Functional study of the Wnt signaling network that coordinates early anterior-posterior and dorsal-ventral axis specification and patterning in the sea urchin embryo

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 2, 2020

Keywords: Wnt signaling, sea urchin, anterior-posterior, dorsal-ventral, Frizzled,
Gene regulatory networks

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Acknowledgments

First, I would like to thank my advisor, Ryan Range, for his guidance and many helpful discussions throughout my PhD. Thank you for giving me the opportunity to discover the fascinating world of developmental biology and for pushing me to be more ambitious. You have made me the scientist I am today and for that, I will always be grateful. I also want to extend my appreciation to my dissertation committee members Brian Counterman, Matthew Brown, Ken Halanych, Aaron Rashotte, and Jennifer Panizzi for all the help provided and great feedback. I am grateful to all past and present members of the Range lab for making the past years unforgettable and transformative. Thanks Anita Khadka for all your help at the beginning of my PhD journey. I would like to thank Sujan Gautam and Che Ka for being such amazing lab mates and made my time in the lab more enjoyable. I will also never forget all the talented undergrads who worked in the Range lab all these years, all of you have a bright future ahead.

I thank the embryology class of 2019. I was specially delighted to meet Llilians, Feña, Ariel, Allan and David who made the time at the MBL so exciting and fun. I would also like to thank Paul for always inspiring me, I hope we continue having fascinating conversations for many more years. I am also lucky to have been welcomed into the sea urchin community and the society of developmental biology. In addition, the Department of Biological Sciences has always provided me immense support.

Finally, I would like to thank all my friends Fernando, Margarita, Luis, Chathu, Adrián, Savera, Prateek, Anay, Aamir, Prabhat, Gerardo, Sara, and Victoria: thank you for the countless memories in the US. I want to especially thank Sanchit Salunkhe for always being my greatest support and for helping me to evolve into a better version of myself. I am also lucky to have Andrea, Joaquina, Paula and Laura in my life, who despite the kilometers they always made me feel so close to them. And lastly, the most important part of my life, my family. Thanks to my mom Piedad Bartolomé, my dad Antonio Martínez and my wonderful sister Lucía Brihuega who have always believed in me. I dedicate this work to them, to my grandfather Bernardo Bartolomé and to my furry family Lucas and Titty.

Abstract

The establishment and patterning of the different embryonic body axes are crucial events in early development of any embryo. Anterior-posterior (AP) and dorsal-ventral (DV) axis specification and patterning are fundamental developmental processes critical for establishing the correct adult body plan in metazoans. In the deuterostome sea urchin embryo AP specification and patterning depends on integrated cross-regulatory information from the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC pathways, forming an interconnected Wnt signaling network. We have previously shown that a non-canonical signaling pathway involving the Fzl1/2/7 receptor antagonizes the progressive posterior-to-anterior downregulation of the anterior neuroectoderm (ANE) gene regulatory network (GRN) by Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling. First, we studied the function of Wnt16 ligand in activating the Fzl1/2/7 signaling during early AP patterning. Our data show that Wnt16 ligand has an early function in activating the Fzl1/2/7 pathway that antagonizes the ANE restriction mechanism and a subsequent role in activating key regulatory endoderm GRN factors and the morphogenetic movements of gastrulation. In addition, our results show that zygotic expression of *wnt16* depends on both Fzl5/8 and Wnt/β-catenin signaling during gastrulation. During early cleavage and blastula stages, the developmental mechanism involved in early AP specification and patterning by the Wnt signaling network appear to be independent of DV patterning. However, a combination of inputs from the Wnt, Nodal and BMP signaling pathways might work together in order to coordinate the proper distribution of the main germ layers along the AP and DV axis in early sea urchin development. Interestingly, our RNA-seq screen data identified several dorsal genes downregulated in Wnt signaling knockdowns at three different developmental stages. Therefore, we used morpholino perturbation approaches to study the function of the Wnt signaling network during AP and DV specification and patterning of the sea urchin embryo. Our data suggest that a non-canonical signaling is required for dorsal specification. In addition, the expression of a ciliary band maker was also affected when we perturb Wnt signaling function. Together, our results show that there is cooperative regulation from both Wnt and BMP2/4 signaling pathways during AP and DV patterning. This study establishes an important connection between the AP and DV gene regulatory networks (GRNs) and provides an important starting point for future comparisons in other deuterostome embryos. In addition, we characterize the distinct roles of the

signaling molecule Siah, the potential transcriptional effector downstream of Wnt16-Fzl1/2/7 signaling NFAT and a dorsal transcriptional factor during early AP and DV axis specification and patterning of the sea urchin embryo. Our spatiotemporal expression experiments of *siah* as well as the Fzl1/2/7 loss-of-function results suggest that the signaling molecule Siah is activated by non-canonical Wnt16-Fzl1/2/7 signaling pathway during anterior-posterior (AP) specification and patterning in the sea urchin embryo. Next, we show that the initial input for the early expression of a specific transcription factor in the dorsal ectoderm is likely to be dependent of the non-canonical Wnt16-Fzl1/2/7 signaling pathway. Our knockdown experiments also indicate that this dorsal gene plays a role in antagonizing the ANE restriction mechanism, in addition to its well-known function in establishing the early specification of the DV GRN. Lastly, we analyze the function of the nuclear factor of activated T-cells, NFAT, as a putative transcriptional effector downstream of non-canonical Wnt16-Fzl1/2/7 signaling pathway. Our functional experiments suggest that NFAT might be required for the antagonism of the ANE positioning mechanism mediated by Wnt1/Wnt8-Fzl5/8-JNK signaling during early body axis specification and patterning of the sea urchin embryo. Taken together, this study provides a better understanding of how coordinated information from different signaling pathways is necessary for early specification and patterning along the AP and DV axes in the sea urchin embryos and provides insights for future comparisons of the shared and novel mechanisms during these crucial events in the early development of metazoan embryos.

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List of Abbreviations

ANE	anterior neuroectoderm
AP	anterior-posterior
APC	adenomatous polyposis coli
ATF2	activating transcription factor 2
BMP	bone morphogenetic protein
CaMKII	calcium calmodulin-dependent protein kinase II
cDNA	complementary DNA
CREB	cAMP response element binding protein
DAG	1,2 diacylglycerol
DIX	dishevelled homologous
Dkk	dickkopf
DNA	deoxyribonucleic acid
DV	dorsal-ventral
Dvl/Dsh	dishevelled
FoxQ	forkhead box Q
Frzb	frizzled related protein
Fzl	frizzled
GRN	gene regulatory network
GSK	glycogen synthase kinase
Hpf	hours post fertilization
IP3	inositol 1,4,5-triphosphate
JNK	c-Jun N-terminal kinase

LEF	lymphoid enhancing factor
LRP	lipoprotein receptor-related
MO	morpholino
mRNA	messenger ribonucleic acid
NFAT	nuclear factor associated with T cells
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSM	non-skeletogenic mesoderm
PCR	polymerase chain reaction
PKC	protein kinase C
qPCR	quantitative polymerase chain reaction
Ror2	receptor tyrosine kinase-like orphan receptor 2
RTK	receptor tyrosine kinase
sFRP	secreted frizzled-related protein
Siah	seven In Absentia Homolog
Six	Sine oculis homeobox
Smad	similar to mothers against decapentaplegic
Tbx	T-box gene
TCF	T-cell factor
TGF-β	transforming growth factor beta
VV	vegetal view
Wnt	Wingless-related integration site

Chapter 1.

Introduction

Wnt signaling pathways

Wnt signaling pathways perform complex and multi-functional roles in the development of metazoan embryos. Numerous ligands, receptors, and co-receptors interact together to activate the three well-characterized Wnt signaling pathways (Wnt/β-catenin, the Wnt/JNK, and the Wnt/PKC pathways) (Fig. 1) (Kestler and Kunhl, 2008; van Amerongen and Nusse, 2009). In the "canonical" Wnt/β-catenin, Wnt secreted glycolipoproteins ligands presented on the surface of signaling cells act on target cells by binding to a Frizzled (Fzl) seven-pass transmembrane receptor and a type of low-density lipoprotein receptor-related protein (LRP), the co-receptor LRP5/6. This Frizzled/LRP receptor complex at the cell surface transduce a signal to several intracellular proteins that include the Disheveled (Dsh) protein, preventing β-catenin degradation. Interestingly, the phosphorylation of cytoplasmic tail of LRP which contains several Pro-ProPro-(SerTrp)Pro [PPP(S/T)P] motifs is an important step for Wnt/β-catenin signaling (Tamai et al., 2004). In addition, Fzls contain seven-transmembrane regions that might be reconfigured by Wnt binding. Next, stabilized β-catenin enters to the nucleus, interacts with the β-catenin/T cell transcription factor (TCF) and activates downstream transcription factors of the Wnt/β-catenin signaling pathway (Macdonald et al., 2007). During this intracellular response, there is an inactivation of a "destruction complex" that contains adenomatosis polyposis coli (APC), Axin, glycogen synthase kinase 3 (GSK3), and Casein kinase I (CKI) that typically sequesters and phosphorylates synthesized β-catenin (van Amerongen and Nusse, 2009). The element of the destruction complex Axin, as well as Dsh, contain a stretch of amino acids called the DIX domain. Since DIX domains of Axin can homodimerize, it is feasible that Wnt binding of Fzl receptor and LRP co-receptor promotes direct interaction between Axin and Dsh through their DIX domains, changing the protein complex that regulates β-catenin levels in the cell (Logan and Nusse, 2004). In the absence of Wnt signaling, the destruction complex targets β-catenin for degradation by the proteasome. Cytoplasmic β-catenin is frequently conserved at low levels through continuous proteasome-mediated degradation controlled by the destruction complex (Logan and Nusse, 2004). Wnt/β-catenin signaling is active when cells received Wnt signals and then the degradation complex is inhibited, allowing the inhibition of β-catenin degradation. Then, β-catenin protein accumulates in the cytoplasm and nucleus and subsequently

interacts with transcription factors such as T cell specific transcription factor /lymphoid enhancer-binding factor 1 (TCF/LEF) to control transcription (Logan and Nusse, 2004).

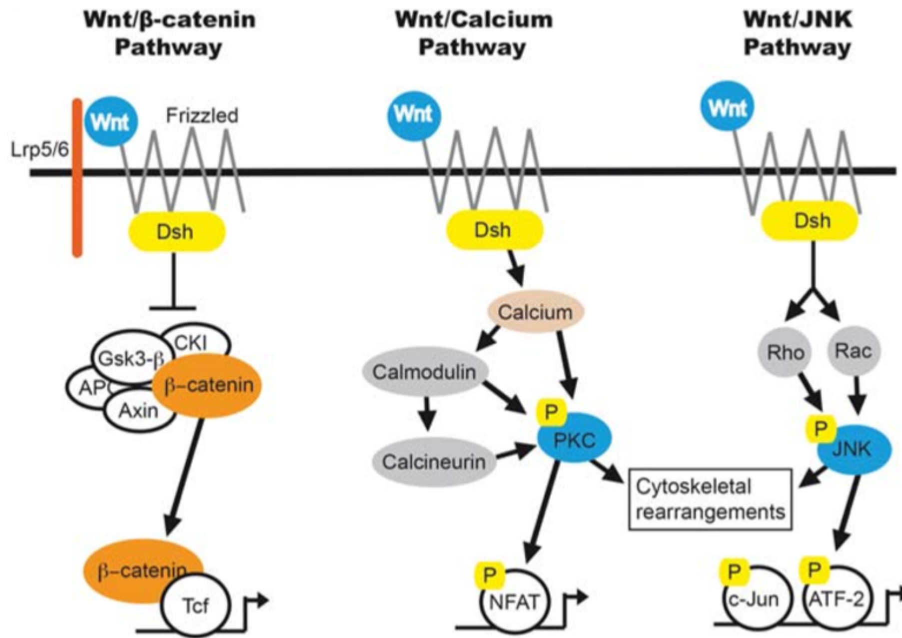

Figure 1. The three Wnt signaling pathways. A schematic representation showing Wnt/β-catenin, Wnt/Calcium, and Wnt/JNK pathways involved in ANE positioning, their extracellular and intracellular players, and the interactions between them (Range 2014).

Wnt ligands are also involved in the activation of other intracellular messengers, including JNK and Src kinases as well as calcium fluxes. The two "non-canonical" or "alternative" pathways, Wnt/JNK and Wnt/Ca2+, implicated in anterior-posterior (AP) specification and patterning also employ Wnt ligands, Fzl receptors, and Dsh proteins; however, they use different intracellular messengers, transcriptional effectors, and downstream transcription factors. For instance, the planar cell polarity (PCP) or Wnt/JNK pathway, which was originally identified in *Drosophila*, uses Wnt ligands, Fzl receptors and Dvl to activate small G proteins, including GTPases Rho and Rac, that turn on c-Jun N-terminal kinases (JNK) and Rho-associated kinase (Rho-kinase) (Kikuchi et al., 2009). Binding of Wnts to receptor tyrosine kinase (RTK) Ror2 can inhibit β-catenin/TCF signaling and activate JNK. Activated JNK can then lead to cytoskeletal rearrangement which further activates downstream gene transcription of

the factors c-Jun and activating transcription factor, ATF2 (Semenov et al., 2007). During regulation of convergent extension movements of gastrulation, a Wnt ligand binds to the receptor tyrosine kinase like orphan receptor 2, Ror2, and activates the JNK signaling which then interacts with the transcription factors ATF2 and c-Jun or filamin A, allowing the proper movements required for cell migration. In addition, several factors such as *Strabismus* and *Flamingo* are involved in this PCP signaling pathway. The Wnt/JNK signaling pathway is usually involved in regulation of the cytoskeleton to coordinate cell migration and polarity.

The activation of another non-canonical Wnt signaling, the Wnt/Calcium signaling, is possible thanks to a combination of Wnt ligands and Frizzled receptors that can induce Ca^{2+} fluxes. Although Wnt/Calcium signaling (Wnt/Ca^{2+}/PKC) has known to regulate cell movements and behavior as well as to induce structural changes (Kuhl et al., 2001; Torres et al., 1996; Okamoto et al., 2007; Witze et al., 2008), this non-canonical signaling pathway has been less described than their canonical counterpart. Thus, further studies characterizing the main players of non-canonical Wnt/Ca^{2+}/signaling are required to gain a better understanding of its potential role as a mediator in many developmental processes and tumorigenesis. The Wnt/Ca^{2+} signaling increases the intracellular calcium (Ca^{2+}) concentrations and activate a variety of intracellular Ca^{2+} factors such as calcium/calmodulin-dependent protein kinase (CaMK) II, calcineurin, and protein kinase C (PKC) (Kuhl et al., 2000; Range 2014; Semenov et al., 2007). These Ca^{2+}-sensitive proteins frequently produce cytoskeletal changes by calcium release. Then, Ca^{2+} releases activate different intracellular signaling molecules, such as 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), as well as several different transcription factors (Semenov et al., 2007). The calcium release causes cytoskeletal changes, disturbing the convergent extension movements and the early development of the embryo during vertebrate gastrulation (Freisinger et al., 2010). Mobilization of calcium ions by IP3 activates the expression of protein phosphatase Calcinurin that in turn can activate the nuclear translocation of transcription factors, such as the Nuclear Factor Associated with T cells (NFAT) by dephosphorylation, leading to gene transcription (De, 2011). When NFAT is activated, it can regulate the expression of several genes in neurons, cardiac and skeletal muscle cells (Feske et al., 2003; Hogan et al., 2003). In addition, IP3 sends diffusion signals through the cytosol and interacts with the calcium channels, releasing calcium ions that further activate CaMKII (De, 2011; Kühl et al., 2000). Interestingly, previously studies showed that CaMKII plays a critical role as a modulator of actin cytoskeleton

and cell motility in prostate cancer cells (Wang et al., 2010). In prostate cancer, activation of Wnt/Ca^{2+} signaling increases the activity of CaMKII which causes cytoskeleton alterations and cells motility (Wang et al., 2010). Both CaMKII and PKC, which is activated by DAG and released calcium, activate several nuclear transcription factors, such as NFkB and CREB (Fig. 2) (De, 2011; Sheldahl et al., 1999).

In addition, several studies in vertebrates have shown that Wnt5a and Wnt11 are involved in the activation the calcium second messenger and that calcium transients in the enveloping layer of the blastodisc depends on the presence of these Wnt ligands (Dejmek et al., 2006; Slusarski et al., 1997; Westfall et al., 2003). In zebrafish embryos, Frizzled-2 (Rfz-2) also induces an increase of the levels of intracellular calcium which is expanded by co-expression of Wnt5a (Slusarski et al., 1997). Thus, different Wnt ligands and Frizzled receptors might have obtained certain specificity to specific signaling pathways, potentially via their co-receptors. In the Wnt/Ca^{2+} signaling, Knypek and Ror2 are co-receptors might stimulate CamKII activity or induce PKC translocation (Hikasa et al., 2002; Topczewski et al., 2001). Thus, Wnt5a can bind to the membrane-bound receptor tyrosine kinase (RTK) Ror1/2 and regulates non-canonical Wnt/Ca^{2+} signaling independent of Wnt/β-catenin pathway (Logan and Nusse, 2004). Interestingly, the non-canonical Wnt5a/Ror signaling activates Ca^{2+}/CaMKII pathway and promotes axonal cone growth via a cleavage of cytoskeleton protein spectrin by calcium-dependent non-lysosomal cysteine protease calpain (De, 2011; Logan and Nusse, 2004; Yang et al., 2011). In addition, Wnt5a/Ror signaling also facilitates the degradation of β-catenin by producing the ubiquitin ligase Siah2 (MacLeod et al., 2007). Calpain is also activated by non-canonical Wnt5a/Ror signaling and has a role in the cleavage to cytoskeleton proteins filamin, spectrin, in a calcium-dependent manner (O'connell et al., 2009; Yang et al., 2011). The production and regulation of the transcription factor CDX2 by the Wnt5a/Ror signaling pathway can lead to the expression of downstream genes (De, 2011; Pacheco and MacLeod 2008). Additionally, specific interactions of Wnt ligands, Frizzled receptors and Knypek and Ror2 co-receptors might activate phosphodiesterase 6 (PDE6) in a calcium-dependent manner which then drives to lower levels of cyclic guanosine monophosphate (cGMP) in the embryo (De et al., 2011; Wang et al., 2004).

All the above examples illustrate that different Wnt signaling pathways display a particular combination of Wnt ligands, Frizzled receptors, co-receptors, and intracellular molecules for the proper activation of a distinct signaling. However, previous studies in vertebrates have suggested that LRP5/6 co-receptors might have an important role in regulating both Wnt/β-catenin and non-canonical Wnt signaling in a complex way (Andersson et al., 2010; Bryja et al., 2009). Thus, it is feasible to speculate that the different Wnt signaling pathways are able to interact with each other using some of the same Wnt players and; therefore, they are not completely distinct from each. Interestingly, non-canonical Wnt/Ca^{2+} signaling seems to inhibit the Wnt/β-catenin signaling during several developmental processes such as cell migration and axis specification and patterning; therefore, we investigated the function of Wnt/Ca^{2+} signaling and how it might antagonize the canonical Wnt signaling during early specification and patterning of the *Strongylocentrotus purpuratus* sea urchin embryo.

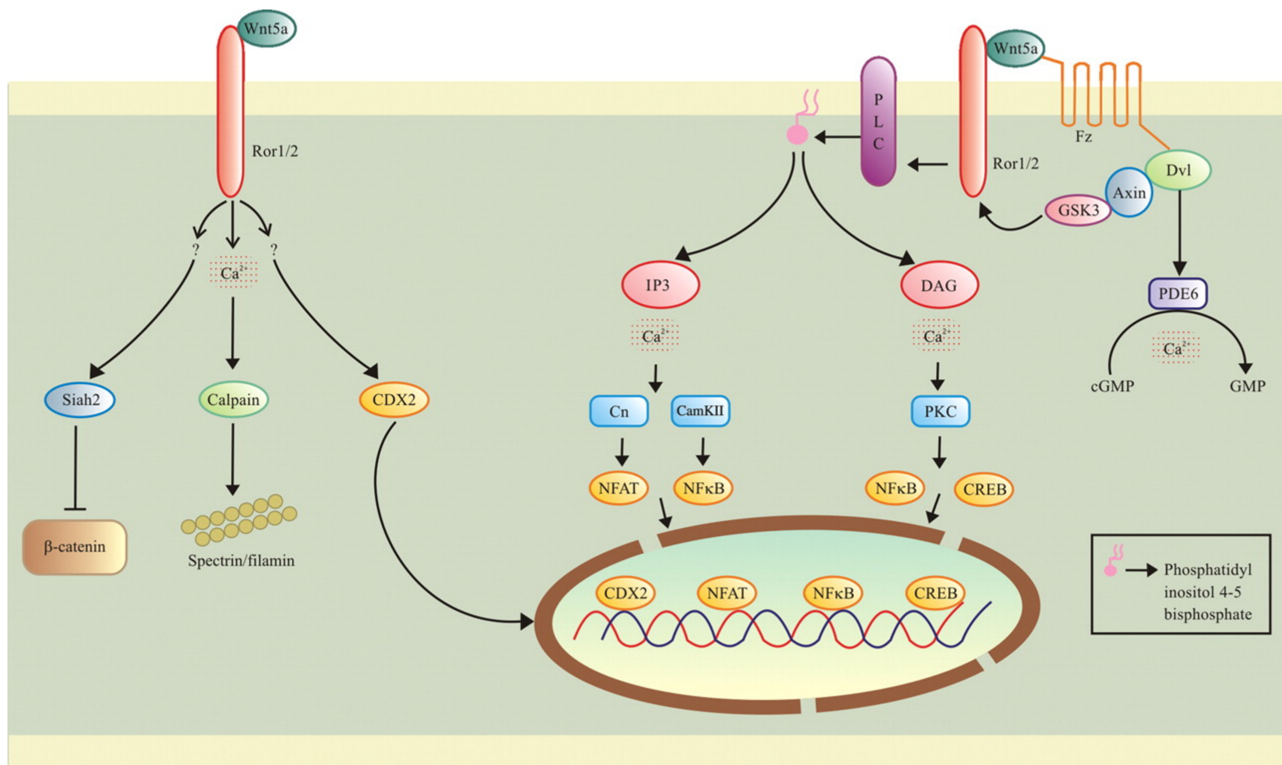

Figure 2. The Wnt/Ca^{2+} signaling pathway. Diagram showing the different Wnt ligands, receptors, coreceptors, intracellular messengers, and downstream transcription factors involved in Wnt/Ca^{2+} signaling (De, 2011).

Wnt networks in animal development

Wnt signaling pathways are required for a wide range of cellular processes during early development of metazoan embryos as well as adult tissue homeostasis. Indeed, signaling by Wnt ligands is a crucial mechanism that directs developmental processes such as cell proliferation, cell polarity, and cell fate determination during early development and tissue homeostasis of the embryo (Logan and Nusse, 2004). A growing body of evidence suggests that two or more Wnt signaling pathways are necessary for the specification of the different cell fates and/or patterning of the main embryonic territories. For example, during the development of the frog, *Xenopus,* one of the roles of Wnt/Ca^{2+} signaling pathway is the inhibition of the Wnt/β-catenin pathway (De, 2011). The CaMKII and PKC downstream factors of Wnt/Ca^{2+} signaling inhibit convergent extension mechanisms, positively regulated by Wnt/β-catenin, by phosphorylating Dishevelled (Dvl) and Tcf/Lef at critical residues, which blocks the Wnt/β-catenin signaling (Kühl et al., 2001). Similarly, the action of Ca^{2+} ions as second messengers downstream on Wnt5a signaling promotes ventral cell fate (Wnt/β-catenin signaling promotes anterior-posterior cell fate) during early embryogenesis (De, 2011; Kühl et al., 2000; Slusarski et al., 1997). In the early blastula stage of *Xenopus* embryos, high levels of CaMKII in the prospective ventral side create a dorsoventral CaMKII asymmetry and overexpression of CaMKII disturbed dorsal structures; whereas downregulation of CaMKII promoted dorsal cell fate (Kühl et al., 2000). Data from other model organisms indicate that GSK3, which is a key component in Wnt/β-catenin signaling, phosphorylates the intracellular messenger NFAT and inhibits Ca^{2+} nuclear entry, preventing the activation of the transcription factors downstream of the Wnt/Ca^{2+} signaling (Beals et al., 1997; Chow et al., 1997; Pandur et al., 2002). In addition, NFAT might be a downstream effector of Wnt/β-catenin-independent signaling since it can work with elements of the non-canonical signaling such as Wnt5a and Rfz2 during dorsal-ventral (DV) axis specification of *Xenopus* embryos (Saneyoshi et al., 2002). Interestingly, NFAT can also repress Wnt8-induced axis duplication and cause a ventralization of *Xenopus* (Saneyoshi et al., 2002). Conversely, studies have shown that the intracellular messenger JNK can also be activated by calcium signaling (Enslen et al., 1996; Zohn et al., 1995), suggesting that the non-canonical Wnt signaling pathways may share and/or compete for intracellular messengers. Enslen et al., (1996)

showed that the downstream factor of Wnt/Ca^{2+} signaling CaM-K can activate MAP kinases (ERK-2, JNK-1, and p38) and activate transcription through Elk-1, c-Jun, and ATF2.
In addition, angiotensin II (Ang II) works in a calcium-dependent manner to stimulate and modulate the JNK pathway in a cell-type specific manner (Zohn et al., 1995). Supporting these results, a recent studies revealed that NFAT and JNK form a context-independent regulatory interactions during hypertrophy induction and osteoclastogenesis regulation (Kang et al., 2017; Lu et al., 2020). CaMKII (δ) regulates NFATc1 activity and the phosphorylation of JNK, ERK, p38, and CREB (Lu et al., 2020). Thus, CaMKII (δ), downstream of Wnt/Ca^{2+} signaling, might regulate processes mediated by JNK, ERK, and p38 MAPKs and CREB signal pathways (Lu et al., 2020).

Taken together, these studies suggest that there is an interdependence among Wnt/β-catenin, Wnt/JNK, and Wnt/ Ca^2 signaling pathways in many important developmental contexts. Further strengthening the idea that two or more Wnt pathways work in the same cell, or territory of cells, recent studies have shown that several combinations of ligands, receptors, and co-receptors are associated with all three pathways (Kestler and Kuhl, 2008; Range 2014; van Amerongen and Nusse, 2009). These results and several other studies have led to a new idea in the field of Wnt signaling that in many, or even most situations of development and adult tissue homeostasis, cells integrate information from a network of two or more integrated Wnt signaling pathways (Kestler and Kuhl, 2008; Range 2014; van Amerongen and Nusse, 2009). Therefore, it is necessary to study the different Wnt signaling pathways as a complex network of protein interactions and its regulatory inputs instead of individual pathways. We propose studying this Wnt signaling network using the sea urchin as a model organism since their embryos are transparent as well as they present simple genomic organization and fewer cell movements which makes them great tractable systems. Our studies will provide a better understanding of the exact mechanism that patterns the anterior neuroectoderm (ANE) along the anterior-posterior (AP) axis in deuterostome embryos.

Anterior-posterior specification and patterning

In the early development of metazoan embryos, mechanisms that specify and pattern cell territories along the primary animal-vegetal (AV) and subsequently form and pattern the AP axis

are responsible for defining the embryonic and, ultimately, the adult body plan. The AV axis is constructed maternally in the egg while the secondary dorsal-ventral axis is established after fertilization. In most metazoan embryos, an ancient AP specification and patterning mechanism establishes posterior and anterior identities by a Wnt/β-catenin signaling gradient (Niehrs, 2010; Petersen and Reddien, 2009; Range 2014). Several studies in deuterostomes (vertebrates, urochordates, cephalochordates, hemichordates, and echinoderms) have shown that a posterior-to-anterior gradient of Wnt/β-catenin signaling is required for the early activation and patterning of the different gene regulatory networks (GRNs) along the AP axis (Darras et al., 2011; Kiecker and Niehrs, 2001; Lekven et al., 2001; Logan et al., 1999; Nordström et al., 2002; Wikramanayake et al., 1998; Yaguchi et al., 2008). In invertebrate deuterostomes, maternally localized components allow β-catenin to stabilized in posterior blastomeres (Darras et al., 2011; Holland et al., 2005; Imai et al., 2000; Logan et al., 1999; Wikramanayake et al., 2003). Then, β-catenin enters the nuclei and activates genes that specify the endomesoderm territory in the vegetal pole of the egg, creating the posterior territory of the embryo that marks the site of gastrulation (Petersen and Reddien, 2009; Range 2014). Thus, high levels of Wnt/β-catenin signaling activates the endomesodermal GRN at the posterior pole of the embryo and low Wnt/β-catenin levels are responsible for the establishment of the ANE GRN which subsequently has the potential to produce several sensory neurons and sensory organs later in development (Niehrs, 2010; Petersen and Reddien, 2009; Range, 2014).

In sea urchin embryos, four major early germ territories are established and positioned along the AP axis at the beginning of gastrulation: posterior endoderm, posterior mesoderm, equatorial ectoderm (that will specify dorsal and ventral ectodermal structures) and the ANE territory around the pole (Angerer et al., 2011; Molina et al., 2013; Range, 2018). One of these germ territories, the ANE, is specified along the AP axis of deuterostome embryos and this territory is derived from the animal pole region of the egg in several metazoan phyla (Angerer et al., 2011; Kozmik et al., 2007; Lagutin et al., 2003; Posnien et al., 2011; Range 2014; Wei et al., 2009). ANE patterning, specification, and positioning are crucial events in the early development of deuterostome embryos (Fig. 3) (Tassia et al., 2017); however, the molecular mechanisms that regulate these fundamental developmental processes are incompletely understood (Foley and Stern, 2001; Wilson and Houart, 2004). Although ANE structures vary among deuterostomes, from the sensory neurons in areas like the apical tuft in sea urchin species to the forebrain and

eye-field in vertebrates, they are originally formed from a flat neuroepithelium during early stages of deuterostome development (Pani et al., 2012; Range 2014). Comparisons of the spatiotemporal gene expression and functional studies on ANE regulatory factors in several species suggest that the signaling pathways and GRNs which include cis-regulatory modules and transcription factors, involved in AP specification and patterning are remarkably shared among many deuterostomes embryos, suggesting that ANE regions are most likely ancient and homologous territories (Range 2014).

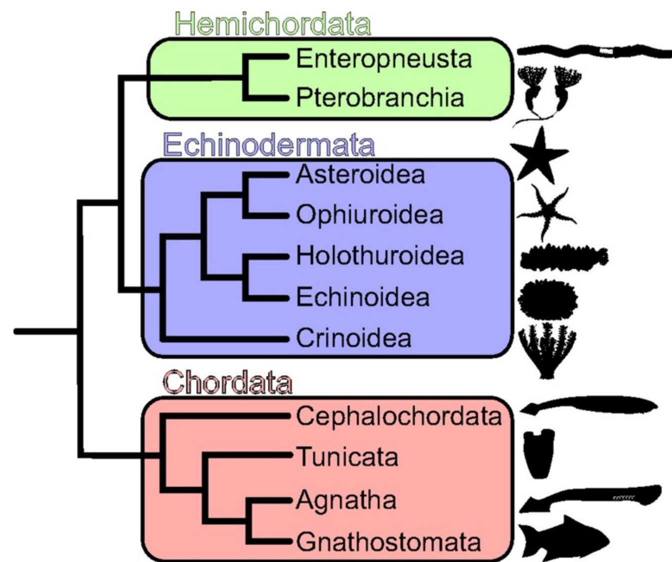

Figure 3. Deuterostome phylogeny. Phylogenetic relationship of the different deuterostome groups based on recent phylogenomic studies. Two clades are represented: the ambulacrarian phyla (hemichordates and echinoderms) and chordates phyla (cephalochordates, tunicates, and vertebrates) (Tassia et al., 2017).

Recent investigations have shown that ANE regulatory genes in deuterostome embryos, such as the cardinal regulators *six3* and *foxq2*, are initially expressed broadly in early ectodermal territories (Range 2014). Therefore, most cells in deuterostome embryos have the potential to become ANE. Next, ANE regulatory genes are subsequently down regulated from more posterior ectoderm in a progressive process, resulting in the ANE territory being positioned around the anterior pole of the embryo (Fig. 4) (Darras et al., 2011; Kiecker and Niehrs, 2001; Kozmik et al., 2007; Nordström et al., 2002; Range 2014; Yaguchi et al., 2008). The downregulation of ANE GRN is possible due to the activity of Wnt/β-catenin signaling pathway which restricts the

ANE potential to specific regions of the embryo (Range 2014; Range, 2018). In addition, Six3 is an antagonist of the posterior-to-anterior gradient of Wnt signaling and, as a result, controls the proper size of the ANE territory (Range, 2018; Wei et al., 2009).

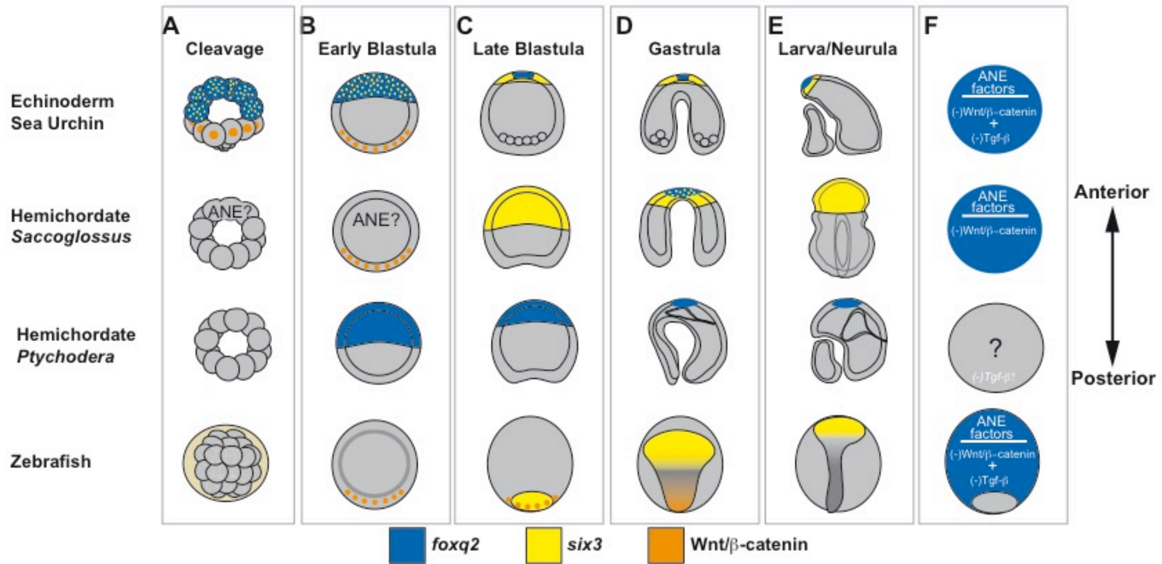

Figure 4. ANE positioning is conserved among deuterostome embryos. (A-E) The development of different deuterostome organisms, from cleavage to larva/neurula stage. Early in the deuterostome development, the ANE factors *foxq2* and *six3* are broadly expressed within the ectoderm, and as development progresses, are restricted to the anterior pole. (F) Diagram showing what occurs to the different hemichordate organisms when Wnt/β-catenin is knocked down (modified from Range 2014).

In the sea urchin, the posterior-to-anterior cascade of Wnt/β-catenin signaling activity is responsible for specification of the endoderm territory to the posterior pole as well as the progressive restriction of ANE territory to the anterior pole (Range et al., 2013; Yaguchi et al., 2008). The first ANE regulatory genes to be expressed in the anterior half of the sea urchin embryo are *six3* and *foxq2* at the 32-cell stage, both acting as cardinal transcriptional regulators of ANE specification because they sit at or near the top of the ANE GRN (Range and Wei, 2016; Range et al., 2013; Wei et al., 2009). Beginning at the 60-cell stage their expression is progressively down-regulated from posterior ectoderm cells in the anterior half of the embryo over the course of approximately 15 hours post-fertilization (hpf). The down regulation of ANE GRN gene expression stops at the beginning of gastrulation (24 hpf), termed the mesenchyme

blastula stage, so that they are positioned around the anterior pole (Fig. 5) (Range and Wei, 2016; Wei et al., 2009; Yaguchi et al., 2008*)*. Recent studies in sea urchins have shown that early specification and patterning of the major embryonic germ layers, including ANE positioning, involve not only Wnt/β-catenin signaling pathway, but three different Wnt signaling branches (Range et al., 2013). In the early sea urchin embryo, positioning of the ANE around the anterior pole depends on the embryo's ability to integrate cross-regulatory information from at least three Wnt pathways: the Wnt/β-catenin, the Wnt/JNK, and the Wnt/PKC signaling pathways, forming an interconnected Wnt signaling network (Range et al., 2013).

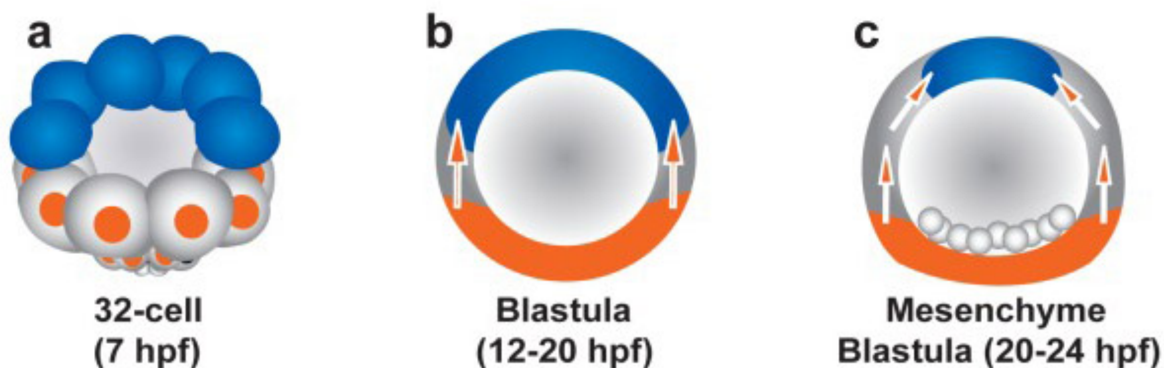

Figure 5. Schematic diagram showing the different development stages of ANE positioning in the sea urchin embryo. (a) At 32-cell stage, ANE is present only in the anterior half of the embryo, (b) At blastula stage (12-20 hours post-fertilization, hpf), there is a down regulation of ANE gene expressions from the posterior ectoderm, (c) At mesenchyme blastula (20-24 hpf) ANE is confined to a small disk territory around the anterior pole of the embryo. Blue = ANE; Orange = Wnt/β-catenin; Orange arrows = positioning mechanism activated by β-catenin (Range et al., 2013).

The role of the sea urchin Wnt signaling network in anterior-posterior specification and patterning

In sea urchin embryos, the specification and patterning of the early germ layers along the AP axis can be described in a four-step model as follows (Fig. 6):

- **Step 1 (1-cell to 60-cell stage):** maternally localized components activate nuclear β-catenin in posterior/vegetal blastomeres as early as the 16-cell stage, allowing the specification of

endomesoderm territories in the posterior territory of the embryo (Logan et al., 1999; Peng and Wikramanayake, 2013; Wikramanayake et al., 2003). At the 32-cell stage, posterior Wnt/β-catenin signaling also prevents ANE GRN activation in the posterior blastomeres, resulting in the expression of the earliest "master ANE regulators" *six3* and *foxq2* only in the anterior half of the embryo in the presumptive ectoderm (Range et al., 2013). Thus, endomesoderm GRN is restricted to the posterior blastomeres and ANE GRN is positioned in the anterior blastomeres of the embryo.

- **Step 2 (60-cell to late blastula stage, 9-24 hpf)**: at 60-cell stage, two Wnt ligands activated by posterior Wnt/β-catenin, Wnt1 and Wnt8, diffuse and interact with a ubiquitously expressed Frizzled 5/8 (Fzl5/8) receptor in the posterior ectoderm, activating the intracellular messenger JNK (Range et al., 2013). Wnt1/Wnt8-Fzl5/8-JNK signaling promotes down regulation of ANE GRN gene expression in all the equatorial ectoderm, restricting the ANE expression to the most anterior region of the embryo during late cleavage and blastula stages (Range et al., 2013; Yaguchi et al., 2008). Simultaneously, another Frizzled receptor, Frizzled 1/2/7 (Fzl1/2/7), starts the activation of the third Wnt signaling pathway, Wnt/Ca^{2+}/PKC, which antagonizes Fzl5/8-JNK signaling. This antagonism mechanism prevents the complete elimination of specification of the ANE factors, resulting in an ANE positioning territory in the anterior pole of the sea urchin embryo (Range et al., 2013). Additionally, recent studies showed that, during early phases of ANE positioning, Fzl1/2/7 activates the expression of the Wnt modulator sFRP1 (secreted Frizzled-related protein 1; vertebrate FrzB homologue), which is necessary to protect the ANE factors from the ANE restriction mechanism of the Fzl5/8 signaling (Khadka et al., 2018).

