"Dorsal–Ventral" Genes Are Part of an Ancient Axial Patterning System: Evidence from *Trichoplax adhaerens* (Placozoa)

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Abstract

Placozoa are a morphologically simplistic group of marine animals found globally in tropical and subtropical environments. They consist of two named species, *Trichoplax adhaerens* and more recently *Hoilungia hongkongensis*, both with roughly six morphologically distinct cell types. With a sequenced genome, a limited number of cell types, and a simple flattened morphology, *Trichoplax* is an ideal model organism from which to explore the biology of an animal with a cellular complexity analagous to that of the earliest animals. Using a new approach for identification of gene expression patterns, this research looks at the relationship of Chordin/TgfB signaling and the axial patterning system of Placozoa. Our results suggest that placozoans have an oral–aboral axis similar to cnidarians and that the parahoxozoan ancestor (common ancestor of Placozoa and Cnidaria) was likely radially symmetric.

Key words: **placozoan, oral–aboral, animal evolution, axial patterning, dorsal–ventral.**

Letter

Placozoa are an unusual group of marine animals found on biofilm surfaces around tropical and subtropical environments (Pearse and Voigt 2007; Eitel et al. 2013). Most phylogenetic analyses place them as the closest outgroup to the cnidarian–bilaterian ancestor (Srivastava et al. 2008; Hejnol et al. 2009; Philippe et al. 2009; Borowiec et al. 2015; Simion et al. 2017) (fig. 1*a*) and more recently as the sister taxa to cnidarians (Laumer et al. 2018). Their simple morphology have led some to speculate that the modern-day placozoan "body plan" is similar to, and descended directly from, that of the last common ancestor of all animals (Bütschli 1884; Dellaporta et al. 2006; Schierwater et al. 2009). They consist of a flagelated upper and lower epithelial layer (fig. 1*b*), originally proposed to be homologous to the dorsal and ventral axis of Bilateria (Grell 1971, 1972; Grell and Benwitz 1971). The top epithelial layer of the animal contains a unique autofluorescent cell type called shiny spheres (fig. 1*c–e*), used in predator defense (Jackson and Buss 2009). The lower epithelial layer contains lipophilic cells (fig. 1*b*) used during feeding to lyse algal cells (Smith et al. 2014, 2015). Placozoans feed on bacteria, yeast, algae, and other byproducts of biofilms (Grell and Benwitz 1971; Wenderoth 1986; Ueda et al. 1999; Smith et al. 2015; Smith and Reese 2016) and can phagocytose small latex particles from the lower epithelial layer (fig. 1*f*). Despite evidence of sexual reproduction in these animals (Grell 1972; Signorovitch et al. 2005; Eitel et al. 2011, 2013), asexual reproduction through binary fission (fig. 1*g*) or the production of swarmers (fig. 1*h*) is common in laboratory cultures (Thiemann and Ruthmann 1988, 1991). Gene expression analyses have raised more questions than they have supplied answers toward understanding the morphology of placozoans (Martinelli and Spring 2003, 2004; Jakob et al. 2004; Hadrys et al. 2005).

Herein, we developed an new RNA in situ hybridization protocol for *Trichoplax*, based on work from the cnidarian, *Nematostella vectensis* (Wolenski et al. 2013), to show gene expression patterns for key genes involved in dorsal/ventral patterning in bilaterians. These results provide new evidence toward understanding the relationship between the body axes of Placozoa and other animals. We anticipate that our new protocol will empower a more complete genetic understanding underpinning the biology of this fascinating animal.

Results

Placozoans are diploblastic animals and are thought to exhibit dorso-ventral polarity along the top and bottom tissue layers. To test whether this axis is driven by the same set of transcription factors that define dorsal–ventral patterning in bilaterians (Sasai et al. 1994; Larraín et al. 2000; Lowe et al. 2006; van der Zee et al. 2006; Lapraz et al. 2009), we characterized the TgfB signaling pathway and looked at the evolution of Chordin-related genes in *Trichoplax*.

