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Expression of *GATA* and *POU* transcription factors during the development of the planktotrophic trochophore of the polychaete serpulid *Hydroides elegans*

Kimberly Suk-Ying Wong,[a] and Cesar Arenas-Mena[b,*]

[a] Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614, USA
[b] Department of Biology, College of Staten Island and Graduate Center, The City University of New York (CUNY), Staten Island, NY 10314, USA

*Author for correspondence (e-mail: cesar.arenasmena@csi.cuny.edu)

SUMMARY The expression of transcription factors with endodermal and mesodermal roles in bilaterians is characterized during the development of *Hydroides elegans*, a serpulid polychaete with planktotrophic trochophore. GATA 4/5/6 is expressed in endodermal and mesodermal precursors during embryogenesis and in the midgut of trochophore larvae. *HeGATA1/2/3a* is expressed in animal hemisphere blastomeres 1d121 and 1d122, in dorsal ectoderm and in 4d endomesodermal derivatives that maintain their expression in trochophore larvae. *HeGATA1/2/3b* is not expressed during embryogenesis, but in several regions of the larva during postembryonic development. During very early gastrulation, *Brn1/2/4* is first expressed in cells associated with the prospective oral/foregut side of the blastopore, and during larval development in 4d blastomere descendants. Comparison with orthologs in other metazoans suggests ancestral expression of *GATA4/5/6* in the midgut of the last common ancestor of protostomes and deuterostomes. The conserved expression of *Brn1/2/4* in the foregut precursors of *Hydroides* and sea urchins suggests an ancestral role in patterning the tripartite gut of planktotrophic larvae. Broader analysis of these and other regulatory genes reveals variability of developmental gene expression among polychaetes with lecithotrophic larvae, suggesting that they are evolutionarily derived from polychaetes with planktotrophic larvae.

INTRODUCTION

In *Hydroides elegans* and similarly developing polychaetes, gastrulation by active invagination of endoderm precursors generates the functional gut epithelium of a planktotrophic trochophore (Hatschek 1885; Shearer 1911; Anderson 1966; Arenas-Mena and Li 2014). In many other polychaetes, gastrulation by epiboly internalizes passive and yolky endoderm precursor cells that do not form a functional epithelial gut during embryogenesis (Arenas-Mena and Li 2014). The old question about the evolution of lecithotrophic (direct) and planktotrophic (indirect) development among bilaterians remains a major enigma that is relevant to life-cycle and body-plan evolution in metazoans (Peterson et al. 1997; Arendt et al. 2001; Sly et al. 2003; Arenas-Mena 2010; Nielsen 2013). The direct/indirect development dichotomy is less dramatic when intermediate developmental modes are taken into consideration (Allen and Pernet 2007; Page 2009; Arenas-Mena 2010). Furthermore, both the ontogeny of indirect developers and their evolution seem more feasible when recent findings on developmental plasticity of differentiated cells are taken into consideration (Arenas-Mena 2010; Arenas-Mena and Coffman 2015). Nevertheless, differences in gene regulation are expected to be associated with the distinct cell-type specifications and morphogenetic events of polychaete embryos that form lecithotrophic or planktotrophic trochophores (Arenas-Mena and Li 2014). In principle, conservation of developmental gene expression is expected among developmental modes that are closer to the ancestral and variability among derived developmental modes.

The GATA family of transcription factors contain one or two zinc-finger DNA-binding domains that interact with the consensus sequence (A/T)GATA(A/G), and they are found in unicellular and multicellular eukaryotes (Patient and McGhee 2002). The gene family has few members in bilaterians, and independent gene duplications have occurred among different lineages (Gillis et al. 2008). GATA factors are involved in distinct developmental processes including embryonic endoderm and mesoderm germ layer specification, cell-fate specification during hematopoiesis, regulation of endoderm differentiation, and control of cell proliferation and morphogenetic movements during heart development (Patient and McGhee 2002). The family is subdivided in two classes, GATA1/2/3 and GATA4/5/6, and the last common ancestor of protostomes and deuterostomes apparently had at least one

member of each class (Gillis et al. 2008). Only one *GATA* gene was isolated in the cnidarian *Nematostella vectensis* (Martindale 2004), suggesting that the two subclasses originated along the lineage leading to bilaterians (Gillis et al. 2008). Endodermal, mesodermal, and ectodermal expression of *GATA* genes has been reported among various spiralians (Boyle and Seaver 2008, 2010; Passamaneck et al. 2015), ecdysozoan (Murakami et al. 2005), and echinoderm representatives (Lee and Davidson 2004; Solek et al. 2013). Midgut-restricted GATA 4/5/6 expression has been reported in spiralian (Boyle and Seaver 2008, 2010; Passamaneck et al. 2015), ecdysozoan (Murakami et al. 2005), and echinoderm representatives (Lee and Davidson 2004). Therefore, the characterization of GATA factors in *Hydroides* will inform about their involvement in endoderm, mesoderm, and midgut development in this embryo.

In addition, we decided to study the transcription factor *Brn1/2/4* in order to better understand the regulation of tripartite gut regionalization in the planktotrophic larvae of *Hydroides*. *Brn1/2/4* is a member of the POU-domain family, characterized by having a DNA-binding domain with two regions: a POU-specific domain and a POU homeodomain (Ryan and Rosenfeld 1977). The family has been subdivided in six classes with deep metazoan origins (Gold et al. 2014). POU transcription factors have very diverse developmental roles, including various neural development roles that have been thoroughly summarized previously (Wollesen et al. 2014). The expression of *Brn1/2/4* in *Hydroides* is relevant in our comparative studies because the ortholog gene is expressed during foregut regionalization of the sea urchin feeding larva (Cole and Arnone 2009), and therefore, could inform about shared regulatory mechanisms between protostome and deuterostome feeding larvae.

The characterization of GATA and *Brn1/2/4* transcription factors in *Hydroides* advances our understanding of their role in endoderm, mesoderm, and gut development in spiralians. GATA 4/5/6 is expressed in the midgut and *Brn1/2/4* in the foregut of *H. elegans*, suggesting an evolutionarily conserved role in tripartite gut subdivision among planktotrophic bilaterian larvae. The results are discussed in a broader regulatory and evolutionary context that explores how lecithotrophic and planktotrophic development could have evolved. The present study supplements previous regulatory gene expression studies in *H. elegans* embryos, currently the best characterized among planktotrophic polychaetes (Arenas-Mena and Li 2014). The combined evidence favors scenarios proposing that lecithotrophic development evolved from planktotrophic development in polychaetes.

MATERIALS AND METHODS

Cloning and characterization of transcription factors

Degenerate oligonucleotides against conserved regions resulted in amplification of fragments of 348 bp for *HeBrn1/2/4*, 274 bp for *HeGATA1/2/3a*, 274 bp for *HeGATA/1/2/3b*, and 271 bp for *HeGATA4/5/6* using as template a mixture of cDNAs prepared from mRNA from unfertilized eggs, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- and 12-h embryos and 24- and 32-h larvae.

The GenElute™ Mammalian Total RNA Miniprep Kit from Sigma® was used for total RNA preparation and the GenElute™ mRNA Miniprep Kit to purify polyadenylated mRNA. Rapid amplification of cDNA ends (RACE) PCR was used to extend in the 5′ and 3′ direction of *HeBrn1/2/4* and *HeGATA1/2/3a*, while only 5′ RACE extensions were obtained for the remaining *HeGATA* genes. Sequences retrieved from the degenerate PCR reaction and the RACE extension reactions were of 2124 bp for *HeBrn1/2/4*, 1164 bp for *HeGATA1/2/3a*, 637 bp for *HeGATA1/2/*3b, and 1337 bp for *HeGATA4/5/6*.

For *HeGATA4/5/6* isolation, forward GGCCGGGAGTGCGTNAAYTGYGG and reverse CGGGTCTGGATGCCGTCYTTNYKCAT primers against conserved domains were used. PCR products were T-cloned using the PCR® II-TOPO® kit. To obtain rapid amplification of cDNA ends (RACE) extensions with the BD® SMART™ cDNA amplification Kit, *HeGATA1/2/3b* and *HeGATA4/5/6* nested gene specific primers were designed in the 5′ and 3′ directions. The reverse primer and nested reverse primer used for *HeGATA1/2/3b* RACE extension were CATTATGTAGCTTGAAGTAGAGCCCGCATGCGTTGCACACTGGGTCACCGTT and CGTCGCCACAGCGTCGTGGTCGTCGTTCC, respectively. The reverse primer and nested reverse primer used for *HeGATA4/5/6* were GTCTGGATGCCGTCCTTTCTCATTGACATGGGGCGGTTCACCTG and GAAGTGGAACAGTTGGCGCAGTTCAATCCGGATCTACGAGAACCCTGCAT, respectively. *HeGATA1/2/3a* 5′ RACE-extension was obtained by using the reverse primer ATGGTAGAGGCCACAGGCGTTGCATAAATA.

For Brn isolation, forward WSNGAYGAYYTNGARCARTTYGCNAA and reverse RTTRCARAACAANACNCKNACNACYTCYTTYTCNARYTG primers against conserved domains were used. Extensions were performed with reverse ACAGTGTCCCTAGCGCTAATC CCACATCCGCTTGCGT, forward TCCTGAAACAACCAAAACCCGCCGCGCAGGA, and nested forward CGCAGGAAATCCTTG GCCTTTCCGA.