- **Step 3 (mid-blastula to early blastula, 15-24 hpf)**: The early ANE regulatory protein FoxQ2 activates a signaling center around the anterior pole that activates the Wnt modulators sFRP1/5 and Dkk3 (Dickkopf-related protein 3). These two Wnt signaling modulators diffuse from the anterior pole and potentiate the Fzl5/8-JNK-mediated down regulation of ANE factors from the posterior ectoderm. sFRP1/5 and Dkk3 are both required and work additively during the later phases of ANE positioning, establishing a correctly sized ANE territory (Range and Wei, 2016).

- **Step 4 (early gastrula, 24 hpf)**: The secreted Wnt antagonist, Dkk1, is activated by Fzl5/8 receptor and inhibits the action of Fzl5/8-JNK signaling by a negative feedback mechanism, preventing the complete down regulation of the ANE factors around the anterior pole and defining the boundaries of the ANE territory (Niehrs 2010; Range and Wei, 2016; Range et al., 2013). Another Wnt modulator, sFRP1 (Bovolenta et al., 2008; Leyns et al., 1997; Wang et al., 1997), is expressed throughout ANE positioning and likely works together with Dkk1 to antagonize the final phase of the ANE positioning, establishing the final ANE region (Khadka et al., 2018).

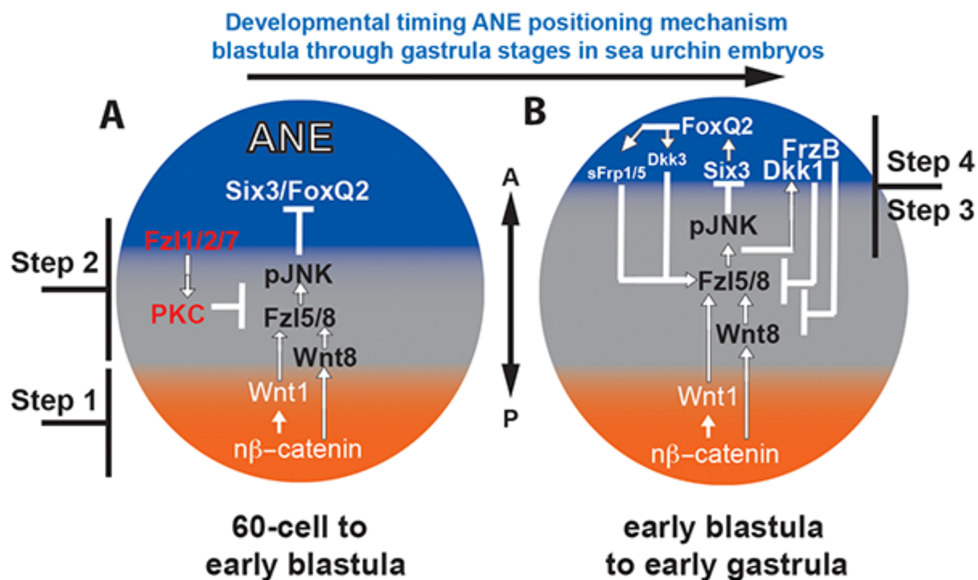

Figure 6. Diagram showing the ANE positioning model. At least three Wnt signaling pathways are necessary for ANE positioning in the sea urchin embryo: Wnt/β-catenin, Wnt/JNK and Wnt/PKC pathways. (A) Steps one and two taking place in 60-cell to early blastula stages. (B) Steps three and four occurring in early blastula to gastrula stages. Orange = endomesoderm; Gray = posterior ectoderm; Blue = anterior ectoderm (ANE) (Khadka et al., 2018; Range and Wei 2016; Range et al., 2013).

The sea urchin is an excellent model organism to study the specification and patterning of the four early germ layers along the AP axis. The easy manipulation of embryonic gene expression as well as its relatively simple morphological features and cell movements provide unique qualities for performing detailed analysis in sea urchin embryos (Angerer et al., 2011; McClay, 2011). More specifically, sea urchins are great model systems to investigate the

development of the endomesoderm and the GRNs governing DV and AP axis formation and to gain further understanding of the Wnt signal communications during development (Ben-Tabou de-Leon et al., 2013; Saudemont et al., 2010). The Wnt signaling network functions early in sea urchin development, from 60-cell to gastrula stage, which allows us to perform functional experiments over the course of approximately 24 hpf. Functional experiments are easily conducted in the sea urchin due to the moderately simple genome compared with other deuterostomes. In addition, interactions within the Wnt signaling pathway can be easily deduced due to the small number of possible Wnt ligand and receptor/co-modulator interactions as well as potential interactions with other signaling pathways (Kikuchi et al., 2009; Houart, 2002). Previous results from our lab have described a detailed regulatory mechanism controlled by a Wnt signaling network that is required for the positioning and patterning of the ANE territory. Thus, we can easily take advantage of our knowledge of ANE territory positioning and use it as a marker for AP axis specification and patterning. During ANE patterning in sea urchin embryos, only a few signaling pathways are activated outside of the three AP Wnt signaling pathways (Range et al., 2013). In addition, the action of Wnt signaling pathways in early patterning of the ANE territory has been shown to operate independently from the DV axis patterning mechanism, unlike in vertebrates, because it takes place before Nodal and BMP2/4 signaling are activated (Angerer et al., 2011). Interestingly, our research results indicate a possible connection between the AP and DV axis specification events, which until now have been shown as independent of one another in the sea urchin (Angerer et al., 2011).

BMP2/4 and Nodal signaling in dorsal-ventral axis patterning

In most deuterostomes, the secondary axis, or dorsal-ventral (DV), is specified and positioned after fertilization by cell interactions (Gilbert, 2010). During gastrulation, the first signs of DV axis in the sea urchin embryo are the formation of a flattening ventral side, an archenteron bent towards the ventral ectoderm, and an elongation of the dorsal region (Fig. 7) (Molina et al., 2013). Then, four arms grow on the ventral side giving the characteristic shape of the pluteus larva (Fig. 7). As development progresses, the left-right axis is established and develops a rudiment on the left side of the sea urchin larva, forming a radially symmetric adult form (Fig. 7) (Molina et al., 2013).

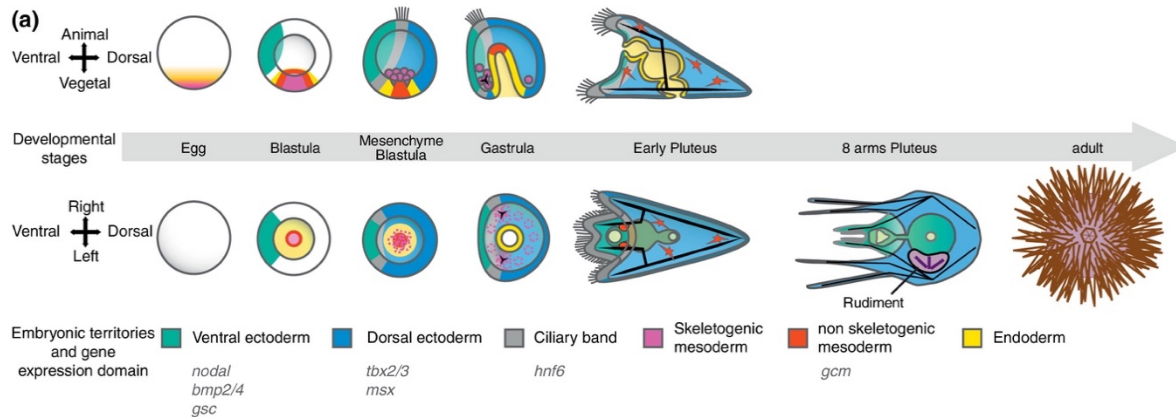

Figure 7. Schematic model showing early development of the sea urchin embryo. The diagram illustrates the patterning and positioning of the major embryonic territories and gene expression domains along anterior-posterior, dorsal-ventral, and right-left axes as development progresses, from egg to sea urchin adult form (Molina et al., 2013).

Specification and patterning of the DV and left-right axes as well as the establishment of the proper DV polarity of the embryo are essential developmental processes coordinated by the Nodal signaling pathway (Duboc et al., 2004; Flowers et al., 2004). Nodal inhibition eliminates DV polarity along the three germ layers (endoderm, mesoderm, and ectoderm) and; therefore, disrupts early DV patterning (Duboc et al., 2004). In the sea urchin embryo, there is a gradient of Nodal activity where nodal self-activates. In addition, Nodal induces the expression of Lefty, a long-range antagonist of Nodal, which diffuses away from the ventral ectoderm (Lapraz et al., 2009; Molina et al., 2013). Then, Nodal signaling on the ventral territory is responsible for the activation of the expression of ventral genes and for the differentiation of the ventral ectoderm. Therefore, high activity of Nodal signaling induces the expression of ventral markers such as *chordin, lefty, brachyury, goosecoid,* and *bmp2/4* and represses the expression of dorsal fates, resulting in ventral ectoderm specification (Molina et al., 2013).

Next, Chordin on the ventral side of the sea urchin embryo inhibits BMP2/4 from signaling in the ventral ectoderm, preventing the binding of BMP2/4 with its receptor (Lapraz et al., 2009). BMP2/4 is then translocated to the dorsal territory, where it is required for the activation of dorsal markers (Molina et al., 2013). Therefore, at blastula stages, BMP2/4-Chordin complexes of BMP2/4 and Chordin proteins diffuse or are transported towards the dorsal side of

the embryo and create a DV gradient of BMP2/4 signaling. The signaling of the TGF-β member BMP2/4 is responsible for controlling dorsal fates and for the specification of dorsal genes by stimulating the expression of *tbx2/3* and other dorsal genes (Duboc et al., 2004). In addition, nuclearized Smad1/5/8 along the dorsal ectoderm suggest that BMP2/4 acts as a morphogen in patterning the DV axis of the sea urchin embryo (Bergeron et al., 2011; Chen et al., 2011; Lapraz et al., 2009; Molina et al., 2013). The schematic diagram of Nodal and BMP2/4 signaling (Fig. 8) shows that Nodal and BMP2/4 are expressed in the same ventral territory but they work in opposite ventral-dorsal territories thanks to the presence of a morphogenetic gradient (Molina et al., 2013). Interestingly, a previous study suggested that early BMP2/4 signaling might be regulated by another unknown mechanism different from Chordin and Nodal (Su et al., 2009). Therefore, further functional studies in the early development of the sea urchin embryo are necessary to obtain a more detailed understanding of the regulatory mechanisms and interactions involved in early AP and DV axis specification and patterning.

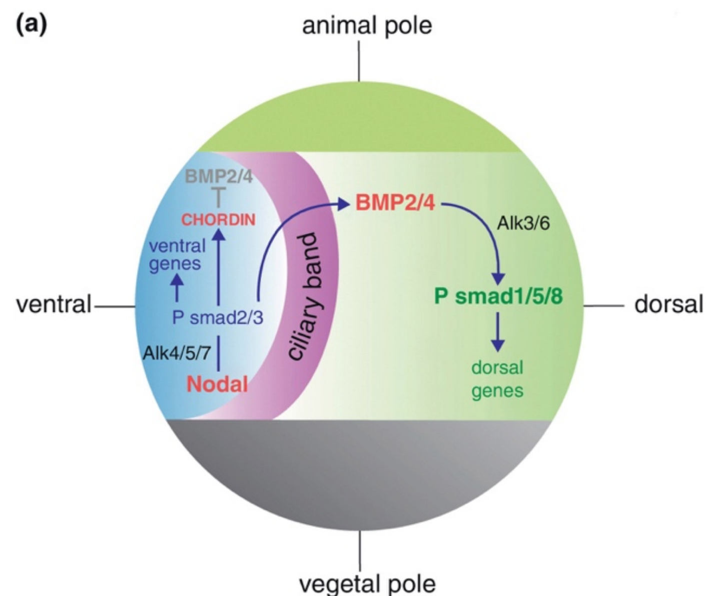

Figure 8. Schematic diagram of the BMP2/4 translocation during dorsal-ventral patterning. The model shows spatiotemporal expression of Nodal and BMP2/4 signaling. Nodal activates the expression of ventral genes. Ventral Chordin inhibits BMP2/4 from signaling in the ventral side. BMP2/4 is translocated from the ventral to the dorsal territory where it activates dorsal genes. (Molina et al., 2013).

The establishment of the primary axis is one of the first developmental decisions in the early embryo, and it enables the latter formation of the three germ layers: endoderm, mesoderm, and ectoderm (Lowe et al., 2015). In deuterostomes, a gradient of Wnt/β-catenin is essential in this fundamental process (Lowe et al., 2015; Range 2014). Interestingly, integrated information from different Wnt signaling pathways are usually required for different developmental processes, including early AP patterning. Wnt networks involve complex protein interactions, with multiple and sometimes simultaneous regulatory inputs at the level of both Wnt-receptor binding and the downstream intracellular response (van Amerongen and Nusse 2009). As an example of Wnt networks in the sea urchin, Range et al. 2013 showed for the first time that three Wnt signaling branches, Wnt/β-catenin, Wnt/JNK, and Wnt/PKC signaling pathways affect and pattern the ANE positioning to the anterior pole of the embryo (Range et al., 2013). These three Wnt pathways are partially dependent and function as an interactive Wnt signaling network. When we compare functional and spatiotemporal expression studies from vertebrates, amphioxus, hemichordates, sea urchins, and sea star embryos it appears that features of the interconnected Wnt signaling network are most likely shared among deuterostome embryos (Range 2014) (Fig. 9). For example, recent studies have found that Fzl5/8 is necessary to position ANE factors to the anterior pole in a hemichordate embryo (Pani et al., 2012). Additionally, the spatiotemporal expression within the ectodermal region undergoing ANE positioning of the putative ANE factors and orthologs of sea urchin Fzl1/2/7, Wnt1, and Wnt8 are similar in all those deuterostomes (Fig. 9) (Holland et al., 2000; McCauley et al., 2013; Onai et al., 2012; Pani et al., 2012; Qian et al., 2013; Range 2014; Range et al., 2013; Röttinger and Martindale, 2011; Sumanas, et al., 2000; Yankura et al., 2013; Yu et al., 2002).

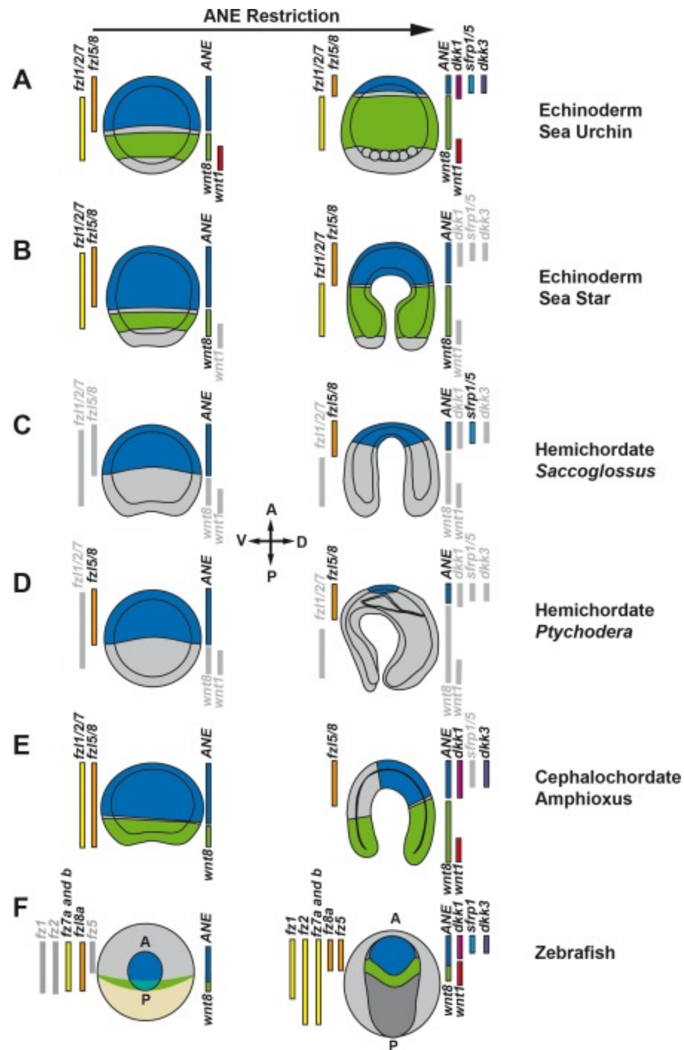

Figure 9. The conserved Wnt signaling network among deuterostomes during ANE positioning. The diagram shows the expression of orthologs necessary for ANE positioning in two echinoderm species (sea urchin and sea start), two hemichordate species (*Saccoglossus* and *Ptychodera*), and two chordate species (Amphioxus and Zebrafish) (Range 2014).

Since the AP specification and positioning is a crucial event in body plan formation in all deuterostomes, here we investigated the role of the non-canonical Fzl1/2/7-PKC signaling pathway in the sea urchin AP positioning mechanism and compared our results to those of other metazoan embryos in order to determine if there are shared aspects of the AP positioning mechanisms among deuterostomes. Additionally, our RNA-seq screen identified several dorsal genes downregulated in Fzl1/2/7 knockdown, leading to the idea that Fzl1/2/7 signaling might be involved not only in AP but also DV axis specification and/or patterning. Previous studies in a

wide range of deuterostome embryos focus on the patterning mechanisms along individual developmental axes. However, little is known about how AP and DV GRNs might be coordinating specification and patterning of the germ layers of the embryo. Most metazoan embryos control cell fate specification along AP and DV body axes with precise timing; consequently, the regulatory mechanism that specifies and patterns AP and DV axes should not be independent. Functional studies in several deuterostome embryos have suggested that inputs from several signaling pathways tightly coordinated AP and DV specification and patterning (Fuentealba et al., 2007; Hashiguchi and Mullins, 2013; Takebayashi-Suzuki et al., 2018; Tucker et al., 2008; Yaguchi et al., 2008). For example, Wnt/β-catenin and TGF-β signaling pathways cooperatively work in order to prevent the activation of neuroectoderm specification in vertebrates and sea urchin embryos (Lapraz et al., 2009; Niehrs, 2010; Range 2014; Yaguchi et al., 2010). Thus, the integrated information from Wnt/β-catenin and TGF-β signaling establishes the proper neuroectoderm patterning along AP and DV axis. Here, we investigated how Fzl1/2/7 signaling might regulate both AP and DV GRNs during early development of the sea urchin embryo.

Functional studies performed in deuterostomes have suggested that secondary DV axis specification and patterning is regulated by remarkably conserved mechanism involving Nodal and BMP signaling pathways. In vertebrates and invertebrates, BMP antagonists such as Chordin repress the activity of BMP signaling (in *Drosophila* BMP homolog is called Dpp) creating a gradient of BMP along the DV axis (De Robertis and Kuroda, 2004; Lowe et al., 2006; O'Connor et al., 2006; Yu et al., 2007). In addition, Nodal signaling is necessary for DV polarity of many embryos (Lapraz et al., 2009; Duboc et al., 2008). Dorsal and ventral signaling centers are observed in many bilaterian embryos. For example, complementary expression patterns of BMP2/4 and Chordin in opposite sites along the DV axis allow dorsal and ventral cells to communicate in most bilaterians (Fig. 10) (Lapraz et al., 2009). The roles of BMP2/4 and Chordin are remarkably conserved between vertebrates and invertebrates but there is an inversion of their expression along the DV axis: *bmp2/4* is expressed ventrally in vertebrates while the expression of *dpp* is in the dorsal region in invertebrates (De Robertis, 2008; Mieko and Bier, 2008). Therefore, although a regulatory mechanism that has inputs from Nodal, BMP2/4 and Chordin is likely to be remarkably conserved among metazoan, differences between organism have occurred in the course of evolution.

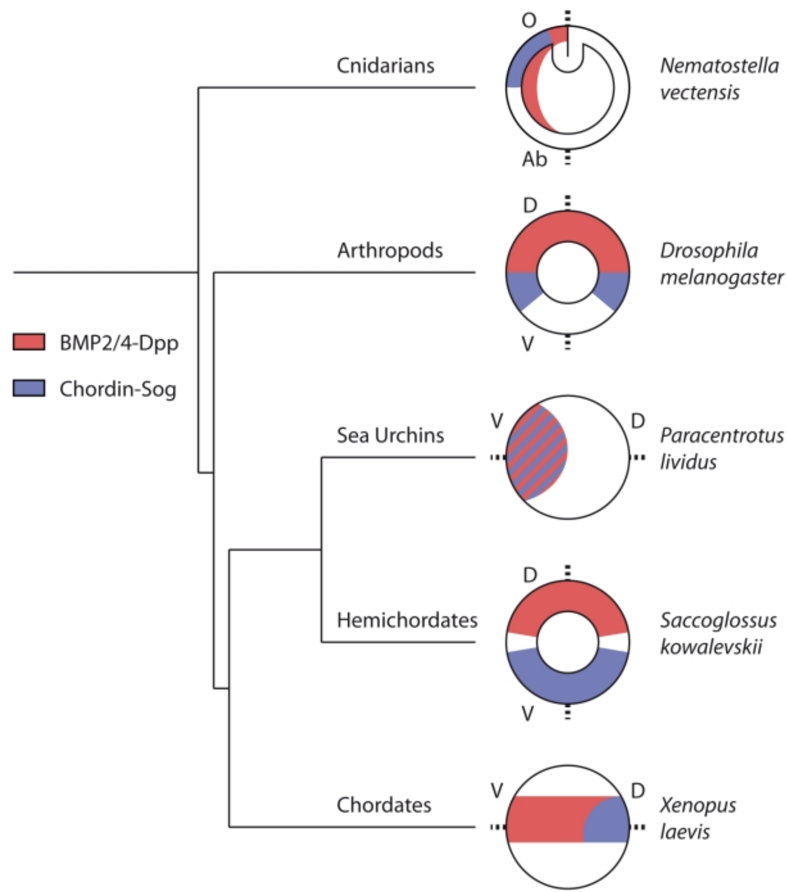

Figure 10. Comparison of the expression domains of BMP2/4 and Chordin among metazoans during DV axis specification and patterning. Schematic diagram compares the expression of homologues necessary for DV axis in several experimental models (Lapraz et al., 2009).

Here, we propose that a detailed investigation of the molecular and cellular architecture controlling the establishment of DV and AP patterning in the sea urchin embryo will provide insights into the deuterostome evolution of the shared mechanisms of body axis patterning. Although there has been a substantial advance in our understanding of body patterning and specification in sea urchins, a number of questions remain unanswered. Therefore, the main goals of this dissertation are as follows: 1) To determine the Wnt ligand(s) necessary to activate the non-canonical Fzl1/2/7-PKC signaling pathway during early anterior-posterior (AP) specification and patterning (Chapter 2); 2) To examine the cooperative functions of Fzl1/2/7 and Nodal signaling pathways in the initial specification of the ectoderm DV GRN (Chapter 3); and 3) To characterize the distinct roles of the signaling transduction molecule Siah, the dorsal

transcription factor Tbx2/3, and the potential transcriptional effector NFAT downstream of Wnt16-Fzl1/2/7 signaling during AP and DV axis specification and patterning of the sea urchin embryo (Chapter 4).

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Chapter 2.

A biphasic role of non-canonical Wnt16 signaling during early anterior-posterior patterning and morphogenesis of the sea urchin embryo

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Key words: Wnt signal transduction, anterior-posterior, deuterostome evolution, gene regulatory networks, Wnt16, Frizzled

Martínez-Bartolomé, M., and Range, R. C. (2019). A biphasic role of non-canonical Wnt16 signaling during early anterior-posterior patterning and morphogenesis of the sea urchin embryo. *Development*, ***146***(24). dev168799 doi: 10.1242/dev.168799

ABSTRACT

Anterior-posterior (AP) patterning of the deuterostome sea urchin embryo depends on integrated information from a Wnt signaling network. We have previously shown that a non-canonical Fzl1/2/7-PKC signaling antagonizes the progressive posterior-to-anterior downregulation of the anterior neuroectoderm (ANE) gene regulatory network (GRN) by canonical Wnt/β-catenin and non-canonical Wnt1/Wnt8-Fzl5/8-JNK signaling. This study focuses on the function of Wnt16 ligand in activating the Fzl1/2/7 signaling during AP patterning. Maternally supplied *wnt16* is expressed ubiquitously during cleavage and zygotic *wnt16* expression is concentrated in the endoderm/mesoderm beginning at mid-blastula stage. Wnt16 antagonizes ANE restriction mechanism and this activity depends on a functional Fzl1/2/7 receptor. Zygotic *wnt16* expression during gastrulation depends on both Fzl5/8 and Wnt/β-catenin signaling. Furthermore, Wnt16 is necessary for the activation and/or maintenance of key regulatory endoderm/mesoderm genes and is essential for gastrulation. Together, our data show that Wnt16 has two functions during early sea urchin AP specification and patterning: (1) an initial role in activating the Fzl1/2/7 pathway that antagonizes the ANE restriction mechanism and (2) a subsequent function in activating key endoderm GRN factors and the morphogenetic movements of gastrulation.

INTRODUCTION

In metazoans, anterior-posterior (AP) axis specification and patterning is one of the first fundamental developmental processes critical for establishing the correct adult body plan. Studies have shown a highly conserved role for Wnt signaling in establishing the AP axis in a number of deuterostome species, from sea urchins to mammals (Loh et al., 2016; Niehrs 2010; Petersen and Reddien, 2009). In these animals, high posterior "canonical" Wnt/β-catenin signaling is necessary to establish the endomesoderm gene regulatory network (GRN) around the posterior pole (Loh et al., 2016; Niehrs 2010; Petersen and Reddien, 2009). In addition, a mechanism that depends on posterior Wnt/β-catenin is required to restrict the initial broadly expressed anterior neuroectoderm (ANE) GRN to a domain around the anterior pole (Angerer et al., 2011; Gaspard et al., 2008; Kiecker and Niehrs, 2001; Lekven et al., 2001; Nordström et al.,

2002; Pani et al., 2012; Yaguchi et al., 2008). Importantly, core members of both the endomesoderm and ANE GRNs have been shown to be conserved in several metazoan taxa (Croce et al., 2006a; Davidson and Erwin, 2006; Hinman and Cheatle Jarvela, 2014; Loh et al., 2016; Wei et al., 2016).

In addition to the Wnt/β-catenin signaling pathway, two conserved “alternative/non-canonical” Wnt signaling pathways, Wnt/JNK and Wnt/Ca^{2+}, have been identified in a variety of metazoan embryos. Recent studies have shown that in many cases during development and adult tissue homeostasis two or more of these three pathways are active in the same cells or territories (Kestler and Kühl, 2008; van Amerongen and Nusse, 2009). These Wnt signaling networks are necessary for various cellular processes such as cell fate specification, neurogenesis, spindle orientation, and maintenance of stem cells (Kestler and Kühl, 2008; van Amerongen and Nusse, 2009). Our lab has discovered that AP specification and patterning in sea urchin embryos depend on integrated cross-regulatory information from all three major Wnt signaling branches (Khadka et al., 2018; Range, 2014; Range, 2018; Range and Wei, 2016; Range et al., 2013), making this one of the few known examples where all three Wnt signaling pathways have been shown to govern a fundamental developmental process. The sea urchin AP axis is initiated when maternally localized Wnt signaling components (e.g. Dishevelled) promote nuclear accumulation of β-catenin around the 16-cell stage resulting in the activation of the endomesoderm GRN (Davidson et al., 2002; Logan et al., 1998; Weitzel et al., 2004; Wikramanayake et al., 1998; Wikramanayake et al., 2003; Wikramanayake et al., 2004). In addition, nuclear β-catenin is necessary to repress the activity of an unknown, broadly expressed regulatory network that drives ubiquitous activity of the ANE GRN, restricting its expression to the anterior half of the embryo at the 32-cell stage (Wei et al., 2009; Yaguchi et al., 2008). Around the same time, Wnt/β-catenin signaling activates the Wnt1 and Wnt8 ligands in posterior cells, which appear to diffuse anteriorly where they interact with the Wnt receptor, Fzl5/8, and activate the intracellular messenger JNK in equatorial ectoderm (an ectoderm territory established between the posterior endomesoderm GRN and the anterior ANE GRN around by mesenchyme blastula stage). The result of Wnt1/Wnt8-Fzl5/8-JNK signaling is the progressive posterior-to-anterior downregulation of ANE GRN activity in ectodermal cells during the late cleavage and blastula stages, ultimately restricting the ANE to a territory around the anterior pole (Range, 2018; Range et al., 2013). During the final phase of this ANE restriction (late blastula/early gastrula stages),

the combined activity of the secreted Wnt modulators sFRP-1, sFRP1/5, Dkk3, and Dkk1 around the anterior pole establishes the correctly sized ANE territory (Khadka et al., 2018; Range and Wei, 2016; Range et al., 2013). Importantly, non-canonical Fzl1/2/7 signaling represents a third Wnt signaling branch in this process and is essential during the entire progressive posterior-to-anterior ANE restriction mechanism. This pathway antagonizes the activity of both the Wnt/β-catenin and Fzl5/8-JNK pathways, governing the rate of ANE restriction (Range, 2018; Range et al., 2013) and allows for the establishment of 4 early regulatory networks along the AP axis by early gastrulation: posterior endoderm and mesoderm GRNs, an equatorial ectoderm GRN, and the ANE GRN around the anterior pole (see Fig. 1A for model). Interestingly, expression and/or functional studies in other deuterostome embryos strongly suggest that major aspects of the AP Wnt signaling network identified in sea urchin embryos may be conserved among deuterostome embryos (Range, 2014).

A combination of the activities of secreted Wnt modulators, trans-membrane Frizzled receptors, and co-receptors is necessary in order to activate one or more Wnt signaling pathways in a given context. It is arguably the spatiotemporal expression of the Frizzled receptors and the particular Wnt ligand(s) they interact with what is the primary determinant of which Wnt signaling branch will be activated, and at what levels these pathways will be activated, in a given cell or tissue territory. Previous studies in sea urchin embryos have defined the composition and spatiotemporal expression patterns of the Wnt ligands and Frizzled receptors, including a set of 4 Frizzleds and 11 or 12 Wnts, depending on the species (Croce et al., 2006b; Robert et al., 2014; Robert et al., 2019). Fzl5/8 and Fzl1/2/7 are the only receptors expressed during the early specification and patterning of the AP axis by the Wnt signaling network (0 to 24 hpf) (Croce et al., 2006b; Lhomond et al., 2012; Range 2018; Range et al., 2013). Both genes are expressed ubiquitously during cleavage and early blastula stages and then progressively restricted to more anterior cells by late blastula stages (Croce et al., 2006b; Lhomond et al., 2012). In addition, six Wnt ligands are expressed during this process (Wnt1, Wnt5, Wnt6, Wnt7, Wnt8, and Wnt16), whereas the other five to six ligands are expressed at or after late blastula/early gastrula stages, i.e. after the completion of early AP patterning (Croce et al., 2006b; Robert et al., 2014; Robert et al., 2019). As mentioned above, we previously demonstrated that two of these six ligands, Wnt1 and Wnt8, activate Fzl5/8-JNK signaling (Range et al., 2013); however, the secreted Wnt ligand(s) required for activation of non-canonical Fzl1/2/7 signaling and antagonism of

Wnt1/Wnt8-Fzl5/8-JNK signaling have not been identified. Fzl1/2/7 signaling appears to be active as early as the 32-cell stage throughout the embryo, suggesting that the Wnt ligand(s) that activate it should also be broadly expressed at or before the 32-cell stage. Of the remaining 4 ligands that could potentially activate Fzl1/2/7, *wnt6*, *wnt7*, and *wnt16* are maternally supplied (Croce et al., 2011; Robert et al., 2014) and only one of these, *wnt7,* has been shown to be expressed ubiquitously during cleavage and blastula stages. In contrast, *wnt5* expression is activated around the 60- to 120-cell stage when it is expressed in cells at the boundary between the endoderm and ectoderm, suggesting that it does not play a role in the antagonism of Wnt/β-catenin and Fzl5/8 signaling throughout the embryo at the 32-cell stage (Cui et al., 2014; McIntyre et al., 2013; Robert et al., 2014). Consistent with this idea, functional studies indicate that Wnt5 acts as a short-range signal necessary for specification of cells along the border ectoderm, not for broad Fzl1/2/7 signaling (McIntyre et al., 2013). Functional analyses also show that Wnt6 is necessary for the activation and/or maintenance of the endomesoderm GRN mediated by Wnt/β-catenin during blastula stages and that overexpression of Wnt6 promotes endomesoderm gene expression throughout the embryo (Croce et al., 2011; Lhomond et al., 2012). These results are inconsistent with a role for Wnt6 in the Fzl1/2/7 signaling-mediated antagonism of Wnt/β-catenin and/or Fzl5/8-JNK signaling during early AP patterning.

In this study, we show that activation of the non-canonical Fzl1/2/7 signaling pathway during cleavage and blastula stages requires Wnt16 activity. Knockdown of Wnt16-Fzl1/2/7 activity allows the Wnt1/Wnt8-Fzl5/8-JNK pathway to eliminate the ANE GRN from around the anterior pole from the beginning of ANE restriction mechanism. We further show that two Wnt network signaling pathways, Wnt/β-catenin and Fzl5/8-JNK, control zygotic *wnt16* expression in the endoderm and mesoderm during late blastula and gastrula stages. At this time, our data indicate that Wnt16 signaling has a temporally distinct second role during early embryogenesis in regulating the expression of critical endoderm and mesoderm GRNs components and the morphogenetic movements of gastrulation.

RESULTS

The spatiotemporal expression of *wnt16* during early AP specification and patterning

Ubiquitously expressed *wnt16* mRNA is the most abundant maternal Wnt mRNA during early cleavage stages in sea urchin embryos (Stamateris et al., 2010). Although the early spatial expression of *wnt16* had previously been examined in the sea urchin embryo (Stamateris et al., 2010), here we present a more detailed *wnt16* expression pattern analysis. We performed whole mount *in situ* hybridization (WMISH) with *wnt16* antisense probe during the early stages of AP specification and patterning. Transcripts were ubiquitously expressed from the zygote and broad expression was detected until the 32-cell stage (Fig. 1Ba-b). Beginning at 60-cell stage, an enrichment of *wnt16* transcripts was detected in posterior endomesoderm cells (Fig. 1Bc-e). Around the mid-blastula stage, the expression levels of *wnt16* transcripts decreased in anterior cells and were strongly upregulated in the endoderm and mesodermal cell territories in blastula and gastrula stage embryos [18-30 hours post fertilization (hpf) in *Strongylocentrotus purpuratus*] (Fig. 1Bf-j). Vegetal views of late blastula stage embryos showed that *wnt16* was expressed broadly throughout posterior/vegetal cells, and then by mesenchyme blastula stages *wnt16* expression was distributed in a concentric ring around the posterior pole (Fig. 1Bg, 1Bi). Consistent with the WMISH data, qPCR analysis at the same developmental time points showed low levels of *wnt16* transcripts in the zygote and at the 32-cell stage (Fig. 1C), then the level of transcripts decreased until zygotic mechanisms activated *wnt16* expression during the blastula stages, reaching maximal levels by the mesenchyme blastula/early gastrula stages (Fig. 1C). These data indicate that *wnt16* is expressed in a spatiotemporal pattern consistent with an early role in the early AP Wnt network, possibly as an activator of the Fzl1/2/7 signaling pathway. Additionally, *wnt16* expression in the posterior endomesoderm cells at mesenchyme blastula stage suggests that this Wnt ligand could also be involved in the specification of the endomesoderm territory and/or the morphogenetic movements of gastrulation.

Wnt16 is necessary to repress early endomesoderm gene expression and for the specification of the ANE territory

In a previous study we showed that Fzl1/2/7 signaling acts broadly throughout early cleavage/blastula stage embryos as an antagonistic buffer to early posterior Wnt/β-catenin signaling. In the absence of Fzl1/2/7 activity, nuclear β-catenin transcriptional activity was upregulated by approximately 2.5 fold and the expression of *wnt8* expanded towards the anterior of the embryo (Range et al., 2013). Based on the broad, early expression pattern of *wnt16*, we hypothesized that Wnt16 may activate Fzl1/2/7 signaling at this time. To test this idea, we used two different morpholino oligonucleotides to perturb Wnt16 function and examined the expression of several core endomesodermal regulatory genes known to be activated by posterior/vegetal Wnt/β-catenin signaling (Emily-Fenouil et al., 1998; Logan et al., 1998; Sethi et al., 2012; Wikramanayake et al., 1998). At the 120-cell stage, the expression of the endomesoderm markers *gataE*, *foxA*, *wnt1*, and *wnt8* was upregulated in Wnt16 morphants (Fig. 2Ah-j, 2An compared with Fig. 2Aa-c, 2Ag). Angle measurements of the surface area occupied by gene expression confirmed the upregulation of these endomesoderm genes in Wnt16 morpholino injected embryos (Fig. 2B). Angle measurements were not performed for *wnt8* expression in Wnt16 knockdowns since it was expressed broadly throughout the embryo (Fig. 2An compared with Fig. 2Ag). Additionally, qPCR analysis confirmed the upregulation of these endomesoderm genes in Wnt16 morphants at 12 hpf (Fig. S2A). In contrast, embryos injected with *wnt16* mRNA showed reduced expression of these endomesoderm genes (Fig. 2Ao-q, 2Au). *wnt16* expression suggested that it could regulate the expression of the other Wnt ligands expressed during early cleavage stages (*wnt4, wnt5*, and *wnt6*). However, manipulating Wnt16 expression did not affect their expression (Fig. 2Ak-m and 2Ar-t compared with 2Ad-f). Together, these data suggest that Wnt16 is necessary to repress posterior endomesoderm GRN activated by Wnt/β-catenin signaling but does not affect the expression of other Wnt ligands during early cleavage stages.

Previous data suggest that the initial nuclear accumulation of β-catenin in posterior blastomeres is ligand independent (Cui et al., 2014; Peng and Wikramanayake, 2013). However, Wnt16 could act in a dominant negative manner to compete with other Wnt ligands during cleavage stages, preventing Wnt ligand/Fzl receptor interactions that inhibit early Wnt/β-catenin

signaling mediated endomesoderm GRN expression. To test this hypothesis, we examined possible interactions among Wnt1, Wnt8, and Wnt16 at the 120-cell stage (Fig. 2C), since Wnt1 and Wnt8 have been implicated in endomesoderm specification during blastula/gastrula stages (Sethi, et al., 2012; Wikramanayake et al., 2004). As expected, overexpression of Wnt1 and Wnt8 did not upregulate the expression of the endomesoderm genes *foxA* and *gataE* at the 120-cell stage (*cf.* Fig. 2Cb-c, 2Ch-i) whereas their expression was eliminated in *wnt16* mRNA-injected embryos (*foxA*: 95%; *n*=60/63; Fig. 2Cd) (*gataE*: 89%; *n*=72/81; Fig. 2Cj). In embryos co-injected with *wnt16* mRNA and either *wnt1* or *wnt8* mRNA the expression of these genes was also strongly downregulated in a majority of embryos (Fig. 2Ce-f, 2Ck-l). Interestingly, some co-injected embryos showed faint expression of *foxA* and *gataE* expression compared with embryos injected only with *wnt16* mRNA (Fig. 2Ce-f, 2Ck-l small panels). As previously reported, *foxq2* expression is severely downregulated in embryos injected with *wnt1* mRNA (*n*=60/66) (Fig. S4B) and *wnt8* mRNA-injected embryos (*n*=70/80) (Fig. S4C) at 120-cell stage compared with control embryos (Fig. S4A). In the large majority of embryos, co-injections of *wnt16* mRNA together with *wnt1* mRNA (*n*=80/94) (Fig. S4E) or *wnt8* mRNA (*n*=65/72) (Fig. S4F) did not rescue *foxq2* expression. Taken together, our data indicate that Wnt16 does not interfere with the nuclear accumulation of β-catenin by interfering with Wnt1 or Wnt8, but that high levels of Wnt1 and Wnt8 could slightly interfere with Wnt16's ability to activate the Fzl1/2/7 receptor.