Chordin is a primary antagonist of Bmp signaling and is composed of chordin (CHD) domains surrounded by cysteine-rich CR-domains. A *Chordin-like* gene was previously identified in the genome of *Trichoplax* (Srivastava et al. 2008; Richards and Degnan 2009), and does not possess Chordin domains (CHD) (supplementary fig. S1, Supplementary Material online). The relationship between bona fide chordin genes and nonbilaterian CR-domain containing genes remains unresolved (e.g., ctenophores [Pang et al. 2011], sponges [Srivastava et al. 2010], and *Hydra* [Rentzsch et al.

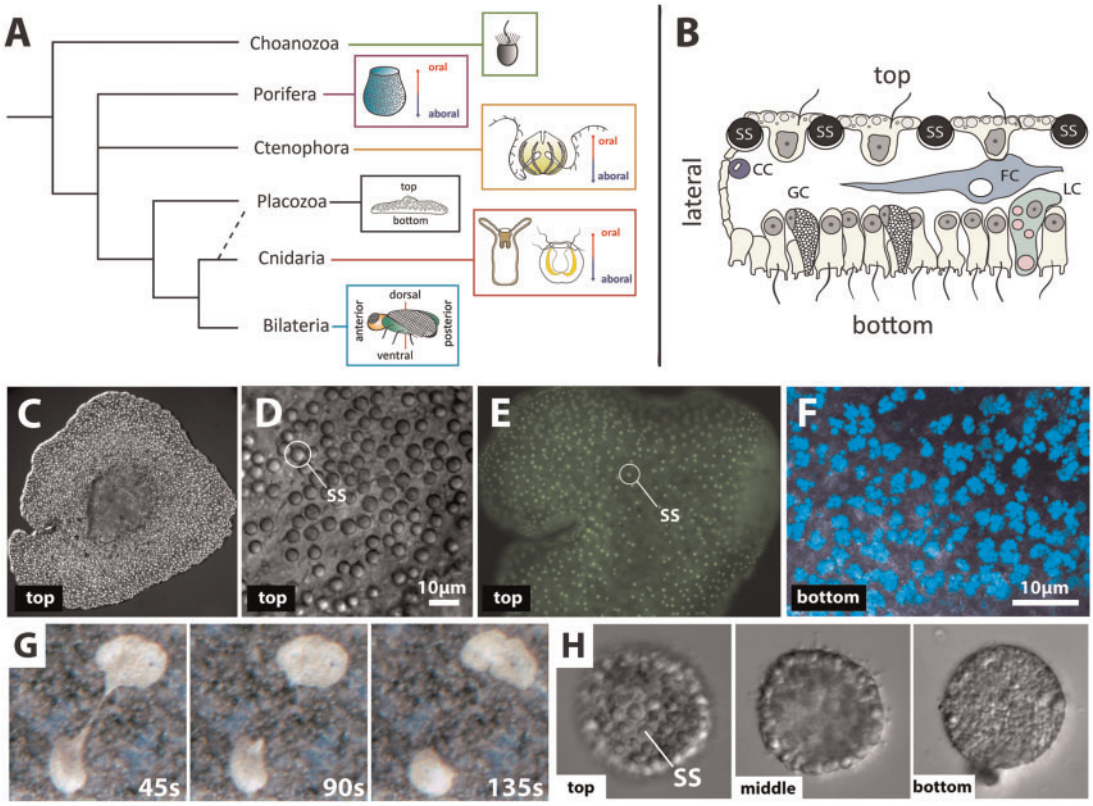

Fig. 1. Phylogenetic relationships and body axes of the five major animal lineages. (*a*) Phylogenetic relationship of animals consistent with the vast majority of animal phylogenomic studies (including Srivastava et al. 2008; Hejnol et al. 2009; Philippe et al. 2009; Borowiec et al. 2015; Simion et al. 2017). The dotted line indicates the most recent finding that placozoans are the sister group to cnidarians (Laumer et al. 2018). Most animals have an obvious oral–aboral axis, where bilaterians have a morphologically distinguishable dorsal–ventral and anterior–posterior axis. It has been speculated that the top–bottom axis of Placozoa is homologous to the dorsal–ventral axis of Bilateria. (*b*) Schematic diagram of the top, middle and bottom tissue layers of placozoans (Grell 1972). The upper layer consists of a thin ciliated epithelial layer, with interspersed shiny spherical cells (SS) thought to be specific to Placozoa. The middle layer has fiber cells (FC) and crystal cells (CC) of unknown functionality. The bottom layer is a thick ciliated epithelial layer interspersed with gland cells (GC) and lipophil cells (LC) used for digestive function. (*c*, *d*) The shiny spheres (SS) along the upper layer of the animal are easily visualized under transmitted light. (*e*) Animal exposed to FITC wavelength of light, showing the distribution of auto-fluorescent shiny spheres along the top tissue layer. (*f*) The lower epithelial layer is capable of phagocytosis of fluorescent latex beads (0.5 and 2 μm). (Beads false-colored blue and were absorbed 4 h after incubation). (*g*) Separation of a *Trichoplax* during asexual reproduction through binary fission can be visualized along the bottom surface of culture bowls (s, seconds). (*h*) Three cross-sectional views of an asexually reproduced ball of cells, "swarmer."

