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Canonical and non-canonical Wnt signaling pathways define the expression domains of Frizzled 5/8 and Frizzled 1/2/7 along the early anterior-posterior axis in sea urchin embryos

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ABSTRACT

The spatiotemporal expression of Frizzled receptors is critical for patterning along the early anterior-posterior axis during embryonic development in many animal species. However, the molecular mechanisms that regulate the expression of Frizzled receptors are incompletely understood in any species. In this study, I examine how the expression of two Frizzled receptors, Fzl1/2/7 and Fzl5/8, is controlled by the Wnt signaling network which directs specification and positioning of early regulatory states along the anterior-posterior (AP) axis of sea urchin embryos. I used a combination of morpholino- and dominant negative-mediated interference to knock down each Wnt signaling pathway involved in the AP Wnt signaling network. I found that the expression of zygotic *fzl5/8* as well as that of the anterior neuroectoderm gene regulatory network (ANE GRN) is activated by an unknown broadly expressed regulatory state and that posterior Wnt/β-catenin signaling is necessary to down regulate *fzl5/8's* expression in posterior blastomeres. I show that zygotic expression of *fzl1/2/7* in the equatorial ectodermal belt is dependent on an uncharacterized regulatory mechanism that works in the same cells receiving the TGF-β signals patterning this territory along the dorsal-ventral axis. In addition, my data indicate that Fzl1/2/7 signaling represses its own expression in a negative feedback mechanism. Finally, we discovered that a balance between the activities of posterior Wnt8 and anterior Dkk1 is necessary to establish the correct spatial expression of zygotic *fzl12/7* expression in the equatorial ectodermal domain during blastula and gastrula stages. Together, these studies lead to a better understanding of the complex interactions among the three Wnt signaling pathway governing AP axis specification and patterning in sea urchin embryos.

1. Introduction

Wnt signaling pathways are used in a large array of cellular processes during embryonic development and adult tissue homeostasis. Three main Wnt signaling pathways have been identified in a variety of organisms: the "canonical" Wnt/β-catenin pathway as well as the "alternative" Wnt/JNK and Wnt/Ca^{2+} pathways. The known molecular components of these pathways are remarkably conserved among metazoan embryos, and in many cases, so are the roles they play during embryonic development. For instance, studies in several deuterostome developmental systems, from echinoderms to mammals, have shown that a posterior-to-anterior gradient of Wnt/β-catenin signaling is necessary to activate and position the activities of early gene regulatory networks (GRNs) along the anterior-posterior (AP) axis (Darras et al., 2011, 2018; Kiecker and Niehrs, 2001; Lekven et al., 2001; Logan et al., 1999; Nordstrom et al., 2002; ten Berge et al., 2008; Wikramanayake et al., 1998; Yaguchi et al., 2008). In these embryos, high Wnt/β-catenin signaling initiates the endomesodermal GRN at the future posterior end of the embryo where the blastopore will form. In contrast, low Wnt signaling levels around the opposite pole allow for the establishment of the anterior neuroectoderm (ANE) GRN that drives formation of several sensory organs (Niehrs, 2010; Petersen and Reddien, 2009; Range, 2014). Despite the fundamental importance of normal AP axis specification and patterning during embryonic development, the exact molecular mechanisms underlying how Wnt signaling positions early GRNs along the primary axis are incompletely understood in any system.

At the beginning of gastrulation in the sea urchin embryo, four major gene regulatory domains are established along the AP axis: the endoderm and mesoderm domains around the posterior pole, an equatorial ectodermal domain that will form ventral and dorsal ectodermal structures separated by the ciliary band and associated nerves, and the ANE domain around the anterior pole (Angerer et al., 2011;

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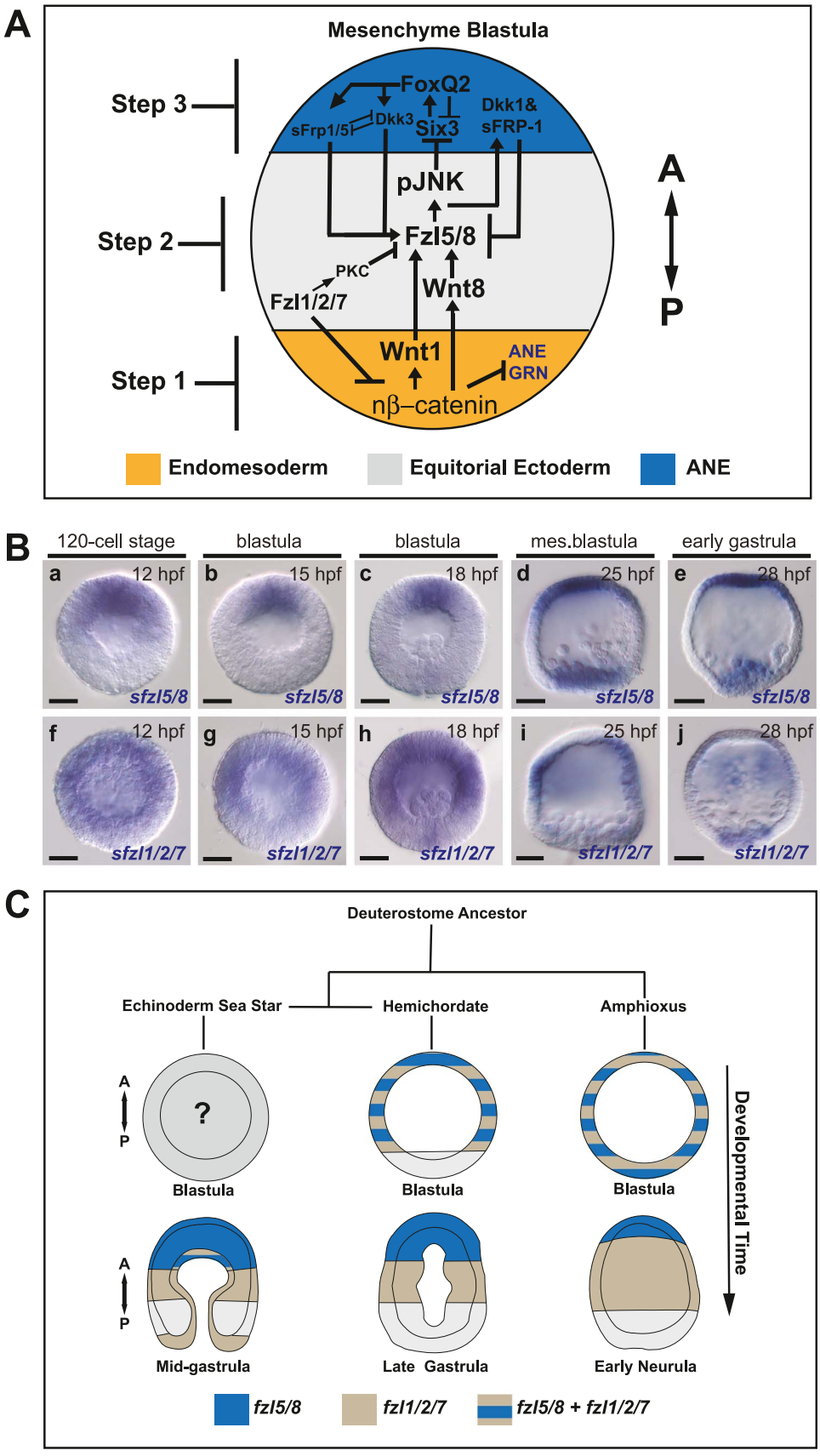

