* development, the β -catenin signaling pathway functions in a reiterative binary mode to specify cell fates (Schneider and Bowerman,

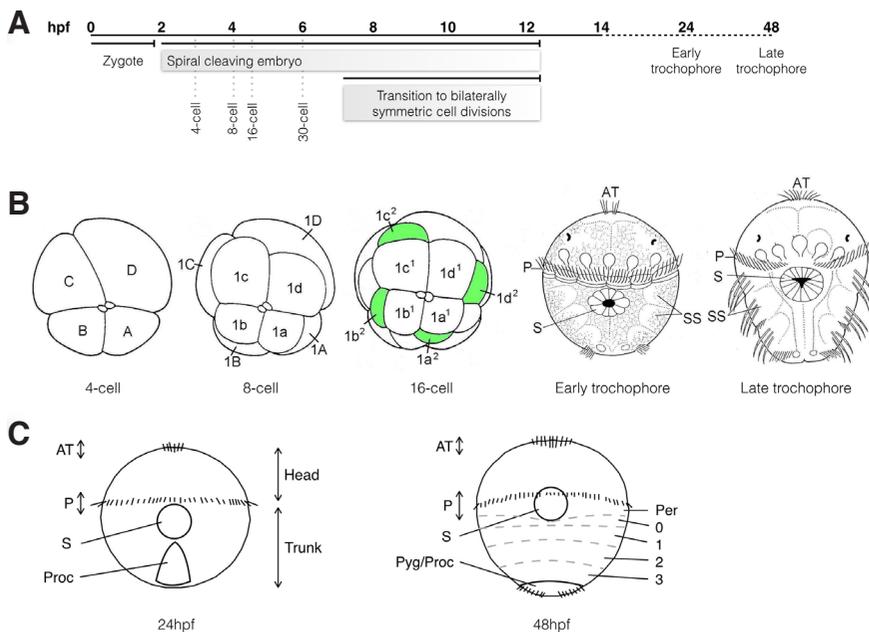


Fig. 1. *Platynereis dumerilii* development. (A)

*Timeline of *Platynereis* development from fertilization (0hpf) to early and late trochophore larvae (24hpf and 48hpf). *Platynereis* embryos exhibit a spiral pattern of cleavages early on, and then transition to a more bilaterally symmetric pattern of cell divisions after 7hpf.*

(B) Schematics are modified from Fischer and Dorresteyn, 2004. 4-, 8-, and 16-cell stage schematics are animal pole views, while the early and late trochophore schematics are ventral views with the anterior to the top. The 4-16-cell schematics show the unequal spiral cleavage pattern seen in *Platynereis* embryos, as well as the nomenclature of the four embryonic quadrants, A, B, C, and D. The two, small circles at the animal pole in the 4-, 8-, and 16-cell stage schematics represent polar bodies. (C) Additional schematics of the trochophore larvae (24hpf and 48hpf) showing structures and areas that were used to describe expression patterns. These are ventral views with the anterior to the top. Abbreviations in the trochophore larvae: AT, apical tuft (apical organ); P, prototroch; S, stomodeum; SS, setal (chaetal) sacs; Proc, proctodeal region; Pyg/Proc, pygidium/proctodeum; Per, peristomium, 0, transient larval segment; and 1-3, larval segments 1-3. The head

region/episphere gives rise to the apical organ, eyes, and brain, and is located anterior to the prototroch. The prototroch is a ciliated ring around the larva. The trunk region/hyposphere is located posterior to the prototroch (and posterior to the peristomium in 48hpf), and contains the chaetal sacs, the larval segments, and the pygidium/proctodeum. Larval segments are named as they are in Janssen et al., 2010.

2007). The binary specification module mediated by β -catenin in *Platynereis* operates between sister cells whenever an embryonic cell division occurs along the animal-vegetal axis. Those sister cells that were located more towards the vegetal pole exhibited high levels of β -catenin protein, while the animal pole sister cells exhibited low levels of β -catenin throughout early development. When β -catenin was ectopically activated through the use of a GSK3 β inhibitor, those animal pole sister cells that usually exhibited low levels of β -catenin accumulated high levels of β -catenin. This accumulation of β -catenin caused the animal pole sister cells to adopt the cell fate of the vegetal pole sister cells. Therefore, reiterative ON and OFF states of β -catenin signaling must play an instructive role for determining cell fates during early development in a binary manner (Schneider and Bowerman, 2007). Whether or not Wnt ligands are involved in the β -catenin-mediated binary cell fate specification in *Platynereis* is unknown. Although studied in several animal embryos, it is currently not understood how the complement of ancient *wnt* subfamilies is utilized to pattern spiral-cleaving embryos. Cho and colleagues have studied the *wnt* complement in the leech *Helobdella robusta*, a spiral-cleaving and direct developing annelid (Cho *et al.*, 2010), and this is the only other publication to date that has studied the *wnt* complement in early development in any spiralian model system. Studies on later roles for *wnt* ligands in *Platynereis* have focused on segmentation (Janssen *et al.*, 2010), and annelid brain development in trochophore larva (Tomer *et al.*, 2010). The expression of *wnt* genes has also been studied in later development of *Helobdella* and *Capitella*, more derived annelids that skip or shorten the trochophore larval stages (Cho *et al.*, 2010).

To identify potential *wnt* signaling centers in spiral-cleaving embryos and early and late trochophore larvae, the expression of all 12 *wnt* genes in *Platynereis* was studied. Transcriptional profiling by RNA-Seq and whole-mount *in situ* hybridization (WMISH) were used to determine the temporal and spatial regulation of *wnt* genes in the early cleavage stages. RNA-Seq results suggest that only a small subset of *wnts* is expressed in early development. WMISH further shows that each of these *wnts* exhibits a distinct cellular pattern of gene expression that can be traced back to individual cells. Surprisingly, none of the 12 *wnt* ligands appears to be expressed in the right place and the right time to be a good candidate to orchestrate the global β -catenin-mediated binary cell fate specification module in early *Platynereis* development. In contrast to early embryos, all 12 *wnt* genes are expressed in distinct patterns in early and late trochophore larvae, suggesting the presence of numerous potential *wnt* signaling centers. Of particular interest is a strong bias for staggered posterior expression of the *wnt* genes in the trochophore reminiscent of similar biased expression domains in cnidarians centered at the blastopore, and the tail organizer in chordate embryos. These similarities support the hypothesis of a common evolutionary origin of posterior organizing centers in bilaterians, and blastoporal signaling centers in cnidarians.