2007]). We identified a true *Chordin* gene in the sponge *Oscarella carmela* (Nichols et al. 2012), and *Chordin-like* genes (lacking CHD domains) among diverse animals (fig. 2). Both species of placozoans, *Trichoplax* and *Hoilungia*, have a *Chordin-like* gene without CHD domains (fig. 2).

We used synteny, or the comparison of gene location between the genomes of two species (e.g., *Trichoplax* and the cnidarian, *N. vectensis*), as additional evidence for orthology of genes from *Trichoplax*. This approach has been applied in many other studies to help resolve gene orthology (Putnam et al. 2007; Hui et al. 2008; Srivastava et al. 2008; DuBuc et al. 2012; Simakov et al. 2013). The *Trichoplax* and *Nematostella Chordin*-containing scaffolds share four other genes that are within close proximity of the *Chordin* gene (supplementary figs. S2 and S3, Supplementary Material online). An additional *Kielin/chordin-like* gene found in the *Trichoplax* genome (supplementary fig. S1, Supplementary Material online) does not share any synteny with scaffolds containing *Chordin* or *Bmp* genes from *Nematostella*. Together, this evidence suggests that Placozoans have a *Chordin-related* gene, and that this gene lacks a CHD domain but contains four CR repeats that are closely related to *Chordin* CR-domains.

Chordin is known to functionally interact with TgfB signaling to specificy the dorsal–ventral axis of diverse bilaterians. Phylogenetic analysis of the TgfB complement of *Trichoplax* is less clear. A number of conditions were tested (see Materials and Methods) yet no tree clearly resolved all *Trichoplax* Bmp ligands (supplementary fig. S4, Supplementary Material online). Our synteny analysis between the cnidarian *N. vectensis* and *Trichoplax* confirms the existence of linkage between orthologs of *Bmp2/4* (Ta57057), *Gdf5* (Ta9164), and *Bmp5/8* (Ta9129) (supplementary fig. S2, Supplementary Material online). We identified a *Trichoplax* gene related to *Bmp3* (Ta58663) that is also present in ctenophores (Pang et al. 2011) and may be a distant relative to both *Bmp3* and *Admp* (and potentially *Nodal*); however, we were unable to confirm the orthology of this gene with synteny.

