

# ***Brachyury, Tbx2/3 and sall* expression during embryogenesis of the indirectly developing polychaete *Hydroides elegans***

CESAR ARENAS-MENA\*

Department of Biology, College of Staten Island and Graduate Center, The City University of New York (CUNY), NY, USA

**ABSTRACT** Expression of the transcription factor genes *brachyury*, *Tbx2/3* and *sall* is characterized in detail for the first time in an indirectly developing spiralian with a feeding trochophore. In *Hydroides elegans*, gut formation proceeds by invagination during embryogenesis and is followed by feeding-dependent posterior growth during larval stages. Posterior growth gives rise to the reproductive and segmented portion of the adult and derives primarily from multipotent dorsal blastomeres. Dorsal fate becomes morphologically evident at the 60-cell stage during spiral cleavage, although the timing of dorsal specification remains uncertain. Expression of *brachyury* anticipates the morphogenetic events associated with both gastrulation by invagination in the endoderm and ventral midline convergent extension in the ectoderm. The absence of *brachyury* expression in endoderm precursors previously reported in annelids that do not have feeding larvae suggests evolutionarily conserved roles associated with morphogenesis rather than endoderm specification. Synexpression of *brachyury* and *FoxA* in the blastopore of eumetazoans as well as in the secondarily formed anus of some protostomes and the mouth of deuterostomes suggests shared regulatory circuits during the formation of both oral and anal openings in protostomes and deuterostomes. Expression of *sall* during gastrulation, in the protonephridium, and in posterior growth zone precursors, also suggests evolutionarily conserved roles. The dorsal sides of the *Hydroides* and sea urchin embryos express *Tbx2/3* in all three germ layer precursors, suggesting evolutionarily conserved dorsal regionalization functions. The results suggest specific gene usage during tubular gut formation, endoderm specification, dorsoventral specification and anteroposterior body elongation in the context of development by feeding larva.

**KEY WORDS:** *indirect development, spiralian, bilaterian body plan evolution, gastrulation, terminal addition*

Development by intermediate feeding-trochophore larva of the spiralian polychaete *Hydroides elegans* provides particular insight into the regulatory entities that control development in spiralian. Modern developmental biology studies have been initiated in various polychaetes that lack a feeding trochophore larva (Irvine & Martindale 2000; Denes *et al.*, 2007; Frobius *et al.*, 2008). Nevertheless, substantial differences in the way feeding trochophores develop make their study relevant to better understand polychaete development and the evolution of its regulation. The tubular gut of feeding-trochophore larvae of indirectly developing polychaetes is formed by active endoderm invagination during embryogenesis (Fig. 1 a) (Anderson 1966). In contrast, in polychaetes with non-feeding larvae, the larval "endoderm" is not an epithelium but consists of large cells full of yolk that are passively internalized

by overlaying of the ectoderm, a process called epiboly, and the true one-way gut only forms long after the foregut and hindgut epitheliums have formed during non-feeding larval stages (Fig. 1 b) (Anderson 1966; Fischer & Dorresteijn 2004; Boyle & Seaver 2008). Polychaetes both with and without feeding larvae have a blastopore that partially closes along the ventral midline (Fig. 1) (Woltereck 1904; Shearer 1911; Anderson 1966; Arenas-Mena *et al.*, 2006; Arenas-Mena & Wong 2007). Formation of the segmented portion of the body in polychaetes with feeding trochophores primarily derives from small multipotent precursors in the larva and is feeding-dependent (Anderson 1966; Arenas-Mena *et al.*, 2007a) (Fig. 1A). In contrast, polychaetes that lack a feeding trochophore

*Abbreviations used in this paper:* PGZ, posterior growth zone.

\*Address correspondence to: Cesar Arenas-Mena. Department of Biology, College of Staten Island, 2800 Victory Boulevard, Staten Island, NY. 10314, USA. Tel: +1-718-982-3869. e-mail: cesar.arenasmena@csi.cuny.edu - web: <http://www.library.csi.cuny.edu/~arenas>

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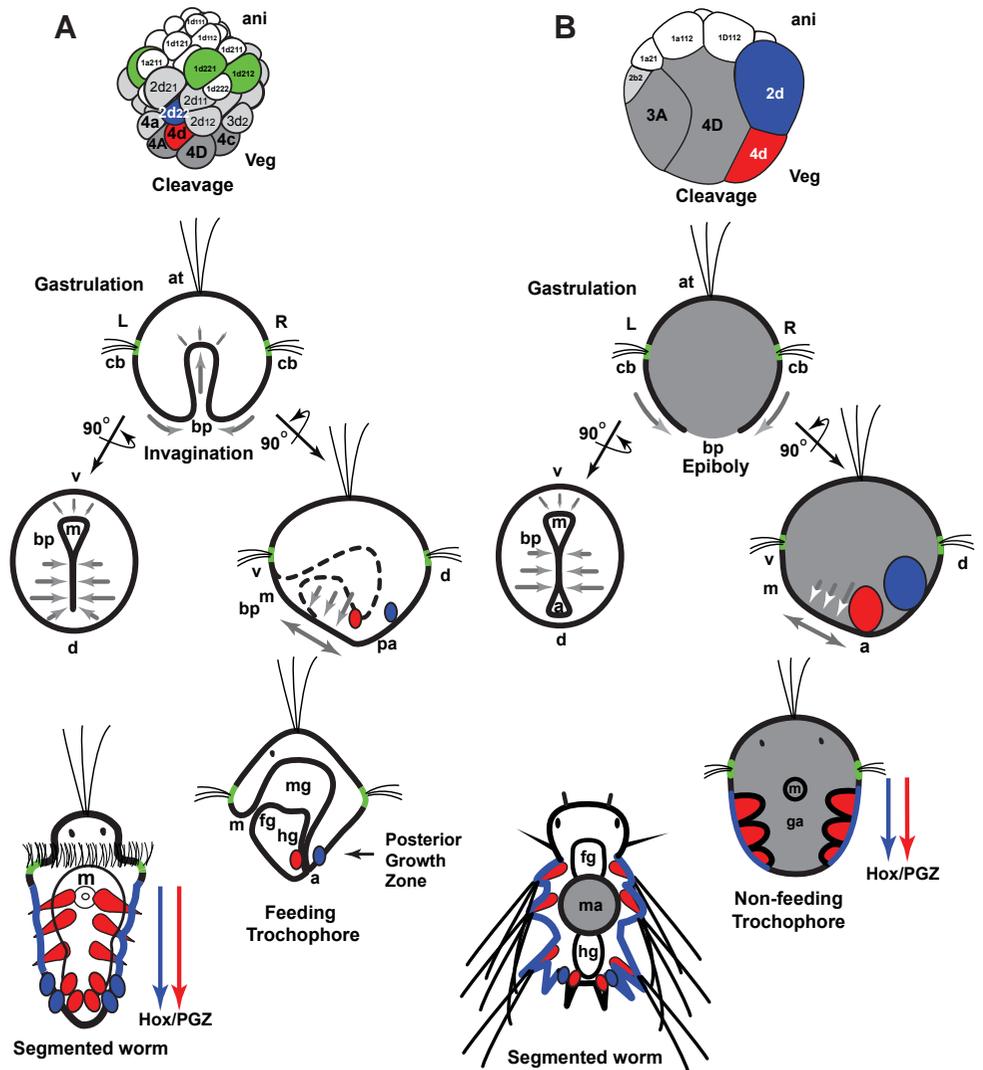
have large yolky eggs that nourish the immediate proliferation of prominent posterior growth-zone (PGZ) precursors in charge of generating the segmented portion of the adult (Anderson 1966) (Fig 1B). These PGZ precursors derive from 4d and 2d blastomeres located in the dorsal/posterior side of the embryo. Similar to the early cleavage in other indirectly developing polychaetes with feeding trochophores, the 4-cell embryo of *H. elegans* has blastomeres of equal size: A, B, C and D (Arenas-Mena 2007b); although, it is unknown if the fate of the dorsal quadrant (D) is already specified among these morphologically indistinguishable blastomeres. In clear contrast, the 4-cell embryos of polychaetes without feeding trochophores have a much larger D blastomere with invariable dorsal fates. These differences in developmental processes among polychaetes with feeding and non-feeding larvae should correlate with distinct temporal deployment of regulatory genes involved in posterior growth, gut formation and dorsal specification. In addition, similar gene deployment may exist among distant animals with similar biphasic development involving feeding ciliated larvae.

