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

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ABSTRACT
Anterior signaling centers help specify and pattern the early anterior neuroectoderm (ANE) in many deuterostomes. In sea urchin the ANE is restricted to the anterior of the late blastula stage embryo, where it forms a simple neural territory comprising 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 that express 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. sFRP1/5 and Dkk3 levels are rigidly maintained via autorepressive 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 molecular mechanism that positions and defines ANE borders. These data strongly support the idea that the sea urchin embryonic anterior neuroectoderm uses an ancient anterior patterning system that was present in the common ambulacrarian/chordate ancestor.

KEY WORDS: Neuroectoderm patterning, Wnt signal transduction, Anterior-posterior, Deuterostome evolution, Gene regulatory networks, Dkk3, sFRP1/5, Strongylocentrotus purpuratus

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. Although 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 that 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 might also 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 downregulates ANE factors from the more posterior ectoderm so that their activities are restricted to a region around the anterior pole (Darras et al., 2011; Kiecker and Niehrs, 2001; Nordström 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, 2011, 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 that 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., 2008). 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 subdomain (Yaguchi et al., 2012, 2010). Remarkably, the ANE positioning mechanism involves much more than the Wnt/β-catenin signaling pathway in the sea urchin. It depends on the ability to integrate information from at least three different Wnt signaling branches, namely Wnt/β-catenin, Wnt/JNK and Wnt/PKC (see Fig. 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 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 anteriormost region of the neural plate, termed the anterior neural ridge (ANR) (Cavodeassi and Houart, 2012; Echevarría 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 subregions within the ANE (Cavodeassi and Houart, 2012). sFRPs comprise 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 the ability of Wnt 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 independently 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 subclasses, 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; Semënov et al., 2001). By contrast, 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 anteriormost ANE territory, 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 ANE genes known to be expressed in the sea urchin embryo are six3 and foxq2 (Wei et al., 2009; Yaguchi et al., 2008). Their expression domains 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 downregulates six3 and foxq2 transcripts in the more posterior ectoderm cells, expression of both transcripts remains very similar within the anterior ectoderm through the blastula stages [as late as 18-20 hours post fertilization (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; Fig. 2) (Li et al., 2014; Wei et al., 2009; Yaguchi et al., 2008).

In a previous study, we showed that Fz5/8 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 downregulation by Fz5/8-JNK signaling, establishing the ANE territory. As a result of this negative-feedback loop fzl5/8 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-label in situ hybridization with fzl5/8 and foxq2 probes revealed that foxq2 is restricted to a central territory around the anterior pole, nested within the fzl5/8 expression domain (Fig. 1Ba-c), 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 downregulated 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,b), which appears to extend to the outer edges of the ANE territory. Interestingly, foxq2 is expressed in the anteriormost territory where six3 expression was downregulated (Fig. 2Aa-d). These observations indicate that, although six3 and foxq2 are both initially restricted to the anterior pole by the Wnt-mediated anteroposterior positioning mechanism, they subsequently respond differentially 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, 2011, 2010), we reasoned that FoxQ2 might play a crucial role in establishing the inner and outer ANE domains. To test this hypothesis, we monitored the expression of several ANE factors by knockdown and measured the expansion and/or upregulation of transcripts at the anterior pole of the embryo (Fig. S1A,B).

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. 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 (7-9 hpf) (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 expression, sfrp1/5 and dkk3 transcripts were progressively downregulated from posterior ectoderm to a region that approximates the central ANE domain, mimicking foxq2 expression. These data suggest that the sfrp1/5 and dkk3 expression domains overlap exactly with that of foxq2 at later stages. To test this hypothesis we performed double-label 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. 3).
(Dkk3 MO1 and MO2) showed posterior expansion of ANE markers (Fig. 4A,B, Fig. S2A,B). In contrast to FoxQ2 morphants, in which \( \textit{six}3 \) was expressed uniformly throughout the ANE territory, \( \textit{six}3 \) was expressed in a thin ring of ectoderm cells near the embryonic equator, presumably because it was downregulated by FoxQ2 throughout the expanded central territory (Fig. 2Ac, Fig. 4Ag,k). Moreover, at the mesenchyme blastula stage (24-26 hpf), the expression of several ANE regulatory factors that depend on \( \textit{Six}3 \) and/or FoxQ2 was upregulated in the absence of either secreted signaling modulator (Fig. 4C).