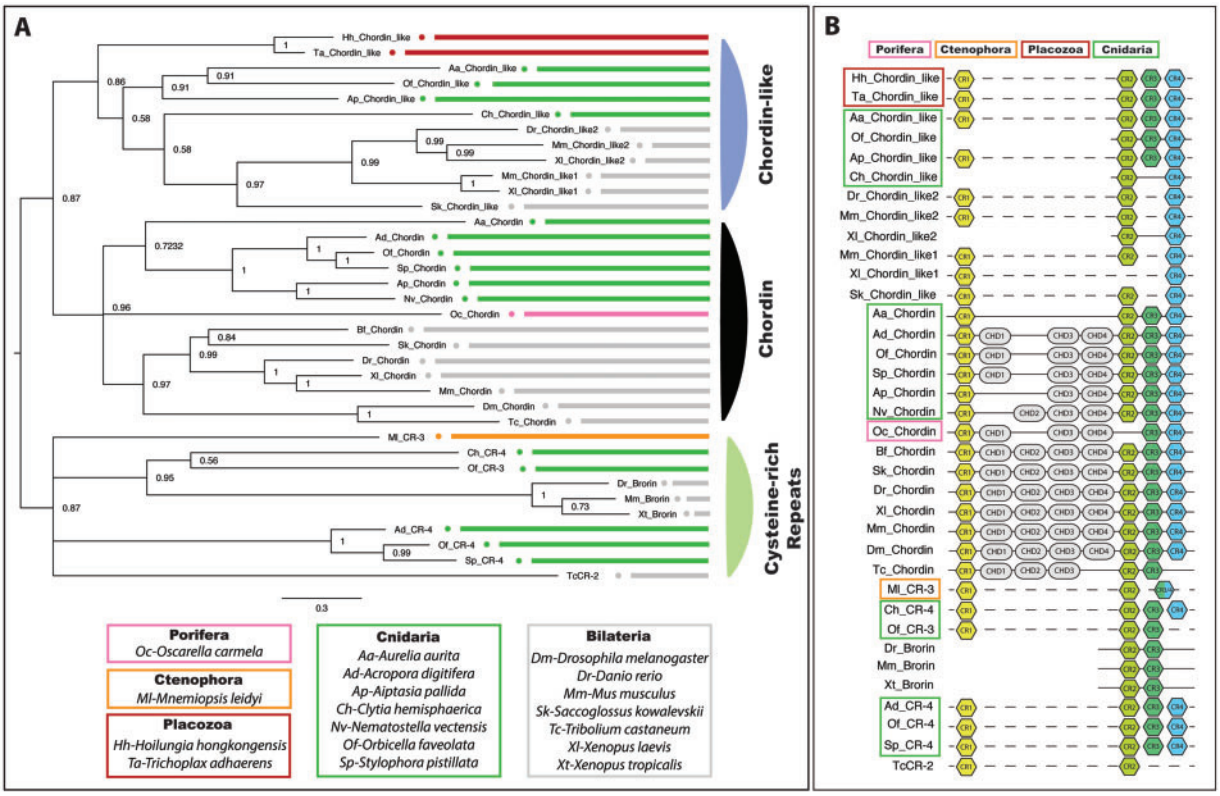

Fig. 2. Placozoans have a Chordin-like gene consisting of only CR domains. (*a*) Bayesian analysis of Chordin, Chordin-related and cysteine-rich (CR) proteins. Posterior probability support is shown at each node. (*b*) *Trichoplax* (Ta) and *Hoilungia* (Hh) have a Chordin-like gene comprised four cysteine rich (CR) domains. The sponge *Oscarella carmela* (Oc) has a *Chordin* gene consisting of both CR and Chordin domains (CHD). Protein domains were predicted using SMART (Schultz and Milpetz 1998; Letunic and Bork 2018) and aligned using MAFFT (Katoh et al. 2017). (Dotted lines between protein domains indicate gaps in the sequence).