Results

Temporal gene expression of the *wnt* complement during early development

To establish the expression levels for each of the 12 *wnt* genes over the course of early development, transcriptional profiles were generated from the one-cell stage (2 hours post fertilization, 2hpf)

to the ~330-cell stage (14hpf) in *Platynereis* (compare to Fig. 1). To do so the transcriptome in early *Platynereis* embryos was determined by deep sequencing of RNA isolated in 2-hour intervals starting with the zygote (2hpf), early spiral cleavage stages (4hpf, 6hpf), the transition to bilateral symmetry (8hpf, 10hpf), and gastrula stages (12hpf, 14hpf) (Chou, Bastin, and Schneider, unpublished data). Transcriptome analysis revealed that 4 of the 12 *wnt* ligands, *wnt4*, *wnt5* (the alternative splice forms *wnt5a* and *wnt5b*), *wnt8*, and *wntA*, are expressed at significant levels in early *Platynereis* development (Fig. 2). Of particular interest is that only 3 of the 12 *wnt* ligands encoded by the *Platynereis* genome are expressed in early *Platynereis* embryos and each *wnt* has an overlapping, yet distinct, temporal pattern of gene expression. Even more

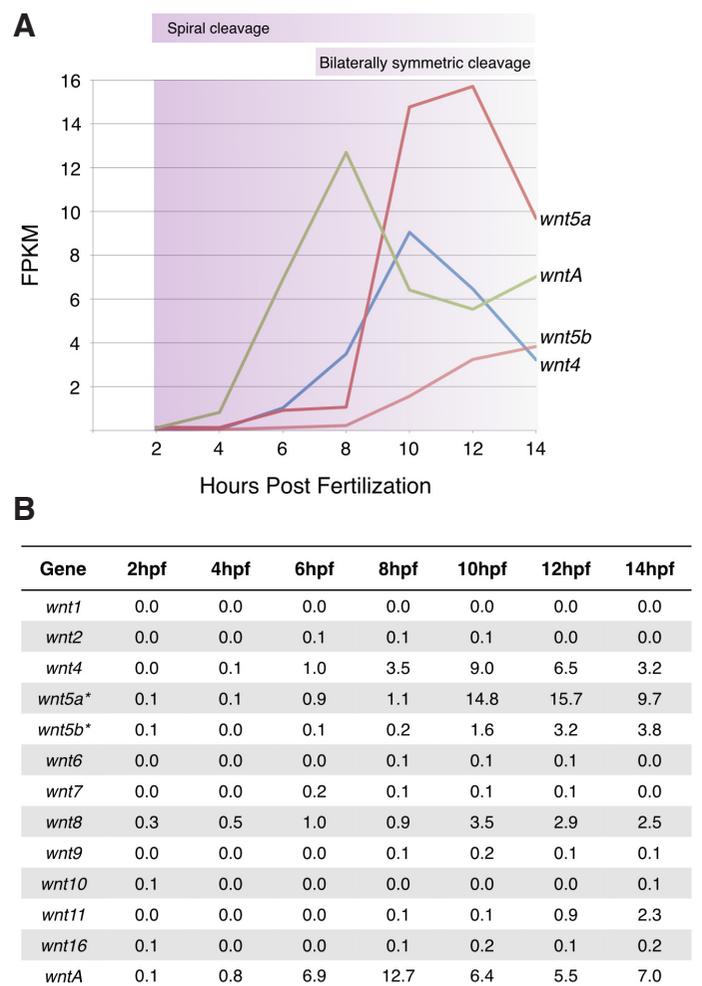


Fig. 2. *wnt* gene expression during early *Platynereis* development based on stage-specific transcriptional profiling during the first 14h of development. (A) The plot illustrates the relative expression levels of *wntA*, *wnt4*, and *wnt5* in FPKM (fragments per kilobase per million mapped reads). Each *wnt* has a distinct temporal pattern of gene expression. *wntA* expression peaks at 8hpf, *wnt4* peaks at 10hpf, and *wnt5a* expression peaks at 12hpf. **(B)** The table displays the relative expression levels of each of the 12 *wnt* genes found in the genome of *Platynereis* in median FPKM values from two biological replicates for each stage (see Sup. Table 1). The values suggest *wntA*, *wnt4*, *wnt5*, and *wnt8* are expressed at significant levels in the early *Platynereis* embryo. Asterisk by *wnt5a* and *wnt5b* denotes that these are two splice forms of a single *wnt5* gene.

intriguing are the lack of significant maternal contribution by any *wnt* gene, and the relatively late onset of some *wnt* gene expression during the transition from a spiral to bilateral symmetrically cleaving embryo (between 6hpf and 8hpf). The RNA-sequencing data revealed that *wnt8* has a slight maternal contribution, but in general, there is no major maternal contribution by the *wnt* genes.

Spatial gene expression in early development

Spatial gene expression of the *wnt* gene complement was characterized at selected stages of *Platynereis* development guided by the quantitative transcriptional data. Initially, the expression for all twelve *wnt* ligands was determined with WMISH at 12hpf to validate the RNA-sequencing results. Only *wntA*, *wnt4*, *wnt5*, and *wnt8* are expressed at detectable levels at 12hpf, while none of the other eight *wnt* genes exhibit any visible expression (Fig. 3-5, Supplementary Fig. 1, and data not shown). All twelve *wnt* genes yielded distinct differential expression patterns by WMISH in later larval stages (Fig. 6, Supplementary Figs. 2,3). Thus, absence

of early expression of the eight *wnt* genes is not due to technical problems with WMISH and confirms the quantitative developmental transcriptional data.

Of the twelve *wnt* ligands present in *Platynereis*, *wntA* has the earliest expression determined by WMISH. At 6hpf, *wntA* is expressed in only one cell, 2d¹ (Fig. 3A). The cell 2d¹ is a descendant of the first somatoblast, 2d, which is the sole progenitor of trunk ectoderm including the neuroectoderm that will form the ventral nerve cord. The expression domain of *wntA* increases during the next two hours of development from one cell to 9-12 cells at 10hpf (Fig. 3 B-G) consistent with a series of fast cell divisions of 2d¹. Thus, *wntA* transcripts are found in patches around nuclei in cells on the dorsal side of the embryo spread out along the animal-vegetal axis. As development continues, *wntA* is more evenly distributed within cells in a broader area, covering most of the dorsal side of the embryo (Fig. 3 H-J, Fig. 7).

wnt4 is the next *wnt* ligand in *Platynereis* where early expression is detected. Transcriptional profiling data shows an increase in *wnt4* expression between 6hpf and 8hpf (Fig. 2). However, the earliest *wnt4* expression detected by WMISH is at 8hpf (Fig. 4). At 8hpf, *wnt4* is expressed in 5-6 cells in the C and D lineages, and by 12hpf there are 7-9 cells with *wnt4* expression. The most anterior expression of *wnt4* at 8hpf is in two lateral patches of cells, just below the most apical region, and likely the descendants of 1c¹¹² and 1d¹¹². Additional individual *wnt4* expressing cells are located along the dorsal midline (Fig. 4 C, F). Similarly to *wntA*, the expression of *wnt4* is found on the dorsal side of the embryo based on patterns of DAPI stained nuclei (data not shown). However, *wnt4* is expressed in specific subsets of dorsal cells, mostly not overlapping with dorsal *wntA* expressing cells, and is not expressed throughout the entire dorsal side of the embryo (compare *wnt4* and *wntA* in Fig. 7).

wnt5 is the last of the *wnt* ligands present in *Platynereis* with a detectable expression pattern within the first 12 hours of development. *wnt5a* is expressed in ~4 cells at 8hpf (Fig. 5 A-C), and 8 to 9 cells by 12hpf (Fig. 5 G-I). At 8hpf and 10hpf, three of the *wnt5a* expressing cells are along the dorsal midline (Fig. 5 B,E). At 12hpf, *wnt5a* is expressed in two cells in the anterior half of the embryo, located bilaterally across from one another (Fig. 5 G,H), and in a number of cells at the posterior/vegetal pole.

While the transcriptome analysis suggests that *wnt8* is also expressed during early development in *Platynereis*, a specific expression pattern could not be detected with WMISH (Supplementary Fig. 1). There was no expression of *wnt8* at 8hpf or 10hpf, and the expression at 12hpf was ubiquitous and just at the detection level.

