An anterior signaling center patterns and sizes the anterior neuroectoderm of the sea urchin embryo

Ryan C Range[1, 2, 3] and Zheng Wei[2]

[1] Current address: Department of Biological Sciences, Mississippi State University, Mississippi State, MS 39762

[2]: National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892

[3]: Corresponding author; range@biology.msstate.edu

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Abstract

Anterior signaling centers are essential to specify and pattern the early anterior neuroectoderm (ANE) of many deuterostome embryos. In the sea urchin embryo the ANE is restricted to the anterior end of the late blastula-stage embryo where it forms a simple neural territory consisting of several types of neurons, as well as the apical tuft. Here, we show that during early development, the sea urchin ANE territory separates into inner and outer regulatory domains expressing the cardinal ANE transcriptional regulators, FoxQ2 and Six3, respectively. FoxQ2 drives this patterning process, which is required to eliminate *six3* expression from the inner domain and activate the expression of Dkk3 and sFRP1/5, two secreted Wnt modulators. Dkk3 and low expression levels of sFRP1/5 act additively to potentiate the Wnt/JNK signaling pathway governing the positioning of the ANE territory around the anterior pole; whereas, high expression levels of sFRP1/5 antagonize Wnt/JNK signaling. Furthermore, the levels of sFrp1/5 and Dkk3 are rigidly maintained *via* auto-repressive and cross-repressive interactions with Wnt signaling components and additional ANE transcription factors. Together, these data support a model in which FoxQ2 initiates an anterior patterning center that implements correct size and positions of ANE structures. Comparisons of functional and expression studies in sea urchin, hemichordate and chordate embryos reveal striking similarities among deuterostome ANE regulatory networks and the individual molecular mechanism that position and define ANE borders. These data provide strong support for the idea that the sea urchin embryo uses an ancient anterior patterning system that was present in the common ambulacrarian/chordate ancestor.

Introduction

The embryonic anterior neuroectoderm (ANE) territory of deuterostomes develops into a variety of neurosensory organs, from the simple apical tuft of sea urchin embryos to the complex vertebrate forebrain and eye field. While there are significant differences in the nervous systems created from this early territory among the deuterostomes, the ANE initially arises from a relatively simple, flat neuroepithelium during the early stages of development and many of the cell types this territory produces are remarkably conserved (Burke et al., 2014; Castro et al., 2015; Cavodeassi, 2013; Garner et al., 2015; Pani et al., 2012; Range, 2014). Based on the spatial and temporal expression of ANE regulatory genes in several species, it appears that the ANE gene regulatory network (GRN) hierarchy is also remarkably conserved among many deuterostome embryos, suggesting that these could be homologous territories (Range, 2014). Strengthening this view, it appears that aspects of the mechanism used to position the ANE territory around the anterior pole also may be shared among deuterostomes (Range, 2014). For example, early ANE genes, such as *six3*, are broadly expressed throughout the presumptive ectoderm in most invertebrate deuterostomes and the presumptive neuroectoderm in vertebrates (Darras et al., 2011; Kozmik et al., 2007; Lagutin et al., 2003; Posnien et al., 2011; Range, 2014; Wei et al., 2009). Then a molecular mechanism that depends on a posterior-to-anterior cascade of Wnt/β-catenin signaling activity down regulates ANE factors from more posterior ectoderm so that their activities are restricted to a region around the anterior pole (Darras et al., 2011; Kiecker and Niehrs, 2001; Nordstrom et al., 2002; Range et al., 2013; Yaguchi et al., 2008).

In the sea urchin embryo *six3* and *foxq2* are the first ANE regulatory genes expressed in the presumptive ectoderm and both transcription factors act as cardinal regulators of ANE territory specification (Wei et al., 2009; Yaguchi et al., 2008; Yaguchi et al., 2011; Yaguchi et al., 2010). Similar to other deuterostome embryos, *six3* and *foxq2* initially exhibit pan-ectodermal expression, but then their expression is progressively restricted to a territory around the anterior pole (Wei et al., 2009; Yaguchi et al., 2008). Based on functional and expression experiments it appears these cardinal regulators are expressed in two concentric domains around this pole with *six3* expressed in the outer ANE and *foxq2* expressed around the central ANE territory (Li et al., 2014; Wei et al., 2009; Yaguchi et al., 2008a). Six3 sits at or near the top of the entire ANE GRN and antagonizes posterior-to-anterior Wnt signaling (Wei et al., 2009). FoxQ2 is necessary for serotonergic neural specification and acts as a cardinal regulator of apical tuft specification in the central ANE sub-domain (Yaguchi et al., 2012; Yaguchi et al., 2010). Remarkably, the ANE positioning mechanism involves much more that the Wnt/β-catenin signaling pathway in the sea urchin. It depends on the embryo's ability to integrate information from at least three different Wnt signaling branches, the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC branches (see figure 8A)(Range et al., 2013), representing one of the few examples of all three being integrated into an overall Wnt signaling network that impinges on the same developmental process. In addition, comparative functional and expression data from vertebrates, amphioxus, hemichordates and sea star embryos suggest that aspects of this Wnt network could be widely shared among deuterostome embryos (Range, 2014).

In vertebrate embryos, specific territories within the presumptive ANE (forebrain and eye field) activate signaling centers that function to subdivide the territory into the telencephalon, diencephalon, and eye fields, once the ANE is positioned around the anterior pole of the early neural plate (Houart et al., 2002; Wilson and Houart, 2004). One of the most important of these signaling centers forms at the anterior-most region of the neural plate, termed the anterior neural ridge (ANR)(Cavodeassi and Houart, 2012; Echevarria et al., 2003). Secreted Frizzled-related proteins (sFRPs) are secreted from the ANR and play key roles in establishing a posterior-to-anterior Wnt signaling gradient within the early anterior neural plate, which is necessary to establish structurally and functionally distinct sub-regions within the ANE (Cavodeassi and Houart, 2012). sFRPs consist of a family of secreted proteins that is the largest family of Wnt modulators in the animal kingdom (Bovolenta et al., 2008). They contain a cysteine-rich domain (CRD) that is almost identical to the CRD of the Wnt receptor Frizzled (Fzl) and several studies suggest that sFRPs could bind Wnt via this domain and inhibit Wnt's ability to activate the Fzl receptor (Bafico et al., 1999; Bhat et al., 2007; Lin et al., 1997). However, recent data inconsistent with this model for sFRP function suggest alternatively that they can act as Wnt signaling agonists. For example, several studies suggest that sFRP may augment Wnt signaling by facilitating interactions between the Fzl receptor and Wnt or by activating Wnt signaling independent of Wnt ligands, since sFRPs can form complexes with Fzl receptors (Bafico et al., 1999; Dufourcq et al., 2008; Esteve et al., 2011; Mii and Taira, 2009, 2011; Rodriguez et al., 2005). Based on these apparently conflicting results, it has been proposed that the functions of sFRPs depend on their expression levels as well as the molecular and cellular context (Bovolenta et al., 2008; Mii and Taira, 2011).

Dickkopf family members are expressed in ANE territories in many deuterostome embryos from sea urchins to vertebrates and appear to play conserved roles in establishing the ANE. The family is divided into two sub-classes with one branch including Dkk1, Dkk2, and Dkk4 and the other Dkk3. Many studies indicate that the most common function for Dkk1, Dkk2, and Dkk4 is to antagonize Wnt signaling by interfering with the Fzl co-receptors Lrp5/6 and Kremen (Bafico et al., 2001; Cruciat and Niehrs, 2013; Mao et al., 2001; Semenov et al., 2001). In contrast, it is not clear that Dkk3 plays a role in modulating Wnt signaling, since secreted Dkk3 does not appear to bind either Fzl receptors or Wnt signaling co-receptors and studies focusing on its function do not point to a clear role for Dkk3 in modulating Wnt signaling (Veeck and Dahl, 2012).

Here we show that as the ANE begins to be established around the anterior pole of the sea urchin embryo, the cardinal ANE regulatory factor FoxQ2 establishes the anterior-most ANE territory in the sea urchin embryo, separating the ANE into two concentric domains by the beginning of gastrulation. We also found that FoxQ2 activates an anterior signaling center that secretes the Wnt modulators sFRP1/5 and Dkk3, which work additively through the Fzl5/8-JNK-mediated ANE positioning mechanism to establish the correct size of the entire ANE territory. In addition, our data indicate that sFRP1/5 and Dkk3 tightly control one another's expression while at the same time regulating their own. Finally, comparison of functional and expression data from several deuterostomes with the data from this study suggests that anterior signaling centers necessary for ANE patterning existed in the last common ancestor of ambulacrarians and chordates.

Results

Two concentric ANE territories

The first known ANE genes expressed in the sea urchin embryo are *six3* and *foxq2* (Wei et al., 2009; Yaguchi et al., 2008). The expression domains of these genes appear to be congruent in the same anterior ectoderm cells during the initial stages of ANE specification, when they are expressed throughout the anterior half of the 32- to 60-cell stage embryo (Fig. 1A)(Range et al., 2013; Wei et al., 2009; Yaguchi et al., 2008). As the Wnt/JNK-mediated ANE positioning mechanism down regulates *six3* and *foxq2* transcripts in the more posterior ectoderm cells, expression of both transcripts remains very similar within in the anterior ectoderm through the blastula stages (as late as 18-20 hpf in *S. purpuratus*)(Fig. 1A, middle)(Range et al., 2013; Wei et al., 2009; Yaguchi et al., 2008). Then, beginning in late blastula stages their transcripts appear to accumulate in separate ANE territories (Fig 1A, right-hand diagram; Fig. 2)(Li et al., 2014; Wei et al., 2009; Yaguchi et al., 2008). In a previous study we showed that Fzl5/8 signaling activates the secreted Wnt signaling antagonist Dkk1 around the anterior pole during the final stages of ANE positioning and in a negative feedback loop Dkk1 blocks further ANE GRN down regulation by Fzl5/8-JNK signaling, establishing the ANE territory. As a result of this negative feedback loop *fzl5/8* gene expression is maintained in the ANE territory and the functional data indicate that along with *dkk1*, it marks the entire presumptive ANE territory at the mesenchyme blastula stage (24 hpf)(Range et al, 2013). In the current study double-labeled in situ hybridization with *fzl5/8* and *foxq2* probes revealed that *foxq2* was restricted to a central territory around the anterior pole, nested

within the *fzl5/8* expression domain (Fig. 1Ba-Bc), consistent with the idea that there are at least two concentric ANE regulatory domains.