Phylogenetic analysis and manipulation of sequences were done using the freely available software package Bioedit 7.0.5.2 (Hall 1999) and Molecular Evolutionary Genetics Analysis MEGA 6.06 (Tamura et al. 2013).

Biological material

Spawning of adult worms was induced by partially breaking the calcareous tubes of wild *H. elegans*. Fertilization and embryo cultures were performed at room temperature, 22°C, in Instant Ocean® sea water reconstituted at 35% salinity. Larvae were fed microalgae *Isochrysis galbana*.

WMISH procedures were basically the same as previously optimized for *H. elegans* (Arenas-Mena 2006). The 5′ RACE-extended gene fragment of *HeBrn1/2/4*, *HeGATA1/2/3b*, *HeGATA4/5/6*, and the 3′ RACE-extended gene fragment of *HeGATA1/2/3a* were used to synthesize antisense digoxigenin-labeled probes for WMISH. *H. elegans* embryos were fixed with 12% formaldehyde, 0.5 M NaCl, and 0.1 M MOPS pH7 solution. After incubation at room temperature for 20 min, the embryos were centrifuged at approximately 450*g*. The fixative was removed by washing four times with MOPS Buffer (MB) (0.1 M MOPS pH7, 0.5 M NaCl, and 0.1% Tween-20) and stored indefinitely in 70% ethanol at −20°C. To rehydrate, the ethanol was removed with five MB washes followed by prehybridization in Hybridization Solution (HS) (70% deionized formamide, 0.5 M NaCl, 0.1 mg/ml BSA, 0.1% Tween-20 in 0.1 M MOPS pH 7) for approximately 3 h. After prehybridization, the specific RNA probe for the gene of interest was added to the embryos at a 0.3–1 ng/μl concentration in HS, and was left to hybridize for 7 days at 50°C. After 7 days, the probe was removed from the solution with five MB washes, and samples were again incubated with HS for 3 h at 50°C. The HS was then removed with five additional MB washes. The samples were then incubated in 1 mg/ml BSA, 10% goat serum, and 1/2000 dilution of Anti-Digoxigenin-AP antibody in MB solution and incubated at room temperature overnight. To remove the antibody, the embryos were washed overnight in MB. Alkaline phosphatase based developing buffer solution (20 × BCIP, 20 × NBT in alkaline phosphatase buffer) was used to stain the targeted location of the specific RNA probe in the embryo. When the embryos showed the appropriate amount of staining contrast, the reaction was halted with three washes in MB. Following these MB washes, DAPI staining in MB was done for all samples. Consecutive serial sections of DIC and DAPI imaging, oftentimes across the whole embryo were taken in order to identify blastomeres.

RESULTS

Developmental expressionof *HeGATA1/2/3a*, *HeGATA1/2/3b*, and *HeGATA4/5/6*

Phylogenetic analysis identified the affiliation of transcription factors *HeGATA1/2/3a*, *HeGATA1/2/3b*, and *HeGATA4/5/6* (Fig. 1) to the GATA family subgroups 1/2/3 and 4/5/6 (Gillis et al. 2008). Two zinc fingers are present in the isolated *GATA* genes (Fig. 1A). We isolated two *HeGATA1/2/3* paralogs in *Hydroides* instead of only one found in polychaetes *Capitella teleta* (Boyle and Seaver 2008) and *Platynereis dumerillii* (Gillis et al. 2007). *HeGATA1/2/3b* is more similar in sequence to homologous genes in these polychaetes than to its *Hydroides* counterpart *HeGATA1/2/3a*. This suggests that *HeGATA1/2/3a* underwent divergence after its clade-specific duplication or that *C. teleta* and *P. dumerillii* lost the *HeGATA1/2/3b* paralogs. The single *Nematostella vectensis GATA* gene (Martindale 2004) is more similar to the GATA1/2/3 subgroup than to the 4/5/6 subgroup, consistent with previous analysis (Boyle and Seaver 2008). Nevertheless, both subgroups are thought to derive from a single gene present in the last common ancestor of cnidarians and bilaterians (Gillis et al. 2008). Because there is no sequenced genome available for *H. elegans*, we do not exclude the possibility of additional *GATA* genes.

HeGATA4/5/6 expression starts in the vegetal quartet 2Q (Q stands jointly for A, B, C, and D quadrants) of 16-cell embryos (Fig. 2a) and it is maintained in the vegetal quartet 4Q of 60-cell stage embryos (Fig. 2b and c); for a detailed description and graphics of *H. elegans* development, please consult previous reports (Arenas-Mena 2006, 2007b; Arenas-Mena and Li 2014). These presumptive gut precursors that express *HeGATA4/5/6* will be the first blastomeres to ingress, and will presumably end located in the prospective midgut area. The expression is not maintained in the animal sister cells 3q and 4q. Later, in 64-cell embryos, 4a, 4b, and 4c blastomeres do express *HeGATA4/5/*, but there is no expression in the dorsal counterpart 4d (Fig. 2e). The expression becomes clearly uneven in 68-cell embryos; it is much stronger in 4a, 4b, and 4c (Fig. 2f) than in the central blastomeres 4Q and 5q. At the beginning of gastrulation, the expression-level further declines in central blastomeres that barely show any expression (Fig. 2h). The strongly expressing cells associated with the forming archenteron locate in foregut and hindgut areas, and possibly have mesodermal fates because they are not integral part of the incipient endoderm epithelium (Fig. 2i, j, m, and n). The fate of these cells remains uncertain because their expression does not continue in differentiated larval organs. In late gastrula embryos, the expression increases along the archenteron (Fig. 2k, l, and o), and in the trochophore larvae *HeGATA4/5/6* expression is restricted to the midgut (Fig. 2p).

HeGATA1/2/3a transcripts are first detected in the animal-cap blastomeres 1d121 and, more strongly, in 1d122 of the almost 72-cell embryo (Fig. 3a). During subsequent cleavage and early gastrulation (Fig. 3c–f), the expression apparently remains restricted to 1d121 and 1d122. During later gastrulation stages, the expression includes additional cells by the dorsal midline in both the animal and vegetal hemispheres (Fig. 3d–j), although the expression remains stronger in animal hemisphere cells, most likely 1d121 and 1d122 and/or their derivatives (Fig. 2e–j), and in blastomeres located by the dorsal side at the base of the blastopore (Fig. 2e–j, white arrowhead); the expressing blastomeres possibly include endomesodermal (4d) derivatives according to earlier anatomical descriptions (Hatschek 1885; Shearer 1911; Arenas-Mena and Li 2014). In 24-h trochophores, expression is maintained in the growth zone in large cells near the anus (Fig. 2k and l, arrowheads), possibly corresponding to 4d descendants (Hatschek 1885; Shearer 1911; Arenas-Mena and Li 2014).

HeGATA1/2/3b expression was not detected during embryogenesis. Expression first appears in trochophore larvae that have

