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REVIEW

Specification and Positioning of the Anterior Neuroectoderm in Deuterostome Embryos

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Summary: The molecular mechanisms used by deuterostome embryos (vertebrates, urochordates, cephalochordates, hemichordates, and echinoderms) to specify and then position the anterior neuroectoderm (ANE) along the anterior–posterior axis are incompletely understood. Studies in several deuterostome embryos suggest that the ANE is initially specified by an early, broad regulatory state. Then, a posterior-to-anterior wave of respecification restricts this broad ANE potential to the anterior pole. In vertebrates, sea urchins and hemichordates a posterior–anterior gradient of Wnt/β-catenin signaling plays an essential and conserved role in this process. Recent data collected from the basal deuterostome sea urchin embryo suggests that positioning the ANE to the anterior pole involves more than the Wnt/β-catenin pathway, instead relying on the integration of information from the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC pathways. Moreover, comparison of functional and expression data from the ambulacrarians, invertebrate chordates, and vertebrates strongly suggests that this Wnt network might be an ANE positioning mechanism shared by all deuterostomes. genesis 52:222–234. © 2014 Wiley Periodicals, Inc.

Key words: development; anterior neuroectoderm; evolution; regulatory networks

Establishment of the anterior neuroectoderm (ANE) is a defining feature of the animal body plan, yet it is still unclear how this territory is created or how it evolved in deuterostome embryos (vertebrates, urochordates, cephalochordates, hemichordates, and echinoderms; Fig. 1). ANE structures range from the simple bundle of sensory neurons in sea urchin larvae to the complex vertebrate forebrain and eye field. In all deuterostome embryos the ANE starts out as a simple, flat neuroepithelium. In vertebrates this simple neuroepithelium later undergoes complicated molecular patterning and morphogenetic movements that produce the forebrain and eye field, which have made it difficult to fully understand the establishment of this territory. In contrast, there are few cell movements during specification and patterning of the ANE in invertebrate deuterostome embryos (urochordate, cephalochordate, hemichordates, and echinoderms), making these tractable model systems for studying the early gene regulatory networks (GRNs) that establish and position this territory. However, this simplicity has made it challenging to identify morphological homologies between the invertebrate deuterostomes and the vertebrates. Moreover, the ANE is placed at the anterior-dorsal side of chordate embryos, whereas it is generally established around the anterior pole in ambulacrarian embryos (hemichordates and echinoderms), complicating comparisons between these two groups. In spite of these significant morphological differences recent comparative gene expression and functional studies, coupled with high-throughput genome-wide assays, have provided new insight into early neuroectoderm specification and patterning in the invertebrate deuterostomes. These studies show that the GRNs underlying the early specification and gross positioning of the ANE along the anterior–posterior (AP) axis are remarkably similar among deuterostomes, suggesting that this territory is both ancient and

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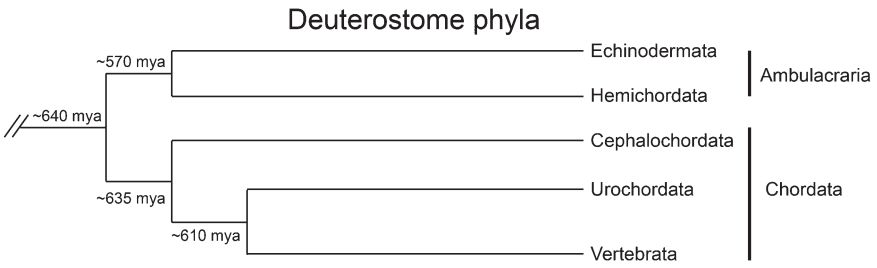

FIG. 1. Deuterostome phylogeny. A diagram showing the relations among deuterostomes. It is not clear when the phyla separated, but the approximate dates have been inserted at each branch point. Data taken from Swalla and Smith (2008).

homologous. This review will provide an overview of the data supporting this hypothesis, focusing on a Wnt signaling network that positions the ANE territory along the AP axis in the sea urchin embryo and the possibility that aspects of this network may be shared broadly among deuterostomes. These studies provide important insight into the shared developmental mechanisms used by deuterostome embryos to specify and position the ANE.