The transcription factor *brachyury* is generally involved in gastrulation (Arendt 2004), *Tbx2/3* is associated with dorsal fate specification in sea urchins (Gross *et al.*, 2003), and *sall* is associated with posterior growth (Copf *et al.*, 2006) and gastrulation (Materna *et al.*, 2006; Sweetman & Munsterberg 2006). In this study, the expression of these transcription factors is characterized in the indirectly developing polychaete *H. elegans*.

## Results

### HeSall expression

The zinc finger transcription factor *sall/spalt* (*sall/salm*) has been characterized in several bilaterians, including *Drosophila* (Copf *et al.*, 1988; Barrio *et al.*, 1996), *C. elegans* (Toker *et al.*, 2003), vertebrates (Hollemann *et al.*, 1996; Kohlhase *et al.*, 1996; Buck *et*



**Fig. 1. Comparison of direct and indirect development in polychaetes. (A)** Development of an indirectly developing serpulid with feeding trochophore (Hatschek 1885; Shearer 1911; Wisely 1958; Anderson 1966; Arenas-Mena 2006; Arenas-Mena 2007b; Arenas-Mena *et al.*, 2007b). **(B)** Development of a nereid with non-feeding trochophore (Wilson 1892; Anderson 1966; Arendt 2004; Fischer & Dorresteijn 2004). First row. Gray and colors denote vegetal hemisphere blastomeres; animal hemisphere blastomeres are in white and green. Embryos depicted at similar size for clarity; the actual relative size of indirectly developing embryos is much smaller (Anderson 1966). Blastomeres fated to midgut (dark gray), posterior growth zone (PGZ) mesoderm (red) and PGZ ectoderm (blue) are of large-size in directly developing nereids, whereas a larger number of blastomeres have larval fates in indirectly developing serpulids. Second row. Gastrulation in indirectly developing polychaetes with feeding trochophore proceeds by active invagination of the endoderm to form the gut epithelium (gastrulation movements indicated by gray arrows). In polychaetes with non-feeding trochophore the passive and yolky endoderm precursors are internalized by epiboly (the ectoderm actively encloses the passive endoderm). Third row. Anal blastopore closure is complete in indirect developers, only the mouth opening remains; in the side view, the internal endoderm is represented by a dashed line; the secondary gut opening (anus) will form after fusion of the ectoderm and endoderm on the dorsal side in the prospective anal region (pa). In both polychaetes there is elongation along the ventral midline by convergent extension of the ectoderm after blastopore closure (double headed arrows). Fourth row. The feeding trochophore of a prototypical indirectly developing serpulid has a functional tripartite gut and precursors of the segmented portion of the worm are inconspicuous (red and blue); only after feeding is the segmented portion formed by posterior growth (fifth row). The non-feeding trochophore of a prototypic nereid lacks a functional epithelial gut. Its endoderm consists of compact and yolky cells that form the gut anlage (ga); posterior growth during non-feeding larval stages starts during embryogenesis. Incipient crawling worms of direct developers still lack feeding capability, and nourishment remains dependent on the yolky midgut anlage (ma). Blastomere designations as previously summarized (Anderson 1966; Arenas-Mena 2007b). a, anus; at, apical tuft; bp, blastopore; cb, ciliary band; d, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; o, oral; pa, prospective anus; R, right; PGZ, posterior growth zone; v, ventral.

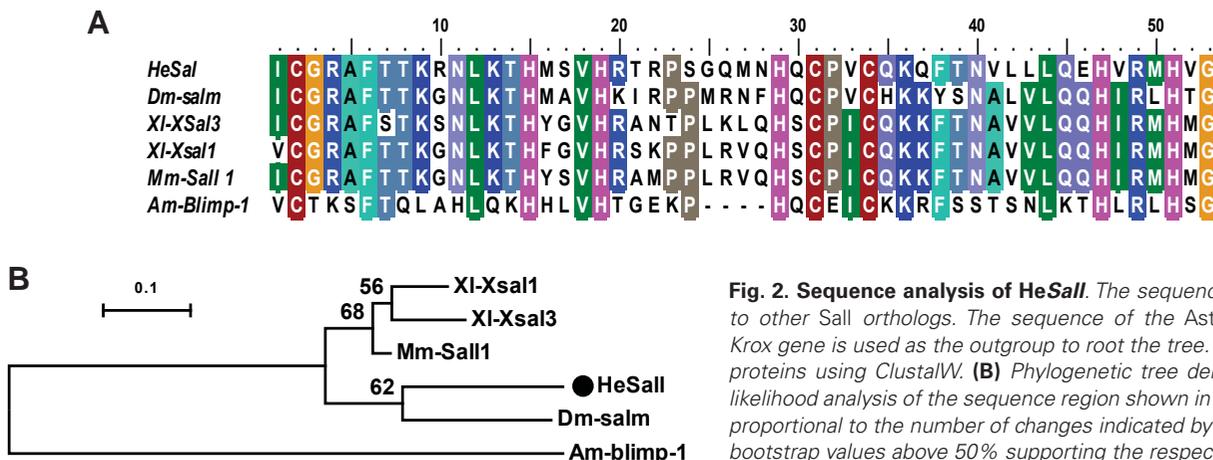
*et al.*, 2001) and an indirectly developing sea urchin (Materna *et al.*, 2006). Virtual translation of a cDNA isolated from *H. elegans* by PCR methods reveals a polypeptide sequence having clear affinity with *Sall/Salm* zinc finger transcription factors (also known as *sal* and *spalt*) (Fig. 2).

Expression of *HeSall* is first detected in all four 2q2 blastomeres of 52-cell embryos (Fig. 3A); at this stage, a, b, c and d quadrants are indistinct (Arenas-Mena 2007a), and are jointly designated as q for quadrant. The simultaneous nuclear staining (Fig. 3E) allows unambiguous identification of the cleavage stage, which has been characterized in detail up to the 80-cell stage (Arenas-Mena 2007b). During later stages, identified by chromatin condensation that anticipates the division of 2q2 blastomeres, *HeSall* expression is also seen in all four 3q2 blastomeres (Fig. 3B). Subsequently, there is a decline in expression in 2q2 blastomeres and their descendants (Fig. 3K, and optical sections not shown). Therefore, there is a shift in gene expression from more animal 2q2 blastomeres to more vegetal and adjacent 3q2 blastomeres. The 3q2 expression is more robust in 60-cell embryos (Fig. 3C). In recently formed 64-cell embryos, expression now includes blastomeres adjacent to 3q2, that is, in 3q1 and 2q22 blastomeres (Fig. 3D). In more advanced 64-cell embryos, when chromatin is fully decondensed (Fig. 3I), a decline in expression is observed in 3d1 and 2a21 (out of focus in Fig. 3I), which occupy nearly symmetrical positions relative to the sagittal plane. During incipient gastrulation stages, a ring of expression is seen in blastomeres that occupy the prospective blastopore rim (Fig. 3J). The central blastomeres, which do not express *HeSall* (Fig. 3J), start to ingress into the blastocoel (serial optical sections not shown); during this stage the identity of individual cells in the prospective blastopore cannot be unambiguously assigned, because gastrulation movements start to blur the invariant spiral-cleavage pattern that has been characterized up to the 80-cell stage (Arenas-Mena 2007a). Nevertheless, the *HeSall*-expressing ring of cells seems to include all 3q2 descendants and at least some of the 2a-c22 descendants. Blastomeres 4q and 2d22 express the gene during late cleavage stages (Fig. 3 L,Q). The blastopore-associated expression of *HeSall* is maintained during early gastrulation stages (Fig. 4 A,E). During later gastrulation stages, the expression declines first from the left and right flanks of the blastopore and remains high in the oral and aboral sides

of the blastopore (Fig. 4 B,F). Eventually, the expression further declines in the oral-side blastopore cells but remains high in the aboral-side blastopore cells (Fig. 4 C,D,G). During these early gastrulation stages, the *HeSall*-expressing blastomeres apparently derive from blastomeres expressing the gene during the invariant spiral cleavage (Fig. 4 H). The dynamic expression of *HeSall* around the blastopore rim is suggestive of morphogenetic functions associated with gastrulation. Similar association of *sall* expression with gastrulation was previously described in vertebrates and sea urchins (Materna *et al.*, 2006; Sweetman & Munsterberg 2006), suggesting an evolutionarily conserved morphogenetic role.