FoxQ2 determines the inner ANE territory and activates the secreted signaling modulators sFRP1/5 and Dkk3

six3 transcripts are expressed broadly and uniformly around the anterior pole from early- to mid-blastula stages (Wei et al., 2009). Then, during later blastula stages, *six3* transcripts are down regulated in the central anterior pole region (Wei et al., 2009), resulting in a ring of *six3* expression by mesenchyme blastula stages (24 hpf)(Fig. 2Aa, Ab), which appears to extend to the outer edges of the ANE territory. Interestingly, *foxq2* is expressed in the anterior-most territory where *six3* expression was down regulated (cf. Fig. 2Aa, Ab and Ac, Ad). These observations indicate that, while *six3* and *foxq2* are both initially restricted to the anterior pole by the Wnt-mediated AP positioning mechanism, they subsequently respond differently to this and/or some other signaling mechanism, resulting in two concentric domains of expression.

Based on the expression patterns of *six3* and *foxq2* at the mesenchyme blastula stage (24 hpf) and the fact that FoxQ2 acts as a cardinal regulator of many cell fates in the central ANE territory (Yaguchi et al., 2012; Yaguchi et al., 2011; Yaguchi et al., 2010), we reasoned that FoxQ2 might play a critical role in establishing the inner and outer ANE domains. To test this hypothesis, we monitored the expression of *six3* at the mesenchyme blastula stage in embryos injected with FoxQ2 morpholino oligonucleotides. In the absence of FoxQ2, *six3* transcripts were no longer expressed in a

ring around the anterior pole, but were instead expressed throughout an expanded ANE territory (Fig. 2Ae, Af). This result indicates that FoxQ2 activity is necessary for establishing a distinct regulatory domain in the central animal pole region confirming similar data previously reported in *S. purpuratus* (Li et al., 2014). Interestingly, the expression domains of both *foxq2* and *six3* also expanded towards the posterior pole of the embryo (cf., Fig. 2Aa, Ac and Ae, Ag). This surprising result suggests that FoxQ2 directly or indirectly limits the range of its own expression and is necessary for sizing the ANE.

We have shown previously that the size of the ANE territory depends on a remarkable balance between Wnt potentiation and Wnt antagonism along the AP axis of the sea urchin embryo (Range et al., 2013). Thus, we hypothesized that FoxQ2 might activate the expression of secreted Wnt modulators, the absence of which would cause the ANE expansion observed in the absence of FoxQ2. We identified from a differential microarray screen two potential Wnt modulators, sFRP1/5 and Dkk3, that were severely down regulated in the absence of FoxQ2. To confirm this result we analyzed the effect of FoxQ2 knockdown by qPCR (Fig 2Be) and *in situ* hybridization assays (Fig 2Ba-d), which showed that both *sfrp1/5* and *dkk3* are severely down regulated in the absence of FoxQ2 (Fig. 2B).

sfrp1/5, dkk3, and foxq2 expression overlap during ANE restriction

We performed in situ hybridization with *sfrp1/5*, *dkk3*, and *foxq2* probes to determine the developmental time course of their expression. Both *sfrp1/5* and *dkk3* transcripts were

first detectable after the initial expression of *foxq2*, which is initiated at the 32-cell stage and extends throughout the presumptive ectoderm by the 32- to-60 cell stage (7-9hfp)(Fig 3A). *sfrp1/5* expression was first detected at the 120-cell stage (12 hpf) and *dkk3* expression at the hatched blastula stage (15 hpf)(Fig. 3). From the onset of their expression, both *sfrp1/5* and *dkk3* transcripts are progressively down regulated from posterior ectoderm to a region that approximates the central ANE domain, mimicking *foxq2* expression. These data suggest that *sfrp1/5* and *dkk3* expression domains overlap exactly with that of *foxq2* at later stages. To test this hypothesis we performed doubly labeled in situ hybridization with probes for these signaling modulators and *foxq2* at the mesenchyme blastula stage (24 hpf) and confirmed that their expression is congruent with that of *foxq2* transcripts at the anterior pole of the embryo (Fig. S1A, B).

FoxQ2 activates an anterior signaling center

FoxQ2 activates *sfrp1/5* and *dkk3* within the central ANE territory during the middle-to-late stages of ANE restriction, consistent with a role for these genes in mediating the function of FoxQ2 in sizing the ANE. If this hypothesis is true, then the ANE territory should expand in the absence of sFRP1/5 and/or Dkk3, as observed in FoxQ2 morphants (Fig. 2Ac,Ag). We tested this possibility by morpholino knockdown and measured the expansion and/or up regulation of several ANE factors by *in situ* hybridization and qPCR at the end of ANE positioning (mesenchyme blastula stage/24-26 hpf in *S. purpuratus*). Embryos injected with either of two different morpholinos targeting sFRP1/5 or two targeting Dkk3 showed posterior expansion of ANE markers (Fig. 4A, B; S2A, B). In

contrast to FoxQ2 morphants, in which *six3* was expressed uniformly throughout the ANE territory, *six3* was expressed in a thin ring of ectoderm cells near the embryonic equator, presumably because FoxQ2 down regulated it throughout the expanded central territory (cf., 2Ae, 4Ag, and 4Ak). Moreover at the mesenchyme blastula stage (24-26 hpf), the expression of several ANE regulatory factors that depend on Six3 and/or FoxQ2 was up regulated in the absence of either secreted signaling modulator (Fig. 4C). Specification of serotonergic neurons requires both Six3 and FoxQ2 activity. By 72 hpf, control plutei normally develop 3-5 serotonergic neurons (control averaged 3.7 serotonergic neurons, n = 23)(Fig. 4Ad). In the absence of either sFRP1/5 or Dkk3 there was a 3- to 4-fold increase in the number of serotonergic neurons and these were distributed over a broader territory (sFRP1/5 MO = 12.3 serotonergic neurons, n = 32; Dkk3 MO = 11.7 serotonergic neurons, n = 27)(Fig. 4Ah and l; S2B). Taken together, these results indicate that sFRP1/5 and Dkk3 are part of an anterior signaling center activated by FoxQ2. This signaling center is essential for establishment of a correctly sized ANE territory and appears to be subsequently required for the correct pattern and number of serotonergic neurons within the ANE.

sFRP1/5 and Dkk3 are required to potentiate the Fzl5/8-JNK mediated ANE positioning mechanism

FoxQ2 activates *dkk3* and *sfrp1/5* expression during the later stages of ANE positioning (15-24 hpf)(Fig. 2Bb). Both ligands have presumptive roles as Wnt signaling modulators, and both morphant phenotypes at the end of ANE positioning (mesenchyme blastula

stage/24 hpf) are similar to the phenotype observed when signaling via Fzl5/8-JNK pathway is inhibited: expansion of the ANE territory (Range et al, 2013). Based on this evidence, we hypothesized that the anterior signaling center secretes Dkk3 and sFRP1/5, forming an anterior-to-posterior diffusion gradient that potentiates down regulation of ANE gene expression by Fzl5/8-JNK signaling. To test this hypothesis, we overexpressed Dkk3, which resulted in the complete elimination of expression of the ANE regulatory factors *foxq2, fzl5/8* and *nkx3.2* (Fig. 5Db; S2D). Similarly, low levels of sFRP1/5 overexpression reduced *foxq2* expression (Fig. 5Aa-c), but surprisingly *foxq2* expression expanded in embryos that were injected with progressively higher concentrations of sFRP1/5 mRNA (Fig. 5Ad, Ae; S2C). Furthermore, injection of relatively low levels of sFRP1/5 mRNA rescued the sFRP1/5 morpholino phenotype (3 different batches of embryos; 84.2 percent rescue of sFRP1/5 MO2; n = 67)(cf., Fig. 5Ba, Bb, Bc), whereas *foxq2* was expanded in embryos co-injected with sFRP1/5 morpholinos and higher levels of sFRP1/5 mRNA (3 different batches of embryos; 95.3% *foxq2* posterior expansion, n = 69)(Fig. 5Bd).

The Dkk3 and low-level sFRP1/5 overexpression phenotypes are identical to the phenotypes observed when the Fzl-5/8-JNK pathway is up regulated in sea urchin embryos embryo (Range et al, 2013), suggesting that they may potentiate signaling through this pathway. To test this idea, we determined whether Dkk3 and/or sFRP1/5 could down regulate *foxq2* expression in the absence of a functional Fzl5/8-JNK signaling pathway. We found that overexpression of either Dkk3 or sFRP1/5 (at low concentrations) no longer down regulated *foxq2* expression when we blocked the function of Fzl5/8 with a dominant negative Fzl5/8 construct (ΔFzl5/8)(3 different batches of

embryos; 88.9% rescue of sFRP1/5 [n=57] and 100% rescue of Dkk3 [n= 71] misexpression phenotypes)(Fig. 5Cd, Dc). Similarly, Dkk3 and sFRP1/5 overexpression did not suppress *foxq2* expression in embryos treated with the JNK inhibitor (3 different batches of embryos; 95.9% rescues FRP1/5 [n = 65] and 90.7% rescue of Dkk3 [n= 61] misexpression phenotype)(Fig. 5Ce, Dd). These data strongly suggest that Dkk3 and low levels of sFRP1/5 are required for Fzl5/8-JNK signaling to fully down ANE gene expression.

sFRP1/5 and Dkk3 act additively to restrict the ANE territory

To gain a more detailed understanding of the role of sFRP1/5 and Dkk3 in sizing the ANE territory, we carefully measured the size of the inner ANE territory (i.e. the region expressing *foxq2*) in each morphant at the completion of the ANE positioning process (24 hpf)(Fig. 6). Injection of either sFRP1/5 or Dkk3 morpholino oligonucleotides caused similar large increases in the area of the inner ANE discoidal domain when compared to control (Fig. cf. 6Aa-c; Ae-g) and the expanded ANE territory was approximately the same size in both morphants (Fig. 6B). Next, we co-injected embryos with Dkk3 and sFRP1/5 morpholino oligonucleotides (Fig. 6d,h). Strikingly, in these double-knockdown embryos the area containing the inner ANE territory increased roughly 2-fold when compared to either morphant alone (Fig. 6B). We conclude that sFRP1/5 and Dkk3 likely work additively to promote down regulation ANE gene expression by Fzl5/8-JNK signaling during the later stages of the positioning mechanism, resulting in an appropriately sized ANE territory.