Next, we analyzed a diverse set of eight different genes to better understand patterning in *Trichoplax*. *Beta-actin*, a house-keeping gene, is expressed throughout the *Trichoplax* body in both top and bottom tissue layers (fig. 3*a*). *Trichoplax* has a single ortholog of the zinc-finger *Snail* transcription factor (Dattoli et al. 2015), a marker for mesoderm in bilaterians (Essex et al. 1993; Carver et al. 2001) and endomesodermal cells in cnidarians (Fritzenwanker et al. 2004; Martindale et al. 2004; Magie et al. 2007). *Snail* is broadly expressed in the bottom tissue layer and exhibits a salt-and-pepper pattern along the top surface (fig. 3*b*). *Bmp2/4* is expressed primarily along the bottom tissue layer, with a few scattered cells around the lateral edge (fig. 3*c*). *Chordin-like*, a Bmp antagonist, is found along the bottom surface (fig. 3*d*) and double in situ hybridization confirms localization to an overlapping region as *Bmp2/4* (fig. 3*e*). *Bmp3* is expressed asymmetrically along one lateral edge of the animal (fig. 3*f*). *Gdf5* is expressed in a ring around the bottom layer (fig. 3*g*). A single *Elav* gene, a broad-neuronal marker in *Nematostella* (Marlow et al. 2009; Kelava et al. 2015), is found expressed throughout the lower epithelial layer (fig. 3*h*). The ParaHox gene *Gsx* (also called *Trox-2* [Jakob et al. 2004]) is broadly expressed throughout the lower epithelial layer (fig. 3*i*). Additional images of variability in gene expression patterns and our sense control can be found in the supplementary fig. S5, Supplementary Material online.

Discussion

Much has been learned about Placozoan biology through the application of electron microscopy techniques (Grell and Benwitz 1971, 1981; Rassat and Ruthmann 1979; Behrendt and Ruthmann 1986; Ruthmann et al. 1986; Wenderoth 1986; Thiemann and Ruthmann 1988, 1990; Buchholz and Ruthmann 1995; Guidi et al. 2011). On the other hand, to date much less has been gleaned through gene expression studies. The in situ protocol developed in this study is derived from cnidarian research (Wolenski et al. 2013) and two crucial changes helped allow for tissue preservation and increased specificity. We added extra sodium chloride to the seawater to balance the osmolarity when seawater was diluted with aldehyde. This step, along with placing individual animals in a 96-well plate, helped preserve tissue throughout the experiment. Additionally, our in situs were conducted at 64 °C, which greatly increased the specificity of the hybridization, where previous studies used 50–55 °C (Martinelli and Spring 2003, 2004; Jakob et al. 2004; Hadrys et al. 2005).

The body axis of *Trichoplax* and the new placozoan species, *Hoilungia hongkongensis* (Eitel et al. 2018), is overtly similar to the oral–aboral axis of cnidarians. The bottom layer expresses *Chordin-like*, *Bmp2/4*, *Gdf5*, *Snail*, and the Parahox gene *Trox-2* (fig. 3*j*), and orthologs of all of these genes are expressed orally or in the endomesoderm of *Nematostella* (Fritzenwanker et al. 2004; Matus, Pang, et al. 2006;