Spatial expression in early (24 hpf) and late (48hpf) trochophore larvae

To determine potential *wnt* signaling centers in trochophore larvae of *Platynereis*, the expression of the twelve *wnt* ligands was determined at 24hpf and 48hpf. Whereas the expression of the *wnt* complement in early trochophore larvae (24hpf) has not been reported yet, two previous publications described patterns of *wnt* expression in late trochophore larvae (48hpf) with a focus on segmentation (Janssen et al., 2010) and brain development (Tomer et al., 2010). In general, the *wnt* gene expressions at 48hpf reported here confirm most of the previously established expression pattern for the *wnt* ligands during larval segmentation and within the developing brain. However, the expression domains for

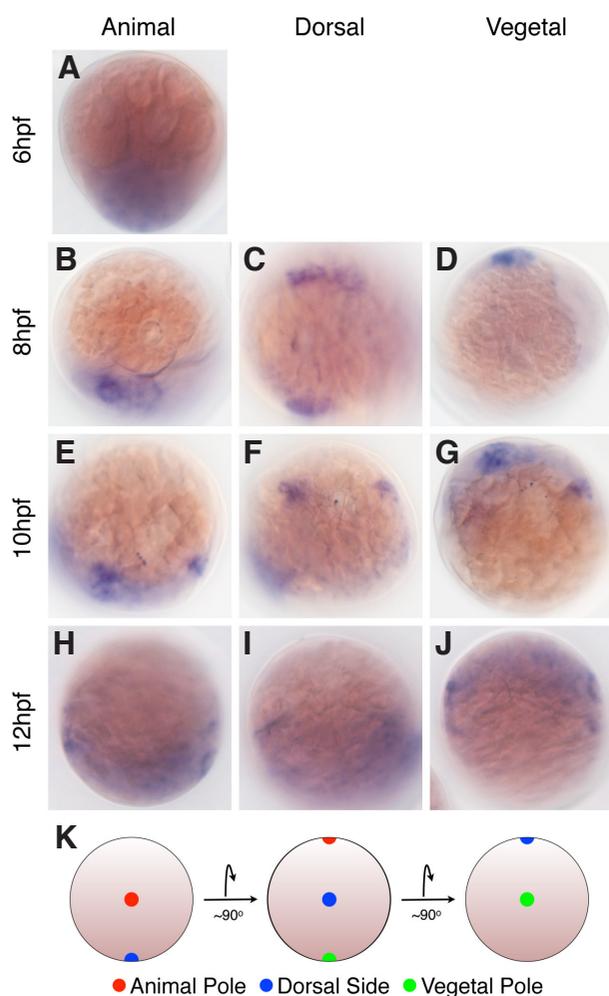


Fig. 3. Spatial gene expression of *wntA* during early development. WMISH of *wntA* on (A) 6hpf, (B-D) 8hpf, (E-G) 10hpf, and (H-J) 12hpf embryos. (A), (B), (E), and (H) are animal pole views with the dorsal side down, (C), (F), and (I) are dorsal views with the animal pole up, and (D), (G), and (J) are vegetal pole views with the dorsal side up. (K) Schematic of the orientation of (A-J) with respect to one another.

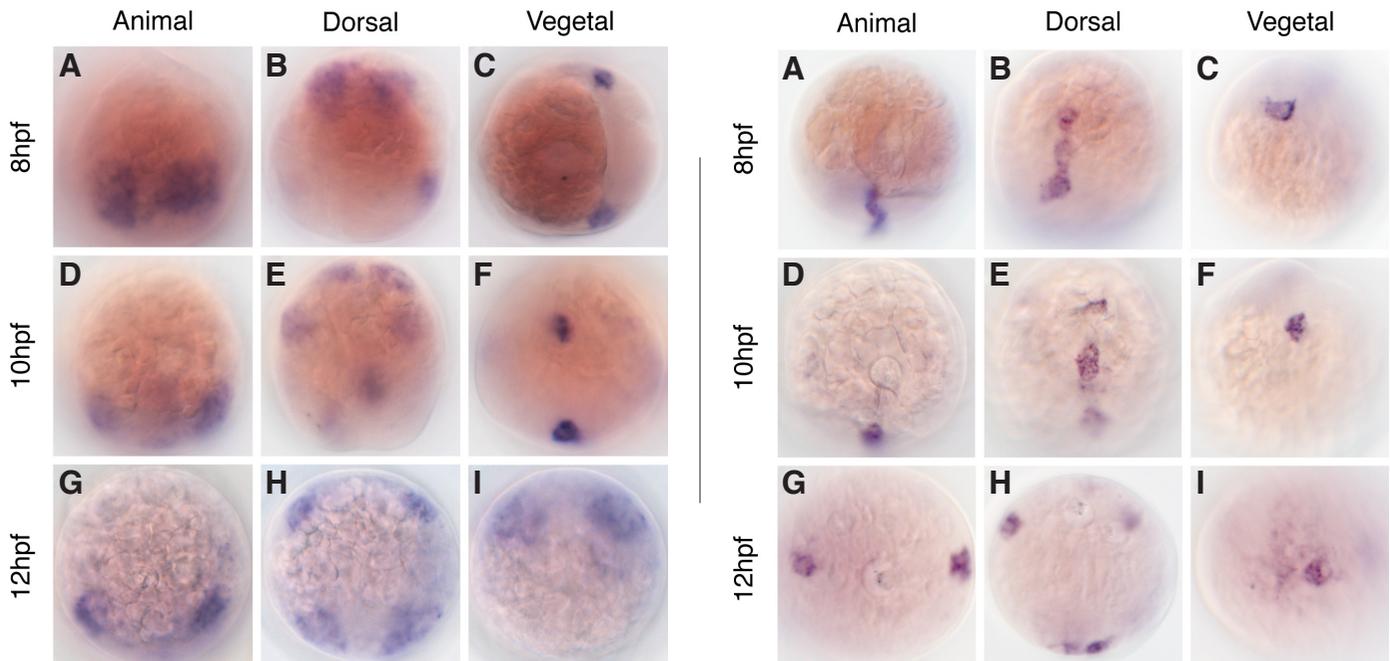


Fig. 4 (Left). Spatial gene expression of *wnt4* during early development. *WMISH* of *wnt4* on (A-C) 8hpf, (D-F) 10hpf, and (G-I) 12hpf embryos. (A), (D), and (G) are animal pole views with the dorsal side down, (B), (E), and (H) are dorsal views with the animal pole up, and (C), (F), and (I) are vegetal pole views with the dorsal side up. Compare to Fig. 3K for embryo orientation.

Fig. 5 (Right). Spatial gene expression of *wnt5a* during early development. *WMISH* of *wnt5a* on (A-C) 8hpf, (D-F) 10hpf, and (G-I) 12hpf embryos. (A), (D), and (G) are animal pole views with the dorsal side down, (B), (E), and (H) are dorsal views with the animal pole up, and (C), (F), and (I) are vegetal pole views with the dorsal side up. Compare to Figure 3K for embryo orientation.

several *wnt* ligands were missing, and some of the patterns seen at 48hpf shown here are different.