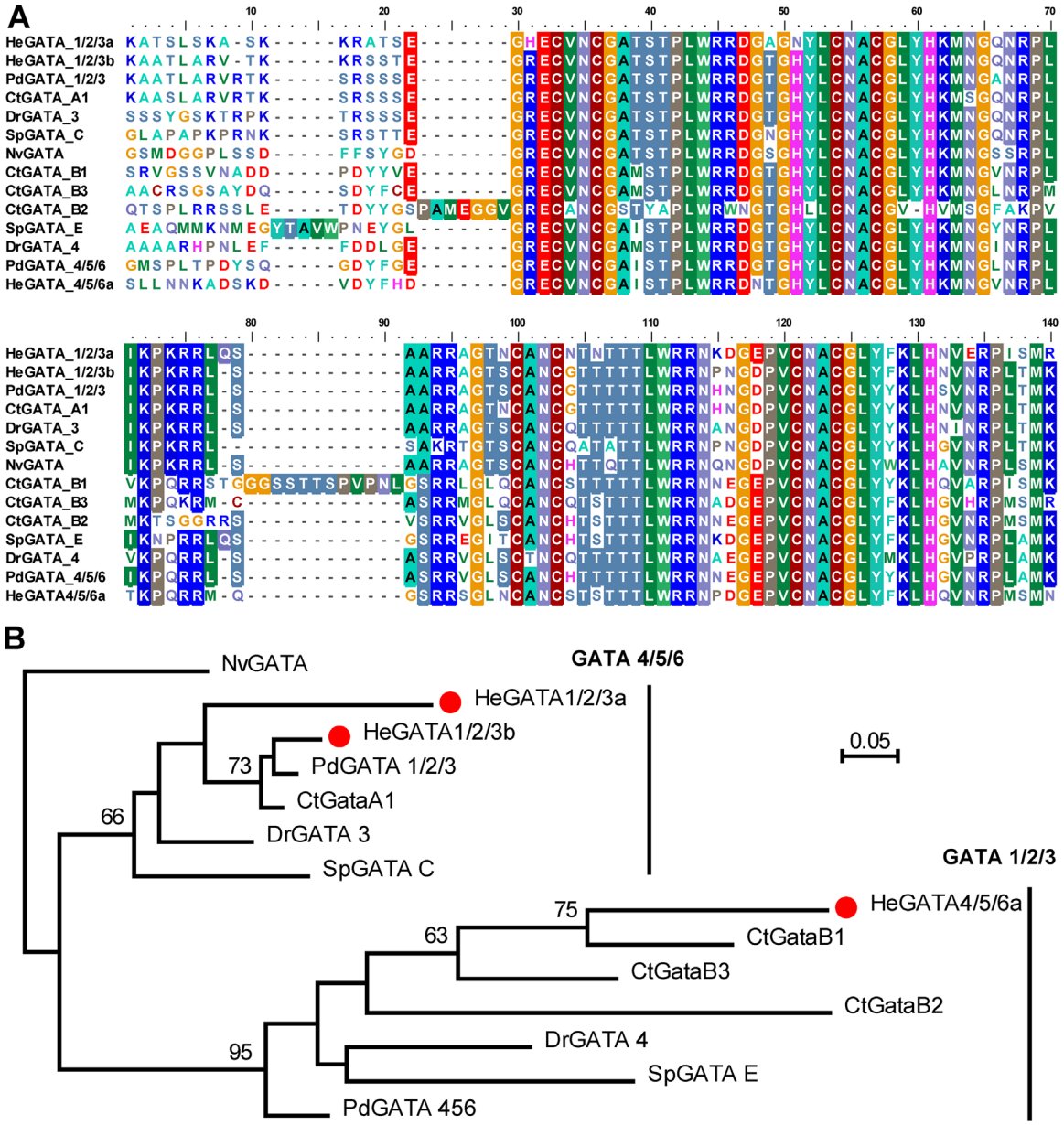

Fig. 1. Orthology assignment of *HeGATA1/2/3a*, *HeGATA1/2/3b*, and *HeGATA4/5/6*. (A) Alignment of GATA proteins from different subfamilies using muscle (Edgar, 2004). Two consecutive zinc-finger DNA-binding domains are shown. (B) Phylogenetic tree derived from maximum likelihood analysis of the sequence region shown in A. Branch lengths are proportional to the number of changes per amino acid indicated by the scale. Percent of bootstrap values above 50% supporting the respective nodes after 1000 replications are shown. Prefixes signify species: Ct, *Capitella teleta*; Dr, *Danio rerio*; Nv, *Nematostella vectensis*; Pd, *Platynereis dumerilii*; Sp, *Strongylocentrotus purpuratus*. Accession numbers: CtGATA_A1, gi|157169245|; PdGATA_1/2/3, gi|117276621|; SpGATA-C, gi|3702856|; DrGATA-3, gi|1245717|; NvGATA, gi|38569869|; CtGATA_B1, gi|157169239|; CtGATA-B2, gi|157169241|; CtGATA-B3 gi|157169243|; PdGata_4/5/6 (Pd), gi|117276623|; SpGATA-E, gi|54262117|; DrGATA-4, gi|114319150|; HeGATA1/2/3a |KU587793|, HeGATA1/2/3b | KU587794|, and HeGATA4/5/6 |KU587792|.

been fed microalgae for several days (Fig. 4). *HeGATA1/2/3b* is strongly expressed in a variety of regions during postembryonic development. Endodermal cells of the foregut and hindgut express the gene, but not homogeneously; lower levels of expression are seen in the dorsal side of the midgut (Fig. 4c and d). The animal ectoderm does not express the gene, except for some larval organs or adult organ precursors possibly involved in postembryonic development (Fig. 4a, c, and d). Some differentiated larval organs, such as the paired protonephridiums, do not have much expression relative to adjacent tissues

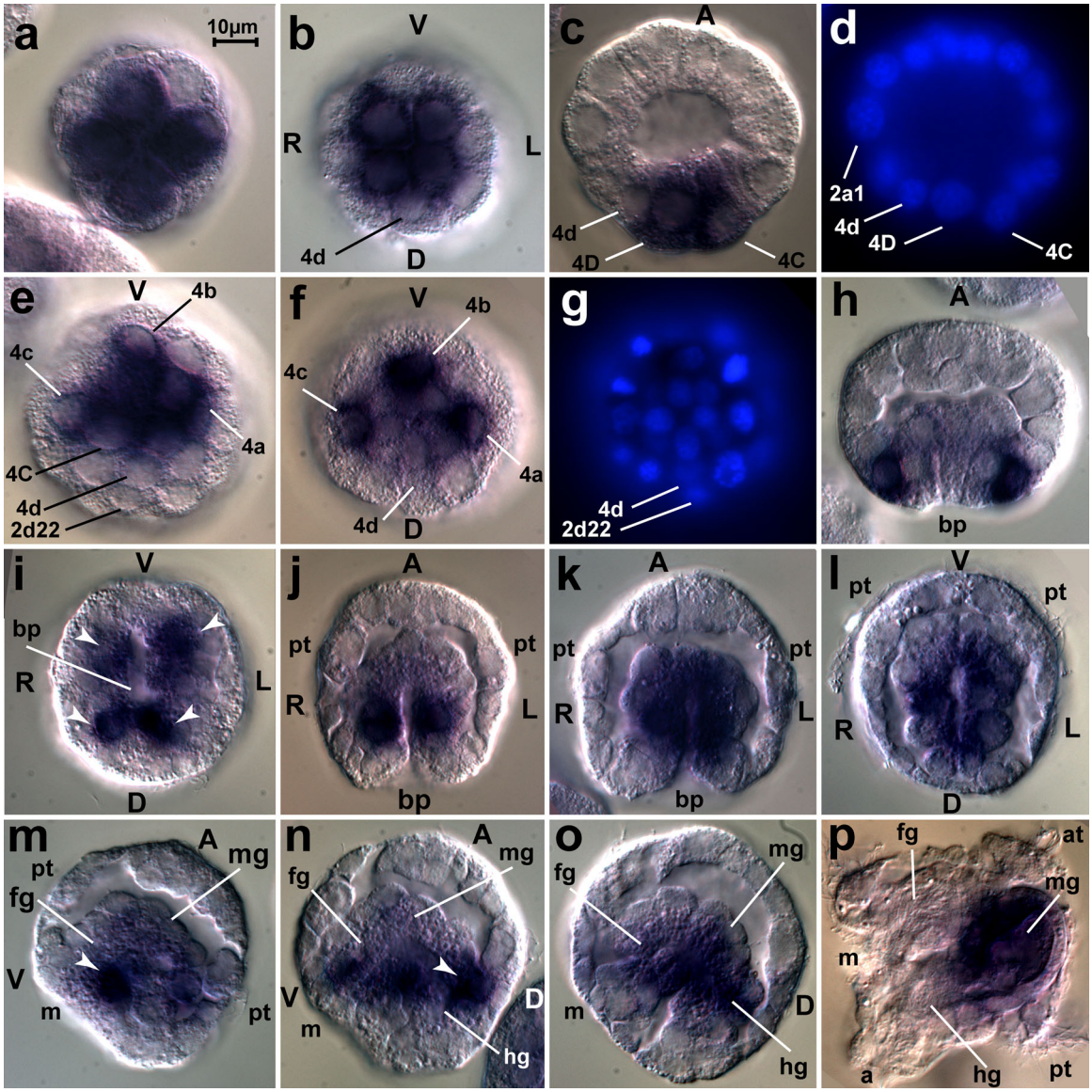

Fig. 2. *HeGATA4/5/6* mRNA expression during embryo and larval stages. (a) Vegetal view of a 16-cell embryo shows vegetal quartet (2Q) expression. Scale bar for this and subsequent panels. (b) Vegetal view of a 60-cell embryo shows expression in the 4Q quartet. (c) Side view of an embryo in a 60-cell embryo illustrates the expression in the vegetal-most blastomeres. (d) DAPI staining corresponds to c. (e) Vegetal view of 64-cell embryo seen from the animal hemisphere with expression in 4Q, 4a, 4b, and 4c. (f) Vegetal view of a 68-cell embryo seen from the vegetal pole shows stronger expression in 4a, 4b, and 4c and declining expression in 5Q and 5q. (g) DAPI staining of the embryo in f. (h) Transversal section of an embryo during early gastrulation, intersecting now relocated blastomeres 4a and 4c. (i) Vegetal view of a mid-gastrula stage embryo shows stronger expression in a subset of cells in the archenteron, see arrowheads. (j) Frontal section of a mid-gastrula embryo shows strong expression in a subset of cells in the archenteron. (k) Frontal section of a late-gastrula embryo shows broader expression in the archenteron. (l) Transversal section of a late-gastrula embryo shows generalized archenteron expression. (m) Side view of an embryo during mid-gastrulation shows expression in mesodermal cells adjacent to the foregut, see arrowhead, oral side to the left. (n) Mid-gastrula stage embryo shows particularly strong expression in mesodermal cells by the hindgut region, see arrowhead. (o) Sagittal section of late-gastrula embryo about to form the anal opening shows generalized endodermal expression. (p) Sagittal view of a 24-h trochophore larva shows expression in the midgut. a, anus; A, animal; bp, blastopore; D, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; pt, prototroch; R, right; V, ventral.