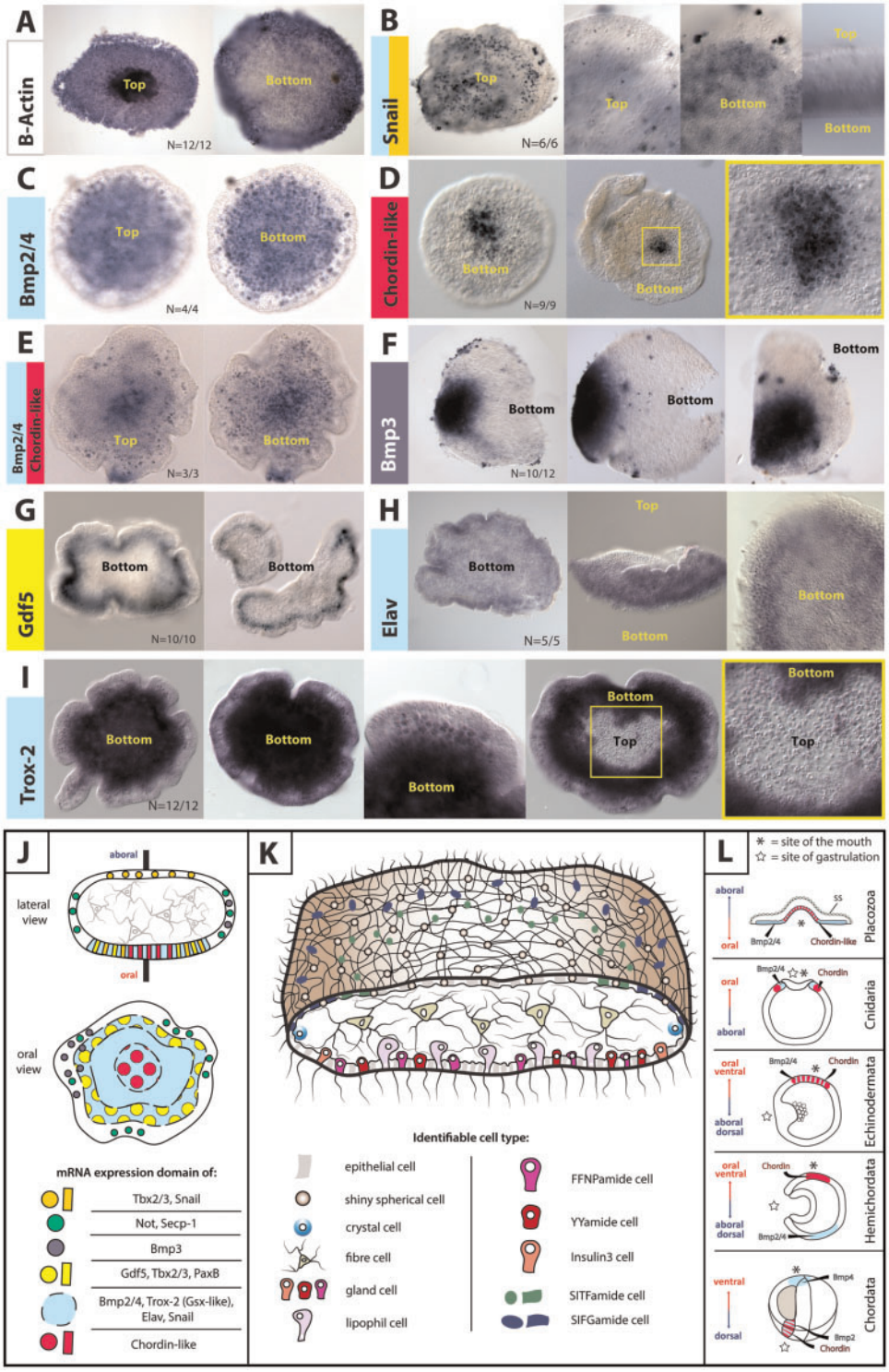

Fig. 3. Asymmetric expression of mRNA transcripts along the body of *Trichoplax*. (*a*–*i*) In situ hybridization of adult *Trichoplax*. (*a*) *Beta-actin* is expressed throughout the tissue layers and appears highly expressed or in a greater number of cells when the animal is contracted (top, left). (*b*) The zinc-finger transcription factor *Snail* is expressed in a punctate (salt-and-pepper) pattern along the upper/lower layers. (*c*) The TgfB ligand, *Bmp2/4* is expressed in the bottom tissue layer. (*d*) The Bmp antagonist, *Chordin-like* gene is expressed in a small subset of cells along the bottom layer. (*e*) Double in situ revealed *Chordin-like* and *Bmp2/4* are in overlapping domains along the bottom tissue layer. (*f*, *g*) Two additional TgfB ligands (*Bmp3* and *Gdf5*) are expressed (*f*) along one side of the animal (*Bmp3*) or (*g*) in a ring around the bottom layer (*Gdf5*). (*h*) The RNA-binding protein, *Elav* is found broadly along the lower layer. (*i*) The Parahox gene related to *Gsx*, *Trox-2* is highly expressed throughout the bottom layer. (*l*) Summary of regional patterning domains from this study (*Beta-actin*, *Bmp2/4*, *Bmp3*, *Chordin*, *Gdf5*, *Elav*, *Snail*, and *Trox-2* (*Gsx*)) and from Martinelli and Spring (2003, 2004) and Hadrys et al. (2005). (*j*) Diverse cell types can be found along top and bottom layers of *Trichoplax* (Smith et al. 2014), with different peptides distributed throughout the bottom layer and along top lateral edges (Varoqueaux et al. 2018). The bottom layer containing many lipophil cells is likely used for digestion (Smith et al. 2014), where wildtype populations maintain shiny

Rentzsch et al. 2006; Magie et al. 2007; Ryan et al. 2007; Saina et al. 2009; Kraus et al. 2016). None of the genes addressed in this study was differentially expressed in a single-cell RNAseq data set from placozoans (Sebé-Pedrós et al. 2018), suggesting that their role is for broad spatial patterning, rather than specifying particular cell types. There is an abundance of evidence that placozoans feed from the lower tissue layer (Grell and Benwitz 1971; Wenderoth 1986; Ueda et al. 1999; Smith et al. 2015; Smith and Reese 2016). This layer has a diversity of peptidergic gland cells (Varoqueaux et al. 2018) (fig. 3*k*) that appear to overlap with the distinct rings of expression within this and other studies (fig. 3*j*). The conserved gene expression domains and evidence for digestion suggest that the bottom layer may be homologous to a primitive mouth and/or endomesodermal digestive surface.