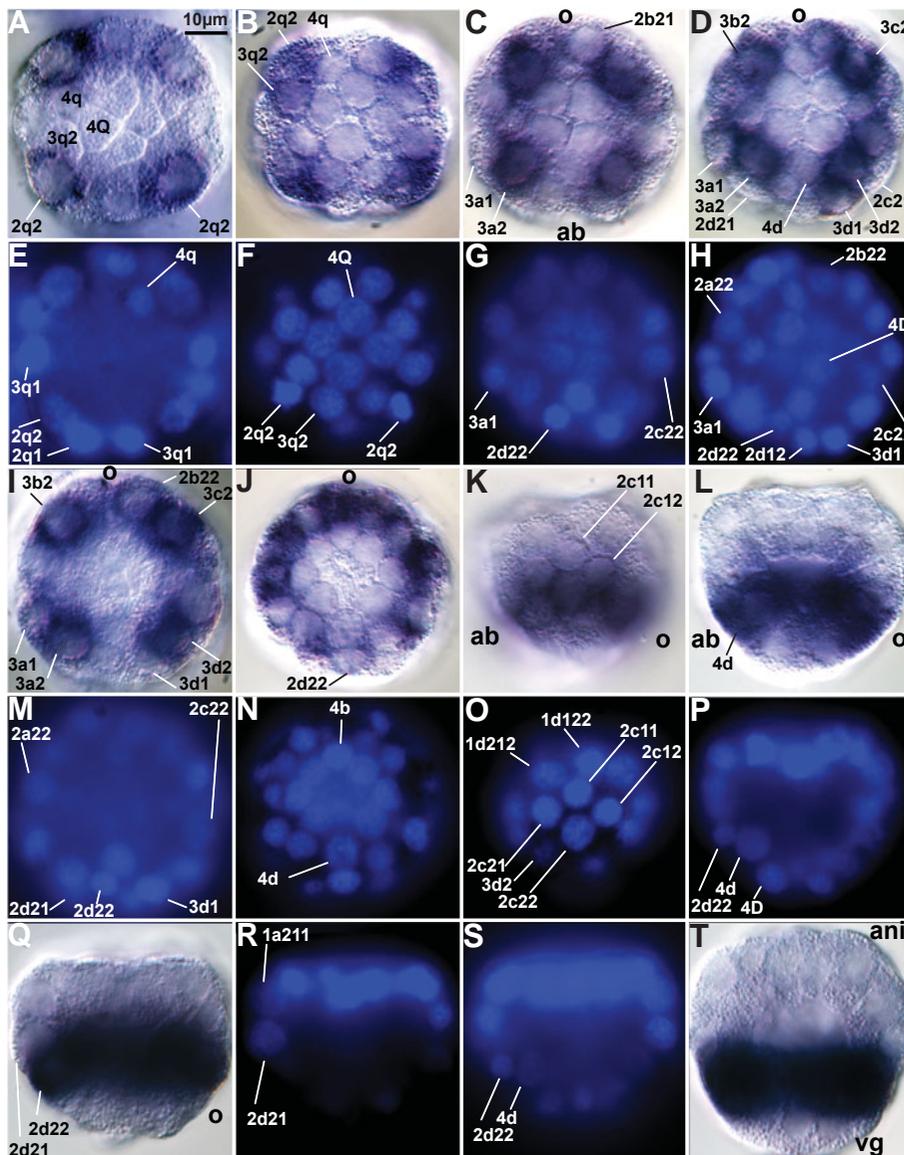
*HeSall* is later expressed in putative protonephridium precursors flanking foregut precursors (Fig. 4I) and in their presumptive descendants in trochophore stages (Fig. 4 M-O). The expression is rarely seen on the left and right sides simultaneously, suggesting transient and/or oscillatory expression (Fig. 4 I). The protonephridia are thought to derive from left and right ectomesoderm derivatives that form a strand by proliferation toward the anal region (Shearer 1911). The *HeSall*-expressing cells flanking the foregut (Fig. 4 M,N) apparently differentiate into the elongated protonephridium cells of early trochophore larvae (Fig. 4O). The expression of *HeSall* in protonephridium precursors has counterparts in other bilaterians; *sall* is involved in complex morphogenetic events related to the formation of diverse tubular organs in *Drosophila* and vertebrates. In vertebrates, one of the *sall* paralogs has an essential role in metanephros development by mediating ureteric tube interactions with nephron mesenchyme precursors (Nishihanakamura & Osafune 2006) (Farrell *et al.*, 2001), and, in the *Drosophila* tracheal system, *sall* is downstream of Wingless, and its absence specifically disrupts dorsal branches (Chihara & Hayashi 2000). The expression of *HeSall* described here would be compatible with developmental functions in the excretory organs of the polychaete larva, which would represent conservation of more general functions in nephridial precursors and tubular organ development in bilaterians.

*HeSall* is later expressed in cells that, by their position, are identified as PGZ precursors (4d and 2d22 descendants) that contribute to form the segmented portion of the adult. After aboral blastopore closure the number of mesenchyme cells expressing *HeSall* on the aboral side increases (Fig. 4 G-K), perhaps by proliferation of



**Fig. 2. Sequence analysis of *HeSall*.** The sequence of *HeSall* is similar to other *Sall* orthologs. The sequence of the *Asterina miniata* *Blimp/Krox* gene is used as the outgroup to root the tree. (A) Alignment of *Sall* proteins using ClustalW. (B) Phylogenetic tree derived from maximum likelihood analysis of the sequence region shown in a. Branch lengths are proportional to the number of changes indicated by the scale. Percent of bootstrap values above 50% supporting the respective nodes after 1000 replications are shown. The following sequences were used for the alignment: *HeSall*, gi|JX457836|; *Am-blimp1*, gi|37781470|; *XI-Xsal1*, gi|1235931|; *Dm-salm* (*spalt*, *sal*), gi|2598394|; *XI-Xsal-3*, gi|6172236|; *Mm-Sall 1*, gi|38566278|; *Am*, *Asterina miniata*; *Dm*, *Drosophila Melanogaster*; *Mm*, *Mus musculus*; *XI*, *Xenopus laevis*.

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**Fig. 3. Expression of *HeSal1* mRNA during cleavage.** DIC optical sections *a-d* and *i-h* with their corresponding nuclear staining *e-h* and *m-p*. **(A)** Animal cap view of a 52-cell embryo at the level of the vegetal hemisphere. Scale bar for this and subsequent panels. **(B)** 52-cell embryo just before the division of 2q2. **(C)** Animal cap view of a 60-cell embryo at the level of the vegetal hemisphere. **(D)** Animal cap view of a 64-cell embryo at the level of the vegetal hemisphere. **(I)** 64-cell embryo at a later stage than the embryo in *d*. **(J)** Vegetal view of an embryo with more than 80 cells, before gastrulation. **(K)** Lateral view of a 64-cell embryo. **(L)** Optical medial section of the embryo shown in *k*. **(Q)** Optical section of a 68-cell embryo. **(R)** Nuclear staining of *q*. **(S)** Consecutive optical section of *r*. **(T)** Optical medial section of a blastula embryo with more than 80 cells, at a stage similar to the embryo in *j*. Superscript has been omitted to improve readability. *ab*, aboral; *ani*, animal; *vg*, vegetal.

already expressing cells. These cells are associated with ectoderm cells that also express *HeSal1* (Fig. 4L). The ectoderm of the PGZ of the trochophore larva expresses *HeSal1* in bilaterally distributed cells (Fig. 4O, double arrowhead and 4P, arrowheads).

