Deployment of Regulatory Genes During Gastrulation and Germ Layer Specification in a Model Spiralian Mollusc *Crepidula*

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Background: During gastrulation, endoderm and mesoderm are specified from a bipotential precursor (endomesoderm) that is argued to be homologous across bilaterians. Spiralians also generate mesoderm from ectodermal precursors (ectomesoderm), which arises near the blastopore. While a conserved gene regulatory network controls specification of endomesoderm in deuterostomes and ecdysozoans, little is known about genes controlling specification or behavior of either source of spiralian mesoderm or the digestive tract.

Results: Using the mollusc *Crepidula*, we examined conserved regulatory factors and compared their expression to fate maps to score expression in the germ layers, blastopore lip, and digestive tract. Many genes were expressed in both ecto- and endomesoderm, but only five were expressed in ectomesoderm exclusively. The latter may contribute to epithelial-to-mesenchymal transition seen in ectomesoderm.

Conclusions: We present the first comparison of genes expressed during spiralian gastrulation in the context of high-resolution fate maps. We found variation of genes expressed in the blastopore lip, mouth, and cells that will form the anus. Shared expression of many genes in both mesodermal sources suggests that components of the conserved endomesoderm program were either co-opted for ectomesoderm formation or that ecto- and endomesoderm are derived from a common mesodermal precursor that became subdivided into distinct domains during evolution. Developmental Dynamics 244:1215–1248, 2015. © 2015 Wiley Periodicals, Inc.

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Introduction

Most bilaterian taxa have three principle germ layers: ectoderm, endoderm, and mesoderm. The invention of mesoderm was intimately tied to the generation of the diverse morphologies of triploblasts (Perez-Pomares and Muniz-Chapuli, 2002; Martindale, 2005; Martindale and Hejnol, 2009). Much effort has centered on deciphering the molecular basis for specifying distinct ectodermal, endodermal, and mesodermal germ layers during development and great progress has been made to understand germ layer specification in deuterostomes (e.g., echinoderms, chordates), ecdysozoans (e.g., fly, nematode), and cnidarians (Byrum and Martindale, 2004; Martindale et al., 2004; Magie et al., 2007; Sawyer et al., 2010; Gorfinkel et al., 2011; Peter and Davidson, 2011; Röttinger et al., 2012; Solnica-Krezel and Sepich, 2012). In contrast, germ layer specification is not well understood in the Spiralia, a major branch of bilaterians (Boyle et al., 2014; Passamanec et al., 2015). The Spiralia contains animals such as annelids, molluscs, brachiopods, phoronids, rotifers, among others, with diverse larval and adult body plans (Hejnol, 2010; Henry, 2014). Many members of the Spiralia exhibit a highly conserved spiral cleavage pattern and cell lineage fate map (Hejnol, 2010; Lambet, 2010; Henry, 2014). This characteristic spiral cleavage program, together with molecular phylogenetic analyses, reveals the close evolutionary relationships amongst members of the Spiralia (Boyer et al., 1996; Henry and Martindale, 1999; Dunn et al., 2008; Gribet, 2008; Hejnol, 2010; Lambert, 2010; Henry, 2014). Thus understanding germ layer specification in the Spiralia, particularly in those with spiral cleavage, which is believed to be the ancestral condition for this clade, should expand our understanding of how this clade evolved.

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Ectoderm, endoderm, and mesoderm are segregated during the process of gastrulation, which occurs as the result of cellular signaling, and morphogenetic rearrangement (Stern, 2004). Morphogenetic events associated with the process of gastrulation and germ layer formation also have a direct bearing on the formation of the digestive tract, including structures such as the mouth and the anus (Hejnol and Martindale, 2008; Lyons and Henry, 2014). Cell lineage and fate mapping studies show that mesoderm and endoderm usually arise from common endomesoderm progenitor cells, and that this condition appears to be conserved across metazoans, including in ctenophores, acelois, ecdysozoans, spiralians, echinodermatics, urochordates, and vertebrates (Nishida and Satoh, 1985; Martindale and Henry, 1999; Logan et al., 1999; Henry et al., 2000; Kimelman and Griffin, 2000; Fukuda and Kikuchi, 2005; Maduro, 2006; Gilles et al., 2007; Ryan et al., 2013). Mesoderm likely evolved from endoderm, since many genes associated with bilaterian mesoderm cell types are expressed within the endoderm of basally branching animals like cnidarians (Martindale et al., 2004; Röttinger et al., 2012). It is now common to build gene regulatory networks (GRNs) for specification of endoderm, and mesoderm in order to understand how endoderm and mesoderm become distinct cell types during development and evolution (Hinman et al., 2003; Maduro, 2006; Peter and Davidson, 2011; Röttinger et al., 2012). Such studies have revealed highly conserved core regulatory components and wiring that are involved in endomesoderm specification and diversification. Comparing GRNs across species can reveal which sub-circuits of the network are most conserved, and how they have diverged over evolutionary time.

The most complete endomesoderm GRN is that of the sea urchin (Oliveri et al., 2008; Peter and Davidson, 2011; http://sugp.caltech.edu/endomes). It often serves as a framework for examining germ layer specification in other organisms. Additional GRNs have been reconstructed for sea stars (Hinman and Davidson, 2003; Maduro, 2006; Peter and Davidson, 2011; Röttinger et al., 2012). Such studies have revealed highly conserved core regulatory components and wiring that are involved in endomesoderm specification and diversification. Comparing GRNs across species can reveal which sub-circuits of the network are most conserved, and how they have diverged over evolutionary time.

Overview of Cleavage, Gastrulation and Organogenesis in Crepidula

Spiral cleavage involves alternating sets of oblique cell divisions that form staggered quartets of micromeres at the animal pole of the embryo. The first two cell divisions generate four blastomeres...
occupying each of the basic embryonic quadrants (termed A, B, C, and D; see Conklin, 1897). Each of these cells subsequently generates a series of typically smaller animal daughter cells termed “micromeres,” which are formed in alternating clockwise and counterclockwise orientations around the animal-vegetal axis, whereas the four vegetal-most cells are typically larger and termed “macromeres.” Animal micromeres are designated with lowercase letters, while the vegetal macromeres are designated with uppercase letters. Hence, the first quartet of micromeres is named 1a, 1b, 1c, and 1d, while the corresponding macromeres are named 1A, 1B, 1C, and 1D. The second quartet is named 2a, 2b, 2c, and 2d, and the corresponding macromeres are named 2A, 2B, 2C, and 2D, and so on.

The process of gastrulation follows early cleavage events and has recently been examined in depth in *C. fornicata* using in vivo imaging and cell lineage analyses (Lyons et al., 2015). Gastrulation occurs by epiboly, and is characterized by expansion of the progeny of the first through third quartet animal micromeres toward the equatorial zone and ultimately the vegetal pole (approximately 48–117 hpf; all staging follows that established in Lyons and Henry, 2014, and Lyons et al., 2015). During this process, animal cap cells surround vegetal endodermal precursors (the fourth quartet micromeres: 4a–4c; and the macromeres: 4A–4D). As epiboly progresses, the leading edge of animal micromeres (i.e., the blastopore lip) begin to spread and cover these underlying cells (round stages, 48–117hpf). Eventually, the circumference of the blastopore lip constricts as it approaches the vegetal pole of the embryo, and the cells around the lip (derived from progeny of the second and third quartet of micromeres; 2a–2d and 3a–3d; Fig. 1) undergo rearrangement around the open blastopore. The blastopore, as it relates to *C. fornicata*, is defined as the site where presumptive endoderm and mesoderm are internalized during gastrulation. In *C. fornicata*, this opening never fully closes and ultimately gives rise to the mouth and ectodermal foregut (or esophagus). Cells of the posterior lip of the blastopore (progeny of 2d) also contribute to the anus (Lyons and Henry, 2014; Lyons et al., 2015). Specifically, during later stages of epiboly (beginning at 97 hpf), the posterior lip of the blastopore closes by a zippering process that involves convergence and extension (CE; Lyons et al., 2015). At this time, the cells that are ultimately involved in the formation of the anus are displaced towards the posterior pole of the embryo, away from the persistent opening of the blastopore (described further below).

Mesoderm in *C. fornicata* is derived from two sources: endomesoderm (derived from one of the fourth quartet micromeres, 4d; Fig. 1), and ectomesoderm (derived from ectodermal bipotential progeny of 3a and 3b at the very anterior-lateral edges of the blastopore; Fig. 1). During epiboly, progeny of 4d reside at a posterior location adjacent to the blastopore lip (Lyons et al., 2015). For the expression data represented in the next section, we refer to any expression (after 90 hpf) in the 4d lineage as either being expressed in the mesodermal derivatives as “endomesoderm,” or in the 4d-derived endodermal progeny, which contributes to the “hindgut precursors” or “hindgut rudiment.” Following blastopore constriction, the embryo undergoes anterior-posterior axial elongation (elongation stages, 117–170hpf) and this axial elongation results in the mouth becoming displaced towards the anterior pole of the embryo (Fig. 1; Lyons and Henry, 2014). By ~145 hpf (later stages of elongation just prior to organogenesis stages), the morphogenetic events that make the mouth and esophagus are largely completed, and subsequent morphogenesis results in axial elongation of the embryo. Posterior closure of the blastopore lip and axial elongation displaces the ectodermal progeny of 2d (including the 2d2 derived anus anlagen), the terminal cells (3c2/3d2-derivatives), and the endodermal hindgut, away from the mouth.

During organogenesis stages, the ciliated prototroch forms perpendicular to the anterior-posterior axis and separates the ectoderm of the developing larvae into different regions. The anterior region consists of pre-trochal ectoderm (derived from the first quartet and includes anterior ectoderm of the velum, apical organ, and apical ganglia, 1q; Fig. 1). The post-trochal region that lies posterior to the mouth consists of ectoderm derived from second and third quartet micromeres (2q, 3a; Fig. 1). During later stages of organogenesis (196hpf), the rudiments of the velar lobes become more pronounced and the ciliated apical plate shifts ventrally within the developing head. Prominent ciliated bands involved in larval feeding and locomotion form at the edge of the developing velum, and include the food groove, and primary and secondary ciliary bands (which Hejnol et al., 2007, describe as the prototroch and metatroch, respectively). In the post-trochal region, the shell gland forms on the dorsal surface, the foot rudiment and operculum develop on the ventral surface, and the paired bilateral larval external kidneys or absorptive cells develop on the left and right lateral sides (Rivest, 1992; Lyons et al., 2012). The hindgut rudiment also forms within the post-trochal region along the posterior ventral midline, but as development progresses this structure shifts towards the right lateral side (see Hejnol et al., 2007; Henry et al., 2010a; Lyons et al., 2012, 2015). The veliger larval stage is reached by 10 days of development. The anus, which will be located at the distal tip of the hindgut, does not open until later, at approximately 12 days of development (veliger larval stage; Lyons et al., 2015).