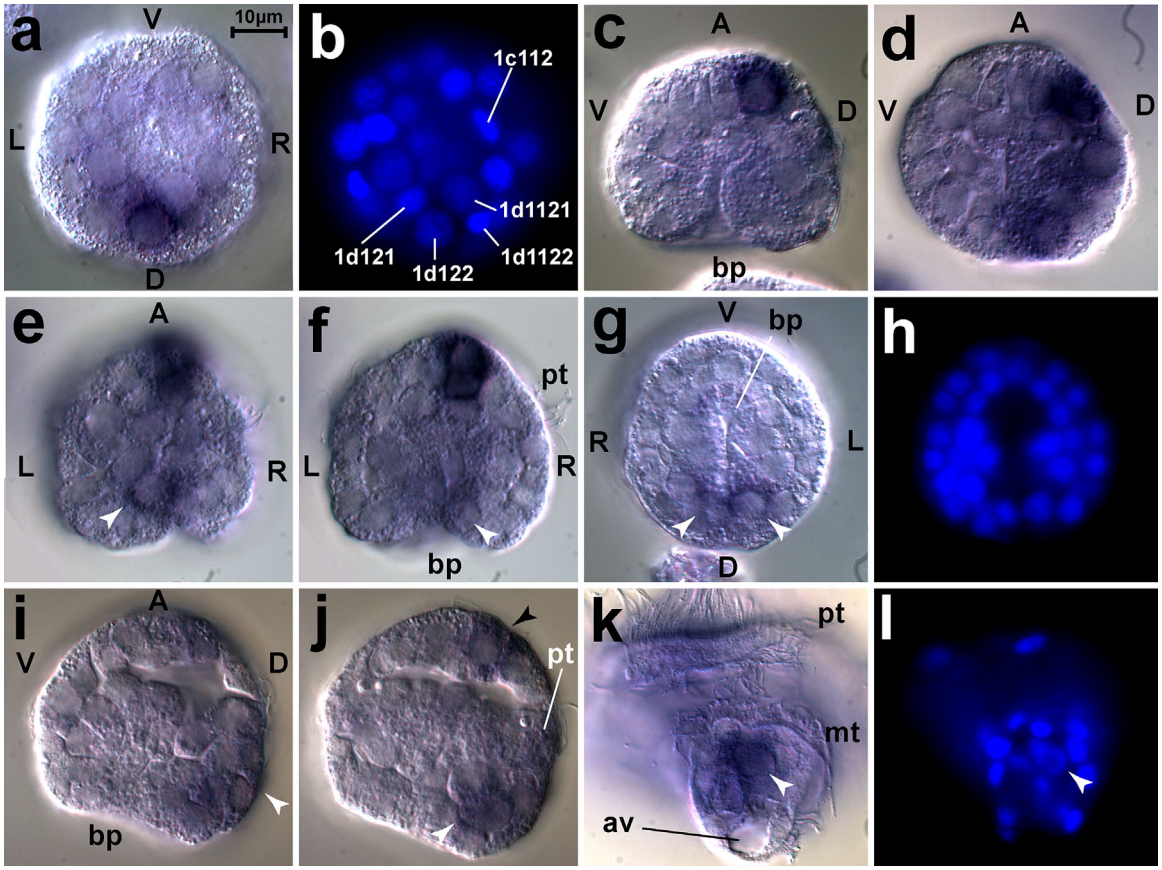

Fig. 3. *HeGATA1/2/3a* mRNA expression during embryogenesis and larval stages. (a) Animal view of a 71-cell embryo shows expression in 1d121, slightly out of focus, and 1d122, more intensely stained. (b) DAPI staining of the embryo in a. (c) Sagittal section of an early-gastrula stage embryo shows expression in a single cell, possibly 1d121 (d) Sagittal optical section of mid-gastrula stage shows expression in additional cells along the dorsal midline. (e and f) Consecutive transversal sections starting from the dorsal side of a mid-gastrula embryo reveal more intense staining in the animal hemisphere (e) and in mesodermal (f) cells at the dorsal side of the blastopore, arrowheads. (g) Transversal section of the blastopore at the vegetal pole of a gastrulating embryo more clearly reveals the extent of expression by the dorsal side of the blastopore. (h) DAPI staining of the embryo shown in g, the 8-shaped blastopore can be observed at this stage. (i and j) Sagittal serial sections of a gastrulating embryo at a stage similar to that shown in g; white arrowhead in i indicates ectodermal expression, and in j it marks an expressing cell that possibly corresponds to the left derivatives of 4d. Animal hemisphere cells maintain their expression, black arrowhead. (k) Posterior view of a 24-h trochophore. (l) DAPI staining of the trochophore shown in k, the arrowhead marks the expressing cell, which possibly corresponds to 4d derivatives. A, animal hemisphere; av, anal vesicle bp, blastopore; D, dorsal; mt, metatroch; L, left; pt, prototroch; R, right; V, ventral.

(Fig. 4e). The growth zone (Fig. 4 f) and adjacent tissues (Fig. 4b) exhibit strong expression, while the differentiated prototroch, the ventral midline, which includes the gastrotroch, its adjacent ectoderm, and the anal vesicle do not express *HeGATA1/2/3b* (Fig. 4b, e, and f). In general, the expression does not associate with particular organs or cell types, and it is clearly excluded from terminally differentiated larval cells such as the prototroch or the apical tuft. Thus, the expression seems associated with general larval development.

Developmental expression of *HeBrn1/2/4*

Transcription factor *HeBrn1/2/4* contains a POU-specific domain and a POU homeodomain, a unique feature of the gene family (Fig. 5A). Phylogenetic analysis reveals that *HeBrn1/2/4* belongs to the class III within the POU family.

HeBrn1/2/4 is first detected in three blastomeres to the ventral, right, and left sides of the prospective blastopore during very early gastrulation (Fig. 6a). Once the blastopore is formed, expression is robust in individual cells in the A, B, and C quadrant cells, but the corresponding blastomere by the dorsal-most side of the D quadrant shows no expression (Fig. 6b). The expression is maintained during early gastrulation (Fig. 6c and d), but it starts to decline once the blastopore adopts its typical slit shape; the weaker expression now involves more than the initial three cells (Fig. 6e). Soon thereafter, expression is seen in two blastomeres by the anal side of the blastopore that became internalized during gastrulation (Fig. 6f–i). The expression

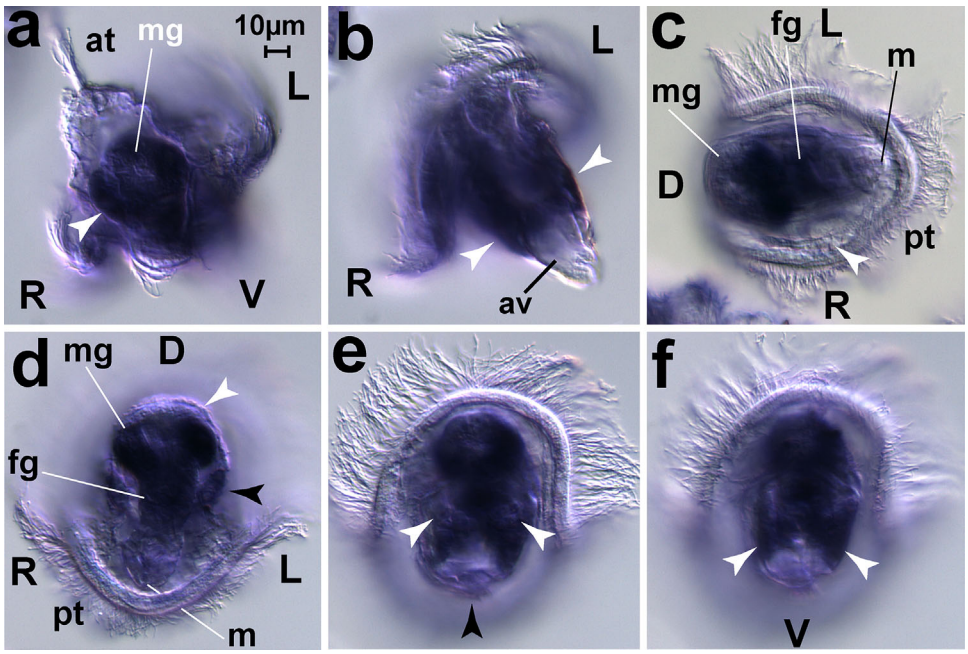

Fig. 4. *HeGATA1/2/3b* mRNA expression in 3-day old larvae. Consecutive oblique sections are shown to maximize optical clarity in the context of broad and strong expression. (a) Animal hemisphere view of a 4-day old trochophore larva shows expression in the midgut and associated mesodermal cells, arrowhead. The apical tuft cells and surrounding animal hemisphere ectoderm do not express the gene. The orientation of this optical section is oblique; it intersects the apical tuft and is tilted toward the mouth in the ventral side. Scale bar for this and subsequent panels. (b) Optical section of the larvae at the level of the anal vesicle. Expression is detected in ectodermal regions that comprise the adoral ciliary zone and the growth zone, arrowheads. (c) Transversal section at the level of the prototroch. The dorsal side of the midgut has lower expression than the side facing the foregut. The ciliary band and associated cells do not express the gene. (d–f) Consecutive sections in oblique orientation (similar to a and b). (d) Shows expression in the ventral side of the midgut and foregut. The ectoderm does not express the gene, white arrowhead, except for some unidentified larval organs or adult precursor cells, black arrowhead. (e) Protonephridium cells seem to have lower expression than surrounding tissues, white arrowheads. The ventral side flanking the central gastrotroch does not exhibit expression, black arrowhead. (f) Arrowheads mark cells by the growth zone that express the gene, arrowhead. at, apical tuft; av, anal vesicle; D, dorsal; L, left; mg, midgut; m, mouth; pt, prototroch; R, right; V, ventral.

starts on the left side (Fig. 6f) and then becomes bilateral (Fig. 6g). Considering previous observational lineage descriptions (Hatschek 1885; Shearer 1911), the expressing blastomeres by the dorsal side should correspond with 4d derivatives. The cells having low expression by the ventral side of the blastopore seem to be integral part of the archenteron and occupy positions that possibly contribute to the foregut (Fig. 6g, arrowhead). As gastrulation continues, *HeBrn1/2/4* has low expression in the foregut area (Fig. 6i and k) and maintains the expression in mesodermal cells flanking the hindgut and just on top of the anal vesicle, which most likely are 4d derivatives (Fig. 6k and l). We did not detect *HeBrn1/2/4* expression above background during postembryonic development other than in a few cells expressing the gene in the growth zone that possibly are derived from 4d (not shown).