#### Brachyury expression

*Brachyury* is a T-box family transcription factor associated with morphogenetic functions across bilaterians and cnidarians (Arendt 2004). *Brachyury* is involved in convergent extension rear-

rangements during gastrulation in bilaterians (Arendt 2004), including convergent extension rearrangements during the formation of the sea urchin tubular gut (Hardin 1989). The corresponding gene, *HeBra*, was isolated in *H. elegans* (Fig. 5), and its expression is also found to be associated with gastrulation. Expression of *HeBra* is first detected in blastomeres that lead gastrulation by invagination, first in the four central vegetal blastomeres (Fig. 6A) and then in the adjacent peripheral blastomeres (Fig. 6 B-D). Subsequently, *HeBra* expression declines in the central vegetal blastomeres while it is further enhanced in the peripheral blastomeres, with particular emphasis in the four 3q2 blastomeres (Fig. 6 C-E). This centrifugal sequence of activation followed by deactivation continues during gastrulation and results in transient *HeBra* expression in cells that enter and leave the blastopore during invagination (Fig. 6 F-N). Thus, the expression declines in cells that are about to invaginate. *HeBra* expression in central vegetal blastomeres declines faster in the ventral-side blastomeres than in the dorsal-side blastomeres (Fig. 6 E-H). This dorso-ventral asymmetry of expression correlates with the greater contribution to endoderm by the ventral side of the vegetal plate (Anderson 1966; Arenas-Mena *et al.*, 2006; Arenas-Mena & Wong 2007), which contributes to the upward shift of the blastopore from its originally central-vegetal position (Fig. 6 N-R). The generalized endodermal expression of *HeBra* correlates with the complex morphogenetic process of gastrulation by invagination, which is consistent with its demonstrated role in morphogenesis rather than endoderm specification as seen in sea urchins (Gross & McClay 2001).

The expression of *HeBra* is also associated with morphogenetic events in the ectoderm. During the final stages of gastrulation, *HeBra* is found in the left and right ectodermal flanks (Fig. 6 G-N) down to the blastopore lips that will eventually merge to seal the ectoderm (not the endoderm) along the ventral midline (Fig. 6 L,M). The initial range of expressing cells has a crescent shape with the broadest section in the ventral midline. The expressing cells generally correspond to the area that apparently rearranges by means of convergent-extension cell rearrangements that separate the mouth from the prospective anus in *H. elegans* and other indirectly developing polychaetes with a feeding trochophore (Anderson 1966; Arenas-Mena 2006). Convergent extension in the ventral midline also moves the prospective mouth away from the prospective anus in the polychaete *Platynereis dumerilii*, and is similarly associated with the expression of *brachyury* (Steinmetz *et al.*, 2007). This second ectodermal phase of expression does have a counterpart in the ventral midline expression during the

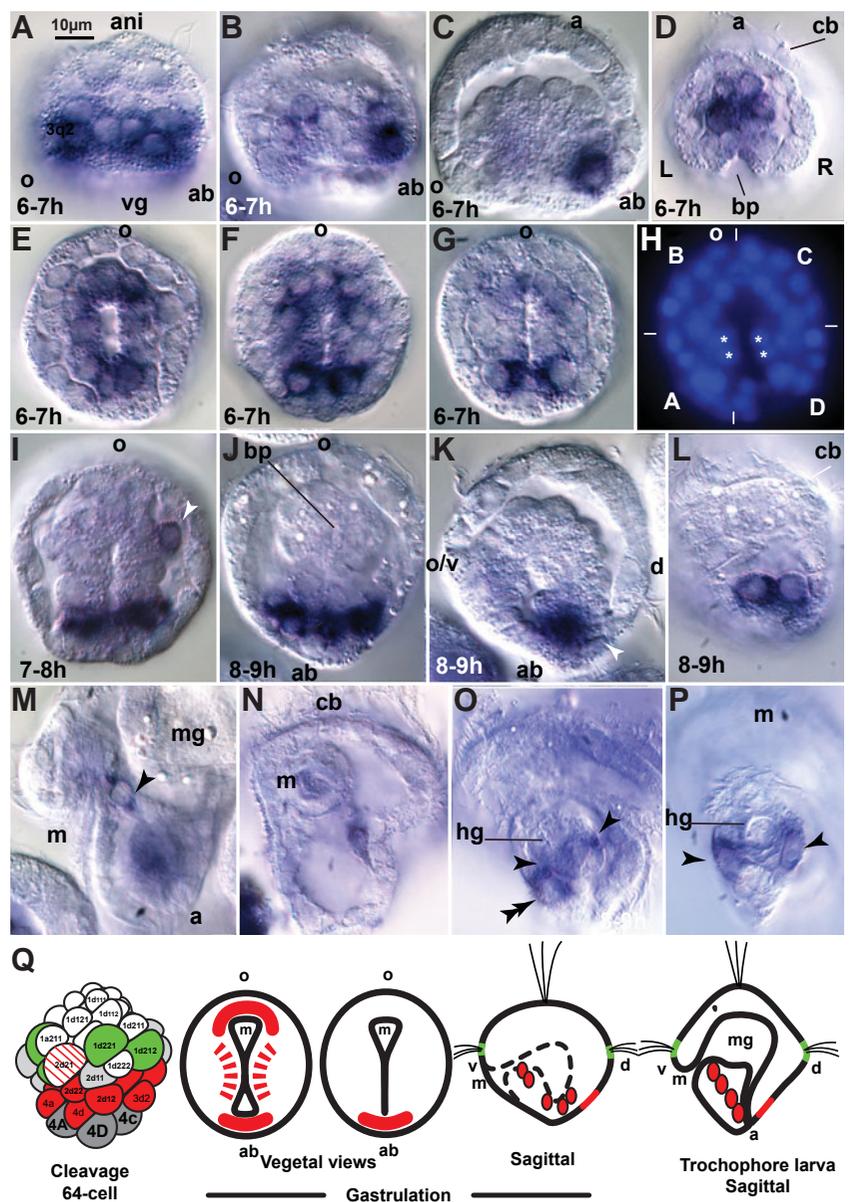
epibolic gastrulation of the annelid *P. dumerilii* (Arendt *et al.*, 2001) and the gastropod *Patella vulgata* (Lartillot 2002). In contrast, the early endodermal phase does not have a counterpart in the yolky and passive endoderm precursors of *P. dumerilii* (Arendt *et al.*, 2001) and *P. vulgata* (Lartillot 2002), further emphasizing a morphogenetic role rather than a germ layer-specification role of *brachyury*. Thus expression of *brachyury* in *H. elegans* could be said to have earlier endodermal and later ectodermal phases, which are continuous in space and time and correlate with extensive morphogenetic rearrangements.