**In situ Expression Patterns**

**Endomesoderm**

**Beta-catenin (**ctnnb**).** Beta-catenin is a multifunctional protein involved in cell adhesion, cell signaling, and transcriptional regulation. It is broadly expressed, given its roles in cell adhesion. It has also been shown to play conserved roles during gastrulation and endomesoderm specification, and serves as a key upstream component of the sea urchin endomesoderm GRN (Wikramanayake et al., 1998, 2003; Logan et al., 1999; Imai et al., 2000; Miyawaki et al., 2003; Kawai et al., 2007; Röttinger et al., 2012). Active **ctnnb** signaling occurs with the nuclearization of this protein that serves as an intracellular signal transducer to regulate downstream targets, such as those involved in the canonical Wnt signaling pathway. The expression of **ctnnb** has been described for *C. fornicata* (Henry et al., 2010), but that study did not specifically describe expression during gastrulation stages. Here we report on the mRNA expression patterns, which do not necessarily indicate active **ctnnb** signaling.

The expression pattern of **ctnnb** at early stages of development is dynamic (Figs. 1 and 2A–F; Table 1; J. Q. Henry et al., 2010). In a previous study we showed that expression is seen weakly in macromeres and animal micromeres (quadrants A–D), and depending on the cell cycle, **ctnnb** becomes more highly localized to a region adjacent to the nuclei, which we presume to be the centrosomes (Henry et al., 2010c). By mid to late gastrulation more intensely labeled cells are observed around the entire edge
of the blastopore (Fig. 2E–I). Signal is noted particularly along the posterior edge of the blastopore lip, along with cells exhibiting higher levels of expression extending from the posterior blastopore along the ventral midline, towards the more posterior regions (ectodermal cells derived from 3c and 3d, Fig. 2G–H).

Fainter label also becomes visible in some posterior progeny, lateral to the ventral midline. During later elongation stages, expression is observed in the anterior ectoderm derived from the 1st quartet micromeres and in posterior ectoderm (mainly progeny of 2d) in a radiating pattern that extends from the blastopore and
Fig. 2. Expression of ctnnb during early development in C. fornicata. A–T: ctnnb expression in embryos ranging from 80–170hpf (early epiboly–early organogenesis). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (an., animal; veg. vent., vegetal/ventral; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Animal view of ctnnb expression (80hpf) (A) and corresponding DAPI labeled nuclei (B). Embryonic quadrants A–D are labeled within each panel of A–D. Vegetal view of same 80hpf specimen in A,B as epiboly begins, showing the localization of ctnnb expression (C) and corresponding DAPI-labeled nuclei (D). Flattened round stage embryo (E) and corresponding DAPI image (F), and early elongation embryo with expression around the blastopore (G) with corresponding DAPI labeling (H). Expression during elongation is represented in I with corresponding DAPI labeling in J. A dorsal view of expression from the same embryo shown in I–J is shown in (K) and corresponding DAPI labeled nuclei (L). Various views of expression are shown from the same late elongating ovoid specimen in M–P, and similarly the same organogenesis specimen in Q–T. Structures are labeled as follows: bp, blastopore; ec, ectomesoderm; hr, hindgut rudiment; mo, mouth; sg, shell gland; st, stomodeum. Scale bar in T = 50 μm.
developing mouth (Fig. 2I–L). Expression is somewhat diminished by early organogenesis stages, but broadens during later organogenesis stages and is observed around the mouth, the shell gland, and a proliferative zone located on the left- and right-lateral post-trochal area (Fig. 2M–T). Pre-larval expression also appears to be somewhat more intense on the right side.

Expression is also seen in the progeny of 4d. *ctnnb* expression was observed in organogenesis specimens, where it is noted more strongly in progeny of 4d, particularly the hindgut rudiment (Fig. 2Q–S).

**Orthodenticle (otx).** Orthodenticle is a homeobox transcription factor with conserved roles in the specification of anterior structures, including those of the CNS and the stomodeum (Cohen and Jürgens, 1990; Finkelstein et al., 1990; Acampora et al., 2000; Boyle et al., 2014).

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**TABLE 1.** Grids Detailing Expression in Specific Tissues/Rudiments During Embryogenesis in *C. fornicata* (3 Representative Time Periods)"
otx does not exhibit a distinct localized expression pattern during early cleavage stages, but there appears to be faint widespread expression throughout the embryo. During early to mid-gastrula stages, more pronounced expression is seen around the blastopore (Figs. 1, 3A–D; Table 1), and later gastrula embryos have expression around the entire blastopore (Fig. 3E–F). Mid to late gastrula embryos display otx labeled cells along the ventral midline posterior to the blastopore (Fig. 3C–F). During organogenesis, expression is noted in the anterior mouth and esophagus, with less intense expression along the posterior edge of the mouth and foregut (Fig. 3G, I–K, M–O). otx expression is widespread in the ectoderm at later stages of development and noted asymmetrically in a patch of cells on the right side of the embryo in the post-trochal region (Fig. 3I–J, L–N, P). Structures are labeled following designations used in Figure 2 and as follows: fr, foot rudiment; np, neural precursors; vr, velar rudiment. Scale bar in P = 50 μm.

Fig. 3. Expression of otx during early development in C. fornicata. A–P: otx expression in embryos ranging from 99–165 hpf (late epiboly through elongation stages). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (veg. vent., vegetal/ventral; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Mid-epiboly embryo (A) and corresponding DAPI image (B), and later epiboly embryo with expression around the blastopore (C) with corresponding DAPI labeling (D). Expression during elongation is represented in E with corresponding DAPI labeling in F. Ventral expression in an elongating oval stage is shown in G with corresponding dorsal view in H. Various views of expression are shown from the same late ovoid specimen in I–L, and similarly the same organogenesis specimen in M–P. Black arrowheads point to asymmetric post-trochal ectodermal patch of expression in I, J, L–N, P. Structures are labeled following designations used in Figure 2 and as follows: fr, foot rudiment; np, neural precursors; vr, velar rudiment. Scale bar in P = 50 μm.
Fig. 4. Expression of foxA during early development in C. fornicata. A–T: foxA expression in embryos ranging from 24-cell-170hpf (early cleavage to early stages of organogenesis). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (an., animal; veg. vent., vegetal/ventral; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Animal view of foxA expression in cleavage embryo (A) and corresponding DAPI labeled nuclei (B). Embryonic quadrants A–D are labeled within each panel of A–D. Animal view of foxA expression in 45hpf specimen (C) and corresponding DAPI-labeled nuclei (D). A dorsal view of a 48hpf specimen (E) and corresponding DAPI image (F) showing expression that is localized to the teloblasts progeny (1mL, 1mR, 3mL, 3mR). Flat round embryo with expression at the posterior region of the blastopore (G) with corresponding DAPI labeling (H). foxA expression during an epiboly stage (I) with corresponding DAPI labeling (J), and an ovoid stage (K) with corresponding DAPI labeling (L). Various views of expression are shown from the same late ovoid specimen in M–P, and similarly the same organogenesis specimen in Q–T. Black arrowheads point to asymmetric post-trochal ectodermal patch of expression in Q, T. Structures are labeled following designations used in Figures 2 and 3. Scale bar in T = 50 μm.
Fig. 5. Expression of *bra* during early development in *C. fornicata*. A–X: *bra* expression in embryos ranging from 8-cell–170hpf (early cleavage to early stages of organogenesis). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (an., animal; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Animal view of *bra* expression in early cleavage embryo (A) and corresponding DAPI-labeled nuclei (B). Embryonic quadrants A–D are labeled within each panel of A–F. Animal view of 48hpf specimen (C) and corresponding DAPI-labeled nuclei (D) showing *bra* expression in the teloblasts (3ML, 3MR) along with the dorsal view of the same embryo (E) and corresponding DAPI labeled nuclei (F). Early oval embryo with expression at the posterior region of the blastopore (G) with corresponding DAPI labeling (H). *bra* expression is shown in an early ovoid stage (I). A later ovoid stage (J) is shown with corresponding DAPI labeling (K), as well as anti-acetylated tubulin labeling (L, green fluorescence). A posterior view of the same specimen from J–L is shown (M) with the corresponding DAPI labeling (N). A late oval stage (elongated) is shown (O) as well as a similarly staged specimen showing anti-acetylated tubulin labeling (P, green fluorescence). Various views of expression are shown from the same late ovoid specimen in Q–T, and similarly the same organogenesis specimen in U–X. Black arrowheads point to asymmetric post-trochal ectodermal patch of expression in U, V, and X. Structures are labeled following designations used in Figures 2 and 3, and as follows: ap, apical plate; nt, neurotroch; tb, teloblasts; tc, terminal cells; vm, ventral midline. Scale bar in X = 50 μm.
Fig. 6. Expression of \textit{cdx} during early development in \textit{C. fornicata}. \textbf{A–X}: \textit{cdx} expression in embryos ranging from 16-cell–170hpf (early cleavage to early stages of organogenesis). Orientation of specimens is located in the bottom right corner of each panel (an., animal; veg., vegetal; veg. vent., vegetal/ventral; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Animal view of \textit{cdx} expression in early cleavage embryo (A) and corresponding DAPI labeled nuclei (B), as well as more advanced cleavage embryo (C) and corresponding DAPI nuclei (D). Embryonic quadrants A–D are labeled within each panel of A–F. Vegetal view of 80hpf specimen (E) and corresponding DAPI labeled nuclei (F). Epiboly specimen is shown with blastopore expression (G) and corresponding DAPI labeling (H). \textit{cdx} expression is shown at an ovoid stage (I) with corresponding DAPI labeling (J), as well as a posterior view of the same embryo (K) with corresponding DAPI labeling (L). An elongating stage is shown (M) with corresponding DAPI labeling (N), as well as a slightly older ovoid stage (O) and DAPI labeling (P). Various views of expression are shown from a single late ovoid specimen in Q–T, and also a single organogenesis stage specimen in U-X. Black arrowheads point to asymmetric post-trochal ectodermal patch of expression in Q, R, T–V, and X. Structures are labeled following designations used in Figures 2, 3, and 5. Scale bar in X = 50 \(\mu\)m.
developing velum expresses otr (Fig. 3G, I–K, M–O), as do cells in the foot rudiment (Fig. 3N) and in the proliferative zone of the shell gland (Fig. 3J, K, L).

*otr* can also be observed in ectomesodermal derivatives (3a<sup>2</sup>, 3b<sup>2</sup>; Fig. 3C–G), as well as the ectomesoderm cells undergoing EMT. Expression is also observed in both the endomesodermal progeny of the mesentoblast (2mL and 2mR) and in the endodermal hindgut rudiment (Fig. 3E–G, I–K, M–O). Relatively intense expression is present in the teloblast cells at certain stages.