Each *wnt* ligand has a distinct pattern of expression in trochophore larvae, and while there are similarities between the *wnts* none of the expression patterns are the same. However, while the expression patterns are different, in many cases there are multiple *wnt* ligands expressed in the same larval region. *wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt11*, and *wntA* are all expressed in the developing brain anterior to the prototroch (Fig. 1C, Fig. 6, Supplementary Figs. 2,3). Each of these *wnt* ligands has a distinct expression pattern, but the expression patterns also have a high degree of overlap. The larval regions with the most overlap of *wnt* ligand expression are the trunk ectoderm and trunk mesoderm. At 24hpf, 10 of the 12 *wnt* ligands are expressed in either the trunk ectoderm or trunk mesoderm (*wnt1*, *wnt4*, *wnt5*, *wnt6*, *wnt7*, *wnt8*, *wnt10*, *wnt11*, *wnt16*, and *wntA*). There are 9 of the 12 *wnt* ligands expressed in these regions at 48hpf (*wnt1*, *wnt4*, *wnt5*, *wnt7*, *wnt8*, *wnt10*, *wnt11*, *wnt16*, and *wntA*). Interestingly, the three *wnt* ligands not expressed in the trunk ectoderm or trunk mesoderm at 48hpf are expressed in a region more posterior, the pygidium/proctodeum (*wnt2*, *wnt6*, and *wnt9*). Similarly, one of the two *wnt* ligands, *wnt9*, not expressed in the trunk ectoderm or trunk mesoderm is expressed in the proctodeal region at 24hpf. The only *wnt* ligand not expressed in the posterior of the *Platynereis* larva at 24hpf is *wnt2*, which is expressed just anterior to the prototroch. From this data, there appears to be a strong bias for *wnt* ligands to be expressed in the posterior, in contrast to a selective expression of a few *wnt* ligands in the anterior.

Wnt1: At 24hpf, *wnt1* is weakly expressed in the trunk ectoderm

and strongly in two areas in the proctodeal region (Fig. 6A and Supplementary Fig. 2). The expression of *wnt1* is expanded and elevated by 48hpf. At 48hpf, *wnt1* is expressed in the trunk ectoderm in the posterior half of segments 1, 2, and 3 as well as the pygidium/proctodeum (Fig. 6G). *wnt1* also has slight expression in the developing brain at 48hpf (Supplementary Fig. 3).

Wnt2: *wnt2* is expressed in two small symmetrical areas anterior to the prototroch at 24hpf (Fig. 6B). By 48hpf, *wnt2* is expressed in parts of the developing larval brain, and in the pygidium/proctodeum area (Fig. 6H and Supplementary Fig. 3). This is the first reported analysis of *wnt2* expression pattern in early and late trochophore larvae, and was missed by Tomer and colleagues (Tomer *et al.*, 2010) in the screen for *wnt* genes expressed in *Platynereis* brain regions.

Wnt4: *wnt4* is expressed in the trunk ectoderm and in two symmetrical areas in the developing brain at 24hpf (Fig. 6C and Supplementary Fig. 2). By 48hpf, *wnt4* is expressed in the stomodeum, anterior half of segments 1, 2, and 3 in the trunk ectoderm, and along the ventral midline along segments 1, 2, and 3 (Fig. 6I and Supplementary Fig. 3).

Wnt5: *wnt5* is expressed in two bilaterally symmetric regions in the developing larval brain at 24hpf, as well as in a segmented-like pattern in the trunk ectoderm and mesoderm, and strongly in the proctodeal region (Fig. 6D and Supplementary Fig. 2). At 48hpf, *wnt5* has the most anterior expression of all of the *wnt* ligands. *wnt5* is expressed in the developing brain, the peristomium, the stomodeum and the anterior half of segments 1, 2, and 3 in the trunk ectoderm and mesoderm (Fig. 6J and Supplementary Fig. 3). The brain expression for *wnt5* at 48hpf is similar to the pattern

shown in Tomer *et al.*, (Tomer *et al.*, 2010).

Wnt6: *wnt6* has a weak expression pattern at 24hpf and 48hpf. At 24hpf, *wnt6* is faintly expressed in the proctodeal region and surrounding mesoderm (Fig. 6E and Supplementary Fig. 2). *wnt6* expression becomes localized to the pygidium/proctodeum at 48hpf (Fig. 6K and Supplementary Fig. 3). The expression pattern shown for *wnt6* by Janssen and colleagues is a broad mesodermal expression domain in the trunk (Janssen *et al.*, 2010). The difference in expression pattern from Janssen and colleagues and the pattern shown here could be due to different regions of *wnt6* used for RNA probe synthesis.

Wnt7: At 24hpf, *wnt7* is expressed in the mesoderm in two small, symmetrical areas posterior to the prototroch in the peristomium.

wnt7 is also expressed slightly in the trunk mesoderm at 24hpf (Fig. 6F and Supplementary Fig. 2). At 48hpf, *wnt7* continues to be expressed in the peristomium and the segmental mesoderm (Fig. 6L and Supplementary Fig. 3). The peristomium expression of *wnt7* at 48hpf was not described previously (Janssen *et al.*, 2010).

Wnt8: *wnt8* is expressed in the developing brain and two additional bands, both anterior and posterior to the prototroch at 24hpf (Fig. 6M and Supplementary Fig. 2). *wnt8* is also expressed in the trunk mesoderm at 24hpf. At 48hpf, *wnt8* is strongly expressed in the developing brain, the peristomium, and in short ectodermal stripes in trunk segments 1, 2, and 3 (Fig. 6S and Supplementary Fig. 3). Similar to *wnt5*, the expression of *wnt8* in the developing brain at 48hpf is in agreement with Tomer *et al.*, (Tomer *et al.*, 2010).

Wnt9: *wnt9* has a small but specific region of expression at 24hpf and 48hpf. At both times in development, *wnt9* is expressed in the proctodeum (Fig. 6N, T, Supplementary Figs. 2,3).

Wnt10: At 24hpf, *wnt10* has slight expression in the trunk mesoderm and ectoderm, and in the proctodeal region (Fig. 6O and Supplementary Fig. 2). At 48hpf, *wnt10* is expressed in the peristomium, the trunk ectoderm in the posterior half of segments 1, 2, and 3, and the pygidium/proctodeum (Fig. 6U and Supplementary Fig. 3). The expression pattern for *wnt10* shown here at 48hpf is more extensive than what was previously shown (Janssen *et al.*, 2010).

Wnt11: *wnt11* has the most anterior expression of all *Platyne-reis* *wnt* ligands at 24hpf. At 24hpf, *wnt11* is also expressed in the stomodeum, the ventral midline, the trunk ectoderm and mesoderm, and proctodeal region (Fig. 6P and Supplementary Fig. 2). The expression of *wnt11* strengthens at 48hpf, and is expressed in the developing larval brain, the peristomium, the stomodeum, the posterior half of segments 1, 2, and 3 in the trunk