### HeTbx2/3 expression

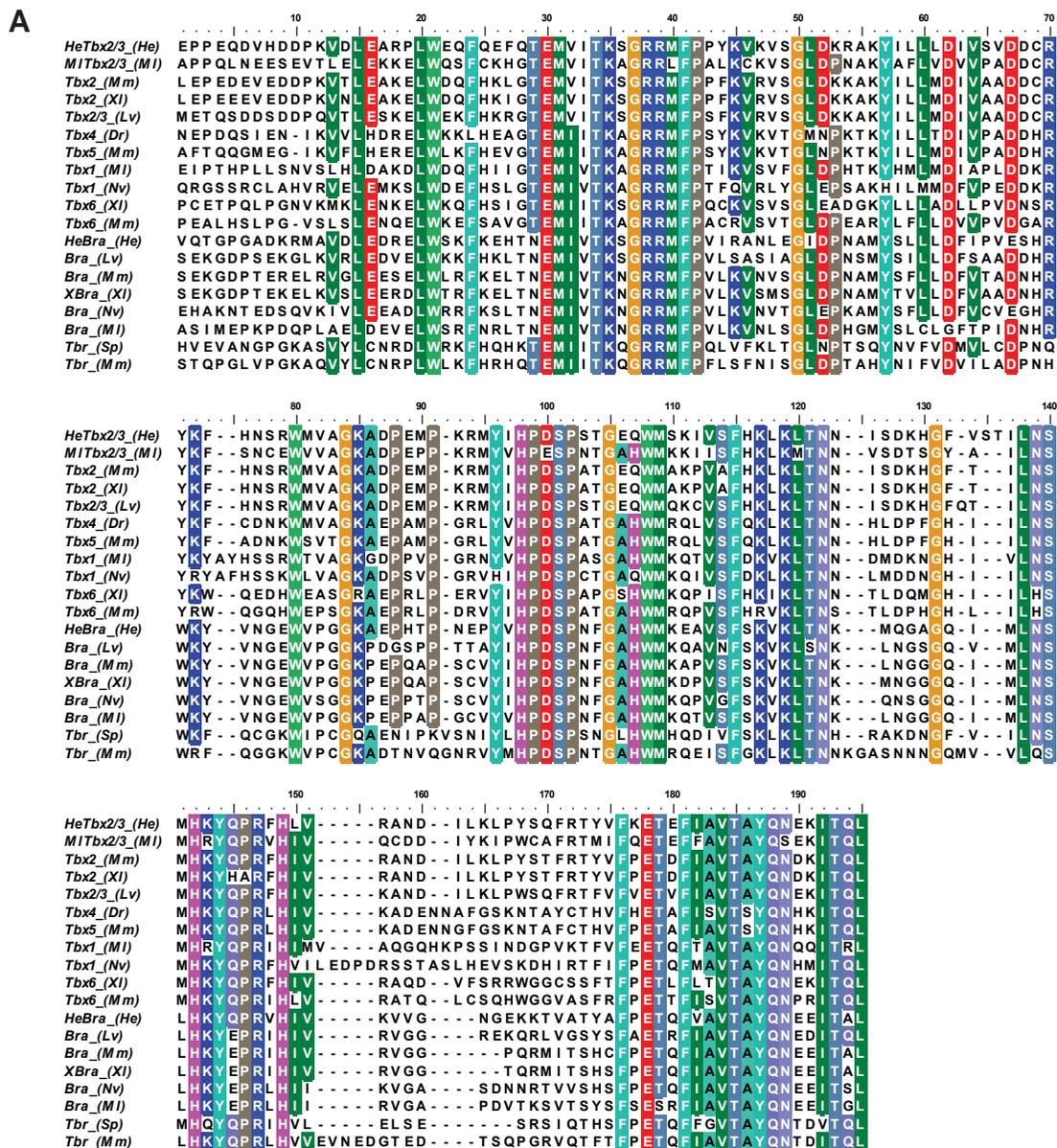
The transcription factor *HeTbx2/3* is expressed in the dorsal half of the embryo in ectodermal, endodermal and mesodermal precursors through development (Fig. 7). Dorsally restricted *HeTbx2/3* staining is robust and generalized in 64-cell embryos (Fig. 7A). Expression is initially detected in a few blastomeres occupying the dorsal midline (Fig. 7 A-D). Later in development, the expression remains centered in the dorsal midline, but is broader and also includes additional blastomeres in the animal and vegetal hemispheres (Fig. 7 E-H). The intensity of staining declines in vegetal areas during gastrulation, although general dorsal restriction continues during gastrulation in all three germ-layer precursors (Fig. 7 I-M), with a slight rightward bias in the endoderm during early gastrulation and enhanced expression in cells at the dorsal side of the archenteron and abutting ectoderm (Fig. 7 I-M). In early trochophore larvae, expression remains high in dorsal-side apical sensory organ cells (Fig. 7N), dorsal endodermal cells (Fig. 7 N-P), putative posterior sensory organ precursors (Fig. 7 O,P) and adjacent hindgut (Fig. 7P). *Tbx2/3* has general dorsal expression in a directly developing hemichordate (Lowe *et al.*, 2006), and *Tbx2/3* is expressed in the dorsal ganglia and optic cup of *Xenopus* embryos (Hayata *et al.*, 1999). The expression of *HeTbx2/3* is generally similar to the expression reported for the corresponding gene in indirectly developing sea urchins, where it is expressed in aboral (dorsal) precursors of all three germ layers and has morphogenetic roles (Gross *et al.*, 2003). The expression of *HeTbx2/3* is compatible with a similar dorsal specification role in the embryo of *Hydroides*.

### Discussion

The expression of genes associated with gastrulation is regulated differently in embryos that generate



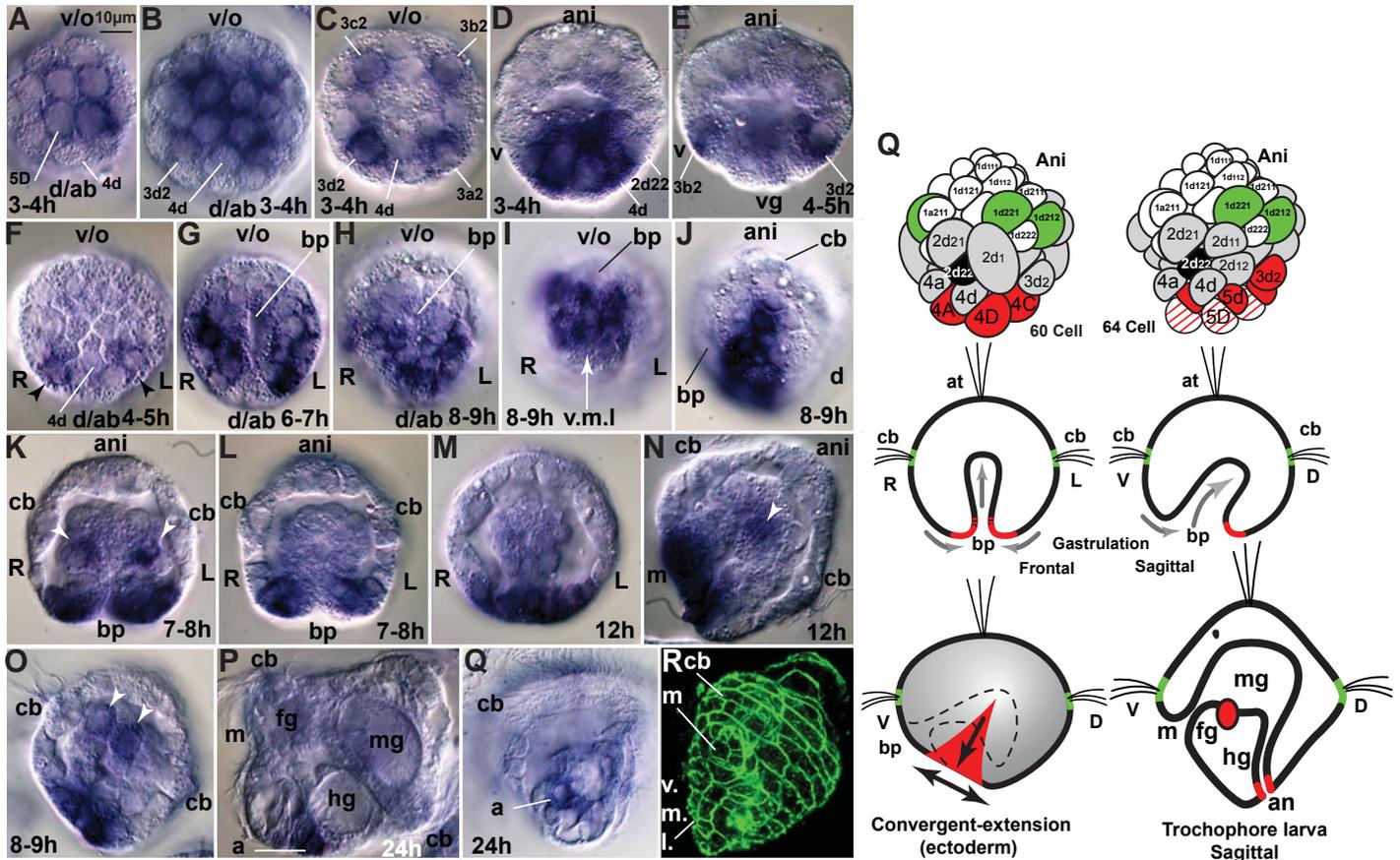
**Fig. 4. Expression of *HeSall* mRNA during gastrulation.** (A) Lateral view of an early gastrula embryo. Central blastomeres already ingressed into the blastocoel. The blastopore orientation, oral to the left, is deduced from its typical rounded shape on the oral side. (B) Lateral view of a gastrulating embryo more advanced than the one shown in a. (C) Lateral optical section of a gastrulating embryo more advanced than the embryo in b. (D) Embryo at a stage similar to that of the embryo in c, seen from the aboral side of the blastopore. (E-G) Blastopore view of embryos at stages similar to those in a-c, respectively. (H) Nuclear staining view of the embryo in g. Asterisks mark merging blastomeres in the blastopore side flanks. Letters and thin lines mark approximate spiral cleavage quadrant boundaries. (I) Oblique optical section intersecting mesenchyme cells flanking prospective foregut precursors, white arrowhead. (J) Optical section of an embryo by the end of gastrulation (after aboral blastopore closure) that intersects foregut and hindgut precursors. (K) Lateral view of a late gastrula embryo. (L) Dorsal surface view of a late gastrula embryo. (M) Lateral view of a 24-hour-old trochophore. Putative protonephridium precursor indicated by arrowhead. (N) Oblique optical section intersecting putative protonephridium precursors. (O) Optical section of a more developed trochophore. Arrowheads point to elongated tubular protonephridium cells flanking the hindgut. Double arrowhead points to an ectoderm cell in the PGZ. (P) Optical section at the level of the PGZ. Arrowheads point to ectoderm cells. (Q) Diagrammatic summary of *HeSall* expression (in red) as indicated. Line filling and dashed lines signify declining expression. See text for detailed description. Oral and aboral designations are restricted to the blastopore and dorsal and ventral extend to the whole embryo-larva, see previous diagrams (Arenas-Mena 2006; Arenas-Mena 2007b; Arenas-Mena *et al.*, 2007b). a, anus; ab, aboral; ani, animal; bp, blastopore; cb, ciliary band; d, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; o, oral; R, right; v, ventral; vg, vegetal.