**Forkhead box A (foxA).** FoxA is a member of the forkhead box, helix-turn-helix class of transcription factors (Kaufmann and Knochel, 1996). Fox genes are known to play roles in specification and differentiation of endodermal structures and are thought to regulate the fates of both ectomesoderm (Arenas-Mena, 2006) and endomesoderm (Olsen and Jeffrey, 1997; Martin-dale et al., 2004; Oliveri et al., 2006; Boyle and Seaver, 2008, 2010).

During early cleavage stages in *C. fornicata*, foxA mRNA is localized to regions adjacent to the nuclei in the A–D macromeres, which we presume to be the centrosomes (Figs. 1, 4A–D). Expression is seen during epiboly in cells of the blastopore lip (Fig. 4G–J; Table 1), and in particular cells of the posterior lip, which undergo convergent extension (Fig. 4I–J). As development continues, expression is seen in all cells of the developing mouth and esophagus, being somewhat more highly expressed in the anterior regions of both structures (Fig. 4K–L). During organogenesis, expression appears in cells posterior to and along the rim of the velum (Fig. 4M–S). Expression is also seen in the foot rudiment and the shell gland (Fig. 4M, O–T).

Expression is detected in progeny of the mesentoblast (4d), including progenitors of the hindgut during early to mid stages of gastrulation (e.g. 1mL, 1mR, 3mL, 3mR; Fig. 4E, F). Expression is also apparent in the progenitors of the ectomesoderm (progeny of 3a<sup>2</sup>, 3b<sup>2</sup>; Fig. 4K, L), and in scattered mesenchymal progeny of 3a<sup>2</sup> and 3b<sup>2</sup>. During stages of organogenesis, expression appears to be lost in ectomesoderm that has migrated away from the region of the mouth, though expression continues in the endoderm of the developing hindgut rudiment (Fig. 4M–O; Q–S). *foxA* is asymmetrically expressed in post-trochal ectoderm with more expression in a patch on the right side (Fig. 4Q, T). Similar to *C. fornicata*, *foxA* is also expressed asymmetrically in one blastomere on the right side of the post-trochal region in *Patella* (possibly the ectodermal midline stem cell; Laritillot et al., 2002a).

**Brachyury (bra).** Brachyury is a T-box transcription factor that plays a key role in specifying cells of the blastopore (Shoguchi et al., 1999; Arendt et al., 2001; Technau, 2001; Gross and McClay, 2002), regulating genes involved in gastrulation-specific morphogenetic movements (i.e., convergent extension; Arendt, 2004), and the development and specification of the adult mesoderm (Peterson et al., 1999; Technau, 2001; Gross and McClay, 2002; Boyle et al., 2014). It also regulates the development of the anterior-posterior axis (Arendt et al., 2001; Laritillot et al., 2002b; Koop et al., 2007). More recently a study in the cnidarian *Acropora* suggests a role for *bra* in demarcating ectoderm from endoderm (Hayward et al., 2015).

In *C. fornicata*, *bra* expression appears to be dynamic and mainly restricted to the vegetal macromeres during early cleavage stages, where it localizes to regions adjacent to the nuclei during interphase (presumably the centrosomes, Figs. 1, 5A, B; Table 1).

Centrosomal localization has been reported previously in the molluscs, *Ilyanassa* (Lambert and Nagy, 2002; Kingsley et al., 2007) and *Crepidula* (Henry et al., 2010). After the 24-cell stage, localization appears mainly in cells of the D-quadrant (Fig. 5C–F). Localization is observed during early gastrulation around the lip of the entire blastopore with more concentrated expression seen along the posterior edge (Fig. 5G, H) and in ectoderm posterior to the blastopore lip. This expression pattern is reminiscent of that seen for *ctnnb*. There is localization of *bra* in some cells derived from 3c<sup>1</sup> and 3d<sup>1</sup> to the sides of the ventral midline (Fig. 5I). Signal is also observed in progeny of 3a<sup>2</sup> and 3b<sup>2</sup>, which give rise to ectomesoderm, and are located on the lateral sides of the blastopore. Unlike in *Capitella*, we did not observe asymmetric *bra* expression to the left side of the blastopore (Boyle et al., 2014). Localization of *bra* in gastrula stage embryos is also noted in what are presumed to be neuronal precursors from scattered anterior 1q progeny. At later stages, expression is seen in the cells that undergo convergent extension (from 3c<sup>2</sup> and 3d<sup>2</sup>), which originally occupied the posterior blastopore lip (including the terminal cells; Fig. 5O–P; Lyons et al., 2015), and to reside along the ventral midline (Fig. 5I, O). During later gastrula stages expression continues in cells around the entire blastopore lip.

During cleavage stages, two areas of expression are also observed in the mesodermal left and right teloblasts derived from 4d (Fig. 5J–N), and at later stages of gastrulation, prominent expression continues in the teloblasts and in endomesodermal progeny. Pre-larval expression appears around the entire mouth, in the esophagus, in both mesodermal and endodermal progeny of 4d, including the hindgut rudiment, in intense spots associated with nuclei in some cells of the anterior/apical plate ectoderm, in the prototroch cells (derived from 1q), and in the more ventral-lateral progeny of 2b<sup>1</sup> (neuronal cells) and 2b<sup>2</sup> (Figs. 5Q–X). Proximal lateral cells of the foot rudiment also express *bra* (derived from 3c<sup>1</sup> and 3d<sup>1</sup>, Fig. 5U–W).

Several *bra* in situ specimens were also labeled with acetylated tubulin antibody, which labels ciliated cells that appear during later stages of gastrulation (during elongation) and organogenesis stages. These ciliated cells are found in ectodermal regions of the mouth, apical plate, and cells located posterior to the mouth along the ventral midline including the two terminal cells (Lyons et al., 2015; Fig. 5L). Acetylated tubulin labeling corresponds with regions along the ventral midline where *bra* expression is also noted. During later elongation stages and organogenesis, the two ciliated terminal cells have migrated towards the posterior pole (Fig. 5P), and lie in a region where *bra* mRNA is also observed (Fig. 5O, P).

**Digestive Tract Markers**

**Caudal (cdx).** Caudal is a hox-related homeobox transcription factor important during early embryonic development and in the process of gastrulation. At later stages, *cdx* is involved in the specification of posterior fates, including the hindgut (Schulz and Tautz, 1995; Wu and Lengyel, 1998; Moreno and Morata, 1999; Laritillot et al., 2002b).

During cleavage stages, *cdx* is localized in specific cells of all four quadrants of the embryo and is particularly localized to regions adjacent to the nuclei, which we presume to be the centrosomes of the macromeres, and to some extent also in the micromeres (Figs. 1, 6A–F; Table 1). At later stages expression appears to be more widespread and is seen in particular around
Fig. 7. Expression of gsc during early development in C. fornicata. A–T: gsc expression in embryos ranging from 16-cell–145hpf (early cleavage to later stages of elongation). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (an., animal; veg. vent., vegetal/ventral; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. Animal view of gsc expression in 16-cell cleavage stage (A) and corresponding DAPI-labeled nuclei (B), as well as a 27-cell cleavage stage (C) and corresponding DAPI nuclei (D). Embryonic quadrants A–D are labeled within each panel of A–F. Animal view of late cleavage specimen (E), and corresponding DAPI-labeled nuclei (F). Flattened round stage embryo (G) and corresponding DAPI image (H), and late epiboly embryo with expression around the blastopore (I) with corresponding DAPI labeling (J). The embryo shown in I, J is represented in a posterior view (K) with corresponding DAPI labeling (L). Expression during elongation is represented in (M) with corresponding DAPI labeling in (N) and in a later specimen (O) with DAPI labeling (P). Various views of expression are shown from the same late ovoid specimen in Q–T. Black arrowheads point to asymmetric post-trochal ectodermal patch of expression in Q, R, and T. Structures are labeled following designations used in Figures 2 and 5. Scale bar in T = 50 μm.
posterior and anterior-lateral cells of the blastopore (Fig. 6G,H).
Though between 120–137hpf (elongating stage), it is excluded from the very anterior region of the blastopore lip.

cdx is observed in posterior ectoderm, and possibly in some cells that are located closest to the ventral midline (Fig. 6I–P). Expression is widespread and includes other ectodermal cells, including an asymmetric patch in the right post-trochal region (Fig. 6I–L). At later stages, cdx is seen in the cells that undergo convergent extension, which originally comprised the posterior blastopore lip. This includes the two terminal cells, but expression at later stages appears to be extinguished in these cells (Fig. 6K, L, O, P).

As development continues, cdx is expressed in all cells of the developing mouth and the esophagus. Pre-larval expression is seen in the cells of the hindgut, shell gland, velar lobes, and foot rudiment (Fig. 6Q, X). Noticeably, cdx is asymmetrically expressed in the ectoderm with more expression in a patch on the right side of the post-trochal region as compared with the left side. Asymmetric expression of cdx was also noted in a similar region in Patella (Lartillot et al., 2002b).

Expression is also seen in progeny of the mesentoblast (4d), including distinct expression in the two teloblasts at certain stages (Fig. 6K–N). At later stages, prominent expression is seen in both endomesoderm and the developing hindgut rudiment (Fig. 6R, U, V). Furthermore, expression is apparent in the progenitors of ectomesoderm (Fig. 6Q–X), but is not apparent once the progeny have undergone EMT.

Goosecoid (gsc). Goosecoid is a homeobox transcription factor expressed during gastrulation. It is expressed during the development of mesendodermal fates in many metazoans, and promotes the development of the dorsal organizer in chordates (Angerer et al., 2001; Lartillot et al., 2002a; Boyle and Seaver, 2008).

During early cleavage stages gsc mRNA is concentrated in regions adjacent to the nuclei, which we presume to be the centrosomes of the A–D macromeres, but is also expressed in animal micromeres (Figs. 1, 7A–F; Table 1). Faint expression is widespread throughout the embryo, but during epiboly it is more concentrated in posterior and lateral regions around the blastopore (Fig. 7G–J). As the blastopore constricts at later stages of epiboly, localization becomes more prominent in cells of the blastopore lip (Fig. 7M, N). Expression becomes less pronounced along the posterior edge of the blastopore (Fig. 7O, R). At later stages, gsc mRNA is seen in the cells that undergo convergent extension, including the two terminal cells, which originally comprised the posterior blastopore lip (Fig. 7M, N). As development continues, gsc is seen in all cells of the developing mouth and the esophagus, and is more prominently expressed in the anterior regions of these structures (Fig. 7Q–S). Additional regions of expression are also noted in post-trochal right and left regions in pre-larval specimens (Fig. 7Q–T).