DISCUSSION

We have identified one GATA4/5/6 and two GATA1/2/3 paralogs in *H. elegans* (Fig. 1). Analysis of a few spiralians generally revealed several GATA4/5/6 homologs and a single GATA1/2/3 member (Gillis et al. 2008), although only one GATA4/5/6 was reported in the brachiopod *Terebratalia transversa* (Passamaneck et al. 2015) and two *GATA1/2/3* genes were identified in the planarian *Schmidtea polychroa* (Martín-Durán and Romero 2011). The orthology of the planarian paralogs cannot be properly analyzed due to the short sequences available. Our analysis suggests that the *HeGATA1/2/3a* and *HeGATA1/2/3b* paralogs originated along the lineage leading to *Hydroides*. Because the genome of *H. elegans* remains to be sequenced, our results do not exclude the possibility of additional GATA factors yet to be discovered.

The embryonic expression of *GATA4/5/6* in endodermal and mesodermal precursors and the expression during midgut differentiation are evolutionarily conserved. The initial expression of *HeGATA4/5/6* in 2Q blastomeres is only maintained in the vegetal-most descendants after cleavage, that is, the 3Q and 4Q quartets, which implies the prompt transcript degradation and/or lack of expression in their animal 3q and 4q descendants (Fig. 2a–d). The expression of *HeGATA4/5/6* at the 64-cell stage includes 4Q, 4a, 4b, and 4c, but not the dorsal-quadrant

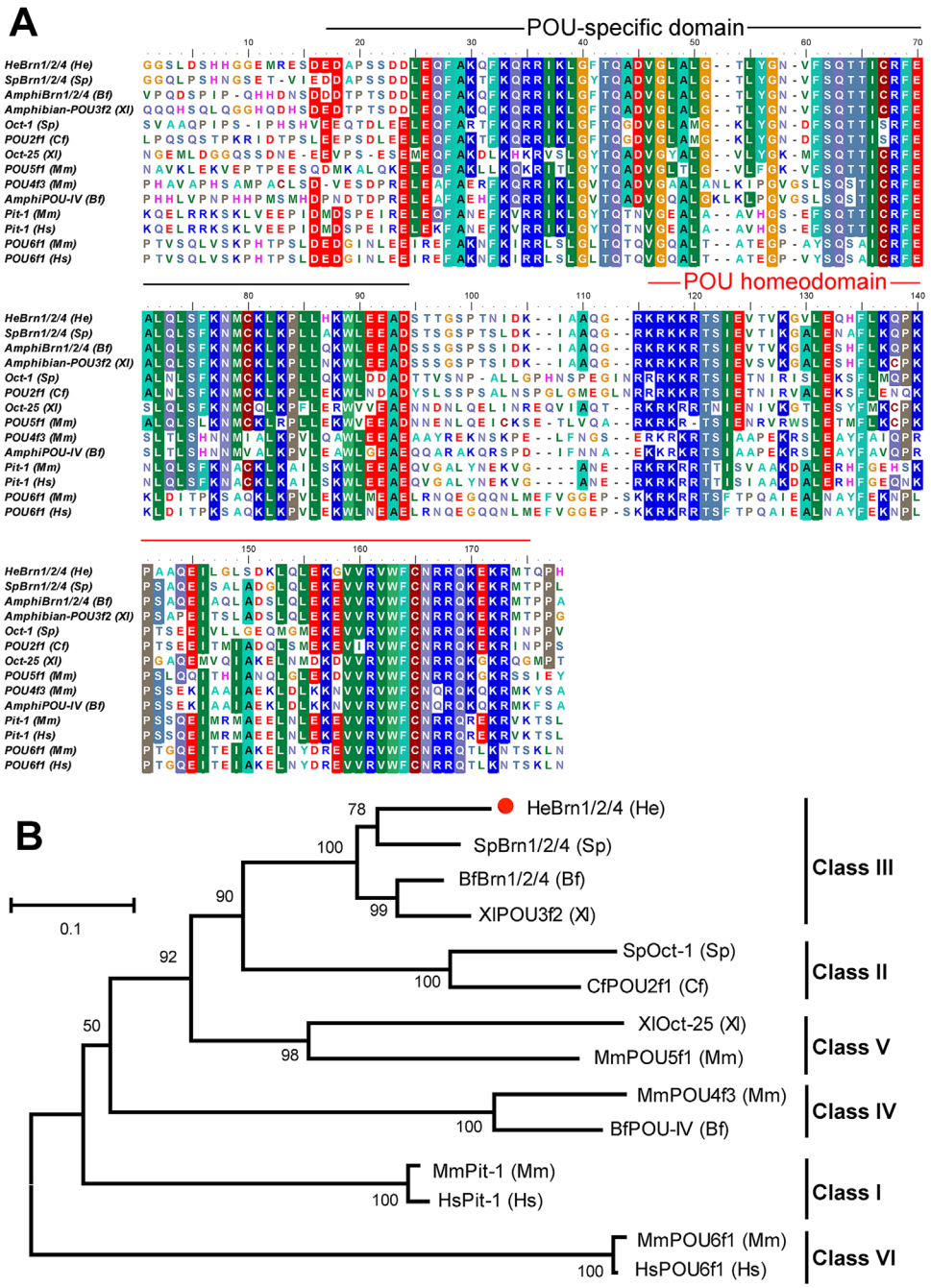

Fig. 5. Orthology assignment of *HeBrn1/2/4*. (A) Alignment of HeBrn1/2/4 with proteins from the six different POU classes using Muscle. DNA-binding domains are indicated. (B) Phylogenetic tree derived from maximum likelihood analysis of the sequence region shown in A. Branch lengths are proportional to the number of changes indicated by the scale. Percent of bootstrap values above 50% supporting the respective nodes after 500 replications are shown. *Strongylocentrotus purpuratus* (Sp), *Branchiostoma floridae* (Bf), *Xenopus laevis* (Xl), *Canis familiaris* (Cf), *Mus musculus* (Mm), *and Homo sapiens* (Hs). Accession numbers: *SpBrn1/2/4* (Sp), gi|115966424|; *Oct-1*(Sp), gi|62821798|; *AmphiBrn1/2/4* (Bf), gi|20384894|; *AmphiPOU-IV* (Bf), gi|83751826|; *POU3f2* (Xl), gi|27370988|; *Oct-25* (Xl), gi|111146889|; *POU2f1* (Cf), gi|73960713|; *POU5f1* (Mm), gi|46189245|; *POU4f3* (Mm), gi|21070954|; *Pit-1* (Mm), gi|53691|; *POU6f1* (Mm), gi|6753746|; *Pit-1* (Hs), gi|4505955|; *POU6f1* (Hs), gi|57163993|; HeBrn1/2/4 |KU587795|.