Negative regulatory interactions control sfrp1/5 and dkk3 expression

During the course of this study, we uncovered two additional regulatory interactions: anterior *dkk3* expression expanded in sFRP1/5 morphants (Fig. 7Ab, Ae) and *sfrp1/5* expression expanded in Dkk3 morphants (Fig. 7Bb, Be). These results suggested that sFrp1/5 and Dkk3 negatively regulate one another's expression. To test this hypothesis we overexpressed sFRP1/5 at high levels (1.0-1.5 μg/μL), which severely down regulated *dkk3* expression in the ANE (cf. Fig. 7Ac, Af); conversely, when we overexpressed Dkk3, *sfrp1/5* expression was completely eliminated (cf. Fig. 7Bc, Bf). To quantitate these effects we measured the expression levels of these factors by qPCR. As expected, the level *sfrp1/5* transcripts increased significantly (4-fold) in Dkk3 morphants (Fig. 7E) and *dkk3* expression increased by ~35 fold in sFRP1/5 morphants (Fig. 7D). We next tested the expression of *dkk3* in a Dkk3 morphant background and *sfrp1/5* in an sFrp1/5 morphant background. In situ hybridization showed that the domains of both transcripts expanded in their respective morphant backgrounds and their expression levels were also markedly up regulated (Fig. 7Ca-Cd). Again, we confirmed these results with qPCR analysis, which showed that *dkk3* expression increased by an average of 32-fold in Dkk3 morphants (Fig. 7E) and in sFRP1/5 morphants *sfrp1/5* expression increased by a remarkable 78-fold on average (Fig. 7D). Collectively, these results show that sFRP1/5 and Dkk3 negatively regulate both their own and each other's expression.

Discussion

We have characterized the roles of two secreted signaling modulators, sFRP1/5 and Dkk3, in the specification and patterning of subdomains of the anterior neuroectoderm (ANE) territory in the sea urchin embryo. Our functional analyses indicate that the cardinal ANE transcriptional regulator FoxQ2 is necessary to subdivide the ANE into two nested concentric domains of gene expression and to activate the expression of sFRP1/5 and Dkk3 within the anterior-most discoidal domain. We show that Dkk3 and low expression levels of sFRP1/5 are both required, and act additively, to stimulate the Fzl5/8-JNK signaling mediated down regulation of ANE gene expression during ANE positioning. Interestingly, higher levels of expression sFRP1/5 appear to antagonize Fzl5/8-JNK signaling. In addition, our data reveal that remarkable negative auto- and cross- regulatory interactions of sFRP1/5 and Dkk3 are necessary to reproducibly establish an appropriately sized ANE around the anterior pole. This is also the first study in deuterostomes outside of the chordate phylum to define functions of sFRP1/5 and Dkk3.

Based on this study we propose an expanded model for ANE positioning in the sea urchin embryo (Fig. 8A). During the early cleavage stages (32- to 60-cell stage) to early-mid-blastula stage, Wnt/β-catenin signaling activates posterior-to-anterior gradients of Wnt1 and Wnt8 that down regulate ANE regulatory factors in the posterior ectoderm via activation of the Fzl5/8-JNK signaling pathway. Then, during the mid-blastula stages, Fzl5/8 signaling in the regressing ANE territory activates Dkk1 expression. At this time Dkk1 antagonizes the posterior-to-anterior repression of ANE cell fate in a

classic negative feedback loop (Range et al, 2013). Our new data suggest that at around the same time FoxQ2, now under the control of Six3 (Wei et al, 2009), drives expression of sFRP1/5 and Dkk3, which diffuse in an anterior-to-posterior gradient from the inner ANE territory, reinforcing the Wnt1/Wnt8/Fzl58/JNK-mediated down regulation of ANE factors at the outer limits of their diffusion gradient. Conversely, high concentrations of secreted sFRP1/5 around the anterior most pole can antagonize Fzl58/JNK signaling, possibly assisting Dkk1 in defining domains within the ANE territory. Finally, our data show that sFRP1/5 and Dkk3 (directly or indirectly) negatively regulate one another's expression, which is also essential to achieve the proper size of the final ANE territory. Thus, the signaling pathways that mediate ANE positioning depend on positive inputs from both primary poles of the embryo. It is the elegant balancing act between the potentiation of the Fzl5/8-JNK positioning mechanism by posteriorly expressed Wnt ligands and anteriorly expressed sFRP1/5 and Dkk3 secreted modulators opposed to the antagonism of Dkk1 that reproducibly establishes a correctly sized ANE territory in the early sea urchin embryo.

FoxQ2 transcriptional activity is necessary for the specification of several cell types within the ANE territory in the sea urchin embryo, including the apical tuft cells and serotonergic neurons (Yaguchi et al., 2012; Yaguchi et al., 2008; Yaguchi et al., 2010). The formation of serotonergic neurons begins during the early pluteus larval stages on the dorsal side of the embryo in a territory around the interface of the inner anterior discoidal domain marked by *foxq2* expression and the outer ANE domain marked by the ring of *six3* expression (Fig. S3). In the absence of either sFRP1/5 or Dkk3, both the inner and outer ANE domains expand towards the posterior pole (see Fig. 4),

resulting in a larger territory around the border between the two domains on the dorsal side of the embryo. Thus, the increased size of the *foxq2/six3* dorsal border territory correlates with the greater number of serotonergic neurons that can be specified. The simplest interpretation of these observations is that the pattern of the ANE is proportionately expanded in sFRP1/5 and Dkk3 morphants, including the area within which serotonergic neurons can form, thus increasing the number of serotonergic neurons there. Alternatively, it is possible that a function of sFRP1/5 and Dkk3 is to antagonize the specification of serotonergic neurons at the *foxq2/six3* interface.

Our functional studies demonstrate that the sea urchin ANE is expanded in sFRP1/5 morphants and embryos overexpressing high levels of sFRP1/5 while ANE gene expression is down regulated in embryos overexpressing low levels of sFRP1/5. These data suggest a biphasic function for sFRP1/5 similar to a model proposed by Uren et al., (2000) based on cell culture studies, which showed that low levels of sFRP1 promoted nuclearization of β-catenin while high levels blocked nuclearization. We propose the higher concentration of sFRP1/5 protein around the anterior pole works with increasing levels of Dkk1 protein to antagonize Fzl5/8-JNK signal mediated down regulation of ANE genes in this anterior-most regulatory domain. Then, as sFRP1/5 protein diffuses in the extracellular space, lower concentrations of sFRP1/5 proteins further from the anterior pole potentiate Fzl5/8-JNK signaling. The exact mechanism by which sFRP1/5 either promotes or antagonizes Fzl5/8-JNK signaling is unclear. It is possible that sFRP1/5 physically interacts with the Fzl5/8 receptor, Wnt ligands and/or other uncharacterized extracellular proteins to directly promote and/or antagonize Fzl5/8 signaling (see Bovolenta et al., 2008). Alternatively, sFRP1/5 could act indirectly by

promoting the diffusion of Wnt1 and Wnt8 ligands by interfering with their interactions with extracellular matrix proteins and Fzl5/8 receptor (see Mii and Taira, 2009, 2011).

We also demonstrate that the ANE territory also expands in the absence of Dkk3, suggesting that Dkk3 and sFRP1/5 diffusing from the anterior signaling center work additively to promote Fzl5/8-JNK signaling. However, some available data suggest Dkk3 may not function in the Wnt signaling pathway. For example, cell culture studies provide evidence that Dkk3 secreted into the extracellular space does not bind Lrp6 or extracellular Kremen (Mao et al., 2002; Nakamura and Hackam, 2010), two extracellular co-receptors that are necessary for both canonical and/or non-canonical Wnt signaling, depending on the context. In contrast, a recent study has shown that Dkk3 can bind to Kremen in intracellular compartments where it can potentiate Wnt3a signaling (Nakamura and Hackam, 2010), representing a potential molecular mechanism by which Dkk3 may stimulate Wnt signaling during the ANE restriction process. Interestingly, blocking the function of the Fzl5/8 receptor or JNK has a more severe effect on ANE restriction than does knockdown of either Wnt1 and Wnt8 (Range et al., 2013) or sFRP1/5 and Dkk3 (this study). Taken together these data are consistent with a model in which sFRP1/5 and Dkk3 diffuse extracellularly in an anterior-to-posterior gradient and Wnt1 and Wnt8 in a posterior-to-anterior gradient and both gradients work in concert to restrict the ANE around the anterior pole.

For many years it was thought that anterior signaling centers were a vertebrate invention that led to the more complex territorial specification and morphogenetic processes of vertebrate forebrain/eye field development (Holland and Short, 2008; Pani et al., 2012). This study, as well as recent studies in invertebrate chordate Amphioxus and

ambulacrarian hemichordate embryos, challenges this idea (Onai et al., 2012; Pani et al., 2012). Similar to early sea urchin embryos, amphioxus and hemichordate embryos express *foxq2* in a territory within the broader *fzl5/8* and *six3* expression domains after these regulatory factors are restricted to a territory around the anterior pole by a Wnt/β-catenin-dependent positioning mechanism (Fig. 8B). In Amphioxus, the expression pattern of *sfrp1/5* has not been determined; however, another sFRP family member, *sfrp*2-like, as well as *dkk3* are expressed at the anterior pole in a pattern similar to that of *foxq2* (Kong et al., 2012), suggesting FoxQ2 might activate an anterior signaling center, as in sea urchin embryos. Interestingly, knocking down Dkk3 protein expression causes a loss of ANE specification in Amphioxus, while over expression causes an expansion of the ANE territory (Onai et al., 2012), presumably because Dkk3 antagonizes the Wnt/β-catenin-driven restriction mechanism. In Hemichordates no functional or expression data are available for Dkk3, but *sfrp1/5* and *foxq2* transcripts overlap in the anterior neuroectoderm towards the end of gastrulation (Pani et al, 2012). The function of sFrp1/5 has not been determined in hemichordates, but Pani et al (2012), showed that expression of another signaling factor, *hedgehog*, appears to overlap with that of *foxq2* in in *Saccoglossus kowalevskii* and is necessary for ANE pattering, suggesting that FoxQ2 may also activate an anterior signaling center in these embryos. Our limited understanding of the signaling landscape (ligands, co-receptors and intracellular modulators) makes it difficult to interpret how the modulators function within the regulatory network that specifies and patterns the ANE in any deuterostome embryo. Yet, collectively the available information strongly suggests that anterior signaling centers are a common feature of deuterostome ANE specification and patterning, suggesting that

studies in non-vertebrate deuterostomes can continue to help inform studies in vertebrate embryos.