During elongation stages, expression is noted in the 4d derived left and right teloblasts (Fig. 7K, L) and in endomesodermal progeny of these cells, as well as the hindgut rudiment (Fig. 7Q–S). Furthermore, expression is apparent in the progenitors of the ectomesoderm (3a², 3b²; Fig. 7R) and seen faintly in scattered mesenchymal cells.

Hex. Hex is a divergent homebox transcription factor involved in anterior-posterior patterning (Thomas et al., 1998). This gene is expressed in vertebrate anterior endomesoderm and is required for anterior development, including the forebrain (Newman et al., 1997; Zorn et al., 1999; Jones et al., 1999; Brickman et al., 2000; Barbera et al., 2000; Zamparini et al. 2006). The literature also suggests a role in migrating hepatic endoderm (Bogue et al., 2000).
Expression of hex is not observed during C. fornicata early cleavage stages. The first detectable expression emerges within mid to late gastrula embryos as faint labeling in a subset of cells of the blastopore lip (Figs. 1, 8A, B; Table 1). During later stages of gastrulation, expression is seen in the more anterior and lateral cells of the developing mouth and esophagus, though it becomes more diffuse at later stages (Fig. 8C–H). During organogenesis stages expression is also noted within two deeper areas in the head, which we presume to be neuronal cells in the vicinity of the photoreceptors (Fig. 8E–G).

Hex is also localized to the hindgut primordium during organogenesis stages (Fig. 8D). This hindgut expression persists during later pre–larval stages (Fig. 8E–G).

**Fig. 9.** Expression of nk2.1 during early development in C. fornicata. A–P: nk2.1 expression in embryos ranging from 24-cell–160hpf (early cleavage through late elongation stage). Refer to the detailed descriptions in the Results section. Orientation of specimens is located in the bottom right corner of each panel (an., animal; vent., ventral). DAPI-labeled nuclei (blue fluorescence) accompany some brightfield images. A–D labeled within each panel in A,B represent the relative position of the embryo quadrants. Animal view of nk2.1 expression in cleavage stage embryo (A) and corresponding DAPI-labeled nuclei (B). Dorsal view (C) of nk2.1 expression in the teloblasts (2ML, 2MR) and their progeny (2mL1, 2mR1) with corresponding DAPI labeling (D). Oval stage specimen (E) with corresponding DAPI labeling (F), as well as ventral view of older oval stage specimen (G) and corresponding dorsal view (H). Various views of expression are shown from the late ovoid specimen (I–L), and also an early organogenesis specimen (M–P). Structures are labeled following designations used in Figures 2, 3, and 5. Scale bar in P = 50 μm.

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**Nk2.1 (ttf1).** Nk2.1 is a homeodomain transcription factor known to regulate the development of endodermal fates, but also plays a role in the specification of the nervous system (Ciona, Ristoratore et al., 1999; Amphioxus, Venkatesh et al., 1999). Additionally, this transcription factor is reported to be a potential downstream target of bra in the sea urchin oral ectoderm GRN (Rast et al., 2002).

Expression is first observed in cleavage stages within each of the four quadrants, and is localized to regions adjacent to the nuclei (presumably the centrosomes of the macromeres, as well as many of the micromeres, Figs. 1, 9A–D; Table 1). This localization of transcripts is dynamic and depends on the cell cycle. Expression is no longer detected during early to mid gastrula stages.
Towards the end of gastrulation and during the formation of the mouth, expression appears in the anterior-most region of the stomodeum (Fig. 9E–G). Expression also expands in an anterior crescent above the mouth (Fig. 9I–K, M–O). These cells presumably include neural precursors of the CNS (Fig. 9M–O).

\(\text{nk2.1}\) is also expressed in what appear to be the terminal cells (Fig. 9I–K; M–O). During organogenesis and pre-larval stages, expression is noted in the shell gland (Fig. 9I, K, L, O, P). Some expression is also seen in the anterior mouth and in the esophagus. Two more intensely labeled lateral clusters of cells reside just outside of the left and right sides of the developing mouth (Fig. 9I–K, M–O). \(\text{nk2.1}\) also continues to be expressed in the terminal cells at these later stages (Fig. 9M–O).

During late cleavage stages, expression is observed faintly in the progeny of the mesentoblast, 4d, including the 2ML and 2MR teloblasts, and more prominently in two daughters (2ML¹, 2MR²; Fig. 9C, D). However during gastrulation and later stages of organogenesis, expression is no longer detected in mesentoblast progeny and no expression is detected in ectomesoderm.

**Orthopedia (otp).** Orthopedia is a homeobox transcription factor that is commonly associated with CNS and brain development in the mouse. Specifically, it controls development of the hypothalamus, a derivative of the forebrain in vertebrates (Acampora et al., 2000; Kaji and Nonogaki, 2013). In *Drosophila*, otp is expressed during gastrulation in the proctodeum, and during later stages in the CNS and posterior fates, including the hindgut and anal plate regions (Simeone et al., 1994).

Expression of \(\text{otp}\) is first observed in the D quadrant during cleavage stages (Figs. 1, 10A; Table 1). During early epiboly stages, expression is present in cells from all four quadrants and becomes more intense in cells along the blastopore lip (Fig. 10C–F). At later stages, expression persists around the constricted blastopore (Fig. 10G, H). During organogenesis stages, labeling is observed along the anterior-lateral edges of the developing mouth and esophagus, but expression in the posterior region of the mouth is visibly diminished (Fig. 10J). Some anterior expression is observed in progeny of the first quartet. Elevated expression is seen in posterior ectoderm cells in the blastopore lip close...
to the ventral midline (Fig. 10G, H). Faint expression of \( otp \) appears along the ventral midline posterior to the blastopore (Fig. 10G, H, J). There appears to be diffuse labeling in the ectoderm at later stages. Interestingly, an asymmetric patch of ectoderm on the right post-trochal region expresses \( otp \) (Fig. 10I, J, L), similar to that described for genes such as \( cdx \), \( otx \), and \( foxA \). Diffuse expression is also noted in the shell gland (Fig. 10I, K, L).

During gastrulation, \( otp \) mRNA is observed in progenitors of ectomesoderm derived from \( 3a \) and \( 3b \) (Fig. 10G, H). Faint expression is seen in both the endomesodermal (2mL, 2mR) and endodermal progeny of the mesentoblast (Fig. 10E–H). During organogenesis, \( otp \) expression is not detected in the ectomesoderm or endomesoderm, nor is it expressed in the developing hindgut rudiment.
Six3/6. Six3/6 is a homeobox transcriptional regulator commonly associated with the development of the forebrain and anterior neural structures including eyes or photoreceptors, and is expressed in the chordate mouth primordium (Niimi et al., 1999; Boorman and Shimeld, 2002; Christiaen et al., 2007; Kumar 2009). It is also involved in the specification of the apical plate in Platynereis (Marlow et al., 2014). Alternatively, six3/6 is involved in the specification of the aboral (posterior) territory in cnidarians (Sinigaglia et al., 2013).

six3/6 expression is not observed at early cleavage stages, but appears anteriorly after the onset of gastrulation when the blastopore is beginning to constrict (Figs. 1, 11A, B; Table 1). During mid to late gastrula stages, expression appears in additional cells around the blastopore lip, being more prominently expressed in the region of the anterior blastopore lip (Fig. 11C, E, G). Expression is also noted as spots in the anterior (animal) with some faint expression observed around the anterior portion of the forming blastopore, which are presumably neural progenitors (Fig. 11A–H). Two
intense focused regions of expression persist around the animal pole through later gastrula stages, which are presumably the forming ocelli. Very faint expression is present along the ventral midline during mid to late gastrula stages. Expression is observed in cells of the ciliary band, which later expands to cells of the developing velum (Fig. 11I–K; M–O). Later stages continue to exhibit expression in the cells of the developing CNS, including the precursors of the apical ganglia and the ocelli (Fig. 11N, P; Hejnol et al., 2007). In these later stages, \textit{six3/6} is noted in the ventral midline cells undergoing convergent extension, which includes the terminal cells (Fig. 11E, H).

During stages of organogenesis, intense anterior signal is observed in a symmetric pattern, which represents components of the developing brain and the ocelli located deeper within the developing head (Fig. 11H, I, N, P). Pre-larval specimens show expression in the mouth, esophagus, left and right velar rudiments, which include the developing ciliated bands, left and right food grooves (Fig. 11I–K; Lyons et al., 2015), the shell gland, central nervous system and neurosensory cells of the foot (which may be the statocysts), with asymmetric expression noted on the right side in the post-trochal ectoderm (Fig. 11I–O).

\textit{six3/6} is detected transiently in both ectomesodermal and endomesodermal cells during mid- to late gastrula stages. \textit{six3/6} is not detected in the developing hindgut rudiment.

**EMT Markers**

\textit{Snail2}. \textit{Snail} genes encode zinc-finger transcription factors commonly associated with development and behavior of vertebrate neural crest. Those cells are of ectodermal origin, which arise along the dorsal midline during closure of the neural tube and undergo epithelial to mesenchymal transition (EMT) to adopt a variety of fates (Baker and Bronner-Fraser, 1997a, b; Nieto, 2002). \textit{snail2} (also known as \textit{slug}) is directly implicated in the process of EMT and serves as a transcriptional repressor of E-cadherin (Knight and Shimeld, 2001). Additionally, \textit{Snail} proteins can also function as regulators of mesodermal invagination (Hemavathy et al., 2000). Only one copy of \textit{snail} has been isolated in \textit{C. fornicata}. According to phylogenetic analysis (not shown), this clone was determined to be orthologous to vertebrate \textit{snail2}.

\textit{snail2} first appears in a small population of anterior ectodermal cells during early stages of gastrulation (Figs. 1, 12A–D; Table 1). During elongation stages, an asymmetric region of
snail2 expression begins to emerge (Fig. 12A–H) and this asymmetry persists into stages of organogenesis in an area posterior to the blastopore on the right lateral side (Fig. 12I–P). Asymmetric expression of another Snail family member (Pv-snail1) was also observed in Patella (Lespinet et al., 2002). At later stages of organogenesis, expression is visible in anterior neuronal precursors that may correspond to the apical ganglia and ocelli (Hejnol et al., 2007; Fig. 12E–G, I–K, M–O), as well as scattered cells in other regions of the embryo. Expression is observed in a subset of cells around the mouth and in bilateral bands of cells that contribute to structures of the velar rim, including the food groove and metatroch (Fig. 12I–K). In the oldest pre-larval specimens, cells in the developing head and more specifically, the developing CNS (ocelli and cranial ganglia) express snail2. Some of those cells are located in the surface ectoderm and may be neuronal precursors, whereas some are deeper cells (presumably ectomesodermal cells that have undergone EMT, Fig. 12I–P). In addition, some cells towards the right side of the foot rudiment also express snail2 (Fig. 12M, N).