Fzl1/2/7 signaling also antagonizes the ANE restriction mechanism mediated by Wnt/β-catenin in posterior cells and Wnt1/Wnt8-Fzl5/8-JNK signaling in anterior ectoderm cells. If Fzl1/2/7 signaling is blocked, these pathways appear to precociously eliminate the ANE GRN as early as the 32- to 60-cell stage (Range et al., 2013). Thus, we examined the expression of the two earliest genes known to be activated in the ANE GRN, *six3* and *foxq2*, in Wnt16 knockdown embryos during early cleavage stages. The expression of both genes was downregulated in 120-cell stage embryos (Fig. 2Dg-h compared with Fig. 2Da-b), suggesting that Wnt16 is necessary for their early activation. Similarly, the expression of ANE GRN genes around the anterior pole at the end of ANE restriction (mesenchyme blastula stage/24 hpf) depended on Wnt16 function (Fig. 2Dc-f versus Fig. 2Di-l). Consistent with the WMISH data, qPCR analysis showed that the transcripts levels per embryo for genes in the 24 hpf ANE regulatory network were consistently downregulated in mesenchyme blastula embryos injected with Wnt16 morpholinos (Fig. S1B). In contrast to these results, embryos injected with Wnt7 morpholino expressed *foxq2*, indicating

that it is not necessary for ANE specification (Fig. S3C). Together, these data indicate that Wnt16 is necessary from the earliest stages for specification of the ANE territory and to antagonize the ANE restriction mechanism.

Wnt16-Fzl1/2/7 signaling is required to antagonize the Wnt1/Wnt8-Fzl5/8-JNK-mediated ANE restriction mechanism

We reasoned that overexpression of Wnt16 should antagonize the ANE restriction mediated by Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling if the Wnt16 ligand is necessary to activate Fzl1/2/7 signaling. Consistent with this idea, expression of the ANE cardinal regulator *foxq2* was expanded throughout the anterior hemisphere in mesenchyme blastula stage embryos injected with *wnt16* mRNA (Fig. 3A). This expansion of ANE factors is remarkably similar to expanded *foxq2* expression observed in embryos lacking functional Fzl5/8-JNK signaling (Kahdka et al., 2017; Range et al., 2013; Range and Wei 2016; Range, 2018). Next, we asked whether blocking Fzl5/8 function could rescue ANE expression in embryos lacking Wnt16. In three different batches of embryos, we injected one set of zygotes with mRNA encoding a dominant negative form of the Fzl5/8 receptor (ΔFzl5/8) (Croce et al., 2006b; Range et al., 2013) (Fig. 3Bc), a second set with Wnt16 MO1 (Fig. 3Bb), and a third with Wnt16 MO1 and ΔFzl5/8 (Fig. 3Bd). When we blocked the function of Fzl5/8 by injecting ΔFzl5/8, the expression of *foxq2* was expanded in the anterior half of mesenchyme blastula embryos, confirming our previous report (Range et al., 2013) (Fig. 3Bc). As shown above, *foxq2* expression was undetectable in 95% of embryos lacking Wnt16 (*n*=70/74; Fig. 3Bb). In contrast, a large majority of zygotes doubly injected with Wnt16 MO1 and ΔFzl5/8 mRNA showed rescue of *foxq2* expression (91% rescue; *n*=63/69; Fig. 3Bd). These results indicate that Wnt16 activity antagonizes Wnt1/Wnt8-Fzl5/8-JNK signaling during the ANE restriction process, allowing the proper size of the final ANE territory.

In addition, we studied the epistatic relationship between Wnt16 and Fzl1/2/7 receptor to investigate whether the Wnt16 ligand functions to activate the Fzl1/2/7 signaling pathway. Similar to the above experiments, in 3 different batches of embryos, we injected one set of zygotes with *wnt16* mRNA, another set with Fzl1/2/7 morpholino, and a third set with both *wnt16* mRNA and Fzl1/2/7 morpholino (Fig. 3C). At the 32-cell stage, the ability of

overexpressed *wnt16* to inhibit the restriction mechanism cannot be assessed, but *foxq2* was expressed in 98% of these embryos (*n*=64/65; Fig. 3Cc). In contrast, *foxq2* expression was completely eliminated in 94% of Fzl1/2/7 morphants (*n*=58/62; Fig. 3Cb), as well as in 91% of the embryos injected with both Fzl1/2/7 morpholino and *wnt16* mRNA (*n*=64/70; Fig. 3Cd). The expression of *foxq2* was expanded broadly in the ectoderm of mesenchyme blastula stage embryos (24 hpf) injected with mRNA encoding *wnt16* (93%; *n*=71/76; Fig. 3Cg compared with 3Ce), but its expression was eliminated in Fzl1/2/7 knockdown embryos (85%; *n*=55/65; Fig. 3Cf) and in embryos co-injected with *wnt16* mRNA and Fzl1/2/7 morpholino (80%; *n*=57/71; Fig. 3Ch). These results strongly support the conclusion from Wnt16 loss-of-function analyses that the Fzl1/2/7-dependent antagonism of Fzl5/8-mediated ANE restriction is activated by the Wnt16 ligand.

Dynamic change in gene expression patterns of *wnt16*, *eve*, *foxA*, and *gcm* during early endoderm and mesoderm patterning

While the zygotic spatiotemporal expression pattern of zygotic *wnt16* has been reported elsewhere (Cui et al., 2014; Robert et al., 2014; Stamateris et al., 2010), it is still unclear in which endoderm and mesoderm cells it is expressed leading up to gastrulation. To better understand the molecular signature of Wnt16 during these stages, we analyzed the spatiotemporal expression relationship between *wnt16* and three well characterized genes in the endoderm and mesoderm GRNs (*eve, foxA*, and *gcm*). As previously reported by Peter and Davidson, 2011, by late blastula (18 hpf) and mesenchyme blastula (24 hpf) stages the endoderm and mesoderm territories have segregated. At these times, *eve* expression defines the anterior most Veg1 ring of endoderm cells (Fig. 4Ab, 4Ad, 4Bb, 4Bd), *fork-head box A* (*foxA)* defines the more posterior endoderm Veg2 ring of cells (Fig. 4Af, 4Ah, 4Bf, 4Bh), and mesoderm regulatory gene *glial cells missing* (*gcm)* expression is detected in the inner Veg2 ring (Fig. 4Aj, 4Al). Here, we show that *wnt16* was co-expressed with *eve, foxA*, and *gcm* at late blastula stage, indicating that it is expressed throughout the endoderm and mesoderm territories (Fig. 4Ac, 4Ag, 4Ak). By mesenchyme blastula stage, *wnt16* was downregulated around the most posterior Veg2 mesoderm region but was expressed in a ring of cells overlapping both *eve* and *foxA* expression, indicating that it was transcribed in both the Veg1 and Veg2 endoderm cells at this time (Fig.

4Bc, 4Bg). These results suggest that Wnt16 may have a role in inducing and/or maintaining the expression of endoderm GRN components by the beginning of gastrulation.

The AP Wnt signaling network regulates *wnt16* expression and its role in the specification of endoderm GRN components

Wnt16's zygotic expression profile, with *wnt16* expression in the endoderm and mesoderm regions, suggests that the Wnt signaling network could control its expression. To test this idea, we used morpholino knockdowns or injected mRNA to perturb each of the three Wnt signaling branches. Embryos injected with mRNA encoding Axin, which prevents nuclearization of β-catenin, showed a severe reduction of *wnt16* expression at mesenchyme blastula stage (Fig. 5Ab). Similarly, *wnt16* expression was downregulated in embryos injected ΔFzl5/8 mRNA at the same developmental stage (Fig. 5Ac; Fig. S1C). In contrast, when we perturbed Fzl1/2/7 signaling, *wnt16* expression appeared similar to that in control embryos (cf. Fig. 5Aa, 5Ad). These analyses demonstrate that two of the three signaling branches in the Wnt network are critical for zygotic *wnt16* expression in the posterior cells that are about to undergo gastrulation at the mesenchyme blastula stage.

Fzl5/8 signaling is necessary for the morphogenetic movements of gastrulation (Croce et al., 2006b) and the activation of Wnt16 by Fzl5/8 in posterior cells correlates with a role in the endodermal GRN governing this process. In addition, a recent study suggested that critical components of the anterior Veg1 and posterior Veg2 endoderm GRNs (*eve, blimp1b*, and *hox11/13b*) were slightly downregulated in the absence of Wnt16 (Cui et al., 2014). Therefore, we examined the role of Wnt16 in the activation and/or maintenance of the endoderm GRN at the beginning of gastrulation. Several endoderm GRN components (*gataE*, *foxA*, *wnt1*, and *wnt8*) were expressed normally in Wnt16 knockdown embryos (Fig. 5Be-h compared with 5Ba-d). However, the expression of *eve*, *blimp1b*, and *hox11/13b* was downregulated in Wnt16 knockdown embryos consistent with the observations in Cui et al. (2014) (Fig. 5Bl-n compared with 5Bi-k). Together, these data suggest that Wnt16 is necessary for the activation and/or maintenance of specific Veg1 and Veg2 endoderm GRN components downstream of Fzl5/8 signaling during gastrulation.

The function of Wnt16 in the morphogenetic movements during gastrulation and mesoderm morphogenesis

The experiments above showing the activation of Wnt16 by Fzl5/8 signaling suggested that Wnt16 might be necessary for the morphogenetic movements involved in gastrulation. Therefore, we observed the morphological phenotypes of embryos injected with Wnt16 morpholino at several stages of development. Cleavage occurred on schedule and embryos developed normally until the mesenchyme blastula stages (Fig. 6Af). At this stage, we observed unorganized mesodermal cells in the blastocoel in Wnt16 morphants (Fig. 6Af compared with 6Aa). During gastrula stages, invagination of the gut was severely disrupted in Wnt16 morphants (Fig. 6Ag-i versus 6Ab-d). By pluteus stages, the gut was small and often partially exogastrulated (cf. Fig. 6Ae, 6Aj). In addition, Wnt16 morphants did not form a skeleton. These phenotypes are similar to those we have observed in Fzl1/2/7 morphants at the pluteus larva stage (Range et al., 2013).

Although it appeared that the mesodermal cells in the blastocoel were disorganized, it is possible that there was an increase in the number of either skeletogenic and/or non-skeletogenic (NSM) mesoderm in Wnt16 knockdown embryos. To test this potential increase, we counted the total number of pigment cells, a subset type of NSM cells. Pluteus stage embryos injected with Wnt16 morpholino did not show an increase in the number of NSM pigment cells produced by the embryo (Control = 74.5±6.7, n=21; Wnt16 MO1 = 71±7.7, n=21), but the arrangement of these cells appeared disrupted (Fig. 6Ba versus 6Bb). To determine the identity of the disorganized cells at mesenchyme blastula stage, we co-immunostained embryos with the skeletogenic marker 1d5 and the general mesodermal marker Meso1. 1d5 staining and Meso1 staining suggested that the number of skeletogenic and NSM cells were normal in both control and Wnt16 morphants (Fig. 7Aa-d, 7Ae-h) but that they were randomly distributed throughout the blastocoel. Finally, to develop a better understanding of how Wnt16 affects the morphogenetic movements of gastrulation, we analyzed the patterns of actin accumulation in the sea urchin embryo. F-actin accumulation measured by Phalloidin binding was present in the invagination of both phenotypic control and Wnt16 morpholino injected embryos at mesenchyme blastula stage (Fig. 7Ba-d). Together, these data suggest that Wnt16 does not affect the actin accumulation around the presumptive blastopore as well as skeletogenic and pigment

cell numbers during gastrulation, but that it is involved in the proper arrangement of mesodermal cells in the blastocoel.

DISCUSSION

The data presented here indicate that the secreted Wnt ligand, Wnt16, plays a critical role in Wnt signaling governing early AP axis specification, patterning, and gastrulation. A posterior-to-anterior wave of sequential Wnt/β-catenin and Wnt/JNK signaling is essential for the proper positioning of the earliest GRNs along the AP axis: posterior endoderm and mesodermal GRNs, equatorial ectodermal GRN, and the ANE GRN around the anterior pole (Khadka et al., 2018; Range, 2018; Range et al., 2013; Range et al., 2017). Importantly, Fzl1/2/7 signaling activity appears to interfere with the level of posterior Wnt/β-catenin signaling as well as the timing and/or level of anterior Wnt/JNK signaling, allowing for the proper positioning of these fundamental embryonic territories (Range, 2018; Range et al., 2013). Here, our data from a series of expression studies, functional perturbations, and epistasis experiments strongly suggest that an early role of Wnt16 is to activate the Fzl1/2/7 signaling pathway during this fundamental AP patterning process. Subsequently, we show that two of the Wnt signaling network pathways, Wnt/β-catenin and Fzl5/8-JNK, activate zygotic *wnt16* expression in the endoderm and mesoderm during mid-to-late blastula stages. At this developmental stage, Wnt16 activates a distinct mechanism necessary for the regulation of a specific set of endoderm and mesoderm regulatory factors and the morphogenetic movements of gastrulation.

Increasing the level of Wnt/β-catenin signaling, either through relieving negative or increasing positive inputs, causes an increase in endomesoderm while also eliminates anterior GRNs during the early embryonic AP axis specification in many metazoan embryos (Angerer et al., 2011; Gaspard et al., 2008; Kiecker and Niehrs 2001; Lekven et al., 2001; Loh et al., 2016; Nordström et al., 2002; Pani et al., 2012; Petersen and Reddien 2009; Range et al., 2013). Our previous functional data showed that if the broad signaling activity of Fzl1/2/7 signaling is eliminated in sea urchin embryos, then Wnt/β-catenin signaling reporter gene activity increases by ~2.5 fold at the 60-cell stage (Range et al., 2013). Also shown was that the expression of *wnt8*, which is activated by Wnt/β-catenin signaling (Minokawa et al., 2005; Wikramanayake et al., 2004), is expanded towards the anterior of early blastula staged embryos (Range et al., 2013).

We show here that *wnt16*, like *fzl1/2/7*, is broadly expressed during early cleavage stages and that, similar to Fzl1/2/7 knockdowns, there is also only a modest increase in the expansion of endomesoderm GRN component expression activated by Wnt/β-catenin in the absence of Wnt16. In addition, when we overexpressed Wnt16, the expression of these genes was suppressed but not completely downregulated. Together, these data suggest that Wnt16-Fzl1/2/7 signaling provides negative, but not prohibitive, inputs into posterior Wnt/β-catenin signaling, suggesting that they are not the only factors necessary to maintain the correct level of Wnt/β-catenin signaling along the AP axis. Interestingly, we also show that by 120-cell stage, *wnt8* expression is expanded towards the anterior in the absence of Wnt16 function. During blastula stages in normal embryos, *wnt8* expression is activated in ectoderm cells where the ANE GRN is downregulated, suggesting that it is driving the Fzl5/8-JNK-mediated downregulation in the equatorial ectoderm. We propose that this precocious ectodermal Wnt8 expression in Wnt16 morphants may drive premature downregulation of the ANE GRN by Wnt/JNK signaling in anterior ectodermal blastomeres.

As mentioned in the introduction, an undefined broadly active regulatory mechanism can activate the ANE GRN throughout the sea urchin embryo (Range 2014; Range 2018; Range et al., 2013). Wnt/β-catenin signaling in posterior cells and, subsequently, Wnt/JNK in the anterior cells restrict this broad ANE potential to an area around the anterior pole (Range et al., 2013). These pathways are connected by a precisely timed relay mechanism. At the 32-cell stage, Wnt/β-catenin activates Wnt1 and Wnt8 in posterior blastomeres and our functional data suggest that these factors diffuse into anterior cells where they stimulate Fzl5/8-JNK-mediated ANE GRN downregulation as early as the 60-cell stage (Range et al., 2013) (Fig. 8A). In this study, our epistasis experiments strongly suggest that Wnt16 is necessary to activate the Fzl1/2/7 signaling pathway as early as the 32-cell stage to regulate this early phase of ANE restriction, most likely at the intracellular level since knocking down the Fzl1/2/7 receptor gives the same phenotype as the Wnt16 ligand. In a recent study, we also showed that a novel, broadly expressed secreted Frizzled-like protein, sFRP-1, is necessary from the beginning of ANE restriction to antagonize Fzl5/8-JNK signaling (Khadka et al., 2018). Similar to the effect of knocking down Wnt16 or Fzl1/2/7, the ANE GRN is completely downregulated in sFRP-1 morphants during early cleavage stages (Khadka et al., 2018). In addition, overexpression of sFRP-1 prevents the downregulation of the ANE GRN by Fzl5/8-JNK signaling from the

anterior half of the embryo (Khadka et al., 2018), mimicking the Wnt16 overexpression phenotype. During early cleavage stages, *wnt16*, *sfrp-1*, *fzl5/8*, and *fzl1/2/7* are expressed in the same cells. This overlap suggests that a complex interplay among these factors operates in the extracellular space. Here, it is important to note that our previous functional data indicate that sFRP-1 does not interfere with Wnt16-Fzl1/2/7 signaling. If it did, then embryos injected with sFRP-1 morpholino or mRNA would show opposite phenotypes, i.e. expansion and elimination of the ANE, respectively. Our model proposes that sFRP-1 interferes with Wnt1 and Wnt8 stimulation of Fzl5/8-JNK at the extracellular level (Khadka et al., 2018), but does not interfere with Wnt16-Fzl1/2/7 signaling which antagonizes the ANE restriction mechanism at the intracellular level. Thus, these two mechanisms work coordinately, but separately, to precisely control the early stages of ANE restriction (Fig. 8A).

As the broadly expressed, maternally supplied *wnt16* mRNA is progressively eliminated from anterior ectodermal cells during early to mid-blastula stages, zygotic *wnt16* expression can be observed around the 120-cell (12 hpf) to mid-blastula stage (15 hpf). Similar to previous studies on zygotic *wnt16* expression that used colorimetric WMISH in *Strongylocentrotus purpuratus* (Cui et al., 2014) and the Mediterranean sea urchin, *Paracentrotus lividus* (Robert et al., 2014), our analyses showed that posterior endoderm and mesoderm cells express zygotic *wnt16*. In Cui et al., (2014), the authors argued that *wnt16* is expressed in the mesodermal territory from 120-cell stage to mid-blastula followed by its expression being cleared from this territory by late blastula stage (18 hpf) when endoderm and mesodermal cells become distinct. Then, *wnt16* is transiently expressed in a ring of posterior-most endodermal/Veg2 cells where it is downregulated by the beginning of gastrulation (24 hpf) and subsequently upregulated in more anterior Veg1 endodermal cells. They hypothesized that Wnt16 is involved in a posterior-to-anterior inductive cascade from Veg2 mesoderm to Veg2 then Veg1 endoderm that ultimately influences the dynamic expression of *hox11/13b, eve, blimp1, and wnt16* (Cui et al., 2014). Both our colorimetric and more detailed two-color FISH analyses contradict this conclusion. Our results indicate that *wnt16* is broadly expressed throughout the entire endoderm and mesoderm territory at late blastula stage (18 hpf), with the most anterior expression overlapping *eve* expression, which marks the more anterior Veg1 endodermal cells that form the hindgut and mid-gut. We also observed a downregulation of *wnt16* in the most posterior mesodermal cells by mesenchyme blastula as did Cui et al., (2014) and Robert et al., (2014). Our results indicate that

there is no transient expression and downregulation of *wnt16* from posterior endodermal Veg2 cells to the more anterior Veg1 cells; instead, *wnt16* remains expressed in a concentric ring throughout the Veg1 and Veg2 endodermal cells at mesenchyme blastula stage (24 hpf). Thus, our observations suggest that Wnt16 is not involved in a posterior-to-anterior inductive cascade in the endoderm and mesoderm. Instead, our data indicate that it can act directly on the Veg2 mesodermal GRN in blastulae (12 - 18 hpf) as well as both Veg1 and Veg2 endodermal cell GRNs from late blastula to mesenchyme blastula stages (18 - 24 hpf).

To get a fuller appreciation of Wnt16 during early development, we also explored the upstream regulation of zygotic *wnt16* expression as well as the downstream GRNs and cellular processes it controls. We show that two of the three AP Wnt signaling network pathways, Wnt/β-catenin and Fzl5/8-JNK, are necessary for *wnt16's* zygotic expression. Wnt/β-catenin is necessary for the activation of the entire endomesoderm GRN, so it is not surprising that *wnt16* is downregulated. It is interesting that Fzl5/8 signaling is necessary for *wnt16* expression in the endomesoderm. Fzl5/8 signaling has been shown to activate the expression of genes, like *brachyury*, that are necessary for gastrulation in the sea urchin embryo (Croce et al., 2006b). Similar to embryos in which Fzl5/8 signaling is inhibited, gastrulation does not occur in the absence of Wnt16. These data suggest that Wnt16 may work downstream of Fzl5/8 and may not feedback into this signaling pathway. Instead, it may signal through the three other Frizzled receptors expressed within the same territory by the onset of gastrulation to influence the morphogenetic movements of gastrulation and gut pattern. Consistent with this idea, gastrulation is severely perturbed in Fzl1/2/7 morphants (Range et al., 2013). Given the conservation of many aspects of the early sea urchin endomesoderm GRN among species and the fundamental role of Wnt signaling during early AP specification and patterning, it is important in the future to perform more detailed explorations of the complex interactions among the several Wnt ligands (WntA, 1, 2, 4, 5, 6, 7, 9, 16, depending on the sea urchin species) and all four Frizzled receptors expressed during the developmental processes leading up to gut patterning and gastrulation.

Downstream of Wnt16, Cui et al., (2014) previously showed that three important endoderm GRN components (*eve*, *hox11/13b*, and *blimp1b*) appeared slightly downregulated in the absence of Wnt16. However, embryos showed light expression of these genes in Wnt16 morphants analyzed by WMISH, suggesting that Wnt16 is only partly necessary for the activity of these genes. Here, we show that all three of these regulatory genes were completely

downregulated in our experiments, reinforcing their data. Importantly, both Blimp1b and Hox11/13b knockdowns show severe defects in gastrulation consistent with a role downstream of Wnt16 (Arenas-Mena et al., 2006; Livi and Davidson, 2006) and; interestingly, Hox11/13b knockdown embryos show disorganized mesodermal cell populations at mesenchyme blastula stage remarkably similar to those seen in Wnt16 morphants (Arenas-Mena et al., 2006). Again, this is consistent with a role downstream of Wnt16. We analyzed this phenotype in greater detail and our results suggest that these cells are primarily mesodermal and that it does not appear that there is an obvious increase in either skeletal or non-skeletogenic mesoderm cells. Interestingly, the larval skeleton does not form in Wnt16 morphants. Wnt16 does not appear to be necessary for the specification of the Veg1 ectoderm GRN (Cui et al., 2014), which is necessary for proper arrangement of the larval skeleton (Duloquin et al., 2007). Thus, we prefer the hypothesis put forth by Arenas-Mena et al. (2006) that cells lacking Hox11/13b could have cell adhesion defects that prevent proper interactions among the mesodermal cells and/or the ectoderm. Alternatively, it could be that these cells have defects in uncharacterized cell-to-cell signaling pathways important for correct cellular migration and/or interactions among themselves as well as the ectoderm. Finally, we tested the idea that Wnt16 and/or the GRNs it activates may affect cytoskeletal rearrangements necessary for gastrulation (Beane et al., 2006); however, our data suggest that Wnt16 affects the directed cellular movements of gastrulation by an unknown mechanism. Together, these data give us a better, but still incomplete, picture of Wnt16's role(s) during the early stages of gastrulation. In the future, it will be interesting to identify more intermediate and terminal GRN components in endoderm downstream of Wnt16 to determine the cellular and molecular mechanism(s) that are necessary for gastrulation and mesodermal cell behavior.

Relatively few studies have been performed on the role of Wnt16 during embryonic development, and all have been performed in vertebrate species (Fokina and Frolova 2006; Movérare-Skrtic et al., 2014; Nalesso et al., 2017). As with most Wnt ligands, Wnt16 is involved in multiple developmental processes, and it can stimulate canonical Wnt/β-catenin as well as non-canonical Wnt signaling. For instance, Wnt16 is expressed in the ciliary margin zone of the chicken embryo and works to maintain retinal progenitor cells in an undifferentiated state through a Wnt/β-catenin-dependent pathway (Fokina and Frolova, 2006). A recent set of interesting studies in *Xenopus* embryos illustrates the complex nature of Wnt16 and Wnt

signaling in general. Movérare-Skrtic et al., (2014) showed that during chondrocyte development, Wnt16 acts as a weak activator of Wnt/β-catenin. Then, Nalesso et al., (2017) showed that Wnt16 can also act as an antagonist of Wnt/β-catenin if other Wnt proteins are administered and that this mechanism works through non-canonical signaling. These studies illustrate the multifunctional roles of Wnt ligands, often in the same cells and territories, and also show that Wnt16 can activate a non-canonical pathway that antagonizes Wnt/β-catenin signaling similar to its role in early AP axis specification in the sea urchin (Fokina and Frolova 2006; Movérare-Skrtic et al., 2014; Nalesso et al., 2017).

In chordates, there are no studies to our knowledge that focus on a role for Wnt16 during early AP axis specification and patterning. However, studies have been performed using qPCR and/or WMISH in invertebrate deuterostomes to analyze the spatiotemporal expression of Wnt and Frizzled gene expression during this fundamental developmental process. The spatiotemporal *wnt16* expression pattern in these embryos is remarkably similar to that in sea urchin embryos. Although *wnt16* is not maternally expressed in sea star embryos, a low level of *wnt16* expression was observed at the hatched blastula stage similar to sea urchins (McCauley et al., 2013). In addition, *wnt16* expression is upregulated in the endomesoderm during late blastula/gastrula stages (McCauley et al., 2013). Similar to sea stars, *wnt16* is not expressed maternally in hemichordates, but low levels are observed throughout the embryo around blastula stages. At gastrula stages, higher expression levels are localized around the exterior edge of the blastopore lip (Darras et al., 2018). Although Wnt16 is not maternally provided in either sea stars or hemichordates, its early broad expression suggests that it could still play a role in early AP axis specification in these embryos. Importantly, ANE GRN restriction appears to initiate during the blastula stages in each of these species as opposed to early cleavage stages in sea urchin embryos (Darras et al., 2018; Pani et al., 2012; Range, 2014; Yankura et al., 2013). In addition, *fzl1/2/7* is expressed maternally and broadly expressed throughout the cleavage and early blastula stages in these embryos (McCauley et al., 2013; Range, 2014; Yankura et al., 2013). Based on these studies, it is tempting to speculate that Wnt16-Fzl1/2/7 signaling may play a similar role in AP patterning and/or gastrulation movements in these organisms.

ACKNOWLEDGEMENTS

We thank Dr. Robert Angerer for helping edit the manuscript, Dr. Athula Wikramanayake for his careful reading of the manuscript and suggestions, Dr. David McClay for the 1d5 antibody, Dr. Gary Wessel for the Meso1 antibody, and Dr. Jia Song for the *eve* construct. NIH R15HD088272-01, as well as the Department of Biological Sciences at Auburn University, provided support to RCR for this project.

MATERIALS AND METHODS

Animals and embryo cultures

Adult *Strongylocentrotus purpuratus* sea urchins were obtained from Monterey Abalone Company (Monterey, CA, USA), Marinus Scientific (Longbeach, CA), and California Institute of Technology (Pasadena, CA, US). The gametes were collected by injecting 0.5 M KCl into the body cavity of adult sea urchins. Fertilized embryos were cultured at 15^0C in artificial seawater (ASW).

RNA extraction and cDNA clone preparation

RNA from embryos at different times of development was extracted and purified using RNeasy Plus Mini kit (Qiagen®). Purified RNA was reverse transcribed to cDNA using SuperScript IV First-Strand Synthesis System (Invitrogen) and random primers for RT-PCR kit. Total RNA samples for qPCR were treated with DNase I from the DNA-free kit (Invitrogen) to remove any possible residual genomic DNA. cDNA from 24 hpf mesenchyme blastula stage embryos was used to obtain full-length clones for *wnt16*. The following primers, based on the sea urchin genome sequence, were used to insert *wnt16* into pGEMT®-easy and pCS2+ for in situ probe and mRNA synthesis, respectively: In situ = Forward 5'-ATATCATGGACTGCGGACTA; Reverse 3'-GTCCATGGTTTAAGCAGACC; pCS2+ vector = Forward 5'-CGCGGATCCACCATGGAGTGTAGCAAT; Reverse 3'-CCGCTCGAGTCATTTACAAGTGTAGAT.

mRNA overexpression and morpholino injections

For overexpression studies the Wnt16-pCS2+, ΔFzl5/8-pCS2+, and Axin-pCS2+ vectors were linearized with Not1 restriction enzyme. mRNA was synthesized using the SP6 mMessage Machine kit (Ambion) according to the manufacturer's protocol, further purified by LiCl precipitation and injected into the embryos at the following concentrations: ΔFzl5/8 mRNA = 1.0 µg/µL; *wnt1* mRNA = 0.05 µg/µL; *wnt8* mRNA = 0.65 µg/µL; *wnt16* mRNA = 0.5 µg/µL; *axin* mRNA 1.5-2.0 µg/µL.

S. purpuratus EST, genomic and cloned *wnt16* sequence was used to generate translation-blocking morpholino-substituted oligonucleotides 1 (MO2) (Gene-Tools LLC, Eugene, OR). The sequences and injection concentrations of all the morpholino oligomers were as follows:
Fzl1/2/7 MO: 5'-CATCTTCTAACCGTATATCTTCTGC -3' (1.3mM) (Range et al., 2013);
Wnt16 MO1: 5'-TCTCAACAAACTCGATAGTTCAACC -3' (0.8 mM) (Cui et al., 2014);
Wnt16 MO2: 5'-CAAAACATCGGTAGCTTAAATCCAT -3' (0.35 mM);
Wnt7 splice MO: 5'-TCCTCGTCGTATATCCTTACCAGCA-3' (1.5 mM).

As a control for morphological and developmental defects related to injections, we used a standard control morpholino: 5'-CCTCTTACCTCAGTTACAATTTATA-3' from Gene-Tools LLC, Eugene, OR. Standard control and experimental morpholinos were injected at the same concentrations. For mRNA and morpholino microinjections, eggs were de-jellied by passing them through 74µm mesh Nitex, plated in rows on a culture dish coated with 25% protamine sulfate and fertilized with diluted sperm. After fertilization, embryos were immediately injected with 15% FITC (2.5 µg/ml), 20% glycerol and mRNA and/or morpholino oligonucleotides and cultured at 15^{0}C until the desired developmental time. For each mRNA or morpholino injection experiment, 50-200 embryos from at least three batches of different mating pairs were used. Only experiments with changes in phenotype or marker expression in at least 85-90% of the injected embryos were considered conclusive.

Quantitative polymerase chain reaction (qPCR)

qPCR assays were performed as described previously (Wei et al., 2009). Each qPCR experiment was repeated with embryos from at least three different mating pairs and each PCR reaction was carried out in triplicate for each developmental stage. The qPCR primer set information for ANE GRN is given in (Range 2013). The endomesodermal and Wnt16 primers are included in

Supplementary Table 1. To calculate the developmental expression levels of *wnt16*, the number of transcripts per embryo was estimated based on the ΔCt value of the z12 transcript (Range et al., 2013; Wang et al., 1995). To compare differential expression between control and perturbed embryos, mitochondrial 12s RNA ΔCt values were used to normalize the relative target gene expression levels. In differential gene expression, 2-fold or higher change in expression level was considered to be significant.

Whole-mount In Situ Hybridization

Antisense RNA probes, complementary to the target mRNA, for each gene analyzed were synthesized from linearized pGEM®-T Easy or pCS2+ plasmids using T7 or SP6 polymerase enzyme. Alkaline phosphatase reporter and two-color fluorescent *in situ* hybridization were carried out as previously described (Wei et al., 2009; Sethi et al., 2014). For the two-color *in situ* hybridization (Fig. 4), *wnt16* was labeled with DIG and detected with fluorescein-TSA and *eve, foxA* and *gcm* were labeled with fluorescein and detected with Cy3-TSA.

Immunohistochemistry

Embryos were fixed in 2% paraformaldehyde in artificial seawater for 20 minutes at room temperature. For 1d5 and Meso1 staining, embryos were washed five times in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Embryos were incubated at 4ºC overnight with primary antibodies against 1d5 (1:25) and Meso1 (1:50) in PBST and 4% normal goat serum. Primary antibodies were detected by incubating embryos for 1 hour at room temperature with 1:1000 Alexa Fluor-coupled 488 goat anti-mouse IgM and 1:1000 Alexa Fluor-coupled 555 goat anti-mouse IgG secondary antibodies (Thermo Fisher Scientific). For actin/phalloidin staining, embryos were washed three times through phosphate-buffered saline containing 0.1% Triton X-100 and kept overnight at 4ºC. Embryos were incubated with 1:2000 Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific) in 3% BSA in PBST 0.1% Triton overnight at 4ºC. Nuclei were stained with DAPI (1:3000).

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FIGURE LEGENDS

Figure 1. Spatiotemporal expression of the *wnt16* ligand during early anterior-posterior specification and patterning. (A) Diagram showing a model for the areas of Wnt/β-catenin, Fzl5/8-JNK, and Fzl1/2/7 signaling during ANE early AP patterning consistent with data from (Khadka et al., 2018; Range, 2018; Range and Wei, 2016; and Range et al., 2013). (B) Whole mount *in situ* hybridization analysis of *wnt16* expression during ANE restriction. (Ba, b) Expression of *wnt16* mRNA transcripts was first detected and they were broadly expressed throughout zygotes and 32-cell stage embryos. (Bd-j) Between 120-cell stage and late gastrula (30 hpf), *wnt16* expression was progressively downregulated from anterior and equatorial ectoderm cells, resulting in a localized expression in the posterior endoderm and mesoderm region of the embryo. (C) qPCR measurements showing the temporal expression of *wnt16* transcripts from three different batches of embryos from egg to mesenchyme blastula stages (24 hpf). The number of *wnt16* transcripts per embryo (*y*-axis) is based on the Ct value of *z12* transcripts. The absolute concentrations of *z12* transcripts are known at each stage (Wang et al., 2004). Scale bars: 20 μm. VV: vegetal view.

Figure 2. Wnt16 represses endomesoderm genes at the 120-cell stage and it is necessary for ANE GRN expression. (A) Expression of the endomesoderm markers *gataE*, *foxA, wnt1*, and *wnt8* and three Wnt ligands (*wn4, wnt5,* and *wnt6*) in control, Wnt16 morpholino-injected, and *wnt16* mRNA-injected embryos at the 120-cell stage. (Ah-u compared with Aa-g) The expression of the endomesoderm markers *gataE*, *foxA, wnt1*, and *wnt8* was upregulated in Wnt16 MO1 injected embryos (Ah-j, n compared with Aa-c, g). *wnt16* mRNA overexpression downregulated the expression of the endomesoderm markers *gataE*, *foxA, wnt1*, and *wnt8* (Ah-j, n compared with Aa-c, g). Solid lines indicate the posterior boundaries of each endomesoderm marker. The expression of *wnt4*, *wnt5*, and *wnt6* ligands was not affected in Wnt16 morpholino-injected and *wnt16* mRNA-injected embryos (Ak-m, and Ar-t compared with Ad-f). (B) The angle α shown in A was measured from three batches using ImageJ. Volume=0.5(1-cos α/2) was used to calculate the percentage of the surface area (± s.e.m) occupied by the endomesoderm territories in control and Wnt16 knockdowns. (C) Wnt1 and/or Wnt8 might interact with Wnt16 to repress endomesoderm genes at the 120-cell stage. The expression of the endomesoderm

markers *foxA* and *gataE* was not affected in *wnt1* or *wnt8* mRNA injected embryos (Cb-c and Ch-i) compared with control (Ca, g). In embryos overexpressing *wnt16* mRNA, *foxA* and *gataE* expression was severely downregulated (Cd, j). Embryos co-injected with *wnt16* mRNA and either *wnt1* or *wnt8* mRNA showed either a strong downregulation (big panels) or a significantly reduced expression (small panels) of both *foxA* and *gataE* genes (Ce-f and Ck-l). Small panels show significantly reduced phenotypes of *foxA* and *gataE* expression in embryos injected with *wnt1* and *wnt16* mRNA (40% and 33%, respectively) and in embryos injected with *wnt8* and *wnt16* mRNA (33% and 43%). The remaining percentages of embryos observed that show the representative phenotypes depicted are indicated in each panel. (D) *foxq2* and *six3* expression at 120-cell stage in control (Da-b) and Wnt16 morpholino-injected embryos (Dg-h). Expression of ANE makers (*foxq2*, *six3*, *dkk3*, and *sfrp1/5*) in control embryos (Dc-f) and Wnt16 MO1 injected embryos at the mesenchyme blastula stage (24 hpf) (Di-l). MO, morpholino; Scale bars: 20 μm.

Figure 3. Wnt16-Fzl1/2/7 signaling antagonizes the Wnt1/Wnt8-Fzl5/8-JNK pathway during ANE restriction mechanism. (A) The expression of the ANE marker *foxq2* expanded in embryos overexpressing *wnt16* mRNA at the mesenchyme blastula stage (Ab-d compared with Aa-c). (B) At mesenchyme blastula stage, the expression of the cardinal regulator *foxq2* was expanded in ΔFzl5/8 mRNA injected embryos (Bc) compared with control embryos (Ba). In the absence of Wnt16, *foxq2* expression was severely downregulated in ANE (Bb), whereas Wnt16 morphants co-injected with ΔFzl5/8 rescued the expression of ANE factors, showing a normal or expanded *foxq2* expression (91%) (Bd). (C) Control embryos showing *foxq2* expression at 120-cell and mesenchyme blastula stage (24 hpf) (Ca and Ce). ANE expression of *foxq2* was completely eliminated in Fzl1/2/7 morpholino-injected embryos (Cb and Cf). At 120-cell stage, *foxq2* was expressed in embryos injected with *wnt16* mRNA (Cc). At mesenchyme blastula stage, *foxq2* expression was strongly upregulated and expanded towards the posterior pole in embryos injected with *wnt16* mRNA (Cg). Overexpression of *wnt16* in a Fzl1/2/7 morphant background produced a completely elimination of *foxq2* expression, mimicking the Fzl1/2/7 knockdown phenotype (Cd and Ch). MO, morpholino; ΔFzl5/8, dominant negative Fzl5/8; Scale bars: 20 μm.

Figure 4. Gene expression patterns of *wnt16*, eve, *foxA*, and *gcm*. (A) Double *in situs* showing posterior/vegetal views that combine *wnt16* and either *eve* (Aa-c), *foxA (*Ae-g) or *gcm* (Ai-k) at late blastula stage (18 hpf). (Ad, h and l) Schematic diagram showing spatial expression patterns in relation to cell lineage (anterior/veg1 endoderm, posterior/veg2 endoderm and veg2 mesoderm). (B) Posterior/vegetal views that combine *wnt16* and either *eve* (Ba-c) or *foxA (*Ae-g) at mesenchyme blastula stage (24 hpf). (Bd, h) Schematic showing spatial patterns of gene expression in relation to cell linage. Endo, endoderm precursors; mes, mesoderm precursors.

Figure 5. Regulation of *wnt16* expression by the AP Wnt signaling network and the role of Wnt16 in activating endoderm genes. (Aa) Control embryo showing *wnt16* expression in the posterior endoderm and mesoderm regions of the embryo at mesenchyme blastula stage (24 hpf). *wnt16* expression was downregulated in Axin mRNA injected embryos (Ab). *wnt16* expression was downregulated in embryos injected with a dominant negative form of Fzl5/8 (ΔFzl5/8) (Ac). *wnt16* expression was unperturbed in Fzl1/2/7 morphants (Ad). (B) Wnt16 knockdown embryos at mesenchyme blastula stage showing that Wnt16 was not necessary for the expression of the endoderm genes *gataE*, *foxA*, *wnt1* and *wnt8* (Be-h compared with Ba-d) but it was necessary for the expression of *eve*, *blimp1b*, and *hox11/13b* expression (Bl-n compared with Bi-k). MO, morpholino; ΔFzl5/8, dominant negative Fzl5/8; Scale bars: 20 μm.