A SHARED DEUTEROSTOME ANE TOOLKIT

Of the studies performed over the last decade, those looking at the function and expression of one transcription factor, Six3, have provided some of the most convincing evidence that the ANE of deuterostomes could be established and patterned by a common genetic mechanism. Six3 is of interest because it is expressed early in the presumptive ANE region in many bilaterian embryos (Steinmetz *et al*., 2010). Moreover, functional studies in diverse deuterostome and protostome embryos (mouse, zebrafish, sea urchins, and beetles) are particularly informative because they suggest it is a critical "master regulator" for the specification of anterior-most neuroectoderm (Lagutin *et al*., 2003; Posnien *et al*., 2011; Wei *et al*., 2009). In both vertebrate and sea urchin embryos Six3 activates a remarkably similar cohort of genes whose temporal sequence of activation is also comparable (Fig. 2). For example, the first genes activated downstream of *six3* in both species appear to be those that control intermediate spatial subdivisions of the ANE [e.g., sFrp1/5, Dkk3, Zic2, FoxQ2 and Hbn in sea urchins (Range and Wei, unpublished results; Yaguchi *et al*., 2008); sFrp1, Zic2, and FoxG1 in vertebrates (Esteve *et al*., 2004; Martynoga *et al*., 2005; Sanek *et al*., 2009)]. As development progresses, intermediate level factors regulate more terminal regulatory and differentiation gene batteries, aspects of which are shared by vertebrates and sea urchins (e.g., Rx, Delta, Ac-Sc, Hairy, etc.; Fig. 2). GRNs are hierarchal in nature and the core structure of some well-studied networks has remained stable for millions of years (Erwin and Davidson, 2009; Peter and Davidson, 2011). Thus, it is tempting to speculate that many aspects of the early ANE regulatory network are conserved in deuterostomes. Strengthening this idea is that in other invertebrate deuterostomes, many of these critical regulatory factors appear to be expressed after Six3 and within corresponding territories in a temporal sequence that suggests they form a similar regulatory hierarchy (Fig. 2). To date, functional studies have not been performed on either Six3 or most of the ANE factors in other invertebrate deuterostome species and the expression patterns of many are not known. Thus, further studies are warranted since functional data are needed from a broad sampling of invertebrate deuterostomes in order to compare GRNs. These comparisons will in turn allow the reconstruction of the ancestral deuterostome ANE GRN and provide mechanism-based insight into the evolutionary relationships of ANE structures in different species.

POSITIONING OF THE ANE ALONG THE AP AXIS

Multiple molecular patterning steps are required to form the mature ANE in different deuterostome embryos (Cavodeassi, 2013; Pani *et al*., 2012; Range *et al*., 2013; Wilson and Houart, 2004). Here, the focus is on the earliest steps in establishing the ANE that appear to be shared among deuterostomes: acquiring ANE fate and restricting this fate to anterior pole.

Upstream ANE Factors Are Expressed Broadly in the Early Presumptive Neuroectoderm

Ambulacraria are a superphylum of marine deuterostome animals that include the sister phyla Echinodermata (e.g., sea urchins and sea stars) and Hemichordata (acorn worms). They sit at the base of the deuterostomes, separated from the chordates by ~650 my (Swalla and Smith, 2008; Fig. 1). Although their adult body plans are very different from one another, ambulacrarians develop from a similar bilaterally symmetrical larval form that is considered to be homologs (Nielson, 2001; Strathmann and Bonner, 1976; Zeng and Swalla, 2005). Consistent with this hypothesis, early activation of ANE gene expression is strikingly similar among the ambulacrarians. In this