Ectomesodermal progenitors express snail2 in mid to late gastrula stages. Though snail2 is expressed in ectomesoderm, it is not expressed in endomesoderm. No expression of snail2 was detected in the hindgut rudiment.

Twist. Twist is a bHLH transcription factor, which is essential for mesoderm development (summarized by Technau and Scholz, 2003). It is involved in specification and patterning of mesoderm and is expressed in the larval head mesoderm, as well as the stomodeum, foregut, and hindgut of polychaetes (Dill et al., 2007). Additionally, it functions to regulate gastrulation, and is an inducer of EMT during gastrulation events, specifically by repressing E-cadherin, which results in modulation of cell adhesion (Gheldof and Berx, 2013; Wong et al., 2014). twist plays a key role in regulating the development of the neural crest in vertebrates (Meulemans and Bronner-Fraser, 2004; Adams et al., 2008; Betancur et al., 2010).

Twist is expressed transiently during early cleavage stages (Henry et al., 2010b), and reappears in mid to late gastrula
embryos in the anterior and lateral edge of the blastopore (Figs. 1, 13A–F; Table 1). Faint label is also seen in the lateral ectoderm and expands at later stages in anterior ectoderm of the head and velum (Fig. 13E–K). twist appears to be very faintly expressed along the posterior blastopore lip and in posterior cells including those that have undergone convergent extension (progeny of 3c^2 and 3d^2; Fig. 13C–F). During stages of organogenesis, twist is also detectable in the developing mouth and esophagus (Fig. 13G). A gradient of expression is observed in the developing mouth, where labeling is more intense along the anterior region and less intense along the posterior edge (Fig. 13G, J). In later stages, asymmetric expression is also noted faintly in the right post-trochal region (Fig. 13I, J, L). twist is expressed more intensely in bilateral bands that run along the edge of the developing velum and include precursors of the prototroch, metatroch, and ciliated food groove (Fig. 13I–K). The ectoderm of the head (neuronal cells) and the proliferative zone of the developing shell also exhibit twist expression (Fig. 13H, L).

While expression is detected in the precursors of the ectomesoderm (3a^2, 3b^2), it is not seen in the mesenchymal progeny later during organogenesis. twist is not detected in the endomesoderm or in the hindgut rudiment.

**Notch Signaling Members**

**Neurogenic locus notch homolog protein (notch2).** Neurogenic locus notch homolog protein (Notch) family members are single pass membrane proteins that serve in contact mediated intracellular signaling and interact with their receptors Delta and Jagged/Serrate (Lawrence et al., 2000; de Celis, 2013). Notch proteins play key roles in regulating many developmental processes including differentiation, proliferation, and apoptosis (Artavanis-Tsakonas et al., 1999). C. fornicata contains 3 variants of Notch. For the purposes of this study, we have examined, C. fornicata notch2, which has relevant expression during gastrulation and particularly in the development of the digestive tract.

Expression for notch2 is seen during cleavage stages in the macromeres. (Figs. 1, 14A–D, Table 1). Further expression is observed during mid to late stages of gastrulation, appearing faintly to the sides of the blastopore. Expression continues to spread to more anterior cells as the embryo undergoes elongation, being excluded from the
posterior lip and developing mouth, with very faint expression along the very posterior edge (Fig. 14E–G). Late elongation stage specimens exhibit more intense expression around the anterolateral edges of the mouth and esophagus (Fig. 14G). Expression is also seen in the ectoderm lateral to the mouth, and the foot rudiment (Fig. 14I–L). Some expression is also seen in post-trochal ectoderm. Expression is also visible in cells of the head that likely include neuronal precursors. During pre-larval stages, there is more intense expression in rudiments of the velar lobes, in the mouth and the foot, as well as the shell gland (Fig. 14H–L). Interestingly there is also an asymmetric patch of ectodermal cells expressing notch2 on the right side of the post-trochal region (Fig. 14G–J, L).

No expression of notch2 was noted in endomesoderm. However, prior to undergoing EMT, progenitors of the ectomesoderm show some faint expression of notch2 (Fig. 14E, F). At later stages during organogenesis, expression is detectable in hindgut endoderm, which is derived from the 4d mesentoblast (Fig. 14I, J).
**Hes.** The Hes family includes basic helix-loop-helix (bHLH) type transcription factors and its members act as repressors, except Hes6, which is an activator protein due to its inhibitory effect on the repressor Hes1. Hes members are downstream targets of Notch signaling (Iso et al., 2003), and are known to be involved in neural differentiation, as well as the regulation and maintenance of stem cells in digestive systems (Bäck et al., 2006; Kageyama et al., 2008). They are also involved in cell fate determination, and this occurs by physical interactions between cells expressing the Notch receptor and Delta/Jagged ligands in adjacent cells. Finally, they are involved in regulating the timing of biological events such as segmentation of the somites (Kageyama et al., 2007). For this study, we report on the expression of two biological events: segmentation of the somites (Kageyama et al., 2007).

When we looked at the expression of these genes, we found that the Hes gene most closely resembles those of the canonical form, including its characteristic basic domain with the proline residue. Expression is not observed during cleavage or early to mid gastrula stages, but appears later during formation of the esophagus in two bilateral clusters of cells (Figs. 1, 15A–D; Table 1). Expression is also detected in neurosensory cells located in the esophagus in two bilateral clusters of cells (Figs. 1, 15A–D; Table 1). Expression is also noted in cells flanking the blastopore (Fig. 16E–G). Expression is also noted in cases where expression is not observed during cleavage or early stages of gastrulation (Figs. 15I–K; Lyons et al., 2015), which could be related to the posterior expression seen at earlier stages (Fig. 15A–C; E-G). In addition, some expression is seen at the base of the foot rudiment anteriorly to the left and right sides.

Expression is not seen in the progeny of the mesentoblast, but the location of expression during later stages of gastrulation suggests that the expression may be associated with some ectomesodermal progenitors at the blastopore prior to their undergoing EMT (Fig. 15E–H).

**HesB.** HesB has a more dynamic expression pattern over a broader range of stages, and is seen first during late cleavage stages within variable cells of all four quadrants of the embryo (Figs. 1, 16A, B; Table 1). Some faint spots of expression are noted in cells of the anterior ectoderm (Fig. 16C, D). Mid to late gastrula stage embryos display expression in a somewhat symmetrical pattern flanking the blastopore (Fig. 16E–G). Expression is also noted in cells along the anterior-lateral blastopore lip (Fig. 16I–K). Some HesB expression is noted during stages of organogenesis in ectoderm, as well as in the right post-trochal region (Fig. 16H–P). Increased expression is also noted along the lateral edges of the foot rudiment and extending toward the medial area of the foot (Fig. 16M–O).

Ectomesoderm expression is noted in the lateral blastopore region during gastrulation stages (Fig. 16C, D). Intense expression is observed in the latest pre-larval stages on either side of the mouth in cells radiating outward towards the anterior and posterior regions. Some of these are ectomesodermal and others appear to be superficial ectodermal cells. Cells expressing HesB seem to be arranged with linear-periodic distributions (Fig. 16E–P). Expression is noted in the velum in scattered mesenchyme cells.

**Discussion**

Relatively little is known about how germ layers are specified in the Spiralia, despite the fact that it is a large branch of the Bilateria. We took advantage of newly generated EST databases in the slipper snail, C. fornicata, and examined expression patterns for fifteen regulatory genes known to be involved in gastrulation and germ layer specification in other metazoans. We present these expression patterns in the context of recently completed fate maps for gastrulation-stage embryos (Lyons et al., 2012, 2015), which allow us to assess expression relative to specific cells and germ layers. It is possible to compare cell-type specific gene expression patterns between species because many spiralians share a homologous, stereotyped cleavage pattern. We discuss these findings within the framework of ongoing debates regarding the evolution of germ layers, and the digestive tract (Technau and Scholz, 2003; Hejnl and Martindale, 2008; Martindale and Hejnl, 2009; Lyons and Henry, 2014; Hejnl and Martín-Durán, 2015).

**Endomesoderm**

ctnnb, bra, cdx, foxA, gsc, nk2.1, otp, otx, and six3/6 were all expressed in the endomesodermal progeny of 4d (Table 1). Four of the genes examined here also exhibited enhanced expression within the 4d-derived teloblasts, which include bra, cdx, gsc, and nk2.1. Two of these genes, bra and cdx, are also known to be expressed in the paired mesoteloblasts in Patella (Lartillot et al., 2002b; Le Gouar et al., 2003). While cttnb, bra, foxA, and otx are known to be involved in the highly conserved sea urchin endomesoderm GRN, in contrast, gsc, nk2.1, and six3 are part of the sea urchin ectoderm GRN (Oliveri et al., 2008; Peter and Davidson, 2011; Li et al., 2014). We were not able to examine expression for orthologs of additional genes often found in metazoan mesoderm, such as Gata-family genes, Blimp/Krox or MeP2, which are either not present in our available ESTs, or have not yet been cloned.

Our results indicate that there is conservation in terms of the deployment of metazoan endomesodermal specification in this representative of the Spiralia. However, while there are similarities in the particular genes that are expressed during endomesoderm specification, it is obvious that the expression domains do not have exactly the same expression patterns, even within the Crepidula endomesodermal lineage. Therefore, these differences could potentially serve as a starting point for understanding specification of sublineages in this tissue.

**A Shared Toolkit for Crepidula Endo- and Ectomesoderm**

In C. fornicata, the 3a and 3b cells are bipotential precursors, and only one daughter cell of each (the 3a2 and 3b2 cells) gives rise to mesoderm. Given that some similar mesodermal fates differentiate from both ectomesoderm and endomesoderm (e.g., muscle cells), one can hypothesize that components of the endomesodermal GRN are shared between the two. Alternatively, it is conceivable that the genes that control ectomesoderm specification are distinct from those controlling endomesoderm specification. There are a few examples of novel origins of mesoderm tissues that are distinct from the eumetazoan endomesoderm, for example the evolution of mesoderm in ctenophores (Ryan et al., 2013), and entocodon of some cnidian medusae (Burton, 2008). Additionally, the neural crest generates some fates in common with both ectoderm and mesoderm (Simões-Costa and Bronner, 2015). Neural crest cells also undergo EMT like cells of the ectomesoderm in Crepidula. In each of these cases, genes typically associated with
endomesoderm are not expressed. Thus, we were interested to compare the expression profiles of C. fornicata 3a², 3b², and 4d-derived lineages to examine any similarity between their repertoires of regulatory factors.

Expression in C. fornicata ectomesodermal progenitors was noted for the following: ctnnb, bra, cdx, foxA, gsc, hesA, hesB, notch2, otp, otx, six3/6, snail, and twist. All of those genes except for snail2, twist, notch2, hesA, and hesB are expressed in both ectomesoderm and endomesoderm. Most of the expression seen in these two populations of cells was coincident over the time intervals we examined (Figs. 1–16; Table 1). Some exceptions were found in the timing (initiation vs. termination) of certain genes such as foxA and gsc (Figs. 4 and 7; Table 1).