The above comparisons among deuterostomes raise the question: When did anterior signaling centers arise in the evolution of metazoans? In animals outside of the deuterostome super phylum, FoxQ2 and Six3 appear to play important regulatory roles during the patterning of anterior/apical territories that develop into sensory organs (Posnien et al., 2011; Sinigaglia et al., 2013; Steinmetz et al., 2010). In cnidarian embryos, which form the sister group to bilaterians, *foxq2* and *six3* are expressed in the ectoderm around the aboral pole of the embryo, opposite to high levels of Wnt/β-catenin signaling that specifiy endoderm within the oral territory and pattern the embryo along its primary, oral-aboral axis (Marlow et al., 2013; Momose and Houliston, 2007; Rottinger et al., 2012; Wikramanayake et al., 2003). Similar to its role in several deuterostome embryos Six3 antagonizes Wnt/β-catenin signaling, and both Six3 and FoxQ2 are necessary for specification and patterning of the apical organ, which is thought to have a sensory function (Rentzsch et al., 2008; Sinigaglia et al., 2013). While the functions of sFRPs and Dkk3 remain unclear in cnidarian embryos, it is interesting that *foxq2* activates a signaling factor, FGFA, which is necessary for patterning the territory that forms the apical organ around the aboral pole (Sinigaglia et al., 2013). In addition to these similarities in cnidarians, a recent study on the bilaterian lophotrochozoan annelid *Platynereis dumerillii* showed that a remarkably conserved set of ANE/apical sensory organ transcription factors and signaling modulators, including *fzl5/8, six3, foxq2,* and *sfrp1/5,* is expressed in the head. Similar to sea urchins, the expression domains of *foxq2* and *sfrp1/5* overlap and are nested within the larger domains of *fzl5/8* and *six3*. Also,

ectopic over activation of the posteriorly localized Wnt/β-catenin pathway severely down regulated the anterior apical sensory organ in these embryos (Marlow et al., 2014), suggesting that control of Wnt signaling could be important for establishing the ANE in these embryos. Taken together, these studies make it tempting to speculate that signaling centers activating around the opposite pole from high Wnt/β-catenin signaling along the primary axis may be an ancient developmental mechanism essential for eumetazoan (cnidarians and bilaterians) body axis specification and patterning.

ACKNOWLEDGMENTS

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MATERIALS AND METHODS

Animals, embryos, and treatments

Strongylocentrotus purpuratus sea urchins were obtained from Point Loma Marine Invertebrate Lab (Lakeside CA), The Cultured Abalone (Goleta, CA), or Marinus Scientific (Garden Grove, CA). Embryos were cultured in artificial seawater at 15^0 C.

Microarray

S. purpuratus genome sequence information was used to prepare the microarray (Wei et al., 2006). mRNA from control and FoxQ2 morphants was isolated and used to synthesize cDNAs that were labeled, hybridized and scanned by Roche Nimblegen microarray services. Data analysis was described previously in Wei et al, 2006.

Preparation of cDNA clones

cDNA from 12-to-24 hpf blastula stage embryos was used to obtain full-length clones for *sfrp1/5* and *dkk3*. The following primers were based on the sea urchin genome sequence:

Sp-dkk3	Forward 5'-AGA**ATG**GCGGCTCCTTCTGC-3'
	Reverse 5'-TCATAATACAGTTAACTGGC-3'
Sp-sfrp1/5	Forward 5'-AGA**ATG**GCTGCCTTCAGTGGAAC-3'
	Reverse 5'-TCACACCTGTACATTTGGTA-3'

mRNA and morpholino injections

Mis-expression studies were performed with full-length *sfrp1/5* and *dkk3 cDNA* sequences inserted into pCS2+ vector. pCS2 constructs were linearized with Not1 and mRNA was synthesized with mMessage Machine kit (Ambion), purified by LiCl precipitation and mRNA was injected at the following concentrations: sFrp1/5 mRNA= 0.1 - 1.5µg/µL and Dkk3 mRNA = 0.75 – 1.0 µg/µL.

S. purpuratus EST sequences for *sfrp1/5 and dkk3* were used to generate translation-blocking morpholino oligonucleotides obtained from Gene-Tools LLC (Eugene, OR) . The sequences and injection concentrations were as follows:

sFrp1/5 MO1: 5'- ACACCACCAACACTCGCTCAATCAT -3' (1.0 mM)

sFrp1/5 MO2: 5'-AAAGCACAGTAATTGATTCCTCACC -3' (1.2 mM)

Dkk3 MO1: 5'-AGGGAGATGCTTACCTAGTGTTCTT-3' (1.0 mM)

Dkk3 MO2: 5'-CGGTGTCCATAATCCGAACCATCTC-3' (1.5 mM)

Zygotes were injected immediately after fertilization with solutions containing FITC, 20% glycerol and mRNA and/or morpholino oligonucleotides. All injected embryos were cultured at 15^0 C. Microinjection experiments were performed using at least three different batches of embryos, and each experiment consisted of 50-250 embryos. Only representative phenotypes present in at least 80% of the injected embryos are presented unless otherwise stated.

Quantitative PCR

Quantitative PCR (qPCR) was performed as previously described (Wei et al., 2009). Embryos from at least three different mating pairs were used for each experiment and

each PCR reaction was carried out in triplicate. The primer set information for ANE GRN genes, including *dkk3* and *sfrp1/5,* is given in Range et al., 2013.

Whole-mount in situ hybridization

Full-length cDNA sequence was used to generate antisense probes for each gene analyzed. Alkaline phosphatase and two-color fluorescent in situ hybridization were carried out as previously described (Sethi, 2012; Wei et al., 2009). For the two-color in situ hybridization, *foxq2* was labeled with fluorescein and detected with Cy5-TSA and *sfrp1/5* was labeled with DIG and detected with fluorescein-TSA in figure in figure S1A. In figure S1B, *sfrp1/5* was labeled with DIG and detected with fluorescein-TSA and *dkk3* was labeled with fluorescein and detected with Cy5-TSA.

Immunohistochemistry

Embryos were fixed in 2-4% paraformaldehyde in artificial seawater at RT for 20 minutes and washed 5 times in phosphate-buffered saline containing 0.1% Tween-20. Embryos were incubated with primary antibodies at 4^0 C overnight at a dilution of 1:1000 for serotonin (Catalog number S5545; Sigma, St. Louis, MO) and synaptotagminB/1e11 (Nakajima et al., 2004) in PBST and 4% normal goat serum. Primary antibodies were detected by incubating embryos with Alexa-coupled secondary antibodies for 1 hour at RT at a dilution of 1:2000. Nuclei were stained with DAPI.

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Figures

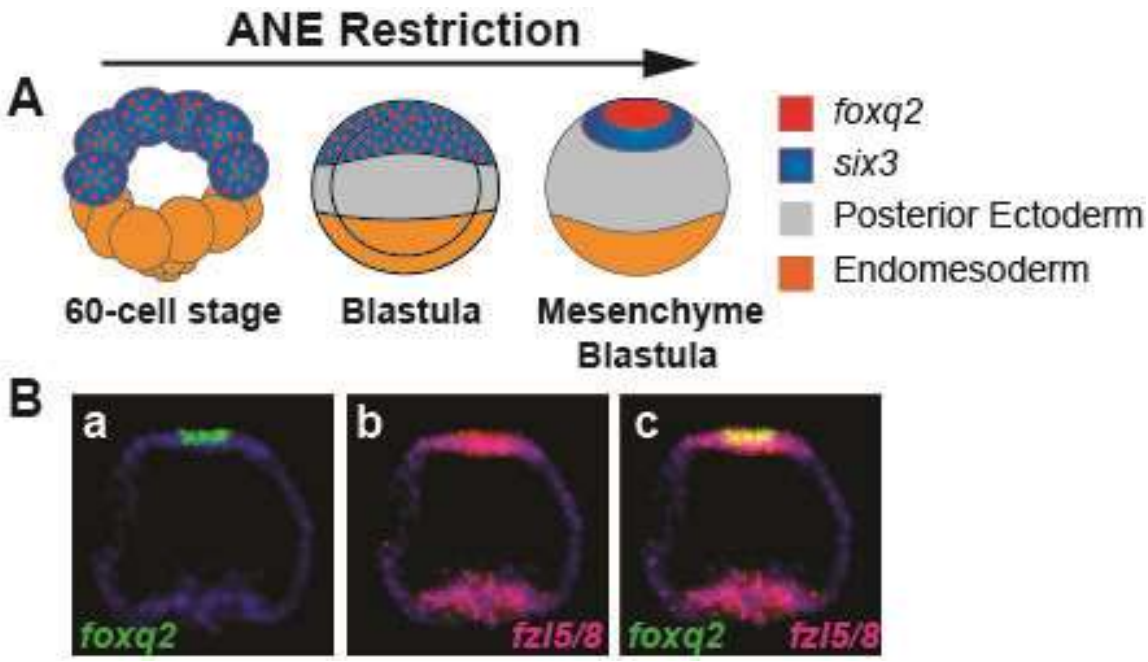


Figure 1. The ANE territory is segregated into two concentric domains. (A) Schematic model showing the progressive positioning of the cardinal ANE regulators *foxq2* and *six3* around the anterior pole of early *S. purpuratus* embryos from the 60-cell to the mesenchyme blastula stage (data taken from Li et al., 2014; Wei et al., 2009; Yaguchi et al., 2008a). (B) *foxq2* expression is nested within the *fzl5/8* expression domain and surrounded by *six3* expression. (a-c) Two-color in situ hybridization for *foxq2* (green) and *fzl5/8* (red) transcript expression at the mesenchyme blastula stage. *foxq2* and *flz5/8* mRNAs appear yellow in overlapping domains.