A
Mesenchyme Blastula
Step 3
Step 2
Step 1
FoxQ2
sFrp1/5
Dkk3
Six3
Dkk1&
sFRP-1
pJNK
Fzl5/8
PKC
Fzl1/2/7
Wnt8
Wnt1
ANE
GRN
nβ–catenin
A
P
Endomesoderm
Equitorial Ectoderm
ANE
B
120-cell stage
blastula
blastula
mes.blastula
early gastrula
a 12 hpf sfzl5/8
b 15 hpf sfzl5/8
c 18 hpf sfzl5/8
d 25 hpf sfzl5/8
e 28 hpf sfzl5/8
f 12 hpf sfzl1/2/7
g 15 hpf sfzl1/2/7
h 18 hpf sfzl1/2/7
i 25 hpf sfzl1/2/7
j 28 hpf sfzl1/2/7
C
Deuterostome Ancestor
Echinoderm Sea Star
Hemichordate
Amphioxus
A
P
?
Blastula
Blastula
Blastula
Developmental Time
Mid-gastrula
Late Gastrula
Early Neurula
fzl5/8
fzl1/2/7
fzl5/8 + fzl1/2/7

Fig. 1. The spatiotemporal expression of *fzl1/2/7* and *fzl5/8* during early AP specification and patterning in invertebrate deuterostomes. (A) In sea urchin early development, the Wnt/β-catenin, Wnt/JNK, and Fzl1/2/7-PKC pathways all converge on the same developmental process: ANE restriction. Step 1(16-to 32-cell stage) Wnt/β-catenin signaling activates the endomesoderm GRN and represses the ANE GRN in posterior blastomeres. Step 2 (60-cell stage to early-mid-blastula stage) Wnt/β-catenin signaling activates posterior-to-anterior gradients of Wnt1 and Wnt8 that activate the Fzl5/8-JNK signaling pathway resulting in the down regulation of the ANE GRN in the posterior equatorial ectoderm. Step 3 (mid-blastula to mesenchyme blastula stage) In the regressing ANE GRN Fzl5/8 signaling activates Dkk1 and sFRP-1 expression. These Wnt antagonists perturb the posterior-to-anterior repression of the ANE GRN by Fzl5/8 signaling via a negative feedback loop. Around the same time, FoxQ2 activates the expression of two Wnt modulators, sFRP1/5 and Dkk3, that potentiate Fzl5/8 signaling (data taken from Range et al., 2013; Range, 2014; Range and Wei, 2016; Khadka et al., 2018). (B) Expression of *fzl12/7* and *fzl5/8* in embryos from the same mating pairs in *S. purpuratus*. (Ba) *fzl5/8* is detected in anterior blastomeres beginning around the 120-cell stage. (Bb, c) In early blastula stages, stage *fzl5/8* expression is progressively down regulated from equatorial ectodermal cells. (Bd, e) *fzl5/8* is expressed around the anterior pole as well as the posterior endomesoderm cells at the mesenchyme blastula and early gastrula stage, as previously shown (Range et al., 2013). (Bf) *fzl1/2/7* is expressed ubiquitously in the cleavage stage. (Bg) In blastula staged embryos, *fzl1/2/7* expression is down regulated in posterior cells. (Bh) *fzl1/2/7* expression is down regulated around the anterior pole at the early mesenchyme blastula stage. (Bi, j) Between mesenchyme blastula stage and early gastrula, *fzl1/2/7* expression is restricted to an equatorial ectodermal belt and activated in the posterior endomesoderm. (C) Diagram of *fzl5/8* and *fzl1/2/7* expression in invertebrate deuterostomes. Data taken from (Darras et al., 2018; McCauley et al., 2013; Qian et al., 2013; Robert et al., 2014).

Molina et al., 2013). Functional studies indicate that posterior Wnt/β-catenin and Notch signaling activate the endoderm and mesoderm GRNs (Davidson et al., 2002; Range et al., 2008; Sherwood and McClay, 1997, 1999; Erkenbrack, 2018), that Nodal and BMP2/4 signaling activate the dorsal and ventral ectoderm GRNs, respectively, in the equatorial ectoderm (Molina et al., 2013), and that Six3 sits at or near the top of the ANE GRN (Wei et al., 2009). Studies from our lab indicate that all three major Wnt signaling pathways operate in an integrated Wnt network that is essential to establish positioning of these early regulatory domains along the AP axis (Khadka et al., 2018; Range, 2014; Range et al., 2013; Range and Wei, 2016; Yaguchi et al., 2008) (For our three step model see Fig. 1A). In the first step of this process maternally localized components activate the Wnt/β-catenin signaling pathway in posterior/vegetal blastomeres as early as the 16-cell stage, resulting in the activation of the endomesoderm GRN (Byrum et al., 2009; Peng and Wikramanayake, 2013; Peng et al., 2017; Weitzel et al., 2004). At the 32-cell stage, Wnt/β-catenin also represses the activation of the ANE GRN in the same blastomeres that would otherwise be activated by an unknown broadly active regulatory mechanism (Range et al., 2013). The result of this mechanism is that the endomesoderm GRN and ANE GRN are restricted to the posterior and anterior blastomeres, respectively. Around the 60-cell stage, Wnt/β-catenin activates the expression of two ligands in posterior/vegetal cells, Wnt1 and Wnt8. Functional analyses suggest these diffuse into more anterior ectodermal blastomeres, activating the Wnt/JNK signaling pathway through interactions with a ubiquitously expressed Fzl5/8 receptor. This Wnt1/Wnt8-Fzl5/8-JNK signaling pathway progressively down regulates the expression of the ANE GRN in cells within the equatorial ectoderm during the late cleavage and blastula stages (Range et al., 2013; Yaguchi et al., 2008). During these stages, zygotic *fzl5/8* becomes integrated into the ANE GRN down regulated by Wnt1/Wnt8-Fzl5/8-JNK signaling. Simultaneously, a different non-canonical Wnt signaling pathway, working through the Fzl1/2/7 receptor, antagonizes Wnt/β-catenin and Wnt/JNK signaling preventing the complete elimination of ANE GRN expression from anterior cells during the initial and middle stages of ANE restriction (Range et al., 2013). In the later stages early AP patterning (mid-blastula to mesenchyme blastula stage) Fzl5/8 signaling in the ANE activates two secreted Wnt signaling antagonists, Dkk1 and sFRP-1. These molecules work in a negative feedback loop to block Wnt1/Wnt8-Fzl5/8-JNK signaling. At the same time, FoxQ2 activates the expression of two more secreted Wnt modulators, sFRP1/5 and Dkk3, that potentiate the Wnt/JNK signaling. It is the balance among the interactions of these molecules that establishes the precise expression of *fzl5/8* and the rest of the ANE GRN around the anterior pole, defining the ANE territory that gives rise to the anterior sensory organ (Khadka et al., 2018; Range et al., 2013; Range and Wei, 2016). Importantly, data from several studies in other deuterostome embryos, including vertebrates, strongly suggest that aspects of this AP Wnt network may have existed in the deuterostome ancestor (Range, 2014).