Figure 6. The role of Wnt16 in the morphogenetic movements during gastrulation.
(A) Morphology of control embryos at 24, 30, 36, 48, and 72 hours post fertilization (hpf) (Aa-e) and embryos injected with morpholino targeting *wnt16* transcripts (Af-j). (B) Control and Wnt16 MO1 injected pluteus larvae. Normal numbers of pigment cells were shown in both control and Wnt16 morphants whereas the arrangement of those cells was disrupted in Wnt16 knockdowns. Control = 74.5±6.7 (21); Wnt16 MO1 = 71±7.7 (21) (Ba-b). Shown are means ± s.d. The total number of embryos counted is in parentheses.

Figure 7. Wnt16 function in mesoderm morphogenesis and gastrulation. (A) 1d5 and Meso1 antibody staining at mesenchyme blastula stage (24 hpf). 1d5 (blue) stains skeletogenic mesoderm cells and Meso1 (orange) is a general mesoderm marker. Neither 1d5 nor Meso1 staining was affected in Wnt16 knockdown embryos (Ae-h) compared with control embryos

(Aa-d). (B) F-actin staining as measured by Phalloidin binding. F-actin accumulation was present in the invagination of both control and Wnt16 knockdown embryos (Ba-d). MO, morpholino.

Figure 8. Model for two phases of Wnt16 activity during early AP axis specification, patterning and morphogenesis of the sea urchin embryo. (A) Broad maternal non-canonical Wnt16-Fzl1/2/7 signaling antagonizes Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling during the ANE restriction process. Illustrated is an extended model for early anterior-posterior axis patterning during sea urchin early development (see intro and discussion for details). (B) Posteriorly localized *wnt16* expression in the endoderm and mesoderm territories is activated by Wnt/β-catenin and Fzl5/8 signaling. In addition, the extended model indicates a role for the activation of key endoderm GRN components, *hox11/13b*, *blimp1,* and *eve*. *hox11/13b* appears to be necessary for both gastrulation and mesoderm morphogenesis (Arenas-Mena et al., 2006) and *blimp1b* is necessary for gastrulation (Livi and Davidson, 2006), both similar to the Wnt16 phenotypes described.

Figure S1. Wnt16 overexpression phenotype and qPCR analysis for ANE GRN members and *wnt16* expression. (A) Phenotypes for control and Wnt16 overexpression at late gastrula stage. (B) qPCR analysis from three different batches of embryos showing the downregulation of ANE regulatory factors at mesenchyme blastula stage (24 hpf) in Wnt16 knockdowns. (C) qPCR data showing that *wnt16* expression was downregulated in embryos injected with a dominant negative form of Fzl5/8 (ΔFzl5/8) at mesenchyme blastula stage (24 hpf). The *y*-axis shows the fold change in gene expression level in Wnt16 knockdowns and *ΔFzl5/8* mRNA injected embryos compared to control embryos at mesenchyme blastula stage (24 hpf). MO, morpholino; Scale bars: 20 μm.

Figure S2. qPCR analysis for endomesoderm GRN factors.
(A) qPCR measurements from three different batches of embryos showing the upregulation of several endomesoderm factors at 120-cell stage (12 hpf) in Wnt16 knockdowns. (B) qPCR measurements from three different batches of embryos showing that the expression of endomesoderm makers was not affected in Wnt16 morphants at mesenchyme blastula stage (24 hpf). The *y*-axis shows the fold change in gene expression level in Wnt16 knockdowns compared

to control embryos at mesenchyme blastula (24 hpf) and 120-cell (12 hpf) stages. MO, morpholino; Scale bars: 20 μm.

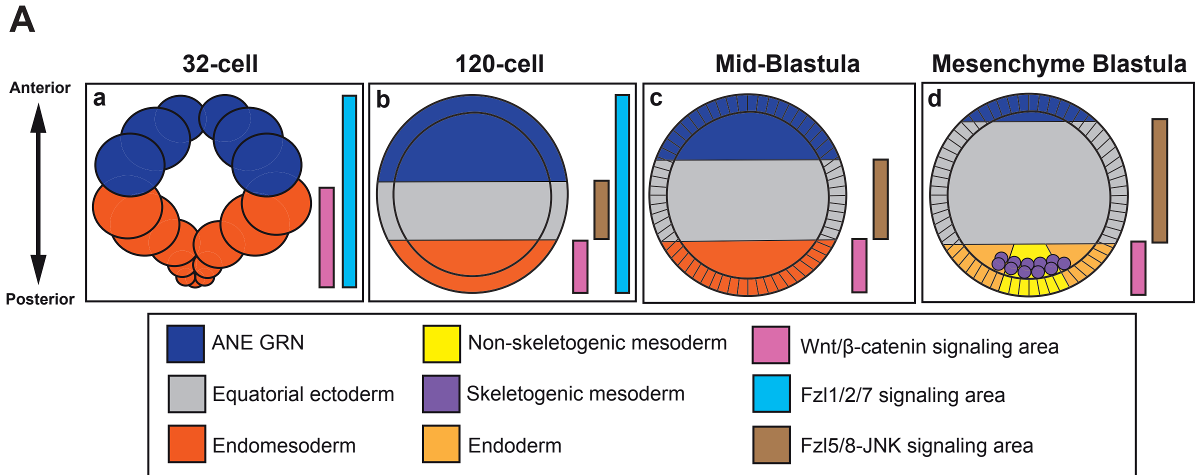
Figure S3. Additional morpholino phenotypes and controls. (A) The ANE makers are downregulated in embryos injected with Wnt16 MO2 (Ad-f) compared with control embryos (Aa-c). (B) Expression of the ANE marker *foxq2* in control (Ba), Wnt16 MO1-injected (92%; *n*=68/74; Bb), and *wnt16* mRNA-injected embryos (90%; *n*=54/60; Bc) at mesenchyme blastula stage. Wnt16 overexpression rescues *foxq2* expression in embryos injected with Wnt16 MO1 (94%; *n*=64/68; Bd), which cannot bind *wnt16* mRNA. (C) *foxq2* expression was unperturbed in embryos injected with Wnt7 morpholino. MO, morpholino; Scale bars: 20 μm.

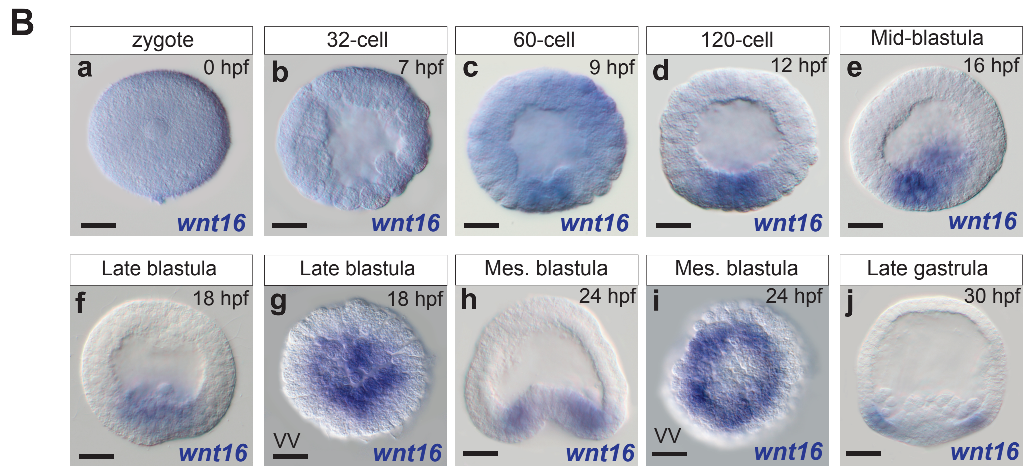
Figure S4. Expression of the ANE marker *foxq2* in embryos co-injected with *wnt16* mRNA and *wnt1* or *wnt8* mRNA at 120-cell stage. The expression of the ANE marker *foxq2* was downregulated in embryos overexpressing *wnt1* (91%) and *wnt8* mRNA (88%) at 120-cell stage (B and C). *foxq2* was expressed in embryos injected with *wnt16* mRNA (97%) (D). In addition, double injected embryos with *wnt16* mRNA and either *wnt1* (85%) (E) or *wnt8* mRNA (90%) (F) downregulated the expression of *foxq2*. Scale bars: 20 μm.

Table S1. qPCR primers pairs used for expression analysis.

FIGURES

Figure 1. Spatiotemporal expression of the *wnt16* ligand during early anterior-posterior specification and patterning.





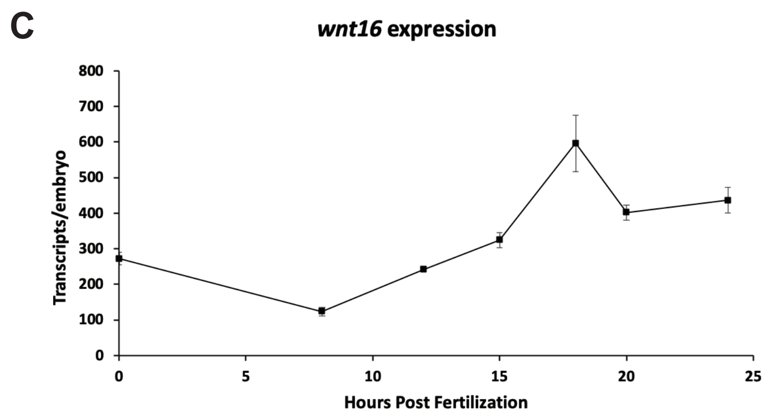
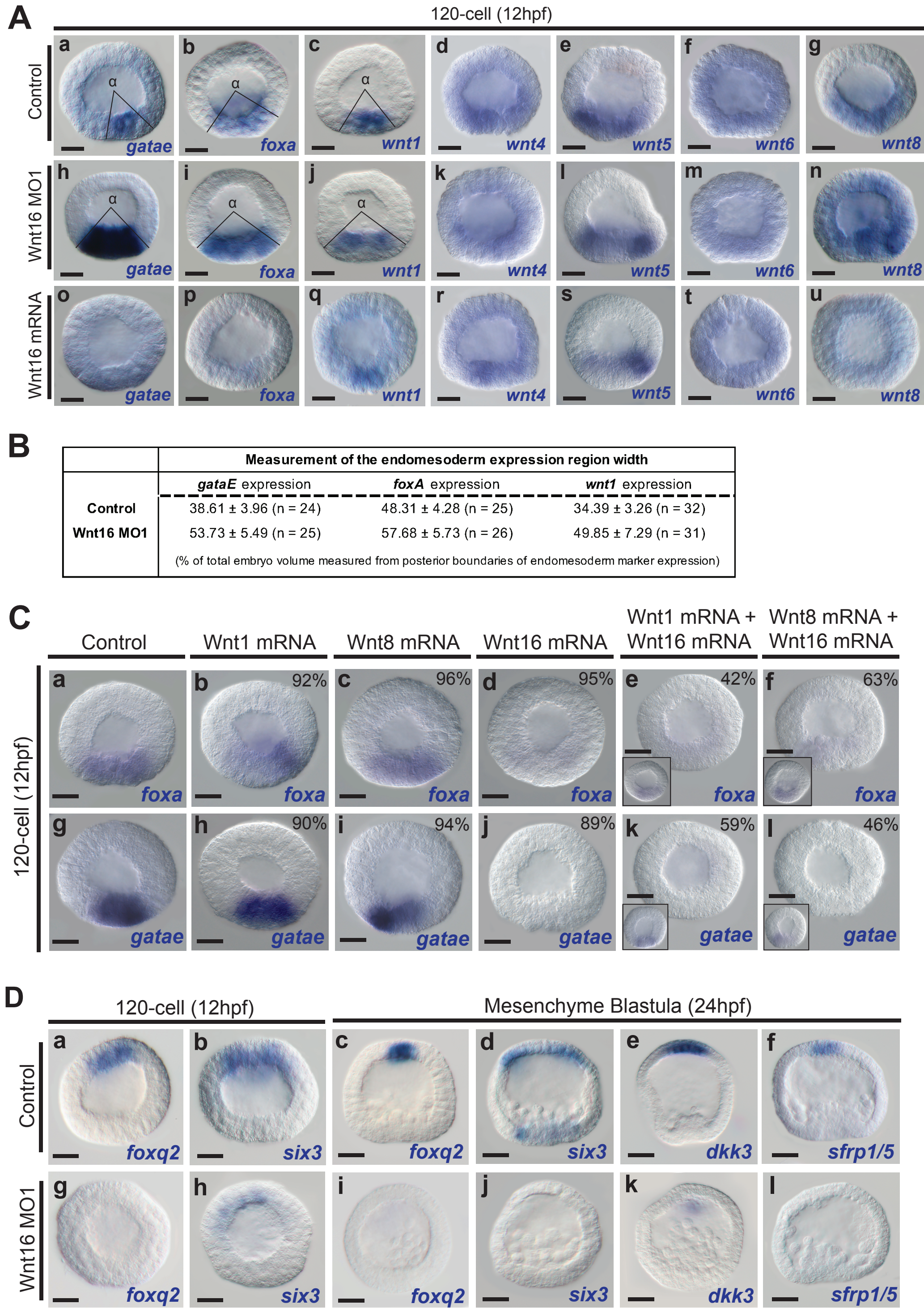


Figure 2. Wnt16 represses endomesoderm genes at the 120-cell stage and it is necessary for ANE GRN expression.



	Measurement of the endomesoderm expression region width		
	gataE expression	***foxA*** expression	***wnt1*** expression
Control	38.61 ± 3.96 (n = 24)	48.31 ± 4.28 (n = 25)	34.39 ± 3.26 (n = 32)
Wnt16 MO1	53.73 ± 5.49 (n = 25)	57.68 ± 5.73 (n = 26)	49.85 ± 7.29 (n = 31)
	(% of total embryo volume measured from posterior boundaries of endomesoderm marker expression)		

Figure 3. Wnt16-Fzl1/2/7 signaling antagonizes the Wnt1/Wnt8-Fzl5/8-JNK-mediated ANE restriction mechanism.

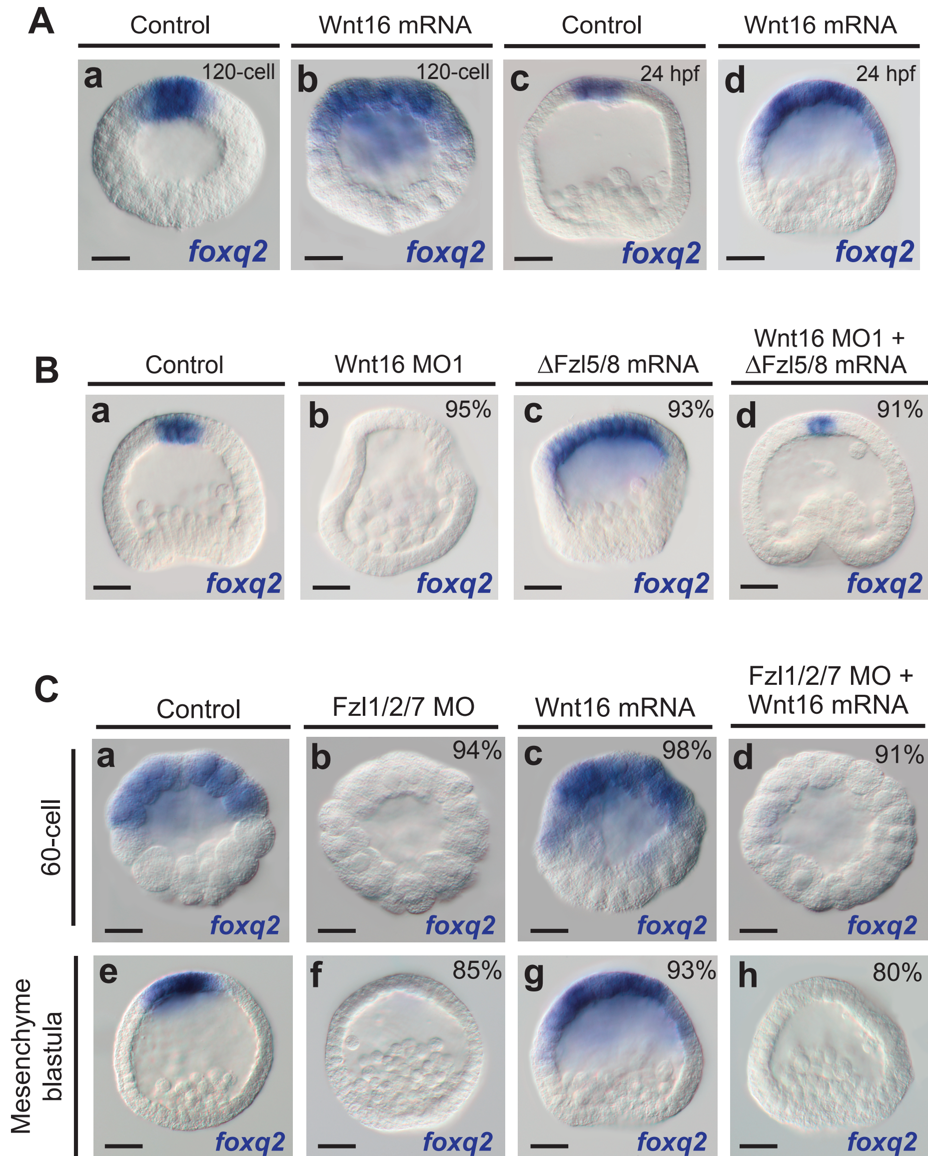


Figure 4. Gene expression patterns of *wnt16*, *eve*, *foxA*, and *gcm*.

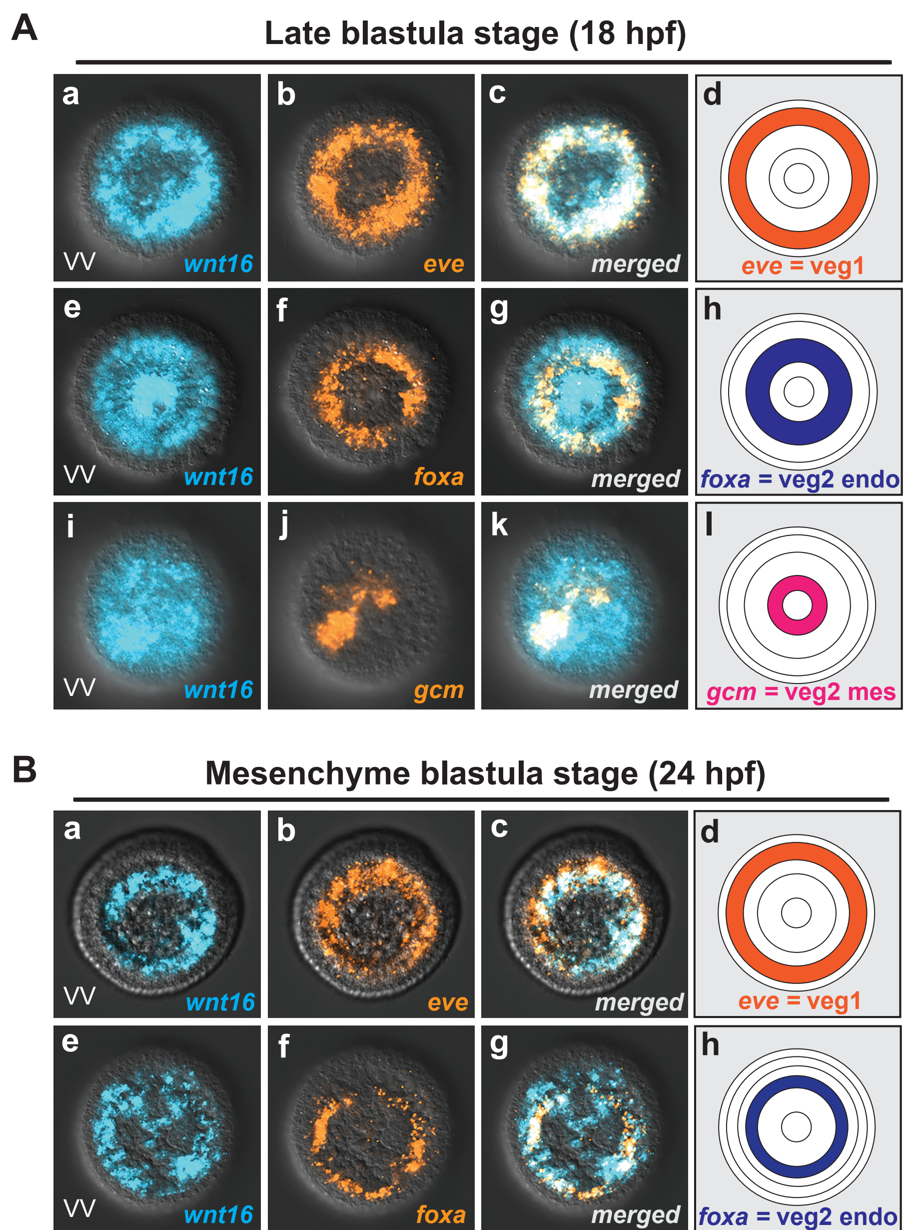


Figure 5. Regulation of *wnt16* expression by the AP Wnt signaling network and the role of Wnt16 in activating endoderm genes.

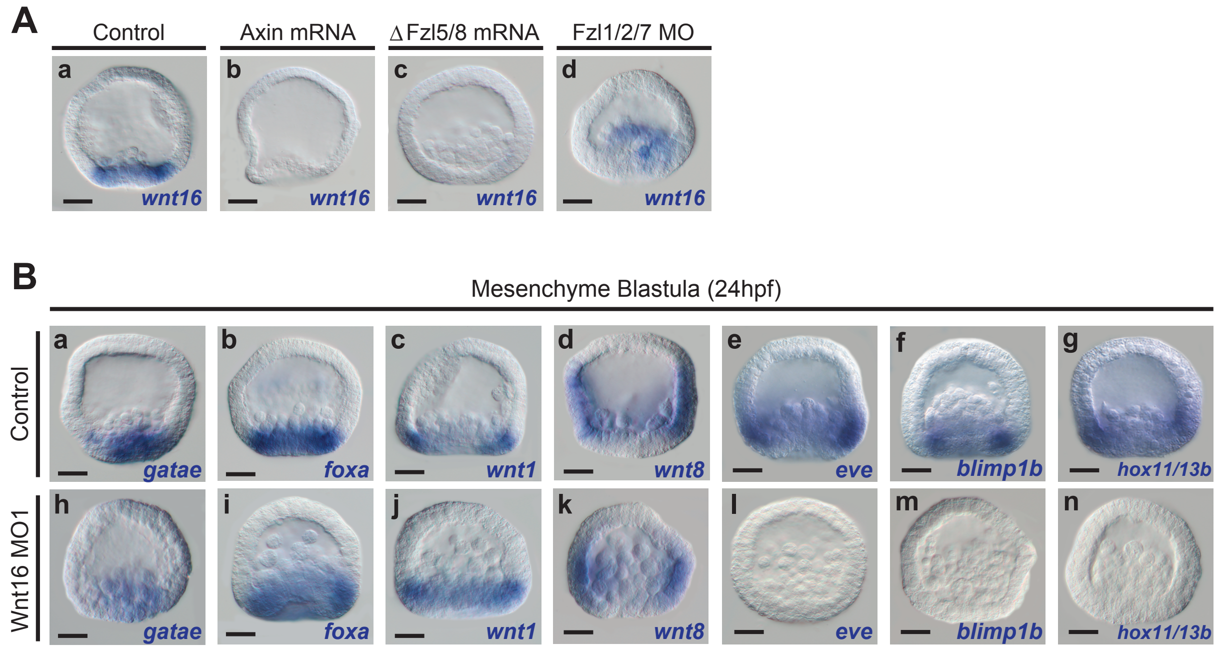


Figure 6. The role of Wnt16 in the morphogenetic movements during gastrulation.

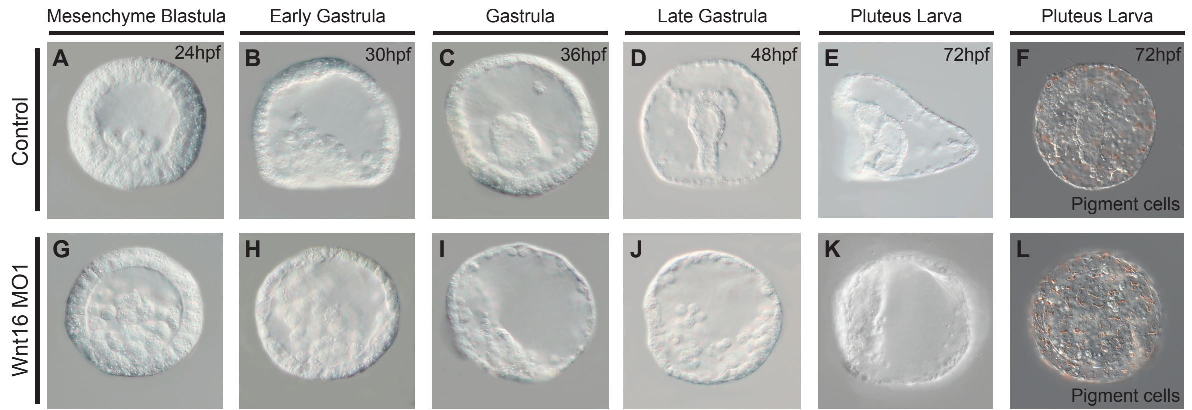


Figure 7. Wnt16 function in mesoderm morphogenesis and gastrulation.

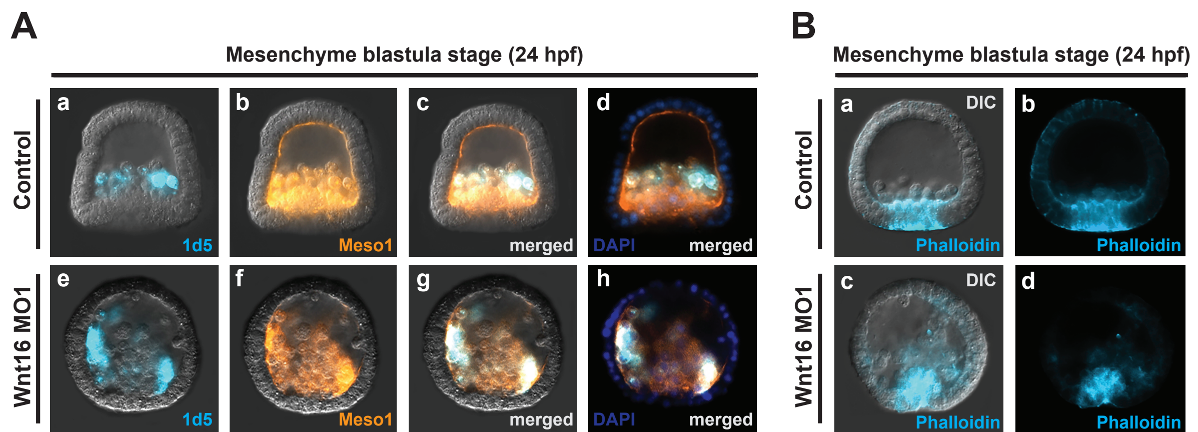
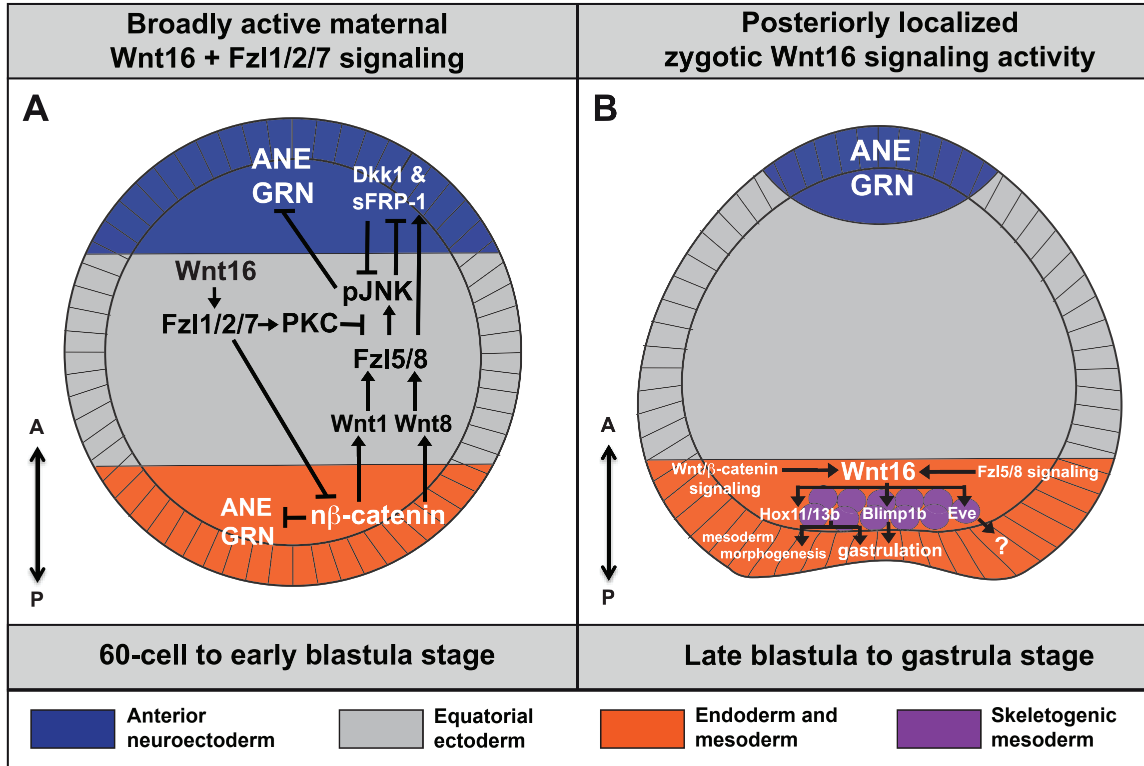
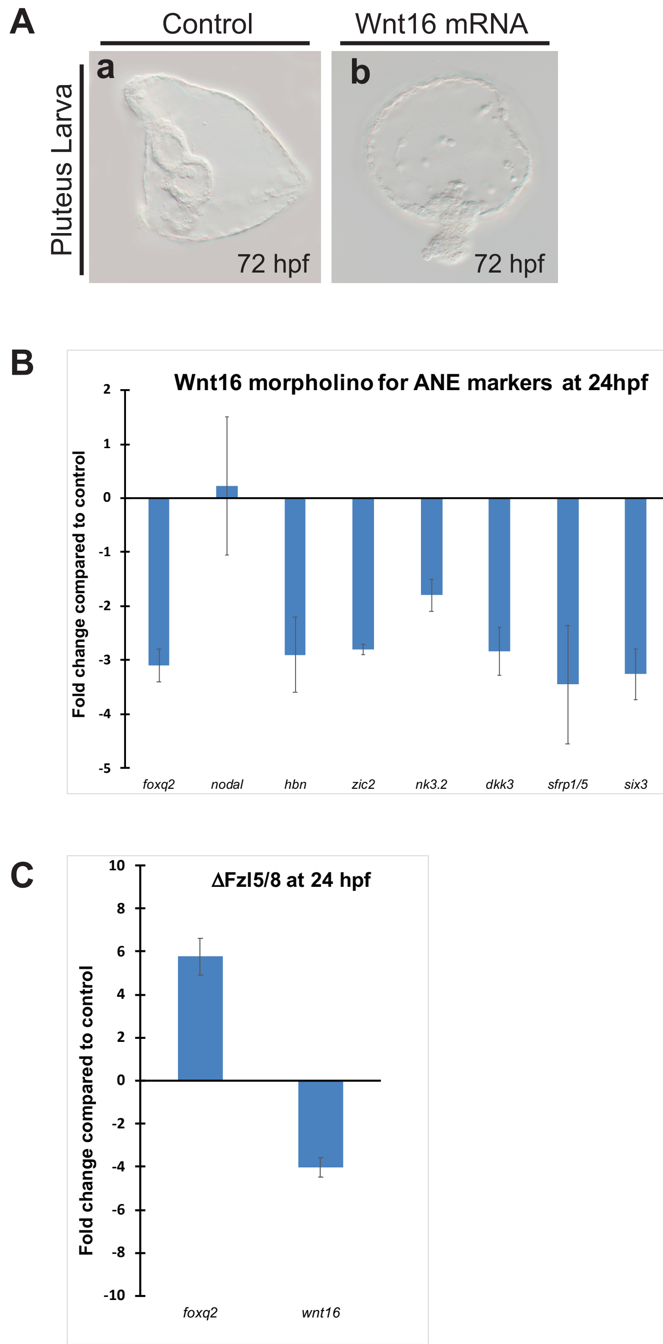


Figure 8. Model for two phases of Wnt16 activity during early AP axis patterning of the sea urchin embryo.



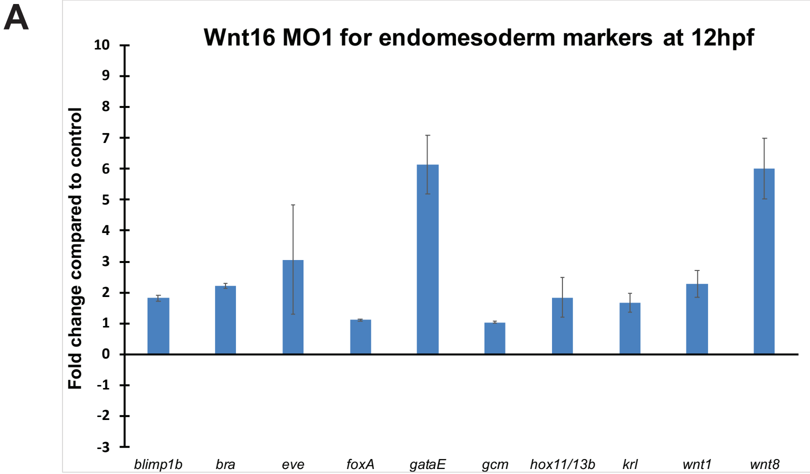
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Wnt16 overexpression phenotypes and qPCR analysis for ANE GRN members and *wnt16* expression

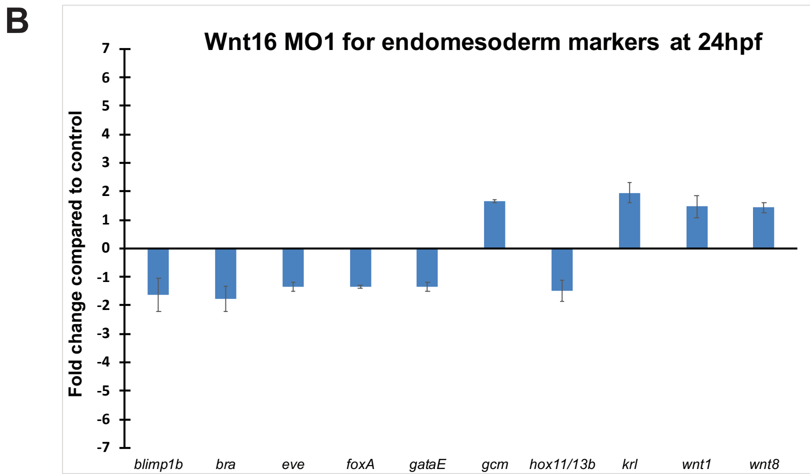


Supplementary Figure 2. qPCR analysis for endomesoderm GRN factors

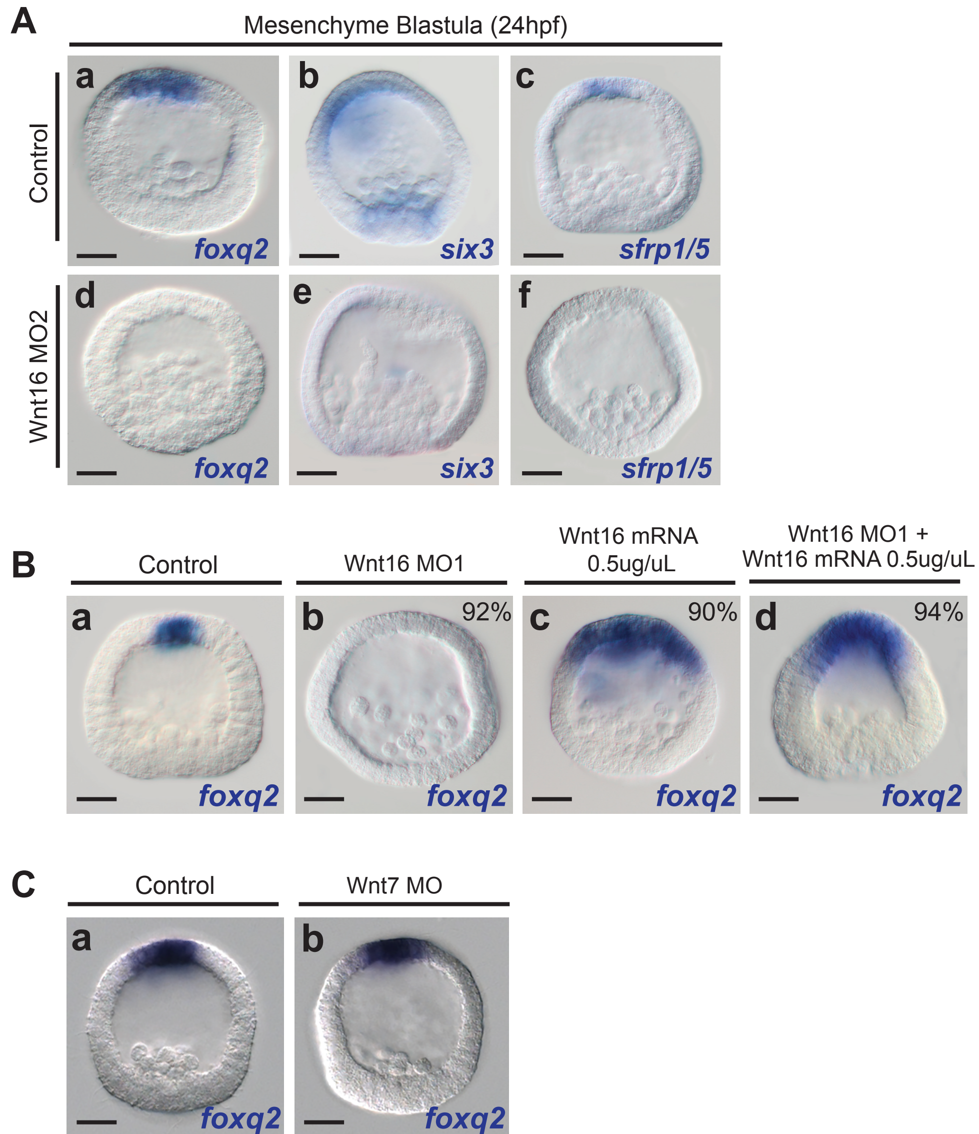
A



B



Supplementary Figure 3. Additional morpholino phenotypes and controls



Supplementary Figure 4. Expression of the ANE marker *foxq2* in embryos co-injected with *wnt16* mRNA and *wnt1* or *wnt8* mRNA at 120-cell stage

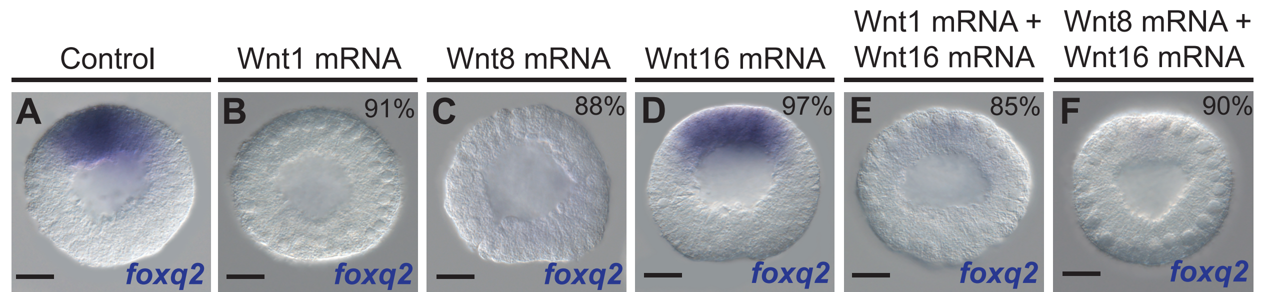


TABLE S1

Sp-mitochondrial 12s rRNA	Forward 5’-ACTCTCTCCTCGGAGCTATA-3’ Reverse 5’-GTATAATTTTGCGTATTCGGC-3’
Sp-zic2	Forward 5’-GAGGGATGTGATCGTCGTTT-3’ Reverse 5’-ACTGCTGTCGTTGGCTTCTT-3’
Sp- blimp1b	Forward 5’-TCGCTATGCGGGATCTCTAC-3’ Reverse 5’-GGGGTCCTTGACCTCGTAA-3’
Sp-bra	Forward 5’-ACACATCGACCCATCATCAA-3’ Reverse 5’-CATGGTGTCGTATCTTGGAAAG-3’
Sp-eve	Forward 5’-CACAGACCCTGGACTTTCGT-3’ Reverse 5’-GACAAACGGTCATCCCACTT-3’
Sp-foxA	Forward 5’-CCAACCGACTCCGTATCATC-3’ Reverse 5’-CGTAGCTGCTCATGCTGTGT-3’
Sp-gataE	Forward 5’-CTGGCTCAAGACGAGAAGGA-3’ Reverse 5’-CCTCTTCCGAGTCTGAATGC-3’
Sp-gcm	Forward 5’-CGACTGATAACCACGCTCAA-3’ Reverse 5’-TTAACGACGTCGGTCGATTC-3’
Sp-hox11/13b	Forward 5’-CACAGGCTCTCGACCTAACC-3’ Reverse 5’-GGTGGATGAGGTGGTAGATGA-3’
Sp-krl	Forward 5’-CACGAACTCTTCGCAATCAA-3’ Reverse 5’-CCAAGGGACAGGAGTGAAGA-3’
Sp-wnt1	Forward 5’-ACCTTCAGAACGGTGGGTGA-3’ Reverse 5’-AGCTTGATTCGGTTTCGCG-3’
Sp-wnt8	Forward 5’-TGTCGTTCATTCAAGCCATC-3’ Reverse 5’-TATCACTCGCCATTCGTTCA-3’
Sp-wnt16	Forward 5’-CTTACCGAATGTGGATGTGC-3’ Reverse 5’-TATCGCCAGAGTCGACAAAC-3’

Chapter 3.

