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, Fz1/2/7 and Fz5/8, 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 downregulate 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 Fz1/2/7 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 nyctic 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.

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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/Ca2⁺ pathways. The known molecular components of these pathways are reasonably 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;
Fig. 1. The spatiotemporal expression of fzd1/2/7 and fzd5/8 during early AP specification and patterning in invertebrate deuterostomes. (A) In sea urchin early development, the Wnt/β-catenin, Wnt/JNK, and Fzd1/2/7/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 Wnt and Wnt8 that activate the Fzd5/8-JNK signaling resulting in the down regulation of the ANE GRN in the posterior equatorial ectoderm. Step 3 (mid-blastula to mesenchyme blastula stage) In the repressing ANE GRN Fzd5/8 signaling activates Dkk1 and sFRP1 expression. These Wnt antagonists perturb the posterior-to-anterior repression of the ANE GRN by Fzd5/8 signaling via a negative feedback loop. Around the same time, FoxQ2 activates the expression of two Wnt modulators, sFRP1/5 and Dkk3, that potentiate Fzd5/8 signaling (data taken from Range et al., 2013; Range, 2014; Range and Wei, 2016; Khadka et al., 2018). (B) Expression of fzd1/2 and fzd5/8 in embryos from the same mating pairs in S. purpuratus. (Ba) fzd5/8 is detected in anterior blastomeres beginning around the 120-cell stage. (Bb, c) In early blastula stages, stage fzd5/8 expression is progressively down regulated from equatorial ectodermal cells. (Bd, e) fzd5/8 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). (Bb) fzd1/2/7 is expressed ubiquitously in the cleavage stage. (Bg) In blastula staged embryos, fzd1/2/7 expression is down regulated in posterior cells. (Bh) fzd1/2/7 expression is down regulated around the anterior pole at the early mesenchyme blastula stage. (Bi, j) Between mesenchyme blastula stage and early gastrula, fzd1/2/7 expression is restricted to the equatorial ectodermal band and activated in the posterior endomesoderm. (C) Diagram of fzd5/8 and fzd1/2/7 expression in invertebrate deuterostomes. Data taken from (Darras et al., 2018; McCauley et al., 2013; Gian 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 S3c 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 Fzd5/8 receptor. This Wnt1/Wnt8-Fzd5/8-JNK signaling pathway progressively 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 fzd5/8 becomes integrated into the ANE GRN down regulated by Wnt1/Wnt8-Fzd5/8-JNK signaling. Simultaneously, a different non-canonical Wnt signaling pathway, working through the Fzd1/2/7 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) Fzd5/8 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-Fzd5/8-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 fzd5/8 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 (Keister 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, Fzd1/2/7, Fzd4, Fzd5/8, and Fzd9/10 (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.

2. Results

2.1. Spatiotemporal expression of fzd1/2/7 and fzd5/8 during early anterior–posterior axis specification

Functional studies have shown that during cleavage and blastula...
stages, Fz5f/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 Fz1/2/7 signaling appears to be active throughout the embryo during cleavage and early blastula stages. Then, similar to Fz5f/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 fzl1f/2f/7 and fzl5f/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, fzl5f/8 expression was down-regulated from posterior endomesoderm blastomeres (Fig. 1Ba). Subsequently, fzl5f/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, fzl5f/8 was also activated in posterior endomesoderm cells (Fig. 1Bd, e). The expression of fzl1f/2f/7 was down regulated in posterior cells during late blastula stages while being maintained in the ectoderm (Fig. 1Bf–h). At mesenchyme blastula stage fzl12f/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 fzl12f/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 fzl5f/8

In a previous study, we showed that fzl5f/8 expression is down regulated from the equatorial ectodermal territory through a negative feedback mechanism mediated by Wnt1/Wnt8-Fz5f/8-JNK signaling (Fig. 2A, B). In addition, we demonstrated that Fz1/2/7-PKC signaling antagonizes Fz5f/8-JNK signaling in these same cells and its down regulation of fzl5f/8 (Fig. 2C) (Range et al., 2013). Although we had not determined how fzl5f/8 expression is down regulated in the posterior endomesoderm territory, we hypothesized that it was due to posterior Wnt/beta-catenin signaling. To test this idea, I injected zygotes with Axin mRNA, which blocks endogenous Wnt/beta-catenin signaling. In these embryos, fzl5f/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 fzl5f/8 expression along the AP axis.

2.3. An early, broadly active regulatory mechanism activates the ANE GRN, which includes fzl5f/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 fzl5f/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 fzl5f/8) in the absence of Wnt/beta-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 fzl5f/8 (n = 56/62) were severely down regulated in Six3-deficient embryos (Fig. 3C, F). In contrast, the ANE regulatory factors were expressed throughout Six3 knockdown embryos when Wnt/beta-catenin signaling was also blocked (n = 51/53 for foxq2 and n = 52/52 for fzl5f/8) (Fig. 3C, F). These results demonstrate that Six3 does not directly activate the expression of foxq2 and zygotic fzl5f/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 fzl5f/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 fzl1f/2f/7 expression during early AP patterning

During early cleavage stages fzl1f/2f/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/beta-catenin signaling is active in the posterior endomesoderm during blastula stages when fzl1f/2f/7 is down regulated in the same region (Fig. 1Bh-i). In addition, fzl5f/8 is expressed within the ANE territory at the same time that fzl1f/2f/7 is down regulated from this territory (cf. Fig. 1Bd, 1Bj). Finally, we previously showed that fzl1f/2f/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 fzl1f/2f/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 fzl1f/2f/7’s spatial expression at mesenchyme
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) (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. 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.

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) fzl1/2/7 expression is similar in control (Ba) and Nodal morphants (Bb). MO, morpholino; ΔFzl5/8, dominant negative Fzl5/8; Scale bar = 20 µm.
that is not activated by Fzl5/8 signaling is necessary for the downregulation 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 fzl1/2/7 expression is downregulated in the ANE territory, and its posterior boundary has shifted more towards anterior (Fig. 5Aa). In Wnt8 morphants, both the anterior fzl1/2/7 domain and the posterior boundary of the ectodermal fzl1/2/7 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 domain. 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 patterning 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 perturbed
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 hierarchy to specify 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. 1A). 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 Fz1/2/7 signaling work in parallel broadly throughout the embryo to antagonize Fz1/2/8-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 as hemichordates, which form a sister phyllum 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 exogenous Wnt and BMP signaling ligands, an intrinsic mechanism broadly activates the ANE GRN throughout (Einaku 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 is necessary for 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 Fz5/8 in hemichordates (Pani et al., 2012) or Fz8a 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 Fz5/8 and Fz1/2/7 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, and Marinus Scientific, Long Beach, CA. 0.5 M KC1 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 ΔPζ15/8 (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′-GTAAGTTTTTTCCATTCTGGAT-3′ (0.7 mM) (Range et al., 2013)
Fz1/2/7 MO: 5′-CATCCTCTAAAGCTATATCTGCTC-3′ (1.3 mM) (Range et al., 2013)
Dkk1 MO: 5′-ATCGTGTAGTGAGGAAATTCTG-3′ (0.7–0.85 mM) (Range et al., 2013)
Nodal MO: 5′-GATGCTCTTGAGTCTGGATAG-3′ (1.0 mM) (Yaguchi et al., 2010)
Six3 MO: 5′-GGCCCGCTCTCATGGCCGGCGGT-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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References