The expression profiles of ectomesoderm and endomesoderm are not identical, which suggests that some aspects of their specification and/or their cellular behaviors are different. The spatial separation of the two sources of mesoderm has been noted in other spiralian species. For example, Lartillot et al. (2002a) and Nederbragt et al. (2002b) questioned any homology between ecto- and endomesoderm in the gastropod Patella. In contrast to Crepidula, Nederbragt et al. (2002b) showed that twist, and Lartillot et al. (2002a) showed that gsc and foxA, are expressed in ectomesoderm, but not endomesoderm. Lartillot et al. (2002a) argued that ectomesoderm may be homologous with anterior prechordal mesoderm of vertebrates, while posterior endomesoderm may be homologous with the tail-trunk mesoderm. An alternative hypothesis is that differences in gene expression might reflect different behaviors of these two forms of mesoderm.

However, since there is some overlap in the expression of genes in both endomesoderm and ectomesoderm, the data may suggest that ectomesoderm is specified by a very similar set of genes that specify endomesoderm. Given these similarities, different scenarios for the evolution of ectomesoderm can be envisioned. When ectomesoderm arose, it may have co-opted the spiralian endomesodermal tool kit. Alternatively, ectomesoderm and endomesoderm may have had a common origin, being separated into distinct domains (e.g., anterior vs. posterior, or ventral vs. dorsal), which subsequently underwent changes during the course of evolution. Depending on the species, ectomesoderm can arise from different combinations of cells from any of the four quadrants (Lyons and Henry, 2014), and 4d has been described as an ectomesodermal cell in the annelid, Capitella (Meyer et al., 2010a, b). Distinguishing between these scenarios will require building GRNs and additional lineage data and lineage-specific expression of “endomesodermal” genes in a wider range of spiralian species.

**Differences in Ecto- Versus Endomesodermal Expression May Be Tied to Morphogenetic Behaviors**

The differences in ecto- and endomesodermal expression may reflect different behaviors of these two derivatives of mesoderm. For example snail2 and twist may be necessary for the EMT that we recently described as a key behavior of the ectomesodermal cells that express these genes (Lyons et al., 2015). snail and twist are necessary for EMT in the sea urchin primary mesenchyme cells (Wu and McClay, 2007; Saunders and McClay, 2014), fly mesoderm (Nieto, 2002), and vertebrate neural crest (Carl et al., 1999; Hall 2000). We note that in C. fornicata, the 4d-derived mesoderm does not undergo EMT, but is born internally (Lyons et al., 2012; Lyons and Henry, 2014). Other species of spiralian gastrulate by invagination, and in those cases the 4d-derived mesoderm may become internalized by EMT (Lyons and Henry, 2014). Examining gene expression patterns in these other spiralian species would allow us to broaden our comparison of expression of snail and twist within the 4d lineage. We note that in Patella, twist is expressed in the ectomesoderm of trochophore-staged embryo, but snail is not (Nederbragt et al., 2002b; Lespinet et al., 2002).

Further, since all of the genes expressed in ectomesoderm (snail2, twist, notch2, hesA, and hesB) start their expression later compared to the expression of the other endomesodermally expressed genes, one could argue that these five genes, and potentially others (Figs. 12–16; Table 1), may be necessary for ectomesoderm differentiation or morphogenesis and not necessary for its initial specification. Alternatively, ectomesoderm specification may simply be delayed in comparison with endomesoderm. Some of these genes could have other roles in segregating cells fates within the 3a and 3b lineages. For example Notch/ Hes signaling is known to be involved in contact mediated cell specification in other systems (Drosophila, Greenwald, 1998; mammals, Kageyama and Ohtsuka, 1999). Likewise, twist expression has been noted later during the specification of anterior mesoderm in brachiopods (Passamaneck et al., 2015). Given the fact that little is known about the morphogenetic behavior of either source of mesoderm in spiralian, it will be very interesting to correlate gene expression and cell behavior in these animals as more detailed descriptions of their development become available.

**Endoderm**

Endoderm is derived from different blastomeres in C. fornicata including the 4th quartet micromeres and macromeres. The hindgut rudiment is a compact structure comprised of many small cells derived specifically from progeny of the mesentoblast 4d (Lyons et al., 2012). We found that many of the genes examined in this study were expressed in the hindgut endoderm, including: ctnnb, bra, cdx, foxA, gsc, hes, notch2, otp, and otx (Tables 1 and 2). While most of these genes were expressed at all stages examined, some were expressed relatively late (hex at ~120 hpf, elongating, short oval stage; notch2 at ~170 hpf, early organogenesis), while others are turned off by ~140 hpf (elongating, late oval stage; six3/6, otp).

Hindgut development in some bilaterian members is often characterized by the expression of conserved genes, which include bra, cdx, otp, foxA, and nkd2.1 (Hejnol and Martindale, 2008; Table 2). Many metazoans display conserved hindgut expression for bra (Kispert et al., 1994; Tagawa et al., 1998; Shoguchi et al., 1999, Woolard and Hodgkin, 2000; Arendt et al., 2001; Croce et al., 2001; Arenas-Mena, 2013). However, unlike C. fornicata, hindgut expression of bra was not observed for the molluscs Patella or Haliotis (Lartillot et al., 2002b; Koop et al., 2007). C. fornicata hindgut expression for cdx (Brooke et al., 1998; Wu and Lengyel, 1998; Edgar et al., 2001; Le gouar et al., 2002; de rosa et al., 2005; Shimizu et al., 2005; Frohius and Seaver, 2006; Arnone et al., 2006), foxA (but not in nematodes or hemichordates; Weigel et al. 1989; Arenas-Mena, 2006; Oliveri et al., 2006; Boyle and Seaver, 2008, 2010) and nkd2.1 (Venkatesh et al., 1999; Takacs et al., 2002; Lowe et al., 2003; Tessmar-Raible, 2007) were also conserved compared to other metazoans.
In contrast to other bilaterians, gsc, hex and hesA are expressed in hindgut tissues in C. fornicata (Figs. 7–15; Table 1). This was surprising, as previous studies highlight the importance of gsc in foregut tissues of arthropods (Drosophila, Goriely et al., 1996), annelids (Platynereis, Arendt et al., 2001), molluscs (Patella, Lartillot et al., 2002a), and sea urchins (Angerer et al., 2001). Likewise, her expression has been noted in the pharyngeal isthmus of C. elegans (Mörck et al., 2004).

The localization of otp to C. fornicata hindgut tissues was also somewhat surprising since this gene is often associated with CNS or brain development (Simeone et al., 1994; Umesono et al., 1997; Acampora et al., 2000). However, in Patella, otp is also expressed in the stomodaemum, as we noted here for C. fornicata (Arendt et al., 2001; Nederbragt et al., 2002a; Steinmetz et al., 2011). Other studies, including one in the spiralian nemertean C. lacteus, reveal an ancestral role for ctnnb in the establishment of endodermal and/or...
endomesodermal fates (Schneider et al., 1996; Rocheleau et al., 1997; Thorpe et al., 1997; Logan et al., 1999; Imai et al., 2000; Miyawaki et al., 2003; Wikramanayake et al., 2003; Lee et al., 2007; Momose et al. 2008; Henry et al., 2008). During earlier cleavage stages, C. fornicata GFP-labeled ctnnb mRNA becomes restricted to the 4d endomesodermal lineage (Henry et al., 2008), which ultimately gives rise to the hindgut. However, the localization of ctnnb to endomesodermal derivatives in other spiralians has not been well characterized to make a sufficient comparison.

It seems somewhat surprising that we did not observe appreciable expression of endodermal genes in the macromeres and fourth quartet micromeres during gastrulation in Crepidula. On the other hand, with the exception of her, notch2, otp, and otr, message was detected in those cells during cleavage stages, where they were generally found to be localized to regions we interpret to be the centrosomes (see Figs. 5–16). Some of these transcripts may become dispersed during gastrula stages. The large, yolky cells could have also hindered visualization of those transcripts. In addition, these cells become covered by ectoderm derived from the animal cap during gastrulation, which also could have obscured their visualization. Alternatively, it is possible that transcripts may have been translated, and/or the RNAs degraded in those cells. Therefore, some of these genes may play a more general role in endodermal development, while others could be more specific to the hindgut.

The Blastopore and Openings of the Digestive Tract

The evolution of the bilaterian gut is still debated (e.g. Martindale and Hejnol, 2009; Martin-Durán et al., 2012; Lyons and Henry, 2014). One question relates to the origin of the mouth and anus in the Deuterostomia, Ecdysozoa, and Spiralia, relative to the entire periphery of the blastopore lip: ctnnb, otx, foxA, goosecoid, otp, six3/6, and notch2. The posterior region of the blastopore showed bra expression, which is similar to that noted in Haliotis (Koop et al., 2007) and Patella (Lartillot et al., 2002a), and the anêlids Capitella (Boyle et al., 2014; also seen in left-lateral blastopore) and Hydrodies (expression noted in left and right sides flanking posterior region of blastopore; Arenas-Mena, 2013). Posterior expression of C. fornicata cdx has also been observed in the mollusc Patella (Le Gouar et al., 2003), and the anêlids Capitella (Fröbius and Seaver, 2006) and Platynercis (de Rosa et al., 2005), but is not detected in early stages of gastrulation for the mollusc, Gibbula varia (Samadi and Steiner, 2010). In contrast, otp expression in the blastopore was not observed in Patella (Nederbragt et al., 2002a).

ctnnb and gsc were also expressed along the posterior blastopore during early epiboly in C. fornicata. In contrast, ctnnb is expressed in many cells of the developing Platynercis embryo, where varying levels of expression determine animal versus vegetal fates during embryonic development (i.e., Schneider and Bowerman, 2007; Pruitt et al., 2014). gsc is not observed around the blastopore for Capitella (Boyle et al., 2014), but is observed in the 3a2 and 3b2 derived cells of the anterior blastopore and several cells along the posterior vegetal plate in Patella (Lartillot et al., 2002b). Anterior blastopore lip expression was noted for hex during early epiboly in C. fornicata, similar to that of amphioxus (Yu et al., 2007).