A novel dorsal ectoderm specification mechanism: Non-canonical Fzl1/2/7 and Nodal signaling cooperate to initiate specification of dorsal territories in sea urchin embryos

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Key words: Wnt signal transduction, dorsal-ventral, deuterostome evolution, gene regulatory networks, Frizzled, BMP2/4, Nodal

ABSTRACT

Establishment of the major body axes is a crucial event in early animal development. In many metazoans, the Wnt and TGF-β signaling pathways play key roles in many developmental processes and are essential for specification and patterning of the anterior-posterior (AP) and dorsal-ventral (DV) axes, respectively. In deuterostome sea urchin embryos, early AP patterning depends on integrated information from the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC pathways, which form an interconnected Wnt signaling network. In addition, Nodal and BMP2/4, both members of the TGF-β superfamily, produce opposing morphogen gradients that pattern the three germ layers (endoderm, mesoderm, and ectoderm) along the DV axis. Although AP and DV body axis specification and patterning mechanisms are closely related spatially and temporally, the current model is that they act independently of one another in early development. Here, we used morpholino perturbation approaches to analyze the function of the non-canonical Wnt16-Fzl1/2/7 signaling pathway during early AP and DV patterning. Our functional data indicate that the Fzl1/2/7 signaling is necessary for the specification of the dorsal ectodermal territory, but not the ventral territory. We also show that during early cleavage stages Fzl1/2/7 signaling controls the initial activation of the dorsal territory specifier, *bmp2/*4, independently of early Nodal signaling, but Fzl1/2/7 signaling is not necessary for its maintenance during later gastrula stages. Taken together, our results suggest that Fzl1/2/7 and Nodal signaling pathways work cooperatively to activate the BMP2/4 signaling pathway that is essential for the specification and patterning of dorsal territories, connecting AP and DV gene regulatory networks (GRNs).

INTRODUCTION

In early animal development, specification and patterning of major embryonic axes is one of the most important events to occur in any embryo. However, the exact molecular mechanisms responsible for this fundamental developmental process are incompletely understood. In many deuterostome embryos (vertebrates, urochordates, cephalochordates, hemichordates, and echinoderms), Wnt signaling pathways play multiple roles in early specification and patterning of embryonic germ layers (endoderm, mesoderm, and ectoderm) (Loh et al., 2016; Niehrs 2010; Petersen and Reddien, 2009). In deuterostomes, "canonical" Wnt/β-catenin signaling is required to specify the endomesoderm territory at the posterior end as well as to restrict the territory of the anterior neuroectoderm (ANE) (Angerer et al., 2011; Gaspard et al., 2008; Kiecker and Niehrs, 2001; Lekven et al., 2001; Loh et al., 2016; Niehrs 2010; Nordström et al., 2002; Pani et al., 2012; Petersen and Reddien, 2009; Yaguchi et al., 2008). Previous studies from our lab have shown that anterior-posterior (AP) specification and patterning in sea urchin embryos depends not only on Wnt/β-catenin but also on integrated cross-regulatory information from Wnt/β-catenin as well as two other Wnt signaling pathways, Wnt/JNK and Wnt/Ca^{2+} (Khadka et al., 2018; Martínez-Bartolomé and Range, 2019; Range, 2014; Range, 2018; Range and Wei, 2016; Range et al., 2013).

Wnt signaling pathways use a combination of transmembrane Frizzled receptors, co-receptors, and secreted Wnt ligands that interact with one another to activate one or more specific Wnt signaling pathways in a given cell or cellular territory. Wnt/β-catenin signaling is necessary for activation of the endomesoderm GRN (Davidson et al., 2002; Logan et al., 1999; Weitzel et al., 2004; Wikramanayake et al., 1998; Wikramanayake et al., 2003; Wikramanayake et al., 2004). Wnt16-Fzl1/2/7 signaling functions broadly throughout early cleavage stages as a negative regulator of posterior Wnt/β-catenin signaling by downregulating Wnt/β-catenin activity (Martínez-Bartolomé and Range, 2019; Range et al., 2013). In addition, the Wnt16 ligand works through the Fzl1/2/7 receptor in order to activate an intracellular signaling cascade involving Protein Kinase C (PKC) (Martínez-Bartolomé and Range, 2019; Range et al., 2013). Wnt16-Fzl1/2/7 signaling is active as early as the 32-cell stage throughout the embryo and it is necessary during early cleavage stages to antagonize Wnt1/Wnt8-Fzl5/8-JNK signaling pathway in the anterior hemisphere of sea urchin embryos (Martínez-Bartolomé and Range, 2019; Range

et al., 2013). This Wnt1/Wnt8-Fzl5/8-JNK signaling pathway progressively downregulates the ANE GRN within the equatorial ectodermal territory, restricting the ANE to the most the anterior pole of the embryo (Range et al., 2013). The antagonism of this ANE restriction mechanism by Wnt16-Fzl1/2/7 signaling is critical for the correct position and size of the ANE territory around the anterior pole (Range et al., 2013). Consequently, integrated information from the Wnt signaling network is necessary to precisely position the early germ layer GRNs of the sea urchin embryo along the AP axis by the early gastrula stage (Martínez-Bartolomé and Range, 2019; Range, 2018; Range et al., 2013).

In most metazoans, the AP axis is the first to be specified during cleavage and blastula stages, followed by the DV axis, and both processes are generally established by the end of gastrulation. However, in many embryos, specification and patterning of these two axes overlap spatiotemporally. Thus, the developmental processes responsible for body axis formation, such as signaling pathways and GRNs, could be integrated. Many studies in vertebrates have focused on the closely aligned AP and DV patterning mechanisms. For instance, maternally Vg1 localized in *Xenopus*, zebrafish, and chick embryos is essential for mesoderm and endoderm induction as well as for the formation of the organizer which has the ability to induce specific fates and pattern the DV axis of these embryos (Dohrmann et al., 1996; Helde and Grunwald, 1993; Thomsen and Melton, 1993). Interestingly, Vg1 cooperates with canonical Wnt/β-catenin to induce *nodal* expression which is necessary for organizer specification (Bertocchini et al., 2004; Skromne and Stern, 2001; Skromne and Stern, 2002). In *Xenopus* and zebrafish embryos, a gradient of Nodal signaling is required for the induction and patterning of the mesoderm along AP and DV axis, for the formation of a dorsally located organizing center, and for establishment of developmental polarities (Agius et al., 2000; Gritsman et al., 2000; Hashimoto-Partyka et al., 2003). In sea urchins, a similar mechanism with integrated maternal inputs from Vg1, and Wnt/β-catenin signaling is necessary to regulate *nodal* expression which in turn initiates DV axis specification and patterning (Duboc et al., 2004; Range et al., 2007). Thus, there is an ancestral regulatory interaction among Vg1, Wnt/β-catenin and Nodal signaling during early establishment of the DV axis in several deuterostome embryos (Range et al., 2007). However, because of complex regulatory mechanisms as well as the complicated and rapid morphogenetic movements occurring during gastrulation when AP and DV axis patterning is occurring in vertebrates, how these GRNs interact is unclear.

Wnt/β-catenin signaling initiates AP specification and patterning (Logan et al., 1999; Wikramanayake et al., 1998) and is necessary for *nodal* activation and DV patterning; however, Wnt/β-catenin signaling is not *directly* involved in *nodal*'s activation (Yaguchi et al., 2008). Instead, the Wnt signaling network detailed above, which is initiated by Wnt/β-catenin signaling, progressively down regulates the transcription factor FoxQ2, a cardinal ANE GRN member, from the equatorial ectoderm (Range et al., 2013). Interestingly, FoxQ2 represses *nodal* transcription (Yaguchi et al., 2008). Thus, Wnt/β-catenin signaling is indirectly necessary for the activation of Nodal by removing a transcriptional inhibitor from the ventral equatorial ectoderm where *nodal* expression is activated by an unresolved mechanism (Duboc et al., 2004; Duboc et al., 2009; Lapraz et al., 2009; Range et al., 2013; Yaguchi et al., 2008). Once activated, Nodal signaling acts as an organizer to pattern the DV axis in the sea urchin embryo and is required for the expression of ventral ectoderm genes such as *bmp2/4* and *chordin* (Angerer et al., 2000; Angerer et al., 2001; Duboc et al., 2004; Flowers et al., 2004). Additionally, *chordin* is an antagonist to *bmp2/4* and is also necessary for the dorsal ectoderm formation. Although *bmp2/4* is expressed in the same presumptive ventral ectodermal cells as *nodal*, these two TGF-βs present antagonistic activities. Interestingly, BMP2/4 diffuses outside its domain of expression, the ventral ectoderm, to induce the specification of the dorsal ectoderm, opposite to the Nodal activity (Duboc et al., 2004). Thus, the effects of Nodal in the dorsal ectoderm are mediated by its role in BMP2/4 regulation. In addition, the presence of the BMP antagonist Chordin inhibits BMP2/4 signaling in the ventral ectoderm but it does not diffuse as far as BMP2/4 signal towards the dorsal territories. BMP2/4 diffuses towards dorsal regions which then induce ventral fates by activating the expression of dorsal genes such as *tbx2/3* and *nkx2.2* (Angerer et al., 2000; Bradham et al., 2009; Duboc et al., 2004; Lapraz et al., 2009; Molina et al., 2013). Therefore, Nodal and BMP2/4 are expressed in the same ventral region but they work in opposite ventral-dorsal territories as a result of the presence of a morphogenetic gradient (Duboc et al., 2004; Molina et al., 2013). Interestingly, large-scale ectoderm GRN analyses in sea urchin embryos suggested that the early input that activates the expression of *bmp2/4* is not controlled by Nodal signaling, as previously thought, but by another unknown mechanism distinct from Nodal signaling (Su et al., 2009). Several perturbation experiments and regulatory level predictions by Su et al., 2009 observed a dorsal-ventral polarity established only gradually; in contrast to the autonomously specified endomesodermal polarity of the sea urchin egg, and suggest that an

unknown signal might feed into the dorsal *bmp2/4* gene. Their direct comparison of their results from qPCR and NanoString nCounter gene expression analysis system, which captures and counts individual mRNA transcripts, showed no effect of Nodal morphants on the expression of several dorsal genes downstream of BMP2/4 signaling such as *tbx2/3* and *dlx* at early stages but not later in development. Thus, Su et al., 2009 proposed that the initial activator of BMP2/4 signaling is likely not dependent on Nodal signaling and suggested that parallel regulatory pathways might exist during the activation of dorsal genes.

Here, we hypothesize that Fzl1/2/7 and Nodal signaling might act together to initiate *bmp2/4* expression and; therefore, specify dorsal fates in sea urchin embryos. The gene regulatory interactions that coordinate AP and DV axes of the sea urchin embryo are still incompletely understood. Previous functional studies from our lab have shown that the position of the three primary germ layers is established by the Wnt signaling network and that non-canonical Wnt16-Fzl1/2/7 signaling is active throughout the sea urchin embryo during the early stages of this mechanism (Martínez-Bartolomé and Range, 2019; Range et al., 2013). Our functional data show that while Fzl1/2/7 signaling pathway is involved in the initial activation of *bmp2/4* expression, Fzl1/2/7 signaling is unnecessary for its maintenance during gastrula stages. As previously shown, this later *bmp2/4* expression depends on the Nodal signaling positive feedback mechanism. In addition, our results indicate that the dorsal ectodermal GRN is not specified in the absence of Fzl1/2/7 signaling. Together, these data support the idea that integrated information from the Wnt, Nodal and BMP2/4 pathways is required for early AP and DV specification and patterning in the sea urchin embryo.

RESULTS AND DISCUSSION

The spatiotemporal expression of *fzl1/2/7*, *bmp2/4*, and *tbx2/3* overlap during early specification of anterior-posterior and dorsal-ventral axes

Our RNA-seq screen from Fzl1/2/7 knockdowns detected a downregulation of two genes from the DV GRN, *bmp2/4* and *tbx2/3*. The spatiotemporal expressions of *fzl1/2/7*, *bmp2/4,* and *tbx2/3* have been previously studied individually (Chen et al., 2011; Gross et al., 2002; Lapraz et al.,

2006; Range, 2018; Saudemont et al., 2010) but here, we demonstrate the spatiotemporal resolution of the *fzl1/2/7*, *bmp2/4*, and *tbx2/3* overlapping expression during the early stages of AP and DV patterning using three-color fluorescent *in situ* hybridizations (FISH) to illustrate the potential interactions among the AP Wnt signaling and TGF-β signaling pathways.

fzl1/2/7 is ubiquitously expressed in early cleavage stages and then it is restricted to an equatorial ectodermal belt (Range, 2018). In addition, BMP2/4 signaling activates the dorsal ectoderm GRN in the same equatorial ectoderm where Wnt16-Fzl1/2/7 signaling is active (Duboc et al., 2004; Molina et al., 2013). At early cleavage stages, maternally activated *fzl1/2/7* was ubiquitously expressed (Range, 2018; Range et al., 2013) while *bmp2/4* and *tbx2/3* expression were not detected in the presumptive ventral and dorsal ectoderm, respectively, until early blastula stage (around 120-cell and mid-blastula stage) (Chen et al., 2011; Gross et al., 2002; Lapraz et al., 2006; Saudemont et al., 2010). Interestingly, *fzl1/2/7* expression was down regulated in the ANE and endomesoderm and maintained in a ring within the more posterior equatorial ectoderm during mid-blastula stage (16hpf), where it is co-expressed with *bmp2/4* in the ventral ectoderm. At the same time, *tbx2/3* expression was first detected in the dorsal side of the sea urchin embryo (Chen et al., 2011; Gross et al., 2002), where it is co-expressed with *fzl1/2/7* in the dorsal ectoderm. Then, we observed a progressive restriction of *fzl1/2/7* expression in an equatorial ectodermal belt due to a downregulation of its expression around the anterior pole (Range, 2018). At mesenchyme blastula (24hpf), the restriction of *bmp2/4* expression in the most ventral territory and *tbx2/3* in the most dorsal region remained (Chen et al., 2011; Gross et al., 2002; Lapraz et al., 2006; Saudemont et al., 2010). Accordingly, we observed a co-expression of *fzl1/2/7* with both *bmp2/4* and *tbx2/3* in the ventral and dorsal equatorial ectoderm, respectively. By early gastrula (30hpf), ventral *bmp2/4* and dorsal *tbx2/3* overlap on opposite sides of the embryo with the restricted *fzl1/2/7* expression in the upper equatorial ectoderm region. These data are consistent with a potential role of Fzl1/2/7 signaling in activating the initial *bmp2/4* expression in the ventral territory and; consequently, controlling the expression of dorsal genes activated by BMP2/4 signaling such as *tbx2/3*.

FISH assays were performed during early cleavage through blastula stages, a period of time when both AP and DV specification and patterning occur. *fzl1/2/7* was co-expressed with *bmp2/4* and *tbx2/3* expression at the time these two genes are activated. Co-expression of

fzl1/2/7, bmp2/4, and *tbx2/3* genes was then restricted to the ventral or dorsal equatorial ectoderm at mesenchyme blastula stage. Since *fzl1/2/7* expression overlaps with both ventral *bmp2/4* and dorsal *tbx2/3* during early stages of development, it is possible that the regulatory mechanisms governing AP and DV axis specification and patterning are connected and dependent upon one another.

Fzl1/2/7 signaling is necessary for dorsal cell fates

Previous analyses of non-canonical Wnt16-Fzl1/2/7 signaling in sea urchin embryos (Martínez-Bartolomé and Range, 2019; Range et al., 2013) observed distinctive phenotypic changes in Fzl1/2/7 morpholino-injected embryos that suggested that Fzl1/2/7 signaling maybe involved in DV axis specification and patterning. In order to test this prediction, we performed a careful examination of Fzl1/2/7 knockdown phenotypes that revealed several striking features. First, we observed the morphological phenotypes of Fzl1/2/7 morpholino-injected embryos during blastula, gastrula, and pluteus stages (Fig. 2A). At mesenchyme blastula stage, we observed a disorganization of mesodermal cells in the blastocoel in Fzl1/2/7 morphants, mimicking our previously reported Wnt16 knockdown phenotypes (Fig. 2Ad compared with 2Aa) (Martínez-Bartolomé and Range, 2019). During late gastrula stage (48hpf), Fzl1/2/7 knockdown embryos showed a severe disruption of the gut invagination (Fig. 2Ae versus 2Ab). By pluteus larva stage, Fzl1/2/7 morphants lacked a thickened columnar epithelium corresponding to the ANE in normal embryos and failed to form a skeleton (Fig. 2Af compared with 2Ac). These phenotypes are remarkably similar to those we have previously observed in Wnt16 knockdowns at late gastrula and pluteus larva stages (Martínez-Bartolomé and Range, 2019). In addition, Fzl1/2/7 morphants showed a proper formation of ventral structures but dorsal structures appear to be disturbed in pluteus larva embryos. Interestingly, a previous study revealed that BMP2/4 knockdowns present a similar phenotype, with a normal ventral region and a disrupted dorsal territory (Lapraz et al., 2009). Thus, Fzl1/2/7 and BMP2/4 knockdowns maintained a DV polarity as ventral fates were not affected. These results suggest that dorsal specification of the embryo is sensitive to both Fzl1/2/7 and BMP2/4 inhibition but that the ventral ectoderm territory is not affected by reductions of Fzl1/2/7 and BMP2/4 signaling.

BMP2/4 knockdown embryos have similar phenotypes to those of Fzl1/2/7 morphants, frequently causing the embryo to develop with a truncated dorsal region (Lapraz et al., 2009). Therefore, we investigated whether Fzl1/2/7 signaling is necessary for DV specification and patterning. We performed immunostaining using antibody markers for proteins that are localized to the dorsal (SpecI) and ventral (Gsc) regions in Fzl1/2/7 knockdown embryos at mesenchyme blastula (24hpf), late gastrula (48hpf) and pluteus larva stages (72hpf) (Fig. 2B) (Carpenter et al., 1984; Kenny et al., 2003). Fzl1/2/7 morpholino-injected embryos at mesenchyme blastula stage showed SpecI downregulation in the dorsal territory compared with control embryos (Fig. 2Bg versus 2Ba). By late gastrula and pluteus larva stages, the protein localization of SpecI was back to normal in the dorsal territory for both control and Fzl1/2/7 knockdowns (Fig. 2Bi,k compared with 2Bc,e). In contrast, the protein localization of Gsc was not disturbed in Fzl1/2/7 morpholino-injected embryos (Fig. 2Bh,j,l compared with 2Bb,d,f).

Previous studies showed that BMP2/4, although expressed in the ventral ectoderm, is necessary for dorsal ectoderm specification (Duboc et al., 2004; Lapraz et al., 2009). Here, we extended these observations by showing that Fzl1/2/7 signaling is also required for the correct specification of dorsal ectodermal territory. First, we showed that Fzl1/2/7 knockdowns affect the specification of dorsal but not ventral ectodermal territory, similar to BMP2/4 morpholino-injected phenotypes (Lapraz et al., 2009). These findings resemble previous results that indicate that an unknown signal activates early BMP2/4 signaling and are compatible with the possibility that Fzl1/2/7 signaling is required for the early activation of dorsal ectoderm territories (Su et al., 2009). Together, our observations from Fzl1/2/7 knockdown phenotypes and immunostaining of dorsal and ventral antibody markers confirm that Fzl1/2/7 signaling is necessary for dorsal but not ventral specification during early development of the sea urchin embryo.

Fzl1/2/7 signaling initiates *bmp2/4* transcription and the dorsal ectodermal GRN

Su et al., (2009) suggested that an additional input(s) may also be necessary to activate the dorsal ectodermal GRN and proposed that *bmp2/4* expression is controlled by a second unknown signal distinct from Nodal and BMP signaling pathways. Supporting this idea, *bmp2/4* expression at early cleavage stages (60-cell and 120-cell stages) was severely downregulated in our RNA-seq screen data of Fzl1/2/7 morpholino-injected embryos (Fig. S1). In addition, we show that spatial

expression of *fzl1/2/7* and *bmp2/4* overlap in the ventral ectoderm during early stages, suggesting that Fzl1/2/7 signaling is at the right place and time to be this additional input responsible for early *bmp2/4* activation and for specification of dorsal ectodermal GRN. Thus, we decided to further investigate the function of non-canonical Fzl1/2/7 signaling pathway in early activation of *bmp2/4* and *nodal* and its role during DV specification and positioning in the sea urchin embryo (Fig. 3A). Expression of *bmp2/4* was slightly detected ubiquitously in early cleavage stages of control but not Fzl1/2/7 knockdown embryos (Fig. 3Ag,h compared with Fig. 3Aa,b). At mid-blastula and late blastula stages, *bmp2/4* was expressed in the presumptive ventral ectoderm in control embryos but Fzl1/2/7 morpholino-injected embryos showed a severe downregulation of *bmp2/4* expression (Fig. 3Ai,j compared with 3Ac,d). The entire *bmp2/4* expression in the ventral territory returned in Fzl1/2/7 knockdown embryos at mesenchyme blastula and early gastrula stages (92%; *n*=47/51) (Fig. 3Ak,l compared with 3Ae,f). These results suggested that Fzl1/2/7 signaling is controlling the early expression of *bmp2/4* in the presumptive ventral ectoderm. Since *bmp2/4* is activated by Nodal signaling (Duboc et al., 2004; Su et al., 2009), we also examined the role of Fzl1/2/7 signaling in controlling early *nodal* expression. At 60-cell stage, *nodal* was broadly expressed throughout the embryo but slightly concentrated towards the ventral side in both control and Fzl1/2/7 knockdown embryos (Fig. 3As versus 3Am), as previously shown (Duboc et al., 2004). From 120-cell stage until early gastrula stage, the ventral expression of *nodal* was also not affected in embryos injected with Fzl1/2/7 morpholino (Fig. 3At-x compared with 3An-r). Thus, whole-mount *in situ* hybridization data for *nodal* indicate that Fzl1/2/7 signaling is not necessary for ventral gene expression. Consistent with these results, previous qPCR data showed that *nodal* transcripts were not affected in Fzl1/2/7 morphants compared with control.

In TGF-β signaling, Smad1/5/8 (receptor-regulated Smad, R-Smad, protein downstream of BMP2/4 signaling) or Smad2/3 (transcriptional modulator R-Smad protein that mediates Nodal signaling) are phosphorylated and, subsequently, they bind the Smad4 protein (Gilbert, 2000). Next, this transcription factor association complex is localized to the nucleus where it is able to bind target cis-regulatory elements, activating transcription (Gilbert, 2000). To better investigate the role of the non-canonical Fzl1/2/7 pathway in both Nodal and BMP2/4 signaling, we performed antibody immunostaining using Smad1/5/8 and Smad2/3 antibodies in Fzl1/2/7 morpholino-injected embryos to detect any change in the nuclear localization of two Smad

proteins. Smad2/3 activity was detected earlier than Smad1/5/8, at 120-cell stage, in the ventral side of the embryo (Fig. 3Bb). We observed that Fzl1/2/7 knockdowns exhibited similar patterns of Smad2/3 nuclearization in the ventral territory compared with control embryos at different stages of development (Fig. 3Bf,h,n,p compared with 3Bb,d,j,l). As previously reported by Chen et al., 2011, Smad1/5/8 activity was not detected until late-blastula stage (18hpf) in control sea urchin embryos (Fig. 3Bi). In contrast, at late-blastula stage, nuclear activity of Smad1/5/8 was not detected in Fzl1/2/7 knockdown embryos (Fig. 3Bm) whereas control embryos showed its activity restricted to the dorsal region of the embryo (Fig. 3Bi). Finally, Fzl1/2/7 morpholino-injected embryos at mesenchyme blastula stage (24hpf) showed a severe downregulation of Smad1/5/8 nuclear activity from the dorsal territory compared with control embryos (Fig. 3Bo versus 3Bk). In summary, we observed changes in the nuclear localization of the Smad downstream of BMP2/4 (Smad1/5/8) but not Nodal (Smad2/3) signaling, corroborating previous results showing that Fzl1/2/7 signaling affects dorsal (SpecI) but not ventral (GscIII) genes.

Consistent with the results above, our RNA sequencing (RNA-seq) data identified a severe downregulation of several dorsal transcription factors (*hmx*, *dlx*, *msx*, and *tbx2/3*) at stages between 12 and 18 hours post fertilization (hpf) in the absence of Fzl1/2/7 signaling (Fig. 1). Many dorsal genes detected in our RNA-seq screen in Fzl1/2/7 knockdowns have been previously reported as downstream of BMP2/4 signaling (Fig. 1). To corroborate these RNA-seq data in Fzl1/2/7 morphants compared with control embryos, we performed additional temporal *in situ* hybridization analyses for dorsal ectodermal genes. The expression of all dorsal markers was severely downregulated in Fzl1/2/7 knockdown embryos (Fig. 3Cg-k compared with 3Ca-e) suggesting an important role of Fzl1/2/7 signaling in the specification of dorsal fates. qPCR analysis confirmed the downregulation of several dorsal makers in Fzl1/2/7 morphants at mesenchyme blastula stage (Fig. 1). In addition, we examined changes in *hnf6* expression, one of the earliest maker genes expressed in the presumptive ciliary band that is down regulated in the dorsal territory by BMP2/4 signaling and in the ventral territory by Nodal signaling, resulting in a band of expression between the two ectodermal territories. Similar to BMP2/4 knockdown embryos (Lapraz et al., 2009), Fzl1/2/7 knockdowns displayed an ectopic expression of the ciliary band marker *hnf6* on the dorsal side of the embryo compared with control (Fig. 3Cl versus 3Cf).

Taken together, our data indicate that Fzl1/2/7 signaling is necessary for the initiation of *bmp2/4* ventral expression but it does not affect the expression of other ventral genes such as *nodal and* Gsc. Before this study, Nodal signaling was the only regulatory factor known to activate *bmp2/4* expression (Molina et al., 2013; Saudemont et al., 2010). However, comparing our results with previous functional studies (Saudemont et al., 2010), Fzl1/2/7 knockdowns showed a similar effect in the ciliary band expression as BMP2/4 morphants, strongly supporting the idea that Fzl1/2/7 signaling may play an essential role in controlling the BMP2/4 signaling during early DV patterning of the sea urchin embryo, as illustrated in Fig. 4. Indeed, our findings confirm that an additional input from non-canonical Fzl1/2/7 is necessary for early activation of *bmp2/4* expression, independent of Nodal and BMP signaling pathways, and for dorsal ectodermal GRN, as suggested by Su et al., 2009.

Implications

Previous functional studies in several deuterostome developmental systems have suggested that Frizzled-7 is necessary for dorsal axis formation (De Robertis et al., 2000; Medina et al., 2000; Nasevicius et al., 1998; Sumanas et al., 2000). For example, Frizzled-7 loss of function experiments in *Xenopus* embryos resulted in disruption of dorsoanterior structures (Medina et al., 2000; Sumanas et al., 2000). Interestingly, Frizzled-7 can work upstream of the canonical Wnt/β-catenin as well as the non-canonical Wnt/Ca^{2+} pathways during dorsal specification, supporting our results that non-canonical Fzl1/2/7 signaling is necessary for dorsal ectodermal territory in the sea urchin embryo (Medina et al., 2000; Sumanas et al., 2000). In addition, several studies in vertebrate and invertebrate embryos propose that distinct signaling pathways involved in AP and DV axis specification might be tightly interconnected (De Robertis et al., 2000; Hashiguchi and Mullins, 2013; Khokha et al., 2005; Yaguchi et al., 2008; Yaguchi et al., 2016; Wei et al., 2009). For instance, during gastrulation of vertebrate embryos, a combination of inputs from TGF-β and Wnt signaling pathways in the Spemann organizer are required for the proper DV and AP specification and patterning (De Robertis et al., 2000; Fuentealba et al., 2007; Harland, 2000; Hashiguchi and Mullins, 2013; Heasman, 2006; Khokha et al., 2005). Similarly, combined inputs from Wnt, Nodal, and BMP2/4 signaling, as well as FoxQ2, work together to position the ANE territory on its ventral and dorsal sides (Yaguchi et al., 2008; Yaguchi et al., 2011). Based on our

results presented here, together with previous functional studies in other deuterostomes, it is tempting to speculate that a conserved regulatory mechanism, with interactions from Wnt signaling and TGF-β signaling pathways, allows early AP and DV GRNs among metazoan embryos.

Sea urchin embryos present an organizing center on the ventral ectoderm which is remarkably similar to the Spemann organizer in vertebrates and is regulated by a similar Nodal signaling (Lapraz et al., 2015). Among deuterostomes, echinoderms are marine invertebrates sister related to hemichordates that also share features with chordates, crucial properties that make them a key phylum to study the evolution of the developmental mechanisms that pattern the early germ layers of the embryo body plan (Saudemont et al., 2010). In addition, remarkably simple cell movements during gastrulation and specification of the AP and DV axes make sea urchins a great and easy model system to study early patterning of the embryo. Interestingly, the developmental mechanisms that regulate AP and DV patterning in deuterostomes, from echinoderms to vertebrates, are remarkably conserved, suggesting that the involvement of a non-canonical Wnt signaling in controlling dorsal specification may be evolutionary conserved. Therefore, it is tempting to speculate that deuterostome embryos shared a regulatory mechanism of AP and DV specification that it may be traced back to the common ancestor of deuterostome. Taken together, our data provide insights of the regulatory machinery during early embryonic patterning and suggest a conserved mechanism among deuterostome embryos to integrate information from distinct signaling pathways to coordinate AP and DV GRNs.

The results of our experimental analysis show that non-canonical Fzl1/2/7 signaling is responsible for the early activation of *bmp2/4* expression. In addition, we have shown that coordinated inputs from Fzl1/2/7 and Nodal signaling control the specification and patterning of AP and DV axes in sea urchin embryos. We propose that non-canonical Fzl1/2/7 signaling is the previously unknown signal that controls early *bmp2/4* expression independent of BMP2/4 in addition to its previous role in AP patterning. However, we need to study in more detail the role of Fzl1/2/7 signaling in early Nodal independent activation of *bmp2/4* expression. Next, we examined the changes in expression of dorsal genes in Fzl1/2/7 knockdown embryos, confirming that Fzl1/2/7 signaling is necessary for the expression of several dorsal genes at mesenchyme blastula stage. Previous studies suggested that AP and DV axes are established by different and

unconnected regulatory mechanisms; however, here we showed that a distinct non-canonical Fzl1/2/7 signaling pathway is essential for the patterning and positioning of both AP and DV axes, building one of the first connections between these two axes in the sea urchin embryo. In addition, we speculate that the key role of Fzl1/2/7 signaling in dorsal-ventral patterning may be conserved in other bilaterian organisms. Taken together, our results suggest that, at least in the sea urchin embryo, the specification and patterning of the AP and DV axes are connected and depend on integrated information of cooperatively signals from both the Wnt and Nodal-BMP2/4 signaling pathways.

MATERIALS AND METHODS

Animals and embryos

Adults sea urchins (*Strongylocentrotus purpuratus*) were obtained from Monterey Abalone Company (Monterey, CA, USA), California Institute of Technology (Pasadena, CA, US), and Marinus Scientific (Longbeach, CA) and maintained in aquaria at 16ºC. Adults were induced to shed gametes by injection of 0.5M KCl into the body cavity. Fertilized embryos were reared at 15ºC in artificial seawater (ASW).

cDNA cloning preparation

cDNA from 24-48 hpf stage embryos was used to obtain *bmp2/4*, *nodal*, *tbx2/3*, *hmx*, *irxA*, *dlx*, *nkx2.2*, and *hnf6* clones. The primers (forward and reverse, 5' - 3'), based on the *S. purpuratus* sea urchin genome sequence, used to generate anti-sense in situ probes are included in Table S1.

Microinjections of morpholino anti-sense oligonucleotides

The sequence and injection concentration of Fz1/2/7 morpholino was as previously described:

Fzl1/2/7 MO: 5'-CATCTTCTAACCGTATATCTTCTGC -3' (1.3mM) (Range et al., 2013). Standard control morpholino: 5'-CCTCTTACCTCAGTTACAATTTATA-3' from Gene-Tools LLC, Eugene, OR, was injected at the same concentration as the experimental morpholino as a control measurement of morphological and developmental defects related to injections.

Morpholino injection solutions contained 20% glycerol, 15% fluorescein isothiocyanate (FITC) (2.5 µg/ml) and morpholino oligonucleotides at working concentrations. Eggs were de-jellied by passing them through 74µm mesh Nitex, plated in rows on a culture dish coated with 25% protamine sulfate and fertilized with diluted sperm. After fertilization, embryos were immediately injected with the morpholino injection solution, washed in ASW and incubated at 15^{0}C until the desired developmental time. Morpholino microinjection experiments were performed using 50-200 embryos from at least three different mating pairs. Only experiments with representative phenotype or changes in gene expression in at least 80% of the injected embryos are presented. Biotapestry (Longabaugh et al. 2009) was used to build the gene regulatory interactions and signaling events of the final GRN model (Fig.4).

Quantitative PCR (qPCR)

qPCR assays were carried out as previously described (Wei et al., 2009). Mesenchyme blastula stage (24hpf) embryos from at least three different mating pairs were used for each qPCR experiment and each PCR reaction was performed in triplicate. Mitochondrial 12s RNA ΔCt values were used to normalize the relative target gene expression levels. In differential gene expression between control and perturbed embryos, 2-fold or higher change in expression level was considered to be significant.

Whole-mount in situ hybridization (WMISH)

Linearized pGEM®-T Easy or pCS2+ plasmids using T7 or SP6 polymerase enzyme were used to generate antisense RNA probes, complementary to the target mRNA, for each gene analyzed. Alkaline phosphatase reporter *in situ* hybridization was carried out as previously described (Wei et al., 2009). Three-color fluorescent *in situ* hybridization (FISH) experiments for *fz1/2/7, bmp2/4,* and *tbx2/3* antisense probes were performed following the previously described protocol by Sethi et al., (2014).

Immunostaining

Embryos were fixed in 3% paraformaldehyde in ASW at room temperature for 20 minutes and washed five times in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Embryos were incubated at 4ºC overnight with primary antibodies against SpecI (1:500; anti-rabbit),

GscIII (1:400; anti-guinea pig), Smad1/5/8 (1:500; anti-rabbit), and Smad2/3 (1:500; anti-rabbit) in PBST and 4% normal goat serum. Primary antibodies were detected by incubating embryos for 1 hour at room temperature with 1:1000 Alexa Fluor-coupled 488 goat anti-rabbit IgG and 1:1000 Alexa Fluor-coupled 488 or 546 goat anti-guinea pig IgG secondary antibodies (Thermo Fisher Scientific). Nuclei were stained with DAPI (1:3000). Embryos were moved to a solution of 70% PBST and 30% glycerol before imaging.

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FIGURE LEGENDS

Figure 1. Analysis of regulatory factors activated by Fzl1/2/7-PKC signaling during early AP patterning. (A) The graph shows transcription factors (*hmx*, *brn1/2/4*, *pax2/5/8*, *myb*, *dlx*, *msx*, and *tbx2/3*), poorly characterized transcription factors (*irxB* and *unc4.1*), and the signaling molecule *siah1* downregulated in the absence of functional Fzl1/2/7 signaling (Fzl1/2/7 knockdown embryos). (B) The diagram displays the spatial expression of transcription factors between blastula (15 hpf) and gastrula (24 hpf) stages, indicating that most of them are in the dorsal side of the sea urchin embryo.

Figure 2. Phenotypic characterization of Fzl1/2/7 knockdown embryos during gastrulation. (A) Morphology of both control (Aa-c) and Fzl1/2/7 morpholino-injected embryos (Ad-f) at mesenchyme blastula (24hpf), late gastrula (48hpf), and pluteus larva (48hpf), and 72 hours post fertilization (hpf). Fzl1/2/7 morphants show a disorganization of mesodermal cells in the blastocoel and a lack of a thickened columnar epithelium. In addition, ventral structures were formed in Fzl1/2/7 knockdowns but dorsal structures appear to be disturbed. (B) SpecI and Gsc antibody staining for control (Ba-f) and embryos injected with Fzl1/2/7 MO1 (Bg-l) at 24hpf, 48hpf, and 72hpf. SpecI (orange) is a dorsal gene marker and Gsc (blue) is a general ventral gene. SpecI staining was affected in Fzl1/2/7 knockdown embryos (Bg) compared with control embryos (Ba) at mesenchyme blastula stage but not at late gastrula or pluteus larva stages. Fzl1/2/7 knockdowns did not affect Gsc staining at any of the observed developmental time points. MO, morpholino; hpf = hours post fertilization; Scale bars: 20 μm.

Figure 3. Fzl1/2/7 signaling is necessary to control BMP2/4 signaling, but not *nodal* expression, and for the expression of dorsal and ciliary band markers during DV patterning. (A) Expression of two ventral genes, *bmp2/4* and *nodal*, in control and Fzl1/2/7 morpholino-injected embryos. The expression of *bmp2/4* was downregulated in Fzl1/2/7 MO1 injected embryos at 120-cell, mid blastula, and late blastula stages (Ah-j compared with Ab-d). Subsequently, *bmp2/4* expression in the most ventral territory was again detected in Fzl1/2/7 knockdown embryos at mesenchyme blastula and early gastrula stage (Ae-f). The ventral expression of *nodal* was not affected in Fzl1/2/7 morpholino-injected embryos (As-x compared

with Am-r). (B) Antibody staining for Smad1/5/8 (protein downstream of BMP2/4 signaling) and Smad2/3 (downstream of Nodal signaling) in control and Fzl1/2/7 knockdown embryos at 120-cell, mid blastula, late blastula, and mesenchyme blastula stages. Smad1/5/8 staining was not detected until late blastula stage as previously reported (Chen et al., 2011) (Bi). Then, at late blastula and mesenchyme blastula stages Smad1/5/8 staining was severely affected in Fzl1/2/7 morpholino-injected embryos (Bm, Bo) compared with control embryos (Bi, Bk). On the ventral side of the embryo, Smad2/3 staining was not affected in Fzl1/2/7 knockdowns at any of the observed developmental time points. (C) Fzl1/2/7 knockdown embryos at mesenchyme blastula stage showing that Fzl1/2/7 was necessary for the expression of the dorsal genes *tbx2/3*, *hmx*, *irxA*, *dlx*, and *nkx2.2* (Cg-k compared with Ca-e). Control embryos showing a restricted expression of the *hnf6* marker in the ciliary band territory (Cf) and Fzl1/2/7 morpholino-injected embryos forming an ectopic ciliary band in the dorsal ectoderm in addition to the normal ciliary band (Cl). MO, morpholino; Scale bars: 20 µm.

Figure 4. An extended GRN model for the proper patterning of the different germ layer territories in the sea urchin embryo. Biotapestry diagram summarizing the provisional gene regulatory interactions responsible for the establishment of the four germ layer territories: endoderm, mesoderm, equatorial ectoderm (ventral and dorsal ectoderm), and anterior neuroectoderm. The proposed gene regulatory network for regionalization of these four germ layer territories of the sea urchin embryo includes signaling events and gene regulatory interactions from previous perturbation studies in addition to a novel function of non-canonical Fzl1/2/7 signaling in dorsal specification. Arrows represent positive transcriptional activation and flat arrows indicate repression. The colored boxes represent the different spatial domains as well as the maternal inputs.