The spatiotemporal expression of secreted Wnt modulators, Frizzled receptors and co-receptors plays a large role in which Wnt signaling pathway will be activated. In many instances, it has been shown that one or more of these pathways are active simultaneously in the same cells or territories (Kestler and Kuhl, 2008; van Amerongen and Nusse, 2009). Of these signal transduction components, the Frizzled receptor arguably plays the most critical role in determining which Wnt signaling pathways will be activated. Phylogenetic analyses suggest that the eumetazoan ancestor possessed a set of four Frizzled receptors, Fzl1/2/7, Fzl4, Fzl5/8, and Fzl9/10 (Lee et al., 2006; Yan et al., 2014). Many metazoan embryos, including species from each deuterostome phylum, still possess this core ancestral group of Frizzled receptors, while others have lost or duplicated one or more of them during evolution. For instance, many vertebrates possess 11 Frizzled receptors due to two rounds of whole-genome duplication (Yan et al., 2014). While many studies have been performed on the spatiotemporal regulation of Wnt ligands in deuterostome embryos, much less is understood about the molecular mechanisms that position Frizzled receptors during early axial specification and patterning.

bib30The spatial expression of the two Frizzled receptors involved in the Wnt network directing early AP specification and patterning in sea urchins, Fzl1/2/7 and Fzl5/8, is remarkably similar along the early anterior-posterior axis in several other deuterostomes (Darras et al., 2018; McCauley et al., 2013; Qian et al., 2013; Robert et al., 2014), including echinoderm sea stars, hemichordates and the chordate amphioxus (see Fig. 1B, C). In most of these embryos, both *fzl5/8* and *fzl1/2/7* are ubiquitously expressed during cleavage stages. Then, depending on the species, the expression of *fzl5/8* is progressively down regulated from posterior endomesoderm and ectoderm during blastula and/or gastrula stages until it is restricted to a territory around the anterior pole. At around the same time in most invertebrate deuterostomes *fzl1/2/7* is initially down regulated from posterior cells so that its expression overlaps with that of *fzl5/8* in anterior ectoderm cells during the early stages of *fzl5/8*'s restriction around the anterior pole. Subsequently, *fzl1/2/7* is down regulated from a territory around the anterior pole, resulting in it being expressed in an equatorial belt. In addition, both *fzl5/8* and *fzl12/7* are re-activated in the endodermal and/or mesodermal cells in many deuterostome species during gastrula stages. Together, these data suggest that the molecular mechanisms that establish the spatial expression of these receptors along the early AP axis may be shared among deuterostomes.

In this study, I made an unexpected discovery that instead of the transcription factor Six3, an uncharacterized and broadly expressed, early regulatory mechanism activates the sea urchin ANE GRN, which includes zygotic *fzl5/8*. In addition, I found that all three Wnt signaling pathways are necessary for the correct spatial expression, and/or activation of both *fzl5/8* and *fzl1/2/7*. Finally, I discovered that a balance between anteriorly and posteriorly secreted Wnt modulators determines the spatial expression of *fzl12/7* in the central ectodermal domain during blastula and gastrula stages.

2. Results

2.1. Spatiotemporal expression of fzl1/2/7 and fzl5/8 during early anterior–posterior axis specification

Functional studies have shown that during cleavage and blastula

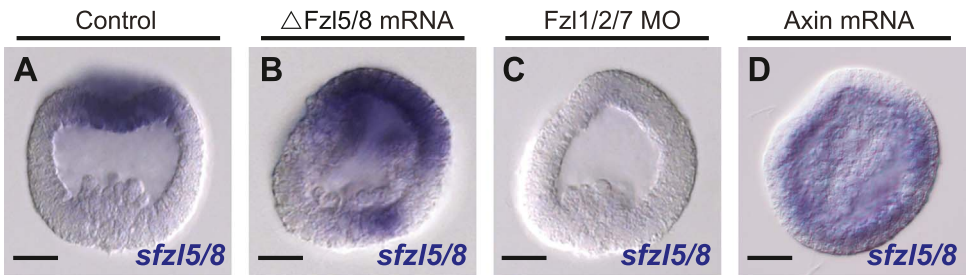


Fig. 2. Zygotic control of *fzl5/8* expression. (A) Control embryo showing anterior expression of *fzl5/8*. (B) *fzl5/8* expression expands throughout the anterior ectodermal territory in embryos without functional Fzl5/8-JNK signaling and (C) is severely down regulated in Fzl1/2/7 knock down embryos. (D) In the absence of functional Wnt/β-catenin (Axin mRNA), *fzl5/8* is expressed throughout the embryo. MO, morpholino; ΔFzl5/8, dominant negative Fzl5/8; Scale bar = 20 μm.

stages, Fzl5/8-JNK signaling is only active in the ectoderm (Range et al., 2013); whereas, it has been shown to have a different role subsequently in posterior endomesoderm cells for the morphogenetic movements involved in gastrulation (Croce et al., 2006). In contrast, non-canonical Fzl1/2/7 signaling appears to be active throughout the embryo during cleavage and early blastula stages. Then, similar to Fzl5/8 signaling, it is necessary for gastrulation (Range et al., 2013). In order to better characterize interactions among the different non-canonical Wnt signaling pathways mediated by these receptors in *S. purpuratus*, I performed a detailed analysis of the spatial expression of *S. purpuratus fzl1/2/7* and *fzl5/8* genes in the same batches of embryos during early AP specification and patterning. Both maternally activated genes show a ubiquitous pattern of expression through the early cleavage stages (Range et al., 2013). Then around the 120-cell stage, *fzl5/8* expression was down-regulated from posterior endomesoderm blastomeres (Fig. 1Ba). Subsequently, *fzl5/8* expression was progressively down regulated from the equatorial band of ectodermal cells during the blastula stages until it was restricted to a territory around the anterior pole of the embryo in mesenchyme blastula and early gastrula stage embryos (Fig. 1Bb-d). During these later stages, *fzl5/8* was also activated in posterior endomesoderm cells (Fig. 1Bd, e). The expression of *fzl1/2/7* was down regulated in posterior cells during late blastula stages while being maintained in the ectoderm (Fig. 1Bf–h). At mesenchyme blastula stage *fzl12/7* was down regulated from around the anterior pole, resulting in a belt of expression in equatorial ectodermal cells (Fig. 1Bi). By early gastrula stages *fzl1/2/7* expression was restricted to the upper equatorial ectoderm territory and it is also expressed in posterior endomesoderm (Fig. 1Bj). These data are consistent with our less detailed previous analysis and similar to results in other sea urchin species (Croce et al., 2006; Range et al., 2013; Robert et al., 2014) suggesting that zygotic expression of both receptors is under complex regulatory control. Interestingly, the spatiotemporal expression of these two receptors is remarkably similar among other invertebrate deuterostome embryos during early development (Fig. 1C).

2.2. The Wnt signaling network determines the spatial expression of zygotic fzl5/8

In a previous study, we showed that *fzl5/8* expression is down regulated from the equatorial ectodermal territory through a negative feedback mechanism mediated by Wnt1/Wnt8-Fzl5/8-JNK signaling (Fig. 2A, B). In addition, we demonstrated that Fzl1/2/7-PKC signaling antagonizes Fzl5/8-JNK signaling in these same cells and its down regulation of *fzl5/8* (Fig. 2C) (Range et al., 2013). Although we had not determined how *fzl5/8* expression is down regulated in the posterior endomesoderm territory, we hypothesized that it was due to posterior Wnt/β-catenin signaling. To test this idea, I injected zygotes with Axin mRNA, which blocks endogenous Wnt/β-catenin signaling. In these embryos, *fzl5/8* transcripts were detected throughout the embryo at the mesenchyme blastula stage (24 hpf) (Fig. 2D). Together with our previous results, these data demonstrate that each Wnt signaling pathway known to be involved in AP axis specification and patterning is necessary for the spatial regulation of zygotic *fzl5/8* expression along the AP axis.