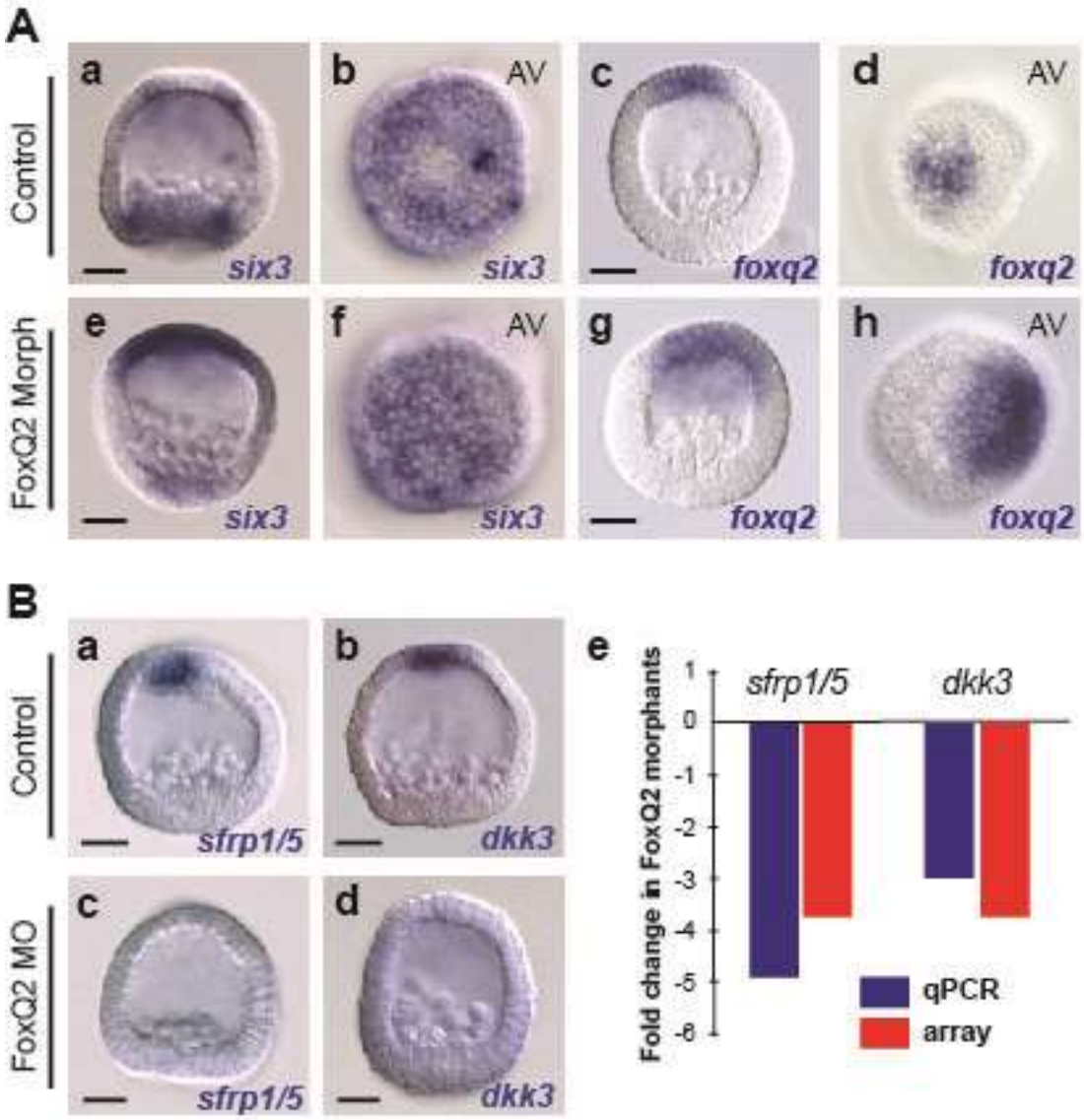


Figure 2. FoxQ2 defines the central ANE territory where it activates sFRP1/5 and Dkk3. (A) (a,b) *six3* is expressed in a ring of ANE cells surrounding the central anterior pole of the embryo. (c,d) *foxq2* expression around the anterior pole of the embryo. AV = anterior view. **(e,f)** Embryos injected with a FoxQ2 morpholino express *six3* throughout

the ANE territory (compare a,b with e,f). *six3* and *foxq2* expression is expanded towards the vegetal/posterior pole in FoxQ2 morphants. MO, morpholino; AV, anterior view. **(B) (a-d)** Injecting a FoxQ2 morpholino into embryos completely eliminates *sfrp1/5* and *dkk3* expression. (e) qPCR analysis of *sfrp1/5* and *dkk3* transcripts in FoxQ2 morphants. Scale bar = 20 μm.

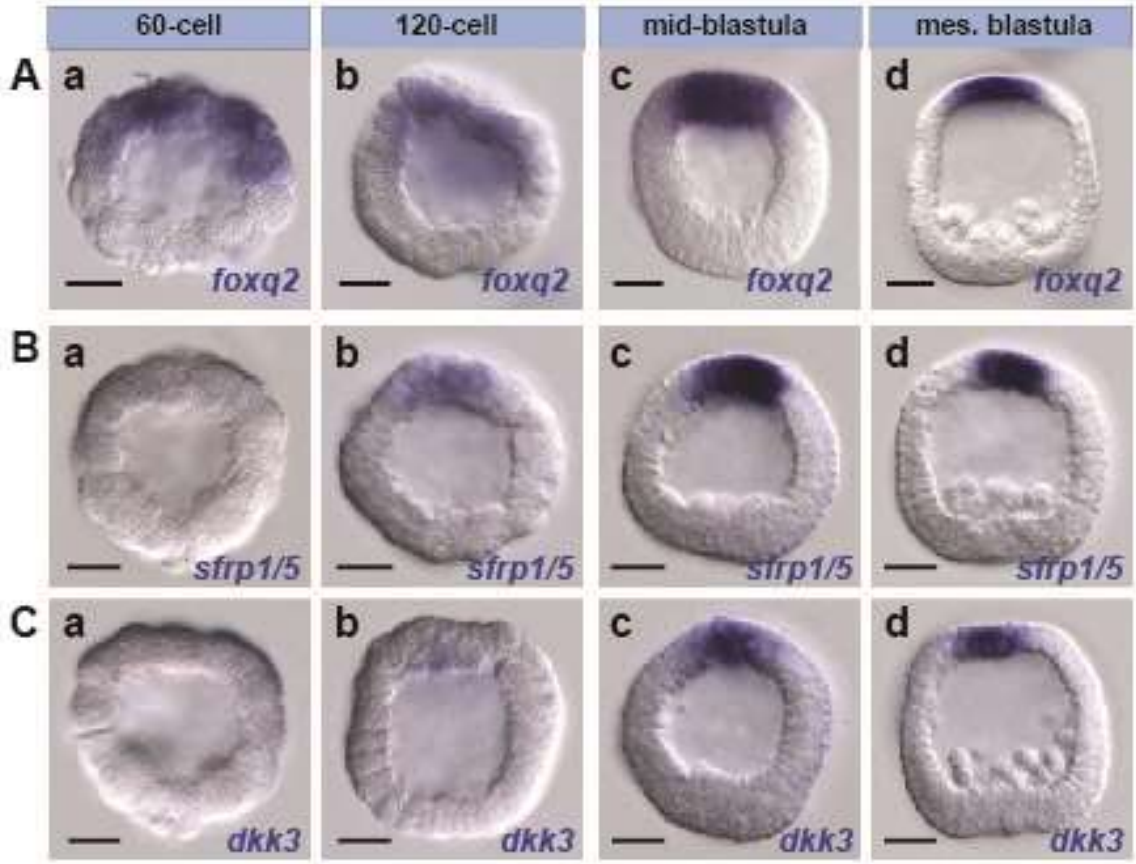
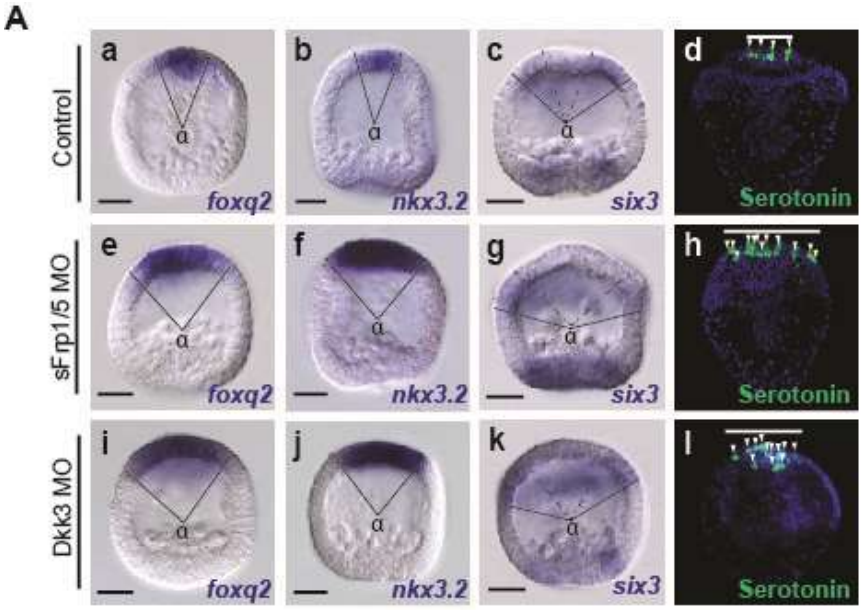


Figure 3. The expression of *sfrp1/5, dkk3,* and *foxq2* transcripts overlaps during ANE restriction. (Aa-d) *foxq2* expression is first detected at the 32- to 60-cell stage in anterior/animal blastomeres (the image in Aa is of a 60-cell embryo). (Ba-d) *sfrp1/5* expression is first detected at the 120-cell stage within the anterior/animal half of the embryo. (Ca-d) *dkk3* expression is first detected 15-16 hpf blastula embryos within the anterior/animal half of the embryo. Scale bar = 20 μm.



B

Treatment	Size of ANE discoidal territories		
	foxq2 expression	*nkx3.2* expression	*six3* expression
Control	5.49 +/- 0.45 (n = 21)	5.61 +/- 0.48 (n = 26)	37.0 +/- 1.10 (n = 20)
sFrp1/5 MO	19.70 +/- 1.90 (n = 20)	22.40 +/- 1.81 (n = 21)	46.0 +/- 0.71 (n = 23)
Dkk3 MO	20.64 +/- 1.43 (n = 23)	16.20 +/- 1.30 (n = 22)	47.3 +/- 0.35 (n = 22)
	(% of total embryo volume measured from posterior boundaries of ANE marker expression)		

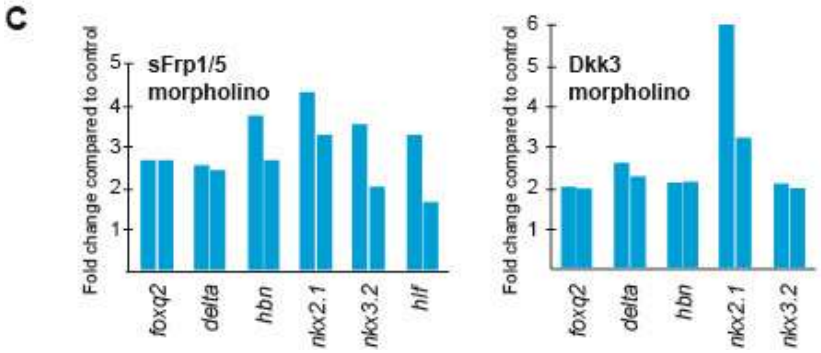


Figure 4. sFRP1/5 and Dkk3 size the sea urchin ANE. (A) *foxq2*, *nkx3.2*, and *six3* expression at mesenchyme blastula stages in control embryos (a-c), *sfrp1/5* morpholino knockdown embryos (e-g), and Dkk3 morpholino knockdown embryos (i-k). **(Ad, h, i)**

Serotonergic neurons (green) in control and sFrp1/5 and Dkk3 morpholino knockdown 80 hpf pluteus larvae. The white line indicates the territory occupied by the serotonergic neurons. **(B)** The angle, α, seen in A was measured for the most posterior boundaries of the selected ANE territory markers for ~2-dozen embryos from 3 batches using ImageJ. Solid white lines indicate the posterior boundaries of each ANE marker used to measure the angle α. The dotted lines show the most anterior boundary of *six3* expression. The angle α and the equation volume = 0.5(1-cos α /2) was used to calculate the percentage of the surface area (+/- SEM) occupied by the inner (*foxq2*, *nkx3*.2) and outer (*six3*) ANE territories in control, sFRP1/5 morphants and Dkk3 morphants. **(C)** qPCR measurements showing that the expression of ANE regulatory genes is up regulated in sFrp1/5 and Dkk3 morphants. The Y-axis shows the fold change in gene expression level in sFrp1/5 and Dkk3 morphants. The bars represent data using two different morpholinos. Scale bar = 20 μm.