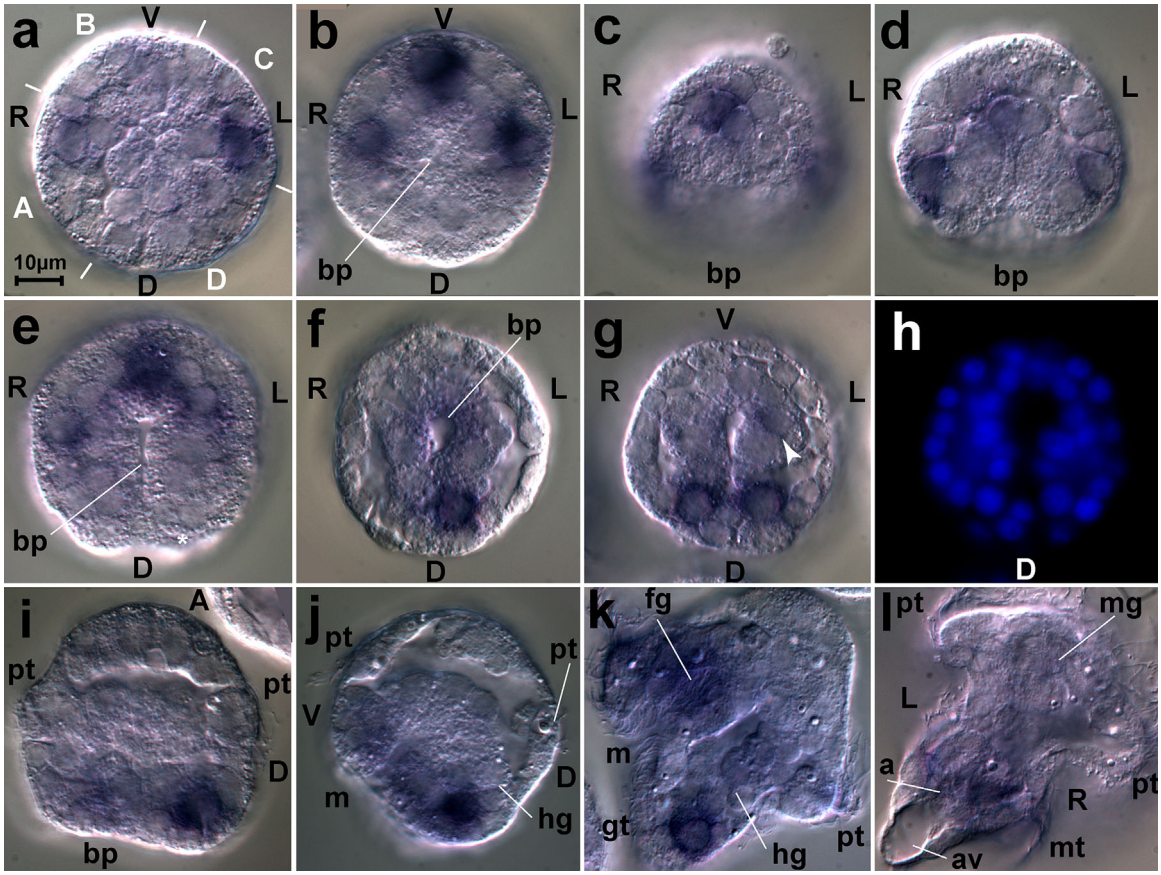

Fig. 6. *HeBrn1/2/4* expression during embryogenesis and larval stages. (a) Vegetal view of an early gastrula embryo shows the earliest expression of *HeBrn1/2/4* detected. White lines indicate the approximate boundaries of A, B, C, and D quadrants. Expressing cell in the B quadrant is weaker and slightly out of focus. Scale bar for this and subsequent panels. (b) An slightly more advanced gastrulating embryo shows robust expression in A, B, and C quadrant cells, but none in the D quadrant. (c) Surface view of an early gastrula stage embryo shows the three expressing cells, two out of the focal plane. (d) Consecutive optical section of c at the level of the expressing cells that flank the blastopore. Two long-bottleneck cells can be seen at the center of the blastopore. (e) Vegetal view of a mid-gastrula embryo shows decreasing expression in the cells flanking the blastopore that seem to have divided. (f) Mid-gastrula embryo shows expression by the dorsal side of the blastopore, in this embryo only the left-side cell expresses the gene. (g) Mid-gastrula embryo shows bilateral expression by the dorsal side of the blastopore and weak expression in cells by the ventral side of the blastopore, arrowhead. (h) DAPI staining of the embryo shown in g. The 8-shaped blastopore can be observed. (i) Optical section parallel to the sagittal plane. (j) Late gastrula embryo has already formed the mouth and maintains stronger expression in cells at the base of the blastopore that corresponds to the prospective hindgut area. (k) Trochophore larva shows strong expression in cells flanking the terminal portion of the hindgut, and weak expression in cells by the foregut area. (l) Detail in the anal area showing expression in cells flanking the hindgut at the level of the metatroch. A, animal; a, anus; av, anal vesicle; bp, blastopore; D, dorsal; fg foregut; gt, gastrotroch; hg hindgut; L left; m mouth; mg midgut; mt, metatroch; pt, prototroch; R right; V ventral.

blastomere 4d (Fig. 2e and f). This exclusion from the dorsal side is similar to the 4d exclusion previously described in the polychaetes *Chaetopterus* (Boyle and Seaver 2010) and *Capitella* (Boyle and Seaver 2008). Blastomeres 4a, 4b, and 4c express the gene more strongly than 4Q and 5q blastomeres, which almost certainly have endodermal fates, suggesting that 4a–c may contribute to distinct fates. The cell-lineage of the *H. elegans* trochophore has not been completed (Arenas-Mena 2007b), and past the 80-cell stage, precise fates can only be assigned with certainty to non-dividing trochoblast and apical tuft cells, because they occupy invariable positions. The expression of *HeGATA4/5/6* during late embryonic stages seems to correspond with mesodermal fates associated with the foregut and hindgut because expressing cells are not integral to the forming epithelium, but the fate of these cells remains uncertain. The midgut expression of *HeGATA4/5/6* during differentiation is also conserved in several spiralian (Boyle and Seaver 2008, 2010; Passamaneck et al. 2015), insect (Murakami et al. 2005), and echinoderm (Lee and Davidson 2004) representatives. The expression in vegetal-most blastomeres (4Q and 5q and their descendants) declines during gastrulation (Fig. 2h–j) and it is regained by their descendants

during late gastrulation and larval midgut differentiation. This suggests the possibility of distinct regulatory inputs controlling *HeGATA4/5/6* expression during early embryonic cleavage and subsequent tripartite gut regionalization and differentiation. Similar biphasic expression has been reported for *GATA-E* (the *GATA4/5/6* homolog) during endomesoderm specification and gut regionalization in sea urchins, where each expression phase is controlled by distinct regulatory inputs (Lee et al. 2007). Similar to *Hydroides*, the mesodermal expression of *GATA4/5/6* in *Platynereis* appears to be localized in 4d mesoderm derivatives (Gillis et al. 2007). Endodermal and mesodermal expression of *GATA4/5/6* homologs was also detected in more distant spiralians, such as the sipunculan *Themiste langeniformis* (Boyle and Seaver 2010), the brachiopod *Terebratalia transversa* (Passamaneck et al. 2015), and the planarian *Schmidtea polychora* (Martín-Durán and Romero 2011), as well as in deuterostomes such as sea urchins (Lee and Davidson 2004).

Comparative analysis reveals variability of *GATA4/5/6* endodermal expression among embryos that generate lecithotrophic larvae. *HeGATA4/5/6* is expressed in endoderm and mesoderm precursors during embryogenesis, and it is eventually restricted to the larval midgut (Fig. 2). Endodermal and mesodermal expression is a bilaterian trait of the *GATA4/5/6* subclass (Patient and McGhee 2002) that has been similarly reported in the polychaetes *Chaetopterus variopedatus* (Boyle and Seaver 2010) and *Capitella teleta* (Boyle and Seaver 2008). In addition, exclusively endodermal expression was reported in the nemertean *Lineus ruber* (Martín-Durán et al. 2015) and exclusively mesodermal expression in the polychaete *Platynereis dumerilii* (Gillis et al. 2007) for *GATA4/5/6* homologs. Absence of *GATA4/5/6* endodermal expression in *Platynereis* embryos may relate to the fact that they do not form a functional gut but a compact and yolky cell mass called "endodermal anlage" (Arenas-Mena and Li 2014). This interpretation would change if additional *GATA4/5/6* homologs with endodermal expression are found in *Platynereis*. The independent evolution of epibolic gastrulation in *Platynereis* and *Capitella* would be consistent with the observed variability of *GATA4/5/6* expression. Similarly consistent with this scenario is the expression of *Otx* and *Brachyury* reported in *Capitella* and *Hydroides* but not in *Platynereis*, as previously discussed (Arenas-Mena 2010; Arenas-Mena and Li 2014). One would expect substantial differences in regulatory gene expression between *H. elegans* embryos and the utterly different epibolic embryos of *Capitella* (Arenas-Mena and Li 2014). The relatively conserved regulatory gene expression may be due to a dual function for *GATA4/5/6* in endoderm germ layer specification and morphogenesis during gastrulation. In other words, endoderm is specified in both cases despite the lack of an epithelial gut in *Capitella* embryos, and this may require the expression of a similar set of transcription factors. We suspect that more substantial differences will be found in the expression of the regulated downstream targets, such as endodermal genes involved in gastrulation by invagination that would not be expressed in epibolic embryos.

Comparative analysis suggests that the expression of GATA1/2/3a is evolutionarily conserved. GATA1/2/3a expression starts in animal hemisphere blastomeres 1d121 and 1d122, which are adjacent to apical tuft precursors 1q111 (Fig. 3a). Expression in similarly located cells was described in *Platynereis* (Gillis et al. 2007). The fate of the expressing cells remains uncertain, but the internalization of the nucleus relative to adjacent cells (results not shown) may relate to neural fates. The cells expressing GATA1/2/3a in the animal hemisphere (1d121 and 1d122) represent a subset of those expressing *Otx*, which is expressed in the equivalent cells in all quadrants and maintains its expression in differentiating neurons associated with the ciliary band and posterior sensory organ (Arenas-Mena and Wong 2007). Nevertheless, the fate of 1d121 and 1d122 remains uncertain. The expression of GATA1/2/3a is also contained within the much broader dorsal expression domain of *Tbx2/3* (Arenas-Mena 2013), although the expression of both genes transiently coincides (Fig. 3d). Anterior GATA1/2/3 expression was also described in *Capitella* (Boyle and Seaver 2008), although in relation to adult brain formation in this lecithotrophic developer rather than to larval development. There are also counterparts to the dorsal ectoderm expression (Fig. 3d) in *Platynereis*. Nevertheless, no neural expression on the ventral side similar to the one reported in *Platynereis* and *Capitella* is found in *Hydroides* during embryogenesis. This suggests that ventral expression in *Platynereis* and *Capitella* belongs to adult neural development and that it has no counterpart during *Hydroides* embryogenesis which ends in a trochophore larva rather than a juvenile worm with a sophisticated ventral nervous system. Expression during larval stages associated to ventral neural development could not be detected above background (results not shown). The expression in the mesoderm that is associated with the posterior side of the blastopore and the hindgut almost certainly corresponds with 4d derivatives, which are posterior growth zone precursors (Hatschek 1885; Shearer 1911; Anderson 1966), and this expression could have a counterpart in the growth zone expression described in *Capitella* (Boyle and Seaver 2008). Mesodermal expression of the sea urchin homolog gene GATAc has been found in the right coelom, skeletogenic mesenchyme, and immune cells (Solek et al. 2013). The expression in the growth zone of *Hydroides* may be homologous to the coelomic expression in sea urchins, because the *Hox* gene expression in the sea urchin coelom (Arenas-Mena et al. 2000) reveals an anteroposterior axis homologous to the anteroposterior axis of the polychaete growth zone (Arenas-Mena 2010).