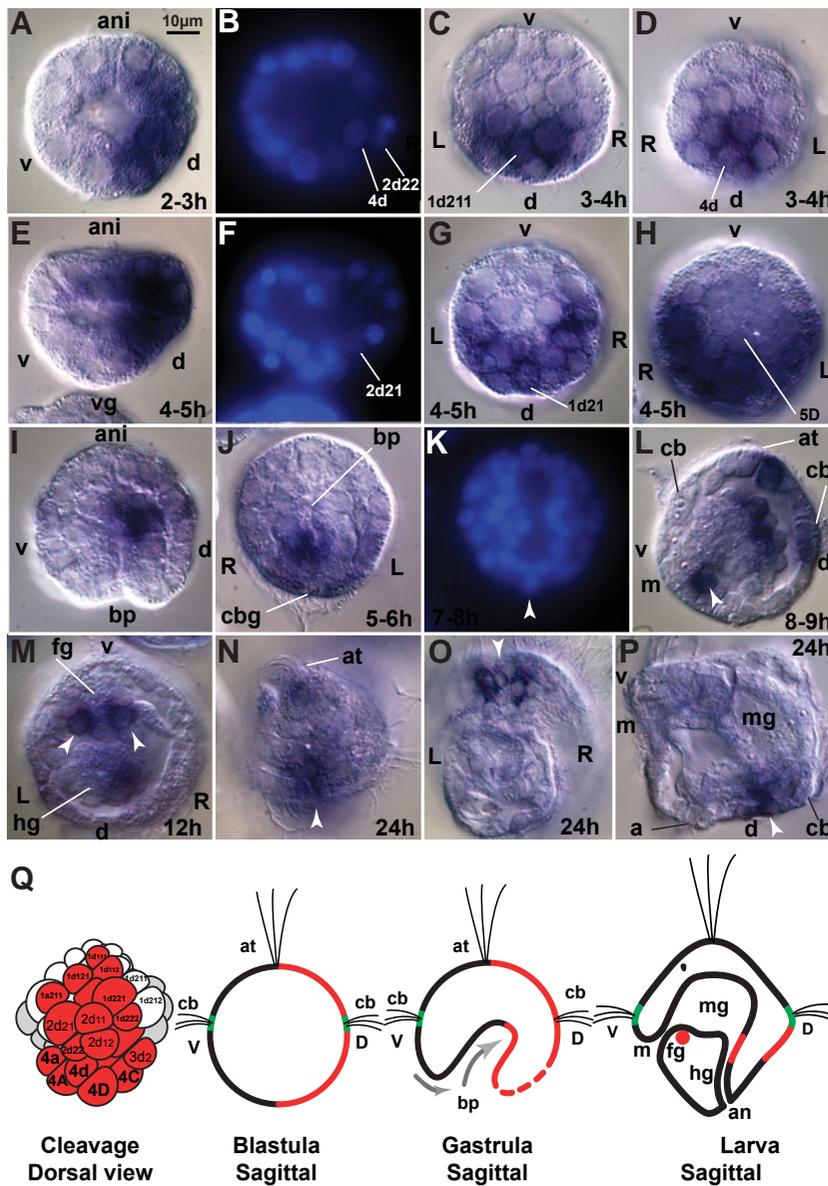
**Fig. 5. Phylogenetic analysis of HeBra and HeTbx2/3. (A)** Alignment of HeBra and HeTbx2/3 with other Tbx subfamily groups indicated in the margin. **(B)** Phylogenetic tree derived from maximum likelihood analysis of the sequence shown in a. Branch lengths are proportional to the number of changes indicated by the scale. Percentages of bootstrap values above 50% supporting the respective nodes after 3000 replications are shown. Abbreviations: Danio rerio (Dr), Lytechinus variegatus (Lv), Hydroides elegans (He), Mnemiopsis leidyi (MI), Mus musculus (Mm), Nematostella vectensis (Nv), Strongylocentrotus purpuratus (Sp), and Xenopus laevis (XI). HeTbx2/3 was assigned the GenBank accession number EU747048, and HeBra the accession number EU747049. Gene identifiers for the proteins used in the alignment: Tbx2 (Mm), gi|120407039|; Tbx2 (XI), gi|148236619|; Tbx2/3 (Lv), gi|23429206|; MITbx2/3 (MI), gi|119370313|; Tbx4 (Dr), gi|18859455|; Tbx5 (Mm), gi|34098933|; Tbx1 (Nv), gi|33621856|; Tbx1 (MI), gi|119370310|; Tbx6 (XI), gi|148226680|; Tbx6 (Mm), gi|48928035|; Bra (Lv), gi|1675550|; Bra (Mm), gi|6678203|; XBra (XI), gi|147902820|; Bra (Nv), gi|122058623|; Bra (MI), gi|119370306|; Tbr (Mm), gi|34328151|; Tbr (Sp), gi|115955121|.

feeding versus non-feeding larvae, as would be expected by their distinct gastrulation modes (Fig. 1). The expression of *HeBra* and *HeSall* reported here is associated with morphogenetic processes of gastrulation. Similarly, the transcription factors *HeFoxA* (Arenas-Mena 2006), *HeOtx*, *HeBlimp* and *HeT-Brain* previously reported (Arenas-Mena & Wong 2007; Arenas-Mena 2008) also have dy-

namic expression during gastrulation by invagination in *H. elegans*. The synexpression of *brachyury*, *Otx* and *FoxA* during gastrulation by invagination was probably present in the most recent common ancestor of bilaterians and cnidarians, because similarly dynamic blastoporal expression has been reported in the sea anemone *Nematostella vectensis* (Fritzenwanker *et al.*, 2004; Mazza *et al.*,