In *Nematostella*, the earliest expression domain of both *Chordin* and *Bmp2/4* is radial along the site of gastrulation and becomes asymmetric during gastrulation (Finnerty et al. 2004; Matus, Thomsen, et al. 2006; Rentzsch et al. 2006; Röttinger et al. 2012) (fig. 3*l*). Although we cannot compare the embryonic pattern of these genes in *Trichoplax*, their patterns in diverse animals (fig. 3*l*) hint that *Chordin* and *Bmp2/4* (*Bmp2* in chordates) may have initially evolved together (rather than on opposite poles) to produce an axial signaling center. The presence of a CHD domain containing Chordin protein in sponges suggests an ancient origin, although these domains are not well conserved throughout evolution.

Our findings, although suggestive, require greater analysis and further study to truly understand how these and other developmental genes shape the body of *Trichoplax*. For example, the fiber layer of these animals has been overlooked in all studies to date and may require better sampling techniques (e.g., histological sectioning) to completely identify transcripts localized to this region. This research has provided a crucial protocol for advancing our knowledge of these enigmatic understudied animals. With new cell-type transcriptome data, these results provide a foundation for understanding the unrealized complexity of placozoans.

Materials and Methods

Collection/Culture of *Trichoplax*

All animals were collected from an outdoor flow-through sea water system at the Kewalo Marine Laboratory (Honolulu, HI). Phagocytosis of latex beads (Cat.# L3280 & L4530, Sigma Aldrich) was conducted by suspending a dilution of 1:100 of stock in seawater, then two droplets from both bead samples were added to each dish and left to settle on the bottom overnight. Animals were then added to each dish and allowed to come in contact with the beads along the surface of the dish for 4 h.

In situ hybridization experiments were visualized using an Axioscope 2 compound microscope with an AxioCam (HRc) camera. Autofluorescence of shiny sphericals could be seen using an FITC filter cube and was visualized using 488 wavelength settings. For fluorescent bead experiments, live animals were placed on glass slides and visualized using a Zeiss 710 scanning laser confocal. Images were cropped and assembled using Adobe Photoshop and Illustrator CS6.