Figure S1. RNA-seq screen heatmap of several signaling molecules in Fzl1/2/7 morpholino-injected embryos. Heatmap showing the changes in expression of signaling molecules in Fzl1/2/7 knockdowns at 60 cell (9 hpf), 120 cell (12 hpf), and late blastula (18 hpf) stages. At early cleavage stages (60-cell and 120-cell stages), the expression of the signaling molecule *bmp2/4* was severe downregulated in Fzl1/2/7 morphants. MO, morpholino.

FIGURES

Figure 1. Analysis of regulatory factors activated by Fzl1/2/7-PKC signaling during early AP patterning

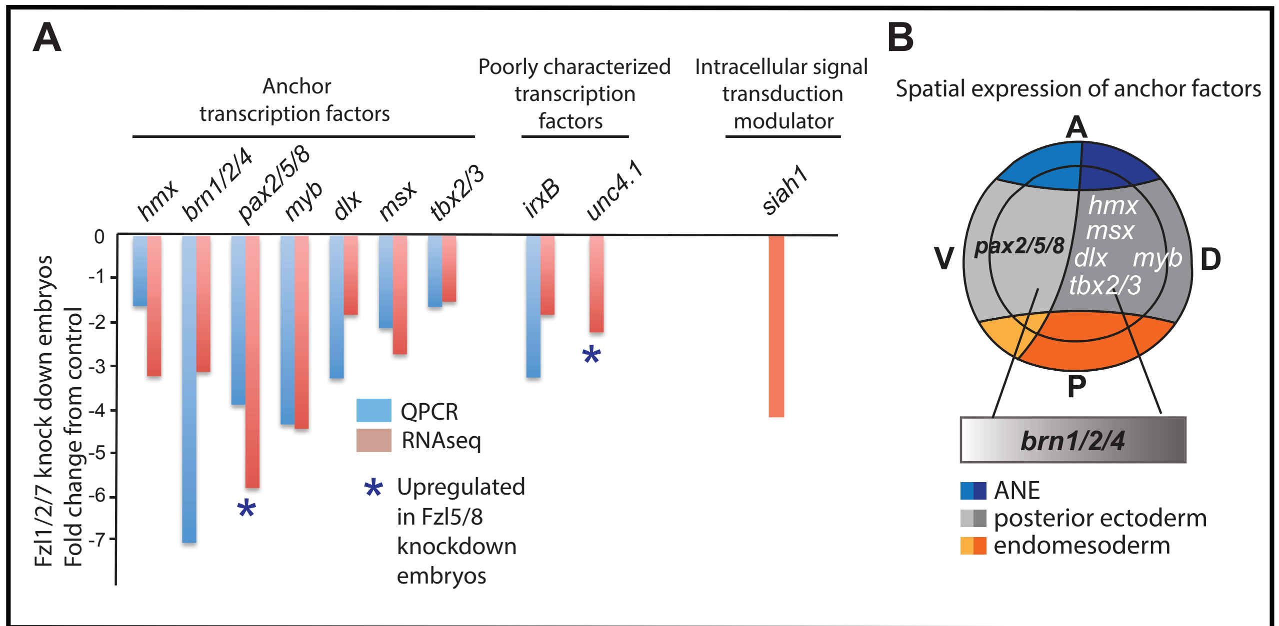
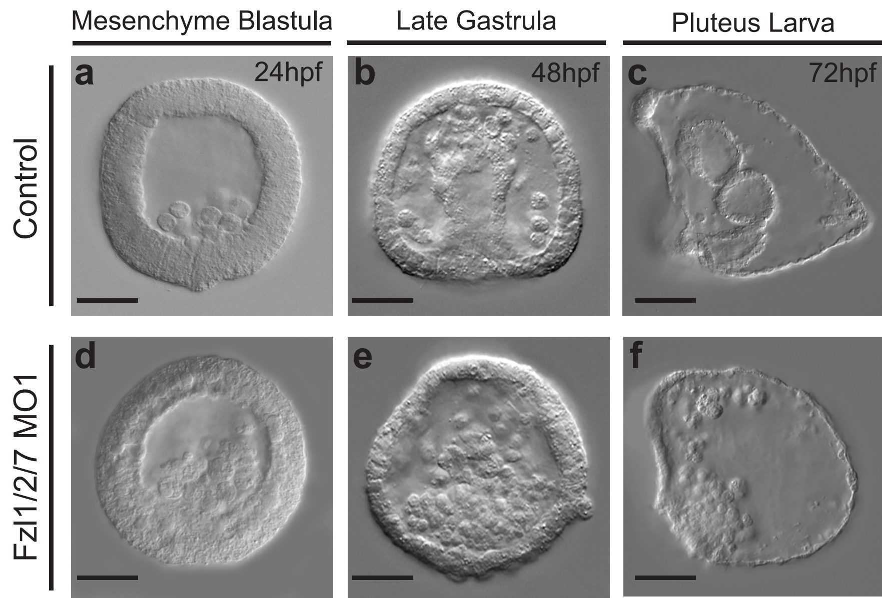


Figure 2. Phenotypic characterization of Fzl1/2/7 knockdown embryos during gastrulation



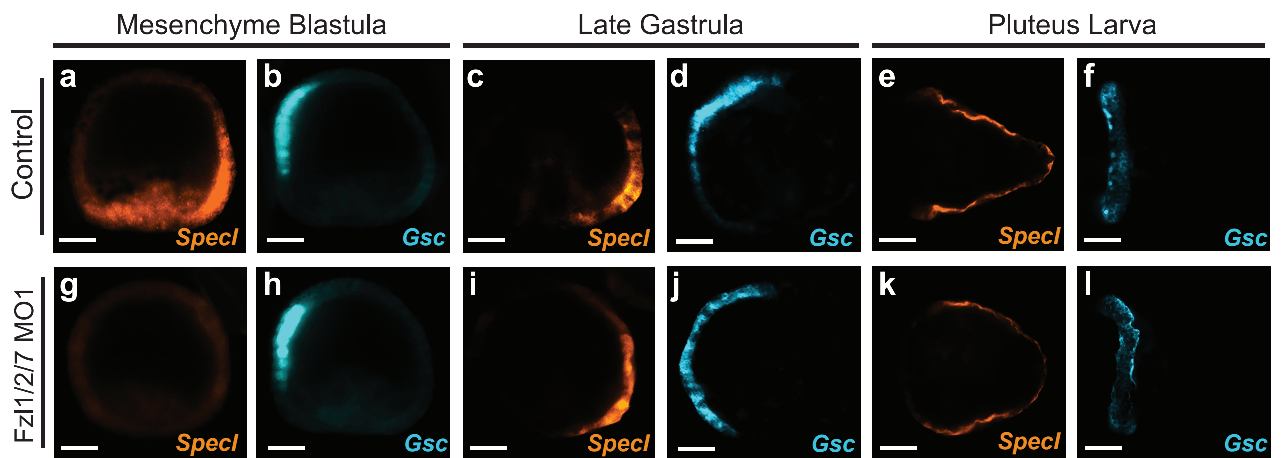
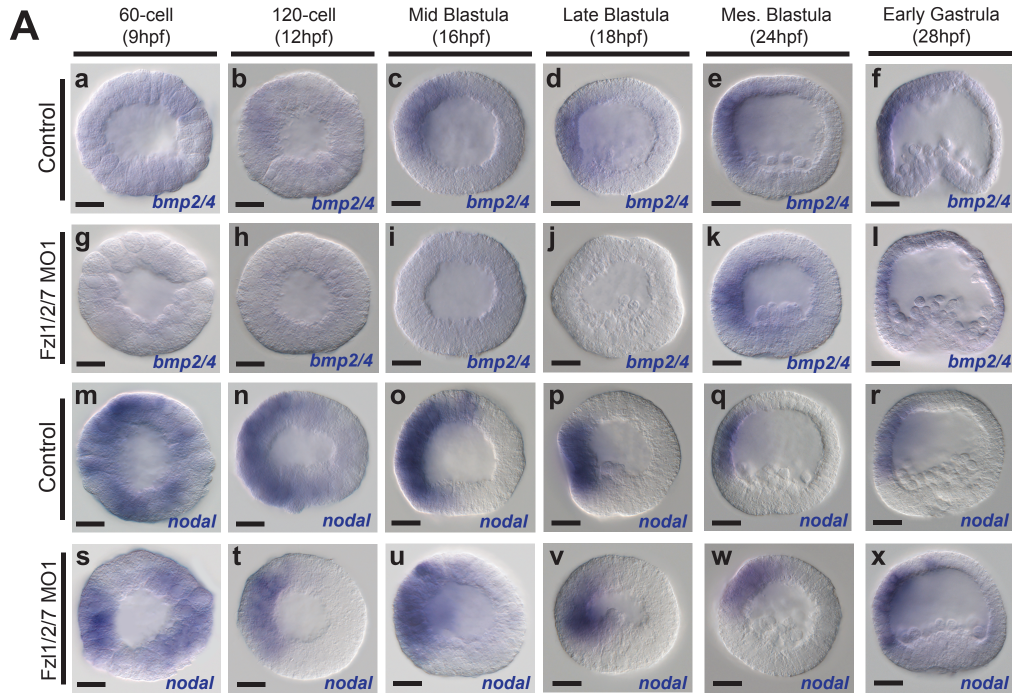


Figure 3. Fzl1/2/7 signaling is necessary to control BMP2/4 signaling but not *nodal* expression, and for the expression of dorsal and ciliary band markers during DV patterning.



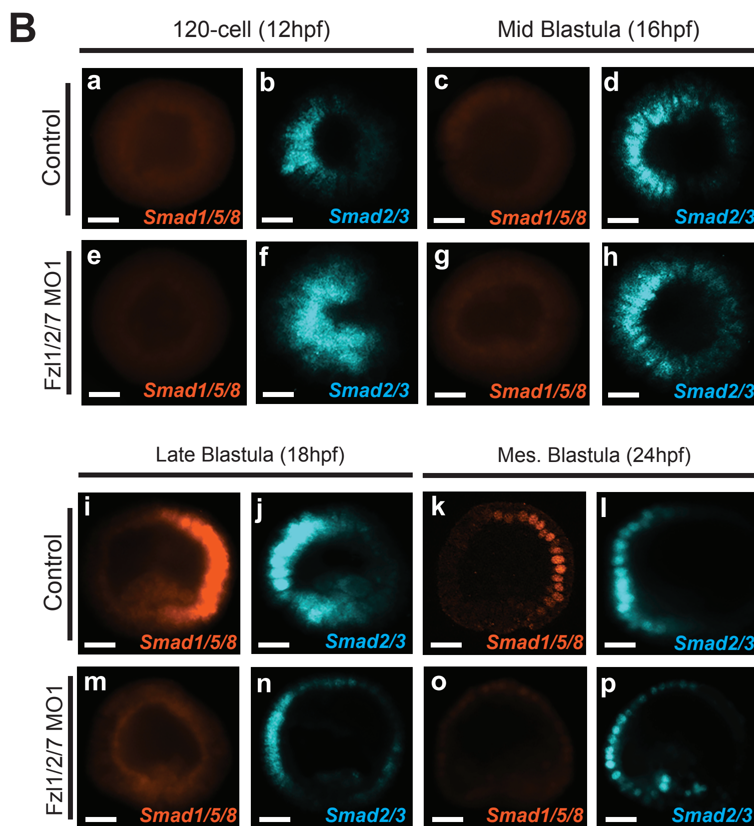


Figure 3. Fzl1/2/7 signaling is necessary to control BMP2/4 signaling, but not *nodal* expression, and for the expression of dorsal and ciliary band markers during DV patterning.

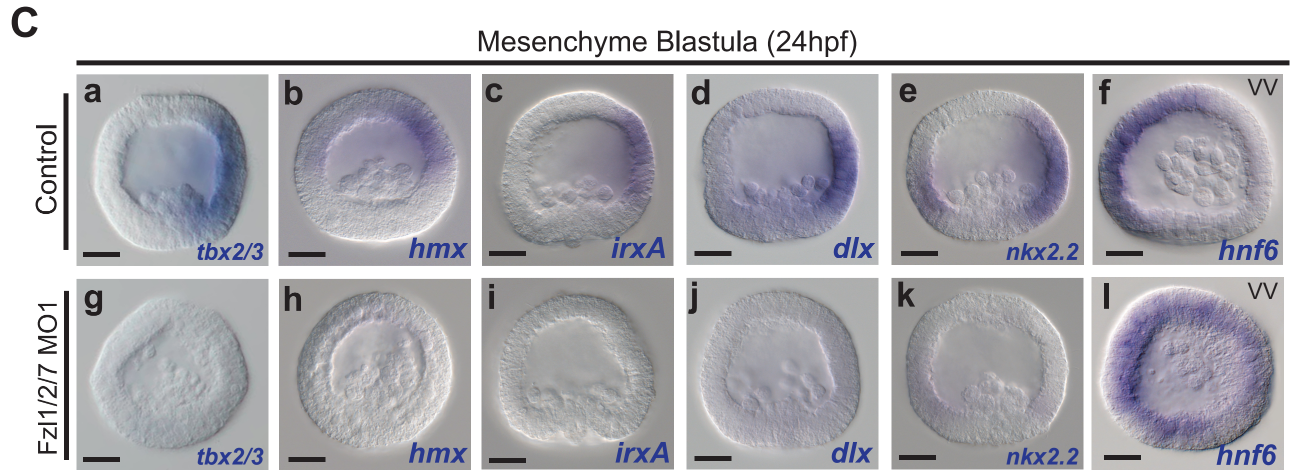
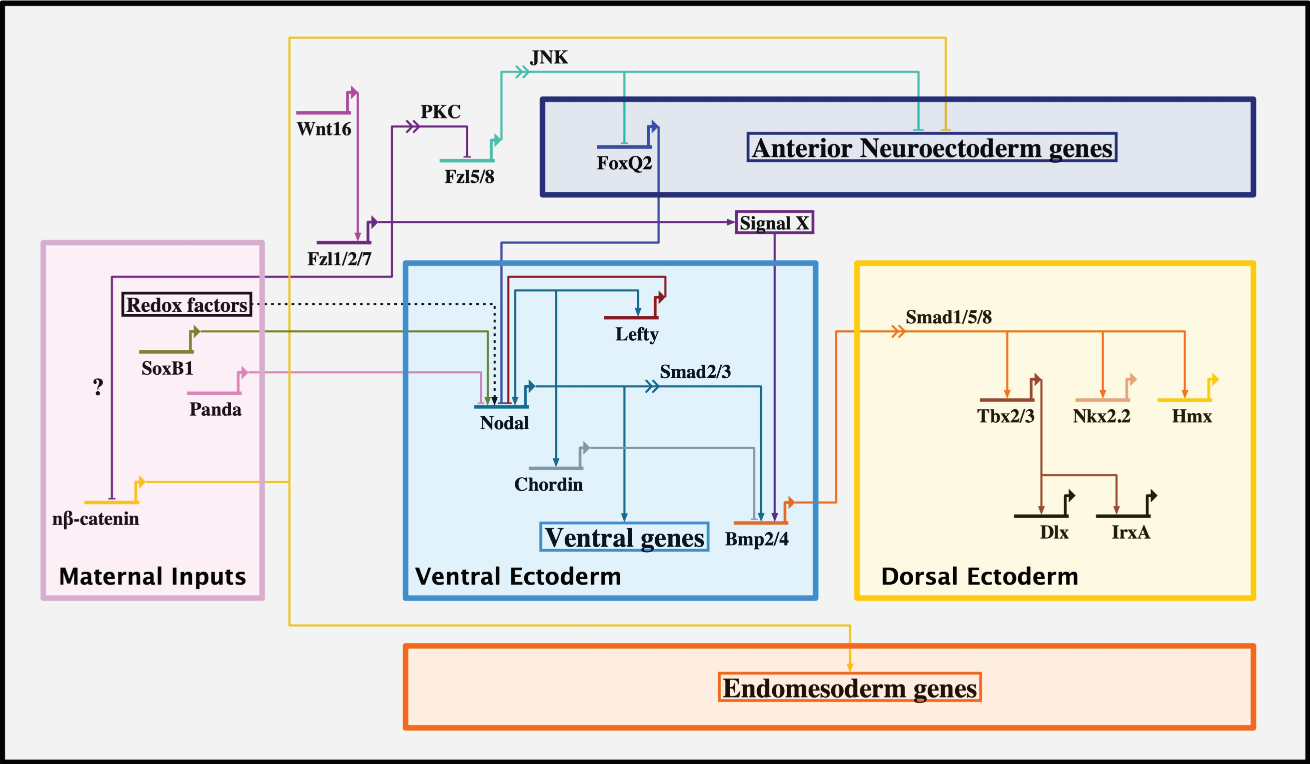


Figure 4. An extended GRN model for the proper patterning of the different germ layer territories in the sea urchin embryo.



SUPPLEMENTARY FIGURES

Supplementary Figure 1. RNA-seq screen heatmap of several signaling molecules in Fzl1/2/7 morpholino-injected embryos.

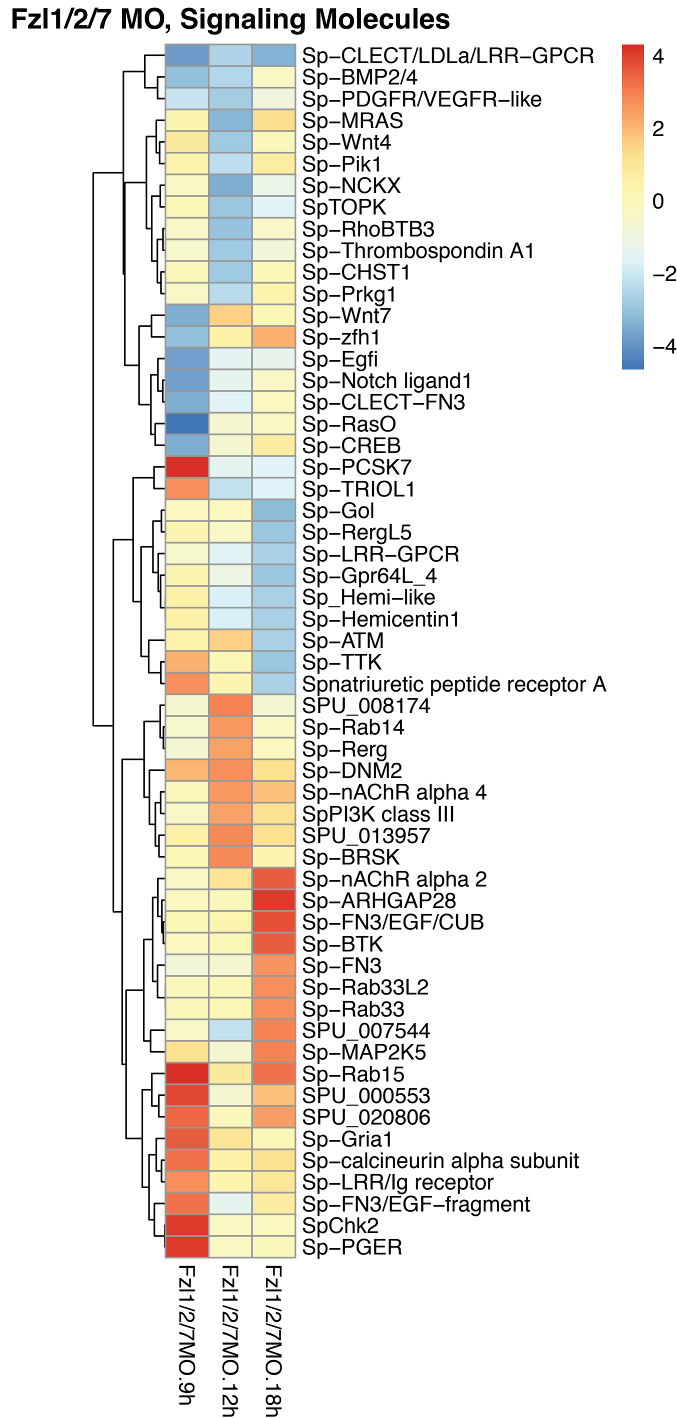


TABLE S1

Sp-mitochondrial 12s rRNA
Forward 5’-ACTCTCTCCTCGGAGCTATA-3’
Reverse 5’-GTATAATTTTTGCGTATTCGGC-3’

Sp-zic2
Forward 5’-GAGGGATGTGATCGTCGTTT-3’
Reverse 5’-ACTGCTGTCGTTGGCTTCTT-3’

Sp-bmp2/4
Forward 5’-ATGGTTACCACCTCACATTGGGGTCAG-3’
Reverse 5’-TAATACGACTCACTATAGGGAGACACTAAGTTCTGTAGGCACGC-3’

Sp-dlx
Forward 5’-GTATGAGGAACATTTACTGCTTGG-3’
Reverse 5’-TAATACGACTCACTATAGGGAGAGTGATGCTGTTGAATGAGATGG-3’

Sp-hmx
Forward 5’-ATGGACAGTAGCCGTGAACTATC-3’
Reverse 5’-ATTTAGGTGACACTATAGAAGNGCTACTGTTGGTTAGTGGCTGGG-3’

Sp-irxA
Forward 5’-CCACAAGTTATTGTTGTTGCTGA-3’
Reverse 5’-TAATACGACTCACTATAGGGAGAAAGTCTCTCAGTCATGGAGTCG-3’

Sp-nkx2.2
Forward 5’-TCTTTTCTTCTCCTGGTTTCCAC-3’
Reverse 5’-TAATACGACTCACTATAGGGAGACTGACATACACGCTGATGCTG-3’

Sp-nodal
Forward 5’-CACAAAGTGTGTTTGTGCAAG-3’
Reverse 5’-GTCGATGAAATTGAAAATATCATGA-3’

Sp-tbx2/3
Forward 5’-TCACAAAAGAGGAACAGAAATGG-3’
Reverse 5’-TAATACGACTCACTATAGGGAGAGGGATGGGTGTCTAAATAACTCG-3’

Chapter 4.

General Conclusions and Future Directions

I. Developmental regulatory mechanisms during early anterior-posterior and dorsal-ventral specification and patterning of metazoan embryos

ABSTRACT

How an animal emerges from a fertilized egg and how the different embryonic body axes are established are some of the most crucial questions in developmental biology. Therefore, it is extremely important to characterize the molecular and cellular mechanisms involved in body axis patterning and how the different body plans have arisen during evolution. Previous studies have shown that Wnt and BMP signaling pathways play crucial regulatory functions during anterior-posterior and dorsal-ventral patterning as well as endomesoderm specification in both Bilaterians and Non-bilaterians. Here, we examine the shared molecular mechanisms of body axis patterning among different organisms and describe potential connections between the primary and secondary axes in several metazoan embryos. Taken together, a better understanding of the common and novel strategies of the different body axis patterning allows us to elucidate how the unique body plans of each organism have evolved from a common ancestor.

INTRODUCTION

The establishment and patterning of cell territories along the different embryonic axes are critical events in the early development of any metazoan. Here, we examine the fundamental molecular mechanisms that organize the asymmetry of an early embryo and the establishment and patterning of anterior (head) and posterior (tail) axis as well as dorsal (back) and ventral (belly) axis during early development. Further studies focusing on how an organism with all the distinct body parts is created from a fertilized egg are essential to better understand the exact mechanisms that position early anterior-posterior (AP) and dorsal-ventral (DV) axes. The early specification and patterning of AP and DV axes along the different embryonic territories are controlled by several sets of polypeptide growth factors such as TGF-β, FGF, and Wnt families (Takebayashi-Suzuki et al., 2018). For example, a combination of maternal and zygotic inductive signals (e.g. a gradient activity of Wnt signaling) is responsible for the first establishment of the embryo's primary anterior-posterior (AP) axis in many organisms (Carron and Shi, 2015;

Petersen and Reddien, 2009). The mechanism of AP axis specification and patterning involves the integration of different signaling pathways to coordinate the specification of the distinct embryonic germ layers, expression of gene markers in the right territories, and morphogenetic movements of gastrulation (Carron and Shi, 2015; Slack, 2014). In addition, a secondary dorsal-ventral (DV) axis perpendicular to the AP axis defines the bilateral symmetry (Martindale, 2005). Cell-cell interactions between AP and DV axes are critical during early development for the proper arrangement and organization of the animal body plan.

Comparison of body plans among metazoans is a fundamental aspect of the field of developmental biology and new molecular tools, e.g. genome editing using CRISPR/Cas9 system, are improving our capability to study metazoan body plan evolution. Moreover, how the different GRNs involved in the formation of the different germ layers, axial specification, and cell differentiation have evolved during development can help us to elucidate how the current diversity of multicellular life has developed throughout time (Martindale, 2005). Previous studies have shown that there is a similar molecular architecture during the specification of anterior-posterior, dorsal-ventral, and left-right body axes among metazoans (Kimelman and Martin, 2012; Loh et al., 2016; Martindale, 2005; Niehrs, 2010; Petersen and Reddien, 2009). Additionally, conserved gene families and gene regulatory networks (GRNs) responsible for those crucial developmental processes have been found across the animal kingdom. Bilaterians are a subkingdom that includes the majority of metazoans except the four early-branching phyla: Porifera, Ctenophora, Placozoa, and Cnidaria (Ryan and Baxevanis, 2007). From all metazoans, Poriferans (sponges) are the only organisms that do not present DV body axis. Interestingly, in contrast to the rest of the animals that show DV axis, Ctenophores are unique organisms that show DV axis patterning but not DV polarity (Martindale, 2005). Therefore, functional studies in Ctenophores will help us to elucidate the evolutionary origins controlling secondary axis specification and patterning. On the other hand, Placozoans are the only phyla with no AP axis, suggesting that the molecular and cellular mechanisms required for the establishment of the primary body axis might be conserved among those other animals. In addition, Hox genes are existent in all metazoans but additional temporal expression and functional studies in Ctenophores and Poriferans need to be performed to obtain a better understanding of how Hox genes might play an important role in AP patterning and the establishment of the endoderm and mesoderm germ layers (Martindale, 2005).

Here, we examine and discuss the current knowledge of body patterning on different species of vertebrates (mouse, chicken, zebrafish, and *Xenopus*), tunicates (ascidians), cephalochordates (*Amphioxus*), echinoderms (sea urchins and sea stars), hemichordates, protostomes (*Drosophila*, *Tribolium*, and planarians), and cnidarians (*Nematostella vectensis* and *Hydra*) to reveal the different strategies of AP and DV specification and patterning in metazoans. Since coordinated and linked mechanisms are required in order to regulate the establishment of the AP and DV axis formation, we discuss distinct cases in different model systems where these two embryonic axes are closely interconnected.

COMPARATIVE PERSPECTIVE OF THE MOLECULAR MECHANISMS CONTROLLING ANTERIOR-POSTERIOR AXIS PATTERNING

Deuterostomes

In the early development of metazoan embryos, the mechanisms that position and pattern cell territories along the primary anterior-posterior (AP) embryonic axis define the embryonic and adult body plan. In most metazoans, the AP axis is the first to be specified during early cleavage stages and it is commonly complete established by the end of gastrulation. First, an asymmetric distribution of maternal components creates an egg polarity during oogenesis and establishes the primary or animal-vegetal (AV) axis in many bilaterians (Lee et al., 2007; Petersen and Reddien, 2009; Wikramanayake et al., 2003). The anterior territory of the adult form is derived from the animal pole and the posterior from the vegetal region (Martindale, 2005). In most metazoans, Wnt signaling pathways play complex and multi-functional roles in early development. In addition, several aspects of the Wnt signaling mechanism that positions the embryonic territories along the AP axis are remarkably conserved among metazoan embryos, including vertebrates (Niehrs, 2010; Range, 2014). Previous studies have discovered that localized Wnt signaling in the posterior region can be inhibited by Wnt antagonists in the anterior territories, forming a gradient of Wnt signaling along the AP axis and inducing anterior structures such as forebrain, heart, and anterior sensory organ (Petersen and Reddien, 2009; Yamaguchi, 2001). Interestingly, the GRNs that control AP axis patterning and specification are also remarkably conserved among

metazoan embryos, suggesting that several of the embryonic territories along this axis could be homologous (Range, 2014).

The posterior canonical Wnt/β-catenin signaling is the most crucial and conserved mechanism that patterns the embryo along AP axis. Wnt/β-catenin pathway presents an ancestral role in specifying posterior territories as well as antagonizing most anterior regions. For example, Wnt/β-catenin is required for the patterning of the neuroectoderm along the AP axis in a wide range of metazoans (Petersen and Reddien, 2009; Schneider et al., 1996). During this signaling pathway, Wnt ligands bind the Frizzled receptors and co-receptor LRP, activating the Disheveled protein and preventing β-catenin degradation (Petersen and Reddien, 2009; van Amerongen and Nusse, 2009). Consequently, the stabilization and nuclearization of β-catenin protein together with TCF/TEF proteins regulates transcription of downstream target genes involved in AP patterning (MacDonald et al., 2007). In addition, a combination of Wnt signaling pathways often work together within the same cell or territory. Extracellular, intracellular, and transcriptional interactions between Wnt branches are remarkably common in deuterostomes, forming interconnected Wnt signaling networks (Petersen and Reddien, 2009; Schneider et al., 1996). For instance, recent model of an integrated Wnt signaling network in sea urchin embryos have proposed that inputs from both canonical and non-canonical Wnt pathways are required for the correct patterning of AP axis (Khadka et al., 2018; Range, 2018; Range and Wei, 2017; Range et al., 2013). Interestingly, the molecular mechanisms for this integrated Wnt signaling network appear to be conserved among deuterostomes (Range, 2014). Therefore, there are multiple interactions between ligands, receptors, and co-receptors in order to activate any of the three different Wnt signaling pathways, the Wnt/β-catenin, the Wnt/JNK, and the Wnt/PKC pathways (Kestler and Kühl, 2008; van Amerongen and Nusse, 2009).

In vertebrates, secreted signaling molecules such as Wnts and TGFβ family BMPs and Nodal are necessary to establish cell fates across the AP axis (Kimelman and Martin, 2012). For example, in mouse and chick, downregulation of Wnt signaling in the anterior territories leads to defects in heart and forebrain formation as well as in the specification of mesoderm and ectoderm territories (Yamaguchi, 2001). Wnt signaling pathways act as posteriorizing signals in all three germ layers (Hikasa and Sokol, 2013; Petersen and Reddien, 2009). Thus, Wnt signaling pathways are necessary to specify posterior fates and their inhibition is regulated by Wnt

antagonists (such as GSK3, Shisa, Dkks, and sFRPs), creating a gradient of Wnt signaling distribution that allows the proper formation of the embryonic anterior and posterior territories (Itoh et al. 1995; Glinka et al. 1998; Yamamoto et al. 2005). Interestingly, previous studies have suggested that early specification and patterning along the AP axis is independent of information from the dorsal organizer. After cortical rotation, early Wnt/β-catenin signaling upregulates Wnt and BMP antagonists in the anterior regions and activation of zygotic *wnt8* creates an accumulation of β-catenin in the ventral and posterior territories during gastrulation (Hikasa and Sokol, 2013). In addition, Wnt/β-catenin and Nodal signaling give integrated information for both AP and DV polarities and; therefore, they are the main responsible in the establishment of the distinct body axes. In addition, there are negative cell-cell interactions between both signaling pathways building an integrated gene regulatory network that controls AP and DV patterning. In contrast to *Xenopus,* in other deuterostomes such as zebrafish, tunicate, and cephalochordate embryos the mechanism that positions AP axis patterning is highly conserved and the connection of AP and DV axes have not been that extensively studied. Taken together, vertebrate embryos present a shared signaling center that dorsalizes all three germ layers and regulates early AP specification and patterning (Hikasa and Sokol, 2013).

Protostomes

Many of the molecular events critical for protostome embryogenesis occur during oogenesis. For example, in *Drosophila,* a long germ-band insect, Bicoid and Nanos are essential proteins for establishing AP polarity of the embryo (Gilbert, 2010). Canonical Wnt/β-catenin has an essential role in AP patterning and AP polarization in most animals, despite limited evidence from arthropods. Wnt/β-catenin signaling has also an essential role in pattern the primary axis in protostomes. Vertebrate embryos present Wnt inhibitors such as Dkks and sFrps in the most anterior region with a clear role in negatively regulating Wnt signaling in the anterior territories of the embryo. On the other hand, Wnt antagonists have not been found in protostomes and; therefore, the Wnt signaling inhibition has not been connected with the specification and patterning of the most anterior regions. In *Drosophila,* Wnt signaling is necessary to define the different boundaries of the distinctive segments but it does not play a function in the polarization along the AP axis (Bejsovec and Arias, 1991). In addition, the proper regulation of Wnt signaling

is necessary for anterior development in arthropods similar to vertebrate embryogenesis and planarian regeneration (Fu et al., 2012).

Anterior specification in *Tribolium,* a short germ-band insect, occurs thanks the localized activity of *hb* and *otd* proteins in the anterior end of the embryo, suggesting that a mechanism which involves both Hb and Otb is likely to be ancient for anterior specification in insects (Kimelman and Martin, 2012; Wolff et al., 1995). Posterior Wnt signaling is also essential for the elongation of germ-bands and the formation of abdominal segments but previous data suggested that this signaling pathway does not seem to be required for the specification of anterior fates (Bolognesi et al., 2008a; Bolognesi et al., 2008b; McGregor et al., 2008). Loss of function experiments showed that Wnt signaling affects segment development in the growth zone but not in the blastoderm area. Interestingly, recent studies in *Tribolium* embryos have shown that Wnt/β-catenin signaling needs to be properly regulated during AP axis specification and presented evidence of its ancestral function in defining AP polarity and patterning in early development of many metazoans (Bolognesi et al., 2008; McGregor et al., 2008). Data from Fu et al., (2012) showed that one of the first asymmetric signals in *Tribolium* embryos is maternally localized Tc-axin mRNA in the anterior pole of the egg, reminiscent to bicoid in *Drosophila*. During early cleavage stages, the expression of Tc-axin, a highly conserved negative regulator of Wnt/β-catenin signaling, disperses towards the posterior pole and; subsequently, when the germ rudiment is formed, Tc-axin is ubiquitously expressed. Perturbation experiments using Tc-axin RNAi showed an expansion of the posterior fates to most anterior territories; for instance, several blastodermal genes presented an altered expression domain (Fu et al., 2012). The effects of blocking Tc-axin are controlled by the canonical Wnt pathway, suggesting that an inhibition of Wnt/β-catenin signaling is necessary for the proper development of the anterior regions in *Tribolium* embryos (Fu et al., 2012). In addition, the activity of β-catenin destruction complex is localized in the anterior region and regulates the early positioning along AP axis (Fu et al., 2012).

During early cleavage stages of the annelid *Platynereis dumerilii* β-catenin localizes to the vegetal cell and creates β-catenin sister-cell asymmetries after each axial division that occur along the animal-vegetal (AV) axis but not after the divisions that establish the bilateral symmetry in the trunk (Schneider and Bowerman, 2007). High levels of β-catenin in the vegetal

pole and low β-catenin in the animal pole create asymmetries of nuclear and cytoplasmic β-catenin of sister-cell pairs in AV oriented cell divisions (Schneider and Bowerman, 2007). The β-catenin accumulation in the animal pole is negatively regulated by GSK-3β. Overexpression of the Wnt/β-catenin signaling induces animal-pole sister cells to adopt the vegetal fate of their vegetal sisters as a result of higher levels of nuclear β-catenin in these cells at the animal pole of the embryo (Schneider and Bowerman, 2007). These studies give evidences that Wnt signaling pathways are essential for the proper regulation of early AP specification in arthropods and annelids. For example, nuclear β-catenin has a crucial function during AP patterning, creating asymmetry in distantly related animals. Taken together, the studies mentioned above suggest an ancestral metazoan mechanism using Wnt signaling to polarize and pattern the AP axis.

COMPARATIVE VIEW OF THE MECHANISMS NECESSARY FOR DORSAL-VENTRAL SPECIFICATION AND PATTERNING

Deuterostomes

Extensive embryological and molecular investigations have contributed to a major advance in the understanding of the mechanisms responsible for DV patterning. In most deuterostomes, DV axis is specified and positioned after fertilization by cell interactions (Gilbert, 2010). During fertilization events, β-catenin localization in a specific region of the egg will induce expression of specific genes and establish AP and DV axis of the embryo. Interestingly, a similar molecular mechanism for patterning of the bilaterian DV axis has been previously identified in several organisms, with members of the TGFβ family such as bone morphogenetic protein (BMP) and Nodal signaling playing conserved roles during DV polarity (De Robertis and Sasai, 1996). BMPs and Nodal are extracellular secreted ligands that bind the specific heterotetrameric transmembrane receptor complex formed by type I and type II receptors (Little and Mullins, 2006). This binding allows the activation and phosphorylation of the intracellular mediators of the Smad protein family (e.g. Smad1/5/8 and Smad2/3) which will subsequently bind to Smad4 and form a Smad complex (Little and Mullins, 2006; Molina et al., 2007).

From vertebrates to echinoderms (Khokha et al., 2005; Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007), the conserved function of BMP signaling is a clear example of similarity in DV patterning in metazoans. BMP antagonists such as chordin, noggin, and follistatin in the Spemann's organizer play important and cooperative functions for the proper mesoderm formation along the DV axis in *Xenopus* (Khokha et al., 2005). In addition, a combined effort between different BMP antagonists as well as early β-catenin and/or FGF signaling is essential for correct positioning of dorsal and ventral fates (Khokha et al., 2005). Previous studies have also reported that loss of BMP antagonism causes a failure in the expansion of dorsal and ventral territories but; interestingly, early specification of the organizer is not disturbed when the BMP antagonists are not presented (Khokha et al., 2005).

The developmental and molecular mechanisms during asymmetry along the DV axis required extra contributions from integrated signaling pathways; therefore, the activation of BMP signaling is usually not the only contribution to this mechanism of DV specification and patterning. In the frog embryo, maternal inputs of β-catenin are essential for mesoderm specification. Then, after zygotic transcription and before gastrulation, the induction of mesoderm is regulated by Nodal signaling thanks to the activity of maternal determinants such as VegT and β-catenin (Carron and Shi, 2015). Following frog fertilization, a cortical rotation leads to the transport of maternal determinants to the dorsal region such as β-catenin activation and establishes the Spemann's organizer in the dorsal side of the embryo, creating the primary dorsoventral asymmetry, that consequently signals BMP inhibitory proteins (Carron and Shi, 2016; De Robertis and Sasai, 1996). In *Xenopus,* an integrated information between VegT and Wnt/β-catenin signaling is responsible for DV axis patterning (Carron and Shi, 2016; De Robertis and Sasai, 1996). VegT regulates the expression of nodal-related genes and high levels of Nodal-related proteins together with the activity of Wnt/β-catenin signaling creates the Nieuwkoop center in the dorsal-ventral territory (Carron and Shi, 2016). Thus, maternally activated Wnt/β-catenin signaling and high levels of Nodal signaling induces the specification of the Nieuwkoop center in the dorsal-vegetal cells, which then triggers the formation of the Spemann organizer in the overlying dorsal marginal zone (Carron and Shi, 2015). In addition, Wnt antagonists are responsible for blocking ventral and posterior activity of zygotic Wnt/β-catenin and; therefore, promote dorsal and anterior fates in early development of *Xenopus* embryos (Glinka et al., 1998; Leyns et al., 1997; Wang et al., 1997).

