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# An organizing role for the TGF-β signaling pathway in axes formation of the annelid *Capitella teleta*

Alexis R. Lanza, Elaine C. Seaver*

*Whitney Laboratory for Marine Bioscience, University of Florida, 9505 Ocean Shore Blvd., St. Augustine, Fl 32080, USA*

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ABSTRACT

Embryonic organizers are signaling centers that coordinate developmental events within an embryo. Localized to either an individual cell or group of cells, embryonic organizing activity induces the specification of other cells in the embryo and can influence formation of body axes. In the spiralian *Capitella teleta*, previous cell deletion studies have shown that organizing activity is localized to a single cell, 2d, and this cell induces the formation of the dorsal-ventral axis and bilateral symmetry. In this study, we attempt to identify the signaling pathway responsible for the organizing activity of 2d. Embryos at stages when organizing activity is occurring were exposed to various small molecule inhibitors that selectively inhibited either the Activin/Nodal or the BMP branch of the TGF-β signaling pathway. Embryos were then raised to larval stages, and scored for axial anomalies analogous to 2d ablated phenotypes. Our results show that interference with the Activin/Nodal pathway through a short three hour exposure to the inhibitor SB431542 results in larvae that lack bilateral symmetry and a detectable dorsal-ventral axis. However, interference with the BMP signaling pathway through exposure to the inhibitors DMH1 and dorsomorphin dihydrochloride does not appear to play a role in specification by 2d of the dorsal-ventral axis or bilateral symmetry. Our findings highlight species differences in how the molecular architecture of the conserved TGF-β superfamily signaling pathway components was utilized to mediate the organizing activity signal during early spiralian development.

## 1. Introduction

Formation of animal body axes (anterior-posterior, dorsal-ventral, left-right) is crucial in the development of an embryo. For many species, this is orchestrated by a central signaling center known as the organizer, which choreographs developmental events essential for body patterning. Both the number of cells and the timing of action that constitute the organizer differ across species. For example, in chordates such as *Xenopus laevis* (Gerhart et al., 1991) and *Danio rerio* (Kimmel et al., 1990; Shih and Fraser, 1996), the cephalochordate *Branchiostoma floridae* (Onai et al., 2010) as well as in the cnidarian *Nematostella vectensis* (Kraus et al., 2016), organizing activity is localized to a group of cells at the blastopore lip during gastrulation. However, in mollusks such as *Ilyanassa obsoleta* (Clement, 1976, 1962), and *Lymnaea stagnalis* (Martindale, 1986), a single cell in the early cleavage stage embryo has organizing activity. Furthermore, the number of axes induced by an organizer differs across species; whereas in some bilaterians all three axes are specified by the organizer, in others it is only dorsal-ventral and left-right axes (Goldstein and Freeman, 1997).

In spiralians, a group of animals that include mollusks, annelids, nemerteans, etc., the organizer is generally localized to a single cell in the early cleavage stage embryo. Spiralian embryonic development follows a highly conserved, stereotypic cleavage program named spiral cleavage. This conservation in cleavage pattern across taxa allows for the identification of individual cells and for intertaxonomic comparisons, which is facilitated by a standard nomenclature. In some spiralian embryos, the first two divisions produce cells of unequal sizes that result in the formation of four blastomeres called A, B, C, and D, the largest of which is the D macromere. Together, descendants of these four blastomeres define the four quadrants of the embryo. During the third cleavage division, each cell gives rise to one daughter cell at the vegetal pole, the macromeres 1A, 1B, 1C, and 1D, as well as typically smaller daughter cells towards the animal pole, called micromeres 1a, 1b, 1c, and 1d. The birth of these micromeres, known as the 1st quartet micromeres, occurs in a clockwise direction with respect to the position of the macromere when viewed from the animal pole. The formation of each subsequent quartet of micromeres occurs in cycles of counter-clockwise and clockwise directions. During the fourth cleavage division from the eight to the sixteen-cell stage, both the macromeres

* Corresponding author.
*E-mail address:* seaver@whitney.ufl.edu (E.C. Seaver).