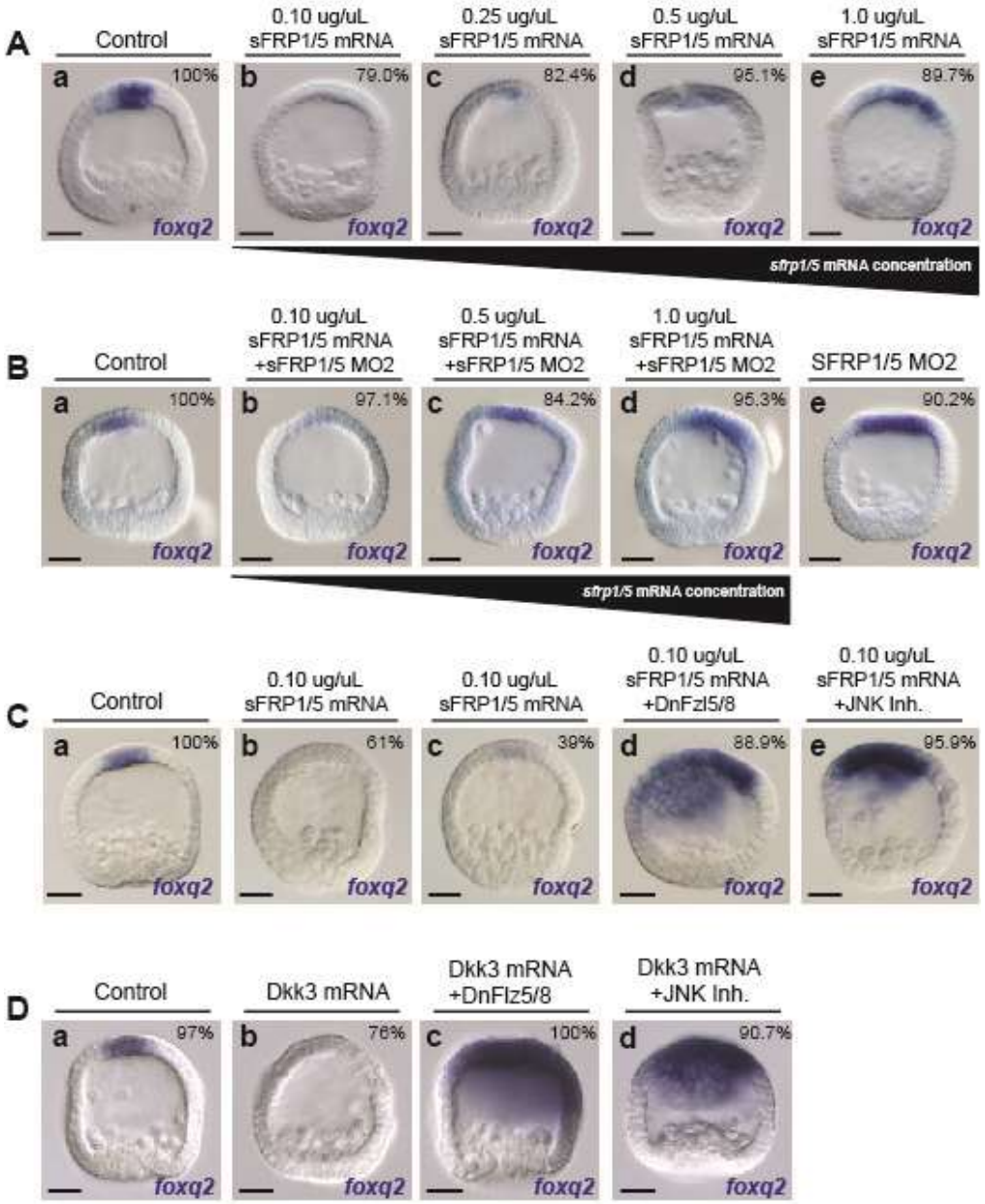


Figure 5. The roles of sFRP1/5 and Dkk3 in Fzl5/8-JNK mediated ANE positioning. The percentage of the representative phenotypes depicted is included in each figure frame. (**A**) The expression of *foxq2* expression is down regulated in embryos injected

with lower concentrations of *sfrp1/5* mRNA (0.10 μg/μL, n = 81; 0.25 μg/μL, n = 86)**(b, c)** and expands in embryos injected with higher concentrations of sFRP1/5 mRNA (0.50 μg/μL, n = 77; 0.25 μg/μL, n = 75) **(d, e)**. **(B)** Low levels of sFRP1/5 expression rescue embryos injected with sFRP1/5 MO2 but increasing concentrations do not. sFRP1/5 MO2 does not bind to exogenous *sfrp1/5* mRNA. (**C**) *foxq2* expression is completely eliminated in embryos injected with relatively low levels of sFRP1/5 mRNA (**b**). The sFRP1/5- mediated inhibition of *foxq2* expression requires functional Fzl5/8 (**c**); the sFRP1/5- mediated inhibition of *foxq2* expression requires functional JNK activity (**d**). (**D**) Dkk3 overexpression down regulates *foxq2* expression (**b**). The Dkk3- mediated inhibition of *foxq2* expression requires functional Fzl5/8 (**c**); the Dkk3-mediated inhibition of *foxq2* expression requires functional JNK activity (**d**). MO, morpholino. Scale bar = 20 μm

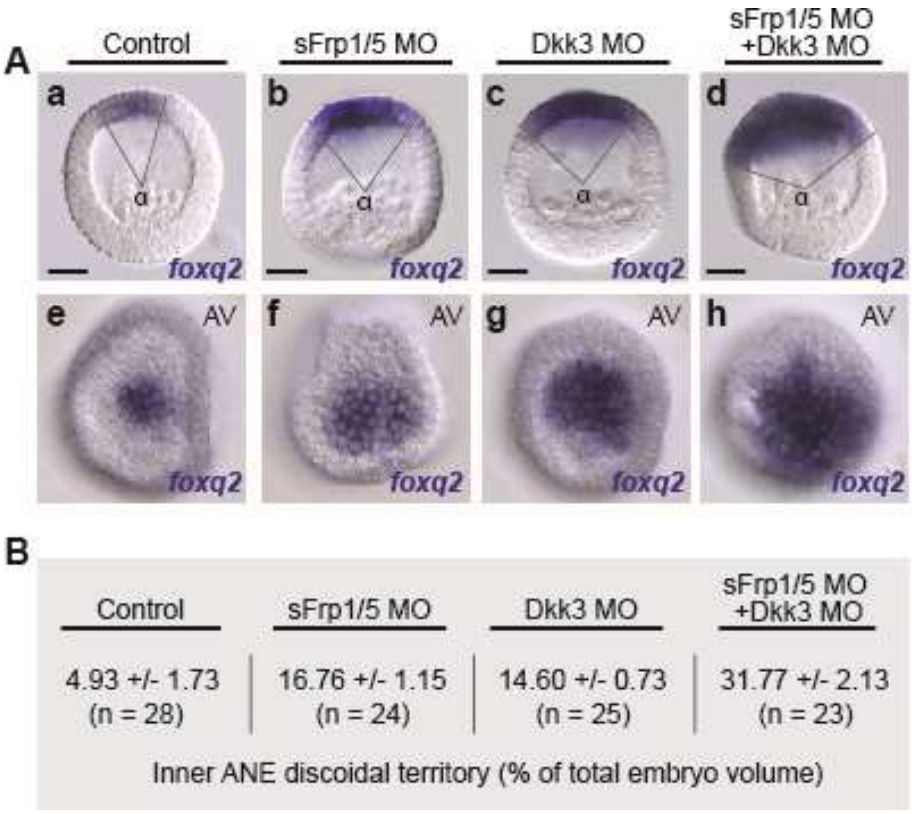


Figure 6. ANE restriction depends on additive inputs from sFrp1/5 and Dkk3.

(A) *foxq2* mRNA expression marks the inner ANE territory in control(a,e), sFRP1/5 morphants (b, f), Dkk3 morphants (c, g), and FRP1/5-Dkk3 double morphants (d, h). (a - d) Representative images showing the distribution of *foxq2* mRNA expression in inner ANE of control and knockdown embryos. Scale bar = 20 μm. **(B)** The angle, α, seen in Aa-d was measured for ~2-dozen embryos from 3 batches using ImageJ. The angle α and the equation volume = 0.5(1-cos α /2) was used to calculate the percentage of the surface area (+/- SEM) occupied by the inner ANE territory in control, sFRP1/5 morphants, Dkk3 morphants, and sFRP1/5+Dkk3 double morphants. .