2.3. An early, broadly active regulatory mechanism activates the ANE GRN, which includes fzl5/8

Wei et al. (2009) showed that knockdown of Six3 results in the complete elimination of the ANE GRN in sea urchin embryos, including the cardinal regulator *foxq2* and also zygotic *fzl5/*8, which joins the ANE GRN during the blastula stages. Conversely, overexpression of Six3 antagonized the posterior-to-anterior gradient of Wnt signaling, allowing for the expansion of the ANE territory. These results led Wei et al. to suggest that Six3 is necessary for the activation of the ANE GRN while also acting as a repressor of Wnt signaling. However, it is also possible that Six3 may only be necessary to repress posterior-to-anterior Wnt signaling. To distinguish between these alternatives, I asked whether Six3 could activate critical ANE GRN components, (e.g.*foxq2* and *fzl5/8)* in the absence of Wnt/β-catenin signaling, which eliminates the ANE GRN (Range et al., 2013). Within each of three batches of embryos, I injected one set of zygotes with Six3 morpholino and another set with Six3 morpholino and Axin mRNA. Both *foxq2* (n = 53/57) and *fzl5/8* (n = 56/62) were severely down regulated in Six3-deficient embryos (Fig. 3B, E). In contrast, the ANE regulatory factors were expressed throughout Six3 knockdown embryos when Wnt/β-catenin signaling was also blocked (n = 51/53 for *foxq2* and n = 52/52 *for fzl5/8*) (Fig. 3C, F). These results demonstrate that Six3 does not directly activate the expression of *foxq2* and zygotic *fzl5/*8. Instead, it appears the major role of Six3 is to antagonize Wnt signaling during the ANE restriction mechanism, allowing for the establishment of the ANE territory. Importantly, these data also indicate that *foxq2* and *fzl5/8* are activated by one or more unknown broadly expressed regulatory factors.

2.4. All three Wnt signaling pathways, but not Nodal and BMP2/4 signaling, control zygotic fzl1/2/7 expression during early AP patterning

During early cleavage stages *fzl1/2/7* is expressed throughout the embryo, then down regulated in the ANE, endoderm and mesoderm territories by the mesenchyme blastula stage. It is well established that Wnt/β-catenin signaling is active in the posterior endomesoderm during blastula stages when *fzl1/2/7* is down regulated in the same region (Fig. 1Bh-i). In addition, *fzl5/8* is expressed within the ANE territory at the same time that *fzl1/2/7* is down regulated from this territory (*cf* Fig. 1Bd, 1Bi). Finally, we previously showed that Fzl1/2/7 signaling appears to be active throughout the embryo during early cleavage and blastula stages (Range et al., 2013). Based on these data, I hypothesized that the dynamic spatial expression of *fzl1/2/7* along the AP axis suggests that it could be regulated by each of the Wnt signaling pathways in the network. To test this idea, I performed knockdowns of each pathway and assayed *fzl1/2/7's* spatial expression at mesenchyme

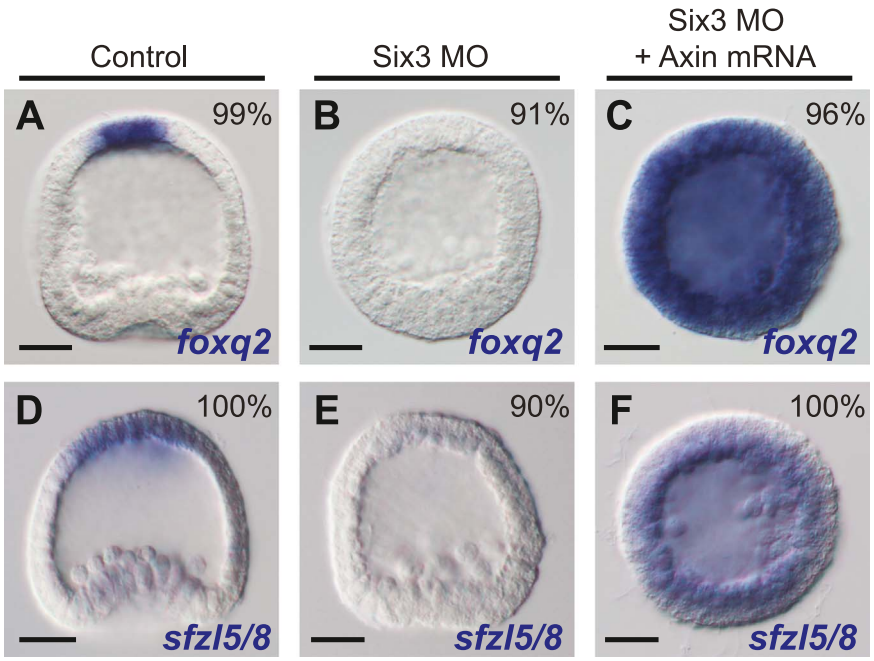


Fig. 3. Initial activation of *fzl5/8* and *foxq2*, a cardinal regulatory of the ANE GRN. The percentage of embryos examined that show the representative phenotypes depicted is indicated in each panel. In Six3 knockdown embryos the cardinal ANE regulator *foxq2* (A, B) and *fzl5/8* (D, E) are down regulated. In contrast, *foxq2* (C) and *six3* (F) are expressed broadly in Six3 morphants in the absence of Wnt/β-catenin signaling. MO, morpholino; Scale bar = 20 μm.

blastula stage (24 hpf). In contrast to control embryos (Fig. 4Aa), the territory of *fzl1/2/7* expression, as well as what we term the "anterior *fzl1/2/7* hole", shifted towards the posterior pole in embryos injected with mRNA encoding a previously characterized dominant negative version of Fzl5/8 (ΔFzl5/8) (Fig. 4Ab) (Croce et al., 2006; Range et al., 2013), suggesting that the correct positioning of the equatorial band of *fzl1/2/7* expression depends on Wnt1/Wnt8-Fzl5/8-JNK signaling. Next, I assayed for *fzl1/2/7* expression in Fzl12/7 morphants and found that it was severely up regulated and expressed in most cells in these embryos (Fig. 4Ac) indicating that Fzl1/2/7 signaling negatively regulates the expression of its own receptor. Finally, I blocked Wnt/β-catenin signaling by overexpressing Axin mRNA, and in these embryos *fzl1/2/7* transcripts were undetectable (Fig. 4Ad). This severe down regulation of Fzl1/2/7 suggests that zygotic expression of *fzl1/2/7* is activated by Wnt/β-catenin. However, it is possible that expansion of the ANE GRN, which in control embryos is necessary for the anterior *fzl12/7 hole*, may be responsible for the downregulation of *fzl1/2/7* in the Wnt/β-catenin (-) embryos. Thus, I injected embryos with mRNA encoding β-catenin that cannot be phosphorylated by GSK3-β, resulting in nuclear localization. I and others term this construct "activated β-catenin". In these embryos, *fzl1/2/7* appeared to be down regulated throughout the embryo (Fig. 4Ae), suggesting that Wnt/β-catenin signaling does not activate zygotic *fzl1/2/7* expression. Collectively, these data indicate that a complex interplay among the three Wnt signaling pathways is required for Fzl1/2/7 in the equatorial ectodermal territory and that a member of the ANE GRN