**Fig. 6. *HeBra* mRNA expression during embryonic and early trochophore larval stages.** For nomenclature and reconstruction of blastomere designations consult our previous report (Arenas-Mena 2007b). Nuclear staining required for blastomere designations not shown. Citations refer to further anatomical descriptions. **(A)** 60-cell embryo shows staining in vegetal 4Q blastomeres. Scale bar for this and subsequent panels. **(B)** 64-cell embryo with expression in 5Q, 5q and 3q2 blastomeres. **(C)** 64-cell embryo with declining 5Q (out of focus) and 5q expression and strong expression in 3q2 blastomeres. Staining in oral-side blastomeres 3a2 and 3b2 is lighter than staining in aboral-side blastomeres 3a2 and 3d2. **(D)** Nearly sagittal section of a 64-cell embryo. **(E)** Oblique optical section of a 68-cell embryo that has already lost expression in 5Q and 5q blastomeres. Expression declines in ventral-side 3b2 (and 3c2, out of focus), but remains high in 3d2 (and 3a2, out of focus). **(F)** 72-cell embryo seen from the vegetal hemisphere. Expression is detected in aboral-vegetal blastomeres in the left and right flanks, arrowheads. **(G)** Early gastrula embryo. Expression is detected in left and right cells that flank the aboral side of the blastopore. **(H)** Embryo where the anal side of the blastopore has already closed and the oral side remains open in the future mouth. **(I)** Detail of the blastopore lips merging at the center of the blastopore in the prospective ventral midline. **(J)** Side view shows the extent of *HeBra* expression in the left and right ectodermal flanks which, through convergent extension in the ventral midline (v.m.l., indicated by the white arrow), contributes to the separation of the anus from the mouth (Anderson 1966; Arenas-Mena 2006). **(K)** Detail of a gastrulating embryo shows a pair of cells that maintain the expression after invagination, arrowheads; these cells probably adopt a mesenchymal fate and correspond to those labeled by arrowheads in n and o. **(L)** Transversal optical section of an early gastrula embryo at the level of the prospective foregut shows the expression in the blastopore and the lack of expression in epithelial endoderm. **(M)** Transversal section after midline ectodermal blastopore closure and epithelial endoderm formation. **(N)** Side view of a late gastrula embryo. The mouth is still adjacent to the area of the prospective anus. Arrowhead, staining from a mesenchymal cell is out of focus. **(O)** Detail of expressing cells that apparently adopt mesodermal fates associated with the midgut-hindgut juncture. **(P)** 24-hour-old trochophore larva exhibits expression in the anus. **(Q)** Detail of anal expression in an oblique-transversal section. **(R)** Cellular surface staining using a  $\beta$ -catenin antibody illustrates the cellular structure of the ventral midline and mouth of a 24-hour trochophore larva. **(S)** Diagrammatic summary of *HeBra* expression (in red) as indicated. Line filling and dashed lines signify declining expression. First row from left to right, 60- and 68-cell embryos; second row, gastrulating embryo, in frontal (left) and sagittal (right) sections (sagittal section similar to the embryo shown in I); third row, left, side view of gastrulating embryo, ectodermal expression indicated in red and ectodermal cell rearrangements indicated by arrows (similar view to the embryo shown in J). Third row, right, diagram of trochophore larva, sagittal section (similar view to the larva in P). a, anus; ab, aboral; ani, animal; at, apical tuft; bp, blastopore; cb, ciliary band; D, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; o, oral; R, right; V, ventral; vg, vegetal; v.m.l., ventral midline.

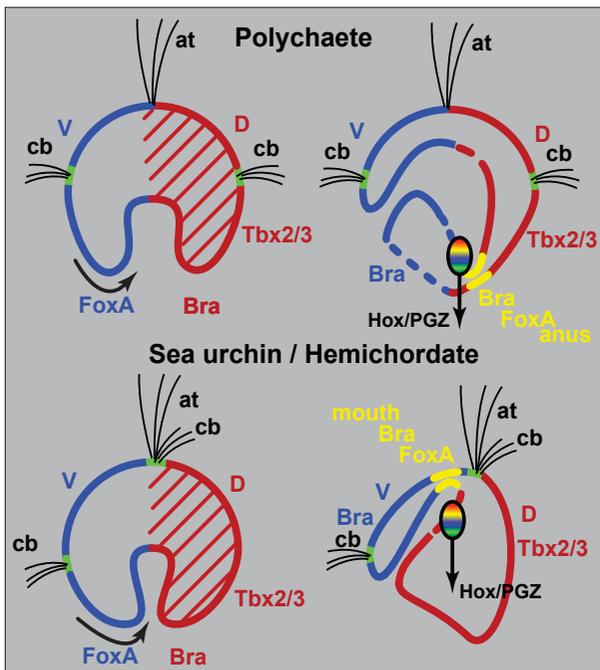


**Fig. 7. *HeTbx2/3* mRNA expression during embryonic and early trochophore larval stages.** For nomenclature and reconstruction of blastomere designations consult our previous report (Arenas-Mena 2007b). Serial optical sections and nuclear staining required for blastomere designations not shown. **(A)** Sagittal optical section of a 64-cell embryo. **(B)** Nuclear staining of the embryo in a. **(C)** Animal cap view of a 64-cell embryo. **(D)** Vegetal view of a 64-cell embryo. **(E)** Nearly sagittal view of a 72-cell embryo. Expression is clearly associated with the dorsal side of the embryo, which is morphologically distinct (Arenas-Mena 2007b). **(F)** Nuclear staining of the embryo in e. **(G)** Animal cap of a 72-cell embryo. The expression has expanded or increased in the left and right flanks. **(H)** Vegetal view of a 68-cell embryo; similar lateral expansion is observed on the vegetal side. **(I)** Nearly sagittal optical section shows expression in endodermal and ectodermal precursors on the dorsal-animal side. The expression has declined in cells in the blastopore area. **(J)** Vegetal view of a gastrulating embryo. The expression remains restricted to dorsal ectodermal and endodermal precursors with a slightly broader domain of expression on the right side. **(K)** Nuclear staining of the embryo in j. Arrowhead points to the ciliary band gap (c.b.g) in the dorsal midline. The 8-shaped blastopore is merging at the middle. **(L)** Slightly oblique optical section. The expression continues in ectodermal, mesodermal and endodermal precursors. The dorsal-most cell of the apical tuft has particularly strong staining. A couple of putative protonephridium precursors (Arenas-Mena 2006) flanking the foregut express the gene, arrowhead. **(M)** Transversal section shows the bilateral arrangement of putative mesenchymal precursors, arrowheads, and the asymmetric expression in the forming hindgut. **(N)** Animal cap view. The dorsal-most apical tuft cells maintain *HeTbx* expression. Cells adjacent to the ciliary band gap (arrowhead) maintain the expression. **(O)** Dorsal-vegetal view. Putative posterior sensory organ precursors (Arenas-Mena & Wong 2007) express the gene. Arrowhead, ciliary band gap. **(P)** Sagittal optical section reveals expression that remains in differentiated dorsal ectoderm and adjacent hindgut of a 24-hour trochophore larva. **(Q)** Diagrammatic summary of *HeTbx2/3* expression (in red) as indicated. an, anus; ani, animal; at, apical tuft; bp, blastopore; cb, ciliary band; c.b.g, ciliary band gap; D, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; R, right; V, ventral; vg, vegetal.

2007). In contrast, endodermal expression of *brachyury* is absent in polychaetes without feeding trochophores that do not form a one-way gut by invagination during embryogenesis. Expression of *brachyury* in endodermal precursors has not been detected in the passive yolk "endoderm" that is internalized by epibolic gastrulation in the mollusk *P. vulgata* (Lartillot 2002) or the polychaete *P. dumerilii* (Arendt *et al.*, 2001) during the formation of its non-feeding trochophore larva. Similarly, the embryonic endodermal expression of *HeOtx* observed in *H. elegans* (Arenas-Mena & Wong 2007) has not been detected in the yolk "endoderm" of *P. dumerilii* (Arendt *et al.*, 2001). In contrast, hemichordates and sea urchins with feeding larvae exhibit a dynamic sequence of activation followed by deactivation of *brachyury* in a ring of endodermal precursors during gastrulation by invagination (Tagawa *et al.*, 1998; Gross & McClay 2001), similar to the pattern of *brachyury* expression in *H. elegans*. Therefore, the evolutionary comparison of gene expression confirms a conserved morphogenetic role, rather than a conserved role in germ layer specification, of *brachyury*.

*Brachyury* and *FoxA* form an evolutionarily conserved synexpression group in the blastopore of protostomes, deuterostomes and cnidarians (Fritzenwanker *et al.*, 2004). Both in *Hydroides* and sea urchin embryos, the areas of endoderm and ectoderm that will fuse to form the respective secondary gut openings are also anticipated by *FoxA* expression (Arenas-Mena 2006; Oliveri *et al.*, 2006). Furthermore, *brachyury* expression is maintained during the early larval stages in the hindgut/anus of *Hydroides* (Fig. 6P) and foregut/mouth of sea urchins (Gross & McClay 2001). Therefore, it seems that *Brachyury* and *FoxA* are required at the boundary of endoderm and ectoderm during the formation of both mouth and anus in protostomes and deuterostomes (Fig. 8).