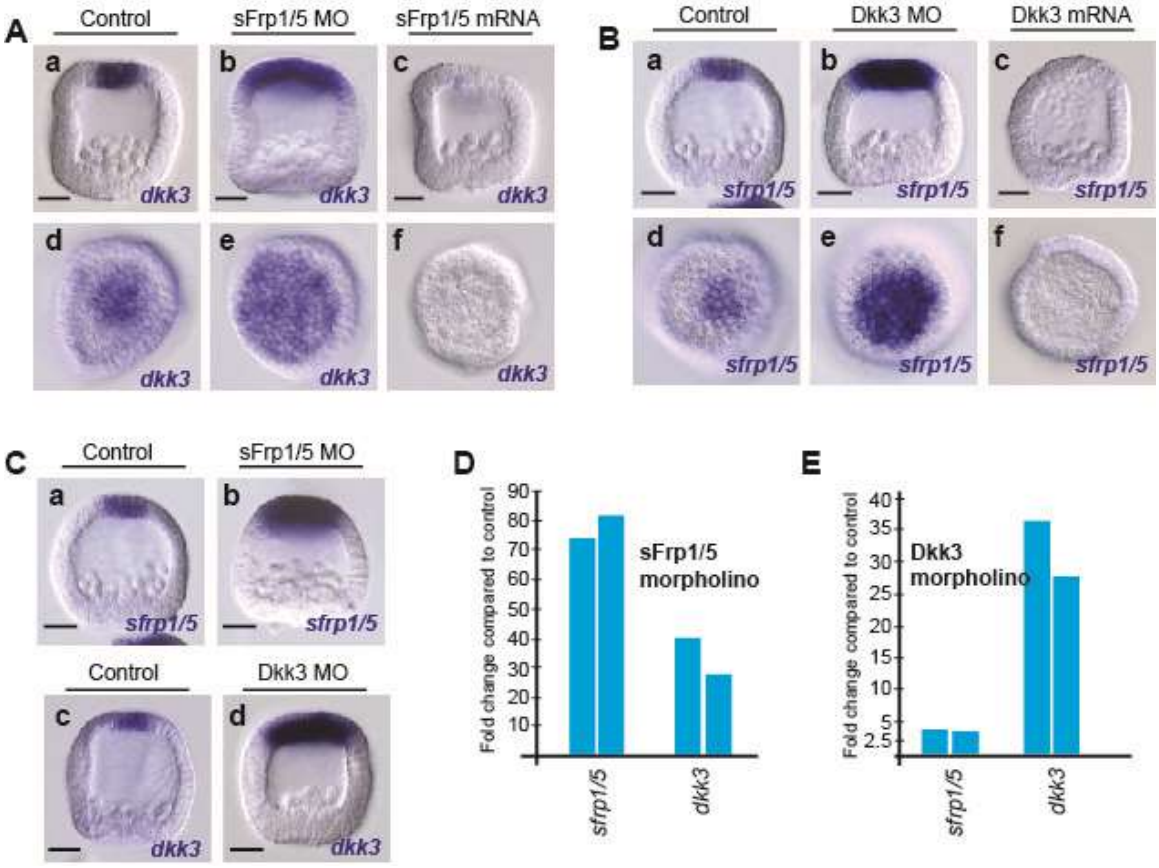


Figure 7. Auto-repressive and cross-repressive regulatory interactions between sFRP1/5 and Dkk3 refine the size of the ANE. (A, D) ANE expression of *dkk3* is expanded towards the posterior pole (Ab,e) and up regulated (D) in sFRP1/5 morpholino-injected embryos. *dkk3* expression is severely down regulated in embryos injected with *sfrp1/5* mRNA (Ac, f). **(B, D)** Embryos injected with Dkk3 morpholinos fail to completely restrict the *sfrp1/5* expression domain to the anterior pole (Bb, e) and the level of *sfrp1/5* is strongly up regulated (D). Expression of *sfrp1/5* is eliminated in embryos injected with *dkk3* mRNA (Bc, f). **(C, D)** Anterior *sfrp1/5* expression domain is

expanded in sFrp1/5 morphants (Ca, b) and strongly up regulated (D). Similarly, Dkk3 morpholino-injected embryos show expanded *dkk3* expression (Cc, d) and severe up regulation of *dkk3* transcripts (D). **(D)** qPCR analysis showing that *sfrp1/5* and *dkk3* expression is severely up regulated in sFRP1/5 morpholino injected embryos. (E) qPCR analysis showing that *sfrp1/5* and *dkk3* expression is severely up regulated in Dkk3 morpholino injected embryos. Scale bar = 20 μm.

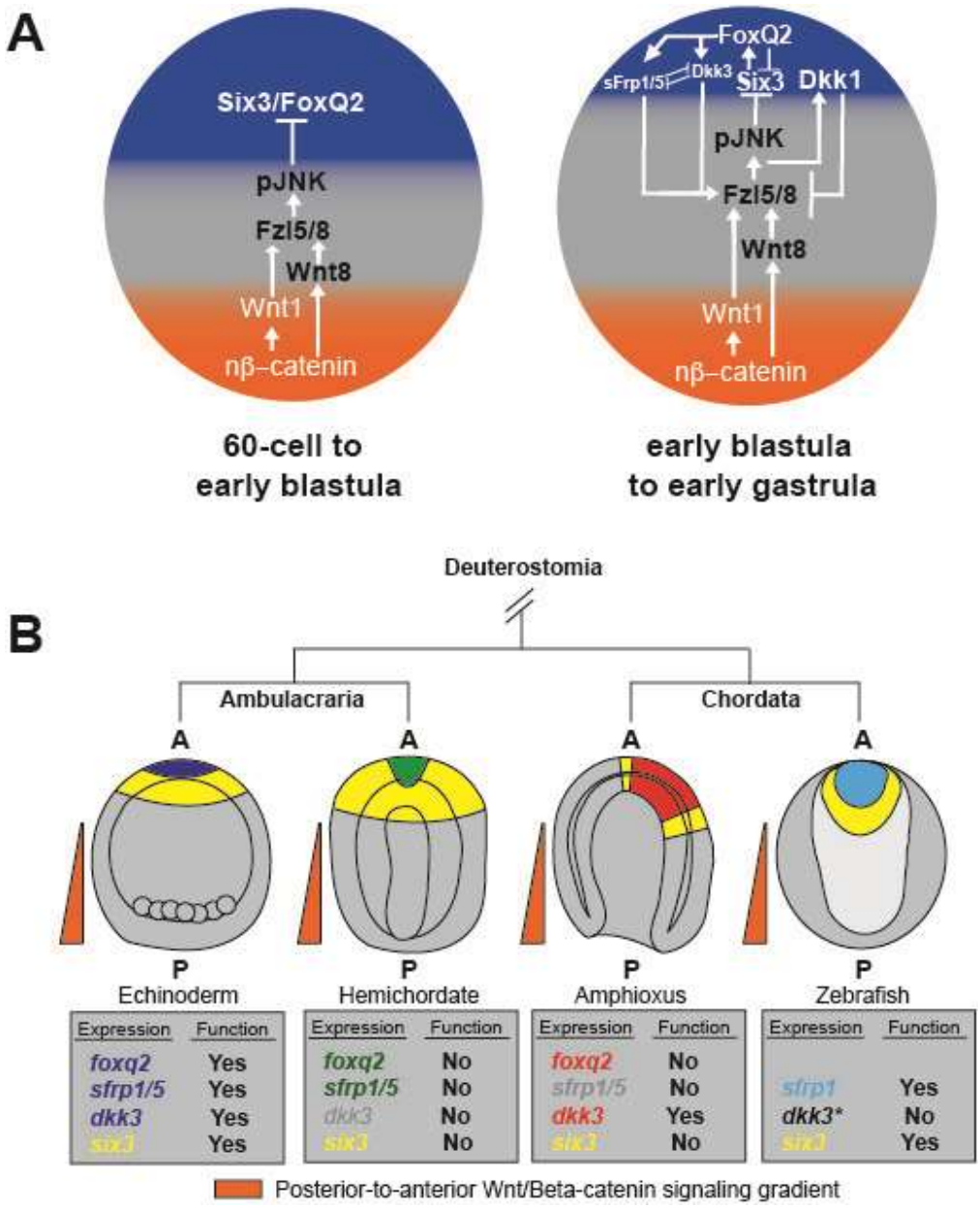


Figure 8. An extended model for ANE restriction in the sea urchin embryo and the conservation of anterior signaling centers among deuterostome embryos. (A) In sea urchin early development, the Wnt/β-catenin, Wnt/JNK, and Fzl1/2/7-PKC pathways all

converge on the same developmental process: ANE restriction. This diagram illustrates an extended model for sea urchin ANE restriction that integrates the FoxQ2, sFRP1/5 and Dkk3 signaling center. The model is detailed in the text. **(B)** The territorial expression of *foxq2*, *six3*, *sfrp1/5*, and *dkk3* orthologues in anterior neuroectoderm territories during the gastrula stages of several deuterostome model species. The diagrams of the embryos are colored to indicate the location of *six3* and *foxq2* in relation to orthologues of sea urchin *sfrp1/5* and *dkk3*. The colors match the regulatory factors in the boxes underneath each diagram; no expression data are available for gene names in grey. The boxes also indicate whether expression or functional studies have been performed on each regulatory factor in each deuterostome model species. Data taken from (Darras et al., 2011; Fritzenwanker et al., 2014; Hsu et al., 2010; Kozmik et al., 2007; Onai et al., 2012; Pani et al., 2012; Range, 2014; Seo et al., 1998; Tendeng and Houart, 2006; Yu et al., 2003).

* Expression studies on *dkk3* have been performed in zebrafish, but it is not clear from the available data where *dkk3* is expressed at the late gastrula stage. At the early shield stage (6 hpf) *dkk3* appears to be expressed broadly throughout the embryo, and expression appears to be restricted to the anterior neuroectoderm region by 24 hpf (~26-somite stage)(Hsu et al., 2010).