When the blastopore begins to constrict further during later stages in C. fornicata (late epiboly through elongating stages, 97–140 hpf), expression patterns changed slightly, but a majority of the genes display some expression in progeny associated with the blastopore lip. The following genes were observed around the entire periphery of the blastopore lip: ctnnb, otx, foxA, goosecoid, otp, six3/6, and notch2. The posterior region of the blastopore showed bra expression, which is similar to that noted in Haliotis (Koop et al., 2007) and Patella (Lartillot et al., 2002a), and to the dorsal/aboral (posterior) region in Hydrodies (Arenas-Mena, 2013). cdx was observed only in the lateral regions of the blastopore in C. fornicata, which differs from the posterior blastopore expression noted in another mollusc, Patella (Le Gouar et al., 2003), but is consistent with the lateral regionalization of cdx following blastopore closure in the annelid, Capitella (Fröbius and Seaver, 2006). Additionally, cdx was localized to a U-shaped area, which extended around the posterior and lateral regions of the blastopore for Platynercis (de Rosa et al., 2005). nk2.1 and hex were observed in the anterior region of the blastopore in C. fornicata, which includes ectomesodermal progenitors. Likewise, nk2.1 was expressed in regions flanking the blastopore for Capitella (Boyle et al., 2014).

During early epiboly to flattened round stages (50–65% epiboly, 48–91 hpf), otx, foxA, bra, cdx, and otp, were expressed in cells of the blastopore lip in C. fornicata (Figs. 1, 3–6, 10; Tables 1 and 2). otx was also observed around the entire blastopore periphery for Patella (Nederbragt et al., 2002a) and Capitella (Boyle et al., 2014). Likewise, blastopore expression was also noted for FoxA1 and FoxA2 in Hydrodies (Arenas-Mena, 2006), and Patella (Lartillot et al., 2002b). Despite labeling of the entire blastopore, enhanced posterior blastopore signal was noted for both C. fornicata bra and cdx. Posterior localization of bra is consistent with that observed during blastopore formation in other molluscs, including Haliotis (Koop et al., 2007), Patella (Lartillot et al., 2002a), and anêlids, Capitella (Boyle et al., 2014; also seen in left-lateral blastopore) and Hydrodies (expression noted in left and right sides flanking posterior region of blastopore; Arenas-Mena, 2013). Posterior expression of C. fornicata cdx has also been observed in the mullusc Patella (Le Gouar et al., 2003), and the anêlids Capitella (Fröbius and Seaver, 2006) and Platynercis (de Rosa et al., 2005), but is not detected in early stages of gastrulation for the mollusc, Gibbula varia (Samadi and Steiner, 2010). In contrast, otp expression in the blastopore was not observed in Patella (Nederbragt et al., 2002a).
definitive arguments about the consecutive, or simultaneous, evolution of the mouth and anus can be made.

**The Posterior Blastopore Lip**

In spiralians the cells that contribute to the mouth and anus are initially located close to one another during early development, but those cells become separated by various morphogenetic events that are not well understood in most species (Lyons et al., 2015). Our recent cell lineage analysis of gastrulation stages in *C. fornicata* revealed that the mouth and esophagus are made by sublineages of each of the second and third quartet micromeres (except the 2d lineage), which make up the blastopore lip at early epiboly stages (Lyons et al. 2015). As the blastopore constricts, clones of micromeres on the anterior and lateral sides largely retain their relative positions along the anterior-posterior axis. Cell re-arrangement largely involves some progeny becoming displaced deeper into the blastopore, contributing to the esophagus, while the rest of the clone stays at the surface, contributing to the anterior and lateral sides of the mouth (Fig. 1). In contrast, we have shown that the posterior lip of the blastopore undergoes closure via a novel zipper process (Lyons et al., 2015). Zippering is a unique example of convergence and extension, which gives rise to an elongated assemblage of eight ciliated cells that extend from the esophagus, anteriorly, towards the posterior end of the embryo.

As closure of the posterior blastopore lip takes place, these ciliated cells displace progeny of 2d (more specifically 2d²) away from the blastopore lip towards the posterior end of the embryo. Progeny of 2d ultimately form the anus later in development. Two cells of the posterior blastopore lip, which were located closest to the ventral midline prior to zipperping (3c, 2d²), are displaced farther towards the posterior pole and become isolated within the clone of cells derived from 2d. Earlier investigators confused these cells with those that make the anus, referring to them as “anal cells” (e.g., Conklin, 1897). These cells do not give rise to the anus in *Crepidula*, which forms much later in development from progeny of 2d² (Lyons et al., 2015). Hence, we renamed the former anal cells as “terminal cells.” Previous interpretations of gene expression in these cells (as being in the anus in other spiralians) must be regarded with caution. Similar events leading to closure of the posterior lip of the blastopore likely occur in the annelids *Polygyrodium* and *Platynereis*, and the molluscs *Ilyanassa* and *Patella* (Wolterreok, 1904; Arendt et al., 2001; Larillot et al., 2002a, Chan and Lambert, 2014; Lyons et al., 2015), though they have not been formally described.

Just two cells from each of the 3c and 3d² lineages remain at the opening of the blastopore to give rise to the posterior-most portion of the esophagus and mouth. The analysis reported here shows that several genes are expressed in the progeny of 3c and 3d² during closure of the posterior blastopore lip, including *ctnmb, otx, foxA, bra, cdx, gsc, otp, six3/6, notch2, hes* (Figs. 1–16; Table 1). This also includes the terminal cells (*bra*, Fig. 5M–O, Q–S, V; *cdx*, Figure 6K, L, O, P). Following gastrulation during later development, *bra* and *cdx* continue to be expressed in these cells along the ventral midline, with *nk2.1* also being expressed in the terminal cells (Fig. 9G–K, M–O). Earlier localization of *otp* to terminal cells in *C. fornicata* contradicts with many studies that usually associate this gene with anterior fates such as the apical organ in *Patella* (Nederbragt et al., 2002a) and the brain in the planarian (*Umesono et al., 1997*), and annelids (*Tessmar-Rai
dle et al., 2007*). However, *otp* is observed in hindgut tissues of *Drosophila melanogaster* (*Simeone et al., 1994*) and posterior ectoderm of hemichordates (*Lowe et al., 2003*).

Cells that are likely homologous to the two terminal cells of *C. fornicata* express *bra* and *cdx* in the mollusc *Patella* (*Larillot et al., 2002a; Le Gouar et al., 2003; see Lyons et al., 2015*). Fist labeling of *bra* was also observed in *Haliotis* in a posterior region judged to be the site of the anal cells (likely the terminal cells), although this was not linked to discrete cells of known lineage origins (*Koop et al., 2007*). It is important to note that *bra* expression is not confined to terminal cells in these species. Whether or not *bra* plays a role in the development of terminal cells is unclear; however, *bra* expression may be involved in convergent extension and closure of the posterior lip of the blastopore in those species where these events likely take place. Others have proposed that *bra* plays a role in regulating morphogenetic processes associated with convergent extension in vertebrates and other bilaterians (*Hardin, 1989; Yamada, 1994; Conlon and Smith, 1999; Arendt, 2004*). For example, although *bra* is expressed in endodermal cells during gastrulation in the annelid *Hydroïdes*, it is also expressed in ectodermal tissue that eventually merges along the ventral midline in *Hydroïdes*, thereby contributing to elongation and displacement of the mouth from the anus (*Arenas-Mena, 2013*). Likewise *bra* is expressed in ventral cells that undergo convergent extension in the annelid *Platynereis* (*Steinmetz et al., 2007*).

**Expression of Genes in the Digestive Tract (Mouth and Esophagus)**

Many of the genes examined in this study are localized to both the *C. fornicata* pre-larval/larval mouth and the esophagus (foregut), including *ctnmb, otx, foxA, bra, cdx, gsc, nk2.1, otp, six3/6, twist*, and *notch2* (Tables 1 and 2). Table 2 reveals that all of the genes expressed in the mouth are expressed earlier in the blastopore. We found that *nk2.1, otp*, and *six3/6* mark the mouth, and not the endodermal hindgut (Table 2). *nk2.1* was found to be expressed in the anterior blastopore lip (Fig. 9E, G), while *otp* and *six3/6* were expressed in the posterior blastopore lip, and the posterior ectodermal ventral midline (Figs. 10G, H, 11E, H), before being restricted to the mouth (Figs. 10J, 11J). Only *otp* was also found in the 2d lineages, which gives rise to the anus, though we did not examine embryos old enough to score *otp* in the anus itself. These data suggest that the morphogenetic events that zipper closed the posterior blastopore lip, and separate the mouth territory from the posterior end of the embryo, involve changes in expression of regulatory factors that could be necessary for proper mouth formation. Functional analyses of these genes will be necessary to test this hypothesis.

A comparison of gene expression in the mouth among spiralians (Table 2), and other bilaterians, reveals interesting similarities and differences. *C. fornicata* ctnmb expression is comparable to that observed in the ectoderm around the stomodeum of the mollusc, *Haliotis* (*Koop et al., 2007*). Likewise, *bra* is also observed in the foregut of annelids (*Arendt et al., 2001; Boyle et al., 2014*), molluscs (*Larillot et al., 2002a; Koop et al., 2007*), echinoderms (*Shoguchi et al., 1999; Croce et al., 2001*) and hemichordates (*Tagawa et al., 1998*). However, some localization of *C. fornicata* cdx in foregut tissue contrasts with more highly conserved hindgut expression across the Metazoa, though *cdx* is also expressed in the hindgut of *C. fornicata* (*Brooke et al., 1998; Wu and Lengyel, 1998; Hinman et al., 2000; Edgar et al., 2001; Le Gouar et al., 2003; de Rosa et al.,...*)
Conserved Expression of Genes in the Digestive Tract (Anus)

Lineage tracing in C. fornicata has demonstrated that the endodermal hindgut arises from several daughter cells of the 4d lineage (Lyons et al., 2012), while the ectodermal anus is derived from the 2d2 lineage (Lyons et al., 2015). At 12 days post fertilization, the opening of the anus forms as it fuses with the endodermal hindgut. The current gene expression analysis stops before the definitive anus forms. While we did not score for expression in the anus itself, we were able to score for expression in the hindgut rudiment, terminal cells, and the posterior 2d clone, which eventually forms the anus in Crepidula. Many of the genes expressed in hindgut endoderm were also expressed in the overlying 2d clone during late epiboly and early elongation stages, but at later stages, ventral midline staining in the ectoderm (derived from 2d, 3c, and 3d) is quite reduced. At later stages, we were only able to see expression of otx in the 2d clone where the anus will eventually form (Fig. 31-K, M–O). Thus, while the ectodermal and endodermal components of the terminal portion of the digestive tract initially express many of the same genes, it is likely that the ectodermal lineages that make the anus and other nearby ectodermal cells (e.g., TCs), express only a subset of these genes.