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and the 1st quartet micromeres divide. Macromeres 1A, 1B, 1C and 1D each divide to form macromeres 2A, 2B, 2C, and 2D respectively, and the 2nd quartet micromeres 2a, 2b, 2c, and 2d, respectively. Meanwhile, the micromeres of the 1st quartet, 1a, 1b, 1c, and 1d each divide to generate two daughter micromeres – $1a^1$ and $1a^2$, $1b^1$ and $1b^2$, $1c^1$ and $1c^2$, $1d^1$ and $1d^2$. Cells with a '1' superscript are positioned towards the animal pole of the embryo with respect to their sister cell (designated by a superscript '2'). Subsequent cleavages of the macromeres occur following a similar spiral cleavage pattern through to the onset of gastrulation.

Aside from a shared cleavage program, spiralians also possess a conserved fate map in which structures are generally derived from homologous cells (Ackermann et al., 2005; Hejnol, 2010; Meyer et al., 2010). For example, larval eyes in many species are derived from the 1st quartet cells 1a and 1c, trunk mesoderm from cell 4d, and a majority of the trunk ectoderm is derived from cell 2d. Furthermore, it has also been demonstrated that the D quadrant possesses the ability to establish dorsal-ventral polarity in embryos (Dorresteijn et al., 1987; Hejnol, 2010; Henry and Martindale, 1987; Render, 1989, 1983).

It is the spiralian D quadrant that generally gives rise to a single cell organizer that functions in patterning the embryo during early cleavage stages. Despite conservation in quadrant identity, the precise timing of the organizing signal and its cellular identity varies among species. In mollusks such as the mudsnail *Ilyanassa obsoleta* and the limpet *Patella vulgata,* the organizing signal is required through the 32 cell stage and is localized to cell 3D (Clement, 1962; Damen and Dictus, 1996; Lambert and Nagy, 2003). In the slipper shell snail *Crepidula fornicata,* the organizing activity is required one cleavage division later at the 64 cell stage as cell 4d (Henry and Perry, 2008; Henry et al., 2006).

Another spiralian in which organizer activity is localized to the D quadrant is in the annelid, *Capitella teleta* (Amiel et al., 2013). In *C.* teleta, organizing activity patterns the dorsal-ventral axis and establishes bilateral symmetry. Amiel et al. demonstrated that cell 2d, which is present in the 16 cell stage embryo a day prior to gastrulation, is required for organizing activity. Descendants of cell 2d give rise to the ectoderm of the larval trunk and pygidium (Meyer and Seaver, 2010). However, laser deletion of cell 2d not only results in the loss of 2d and its descendants, but also leads to disorganization of the head (Amiel et al., 2013). The structures of the head arise from first quartet micromeres (Meyer et al., 2010; Meyer and Seaver, 2010), suggesting that cell 2d is capable of influencing the fate of its neighbors and is ultimately essential for bilateral symmetry and dorsal-ventral axis formation. Such an effect on the developmental fate of surrounding cells is not seen following the deletion of both daughter cells of 2d, $2d^1$ + $2d^2$ at the 32 cell stage (Amiel et al., 2013). This further indicates that the requirement for organizing activity ceases after the 16-cell stage.

Initial investigations into the nature of the molecular signals mediated by the organizer cell suggest that D quadrant specification and axes formation has been linked to activation of the ERK/MAPK signaling pathway in mollusks. For example, in mollusks such as *Ilyanassa fornicata* (Lambert and Nagy, 2001), *Crepidula fornicata* (Henry and Perry, 2008), *Tectura scutum* (Lambert and Nagy, 2003), and *Haliotis asinina* (Koop et al., 2007), activation of MAPK is observed in D quadrant blastomeres, and inhibition of ERK phosphorylation in these embryos leads to loss of axial organization in larvae. In contrast, in the annelids *Platynereis dumerilii* (Pfeifer et al., 2014) and *C. teleta* (Amiel et al., 2013), it appears that a distinct molecular mechanism is used. When either *P. dumerilii* or *C. teleta* embryos are exposed to U0126, a MEK-1 and MEK-2 inhibitor, the resulting larvae have normal body axes organization (Amiel et al., 2013; Pfeifer et al., 2014). Therefore, there appears to be variation in the molecular mechanisms utilized by spiralians to mediate the organizer signal.