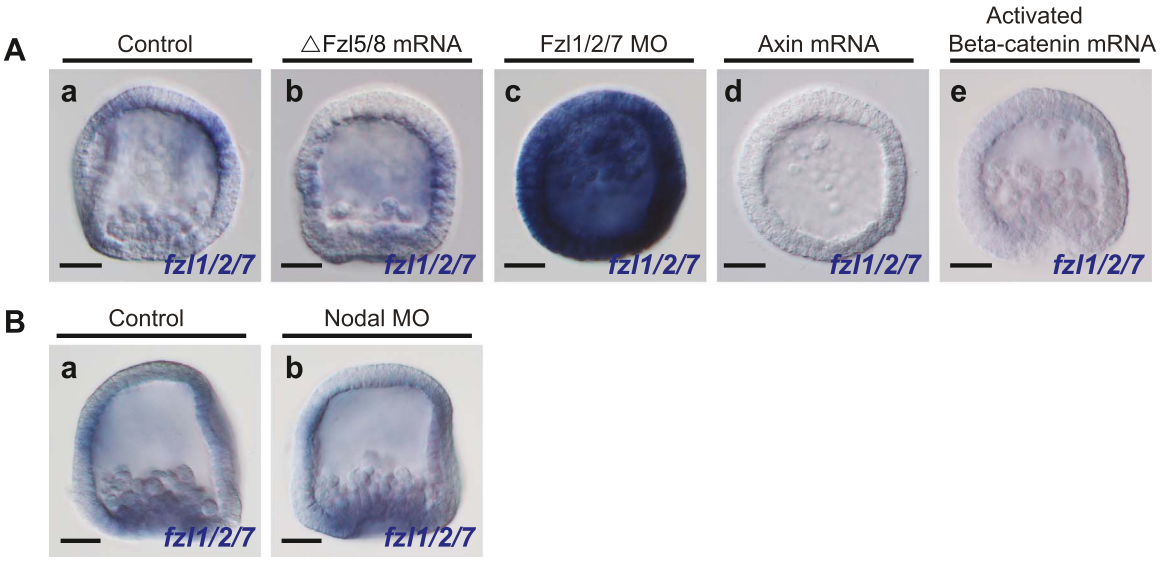


Fig. 4. Control of early zygotic *fzl1/2/7* expression by the Wnt signaling network. (A) Compared to control embryos (Aa), the anterior domain of *fzl1/2/7* down regulation expands and the belt of *fzl1/2/7* expression shifts toward the posterior/vegetal pole in embryos injected with ΔFzl5/8 mRNA (Ab). (Ac) In the absence of Fzl1/2/7, the expression of *fzl1/2/7* expands throughout the entire embryo. *fzl1/2/7* expression is down regulated in embryos lacking Wnt/β-catenin signaling (Ad) and in embryos with up regulated Wnt/β-catenin signaling (Ae). (B) *fzl12/7* expression is similar in control (Ba) and Nodal morphants (Bb). MO, morpholino; ΔFzl5/8, dominant negative Fzl5/8; Scale bar = 20 μm.

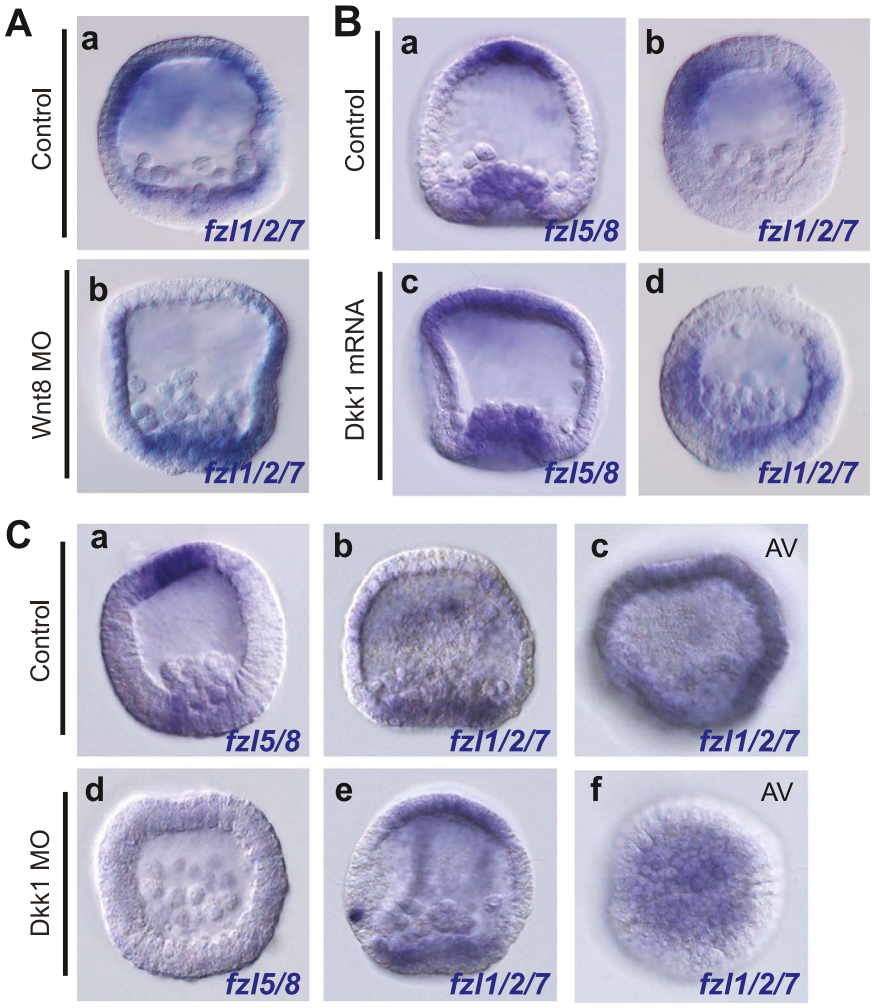


Fig. 5. Wnt8 and Dkk1 regulate the spatial expression of *fzl1/2/7* in the ectoderm. (A) Compared to control (a), ectodermal *fzl1/2/7* expression shifts towards the posterior of mesenchyme blastula embryos in Wnt8 morphants (b). (B) *fzl5/8* expression expands towards the vegetal/posterior pole in embryos injected with Dkk1 mRNA (a, c) coincident with the expanded downregulation of *fzl1/2/7* around the anterior pole and shift of the *fzl1/2/7* expression belt towards the posterior/vegetal pole (c, d). (C) Consistent with previous experiments (Range, 2013), *fzl5/8* expression around the anterior pole requires Dkk1 (a, d). The expression of *fzl1/2/7* is shifted towards the anterior pole (b, e) and expressed throughout the anterior territory (e, f). MO, morpholino; AV, anterior view; Scale bar = 20 μm.

that is not activated by Fzl5/8 signaling is necessary for the down regulation of *fzl1/2/7* in the ANE territory.