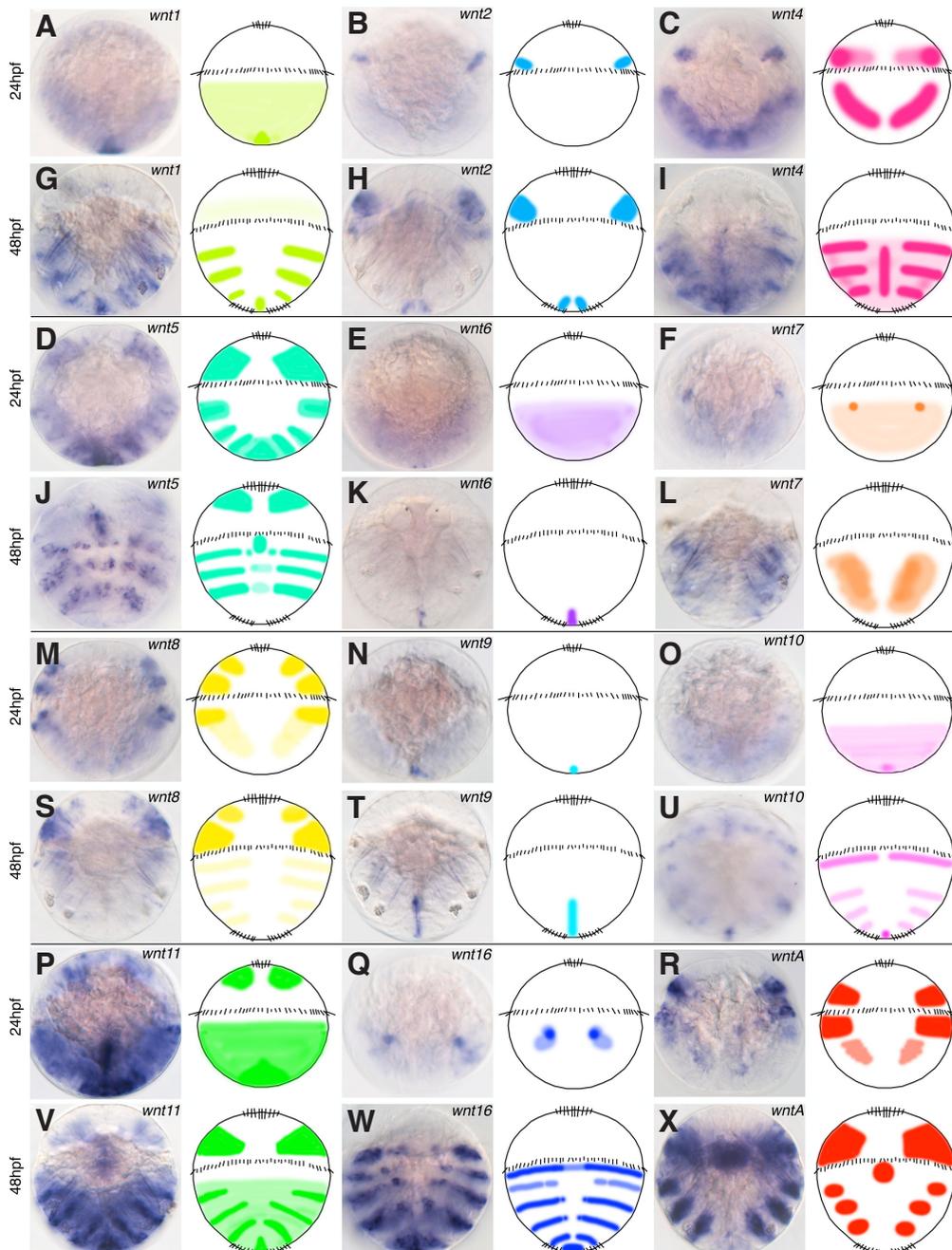


Fig. 6. Expression of the *wnt* gene complement in early (24hpf) and late (48hpf) trochophore larvae. Both in situ images and schematics are ventral views with the anterior of the specimen up. Gene expression analysis was performed with *WMISH*, and the neighboring schematic represents the expression pattern as a projection of all focal planes of the larva. Rows 1, 3, 5, and 7 are early trochophore larvae (24hpf), and rows 2, 4, 6, and 8 are late trochophore larvae (48hpf). Refer to the text for details on the expression patterns as well as Sup. Fig. 2 and Sup. Fig. 3.

ectoderm and mesoderm, the pygidium/proctodeum (Fig. 6V and Supplementary Fig. 3).

Wnt16: At 24hpf, *wnt16* is expressed in two symmetrical areas in the trunk ectoderm (Fig. 6Q and Supplementary Fig. 2). By 48hpf, *wnt16* expression has strongly increased. *wnt16* is expressed in the peristomium, a transient segment posterior to the peristomium, the posterior half of segments 1, 2, and 3 in the trunk ectoderm, and the pygidium/proctodeum (Fig. 6W and Supplementary Fig. 3).

WntA: The most anterior expression of *wntA* at 24hpf is in the developing brain (Supplementary Fig. 2). At 24hpf, *wntA* is also expressed posterior to the prototroch in the peristomium, and weakly in the trunk ectoderm (Fig. 6R). At 48hpf, *wntA* is expressed in the developing larval brain, the peristomium, the stomodeum and the parapodial primordia (Fig. 6X and Supplementary Fig. 3).

Discussion

Do Wnt ligands trigger β -catenin-mediated binary cell fate specification?

Extensive reiterative asymmetric β -catenin localization patterns have been observed between sister cells in early embryos of *Platynereis dumerilii* after every embryonic cell division that occurs along the animal-vegetal axis. The animal pole sister cell has lower levels of nuclear β -catenin while the vegetal pole sister cell has high levels of nuclear β -catenin. Ectopic activation of β -catenin causes the animal pole sister cell to accumulate high levels of nuclear β -catenin and adopt the cell fate of the vegetal sister cell. This β -catenin asymmetry between sister cells from animal-vegetal cell divisions is reiterative and continues to specify cell fates until at least 12hpf (Schneider and Bowerman, 2007). In the canonical Wnt/ β -catenin signaling pathway, the binding of a Wnt ligand to its receptors causes a stabilization of cytoplasmic β -catenin and allows β -catenin to move to the nucleus to begin transcription of Wnt target genes. As this is a well-studied pathway, and there is a clear stabilization of β -catenin in the nuclei of vegetal pole sister cells in *Platynereis*, the identification of candidate *wnt* genes involved in the binary specification mediated by β -catenin was an important next step. If a *wnt* gene was involved in β -catenin-mediated binary specification, one could expect it to be ubiquitously expressed, localized symmetrically along the animal-vegetal axis, or expressed in a distinct pattern reminiscent of the β -catenin activation pattern, maybe expressed in the animal pole sister cells to signal to the vegetal pole sister cells.

To test the involvement of the twelve *wnt* ligands present in the *Platynereis* genome (Janssen *et al.*, 2010) in the β -catenin-mediated binary cell fate specification, the temporal and spatial expression patterns of the *wnt* ligands were examined. Temporal expression of the *wnt* complement through transcriptome analysis of early *Platynereis* development revealed a lack of significant maternal contribution by any *wnt* ligand (Fig. 2). Additionally, the transcriptome analysis established that few *wnt* ligands are expressed during early development (*wntA*, *wnt4*, *wnt5*, and *wnt8*), but these *wnts* are not expressed during the early spiral cleavage stage (Fig. 2). WMISH confirmed the temporal expression pattern of the *wnt* ligands (Fig. 3-5), and that the zygotic expression begins primarily in cell lineages on the dorsal side, which does not explain the β -catenin asymmetries in the ventral cell lineages. Together, the temporal and spatial expression patterns suggest a *wnt*-independent mechanism for the binary specification through

β -catenin. Another possibility is that very low transcript levels of a *wnt* ligand, below the RNA-Seq and *in situ* detection level, like *wnt8*, could be sufficient to trigger early β -catenin activation. Further analysis by loss-of-function experiments is necessary to exclude this possibility.

The likely *wnt*-independent mechanism for the binary specification through β -catenin in *Platynereis* might be in contrast to what is seen in the nematode *C. elegans*. *C. elegans* is another species that exhibits β -catenin activation asymmetries throughout early development. In *C. elegans*, most divisions that take place along the anterior-posterior axis show asymmetric activation of β -catenin, with high levels of β -catenin in the posterior sister cell (Park and Priess, 2003), and part of the asymmetry is due to Wnt signaling. At the 4-cell stage, MOM-2/Wnt signals from a cell, P_2 , to the adjacent cell, EMS. EMS will divide along the anterior-posterior axis to become the anterior MS cell (mesoderm precursor) and the posterior E cell (endoderm precursor). The Wnt signal from P_2 is essential for β -catenin activation in the posterior cell, E. Asymmetries up to the AB⁹ stage in *C. elegans* are regulated, at least in part, by MOM-2/Wnt signaling. Cell divisions after AB¹⁶ seem to be independent of Wnt signaling and require MOM-5/Frizzled instead (Park and Priess, 2003).