TGF-β superfamily signaling regulates a variety of developmental processes, and has a conserved role in patterning the dorsal-ventral body axis (Wu and Hill, 2009). There are two distinct branches within the TGF-β superfamily; the branch activated by ligands such as TGF-β, Nodal, and Activin will here on be referred to as the Activin/Nodal pathway and the branch activated by ligands such as BMP5-8, BMP2/4, and ADMP will here on be referred to as the BMP pathway. Furthermore, both possess branch-specific signal transduction components. Dimerized ligands in both the Activin/Nodal and BMP branches mediate signaling by binding to a type II transmembrane serine/threonine kinase receptor. Upon ligand binding, a heterotetramer forms with a type I serine/threonine kinase receptor, which is subsequently activated via a transphosphorylation event. This results in the intracellular activation of downstream Smads via phosphorylation, and complex formation with the mediator Smad4. Following translocation into the nucleus, the complex regulates gene expression, in some cases by binding to DNA directly. Regulation of the TGF-β superfamily is also facilitated by secreted agonists and antagonists, such as chordin and gremlin, and by membrane-associated co-receptors (Weiss and Attisano, 2013). In *C. teleta,* all components necessary for signaling via both branches of the TGF-β superfamily are present within the genome, as was recently reported by Kenny et al. (2014).

In this study, we investigate the identity of the molecular signal sent by 2d that induces the formation of the dorsal-ventral axis and bilateral symmetry in *C. teleta*. The Activin/Nodal and BMP signaling pathways are investigated using small molecule chemical inhibitors that target the type I receptors in each of the two branches of this signaling pathway. Embryos at stages during which organizing activity occurs were exposed to either an Activin/Nodal (SB-431542) or BMP (dorsomorphin dihydrochloride; DMH1) pathway inhibitor, raised to larval stages, and scored for axial anomalies using morphological and molecular markers. Further analyses of larval phenotypes were also conducted using lineage tracing.

## 2. Materials and methods

### 2.1. Animal care

A laboratory colony of *C. teleta* adults were kept and maintained as previously described (Seaver et al., 2005). Broods of early stage embryos were obtained by mating gravid females with sexually mature males as previously described (Yamaguchi et al., 2016).

### 2.2. Drug treatments

Each brood of early stage embryos was divided into experimental and control conditions. Embryos were exposed to a DMSO solvent control or drug inhibitor for approximately 3 h during two early-cleavage-stage time windows: the 4–32 cell stage and the 32–256 cell stage. For inhibition of the Activin/Nodal signaling pathway, SB431542 (Santa Cruz; Cat No: SC-204265A), a chemical inhibitor that prevents the phosphorylation of ALK4, ALK5, ALK7 Activin/Nodal type 1 receptors, was used. For BMP inhibition, two different chemical inhibitors were used: DMH1 (Sigma; Cat No: D8946), an inhibitor of ALK2 a BMP type 1 receptor, or dorsomorphin dihydrochloride (Tocris; Cat No: 3093), an inhibitor of both ALK2 and ALK3 BMP type 1 receptors. SB431542, DMH1, and dorsomorphin dihydrochloride were each diluted in 100% Dimethyl sulfoxide (DMSO) to a 10 mM stock. Working concentrations were diluted in filtered seawater (FSW). Initial experiments included exposure to the following range of drug concentrations: 1–100 μM for SB43154; 10–40 μM for DMH1; 5–10 μM for dorsomorphin dihydrochloride. The lowest concentration at which there was a consistently reproducible larval phenotype was selected for detailed analysis. To monitor for any potential toxic effects of the solvent, control embryos were exposed to DMSO in FSW at a concentration equivalent to the experimental condition (0.01–1% DMSO). Experimental drug concentrations used were SB431542 at 40 μM, DMH1 at 40 μM, and dorsomorphin dihydrochloride at 5 μM. Following exposure, embryos were washed with FSW for 4 × 1 min,

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