The expression of *HeSall* in the PGZ suggests there are ancestral regulatory roles associated with posterior growth, which may include conserved Hox cluster gene regulatory functions. Hox cluster gene expression is associated with patterning and controlling anteroposterior elongation across bilaterians, but Hox cluster expression is relegated to the postembryonic feeding-dependent



**Fig. 8. Developmental similarities between embryos of indirectly developing sea urchins, hemichordates and polychaetes.** The transcription factors indicated have polarities in their blastopore expression that are similar between polychaetes and sea urchins; the color boundaries in the diagrams do not always signify gene expression domains. The black arrows symbolize oral-aboral morphogenetic asymmetries during gastrulation. *Tbx2/3* exhibits generalized dorsal expression in the animal and vegetal hemispheres. Development of the secondary archenteron opening is preceded by *FoxA* expression in the ectoderm and endoderm that will eventually fuse (yellow); brachyury expression is maintained in the secondary gut opening during early larval stages (yellow). Brachyury persists longer in the dorsal side (auburn) of the blastopore. Dashed lines in the emerging larvae indicate regions that likely originate after cell rearrangements across initial dorsal-ventral subdivisions. The multicolored circles indicate the position of adult mesodermal precursors whose proliferation results in growth along the anteroposterior direction, indicated by the arrow that symbolizes the *Hox/PNZ* vector. at, apical tuft; cb, ciliary band; D, dorsal; V, ventral.

phase and does not participate in the formation of ciliated larvae during the embryogenesis of deuterostomes (Arenas-Mena *et al.*, 1998; Arenas-Mena 2010) and protostomes (Irvine & Martindale 2000; Peterson *et al.*, 2000) (Fig. 8). The regulatory functions of *sall* and *Hox* transcription factors apparently overlap and/or interact during developmental processes in several bilaterians. In *Drosophila*, *sall* is involved in the regional specification of head and tail structures in conjunction with *Hox* genes (Kuhnlein *et al.*, 1994; Toker *et al.*, 2003); in chicken, *sall* is expressed in the tail bud (Farrell *et al.*, 2001); in *C. elegans*, a posterior role of a *sall* homolog is mediated by repression of the posterior *Hox* gene *egl-5* (Jurgens 1988); in the crustacean *Artemia franciscana*, *sall* depletion triggers derepression of various *Hox* genes in the PGZ (Copf *et al.*, 2006). The expression of *Hox* cluster genes has not been characterized in *Hydroides*, but extrapolation from previous characterizations in nereids (Kulakova *et al.*, 2007) anticipates their expression in the PGZ or in immediate derivatives.

*HeSall* is associated both with gastrulation and PGZ precursors, but the developmental processes involved do not seem continuous

in space and time. Gastrulation and anteroposterior elongation are clearly independent from each other in bilaterians with feeding ciliated larvae (Fig. 8). Gut formation precedes posterior growth in polychaetes with feeding larva. On the contrary, in polychaetes without feeding larvae, posterior growth precedes gut formation, which is postponed to late larval stages (Fig. 1). Accordingly, the embryos of polychaetes without feeding larvae have a relatively larger investment in adult fates, and enlarged 4d and 2d adult precursors that form the proliferative PGZ during embryogenesis (Fig. 1B), whereas the embryos of polychaetes with feeding larvae have a relatively larger investment in larval fates: nearly all blastomeres contribute to the larva except for the small blastomeres 4d and 2d that contribute primarily to the adult during the feeding-dependent developmental phase (Fig. 1A). The distinction between gastrulation and posterior growth may not be so apparent in polychaetes without feeding trochophores because posterior growth is initiated during embryogenesis (Anderson 1966). *Platynereis* is a polychaete without a feeding trochophore, and it has been shown that its early elongation along the ventral midline proceeds by convergent extension (Steinmetz *et al.*, 2007). Nevertheless, it remains uncertain if the simultaneously initiated posterior growth is a continuation of the midline convergent extension or a completely different developmental process in *Platynereis*.

The expression of *Tbx2/3* suggests the possibility of ancestral regulatory entities that subdivide protostome and deuterostome embryos into homologous ventral and dorsal sides (Fig. 8). The dorsal expression of *HeTbx2/3* in all three germ layers is similar to the expression reported in sea urchins where its morphogenetic function has been demonstrated (Gross *et al.*, 2003). Similarly, the ventral side of the blastopore also has a clear bias for the expression of the transcription factor *FoxA* in *H. elegans* and in indirectly developing sea urchins (Arenas-Mena 2006; Oliveri *et al.*, 2006). Furthermore, protostome (Arendt *et al.*, 2001) and deuterostome (Duboc *et al.*, 2004) embryos express the transcription factor *gooseoid* in their ventral-oral side, and in sea urchins *gooseoid* represses *Tbx2/3* expression (Croce *et al.*, 2003). The nerve chord is dorsal in chordates and ventral in many protostomes, and it has been suggested that regulatory gene usage favors their homology (Denes *et al.*, 2007), although alternative interpretations have been proposed (van den Biggelaar *et al.*, 2002). The Bmp-Chordin system in charge of setting dorsoventral specifications is also present in ciliated larvae of protostomes and deuterostomes and it is similarly involved in neural cell-type specifications in sea urchin embryos that, nevertheless, do not contribute to a central nerve chord (Angerer *et al.*, 2000; Yaguchi *et al.*, 2006). The signaling ligand *Bmp2/4* is expressed in the ventral (oral) side of sea urchin embryos, but its overexpression promotes dorsal (aboral) fates (Angerer *et al.*, 2000). Therefore, *Bmp2/4* remains a dorsal-promoting signal in the larval context of protostomes (Denes *et al.*, 2007) and deuterostomes (Duboc *et al.*, 2004), although its source originates from dorsal and ventral sides, respectively. Thus the functions and expression of *Bmp2/4* and *Tbx2/3* apparently relate to dorsoventral specification systems that seem generally conserved between protostome and deuterostomes. Nevertheless, because there is no obvious equivalent of a nerve chord in protostome and deuterostome larvae, this dorsoventral specification system should be distinct from regulatory entities controlling the formation of nerve chords in protostomes and deuterostomes when present.

The modular nature of development (Carroll 2001) allows the

independent evolution of developmental processes. Tubular gut formation, endoderm specification, dorsoventral specification, neural tube development and anteroposterior body elongation are associated with particular sequences of regulatory gene deployment in the context of feeding and non-feeding larval development among bilaterians. Future functional analysis will decipher the regulatory entities controlling these processes and perhaps allow their evolutionary reconstruction along lineages leading to indirect and direct development (Sly *et al.*, 2003).

## Materials and Methods

Protocols, reagents and methods utilized in this manuscript have been previously described (Arenas-Mena 2006; Arenas-Mena & Wong 2007), including the sequences of degenerate primers against conserved T-box and zinc finger sequences used for PCR-based cloning (Arenas-Mena 2006). Whole Mount *In situ* Hybridization (WMISH) methods also have been described previously (Arenas-Mena 2006; Arenas-Mena & Wong 2007). In short, embryos were fixed in 0.1 M MOPS pH 7, 0.5 M NaCl, and 4% formaldehyde for 3 h at room temperature. After five washes in at least 10 volumes of 0.1 M MOPS, 0.5 M NaCl and 0.1% Tween-20 (MOPS buffer), the samples were stored indefinitely in 70% ethanol at -20°C. Rehydration in MOPS buffer was followed by a 3-hour prehybridization at 50°C in a solution of 70% deionized formamide, 0.5 M NaCl, 0.1 M MOPS (pH 7), 0.1 mg/ml BSA and 0.1% Tween-20. Hybridization was performed in the same buffer with digoxigenin-UTP-labeled probes at 0.1 ng/μl for one week at 50°C. Excess probe was removed after 3 washes in MOPS buffer with a 3-hour incubation under hybridization conditions. The samples were blocked with 10 mg/ml BSA in MOPS buffer for 20 minutes at room temperature and then with 10% goat serum plus 1 mg/ml BSA at 37°C for 30 minutes in MOPS buffer. Incubation of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN) was done at 1/2000 dilution, and the samples were developed in NBT/BCIP buffer until sufficient contrast of staining was obtained. Simultaneous nuclear staining of the embryos with DAPI and visualization of multiple optical serial sections (not shown) revealed in each case the blastomere designations indicated in the figures according to the invariant spiral cleavage previously described in this species (Arenas-Mena 2007b). Additional diagrams that help understand the anatomy of *H. elegans* and its invariant cleavage can be found in previous publications (Arenas-Mena 2006; Arenas-Mena 2007b; Arenas-Mena *et al.*, 2007b).

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