Wnt signaling centers in early *Platynereis* development

Although the extensive β -catenin-mediated binary cell fate specification mechanism may act independently of the *wnts*, some *wnt* ligands are expressed in specific subsets of cells during early *Platynereis* development. These 'early' *wnts* are first expressed

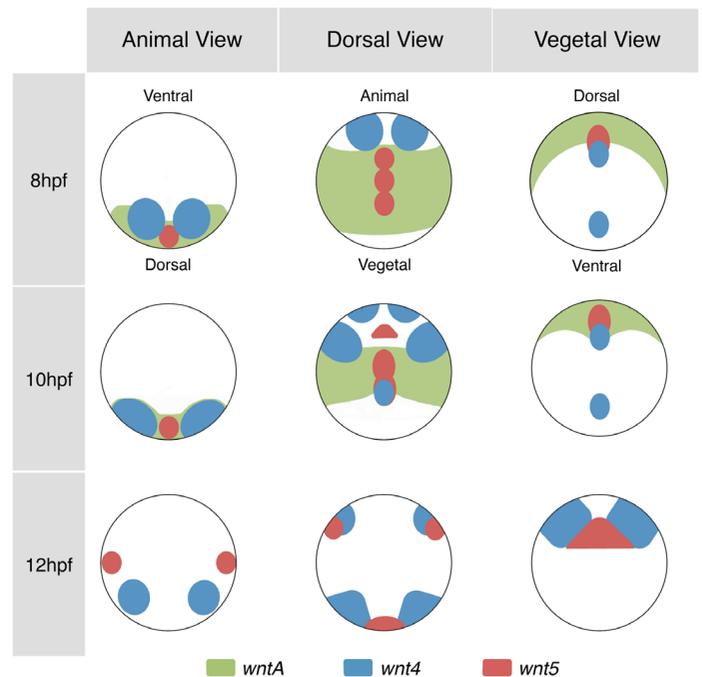


Fig. 7. Interpretive illustration for early *wnt* gene expression in *Platynereis dumerilii*. The schematics depict the hypothesized overlap of *wntA*, *wnt4*, and *wnt5a* in 8, 10, and 12hpf embryos. *wntA* is shown in green, *wnt4* is shown in blue, and *wnt5a* in red. The orientations of the schematics are the same as Fig. 3-5. *wntA*, *wnt4*, and *wnt5a* are predominantly expressed in dorsal cell lineages and absent on the ventral side of the embryos.

at the transition to bilateral symmetrical cleavages after 6hpf and are initially confined to dorsal cell lineages. As the 'early' *wnts* are expressed in dorsal midline cells and/or in a bilateral symmetrical pattern on the dorsal side of the embryo (Fig. 7), these *wnts* may function as early *wnt* signaling centers and organizers to pattern the dorsal-ventral axis.

wntA is the first *wnt* ligand expressed with a distinct expression pattern. At 6hpf, *wntA* is expressed in 2d¹. *wntA* expression increases during the next two hours of development, with the peak expression at 8hpf (Fig. 2). 2d¹ is a dominant cell lineage involved in establishing bilateral symmetry and dorsal-ventral axial tissues in the trunk ectoderm, and could act as a potential dorsal trunk organizer in *Platynereis*. 2d¹ is one of the progeny from the somatoblast 2d, that is the sole progenitor of the trunk ectoderm, and one progeny of 2d¹, 2d¹¹², is one of the first cells in *Platynereis* to divide with bilateral symmetry. It is possible that 2d¹ could act as a signaling center to coordinate bilateral symmetry in the dorsal trunk. Interestingly, in another polychaete annelid *Capitella teleta*, the somatoblast 2d acts as a signaling center and is required for bilateral symmetry and dorsal-ventral axial tissue organization (Amiel et al., 2013). Single-cell laser ablation was used in *Capitella* to determine whether or not a cell had organizing activity, and this type of experiment would be needed to determine if 2d¹ is indeed a dorsal trunk organizer in *Platynereis* embryos. However, the observed localization pattern makes *wntA* a prime candidate to convey dorsal organizing activity to the 2d cell lineage in both species.

Wnt signaling centers in the animal hemisphere and along the dorsal midline could be elicited by *wnt4* and *wnt5*, respectively. The earliest expression of *wnt4* is confined to two bilateral symmetrical expression domains in the anterior, and additional cells along the dorsal midline. Early *wnt4* localization (most likely descendants of 1c¹¹² and 1d¹¹²) is the most anterior expression of any of the 'early' *wnts*, and the cells with early *wnt4* expression will form dorsal tissues within the animal hemisphere of the trochophore larvae. Therefore, *wnt4* may convey dorsal organizing property within the head region. *wnt5* is expressed first at 8hpf in ~4 cells. Three of the four *wnt5* expressing cells are forming the dorsal midline, and three cells continue to express *wnt5* in this region until 10hpf (Fig. 5 and Fig. 7). Thus, *wnt5* may have important functions to define and maintain the dorsal midline and adjacent dorsal tissues.

In total, the combination of early *wnt* expression delineates most major dorsal cell lineages (Fig. 7). It is possible that the 'early' *wnts* act as dorsal signaling centers and are involved in patterning the dorsal-ventral axis.

Wnt signaling during early development in other species

Wnt signaling during early development has been directly or indirectly implicated in cell fate specification in other species. The expression of the 13 *wnt* genes (representing 9 *wnt* subfamilies) has been studied in the leech *Helobdella robusta*. In *Helobdella*, there is extensive overlap of *wnt* expression during the spiral cleavage stage, and most *wnts* are expressed in cells that will give rise to the mesoderm and ectoderm (Cho et al., 2010). In other spiralian, like the nemertean *Cerebratulus lacteus* and the gastropod *Crepidula fornicata*, the expression patterns of *wnt* ligands has not been determined, but the localization of β -catenin has been studied. In *Cerebratulus lacteus*, β -catenin is specifically localized to the nuclei of the vegetal-most cells at the 64-cell stage that give rise to the endoderm, suggesting that β -catenin is required

for endoderm formation in *Cerebratulus* (Henry et al., 2008). In *Crepidula fornicata*, β -catenin is stabilized in the progeny of the mesentoblast, the progenitor of the endomesoderm (Henry et al., 2010). Thus, the currently limited knowledge of *wnt* expression and β -catenin function in early spiral-cleaving embryos including *Platynereis* provides only little evidence for common conserved mechanisms, and may point to a divergence of *wnt*-related mechanisms among spiralian.

Early *wnt* signaling has been observed in a wide variety of species like *C. elegans*, sea urchins, and frogs (for review, see (Petersen and Reddien, 2009; Schneider and Bowerman, 2007)). Similar to *Platynereis*, β -catenin has a binary mode of specification during early *C. elegans* development. In divisions taking place along the anterior-posterior axis, those posterior sister cells have high levels of SYS-1/ β -catenin, and Wnt signaling via LIN-17/Frizzled is required in the posterior cells for SYS-1/ β -catenin and POP-1/TCF mediated activation of target genes (Kidd et al., 2005). In sea urchin, the *wnt* signaling pathway is involved in the specification of the animal-vegetal axis. The maternally provided β -catenin moves into the nuclei of the vegetal cells and there it functions to specify the skeletogenic fates of the micromeres and the endomesoderm fates of the macromeres (Logan et al., 1999). The maternally provided *wnt6* and *wnt* receptor *frizzled1/2/7* are required for endoderm specification (Croce et al., 2011; Lhomond et al., 2012). In *Xenopus*, the *wnt* pathway is involved in the specification of the dorsal-ventral axis. The maternally provided *wnt11* is required for the nuclear localization of β -catenin on the future dorsal side of the embryo (Tao et al., 2005). In *Platynereis*, *wnt6* and *wnt11* are not maternally provided, nor are they expressed during early development until at least late gastrula stages (~330 cells). Thus, although maternal contribution of *wnt* ligands and *wnt*-dependent β -catenin-mediated mechanisms to specify early embryonic cell fates appear to be widespread among various species, there are no apparent similarities in the type of *wnt* ligands that triggers Wnt/ β -catenin-mediated cell fate specification mechanism in early embryos between *Platynereis* and other species.