The expression of GATA1/2/3b correlates with general postembryonic development in various regions. GATA1/2/3b is not expressed during embryogenesis, but only during the feeding-dependent developmental phase (Fig. 4). There is no

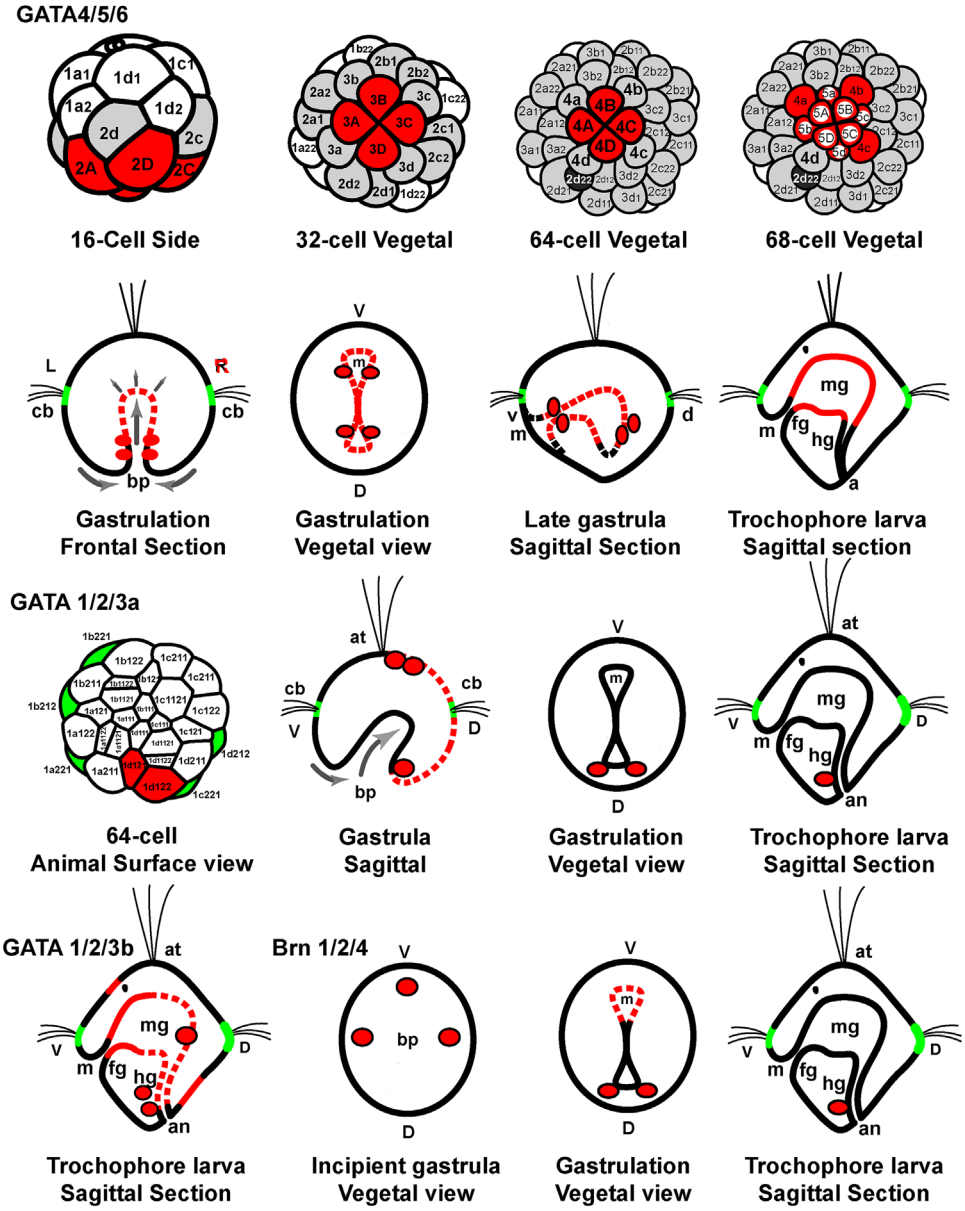

Fig. 7. Expression summary of *GATA4/5/6*, *GATA1/2/3a*, *GATA1/2/3b*, and *Brn 1/2/4*. Diagrams summarize the most relevant expression patterns described in the text. For additional anatomical orientation see previous diagrams (Arenas-Mena 2006, 2007b; Arenas-Mena et al. 2007a). Expression is indicated in red; dashed lines and white-dotted blastomeres signify lower expression; in green, primary trochoblasts and prototroch. Gray arrows indicate morphogenetic events. All figures diagrammatic except for *GATA 1/2/3a* 64-h embryo, which is a realistic surface rendering of a fixed embryo (Arenas-Mena 2007b). a, anus; at, apical tuft; bp, blastopore; D, dorsal; fg, foregut; hg, hindgut; L, left; m, mouth; mg, midgut; pt, prototroch; R, right; V, ventral.

clear cell-type or organ association to its expression profile other than postembryonic development. The expression in the growth zone and adjacent tissues (Fig. 4b and f, arrowheads) may relate to the dorsal-posterior expression of GATA1/2/3 homologs described in *Platynereis* (Gillis et al. 2007) and *Capitella* (Boyle and Seaver 2008). The postembryonic expression of GATA1/2/3b is reminiscent of the expression of histone variant *H2A.Z* in multipotent cells of *Hydroides* (Arenas-Mena 2007a; Arenas-Mena et al. 2007b). As previously discussed, in addition to the growth zone, many of the differentiated larval cells in *Hydroides* apparently transform during the feeding-dependent phase (Arenas-Mena 2010; Arenas-Mena and Li 2014; Arenas-Mena and Coffman 2015). The expression of GATA1/2/3 seems associated with regions, such as the endoderm, that almost certainly do not derive from undifferentiated cells but from non-terminally differentiated larval cells.

The only GATA transcription factor present in the sea anemone *Nematostella vectensis* is expressed in ectodermal and endodermal cells (Martindale 2004). This suggests that GATA1/2/3 and GATA4/5/6 expression differentially partitioned in bilaterians the preexisting regulatory inputs by a process of subfunctionalization (Kleinjan et al. 2008). Nevertheless, only the endodermal expression in cnidarians has a clear counterpart in *Hydroides* given the uncertain fate of the other expressing cells in both organisms.

The expression of *HeBrn1/2/4* in foregut precursors is evolutionarily conserved. *HeBrn1/2/4* is expressed first in three blastomeres around the ventral side of the blastopore and later in cells by the dorsal side that almost certainly correspond to 4d derivatives. The ventral expression generally corresponds to the prospective foregut area, which maintains weak expression in early trochophores (Fig. 6k). The early expression of *HeBrn1/2/4* is similar to the vegetal pattern previously reported for *HeOtx* (Arenas-Mena and Wong 2007), but it is not currently known if the three blastomeres expressing *HeBrn1/2/4* correspond to the same blastomeres, or if any of these correspond to ectomesoderm precursors. Similar foregut expression has been reported for the ortholog gene during sea urchin development (Cole and Arnone 2009) and in the pharynx of amphioxus, suggesting an ancestral role in bilaterian anterior gut development (Candiani et al. 2002). The expression of *HeBrn1/2/4* in 4d derivatives (Fig. 6g, i, and j) is very similar to the expression of *HeGATA1/2/3a* (Fig. 3g, j, and k), and to the expression of *HeSal* (Fig. 4c and g; (Arenas-Mena 2013), suggesting their shared involvement in endomesoderm specification. The early expression in 4d derivatives shows left-right asymmetry; it is initiated in the left side (Fig. 6f), similarly to transcription factors *HeTbx2/3*, *HeSal*, and *HeBlimp* (Arenas-Mena 2008). To our knowledge, in spiralians, the expression of homologous genes has only been characterized in a cephalopod (Wollesen et al. 2014) and a gastropod (O'Brien and Degnan 2002) where it is primarily related to neural tissues that do not exhibit any direct relation to the embryonic expression identified in *Hydroides*. No signal above background was detected during postembryonic stages studied in *Hydroides* that could relate to adult neural tissues described in these spiralians.