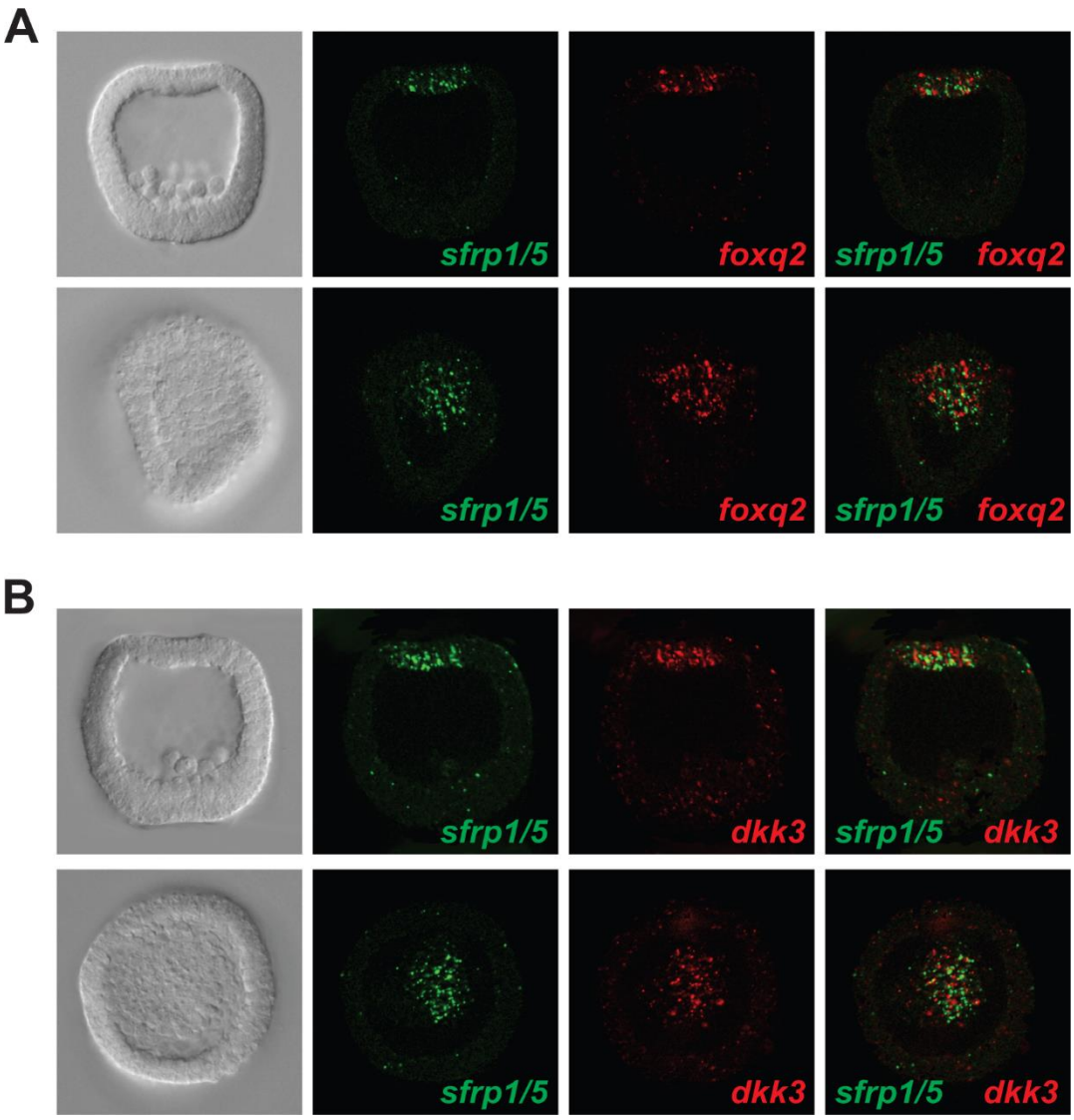


Figure S1. Congruent expression of *foxq2*, *sfrp1/5* and *dkk3*. (A) Two-color in situ hybridization assay for *sfrp1/5* (green) and *foxq2* (red) mRNA transcripts in mesenchyme blastula staged embryos (24 hpf). **(B)** Two-color in situ hybridization assay for *sfrp1/5* (green) and *dkk3* (red) mRNA transcripts in mesenchyme blastula staged embryos.

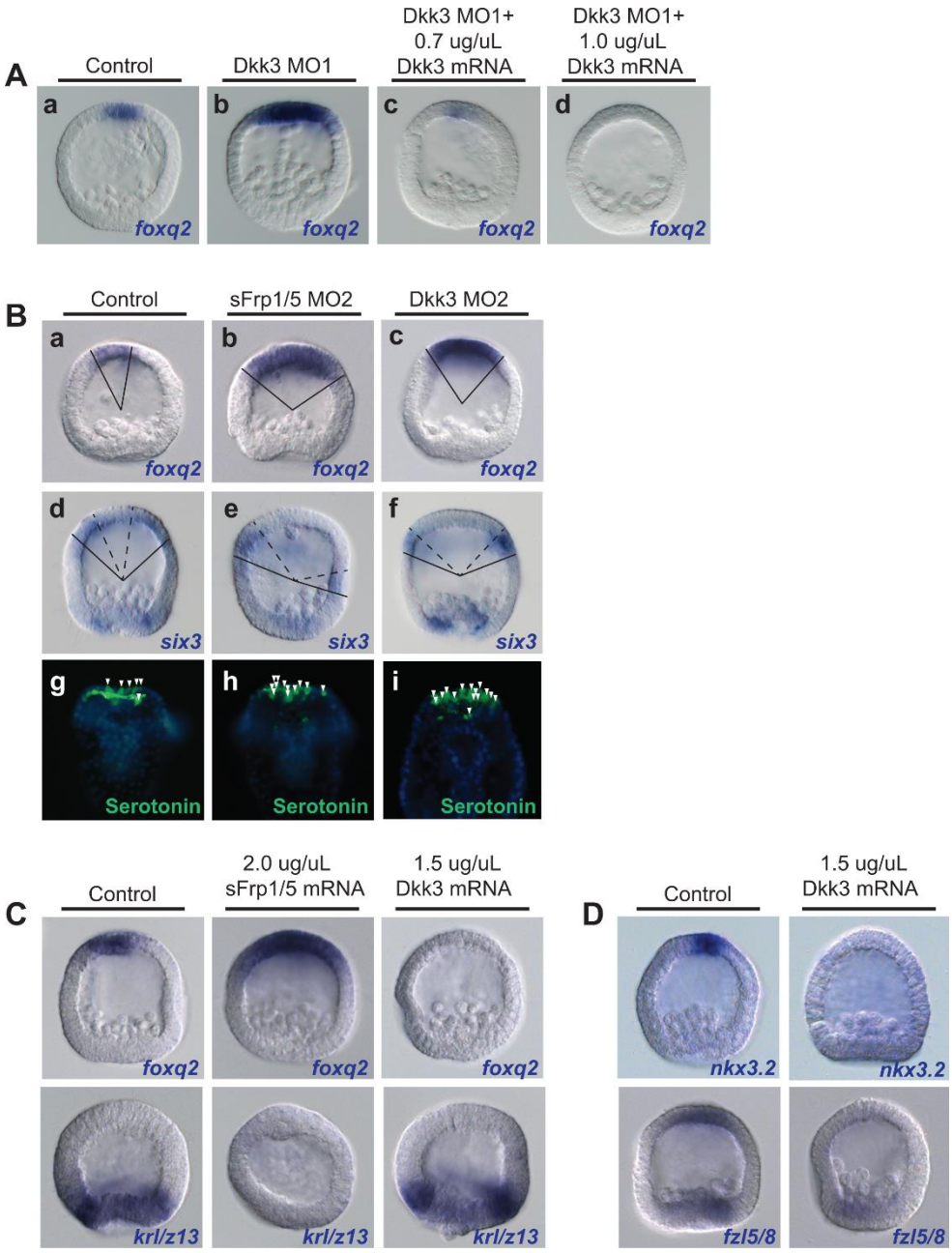


Figure S2. Additional morpholino and overexpression phenotypes. (A) Dkk3 overexpression rescues *foxq2* expression in embryos injected with Dkk3 MO1, which cannot bind to *dkk3* mRNA. **(B)** The ANE territory is expanded in embryos injected with sFrp1/5 Morpholino 2 and Dkk3 Morpholino 2. **(Ba-f)**The solid lines emanating from the center of the embryo mark the most posterior expression of *foxq2* and *six3*. The dotted lines originating from the center of the embryo indicate the anterior-most expression of the *six3* ring. **(Bg-i)** Serotonergic neurons (green) in control. sFrp1/5 and Dkk3 morpholino 2 knockdown 96 hpf pluteus larvae. Control = 5.95, n = 21; sFRP1/5 MO2 = 10.5 serotonergic neurons, n = 23; Dkk3 MO2 = 11.52 serotonergic neurons, n = 23. (**C**) *foxq2* is expanded (a, b) and the endomesoderm marker *krl/z13* is down regulated **(d,e)** in in embryos injected with sFRP1/5 mRNA. *foxq2* is down regulated **(a, c)** and the endomesoderm marker *krl/z13* is unaffected **(d, f)** in embryos injected with Dkk3 mRNA**. (D)** The ANE regulatory network genes, *nkx3.2* and *fzl5/8*, are down regulated in embryos injected with Dkk3 mRNA. MO; morpholino.

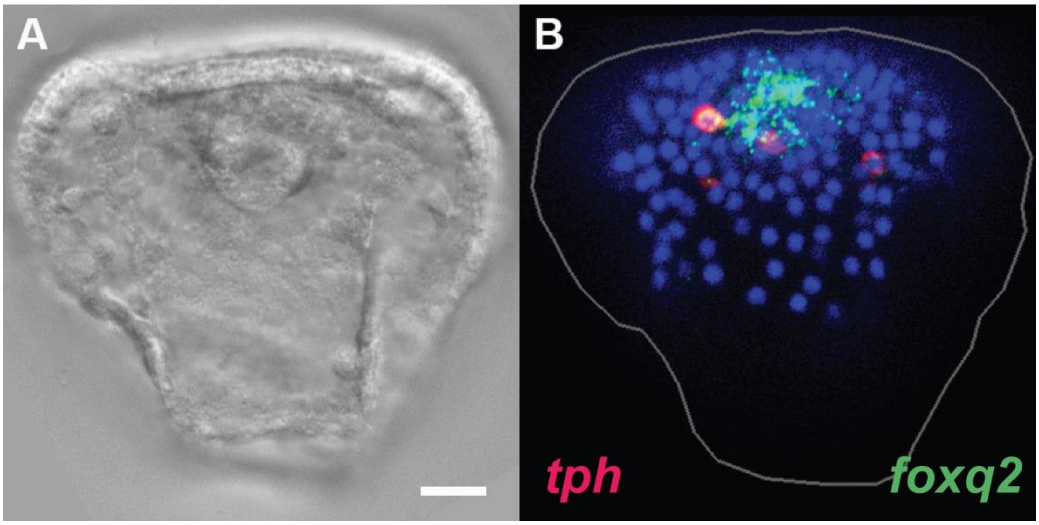


Figure S3. Serotonergic neural cells appear at the dorsal edge of FoxQ2-expressing cells in the ANE. A) DIC image of a gastrula stage (45hpf) embryo. Anterior view, dorsal is to the bottom. B) Whole mount fluorescence in situ hybridization for a serotonergic neural cell marker tryptophan hydroxylase (Tph) (red) and FoxQ2 (green) in the same embryo. Nuclei were labeled with DAPI (blue). Shown is a stack of optical sections through the animal pole. The white bar in A represents 20 μm .