Nodal and BMP2/4 work together during the blastula stages to establish the DV axis in the sea urchin embryo and are critical for the spatial expression of genes in the ventral and dorsal territories respectively within the equatorial ectoderm belt (Molina et al., 2013). To examine the idea that these signaling pathways regulate zygotic *fzl1/2/7* expression within the equatorial ectoderm, I injected previously characterized morpholinos targeting Nodal (*bmp2/4* transcription is not activated in the absence of Nodal signaling). The zygotic ectodermal *fzl1/2/7* expression was unperturbed at the mesenchyme blastula/early gastrula stage (25–26 hpf) in Nodal morphants (4Ba, Bb), suggesting that Nodal and BMP2/4 signaling are unnecessary for patterning zygotic *fzl1/2/7* expression during early stages of development.

2.5. A balance among Wnt modulators secreted from the posterior and anterior poles establishes fzl1/2/7 expression in the equatorial ectodermal domain

Wnt8 is initially activated by Wnt/β-catenin in posterior endomesoderm cells, then an unknown mechanism activates its expression in equatorial ectoderm cells at the same time that it is necessary for the down regulation the ANE GRN in those cells (Range et al., 2013; Wikramanayake et al., 2004). This spatiotemporal expression profile suggests that Wnt8 could also play a role positioning *fzl1/2/7* expression along the AP axis. To test this hypothesis, I injected zygotes with previously characterized Wnt8 morpholinos and assayed *fzl1/2/7* expression at the mesenchyme blastula stage. In control mesenchyme blastula/early gastrula stage (24–26 hpf) ectodermal *fzl12//7* expression is down regulated in the ANE territory, and its posterior boundary has shifted more towards anterior (Fig. 5Aa). In Wnt8 morphants, both the anterior *fzl1/2/7* hole and the posterior boundary of the ectodermal *fzl12/7* expression shifted towards the posterior. In contrast, the endomesoderm expression of *fzl1/2/7* was unaffected (Fig. 5Ab). Together, these assays show that Wnt8 is important for the correct spatial expression of the equatorial ectodermal band of *fzl1/2/7* expression.

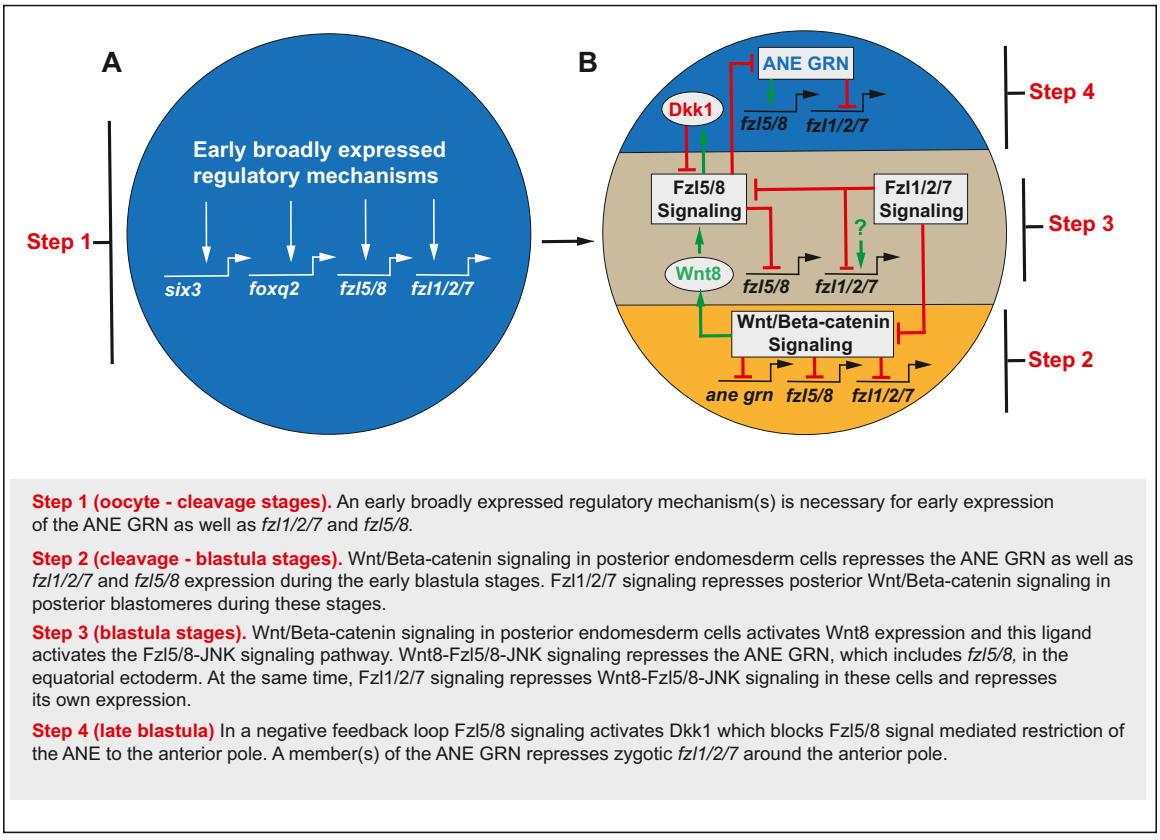
As mentioned in the introduction, a negative feedback mechanism involving Fzl5/8 and Dkk1 defines the outer boundary of the ANE territory (Range et al., 2013). When Wnt1/Wnt8-Fzl5/8-JNK signaling is perturbed, the ANE GRN expands and, as shown above, so does the anterior *fzl1/2/7* hole. Based on these observations, I hypothesized that the Fzl5/8-JNK-Dkk1 negative regulatory mechanism is necessary for the down regulation of *fzl1/2/7* from the ANE territory. Consistent with previous results, when I overexpressed Dkk1 mRNA anterior *fzl5/8* expression expanded towards the posterior of the embryos (Fig. 5Ba, c). In addition, *fzl1/2/7* expression was restricted to a narrower belt in

Dkk1 mRNA injected embryos with an expanded *fzl1/2/7* hole and a lower level of overall expression (Fig. 5Bb, d). Next, I knocked down Dkk1 and observed *fzl5/8* and *fzl1/2/7* expression. *fzl5/8* was severely down regulated in embryos injected with Dkk1 morpholino (Fig. 5Ca, d) indicating that the ANE territory is not specified in these morphants. In contrast, the spatial expression of *fzl1/2/7* along the AP axis changed dramatically, shifting from the equatorial ectodermal band to a contiguous territory around the anterior pole instead (*cf.* Fig. 5Cb, c; Ce, f). Interestingly, posterior expression of *fzl1/2/7* in the endomesoderm remained unchanged (*cf.* Fig. 5Cb, e). Taken together, these data demonstrate that a balance between the posterior-to-anterior signaling activity of Wnt8 and anterior-to- posterior activity of Dkk1 is essential for the correct ectodermal expression of *fzl1/2/7*.