In the sea urchin and sea star embryos, the mechanism of DV patterning is remarkably similar to the mechanisms that pattern DV axis of other deuterostomes. Interestingly, sea urchin embryos present an organizing center, caused by Nodal signaling, on the ventral ectoderm which is remarkably similar to the Spemann organizer in vertebrates. After fertilization, TGF-β Nodal signaling and its downstream target BMP 2/4 are required for the establishment of the three embryonic layers along the DV axis of these embryos (Duboc et al., 2004; Molina et al., 2013; Saudemont et al., 2010). Nodal specifies ventral fates and it is necessary for the expression of ventral ectoderm genes such as *bmp2/4* and *chordin* (Angerer et al., 2000; Angerer et al., 2001; Duboc et al., 2004; Flowers et al., 2004), patterning the DV axis of the sea urchin embryo. Nodal signaling synthesizes *bmp2/4* in the ventral ectoderm which subsequently translocates to the dorsal side of the embryo where it activates the expression of other dorsal genes (Duboc et al., 2004; Molina et al., 2013). Therefore, Nodal and BMP2/4 are expressed in the same ventral territory but they work in opposite ventral-dorsal territories thanks to the presence of a morphogenetic gradient (Molina et al., 2013). This morphogenetic gradient is responsible for the patterning of the three main germ layers (endoderm, mesoderm, and ectoderm) along the dorsal-ventral (DV) axis. In addition, the BMP antagonist Chordin, activated by Nodal signaling, is required to inhibit BMP2/4 signaling in the ventral ectoderm in such a way that ventral *bmp2/4* diffuses towards dorsal regions to induce ventral fates by activating the expression of dorsal genes (Angerer et al., 2000; Bradham et al., 2009; Duboc et al., 2004; Lapraz et al., 2009; Molina et al., 2013). Interestingly, a recent large-scale ectoderm GRN analysis suggested that BMP2/4 signaling might not be the early input that activates the expression of particular dorsal markers, suggesting that another mechanism is likely required for their activation (Su et al., 2009). In sea star embryos, TGFβ signaling, but not Nodal, plays a crucial role in DV axis patterning, suggesting that a different use of signaling molecules during early echinoderm development (Sasaki and Kominami, 2017). Together, these findings indicate once again that a combination of multiple signaling pathways is frequently used for patterning the secondary axis.

Interestingly, DV axis is inverted in chordates compared with other non-chordate invertebrates, while still determined by expression of BMP signaling members and their antagonists (De Robertis and Sasai, 1996; De Robertis et al., 2000). Although the molecular and cellular mechanisms involved in body axis specification and patterning in deuterostomes have been extensively investigated, a number of questions remain unanswered. For example,

cooperative inputs from Nodal, BMP and Wnt signaling pathways activates the formation of mesoderm and endoderm, patterns ectoderm along the AP and DV axis, and induce the Spemann organizer during gastrulation of several vertebrate embryos (Agius et al., 2000; Hashimoto-Partyka et al., 2003; Zorn et al., 1999). However, the exact machinery that regulates the integrated information from different signaling pathways is still not well understood. Therefore, future studies on deciphering the genetic cascades that control DV patterning in diverse deuterostome and protostome embryos will advance our understanding of the shared and novel mechanisms in an evolutionary perspective.

Protostomes

Protostomes present slightly different molecular mechanisms of DV patterning during early development. Drosophila embryos require the activity of Toll-Dorsal signaling pathway to generate a gradient of dorsal activity of the BMP-like Decapentaplegic (Dpp) protein (Moussian and Roth, 2005). Toll receptors expressed on the ventral side but not on the dorsal side of the oocyte transduce a signal into the egg and; therefore, activated a localized DV polarity (Gilbert, 2010). However, in contrast to most deuterostomes, BMP signaling is not polarized in Drosophila and other protostomes. After fertilization, *dorsal* gene is translated throughout the embryo but it is only translocated into nuclei on the ventral side of the *Drosophila* embryo, acting as a morphogen (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Dorsal protein in the nucleus works as a transcription factor activating or repressing the expression of dorsal and ventral genes. Specification of ventral fates and repression of dorsal cell type are dependent on Dorsal entering the nucleus (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). When Dorsal does not enter the nucleus, it cannot transcribe ventral genes, forming completely dorsalized embryos.

On the other hand, BMP signaling is a conserved pathway with two main roles in development of planarian embryos: first, it controls DV axis patterning and second, plays an essential role in the regeneration of this axis (Molina et al., 2007). Taken together, these data show that a gradient of BMP activity is necessary to pattern the DV axis and this is a highly conserved mechanism among metazoans. However, the machinery required to establish the BMP gradients during DV axis patterning can be highly divergent and more comparative analysis need

to be performed among diverse organisms in order to elucidate the similarities and novelties related to the proper formation of the secondary axis.

THE ENIGMATIC CNIDARIANS – THE SIGNIFICANCE OF NON-BILATERIANS

Axial patterning: anterior-posterior and directive axis positioning in Cnidarians

Cnidarians are ancient metazoans (>500 million years old) and sister-group to the triploblastic bilaterians that share a simple body plan. They are diploblastic organisms, with only two germ layers (an outer ectoderm and inner endoderm) and lacking the mesoderm territory (Guder et al., 2006). They are considered to have less cell type and be less specialized along AP axis but they are still extremely useful model organisms to study how evolutionary changes in the genetic toolkit have leaded to the diverse and complex body plans of metazoans (Ryan and Baxevanis, 2007). Therefore, the study of the early molecular mechanisms in cnidarians is of crucial importance for unravelling the evolution and origin of the different body plans in earth. For example, a fundamental question in developmental biology is how the oral–aboral primary body axis of cnidarians relates to the AP axis of bilaterians.

A role of Wnt signaling responsible for primary axis polarity in pre-bilaterian animals such as cnidarians has been shown, indicating that an axial patterning function for Wnt signaling could preexist in the common ancestor of bilaterally symmetric organisms during evolution (Petersen and Reddien, 2009). In cnidarians, β-catenin protein is localized to the nucleus in the blastomeres of the animal pole where the presumptive endomesoderm is formed (Lee et al., 2007; Röttinger et al., 2012; Wijesena et al., 2017; Wikramanayake et al., 2003). In addition, a cytoplasmatic component of the Wnt signaling, Disheveled, is important in the development of early asymmetry since it functions with β-catenin in the animal pole where it is localized (Wijesena et al., 2017). In the cnidarians *Nematostella vectensis* and *Hydra,* high levels of Wnt/β-catenin signaling promote oral pole identity. In addition, stabilization of β-catenin and its coactivator TCF activates endomesoderm gene markers (Lee et al., 2007; Röttinger et al., 2012; Wijesena et al., 2017; Wikramanayake et al., 2003). In *Nematostella* embryos, TCF knockdowns

expand the expression of the aboral marker *fgfa1* and downregulate *wnt2* and *chordin* oral ectoderm genes.

Similar to deuterostome embryos, the establishment and patterning of the directive axis depend on BMP/Chordin signaling. In *Nematostella*, the directive axis is mainly controlled by BMP2/4 signaling (Genikhovich et al., 2015; Rentzsch et al., 2006; Saina et al., 2009; Wijesena et al., 2017). These embryos also show a combination of both BMPs (e.g. BMP2/4 and BMP5/8) and BMP inhibitors (e.g. Chordin) with a partially overlapping expression domains along the directive axis (Genikhovich et al., 2015; Rentzsch et al., 2006; Saina et al., 2009). In contrasts to most bilaterians, there is a negative feedback loop of BMPs regulating their own expression (Genikhovich et al., 2015; Rentzsch et al., 2006; Saina et al., 2009). In addition, Hox genes are also expressed along the directive axis but their expression is controlled by BMP signaling (Genikhovich et al., 2015). Importantly, recent work has demonstrate that Wnt/β-catenin and BMP2/4 signaling interact in order to pattern the endomesoderm along the AP and directive axes, corroborating that usually in early development different signaling pathways work together to regulate both primary and secondary axes (Wijesena et al., 2017).

Although the molecular mechanisms that connect AP and DV axes in deuterostomes and protostomes with the AP and directive axes in cnidarians have not been well-established yet, several studies have improved our understanding of the evolutionary aspects of body axis patterning. The fundamental molecular basis for primary and secondary axis is likely to be conserved among bilaterians and cnidarians. For example, previous functional work has shown that the bilaterian heart mesoderm GRN is functioning in *Nematostella*, supporting that endoderm and mesoderm in triploblastic bilaterians evolved from a diploblastic ancestor (Rentzsch et al., 2006; Saina et al., 2009). Future work in *Nematostella* and *Hydra* will need to focus on the exact molecular mechanisms that stabilize Disheveled at the animal pole in order to discover the most upstream signals that regulate AP patterning in cnidarians. A better understanding of the molecular and cellular architecture that control the establishment of AP patterning and the directive axis in cnidarian embryos will provide insights into the shared evolutionary mechanisms of body axis specification and patterning among metazoan embryos.

MOLECULAR CONNECTIONS BETWEEN ANTERIOR-POSTERIOR AND DORSAL-VENTRAL BODY PATTERNING IN EARLY DEVELOPMENT

In *Xenopus* embryos, formation and establishment of AP and DV axes are strongly linked. The canonical Wnt/β-catenin signaling functions in both the establishing the DV and AP axis patterning in these organisms. On the dorsal side of the embryo, activation of Wnt signaling localizes β-catenin protein into the nuclei, producing a molecular gradient of Nodal signaling that regulates induction and patterning of the endoderm territory (De Robertis et al., 2000). Antagonists of both Wnt and BMP signaling pathways, such as Noggin, Chordin, Cerberus, Crescent, Frzb-1, and Dkks also play important roles in regulating cell differentiation and position along primary and secondary axis. The antagonistic interactions between Wnt, BMP, and Nodal signaling secreted in the Spemann organizer and ventral territories pattern frog embryos along the DV and AP axis during gastrulation (Takebayashi-Suzuki et al., 2018). In addition, FGF and retinoic acid (RA) signaling together with the expression of Hox genes pattern the most posterior regions of *Xenopus* embryos (Takebayashi-Suzuki et al., 2018).

In addition, the BTB/POZ zinc finger protein Zbtb14 has been shown to play an essential function in regulation of AP and DV patterning during early *Xenopus* development. Zbtb14 regulates both the BMP and Wnt signaling pathways during early embryogenesis by downregulating the levels of pSmad1/5/8 to suppress BMP signaling and promoting Wnt signaling mediated an stimulation of β-catenin accumulation (Takebayashi-Suzuki et al., 2018). In addition to Zbtb14, previous data have revealed that FGF and Wnt signaling might also work together to regulate BMP signaling pathway in dorsal territories (Hashiguchi and Mullins, 2013). Therefore, it is feasible that an integration of these three pathways is required for the proper arrangement of the dorsal fates along both DV and AP axis in these embryos. Consistent with this idea, Fuentealba et al. 2007 proposed that FGF/MAPK and Wnt/GSK3 are necessary to phosphorylate, and consequently degradate, Smad1, a downstream component of BMP signaling. Taken together, polarity and cell-cell interactions along AP and DV axes are highly interconnected and same regulatory networks operate in both DV and AP patterning in frogs.

Similar to *Xenopus*, recent studies have suggested that a coordinated information of signaling pathways allows the position of the distinctive embryonic cell types during DV and AP

axis patterning in the early development of zebrafish embryos (Hashiguchi and Mullins, 2013). During gastrulation, combined signals of FGF, Wnt, and retinoic acid (RA) pathways negatively interact with BMP signaling in order to temporally control DV axis patterning (Hashiguchi and Mullins, 2013). Thus, a combination of four signaling pathways regulates DV positioning directly coordinated by AP patterning, forming a synchronized mechanism that coordinates the establishment of these two axes in a similar patterning clock. This suggests that a connection of AP and DV axis patterning is necessary for the proper position of the cells as well as a simultaneous temporal information in zebrafish embryos (Hashiguchi and Mullins, 2013). Thus, a combination of four signaling pathways regulates DV positioning directly coordinated by AP patterning, forming a synchronized mechanism that coordinates the establishment of these two axes in a similar patterning clock. This suggests that a connection of AP and DV axis patterning is necessary for the proper position of the cells as well as a simultaneous temporal information in zebrafish embryos (Hashiguchi and Mullins, 2013). Similar to *Xenopus*, recent studies have suggested that a coordinated information of signaling pathways allows the position of the distinctive embryonic cell types during DV and AP axis patterning in the early development of zebrafish embryos (Hashiguchi and Mullins, 2013).

In chicken and mice embryos, FGF, BMP, Hedgehog and Wnt signaling pathways are interconnected, interacting with each other in order to specify the right locations of the different embryonic territories. A recent study in chicken, frog, zebrafish, and humans have revealed a coordinate role of Apcdd1 in body axis patterning and formation as well as the specification of the nervous system (Vonica et al., 2019). Apcdd1 is a taxon-restricted gene present in a subset of metazoan embryos that negatively regulates both BMP and Wnt intracellular signaling pathways (Vonica et al., 2019). Apcdd1 is bi-functional protein with a novel protein domain that can bind to Wnt and BMP receptors and block downstream signaling. At the beginning of metazoan evolution, Apcdd1 might have evolved at the same time as BMP and Wnt signaling with a distinct function in organizing and integrating the outputs of these two signaling pathways. Interestingly, Apcdd1 has been preserved in 15 phyla in which BMP and Wnt signaling have important functions in the establishment of the primary and secondary embryonic axes (Vonica et al., 2019). On the other hand, this protein has been lost in several organisms that do not use BMP for axial body patterning, suggesting a correlation between Apcdd1 and an integrated information of BMP and Wnt signaling during early body patterning. Taken together, the

discovery of the taxon-restricted Apcdd1 presents one of the very first connections of AP and DV in many organism. Future directions will need to further examine how Apcdd1 coordinates the functional outputs of BMP and Wnt signaling pathways during early AP and DV patterning in a variety set of metazoan embryos.

In summary, integrated inputs from diverse signaling pathways during early AP and DV axis patterning and positioning are observed in several metazoan organisms. Recent unpublished data from our lab suggest that a Wnt signaling network regulates Nodal and BMP2/4 signaling pathways during early AP and DV positioning of sea urchins. Therefore, canonical and non-canonical Wnt and BMP2/4 signaling could work collectively in echinoderms and other deuterostomes during primary and secondary axis patterning, connecting AP and DV GRNs. Future comparative and functional studies in a wide range of deuterostome and protostome embryos will help us to elucidate the connected molecular mechanisms of AP and DV axis patterning and specification. Finally, comparing the molecular mechanisms in different bilaterian and non-bilaterian organisms is essential to better understand the evolution of the remarkably diverse metazoan body plan.

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II. The role of Siah during early anterior-posterior axis specification and patterning

ABSTRACT

Members of the Siah family modulate signaling pathways and act as E3 ubiquin ligase, binding proteins destined for degradation. Previous studies have shown that the signaling molecule Siah plays critical roles in vertebrate body axis formation and may function as an antagonist of the Wnt signaling by downregulating Wnt/β-catenin. Here, we identified a sea urchin homologue of the mammalian Siah family from our whole-transcriptome differential screen and tested its role as a putative Wnt/β-catenin antagonist. Siah is broadly expressed during early cleavage and blastula stages in the sea urchin embryo and then, it appears to be restricted to the anterior neuroectoderm (ANE) and endomesoderm. In addition, Fzl1/2/7 knockdowns downregulated Siah expression, confirming previous data from our differential screen results. Taken together, our spatiotemporal expression and loss-of-function analyses suggest that Siah is activated by the non-canonical Wnt16-Fzl1/2/7 signaling pathway during anterior-posterior (AP) specification and patterning in the sea urchin embryo. Siah might also play a key role as an inhibitor of the Wnt/β-catenin signaling during ANE positioning, preventing the premature elimination of ANE specification around the anterior pole.

INTRODUCTION

Mammalian homologs of *Drosophila* Sina (Siah) act as E3 ubiquin ligases by binding proteins destined for degradation (House et al., 2009; Qi et al., 2013; Wong and Möller 2013). The structure of Siah family usually have a divergent N-terminal and a high conserved RING domain and C-terminal (Della et al., 1993). Proteins from the Siah family have been found to interact with numerous cellular proteins such as scaffold proteins, transcriptional repressors, the motor protein Kid, the oncogene β-catenin, and the tumor suppressor TGF-β (House et al., 2009; Ji et al., 2017; Nakayama et al., 2009). In addition, Siah family modulates a variety of signaling pathways and appears to play critical roles in vertebrate body axis formation (Kang et al., 2014).

However, more functional studies of Siah in deuterostome development are necessary in order to understand the specific role of this signaling factor.

Interestingly, studies have shown that Siah may function as a negative regulator of the Wnt signaling by targeting β-catenin for degradation in a GSK3-mediated phosphorylation independent manner and; therefore, down regulating β-catenin stability (Fig. 1) (Jumpertz et al., 2014; Krämer et al., 2013; Liu et al., 2001; Matsuzawa and Reed, 2001). Interactions between Siah and β-catenin as well as other molecular and cellular components (e.g. CSN5) have been reported in cell cultures and animal embryos. For example, Siah-containing β-catenin destruction complex can play an alternative role in β-catenin degradation and; in addition, alternative APC-independent β-catenin degradation pathways can play important roles in development (Fig. 1) (Jumpertz et al., 2014); Topol et al., 2003). In addition, Siah also modulates other signaling pathways during early development. In zebrafish embryos, recent studies have revealed that Siah can also modulate Nodal signaling to pattern the mesendoderm territory (Kang et al., 2014). In these embryos, a functional RING domain of Siah is necessary to antagonize the function of Nodal antagonists (Kang et al., 2014). Given our previously results showing that Wnt16-Fzl1/2/7 signaling is necessary for AP and DV patterning in sea urchin embryos, it is also worth consideration that Siah might interact with intracellular inhibitors of Nodal and/or BMP2/4 signaling pathways to establish the correct positioning of the different embryonic territories. Thus, future studies exploring the nature of Siah-degrading E3 ligase and its specific roles in the downregulation of Wnt/β-catenin by degradation and the modulation of Nodal signaling will be essential in order to better characterize the molecular components involved during body axis patterning in deuterostome embryos.

In metazoan embryos, numerous developmental processes share a conserved toolkit of transcription factors and intracellular signaling molecules. Previously, we have identified transcription factors involved in the Wnt16-Fzl1/2/7 GRN that antagonizes the Wnt/β-catenin and Fzl5/8-JNK mediated ANE positioning mechanism (Fig. 1; Chapter 3). Intracellular signal transduction modulators are also frequently responsible for multiple roles during body axis patterning and specification. Interestingly, we observed in our RNA-seq screen that a known intracellular modulator of Wnt signaling, Siah, was severely downregulated in our differential screens in mid-blastula (18 hpf) Fzl1/2/7 knockdown embryos (10 fold difference compared with

control embryos) (Fig. 1; Chapter 3). Here, we identified two duplicate sequences of Siah sea urchin protein homologue of the mammalian Siah family. The main focus of this study is to investigate the potential functions of Siah during anterior-posterior (AP) patterning in the early sea urchin development. Since Wnt16-Fzl1/2/17 appears to antagonize the Wnt/β-catenin-dependent ANE restriction mechanism, we hypothesize that the signal transduction molecule Siah may mediate this antagonism downstream of the Fzl1/2/7-PKC signaling pathway. Taken together, our results suggest that the signaling molecule Siah may act as a putative Wnt/β-catenin antagonist in the sea urchin embryo.

RESULTS

Our RNA-seq differential screens identified *siah* as a signaling molecule downregulated in Fzl1/2/7 knockdown embryos (more than 10 fold difference compared with control embryos) (data shown in Fig. 1; Chapter 3). We performed in situ hybridization analysis to identify when and where *siah* gene is expressed in the neuroectoderm territory of the sea urchin embryo. The spatiotemporal expression pattern of *siah* mRNA in the different developmental stages showed that it is ubiquitously expressed as early as the 120-cell stage (12 hpf) through mid-blastula stages (15-18 hpf) (Fig. 2). As development progresses, *siah* mRNA expression seems to be up regulated in the skeletogenic mesenchyme cells that ingress into the blastocoel during the first gastrulation event in the posterior territory as well as the ANE territory (19-27 hpf) (Fig. 2). The localization of *siah* expression to the ANE territory presents a similar pattern as seen with other Wnt modulators, such as *dkk1*, *dkk3, sFRP1,* and *sFRP1/5*, during the final phase of ANE restriction (late blastula/early gastrula stages) (Khadka et al., 2018; Range and Wei, 2016; Range et al., 2013). Thus, it is reasonable to speculate that a combined activity of secreted modulators and signaling transduction factors such as *siah* around the anterior pole establish the correct patterning of the AP axis.

To confirm that Siah may act as a signaling transduction factor downstream of Fzl1/2/7 we carried out Fzl1/2/7 morpholino injections and evaluate any change in *siah* mRNA expression. Our results showed that at mid-blastula stage (18 hpf) expression of both *foxq2* ANE marker and *siah* was downregulated in Fzl1/2/7 knockdown embryos, confirming the results from the differential screens (Fig. 3).

In addition, we used CD-search from NCBI to search the conserved domain database with the two duplicates of Siah protein sequences in the sea urchin genome (Marchler-Bauer et al., 2017; Marchler-Bauer et al., 2015; Marchler-Bauer et al., 2011; Marchler-Bauer and Bryant 2004). The graphical display of the results for the protein domains is presented in Figure 4. Only one of the Siah protein duplicates presents a RING HC finger found in Siah1 homolog. In the future, we will attempt to better characterize both duplicates of Siah and compare these sea urchin duplicates with Siah proteins of other organisms in order to better understand the evolutionary similarities and differences among deuterostomes.

DISCUSSION AND FUTURE DIRECTIONS

We propose to better characterize the signaling molecule Siah in the sea urchin embryo using further phylogenic, molecular, and developmental approaches. Using phylogenic methods, it will be possible to carry out a better analysis of the protein and nucleotide sequences of the several Siah duplicates in sea urchins and close related species. In the purple sea urchin genome, we found two Siah duplicates as well as a potential Siah repressor. Our preliminary analysis of the conserved domains of Siah proteins have found that only one of the Siah duplicates contains a N-terminal RING-HC finger. The RING finger domain found in Siah1 is an inducible E3 ubiquitin-protein ligase that interacts with different substrates for ubiquitination and; subsequently, contributes proteasomal degradation of numerous targets in multiple cellular processes including transcription regulation (House et al., 2006; House et al., 2009; Qi et al., 2013; 2009; Wong and Möller, 2014). Siah1 also contains two zinc-finger subdomains and a C-terminal tumor necrosis factor (TNF) receptor associated factor (TRAF)-like substrate-binding domain (SBD) responsible for dimer formation (House et al., 2006; Polekhina et al., 2002). Therefore, it would also be interesting to identify the potential receptors with binding domains for Siah in the sea urchin genome. In addition, we found Siahbp1, a fuse-binding protein-interacting, that might act as a repressor of the Siah signal. Future work exploring the functions of the two Siah duplicates together with Siahbp1 will help to better understand how these signaling molecules might be antagonizing Wnt/β-catenin and/or Wnt/JNK signaling pathways during AP and DV patterning in the sea urchin embryo. In addition, further phylogenetic analyses of the Siah protein in a more detail level will facilitate to elucidate the conserved mechanisms in an evolutionary context.

Interestingly, studies have shown that Siah may function as a negative regulator of the Wnt signaling by down-regulating β-catenin stability as well as a modulator of Nodal signaling. Therefore, future studies in the lab will focus on confirming the potential role of the signaling molecule Siah in the Wnt signaling network as a possible antagonist of Wnt/β-catenin and Wnt/JNK signaling mediated ANE positioning mechanism. Additionally, it would be interesting to investigate the function of the intracellular signaling Siah in regulating Nodal and/or BMP2/4 signaling during early AP and DV specification and patterning. First, a better characterization of *siah* spatiotemporal expression in the sea urchin embryo using qPCR and *in situ* techniques will be extremely useful. Our preliminary data show that *siah* mRNA expression is ubiquitously expressed at 120-cell stage (12 hpf) but earlier developmental stages need to be analyzed by qPCR and *in situ* hybridization in order to confirm initial *siah* expression. As development progresses, *siah* expression is upregulated in the most posterior region and potentially in the ANE territory at mesenchyme blastula stage (24 hpf). More careful characterization of *siah* expression by *in situ* hybridization will confirm those results and will allow the comparison of the *siah* expression pattern with other Wnt modulators.

Second, the Range lab will confirm the RNA-seq data of Siah with supplementary qPCR analysis at different time points to corroborate the severe downregulation of the expression of the *siah* signaling factor in Fzl1/2/7 knockdown embryos. Third, injections with Fzl1/2/7 morpholino into the embryos at developmental stages different than mid-blastula should be performed in order to observe if Fzl1/2/7 signaling is necessary early and late in development for *siah* gene expression. In addition, Wnt16 morpholino injections at mesenchyme blastula stage where *siah* expression is upregulated in the posterior territory as well as in the ANE region will provide insights into the role of Siah in AP patterning. Those results will confirm if *siah* is regulated by the Wnt16-Fzl1/2/7 signaling pathway during AP axis patterning of the sea urchin embryo. Lastly, further experiments with an antisense morpholino against *siah* using the *foxq2* transcriptional readout system will be crucial to test its potential role in ANE positioning. If *siah* is activated and involved in Fzl1/2/7 signaling, we expect a severe or complete elimination of the expression of ANE marker *foxq2*, preventing the antagonizing of the ANE positioning. To test if *siah* also antagonizes the Fzl5/8-JNK-dependent ANE positioning, we could block Fzl5/8 or JNK function and observe if there is a rescue of ANE positioning in embryos lacking *siah*. This

rescue experiment will indicate if Siah is necessary to antagonize Fzl5/8-JNK-mediated ANE positioning. Taken together, our results suggest that we have identified the signaling molecule Siah as a putative Wnt/β-catenin antagonist in the sea urchin embryo. In the future, further functional studies on Siah need to be performed to determine its function in the Wnt signaling network as a possible antagonist of Wnt/β-catenin signaling. In addition, functional studies on Siah in different deuterostome species will be essential to better understand its specific role(s) in fundamental developmental mechanisms such as the patterning and specification of the primary and secondary axes.

MATERIALS AND METHODS

Animals and embryos

Strongylocentrotus purpuratus adults were obtained from Monterey Abalone Company (Monterey, CA, USA), Marinus Scientific (Longbeach, CA), and California Institute of Technology (Pasadena, CA, US). Adult sea urchins were maintained in aquaria at 16ºC and were induced to shed gametes by injecting 0.5M KCl into the body cavity. Fertilized embryos were cultivated at 15ºC in artificial seawater (ASW).

Preparation of cDNA clones

cDNA from mesenchyme blastula stage (24 hpf) embryos was used to obtain *siah* clone. The *siah* primers (forward and reverse, 5' - 3'), based on the *Strongylocentrotus purpuratus* sea urchin genome sequence, used to generate anti-sense in situ probes are included:
Sp-siah in situ forward, 5'-ATCACCCTGGAACCCAAACC;
Sp-siah in situ reverse, 3'-TGTAGCCGCTTTCAGCACAT;
Sp-siah in situ reverse SP6 site,
3'-ATTTAGGTGACACTATAGAAGTGTAGCCGCTTTCAGCACAT.

Morpholino microinjections

The sequence and injection concentration of Fz1/2/7 morpholino were as previously described: Fzl1/2/7 MO: 5'-CATCTTCTAACCGTATATCTTCTGC -3' (1.3mM) (Range et al., 2013).

The morpholino injection solution contained 15% fluorescein isothiocyanate (FITC) (2.5 μg/ml), 20% glycerol, and morpholino oligonucleotides at the appropriate concentration. Eggs were de-jellied by passing them through 74μm mesh Nitex, plated in rows on a culture dish coated with 25% protamine sulfate and fertilized with diluted sperm. Next, fertilized eggs were directly injected the morpholino injection solution and incubated at 15^0C until embryos reached the adequate developmental time. Morpholino microinjection experiments were performed using 50-100 embryos from different batches of embryos. Only experiments with representative phenotype or changes in gene expression in at least 80% of the injected embryos are presented.

Whole-mount in situ hybridization

Antisense RNA probe, complementary to the target *siah* mRNA, was generated using SP6 polymerase enzyme. Alkaline phosphatase reporter *in situ* hybridization was performed as previously described (Wei et al., 2009).

Phylogenetic analysis

For the domain analysis, we used amino acid sequences from each protein in order to identify the different domains in Siah proteins (Fig. 4).

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FIGURE LEGENDS

Figure 1. Schematic model of Siah-mediated degradation pathway. Siah regulates the levels of β-catenin via degradation. The model suggests the formation of a Siah based β-catenin degradation complex containing APC, SIP, SKP1, and EBI, disturbing associated Wnt signaling (Jumpertz et al., 2014).

Figure 2. Spatiotemporal *siah* mRNA expression profile. The signaling molecule *siah* is expressed broadly during early cleavage (12 hpf) and early blastula stages (15 hpf); then appears to be restricted to the ANE territory and endomesoderm regions at mesenchyme blastula stage (24 hpf). hpf, hours post fertilization.

Figure 3. *siah* and *foxq2* expression profiles in Fzl1/2/7 morpholino-injected embryos. At blastula stage (18 hpf), the mRNA expression for both *foxq2* and *siah* was down regulated in Fzl1/2/7 knockdown embryos, confirming that Siah is downstream of the Wnt16-Fzl1/2/7 signaling during the early AP patterning of the sea urchin embryo. hpf, hours post fertilization.

Figure 4. Conserved domains of Siah and Siah1 in the sea urchin. Both protein sequences present a seven in absentia (SINA) superfamily substrate binding site but only one shows a RING finger, HC subclass found in seven in absentia homolog 1 (Siah1).

FIGURES

Figure 1. Schematic model of Siah-mediated degradation pathway.

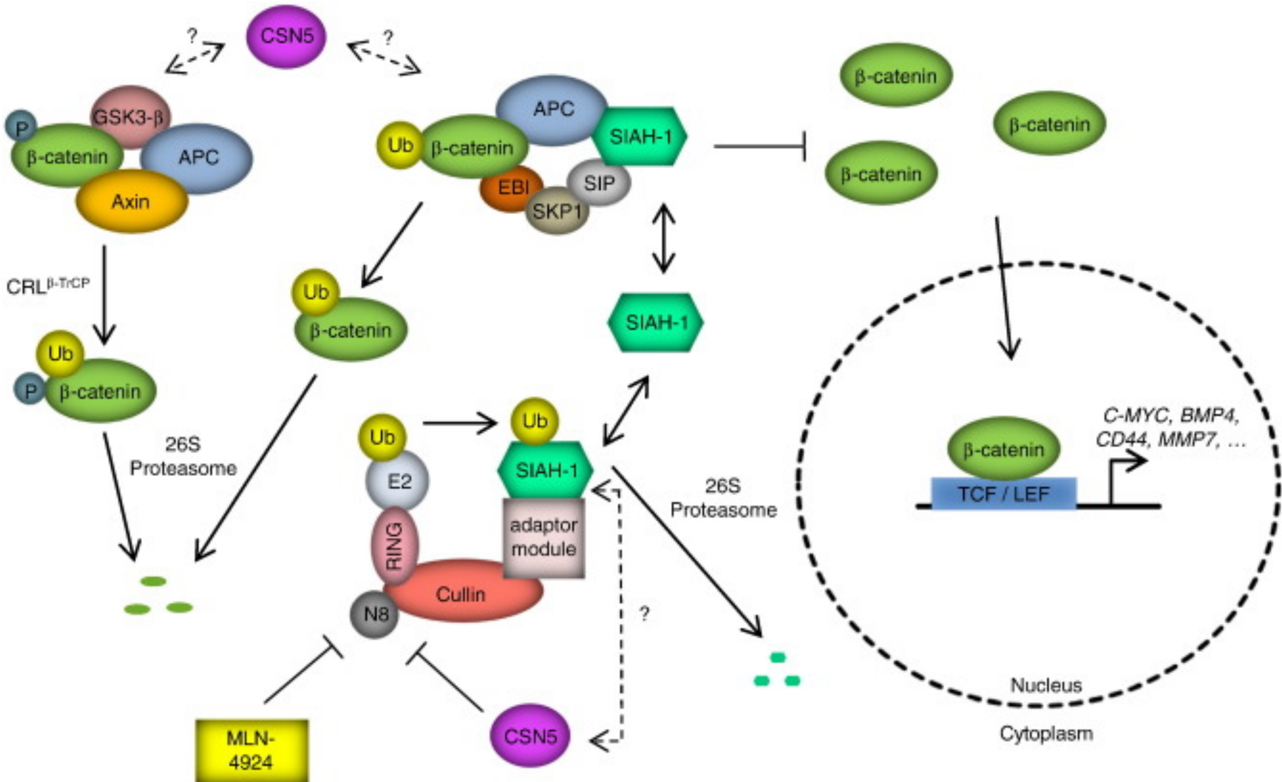


Figure 2. Spatiotemporal *siah* mRNA expression profile.

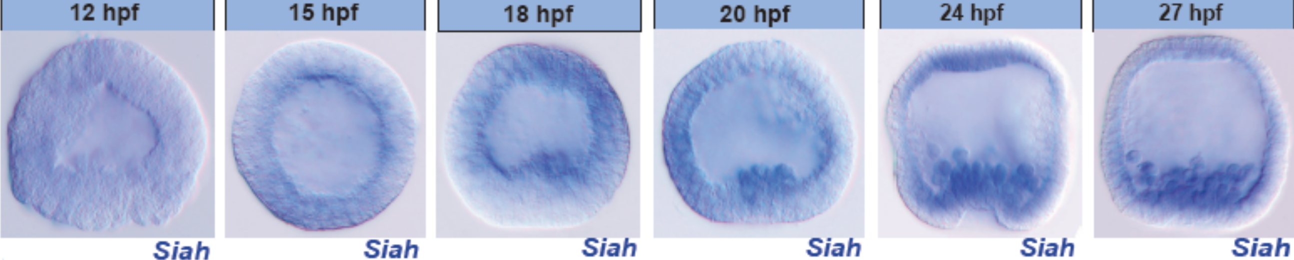


Figure 3. ***siah* and *foxq2* expression profiles in Fzl1/2/7 morpholino-injected embryos.**

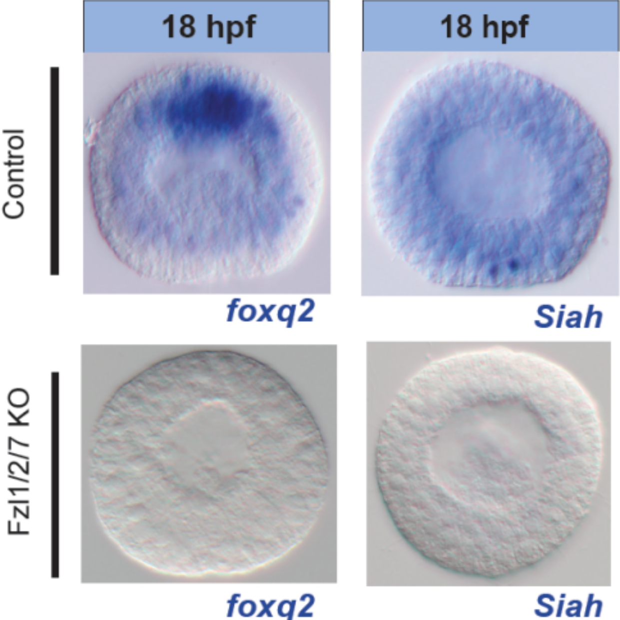
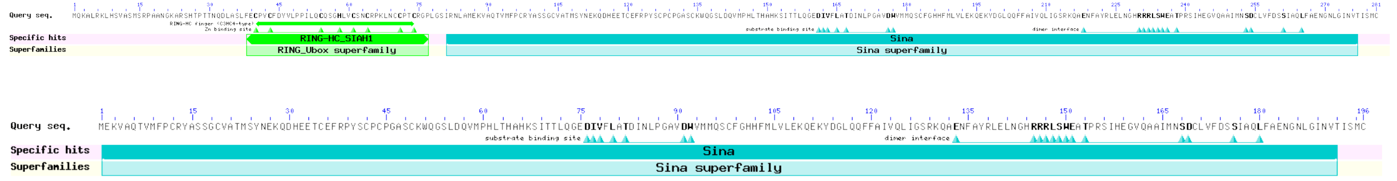


Figure 4. Conserved domains of Siah and Siah1 in the sea urchin.



III. Functional role of Tbx2/3 in anterior-posterior and dorsal-ventral patterning

ABSTRACT

In most metazoans, the specification of the anterior-posterior (AP) axis is one of the first crucial events during early cleavage and blastula stages, followed by the formation of the dorsal-ventral (DV) axis, and both developmental processes are mostly established by the end of gastrulation. Interestingly, the molecular and developmental mechanisms, including signaling pathways and gene regulatory networks (GRNs), responsible for AP and DV axis formation seem to be tightly integrated. In the early development of the sea urchin embryo, Nodal signaling is absolutely necessary for the specification and patterning of both ventral and dorsal ectoderm territories. Thus, the ventral region of the sea urchin embryo contains a signaling center that remembers to the vertebrate organizer with signals that pattern the embryo along the DV axis. BMP2/4 activity is exactly the opposite of Nodal activity, BMP2/4 promotes dorsal fates and represses ventral development. In addition, BMP2/4 signaling is downstream of Nodal but it is necessary in the ventral ectoderm for the specification of the dorsal ectoderm. A T-box transcription factor, Tbx2/3, is a regulatory gene controlled by BMP2/4 signaling in the dorsal ectoderm. Interestingly, previous large-scale ectoderm gene regulatory (GRN) analysis suggested that the dorsal *tbx2/3* activation is independent of Nodal signaling and that an unknown signaling might be responsible for its early activation. Here, we show that the early expression of *tbx2/3* requires the activity of the non-canonical Wnt16-Fzl1/2/7 signaling pathway, suggesting that this signaling might be the initial input for *tbx2/3* expression. In addition, our functional analyses in Tbx2/3 morpholino-injected embryos indicate that the dorsal gene Tbx2/3 is necessary to antagonize the mechanism that positions the anterior neuroectoderm (ANE) along the AP axis, as well as to set up the initial aspects of DV specification. Taken together, our data suggest that integrated inputs from the AP Wnt signaling and TGF-β signaling pathways such as Nodal and BMP2/4 are required for the correct establishment and early patterning of the main embryonic germ layers along the AP and DV axes of the sea urchin embryo.

INTRODUCTION

The regulatory developmental mechanisms during primary anterior-posterior (AP) and secondary dorsal-ventral (DV) axis formation and patterning are mechanistically linked in many deuterostome embryos as discussed in Chapter 3. Thus, the developmental processes responsible for body axis formation, such as signaling pathways and GRNs, should be tightly integrated. For example, Nodal/BMP and Wnt signaling pathways work together during gastrulation to induce the formation of the Spemann organizer and specify AP and DV territories in several vertebrate embryos (Agius et al., 2000; Hashimoto-Partyka et al., 2003; Khokha et al., 2005; Zorn et al., 1999). In the sea urchin embryo, Nodal signaling acts as an organizer to pattern the DV axis (Angerer et al., 2000; Angerer et al., 2011; Duboc et al., 2004; Flowers et al., 2004; Molina et al., 2013). Nodal signaling in the ventral territory induces ventral *bmp2/4* expression which then diffuses towards dorsal regions to induce ventral fates by activating the expression of dorsal genes (Molina et al., 2013). Therefore, Nodal and BMP2/4 are expressed in the same ventral territory but they work in opposite ventral-dorsal territories thanks to the presence of a morphogenetic gradient (Molina et al., 2013). In addition, Nodal signaling on the ventral side of the animal plate also represses serotonergic neurons and, at the same it, it is suppressed from the ANE territory by Lefty, an extracellular antagonist, and FoxQ2, an intracellular transcription factor (Yaguchi et al., 2008; Yaguchi et al., 2011). Thus, Nodal signaling and DV polarity is coordinated by signals of a Wnt signaling network and FoxQ2 by a double-negative mechanism (Yaguchi et al., 2008). Similarly, it has been proposed that after ANE restriction by Wnt/β-catenin signaling, further inputs from BMP2/4 signaling on the dorsal side of the animal plate control the size of the ANE territory (Yaguchi et al., 2008; Yaguchi et al., 2011).

Taken together, these data suggest that AP and DV GRN involves combined information from Wnt, Nodal, and BMP2/4 signaling as well as FoxQ2 inputs for the proper positioning and size of the neurogenic animal plate on its ventral and dorsal sides. It is likely that the same signaling pathways are required for the correct patterning of all the germ layers of the embryo along the AP and DV axes. However, the exact molecular mechanisms as well as the signaling and gene regulation interactions necessary to coordinate the correct timing of GRN activation along the AP and DV axes are still unclear. Interestingly, several dorsal markers have been previous studied in other contexts and found throughout or within sub-domains of the ectoderm

where ANE GRN genes are expressed or downregulated (Howard-Ashby et al., 2006; Li et al., 2014; Materna et al., 2010; Su et al., 2009). One of the most interesting anchor factors identified in our RNA-seq screens for transcription factors activated by the Fzl1/2/7 signaling was a T-box dorsal gene, Tbx2/3. In the sea urchin, *tbx2/3* is activated zygotically and expressed in all three germ layers (ectoderm, mesoderm, and endoderm) in the presumptive dorsal region of the embryo during the ANE positioning mechanism, starting around mid-blastula stages (14-15 hpf) (Croce et al., 2003; Gross et al., 2003; Molina et al., 2013). Previous studies have identified Tbx2/3 as an early dorsal gene critical for differentiation of the dorsal ectoderm, acting downstream of BMP2/4 signaling (Croce et al., 2003; Duboc et al., 2004; Gross et al., 2003; Lapraz et al., 2009). Interestingly, Tbx2/3 also activates a subset of other dorsal transcription factors, such as *dlx*, *msx*, and *irxA* (Fig. 5) (Molina et al., 2013; Saudemont et al., 2010), which we also identified in our Fzl1/2/7 screens. A recent large-scale ectoderm GRN analysis showed that BMP2/4 signaling, essential for dorsal fates, is required for general expression of *tbx2/3* expression but it may not be necessary for early activation, suggesting that another mechanism is responsible for the activation of *tbx2/3* and dorsal genes downstream of it (Su et al., 2009).