Phylogenetic and Synteny Analysis

Protein sequences from a diverse set of taxa were collected using the NCBI protein databases (http://www.ncbi.nlm.nih.gov; last accessed February 24, 2019). A list of the different species used in our phylogenetic analyses can be found in supplementary figure S2, Supplementary Material online. Protein-coding domains were predicted using SMART (Schultz et al. 1998), and all sequences were aligned using MUSCLE (Edgar 2004). Trees were constructed using MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using five independent runs, consisting of 5,000,000 generations using "mixed" models. A second tree was constructed using maximum-likelihood analysis using RaxML (version 7.2.8) as described (Ryan et al. 2013). Maximum-likelihood tree bootstraps were based on 100 replicates. Four different alignments were run using each analysis. They included conditions listed below each tree. Trees were imported and edited using FigTree (version 1.4.0) (Rambaut 2007).

Whole Mount In Situ Hybridization

Animals were transferred from glass slides to gelatin-coated dishes using a glass Pasteur pipette (Cat.#CLS7095D5X, Sigma-Aldrich) and allowed to settle on the bottom of the dish overnight. Fixation was achieved by gently adding ice-cold fix (4% PFA, 0.2% glutaraldehyde in high-salt seawater; 0.5 g NaCl/50 ml seawater) to the dish for 90 s. Following this, the solution was gently removed and ice-cold 4% PFA (in high-salt seawater) was added and dishes placed at 4 °C for 1 h. Fix was removed and animals were washed three times in ice-cold DEPC-treated H_2O, then dehydrated to 100% methanol over several steps (25% methanol:DEPC-H_2O for 5 min, 50% methanol:DEPC-H_2O for 5 min, and 75% methanol:DEPC-H_2O for 5 min). Animals were washed in 100% methanol for 1 h at 4 °C and then used immediately for in situ hybridization.

Animals were transferred to a 96-well plate (one animal per well) and rehydrated into 1× phosphate buffered saline

Fig. 3 Continued

sphericals (proposed defensive cells) in the top layer. Shiny sphericals are thought to be dispensable in lab-maintained animals (Smith et al. 2014). (*k*) *Chordin* is expressed along the oral side of many invertebrates during embryonic development. Invertebrates have a single homolog of *Bmp2/4*, that is expressed in close proximity to *Chordin* (Duboc et al. 2004; Rentzsch et al. 2006; Lapraz et al. 2009), except in hemichordates where they are expressed on opposite sides during embryonic development (Lowe et al. 2006; Röttinger and Martindale 2011; Rottinger et al. 2015). Chordates have two TgfB ligands, *Bmp2* and *Bmp4* that are expressed on opposite sides during early development. *Bmp2* is coexpressed with *Chordin* in the Spemann–Mangold organizer, and *Bmp4* is expressed along the future mouth (De Robertis 2009). Images in (*l*) are orientated to have the oral side up except in *Trichoplax* (SS, shiny spherical cells).

(PBS) pH7.4 + 0.1% Tween-20 (PTw) using a series of washes (150 μl per wash; 75% methanol:25% PTw, 50% methanol:50% PTw, 25% methanol:75% PTw), before washing five times in PTw. Proteinase K was added (0.01 mg/ml) for 5 min before digestion was stopped by two, 5-min washes in PTw + 2 mg/ml glycine. The remaining steps for in situ hybridization are the same as established protocols for *N. vectensis* (Wolenski et al. 2013). Probes were hybridized at 64 °C. For our double in situ experiment (fig. 3*e*), two DIG-labeled probes were hybridized and developed at the same time because attempts to extend the number of washes in a traditional two-probe protocol resulted in tissue degradation. The reaction was stopped by washing in PBS or PTw for 3–5 times, and animals mounted in 70% glycerol:PBS for analysis.

Data Availability

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Material online, or from the corresponding author upon reasonable request.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

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