A posterior bias: *wnt* signaling in *Platynereis trochophore larvae*

To determine potential *wnt* signaling centers in later development of *Platynereis*, the expression patterns of the *wnt* complement were investigated in early and late trochophore larvae. Plotting relative expression levels and positions of all 12 *wnts* along the anterior-posterior axis (Fig. 8) reveals three subdivisions in early and late trochophore larva: 1) anterior to the prototroch, 2) the prototroch, and 3) posterior to the prototroch.

1) Wnt signaling centers anterior to the prototroch. Only 6 of the 12 *wnt* ligands are expressed anterior to the prototroch in the developing brain, *wntA*, *wnt11*, *wnt8*, *wnt5*, *wnt4* and *wnt2*. This is an interesting group of *wnt* ligands as half of those, *wntA*, *wnt4*, and *wnt5*, are the *wnt* ligands with early expression patterns, part of which were expressed in progenitor cells of the anterior/episphere cell lineages including lineages that give rise to the developing brain. This is an intriguing observation as this could mean that a *wnt* gene that is expressed earlier in the progenitor cells can more easily function later within the same cell lineage or that a *wnt* gene that has assumed a role in later development can be more easily redeployed in earlier roles in progenitor cells. The *wnt* ligands that are expressed anterior to the prototroch appear to act as a

lateral signaling center to pattern the developing brain, and these results are consistent with another publication studying conserved patterning mechanisms between vertebrates and annelids (Tomer *et al.*, 2010). The expression of the *wnt* ligands in the developing brain is excluded from the most anterior region, and is mostly expressed in lateral regions (Supplementary Figs. 2,3). Vertebrates also have *wnt* signaling centers in the developing brain, however in vertebrates, the signaling center is from the dorsal side as opposed to the lateral signaling center seen in *Platynereis* (Danesin *et al.*, 2009). In both vertebrates and *Platynereis*, there are two opposing signaling centers in the brain, hedgehog signaling and *wnt* signaling. In vertebrates, hedgehog signaling comes from the ventral side and *wnt* signaling comes from the dorsal side. In

Platynereis, hedgehog signaling comes from a medial location, and *wnt* signaling from a more lateral location (Tomer *et al.*, 2010).

2) A lack of Wnt signaling centers at the prototroch. At both 24hpf and 48hpf, there is a lack of *wnt* expression in the prototroch. The ciliated cells of the prototroch are the first cells to terminally differentiate in *Platynereis* embryos, and it is interesting that these differentiating cells do not express any of the 12 *wnt* ligands, and separate two major larval domains that harbor potentially active *wnt* signaling centers.

3) Wnt signaling posterior to the prototroch. Most significantly, all of the *wnt* ligands in *Platynereis* are expressed in staggered domains with a bias to the posterior at 24hpf and 48hpf with the exception of *wnt2* at 24hpf (Fig. 8). At both 24hpf and 48hpf in *Platynereis*, the entire trunk region from posterior to the prototroch to the pygidium has the expression of at least one *wnt* gene. The expression domains of the different *wnts* have a clear staggered appearance, and a number of *wnts* are also expressed in segmental patterns. The earliest segmental pattern detected was *wnt5* at 24hpf (Fig. 6 and Fig. 8). Later, at 48hpf, *wnt1*, *wnt4*, *wnt5*, *wnt8*, *wnt10*, *wnt11*, and *wnt16* have segmental expression domains. The segmental expression domains at 48hpf are consistent with previous work on 48hpf larvae and juvenile worms studying the role of the *wnt* ligands in segmentation and the posterior growth zone (Janssen *et al.*, 2010). The segmental expression domains seen in *Platynereis* are also seen in other animals, like the two annelids *Helobdella* and *Capitella* (Cho *et al.*, 2010). While segmental expression domains for *wnts* are seen in a number of animals, it is even more common to see a staggered *wnt* expression pattern in the posterior of numerous animal species (Holland, 2002; Kusserow *et al.*, 2005; Lee *et al.*, 2006; Lengfeld *et al.*, 2009; Petersen and Reddien, 2009).

Polarized expression of *wnts* and/or β -catenin are seen in a number of different metazoans from deuterostomes to protostomes to pre-bilaterians, but the expression of *wnts* and/or nuclear β -catenin is most commonly biased towards the posterior (Petersen and Reddien, 2009). In contrast to the work presented by Petersen and Reddien (Petersen and Reddien, 2009) who point out posterior signaling centers based on the expression of a few *wnt* genes and β -catenin activation, *Platynereis* exhibits a posterior bias in the expression for all *wnt* genes. This is a significant difference as this feature is very similar to observation of *wnt* expression in the cnidarians *Nematostella vectensis* and *Hydra magnipapillata*. *Nematostella* does not have an anterior-posterior axis but instead an oral-aboral axis, and in *Nematostella* there is a bias for all *wnt* genes to be expressed at the oral end (Kusserow *et al.*, 2005; Lee *et al.*, 2006). While the oral is thought to be anterior and the bias is in the opposite orientation as *Platynereis*, the expression of the