Here, we suggested that non-canonical Wnt16-Fzl1/2/7 signaling pathway may be involved in activating *tbx2/3* expression, which could be necessary to antagonize the ANE positioning mechanism as well as initiate aspects of DV specification. Therefore, this study illustrates how integrated information and interactions among the AP Wnt signaling and TGF-β signaling pathways are essential for the proper establishment of the embryonic germ layers along the AP and DV axes.

RESULTS

Our RNA-seq screen in Fzl1/2/7 knockdown embryos at three different developmental stages identified several dorsal genes downregulated when the function of Fzl1/2/7 was blocked (Fig. 1; Chapter 3). First, we performed whole-mount *in situ* hybridization experiments to observe the temporal and spatial expression of three of the identified dorsal genes, *tbx2/3*, *hmx*, and *myb,* during early AP and DV axis specification and patterning in the sea urchin embryo (Fig. 6). At mid-blastula stage (16 hpf) expression of *tbx2/3* was first detected in the dorsal ectodermal territory (Fig. 6A). The dorsal expression of *tbx2/3* remained in the dorsal ectoderm at

mesenchyme blastula stage (24 hpf) (Fig. 6B). The mRNA expression of another dorsal transcription factor, *hmx*, was broadly expressed throughout the embryo at early cleavage stages (60-cell and 120-cell stages) (Fig. 6C-F). *hmx* expression was then downregulated from the ventral area of the ectodermal territory and its expression progressively became localized in the dorsal ectodermal cells at mid-blastula and mesenchyme blastula stages (Fig. 6E-F). The expression of a less characterized dorsal gene, *myb,* was faint but broad throughout the embryo at 60-cell stage (9 hpf) (Fig. 6G). During early cleavage stages, expression of *myb* was maintained broadly throughout the embryo but a higher transcript level (Fig. 6H-I). At mesenchyme blastula stage, a downregulation of *myb* expression from the ventral ectodermal territory was observed, with a localization of its expression in the posterior territory as well as in dorsal equatorial ectoderm (Fig. 6J).

In our lab, we have developed a *foxq2* transcriptional readout system that allows us to quickly examine the phenotype of a potential gene player in AP specification and positioning and make predictions of the function that the gene might have and which Wnt signaling pathway is upstream of it (Fig. 7). Taking advantage of this *foxq2* transcriptional readout system, we have begun careful analyses of several novel genes in order to generate an extended and more detailed AP GRN during early AP patterning. Since our results from RNA-seq and qPCR analyses showed that Fzl1/2/7 signaling might be involved in early DV patterning, we confirmed the RNA-seq and qPCR data injecting fertilized embryos with a Fzl1/2/7 morpholino and performed *in situ* analysis with antisense *tbx2/3* RNA probes (Fig. 2; Chapter 3 and Fig. 8; Chapter 4). As expected, the dorsal expression of the dorsal gene *tbx2/3* is severely downregulated in Fzl1/2/7 knockdown embryos at mesenchyme blastula stage (18-24 hpf) (Fig. 8B compared with 8A). Thus, our preliminary results are consistent with the hypothesis that Wnt16-Fzl1/2/7 signaling is necessary for the specification and patterning of dorsal genes and for the initiation of *tbx2/3* expression. In addition, we injected Tbx2/3 morpholino in sea urchin embryos at mesenchyme blastula stage and; interestingly, we observed a severe downregulation in the expression of the ANE marker *foxq2*, mimicking the phenotype of Fzl1/2/7 morpholino-injected embryos (Fig. 8D compared with 8C). Therefore, it is feasible to speculate that the transcription factor Tbx2/3, downstream of the BMP2/4 signaling, might play a key function in regulating the proper positioning of the ANE along the AP axis, probably by antagonizing somehow the Wnt/β-catenin

and/or the Wnt1/Wnt8-Fzl5/8-JNK signaling pathways during early development of the sea urchin embryo.

DISCUSSION AND FUTURE DIRECTIONS

BMP2/4 signaling and its downstream transcription factor Tbx2/3 are necessary for dorsal specification (Angerer et al., 2000; Duboc et al., 2004; Su et al., 2009). Interestingly, Tbx2/3 activates the expression of other dorsal factors (*irxA, dlx*, and *msx*) (Fig. 5) (Molina et al., 2013); therefore, we can simplify our analyses by knocking down Tbx2/3 and BMP2/4 to determine if the others are necessary for ANE positioning or not. Therefore, future studies injecting BMP2/4 and Tbx2/3 morpholinos will be crucial in order to better examine the expression of the two earliest genes known to be activated in the ANE GRN, *six3* and *foxq2*, during early cleavage stages (120-cell stage embryos). These results will suggest if BMP2/4 and/or Tbx2/3 are necessary for ANE early activation. Similarly, it will be extremely useful to observe the expression of *foxq2* and *six3* as well as other ANE GRN genes around the anterior pole at the end of the ANE restriction mechanism in BMP2/4 and Tbx2/3 knockdown embryos. Additionally, the lab will support the WMISH data with qPCR analysis showing changes in the transcript levels per embryo for ANE markers at mesenchyme blastula embryos injected with BMP2/4 and Tbx2/3 morpholinos.

T-box family transcription factor Tbx2/3 is involved in DV axis specification and patterning of sea urchin embryos (Croce et al., 2003; Gross et al., 2003). Interestingly, Chen et al., 2011 observed the activation of the dorsal gene *tbx2/3* as early as 16 hpf but the nuclearization of pSmad1/5/8 was observed two hours later at 18 hpf in the dorsal side of the embryo. Because *tbx2/3* expression was detected before pSmad1/5/8 nuclearization, it is tempting to speculate that another initial input different than the Smad-mediated BMP signaling is responsible for *tbx2/3* expression (Chen et al., 2011). Therefore, we propose to compare the initial activation of *tbx2/3* expression in Fzl1/2/7 knockdown embryos and BMP2/4 knockdown embryos. In the future, Range lab will perform epistasis experiments in order to study the role of Wnt16-Fzl1/2/7 signaling in early BMP2/4 independent activation of *tbx2/3* expression. Thus, in three different batches of embryos, there will be injections of one set of zygotes with Fzl1/2/7 morpholino, a second set with BMP2/4 morpholino, and a third with Fzl1/2/7 MO and BMP2/4

MO. Then, we propose to examine the expression of *tbx2/3* as well as pSmad1/5/8 nuclearization at 16 hpf and 18-24 hpf developmental stages. The analysis of both *in situ* hybridization showing *tbx2/3* expression together with Smad1/5/8 antibody immunostaining will allow to better examine their correlation in order to elucidate the molecular mechanisms involved in the regulation and/or activation of Tbx2/3. These assays will tell provide a better knowledge of whether Fzl1/2/7 alone is necessary for the early, BMP2/4 independent, activation of Tbx2/3. In addition, these analyses will indicate whether later in the sea urchin development non-canonical Wnt16-Fzl1/2/7 signaling is necessary to antagonize ANE positioning and activate the Tbx2/3 subcircuit or, in contrast, if the Wnt signaling pathway is only necessary for the Tbx2/3 subcircuit and DV territory specification.

In addition, the Range lab will perform additional morpholino injections against other dorsal transcription factors that show downregulation in Fzl1/2/7 knockdown embryos (e.g. Hmx). The *foxq2* transcriptional readout system will be used to examine the influence of each potential anchor in ANE positioning (Fig. 7). A complete or partial downregulation of *foxq2* will indicate that a particular anchor factor is necessary to antagonize the ANE positioning mechanism. In addition, the lab will examine the different spatiotemporal expression patterns for the "poorly characterized transcription factors" activated by Fzl1/2/7 signaling, using the same approach as the one used for the anchor factors, and test their possible roles in the GRNs of both AP and DV positioning. In an evolutionary context, all this data will provide insights to facilitate future comparative studies in other deuterostome embryos. Together, our results will contribute to a better understanding of the molecular and developmental mechanisms that pattern the primary and secondary axes in the sea urchin embryo and will allow the identification of additional connections between the AP and DV GRNs.

MATERIALS AND METHODS

Animals and embryo cultures

Adult *Strongylocentrotus purpuratus* sea urchins were obtained from Monterey Abalone Company (Monterey, CA, USA), Marinus Scientific (Longbeach, CA), and California Institute of Technology (Pasadena, CA, US). Adult sea urchins were maintained in aquaria at 16ºC and

were induced to shed gametes by injecting 0.5M KCl into the body cavity. Fertilized embryos were cultured in artificial seawater (ASW) at 15ºC.

cDNA clone preparation

cDNA from 24 - 48 hpf embryos was used to obtain *tbx2/3, hmx,* and *myb* clones. The primers (forward and reverse, 5' - 3'), based on the *Strongylocentrotus purpuratus* sea urchin genome sequence, used to generate anti-sense in situ probes are included:

Sp-hmx in situ forward, 5'- ATGGACAGTAGCCGTGAACTATC
Sp-hmx in situ reverse, 3'- CTACTGTTGGTTAGTGGCTGGG
Sp-hmx in situ reverse SP6 site, 3'-
ATTTAGGTGACACTATAGAAGNGCTACTGTTGGTTAGTGGCTGGG

Sp-myb in situ forward, 5'- ATGAATAATGAATTCCAGAAAATTGATGGGTG
Sp-myb in situ reverse, 3'- TGTGAGCATTGAGGGAGTGG

Sp-tbx2/3 in situ forward, 5'- ATTTAGGTGACACTATAGAATCACCGCCTACCAGAACG
Sp-tbx2/3 in situ reverse, 3'- TAATACGACTCACTATAGGGCCAAAAGCGAAGGGATGG
Sp-tbx2/3 in situ reverse SP6 site, 3'-
ATTTAGGTGACACTATAGAAGNGTAATACGACTCACTATAGGGCCAAAAGCGAAGG
GATGG

Injections of morpholino oligomers

The sequence and injection concentration of Fz1/2/7 morpholino oligomers were as previously described: Fzl1/2/7 MO: 5'-CATCTTCTAACCGTATATCTTCTGC -3' (1.3mM) (Range et al., 2013). In addition, Tbx2/3 morpholino oligomers sequence and injection concentration were as follows: Tbx2/3 MO1: 5'-TTCGATGCCGGTTTCATAGAGAAAG -3' (0.6 mM) (Su et al., 2019).

The injection solutions containing the desired morpholino were prepared as previously described (Martínez-Bartolomé and Range, 2019). Preparation of fertilized embryos and morpholino injections were carried out as described in materials and methods of previous

Chapters. Perturbation experiments were performed using at least 50 embryos from different mating pairs. Only representative changes in gene expression in at least 80% of the morpholino-injected embryos are presented.

Whole-mount in situ hybridization

Antisense RNA probes, complementary to the target mRNA, for *tbx2/3, hmx,* and *myb* genes were generated using SP6 or T7 polymerase enzyme. Alkaline phosphatase reporter *in situ* hybridization was performed as previously described (Wei et al., 2009).

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FIGURE LEGENDS

Figure 5. Gene regulatory Network (GRN) of Nodal and BMP2/4 showing the responsible players for the specification of the ectoderm territory along the DV axis in the sea urchin embryo. Nodal is the regulatory factor responsible for ventral territories. Nodal signaling also synthesizes the ventral gene *bmp2/4* that subsequently activates the specification of dorsal territory. Although *bmp2/4* is expressed in the ventral region, a diffusion of BMP2/4 signaling towards dorsal regions induces ventral fates by activating the expression of dorsal genes. Tbx2/3 is one of these dorsal genes that is expressed in the dorsal ectoderm and is necessary for the expression of two transcription factors (*dlx* and *msx*) presented in our Fzl1/2/7 knockdown screen (Molina et al., 2013).

Figure 6. Spatiotemporal expression of *tbx2/3*, *hmx*, and *myb* dorsal genes during early AP and DV specification and patterning. Whole-mount *in situ* hybridization analysis of the expression of several dorsal genes. (A-B) The expression of *tbx2/3* was not detected until mid-blastula stage (16 hpf). At mid-blastula and mesenchyme blastula stages (16hpf and 24hpf) the expression of *tbx2/3* was restricted to the dorsal ectodermal territory. (C-F) Expression of the dorsal gene *hmx* mRNA was broadly expressed throughout the embryo at early cleavage stages (60-cell and 120-cell stages) and progressively downregulated from ventral ectoderm cells, resulting in a localized expression in the dorsal ectodermal territory. (G-J) Dorsal transcription factor *myb* was slightly expressed broadly throughout the embryo at 60-cell stage (9 hpf). At 120-cell and mid-blastula stage (12-16 hpf) high transcript levels of *myb* were observed all over the sea urchin embryo. Then, *myb* expression was downregulated from the most ventral territories, showing a localized *myb* expression in the dorsal equatorial ectoderm and posterior region of the embryo at mesenchyme blastula (24 hpf). AP: anterior-posterior; DV: dorsal-ventral. hpf, hours post fertilization.

Figure 7. *foxq2* transcriptional readout system. Whole-mount *in situ* hybridizations showing the expression of *foxq2* mesenchyme blastula stage (24 hpf) in control (A), Wnt/β-catenin knockdown embryos (B), Wnt/JNK (Fzl5/8-JNK) knockdown embryos (C), and Wnt/PKC (Fzl1/2/7-PKC) knockdown. embryos (D). KD, knockdown.

Figure 8. The functional role of Fzl1/2/7 and Tbx2/3during AP/DV patterning.

(A-B) Fzl1/2/7 morpholino-injected embryos at mesenchyme blastula stage (24 hpf) showing that Fzl1/2/7 was required for the expression of the dorsal gene *tbx2/3* (as previously described in Chapter 3). (C-D) The expression of the ANE gene *foxq2* at mesenchyme blastula stage in control and Tbx2/3 knockdown embryos showing that Tbx2/3 was necessary for elements of the ANE GRN. MO, morpholino; hpf, hours post fertilization; Scale bars: 20 μm.

FIGURES

Figure 5. Gene regulatory Network (GRN) of Nodal and BMP2/4 showing the responsible players for the specification of the ectoderm territory along the DV axis in the sea urchin embryo.

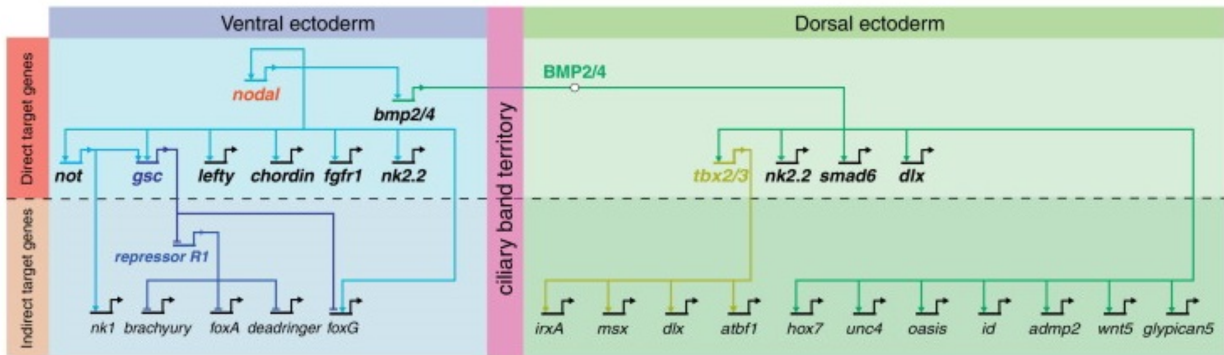


Figure 6. Spatiotemporal expression of *tbx2/3*, *hmx*, and *myb* dorsal genes.

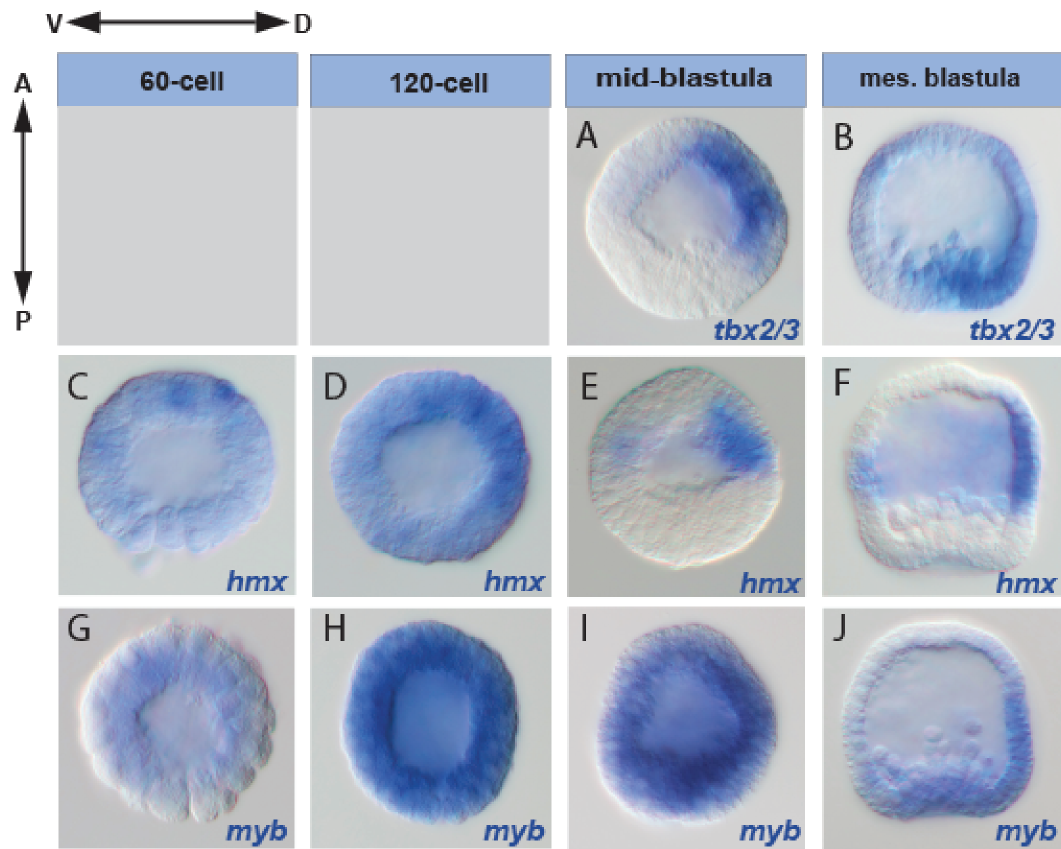


Figure 7. *foxq2* transcriptional readout system.

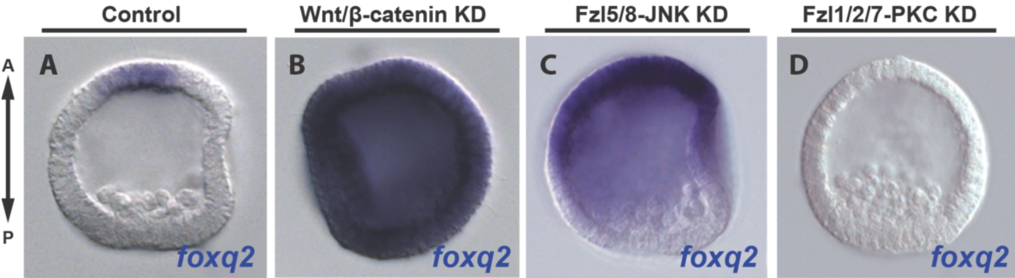
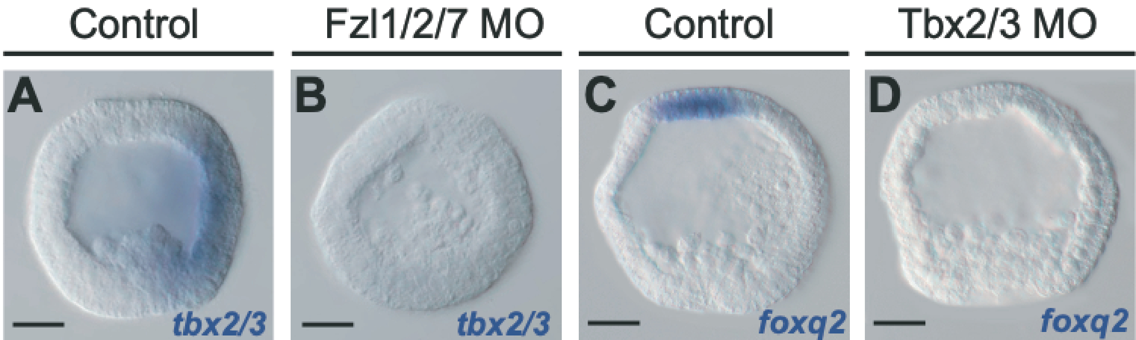


Figure 8. The functional role of Fzl1/2/7 and Tbx2/3 during AP/DV patterning.



IV. Nuclear Factor of Activated T-cells (NFAT) is a potential downstream transcriptional effector of Wnt16-Fzl1/2/7 signaling

ABSTRACT

NFAT, nuclear factor of activated T-cells, is a transcriptional factor with important roles in immune system response, several types of cancer and regulation of many other developmental processes in metazoan embryos. Wnt/Ca^{2+} signaling pathway is frequently required for NFAT activation as a result of the activity of calmodulin (CaM). In early vertebrate DV axis specification, Wnt/Ca^{2+} signaling has been shown to repress canonical Wnt/β-catenin signaling. Our lab has previously proposed that non-canonical Wnt16-Fzl1/2/7 signaling which antagonizes Wnt/β-catenin signaling in early development of the sea urchin embryo, transduces signals through the Wnt/Ca^{2+} pathway. Thus, we investigated the role of the transcription factor NFAT as a potential downstream transcriptional effector of the Wnt/Ca^{2+}/PKC signaling pathway in early AP and DV patterning of the sea urchin embryo. Our preliminary data suggest that NFAT plays a similar function as the non-canonical Wnt16-Fzl1/2/7 signaling, since NFAT morpholino-injected embryos resulted in a downregulation of the anterior neuroectoderm (ANE) territory, similar to Wnt16 and Fzl1/2/7 knockdowns. Further functional experiments from our lab will confirm NFAT's function as a transcriptional effector of Wnt16-Fzl1/2/7 signaling necessary for Wnt/β-catenin negatively regulation and/or for the antagonism of the ANE positioning mechanism mediated by Wnt1/Wnt8-Fzl5/8-JNK signaling during early specification and positioning of the AP and DV body axes in the sea urchin embryo.

INTRODUCTION

Previous functional studies from our lab have demonstrated that PKC is necessary downstream from Fzl1/2/7 signaling to antagonize Fzl5/8-JNK-mediated down regulation of the ANE GRN (Range et al., 2013). Due to the fact that PKC is often involved in the Wnt/Ca^{2+} pathway, we reasoned that Fzl1/2/7 likely activates the non-canonical Wnt/Ca^{2+} pathway. Using a candidate gene approach, we have focused on a potential transcriptional effector, the Nuclear Factor of

Activated T-cells (NFAT), as downstream player of the Wnt/Ca^{2+} pathway during early AP specification and patterning (Kestler and Kühl, 2008). NFAT is a transcription factor that is activated and translocated to the nuclei in response to Wnt/Ca^{2+} signaling (Schwartz et al., 2009). Previous studies in *Xenopus* embryos have suggested that interactions between Wnt/Ca^{2+} pathway and Wnt/β-catenin signaling allow dorsoventral axis formation and that NFAT is a direct target of a calcium signal downstream of the Wnt/Ca^{2+} pathway (Saneyoshi et al., 2002). Additionally, NFAT transcription factor is mainly expressed in the neurogenic region and plays an important role in the regulatory network and development of the neuronal territory (Graef et al., 2003; Groth and Mermelstein, 2003).

Our preliminary data suggest that the transcription factor NFAT might work as a downstream transcriptional effector of the Wnt/Ca^{2+}/PKC signaling pathway in the sea urchin embryo. Therefore, here we suggest that non-canonical Wnt16-Fzl1/2/7 signaling is likely using the NFAT transcription factor as a downstream transcriptional effector during early AP patterning. However, additional functional experiments are required to confirm the function of NFAT in the AP GRN. Taken together, our data show that NFAT is a putative transcriptional effector necessary for the antagonism of the ANE positioning mechanism mediated by Wnt1/Wnt8-Fzl5/8-JNK signaling during early body axis specification and patterning of the sea urchin embryo.

RESULTS

The regulatory mechanisms required for early AP specification and patterning comprise integrated inputs from extracellular and intracellular factors. The transcriptional factor NFAT is activated and translocated to the nuclei in response to Wnt/Ca^{2+} signaling (Schwartz et al., 2009). Thus, we first performed qPCR analysis in order to obtain a better temporal resolution of *nfat* transcripts (Fig. 9). Interestingly, high levels of *nfat* transcripts paired with the temporal window of ANE restriction, supporting the idea that NFAT is the downstream transcriptional effector of a distinct Wnt signaling pathway involved in early AP patterning. Next, we analyzed the phenotypic morphology of NFAT morpholino-injected and compared its morphology with knockdowns of the different Wnt signaling pathways (Fig. 10). Embryos injected with NFAT morpholino presented disturbed dorsal structured similar to the previously reported phenotypes

of Fzl1/2/7 knockdowns at pluteus larva stages (90 hpf) (Fig. 10B compared with 10A and 10C). In addition, NFAT knockdowns showed disorganized mesoderm cells similar to previously described results in Fzl1/2/7 and Wnt16 morpholino-injected embryos (Martínez-Bartolomé and Range, 2019; Range et al., 2013).

Next, we injected antisense morpholino against NFAT and used the *foxq2* transcriptional readout system to determine if NFAT is a transcriptional effector downstream of Fzl1/2/7 signaling (Fig. 7 and Fig. 11). If NFAT functions downstream of this Wnt signaling pathway, the expression of the ANE territory should be completely or partial eliminated during early stages of the sea urchin development. Interestingly, NFAT morpholino-injected embryos showed a severe downregulation in the expression of the two cardinal ANE genes, *foxq2* and *six3,* at mesenchyme blastula stage (24hpf) (Fig 11C-D compared with 11A-B). Taken together, NFAT knockdown experiments confirmed that NFAT has an important function in antagonizing the ANE positioning mechanism along the AP axis and it is likely necessary for specification and/or patterning of the early ANE GRN in sea urchin embryo (Fig. 11). In addition, we performed NFAT antibody staining for embryos during early cleavage stages (8–14 hpf) (Fig. 12). NFAT antibody recognizes a protein that is localized to nuclei in control embryos but not in Fzl1/2/7 signaling knockdown embryos, suggesting that nuclear localization of NFAT is controlled by the non-canonical Wnt16-Fzl1/2/ signaling (Fig. 12). Lastly, we propose an extended model for AP specification and patterning in early development of sea urchin embryos (Fig. 13). This final model suggests that NFAT is the transcriptional activator of the non-canonical Wnt16-Fzl1/2/7 signaling and it is responsible to antagonize the ANE restriction process mediated by Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling, allowing the proper formation of the different germ layers of the sea urchin embryo.

DISCUSSION AND FUTURE DIRECTIONS

In order to strengthen our hypothesis that NFAT is necessary to antagonize the ANE positioning mechanism downstream of Fzl1/2/7 signaling, future experiments in Range lab will focus on inhibiting its function by using a small molecule NFAT inhibitor. The phenotypes resulting from this NFAT inhibition and its comparison with the phenotypes of Fzl1/2/7 knockdown embryos can provide a better understanding of the function of NFAT in ANE positioning. If our

hypothesis that NFAT is necessary to antagonize the ANE positioning is correct, we will expect, based on phenotypic morphology, a completely or partial elimination of the ANE territory. Additionally, from our results of the Wnt ligands involved in Fzl1/2/7 signaling, we will knockdown the function of the Wnt ligand (Wnt16) responsible for activation of this Wnt signaling branch and look at changes in NFAT (Martínez-Bartolomé and Range, 2019). If nuclear localization of NFAT is controlled for Wnt16 ligand, we will be able to conclude that NFAT is the downstream transcriptional effector of this Wnt signaling.

MATERIALS AND METHODS

Animals and embryos

Strongylocentrotus purpuratus were obtained from Monterey Abalone Company (Monterey, CA, USA), California Institute of Technology (Pasadena, CA, US), and Marinus Scientific (Longbeach, CA). Adult sea urchins were maintained at 16ºC in aquaria. Injections of 0.5M KCl into the body cavity of the adults were carried out to collect gametes. Gametes were fertilized with diluted sperm and embryos were then cultured at 15ºC in artificial seawater (ASW).

Injections of morpholino oligomers

Fz1/2/7 morpholino sequence and injection concentration were as previously described: Fzl1/2/7 MO: 5'-CATCTTCTAACCGTATATCTTCTGC -3' (1.3mM) (Range et al., 2013). The sequences and injection concentration for NFAT morpholino oligomers were as follows: NFAT MO1: 5'- ATCAATGGCTGGAGCATAAAAAGTC-3' (0.5 mM); NFAT MO2: 5'-AATGACTGAGATTCCATAGACATGT-3' (1.35 mM);

Preparation of injection solutions containing the desired morpholino was carried out as previously described (Martínez-Bartolomé and Range, 2019; Range et al., 2013). Fertilized embryos and morpholino injections were also performed as previously described (Martínez-Bartolomé and Range, 2019; Range et al., 2013). Perturbation experiments were performed using 50-100 embryos from different batches of embryos from different mating pairs. Changes in gene expression were considered representative when at least 80% of the morpholino-injected embryos were affected.

Quantitative PCR (qPCR)

qPCR experiments were performed as previously described (Wei et al., 2009).

The qPCR primers (forward and reverse, 5' - 3'), based on the *Strongylocentrotus purpuratus* sea urchin genome sequence, used are included:

Sp-nfat qPCR forward, 5'- GGGAAGGGAATTGTGAGATC

Sp-nfat qPCR reverse, 3'- GATACACAATAGGATGGGCG

Whole-mount in situ hybridization (WMISH)

Alkaline phosphatase reporter *in situ* hybridization was performed as previously described (Erkenbrack et al., 2019; Wei et al., 2009).

Immunohistochemistry

Fixative solution containing 3% paraformaldehyde diluted in ASW was added to the embryos for 20 minutes at room temperature followed by five washes in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Next, embryos were incubated at 4ºC overnight with primary antibodies against NFAT in PBST and 4% normal goat serum. The primary antibody detection was performed by incubating embryos with Alexa Fluor-coupled 488 goat IgG (Thermo Fisher Scientific) for 1 hour at room temperature or overnight at 4ºC. Nuclei were stained with DAPI.

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FIGURE LEGENDS

Figure 9. NFAT is a putative downstream transcriptional effector of Fzl1/2/7-PKC signaling during AP patterning. qPCR analysis showing the temporal expression of *nfat* transcripts from three different batches of embryos from egg to mesenchyme blastula stages (24 hpf). *y* axis represents the number of *nfat* transcripts per embryo, based on the Ct value of *z12* transcripts. At each stage, the absolute concentrations of *z12* transcripts are known as previously described in Wang et al., 1995.

Figure 10. Phenotypic morphologies of NFAT and Fzl1/2/7 perturbations during AP patterning. (A) DIC images of 90 hpf control embryos. (B) Embryos injected with morpholino targeting *nfat* transcripts showed a disrupted dorsal structures similar to Fzl1/2/7 knockdown phenotypes previously reported in Chapter 2. (C) Phenotypic morphology of Fzl1/2/7 morpholino-injected embryos at 90 hpf, showing a disorganization of mesoderm cells, disturbed dorsal structures, and a lack of thickened columnar epithelium (arrowhead) (Range et al., 2013). MO, morpholino; hpf, hours post fertilization.

Figure 11. NFAT is necessary for ANE GRN expression. Control and NFAT morpholino-injected embryos at mesenchyme blastula stage (24 hpf). (A-B) Whole-mount *in situ* hybridizations showing the expression of the ANE genes *foxq2* and *six3* in control embryos. (C-D) The expression of the ANE genes *foxq2* and *six3* in NFAT knockdown embryos showing that NFAT was necessary for specification and/or patterning of the ANE GRN. MO, morpholino; hpf, hours post fertilization.

Figure 12. NFAT antibody staining in Fzl1/2/7 morpholino-injected embryos. NFAT antibody recognizes a protein that is localized to nuclei in control embryos, but not in Fzl1/2/7 knockdown embryos at mesenchyme blastula stage (24 hpf). Nuclei were stained with DAPI. DAPI (left) and GFP (right) filters were used.

Figure 13. Final model of NFAT function during AP axis patterning of the sea urchin embryo. Proposed model for early AP axis specification and patterning during sea urchin early

development, extending previous data from Range et al., 2013 and Martínez-Bartolomé and Range, 2019 (Chapter 2). Non-canonical Wnt16-Fzl1/2/7 signaling is necessary to antagonize Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling during early AP restriction process. In addition, the extended model shows a role of NFAT in antagonizing the ANE restriction process mediated by Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling. Legend indicates the different embryonic territories: blue, anterior neuroectoderm; gray, equatorial ectoderm; and orange, endoderm and mesoderm.

FIGURES

Figure 9. NFAT is a putative downstream transcriptional effector of Fzl1/2/7-PKC signaling during AP patterning

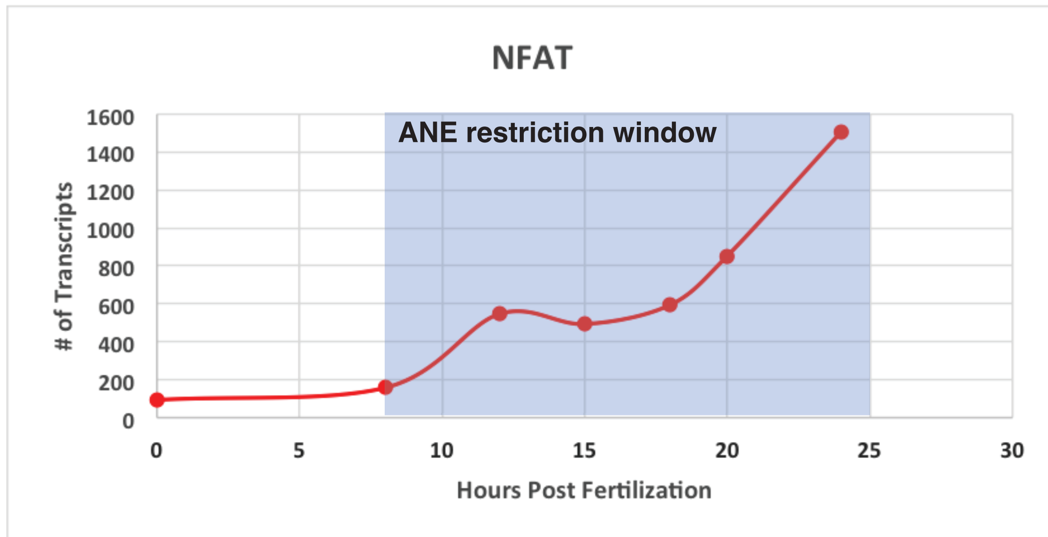


Figure 10. Phenotypic morphologies of NFAT and Fzl1/2/7 perturbations during AP patterning.

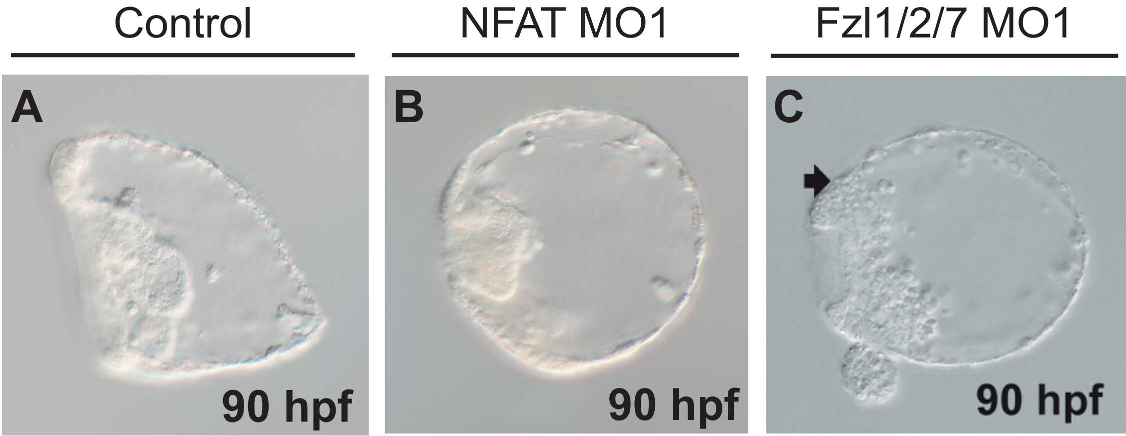


Figure 11. NFAT is necessary for ANE GRN expression.

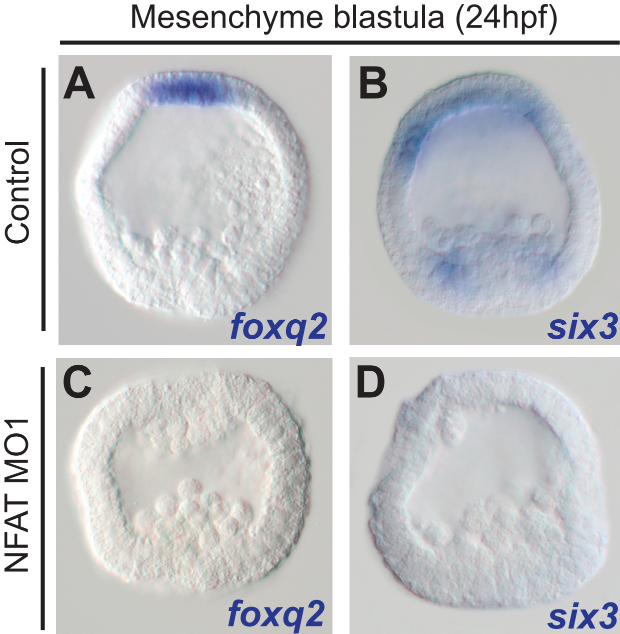
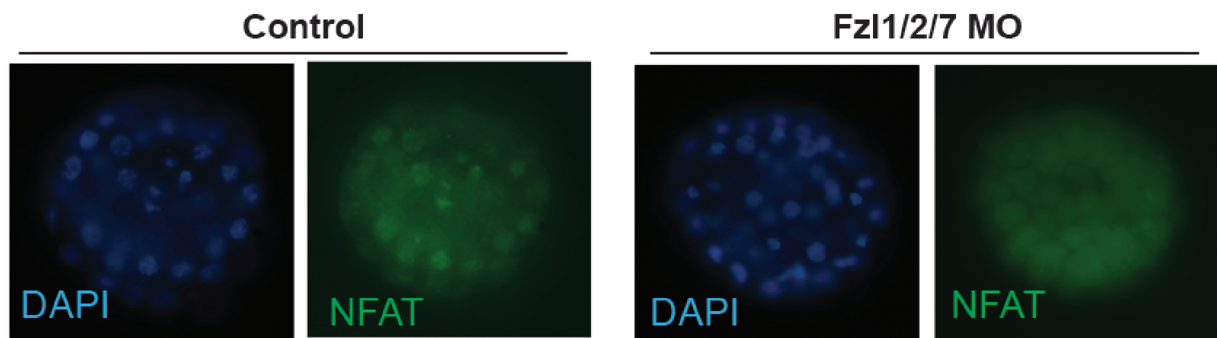


Figure 12. NFAT antibody staining in Fzl1/2/7 morpholino-injected embryos.



NFAT antibody staining: nuclear localization of NFAT seems to be controlled by Fzl1/2/7 signaling

Figure 13. Final model of NFAT function during AP axis patterning of the sea urchin embryo.