3. Discussion

Frizzled receptors play crucial roles in determining when and where various regulatory networks are established along the early AP axis in a variety of species as well as in the subsequent developmental processes that depend on these receptors. I present an analysis of the early zygotic spatiotemporal expression of two receptors, Fzl1/2/7 and Fzl5/8, both of which are essential for the specification and patterning of the early anterior-posterior axis in sea urchin embryos. During the course of this study I made a novel discovery: the sea urchin ANE GRN, which includes Fzl5/8, is not activated by Six3 as previously thought. Instead the zygotic ANE GRN appears to be activated by an uncharacterized broadly expressed, possibly maternally driven, regulatory mechanism that exists during early cleavage stages. Thus, it appears that one of the primary early roles for Six3 is to repress the posterior-to-anterior Wnt signaling gradient that patterns the AP axis. I also show that all three Wnt signaling pathways involved in the signaling network that controls AP specification and patterning in the sea urchin also control the zygotic expression patterns of Fzl1/2/7 and Fzl5/8, adding another level of complexity to the remarkable balance of Wnt signaling activity necessary for early anterior-posterior pattering during sea urchin embryogenesis (See Fig. 1A and 6).

Studies in metazoan species from all major clades (cnidarians, lophotrochozoans, ecdysozoans, echinoderms, hemichordates, and chordates) indicate that Six3 sits at or near the top of a highly conserved anterior GRN necessary for head and/or anterior neural structures in these embryos (Darras et al., 2011; Lagutin et al., 2003; Posnien et al., 2011; Steinmetz et al., 2010; Wei et al., 2009). In several of these embryos, including mammalian, the anterior-most head and/or sensory structure is lost in the absence of functional Six3 (Kitzmann et al., 2017; Lagutin et al., 2003; Wei et al., 2009). In addition, studies in vertebrates, including mice, *Xenopus*, chickens and zebrafish (Carlin et al., 2012; Lagutin et al., 2003), sea urchins (Wei et al., 2009), and cnidarians (Leclere et al., 2016) have shown that overexpression of *six3* expands the anterior/head GRN through antagonizing Wnt signaling along the primary axis. Based on these studies it has been concluded that Six3 is critical for the existence of anterior GRNs, but whether it functions to activate ANE GRNs and/or repress Wnt signaling was unclear. In this study, I designed experiments to address these alternatives and to better understand how *fzl5/8* transcription is initiated in the sea urchin embryo. To my knowledge, I show here for the first time in any embryo that while Six3 is necessary to activate a metazoan ANE GRN, its function is indirect. Instead, this study, in combination with the findings from Wei et al. (2009), strongly suggest that a major role of Six3 in sea urchin embryos, and possibly other species, is to antagonize the ANE restriction mechanism at the level of transcription, *allowing* for, but not directly activating, the ANE GRN. Interestingly, Wei et al. (2009) showed that neurogenesis is purturbed



Step 1 (oocyte - cleavage stages). An early broadly expressed regulatory mechanism(s) is necessary for early expression of the ANE GRN as well as *fzl1/2/7* and *fzl5/8*.

Step 2 (cleavage - blastula stages). Wnt/Beta-catenin signaling in posterior endomesderm cells represses the ANE GRN as well as *fzl1/2/7* and *fzl5/8* expression during the early blastula stages. Fzl1/2/7 signaling represses posterior Wnt/Beta-catenin signaling in posterior blastomeres during these stages.

Step 3 (blastula stages). Wnt/Beta-catenin signaling in posterior endomesderm cells activates Wnt8 expression and this ligand activates the Fzl5/8-JNK signaling pathway. Wnt8-Fzl5/8-JNK signaling represses the ANE GRN, which includes *fzl5/8,* in the equatorial ectoderm. At the same time, Fzl1/2/7 signaling represses Wnt8-Fzl5/8-JNK signaling in these cells and represses its own expression.

Step 4 (late blastula) In a negative feedback loop Fzl5/8 signaling activates Dkk1 which blocks Fzl5/8 signal mediated restriction of the ANE to the anterior pole. A member(s) of the ANE GRN represses zygotic *fzl1/2/7* around the anterior pole.

Fig. 6. A four-step model for the activation of the ANE GRN and spatial regulation of *fzl1/2/7* and *fzl5/8* along the AP axis during early development. (A) A diagram illustrating that maternal regulatory mechanisms are necessary for the expression of *fzl1/2/7* and *fzl5/8* and that an unknown broadly expressed regulatory mechanism is necessary for the zygotic activation of the ANE GRN, which includes *fzl5/8*. (B) From the early cleavage stage to the mesenchyme blastula stages in the sea urchin, a balance among the activities of the Wnt/β-catenin, Wnt/JNK, and Fzl1/2/7 signaling pathways determine the spatial expression of *fzl1/2/7* and *fzl5/8* along the AP axis. The model is detailed in the figure.

in embryos lacking both Wnt/β-catenin signaling and Six3. Together with my results, these data suggest that Six3 may function later in the ANE GRN network heiracrchy to specifiy neurons. As mentioned in the introduction, multiple negative regulatory inputs are necessary to limit the rate of ANE GRN down regulation by Wnt1/Wnt8-Fzl5/8-JNK signaling in anterior ectodermal blastomeres in sea urchin embryos (see Fig. 1 A). As early as the 32- to 60-cell stage, we have shown that a secreted Wnt modulator, sFRP-1, and a mechanism dependent on non-canonical Fzl1/2/7 signaling work in parallel broadly throughout the embryo to antagonize Fzl5/8-JNK signaling in anterior blastomeres (Khadka et al., 2018; Range et al., 2013). Because *six3* is strongly expressed throughout anterior blastomeres at the same stage, I propose that these three separate mechanisms work together to moderate Wnt1/Wnt8-Fzl5/8-JNK signaling in anterior blastomeres during early cleavage stages in sea urchin embryos.

We had previously shown that when we block Wnt/β-catenin signaling (Range et al., 2013), which also blocks subsequent TGF-β signaling (Duboc et al., 2004), the ANE GRN, including the cardinal regulators *six3* and *foxq2*, was expressed throughout sea urchin embryos. Similarly, blocking Wnt/β-catenin signaling in the echinoderm sea star as well in hemichordates, which form a sister phylum to echinoderms, results in activation of *six3* and other ANE GRN genes throughout the embryo (Darras et al., 2011, 2018; Yankura et al., 2013). Importantly, if mammalian ESCs are deprived of exogenenous Wnt and BMP signaling ligands, an intrinsic mechanism broadly activates the ANE GRN throughout (Eiraku et al., 2008; Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005). Together these data indicate that most cells in deuterostome embryos have the potential to become ANE and that one of the major roles of Wnt, and in many cases TGF-β, signaling, is to restrict this potential to specific regions of the embryo. The results from this study strongly suggest that the activation of the ANE GRN in the sea urchin embryo is more complex than the previous idea that the broadly expressed regulatory state simply feeds into the cis-regulatory elements of *six3* and that this transcription factor then acts as the primary driver of the rest of the early ANE GRN. Instead, the activation of *six3* as well as the expression of many of the other early ANE GRN components appears to be dependent on this uncharacterized regulatory mechanism (Fig. 6 A).