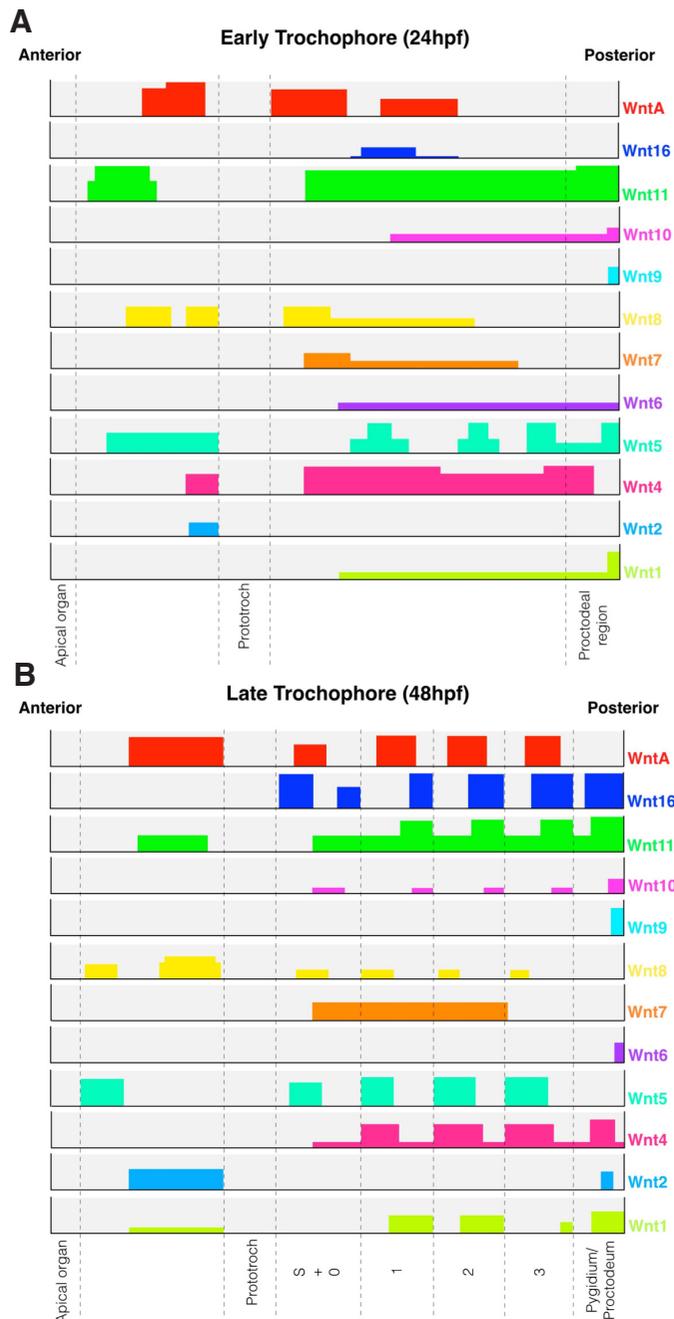


Fig. 8. Schematic illustration of *wnt* expression domains in early and late trochophore larva. Anterior is to the left and posterior is to the right. Relative heights of the bars represent higher and lower expression levels. The placement of the bars refers to *wnt* expression along the anterior (apical organ) to posterior axis (pygidium/proctodeum), and is based on WMISH data analyzed from Fig. 6, Sup. Fig. 2, and Sup. Fig. 3. The location of the apical organ, prototroch, and proctodeal region are indicated in (A) 24hpf, and the location of the apical organ, prototroch, stomodeum (S), the transient segment 0, larval segments 1, 2, and 3, and the pygidium/proctodeum are indicated in (B) 48hpf. See Fig. 1C for schematics of these structural features. These plots show the strong bias for posterior expression of the *wnt* complement in *Platynereis* in both early and late trochophore larva.

complete *wnt* gene complement is polarized across the oral-aboral axis and is expressed in staggered domains, similar to *Platynereis*. The *Hydra* genome contains 8 of the 13 *wnt* subfamilies, and 6 of the *wnt* subfamilies have expression in the adult hypostome (apical half of the head) and act there as a *wnt* signaling center, a head organizer (Lengfeld et al., 2009). It is interesting that there are similar, polarized signaling centers expressing multiple *wnts* in animals as different as *Platynereis*, *Nematostella*, *Hydra*, and deuterostomes like *Xenopus* and *Amphioxus*, an observation that was drawn attention to by Holland and others (Holland, 2002). The data presented here, and previous data from many other labs, points to biased signaling centers towards a major body axis with staggered *wnt* expression domains as an evolutionarily conserved feature. These biased staggered *wnt* domains may function in cell fate specification and axis formation, and might have been exploited during animal evolution to establish novel features like segmentation along the anterior-posterior axis.

In *Nematostella*, *wnts* are expressed around the blastopore (Kusserow et al., 2005; Lee et al., 2006); in *Hydra*, the *wnts* are expressed in the hypostome (Lengfeld et al., 2009); in *Platynereis* and arthropods, *wnts* are expressed in the posterior growth zones (Janssen et al., 2010). *wnts* are also expressed in the tail organizer in frog, fish, and amphioxus (Holland, 2002). While these may seem like different structures, they might be all related to the blastopore as suggested previously by Haeckel and elaborated by others (see Supplementary References). Perhaps the polarized *wnt* expression domains observed in extant species are a legacy of the common cnidarian/bilaterian ancestor, and share a common evolutionary origin that relate the body plans from cnidarians to annelids and vertebrates.

Materials and Methods

Animal culture

Platynereis dumerilii cultures were maintained using established procedures (www.platynereis.de) (Fischer and Dorresteijn, 2004). Synchronously developing embryos and larvae were kept at 18°C until the time of fixation. Prior to fixation, embryos younger than 14hpf were treated twice for 3 min in 25mL TCMFSW (50mM Tris, 495mM NaCl, 9.6mM KCl, 27.6mM Na₂SO₄, 2.3mM NaHCO₃, 6.4mM EDTA [pH 8.0]) to remove the eggshell (Schneider and Bowerman, 2007). Embryos and larvae were transferred to fixative (4% paraformaldehyde, 0.1M MOPS, 2mM EGTA, 1mM MgSO₄, 0.1% Tween20) and fixed overnight at 4°C on a nutator. After fixation, embryos and larvae were washed in phosphate-buffered saline with 0.1% Tween20 and stored in methanol at -20°C.

Transcriptional profiling using RNA-Seq

Total RNA was extracted from batches of synchronously developing embryos in Trizol (Ambion) according to manufacturer's instruction. Extracted RNA was treated with the RNase-free DNase Set (QIAGEN) and purified using an RNeasy Mini Kit (QIAGEN). The total RNA was extracted from seven embryonic stages in two-hour intervals, from 2hpf – 14hpf, with biological replicates. For four embryonic stages (4hpf, 6hpf, 8hpf and 14hpf), including biological replicates, additional technical replicates were generated. For all the expressed coding genes, the correlation coefficient (using Spearman method) for the biological and technical replicates is 0.93 and 0.99 respectively, showing good reproducibility of these replicates. mRNA deep-sequencing was performed with 75bp-100bp paired-end reads using an Illumina HiSeq sequencing system at the IGSP at Duke University. A total of 2.1 billion reads were sequenced. Trimmomatic (Lohse et al., 2012) was used to remove the adaptor sequences and the low quality regions. Around 93% of the reads remained after the pre-processing and

were mapped to the gene models using Bowtie1.0 aligner (Langmead et al., 2009). 88% of the filtered reads can be successfully mapped to the reference transcriptome. The read count of each sample was estimated by RSEM (Li et al., 2010), and the FPKM (fragments per kilobase per million mapped reads) was calculated by the normalization of the total mappable reads and the transcript length.

Whole-mount *In situ* hybridization

The *wnt* clones were provided by Guillaume Balavoine (IJM, Paris, France). RNA probes were synthesized with the DIG RNA labeling kit SP6/T7 (Roche), according to the manufacturer's protocol. WMISH was carried out using established protocols (Asadulina et al., 2012), with the following modifications: procedure was performed in 1.5mL tubes until visualization of the probe, hybridization with the RNA probe occurred at 65°C for at least 40 hours (hybridization buffer: 50% formamide, 5X SSC, 50µg/mL Heparin, 0.25% Tween20, 1% SDS, 0.2mg/mL Salmon Sperm DNA), the alkaline phosphatase buffer did not contain polyvinyl alcohol, and embryos and larvae were stored in 87% glycerol at 4°C. After WMISH, embryos were co-stained with DAPI to aid in cell identification and number of *wnt* expressing cells. At least 100 embryos were analyzed per stage and the combined stereotypic patterns of expression are reported here. Images were taken on an LSM700 Microscope with an AxioCam MRc5 camera, and adjusted in Adobe Photoshop.

Note added in proof

In the Results section Spatial expression in early (24hpf) and late (48hpf) trochophore larvae, it is stated that the expression patterns for the *wnt* ligands have only been reported for late trochophore larvae in *Platynereis*. While this manuscript was under revision, Marlow and colleagues published a paper with a description of *wnt4* expression at 16hpf and 20hpf in *Platynereis* (Marlow et al., 2014). While 16hpf and 20hpf embryos were not used in this study, the expression pattern is similar to what is shown here at 24 hpf.

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