While the bilaterian mouth has been argued to be homologous (Arendt et al., 2001; Hejnol and Martindale, 2008), the anus may have evolved independently on multiple occasions (Hejnol and Martindale, 2008; Hejnol and Martin-Durán, 2015). Hejnol and Martindale (2008) argued that when an anus evolves it might co-opt components of the endodermal hindgut GRN. In fact, most of the genes that are reported to be expressed in the anus in spiralians are also expressed in the hindgut. The ability to compare gene expression in the context of homologous cell lineages makes the spiralian anus particularly useful. For example, lineage tracing in C. fornicata revealed that the ectodermal anus arises from 2d2. In Capitella both anus and hindgut arise from the 4d lineage as a common ectodermal structure, and so it may not be surprising that many of the genes expressed in the anus are also expressed in the hindgut (Meyer et al., 2010b).

Conclusion

This study lays the groundwork for building GRNs during the process of germ layer specification in C. fornicata. If we are to tease apart how the gene regulatory networks controlling mouth and anus formation evolved, we must understand how gastrulation takes place and how specific cell lineages contribute to these structures. While some regard regulatory factors as “markers” for cell fate specification, they also play roles in controlling morphogenetic events. Given that spiralian exhibit a wide range of gastrulation modes (Table 2; Lyons and Henry, 2014; Arenas-Mena, 2013), they present an opportunity to assess the role of specification versus cell behavior in the evolution of gastrulation, germ layer formation (e.g. mesoderm) and gut development.

Experimental Procedures

Animal Care and Handling

Adult C. fornicata were harvested from local waters near Woods Hole, MA, by the Marine Resources Center at the Marine Biological Laboratory. Embryos were collected and reared, as previously described (Henry et al., 2006, 2010a, 2010b; Lyons et al., 2012, 2015).

Fixation and Histology

Embryos and larvae were fixed for one hour at room temperature in a 3.7% solution of ultrapure formaldehyde (Ted Pella, Inc., Redding, CA) dissolved in filtered sea water (FSW), with added Instant Ocean Aquarium Sea Salt Mixture (United Pet Group, Blacksburg, VA), as previously described (Lyons et al., 2015). Following fixation, embryos were rinsed with three sterile 1X PBS washes (1X PBS: 1.86mM NaH₂PO₄, 8.41mM Na₂HPO₄, 175mM NaCl, pH 7.4), followed by three washes in 100% methanol. Embryos were stored in 100% methanol at -80°C. Following in situ hybridization (see below), embryos were incubated in a solution of 0.5 µg/ml DAPI (Life Technologies, Grand Island, NY)
dissolved in 1X PBS. DAPI incubation lasted for 10 min in the dark, followed by 3 washes in 1X PBS/0.1% Tween. Specimens were stored at 4degC in 80% glycerin/20% 1X PBS until imaged (see below).

**cDNA Library Preparation and Analysis**

Three different *C. fornicata* EST databases were used to identify and clone various genes known to be involved in germ layer specification, gastrulation and development of the digestive tract (Lengyel and Iwaki, 2002; Technau and Scholz, 2003; Heath, 2010; Hejnol and Martin-Durán, 2015). All the developmental material used to prepare these libraries came from animals collected at the Marine Biological Laboratory in Woods Hole, MA. These include a previously published *C. fornicata* 454 EST library prepared by Henry et al. (2010a), and two new libraries described below.

A second EST library was prepared in the lab of Dr. Cristina Grande (Centro de Biología Molecular Severo-Ochoa, UAM-CSIC, Madrid, Spain). The RNA used to prepare this library was obtained from early cleavage stage embryos up through advanced veliger larval stages (two to four weeks of age, but prior to hatching). These embryos and larvae were collected and stored in RNA later at -80degC. Total RNA was prepared using Trizol following the manufacturer’s instructions (Life Technologies). The embryos and larvae were homogenized using sterile plastic pestles. To prepare the cDNA, approximately 3 μg of extracted RNA was combined from each stage collected. The RNAseq library was constructed using TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CAA) following the manufacturer’s instructions. The RNAseq library was quantified using the Qubit HS DNA Assay and sequenced in 3 HiSeq 2000 PE100 lanes (Illumina). Assembly was done with Trinity assembler software (Grabherr et al., 2011). After assembly, contigs less than 200 bp in length were removed from our dataset. Assessments of the quality of read data were performed using FastQC (available at: http://www.bioinformatics. bsbc.ac.uk/projects/fastqc/) and the statistics on the final trimmed assembly are as follows: Trinity transcript reads (contigs) = 326,118, mean read (contig) length = 564 bp, N50 read (contig) length = 717 bp; minimum read (contig) length: 201; maximum read (contig) length: 28,997; median read (contig) length: 350; number of reads (contigs) > = 1kb: 37,997; number of bases in all reads (contigs): 183,902,140.

Finally, a third set of EST libraries was prepared in the lab of Dr. Joel Smith (Marine Biological Laboratory, Woods Hole, MA). A total of 24 Illumina RNAseq libraries from various embryonic stages were prepared and the sequence data was pooled prior to assembly. The 18 libraries were collected from a single brood at 22 hpf after treatment with either U0126 (a MAPK inhibitor) or DMSO at the 4-cell stage. These samples contained 115 to 139 embryos each. Total RNA extraction from Trizol and Ribo-Zero low input (Illumina) was performed as recommended by the manufacturer’s instructions. The RNA quality before and after the removal of ribosomal RNAs was assessed on a Bioanalyzer using RNA pico chips (Agilent Technologies, Santa Clara, CAA). The RNAseq libraries were constructed using ScriptSeq (Illumina), with a 60-sec fragmentation time, and otherwise following the manufacturer’s instructions. ERCC control RNA (available from Invitrogen/Life Technologies) was added before library preparation. The libraries were size selected with a 2% Pippin prep gel (Sage Science, Beverly, MA) for 350–600 bp. The quality of the libraries before and after the

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**Table 3. List of Primers Used to Prepare ISH Probes**

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<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe length</th>
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</table>

The NCBI accession number is presented along the far left column for each of the clones examined in this study. Gene-specific forward and reverse primers were isolated for each clone used for ISH, the DIG-labeled probe length is listed in the far right column.
size selection was assessed on a Bioanalyzer using DNA high sensitivity chips (Agilent). Bi-directional sequencing was done using the Illumina HighSeq 1000 platform, and the assembly of this combined library was done with Trinity assembler software (Grabherr et al. 2011): Trinity transcript reads (contigs) = 285,503, mean read (contig) length = 712 bp, N50 read (contig) length = 1145 bp; minimum read (contig) length: 201; maximum read (contig) length: 30,863; median read (contig) length: 389; number of reads (contigs) >=1kb: 71,337; number of bases in all reads (contigs): 203,168,019.

Sequence Analysis

Assembled consensus sequence files from each EST database were uploaded to the software program Geneious (Auckland, New Zealand). Using publicly available invertebrate homologous protein sequences from NCBI or UniProtKB, tblastn alignments against each C. fornicata EST database were performed individually for each of the genes of interest in the Geneious program. Any C. fornicata EST that aligned with a gene of interest was then submitted to BlastX (NCBI) to verify the identity of the EST. Each identified C. fornicata nucleotide sequence was also used as a query sequence for additional Blastn alignments in Geneious against the C. fornicata ESTs to further extend the nucleotide sequence in the 5'- or 3'-direction. Extended sequence information was compiled and assembled using Sequencher software (Gene Codes Corporation, Ann Arbor, MI) and consensus sequences verified again for identity against the NCBI database using BlastX. Only one discrete version of each gene was found in the EST databases, with the exception of notch (three copies), twist (two copies), and hes (two copies). Phylogenetic analysis was conducted for snail2, hesA and hesB sequences (data not shown). snail2 was analyzed and confirmed to be orthologous to the vertebrate Snail2, which is reflected in the naming scheme, even though snail1 has not been identified in the available C. fornicata EST database. We also thank Dr. Nathan Kenny for supporting this research.

Cloning

Using specimens stored in RNAlater (Life Technologies), C. fornicata RNA was isolated from both gastrulating embryos and pre-veliger larvae. High quality total RNA was extracted using TriZol, and purification methods followed the manufacturer’s suggested protocol. The purity and concentration of total RNA was verified with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and approximately 1μg of total RNA from each developmental stage (epiboly and pre-veliger stages) was used to synthesize cDNA (iScript cDNA Synthesis kit, Bio-Rad, Hercules, CA).

Gene-specific primers were designed for each gene of interest and those can be found in Table 3. Due to the presence of GC-rich regions, PCR amplification reactions were performed with Q5 High Fidelity DNA polymerase and Q5 High GC enhancer buffer (New England Biolabs, Ipswich, MA), according to manufacturer suggested ratios. Amplified PCR products were run on 1% agarose gels, gel purified (GeneClean Turbo kit, MP Biomedicals, Solon, OH) and cloned into pGem-T Easy vector (Promega, Madison, WI). All clones were verified by sequencing at the University of Illinois’ Carver Biotechnology Center (Urbana, IL) and sequences assembled using Sequencher software (Gene Codes Corp., Ann Arbor, MI).

Gene Expression Analyses

Gene specific DIG-labeled probes ranged from 290 nt to 1.2 kb in length, depending on available sequence data (see Table 3). Linearized template DNA (amplified from plasmid DNA with T7/SP6 primers) was used to synthesize RNA probes with either T7 or SP6 RNA polymerase (Life Technologies) and DIG-labeling mix (Roche, Indianapolis, IN). Reactions were purified with RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA) and probe concentrations were verified on a NanoDrop ND-1000 Spectrophotometer. The in situ hybridization protocol was modified from Finnerty et al. (2003) and is similar to that described by Henry et al. (2010b), but with the following modifications. The proteinase K digestion was eliminated and following rehydration in PTw (1X PBS, 0.5% Tween 20), embryos were washed in two acetic anhydride washes. Hybridizations were conducted overnight at 61degC with a probe concentration of 1 ng/μl followed by graduated washes into 2X SSC at 61degC, 0.2X SSC at 61degC, and finally PTw at room temperature. The alkaline phosphatase (AP) buffer was modified slightly to eliminate salt precipitation (5 mM MgCl2, 100 mM NaCl, 100 mM Tris pH 9.5, 0.5% Tween 20) and NBT/BCIP was used for probe visualization, by incubation at room temperature in the dark. Following in situ hybridization, specimens were incubated in Hoescht 33342 (Life Technologies, 1:10,000 dilution in PTw) for 10 min at room temperature for visualization of nuclei, followed by three washes in 1X PBS.

Microscopy

Fixed embryos processed for in situ hybridization, were mounted on Rain-X-coated (ITW Global Brands, Houston, TX) glass slides in 80% glycerol/20% 1X PBS. Coverslips were prepared as described in Lyons et al. (2015). Specimens were visualized on a Zeiss Axioplan microscope (Carl Zeiss Inc., Munich, Germany) and a Spot Flex camera (Spot Imaging Solutions, Sterling Heights, Michigan) was used for imaging. Multifocal stacks of brightfield images were combined and flattened using Helicon Focus stacking software (Helicon Soft Ltd., Kharkov, Ukraine).

Acknowledgments

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