Our previous studies have shown that a remarkable balance is realized among the unknown regulatory mechanism(s) that activates the ANE GRN, the suppression of this regulatory state by Wnt/β-catenin and Wnt1/Wnt8-Fzl5/8-JNK signaling, as well as the antagonism of this posterior-to-anterior restriction mechanism by broad Fzl1/2/7 signaling and anteriorly expressed secreted Wnt antagonists, such as Dkk1 (Khadka et al., 2018; Range et al., 2013; Range and Wei, 2016). If any molecule involved in this process is perturbed, then this balance is disrupted, resulting in dramatic changes in the various early AP regulatory states, not just the ANE GRN. As expected, I found in this study that early Wnt/β-catenin signaling is necessary to repress *fzl5/8* expression in posterior cells. In addition, I show that both the anterior and posterior boundaries of *fzl1/2/7* expression shift towards the posterior of the embryo when the function of Wnt8 or Fzl5/8 is perturbed. In contrast, *fzl1/2/7* is expressed in a contiguous domain around the anterior pole in Dkk1 knockdown embryos, and Dkk1 over expression causes the equatorial band of *fzl1/2/7* to shift towards the posterior pole. Together, these data indicate that the same ANE restriction mechanism that establishes *fzl5/8* expression around the anterior pole is also necessary for establishing the anterior and posterior boundaries of *fzl1/2/7* expression in the equatorial ectoderm. Interestingly, the data also suggest that a component(s) of the ANE GRN is critical to down regulate *fzl1/2/7* expression in the ANE territory, since interfering with Wnt/β-catenin signaling, which causes the ANE GRN to be expressed throughout the embryo, results in complete elimination of zygotic *fzl1/2/7* expression. It is important to note that Fzl5/8 signaling in the ANE territory is not involved in this process, since the *fzl1/2/7* expression hole expands when we block Fzl5/8 signaling. Finally, I discovered another level of regulatory balance in the AP Wnt network since Fzl1/2/7 signaling represses zygotic *fzl1/2/7* expression. Together, these results and those from previous studies indicate that all three Wnt signaling pathways involved in AP specification and patterning in the sea urchin control the zygotic spatiotemporal expression patterns of Fzl1/2/7 and Fzl5/8 in the early ectoderm (Fig. 6B). It is possible that this control is indirect, however it has been shown that two of the known signaling pathways active during early cleavage and blastula stages, Nodal, and BM2/4, do not appear to interact with the AP Wnt signaling network. The third signaling pathway active during late blastula stages, Notch, only appears to interact with the Wnt Wnt/β-catenin signaling pathway (see Angerer et al., 2011).

Similar to the complex interactions among the Wnt signaling pathways involved in AP patterning, several TGF-β signaling pathways work together to pattern all three early germ layers along the DV axis (Duboc et al., 2010; Molina et al., 2013). In brief, ventrally localized Nodal signaling is necessary and sufficient for establishing the DV axis during blastula stages. In equatorial ectodermal cells, it activates the ventral ectoderm GRN while at the same time activating the expression of BMP2/4 which subsequently diffuses and activates the dorsal ectoderm GRN (Duboc et al., 2004; Lapraz et al., 2015). Together, these signaling pathways establish appropriate gene expression throughout the equatorial ectoderm belt. Because zygotic *fzl1/2/7* is activated around the same time that TGF-β signaling establishes the DV axis and is expressed in the equatorial ectodermal belt, I reasoned that it could be regulated by these pathways. However, *fzl1/2/7* expression was normal when I knocked down Nodal signaling, which also eliminates BMP2/4 signaling (Lapraz et al., 2009). Together with the data indicating that TGF-β signaling does not activate *fzl1/2/7* signaling. In the future it will be important to identify this uncharacterized regulatory mechanism that is necessary for the zygotic expression of *fzl1/2/7* and works in the same ectoderm cells as TGF-β signaling.

The fact that a broadly expressed regulatory state is necessary for the activation of the ANE GRN in many deuterostome embryos, including mammals, strongly suggests that aspects of this mechanism may be conserved in these animals. To date, the only studies to address this regulatory mechanism have been performed in mammalian embryonic stem cells showing that several intrinsically activated transcription factors, including Zfp521, Pou3f1, Sox2, Otx2 and Zic2 (Iwafuchi-Doi et al., 2012; Kamiya et al., 2011; Thomson et al., 2011; Zhu et al., 2014), are necessary for the activation of the mammalian ANE GRN. However, these genes all appear to be zygotically activated, strongly suggesting that the early broad regulatory mechanism necessary for activation of the ANE GRN in mammals is still uncharacterized. It will be interesting in the future to identify the early regulatory mechanism that initiates the ANE GRN in sea urchin embryos and then to expand studies into other deuterostome embryos to determine if it is a conserved mechanism. We have previously described how expression and/or functional studies in several deuterostomes suggest that aspects of the Wnt network that governs AP axis specification and patterning in sea urchins is conserved (Range, 2014). For example, knockdowns of Fzl5/8 in hemichordates (Pani et al., 2012) or Fzl8a in zebrafish embryos (Kim et al., 2002) result in the expansion of the ANE GRN towards the posterior pole in these embryos. Thus, I find it remarkable that the spatiotemporal expression profiles of Fzl5/8 and Fzl1/2/7 during early AP specification and patterning in sea urchins, sea stars, hemichordates, and amphioxus are similar and believe that this study adds more evidence to the argument that aspects of the Wnt network that governs AP axis specification and patterning in the sea urchin may have existed in the deuterostome ancestor.

4. Materials and methods

4.1. Animals and embryo cultures

Strongylocentrotus purpuratus were obtained from Monterey Abalone Company, Monterey, CA, California Institute of Technology, Pasadena, CA, and Marinus Scientific, Long Beach, CA. 0.5 M KCl was injected into the body cavities of adult sea urchins to collect eggs and sperm. Artificial sea water was used to wash the eggs two to three times and eggs were subsequently fertilized in a glass beaker or a plastic culture dish by adding a 1:1000 dilution of sperm. Embryos were cultured in artificial seawater at 15 °C.

4.2. mRNA and morpholino injections

Overexpression studies were performed by injecting ~20 pL of full-length *dkk1* (3 μg/μL) and Δ*Fzl5/8* (2.0 μg/μL) mRNA into zygotes. The morpholinos were produced by Gene-Tools LLC (Eugene, OR). All morpholinos have been previously characterized (Range et al., 2013; Yaguchi et al., 2010). The sequences and injection concentrations were:

Wnt8 splice MO: 5′-GTAAAGTGTTTTTCTTACCTTGGAT-3′ (0.7 mM) (Range et al., 2013)

Fzl1/2/7 MO: 5′-CATCTTCTAACCGTATATCTTCTGC-3′ (1.3 mM) (Range et al., 2013)

Dkk1 MO: 5′-ATCGTTGGTAGTTGCAGAAATTCGT-3′ (0.7–0.85 mM) (Range et al., 2013)

Nodal MO: 5′-GATGTCTCAGCTCTCTGAAATGTAG-3′ (1.0 mM) (Yaguchi et al., 2010)

Six3 MO: 5′- GGGCCGCTCTCATGGCGCCCCGGTC-3′ (1.0 mM) (Wei et al., 2009)

Zygotes were injected after fertilization with solutions containing 20% FITC (2.5 mg/mL), 20% glycerol, mRNA or morpholino oligonucleotides. Embryos from at least three different mating pairs were used for each experiment that consisted of 25–150 embryos unless otherwise stated. Experiments were scored only if a change in gene expression or morphological phenotype was seen in at least 85–90% of the manipulated embryos. All injected embryos were cultured at 15 °C.

4.3. Whole-mount in situ hybridization

In situ hybridization and detection by alkaline phosphatase staining were carried out as previously described (Sethi et al., 2012). The antisense RNA probes for each gene analyzed correspond to the full-length cDNA sequence.

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