Our analysis adds to the repertoire of regulatory genes characterized during the development of *H. elegans* (Arenas-Mena and Li 2014). The early embryonic expression of *HeGATA4/5/6*, *GATA1/2/3a*, and *HeBrn1/2/4* is invariably initiated in specific blastomeres during the invariant spiral cleavage (Fig. 7), like all other transcription factors with embryonic expression described so far in *Hydroides* (Arenas-Mena and Li 2014). Eventually, the functional characterization of all these transcription factors during the embryonic development of *Hydroides* will reveal the specific regulatory mechanisms that control the formation of its feeding trochophore and those shared with distinct bilaterian embryos (Arenas-Mena 2010). Our characterizations allow a more complete analysis of regulatory similarities and differences among lecithotrophic and planktotrophic larvae of protostomes and deuterostomes that is relevant to understand the evolution of these different developmental modes in metazoans (Arenas-Mena 2010). Our comparative analysis reveals evolutionary conservation of expression of *Brn1/2/4* in the foregut and *GATA4/5/*6 in the midgut of the last common ancestor of protostomes and deuterostomes. It will be of particular interest of future studies to resolve if there is conservation among the regulatory mechanisms of tripartite gut subdivision in planktotrophic larvae of protostomes and deuterostomes.

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REFERENCES

Allen, J. D., and Pernet, B. 2007. Intermediate modes of larval development: bridging the gap between planktotrophy and lecithotrophy. *Evol. Dev*. 9: 643–653.

Anderson, D. T. 1966. The comparative embryology of the Polychaeta. *Acta Zool. Stockh*. 47: 1–42.

Arenas-Mena, C. 2006. Embryonic expression of HeFoxA1 and HeFoxA2 in an indirectly developing polychaete. *Dev. Genes Evol*. 216: 727–736.

Arenas-Mena, C. 2007a. Developmental transcriptional-competence model for a histone variant and a unicellular origin scenario for transcriptional-multipotency mechanisms. *Evol. Dev*. 9: 208–211.

Arenas-Mena, C. 2007b. Sinistral equal-size spiral cleavage of the indirectly developing polychaete Hydroides elegans. *Dev. Dyn*. 236: 1611–1622.

Arenas-Mena, C. 2008. The transcription factors HeBlimp and HeT-brain of an indirectly developing polychaete suggest ancestral endodermal, gastrulation, and sensory cell-type specification roles. *J. Exp. Zool. B Mol. Dev. Evol*. 310B: 567–576.

Arenas-Mena, C. 2010. Indirect development, transdifferentiation and the macroregulatory evolution of metazoans. *Philos. Trans. R. Soc. B Biol. Sci*. 365: 653–669.

Arenas-Mena, C. 2013. Brachyury, Tbx2/3 and sall expression during embryogenesis of the indirectly developing polychaete Hydroides elegans. *Int. J. Dev. Biol*. 57: 73–83.

Arenas-Mena, C., Cameron, A. R., and Davidson, E. H. 2000. Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. *Development* 127: 4631–4643.

Arenas-Mena, C., and Coffman, J. A. 2015. Developmental control of transcriptional and proliferative potency during the evolutionary emergence of animals. *Dev. Dyn*. 244: 11093–11201.

Arenas-Mena, C., and Li, A. 2014. Development of a feeding trochophore in the polychaete Hydroides elegans. *Int. J. Dev. Biol*. 58: 575–583.

Arenas-Mena, C., and Wong, K. S.-Y. 2007. HeOtx expression in an indirectly developing polychaete correlates with gastrulation by invagination. *Dev. Genes Evol*. 217: 373–384.

Arenas-Mena, C., Wong, K. S.-Y., and Arandi-Forosani, N. 2007a. Ciliary band gene expression patterns in the embryo and trochophore larva of an indirectly developing polychaete. *Gene Expr. Patterns* 7: 544–549.

Arenas-Mena, C., Wong, K. S.-Y., and Arandi-Foroshani, N. R. 2007b. Histone H2A. Z expression in two indirectly developing marine

invertebrates correlates with undifferentiated and multipotent cells. *Evol. Dev*. 9: 231–243.

Arendt, D., Technau, U., and Wittbrodt, J. 2001. Evolution of the bilaterian larval foregut. *Nature* 409: 81–85.

Boyle, M. J., and Seaver, E. C. 2008. Developmental expression of foxA and gata genes during gut formation in the polychaete annelid, Capitella sp. I. *Evol. Dev*. 10: 89–105.

Boyle, M. J., and Seaver, E. C. 2010. Expression of FoxA and GATA transcription factors correlates with regionalized gut development in two lophotrochozoan marine worms: Chaetopterus (Annelida) and Themiste lageniformis (Sipuncula). *EvoDevo* 1: 2.

Candiani, S., Castagnola, P., Oliveri, D., and Pestarino, M. 2002. Cloning and developmental expression of AmphiBrn1/2/4, a POU III gene in amphioxus. *Mech. Dev*. 116: 231–234.

Cole, A. G., and Arnone, M. I. 2009. Fluorescent in situ hybridization reveals multiple expression domains for SpBrn1/2/4 and identifies a unique ectodermal cell type that co-expresses the ParaHox gene SpLox. *Gene Expr. Patterns* 9: 324–328.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 32: 1792–1797.

Gillis, W. J., Bowerman, B., and Schneider, S. Q. 2007. Ectoderm-and endomesoderm-specific GATA transcription factors in the marine annelid Platynereis dumerilli. *Evol. Dev*. 9: 39–50.

Gillis, W. Q., Bowerman, B. A., and Schneider, S. Q. 2008. The evolution of protostome GATA factors: molecular phylogenetics, synteny, and intron/exon structure reveal orthologous relationships. *BMC Evol. Biol*. 8: 112.

Gold, D. A., Gates, R. D., and Jacobs, D. K. 2014. The early expansion and evolutionary dynamics of POU class genes. *Mol. Biol. Evol*. 31: 3136–3147.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser*. 41: 95–98.

Hatschek, B. 1885. Entwicklung der trochophora von Eupomatus uncinatus. *Arbt. Zool. Inst. Wien*. 6: 121.

Kleinjan, D. A., et al. 2008. Subfunctionalization of duplicated zebrafish pax6 genes by cis-regulatory divergence. *PLoS Genet*. 4: e29.

Lee, P. Y., and Davidson, E. H. 2004. Expression of Spgatae, the Strongylocentrotus purpuratus ortholog of vertebrate GATA4/5/6 factors. *Gene Expr. Patterns* 5: 161–165.

Lee, P. Y., Nam, J., and Davidson, E. H. 2007. Exclusive developmental functions of gatae cis-regulatory modules in the Strongylocentrorus purpuratus embryo. *Dev. Biol*. 307: 434–445.

Martindale, M. Q. 2004. Investigating the origins of triploblasty: 'mesodermal' gene expression in a diploblastic animal, the sea anemone Nematostella vectensis (phylum, Cnidaria; class, Anthozoa). *Development* 131: 2463–2474.

Martín-Durán, J. M., and Romero, R. 2011. Evolutionary implications of morphogenesis and molecular patterning of the blind gut in the planarian Schmidtea polychroa. *Dev. Biol*. 352: 164–176.

Martín-Durán, J. M., Vellutini, B. C., and Hejnol, A. 2015. Evolution and development of the adelphophagic, intracapsular Schmidt's larva of the nemertean Lineus ruber. *EvoDevo* 6: 1.

Murakami, R., Okumura, T., and Uchiyama, H. 2005. GATA factors as key regulatory molecules in the development of Drosophila endoderm. *Dev. Growth Differ*. 47: 581–589.

Nielsen, C. 2013. Life cycle evolution: was the eumetazoan ancestor a holopelagic, planktotrophic gastraea? *BMC Evol. Biol*. 13: 1–18.

O'Brien, E. K., and Degnan, B. M. 2002. Pleiotropic developmental expression of HasPOU-III, a class III POU gene, in the gastropod Haliotis asinina. *Mech. Dev*. 114: 129–132.

Page, L. R. 2009. Molluscan larvae: pelagic juveniles or slowly metamorphosing larvae? *Biol. Bull*. 216: 216–225.

Passamaneck, Y. J., Hejnol, A., and Martindale, M. Q. 2015. Mesodermal gene expression during the embryonic and larval development of the articulate brachiopod Terebratalia transversa. *EvoDevo* 6: 10.

Patient, R. K., and McGhee, J. D. 2002. The GATA family (vertebrates and invertebrates). *Curr. Opin. Genet. Dev*. 12: 416–422.

Peterson, K. J., Cameron, R. A., and Davidson, E. H. 1997. Set-aside cells in maximal indirect development: evolutionary and developmental significance. *BioEssays* 19: 623–631.

Ryan, A., and Rosenfeld, M. 1977. POU domain family values: flexibility phylogeny. *Genes Dev*. 11: 1207–1225.

Shearer, C. 1911. On the development and structure of the trochophore of Hydroides uncinatus (Eupomatus). *Q. J. Microsc. Sci*. 56: 543–590.

Sly, B. J., Snoke, M. S., and Raff, R. A. 2003. Who came first-larvae or adults? Origins of bilaterian metazoan larvae. *Int. J. Dev. Biol*. 47: 623–632.

Solek, C. M., et al. 2013. An ancient role for Gata-1/2/3 and Scl transcription factor homologs in the development of immunocytes. *Dev. Biol*. 382: 280–292.

Tamura, K., Stecher, G., Peterson, D., Filipsk, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol*. 30: 2725–2729.

Wollesen, T., McDougall, C., Degnan, B. M., and Wanninger, A. 2014. POU genes are expressed during the formation of individual ganglia of the cephalopod central nervous system. *EvoDevo* 5: 41.