Specification of serotonergic neurons requires both \( \textit{Six}3 \) and FoxQ2 activity. By 72 hpf, control plutei normally develop three to five 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 morphant, 12.3 serotonergic neurons, \( n=32 \); Dkk3 morphant, 11.7 serotonergic neurons, \( n=27 \); Fig. 4Ah,l, Fig. 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 the 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 Fz\( l5/8 \)-JNK-mediated ANE positioning mechanism

FoxQ2 activates \( \textit{dkk}3 \) and \( \textit{sfrp}1/5 \) 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 the Fz\( l5/8 \)-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 downregulation of ANE gene expression by Fz\( l5/8 \)-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 nkn3.2 (Fig. 5Dh, Fig. 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,e, Fig. S2C). Furthermore, injection of relatively low levels of sfrp1/5 mRNA rescued the SFRP1/5 morpholino phenotype (three different batches of embryos; 84.2% rescue of SFRP1/5 MO2, n=67; Fig. 5Ba-c), whereas foxq2 was expanded in embryos co-injected with SFRP1/5 morpholinos and higher levels of sfrp1/5 mRNA (three 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 those observed when the Fzl-5/8-JNK pathway is upregulated in sea urchin embryos (Range et al., 2013), suggesting that they might potentiate signaling through this pathway. To test this, we determined whether Dkk3 and/or sFRP1/5 could downregulate foxq2 expression in the absence of a functional Fzl5/8-JNK signaling pathway. Overexpression of either Dkk3 or sFRP1/5 (at low concentrations) no longer downregulated foxq2 expression when the function of Fzl5/8 was blocked with a dominant-negative construct (ΔFzl5/8) [three different batches of embryos; 88.9% rescue of SFRP1/5 (n=57) and 100% rescue of Dkk3 (n=71) misexpression phenotypes; Fig. 5Cd,Dd]. Similarly, Dkk3 and SFRP1/5 overexpression did not suppress foxq2 expression in embryos treated with JNK inhibitor [three different batches of embryos; 95.9% rescue of SFRP1/5 (n=65) and 90.7% rescue of Dkk3 (n=61) misexpression phenotypes; Fig. 5Ce,Dd]. These data strongly suggest that Dkk3 and low levels of SFRP1/5 are required for Fzl5/8-JNK signaling to fully downregulate 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...
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 that their expression levels were also markedly upregulated (Fig. 7Ca-d). 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 that 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 the secreted signaling modulators sFRP1/5 and Dkk3 in the specification and patterning of subdomains of the 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 anteriormost discoidal domain. We show that Dkk3 and low levels of sFRP1/5 expression are both required and act additively to stimulate the Fz15/8-JNK signaling-mediated downregulation of ANE gene expression during ANE positioning. Interestingly, higher levels of sFRP1/5 expression appear to antagonize Fz15/8-JNK signaling. In addition, our data reveal that remarkable negative autoregulatory 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 the functions of sFRP1/5 and Dkk3.

Based on our findings 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- to mid-blastula stage, Wnt/β-catenin signaling activates posterior-to-anterior gradients of Wnt1 and Wnt8 that downregulate ANE regulatory factors in the posterior ectoderm via activation of the Fz15/8-JNK signaling pathway. Then, during the mid-blastula stages, Fz15/8 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 classical 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 Wnt1/8-Fz15/8-JNK-mediated downregulation of ANE factors at the outer limits of their diffusion gradient. Conversely, high concentrations of secreted sFRP1/5 around the anteriormost pole can antagonize Fz15/8-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 Fz15/8-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 serotoninergic neurons.
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, whereas ANE gene expression is downregulated in embryos overexpressing sFRP1/5 at low levels. 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 promote nuclear localization of β-catenin, whereas high levels block nuclear localization. We propose that 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 downregulation of ANE genes in this anteriormost regulatory domain. Then, as sFRP1/5 protein diffuses into 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 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 that Dkk3 might 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 canonical and/or non-canonical Wnt signaling, depending on the context. By 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),

(Yaguchi et al., 2012, 2008, 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.
Fig. 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 Fz1/2/7-PKC pathways all converge on the same developmental process: ANE restriction. Illustrated is an extended model for sea urchin ANE restriction that integrates the FoxQ2, sFRP1/5 and Dkk3 signaling center (see main text for details). nβ-catenin, nuclear β-catenin. (B) The territorial expression of foxq2, six3, sfrp1/5 and dkk3 orthologs in ANE territories during the gastrula stages of several deuterostome model species. The embryos are colored to indicate the location of six3 and foxq2 in relation to orthologs of sea urchin sfrp1/5 and dkk3; colors match the regulatory factors in the boxes beneath (no expression data are available for genes in gray). The boxes also indicate whether expression or functional studies have been performed on each regulatory factor in each deuterostome model species (data are from Darras et al., 2011; Fritztenwanker 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 ANE region by 24 hpf (~26-somite stage) (Hsu et al., 2010). A, anterior; P, posterior.

representing a potential molecular mechanism by which Dkk3 may stimulate Wnt signaling during the ANE restriction process. Interestingly, blocking the function of the Fz1/5/8 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 (Onai et al., 2012; Pani et al., 2012), challenge this idea. Similar to early sea urchin embryos, amphioxus and hemichordate embryos express foxq2 in a territory within the broader fz1/5/8 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, sfrp2-like, as well as dkk3 are expressed at the anterior pole in a pattern similar to that of foxq2 (Kong et al., 2012), suggesting that FoxQ2 might activate an anterior signaling center, as in sea urchin embryos. Interestingly, knocking down Dkk3 protein expression causes loss of ANE specification in amphioxus, whereas overexpression 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 ANE 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 Saccoglossus kowalevskii and is necessary for ANE patterning, suggesting that FoxQ2 might 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, advocating that studies in non-vertebrate deuterostomes can continue to help inform studies in vertebrate embryos.

The above comparisons among deuterostomes raise the question of when anterior signaling centers arose in the evolution of...
metazoans. In animals outside of the deuterostome superphylum, 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 cnidian 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 specify endoderm within the oral territory and pattern the embryo along its primary oral-aboral axis (Marlow et al., 2013; Momose and Houliston, 2007; Röttinger et al., 2012; Wikramanyake 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). Although the functions of sFRPs and Dkk3 remain unclear in cnidian embryos, it is interesting that foxq2 activates a signaling factor, FGFα, 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 bilaterarian lophotrochozoan annelid Platynereis dumerilii showed that a remarkably conserved set of ANE/apical sensory organ transcription factors and signaling modulators, including fz5/8, 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 fz5/8 and six3. Also, ectopic overactivation of the posteriorly localized Wnt/β-catenin pathway severely downregulated 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 that are active around the opposite pole to that of high Wnt/β-catenin signaling along the primary axis might be an ancient developmental mechanism essential for eumetazoan (cnidarians and bilaterians) body axis specification and patterning.

MATERIALS AND METHODS

Animals and embryos

Strongylocentrotus purpuratus sea urchins were obtained from Point Loma Marine Invertebrate Lab (Lakeside, CA, USA), The Cultured Abalone (Goleta, CA, USA) or Mariner Scientific (Garden Grove, CA, USA). Embryos were cultured in artificial seawater at 15°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 and scanned by Roche Nimblgen microarray services. Data analysis was described previously (Wei et al., 2006).

Preparation of cDNA clones

cDNA from 12-24 hpf blastula stage embryos was used to obtain full-length clones for sfrp1/5 and dkk3. The following primers (forward and reverse, 5′-3′; bold indicates the translational start codon) were based on the sea urchin genome sequence: Sp-dkk3, AGAATGGCGGCTCTCTGTC and TCAATAACAGTTAACTGGC; Sp-sfrp1/5, AGAATGGCGCCTCTGTC and TCAATAACAGTTAACTGGCC. In Fig. S1A, sfrp1/5 was labeled with fluorescein and detected with Cy5-TSA and dkk3 was labeled with fluorescein and detected with Cy5-TSA. In Supplementary information, sfrp1/5 is given in Range et al. (2013).

mRNA and morpholino injections

Misexpression studies were performed with full-length sfrp1/5 and dkk3 cDNA sequences inserted into the pcS2+ vector. pcS2 constructs were linearized with NotI and mRNA was synthesized with the mMessage mMachin Kit (Ambion), purified by LiCl precipitation and injected at the following concentrations: sfrp1/5 mRNA, 0.1-1.5 µg/µl; 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 (Gene Tools). Sequences (5′-3′) and injection concentrations were as follows: sFRP1/5 MO1, ACACACCACAAACCTGGCCTCATCAT, 1.0 mM; sFRP1/5 MO2, AAAGCAGATGAATGGATTCCCTACC, 1.2 mM; Dkk3 MO1, AGGGATGAGCTTACATGTGTTCAT, 1.0 mM; Dkk3 MO2, CCGTGCTCATAATCCGAACCATCTC, 1.5 mM.

Zygotes were injected immediately after fertilization with solutions containing fluorescein isothiocyanate (FITC), 20% glycerol and mRNA and/or morpholino oligonucleotides. Injected embryos were cultured at 15°C. Microinjection experiments were performed using at least three 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 (qPCR)

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. 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 et al., 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 Fig. S1A. In Fig. 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 room temperature for 20 min and washed five times in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Embryos were incubated at 4°C overnight with primary antibodies (1:1000) against serotonin (Sigma, S5545) and synaptotagmin B/1e11 (Nakajima et al., 2004) in PBST and 4% normal goat serum. Primary antibodies were detected by incubating embryos for 1 h at room temperature with 1:2000 Alexa Fluor-coupled secondary antibodies (Thermo Fisher Scientific). Nuclei were stained with DAPI.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.C.R. and Z.W. planned, performed and analyzed experiments. R.C.R. prepared the manuscript.

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Data availability

The microarray expression data have been deposited at Gene Expression Omnibus with accession number GSE860012.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/suppl;doi:10.1242/dev.128165/DC1
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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 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.