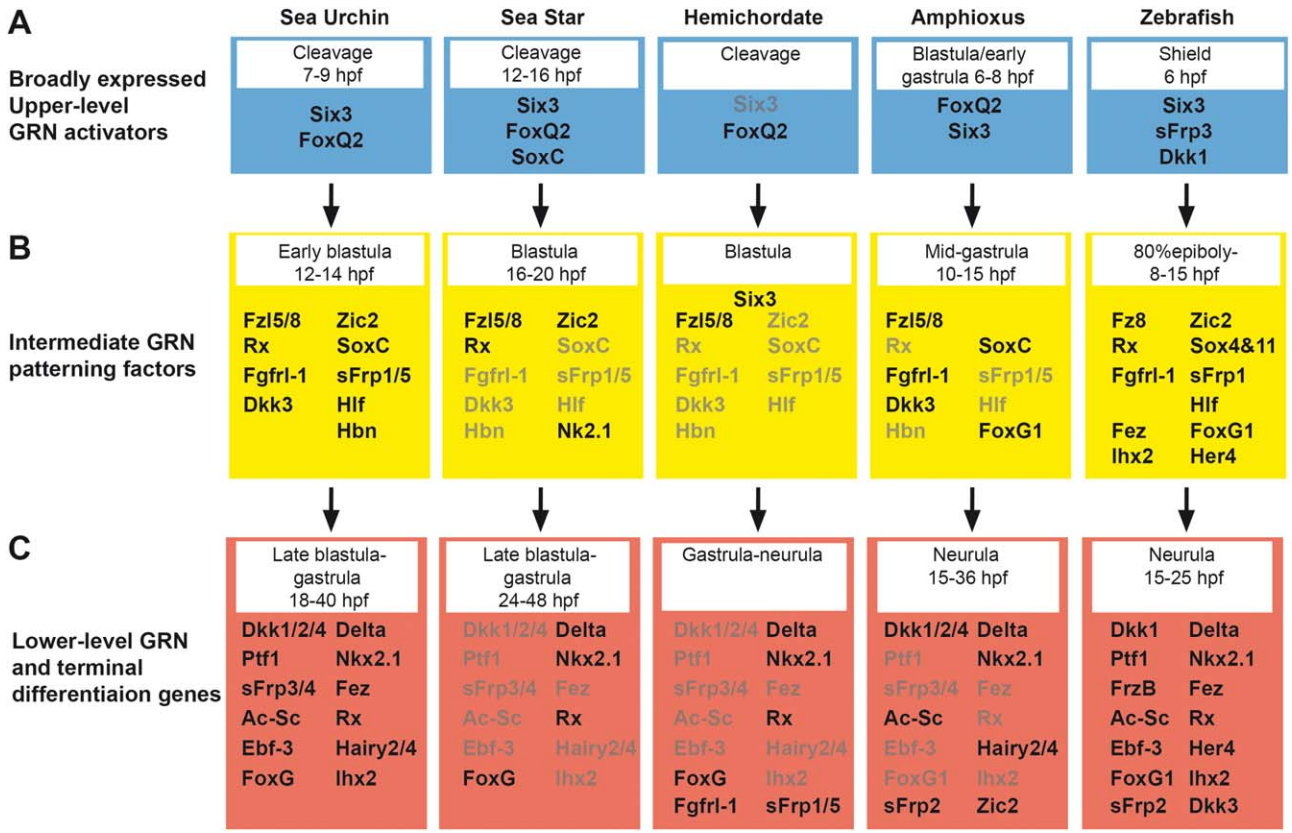

FIG. 2. Shared ANE regulatory hierarchies in deuterostome embryos. The focus of this diagram is on the shared expression of orthologs among deuterostome embryos during ANE specification. In cases where there is no information concerning the expression pattern of a particular ortholog, it is represented in light gray. (**a**) In the initial phases of development genes are expressed that establish broad territories within the embryo. These genes activate gene regulatory network hierarchies that progressively pattern the territories and specify specific cell fates within the territory. The early, broad expression of Six3, as well as FoxQ2 in invertebrate deuterostomes, in the presumptive neuroectoderm suggests that they likely function as activators of the ANE GRN in deuterostomes. Functional studies in the sea urchin and vertebrates support this hypothesis. Data taken from Darras *et al*. (2011), Kobayashi *et al*. (1998), Kozmik *et al*. (2007), Pani *et al*. (2012), Rottinger and Martindale (2011), Shinya *et al*. (2000), Tendeng and Houart (2006), Wei *et al*. (2009), Yaguchi *et al*. (2008), Yankura *et al*. (2010), and Yu *et al*. (2003). (**b**) Upper-level GRN factors like Six3 and FoxQ2 activate genes that subdivide the larger territory, many of which are conserved. In addition, many of the same factors are expressed soon after Six3 and Foxq2 in the other invertebrate deuterostomes, suggesting conservation of the basic ANE regulatory hierarchy. Data taken from Ando *et al*. (2005), Bertrand *et al*. (2009), Darras *et al*. (2011), Grinblat and Sive (2001), Hall *et al*. (2006), Kim *et al*. (2002), Lin *et al*. (2009), Materna *et al*. (2010), McCauley *et al*. (2013), Onai *et al*. (2012), Qian *et al*. (2013), Rimini *et al*. (1999), Rottinger and Martindale (2011), Stigloher *et al*. (2006), Takke *et al*. (1999), Tendeng and Houart (2006), Toresson *et al*. (1998), Wei *et al*. (2009), Yang *et al*. (2001), Yankura *et al*. (2010), and Zhao *et al*. (2009). (**c**) At the termini of GRN hierarchies, lower level regulatory subcircuits activate terminal differentiation gene batteries. There is a high degree of conservation among deuterostomes at this level as well. Data taken from Allende and Weinberg (1994), Darras *et al*. (2011), Gostling and Shimeld (2003), Li *et al*. (2010), Lowe *et al*. (2003), Lu *et al*. (2012), Materna *et al*. (2010), Minguillon *et al*. (2003), Pani *et al*. (2012), Range *et al*. (2013), Rohr and Concha (2000), Rottinger and Martindale (2011), Tendeng and Houart (2006), Untergasser *et al*. (2011), Venkatesh *et al*. (1999), Wei *et al*., 2009), Yang *et al*. (2001), Yankura *et al*. (2013), Yankura *et al*. (2010), Yu *et al*. (2007), Zecchin *et al*. (2004), Zhang and Mao (2010), and Zhao *et al*. (2009).

group, ANE specification and patterning are best understood in sea urchin embryos in which the first ANE factors to be expressed are *six3* and *foxq2* at the 32-cell stage (Wei *et al*., 2009; Yaguchi *et al*., 2008; Fig. 3, Column A). These critical transcriptional regulators are expressed broadly throughout the anterior half of the embryo in the presumptive ectoderm. Because they sit at the top of the ANE GRN, they give this territory an early ANE bias. In echinoderm sea star and the hemichordate embryos *foxq2* and *six3* also are broadly expressed in the anterior ectoderm during early embryogenesis (Pani *et al*., 2012; Yankura *et al*., 2010; Fig. 3, Columns B and C). The precise developmental stage at which these factors are activated and their functional roles in the ANE regulatory network have not been determined in either embryo, but their spatiotemporal expression profiles suggest they may play roles similar to their sea urchin homologues.

The adult body plans of chordates and ambulacrarians are clearly divergent. However, cephalochordate embryos, which sit at the base of the chordate lineage, develop more like ambulacrarians up until the late

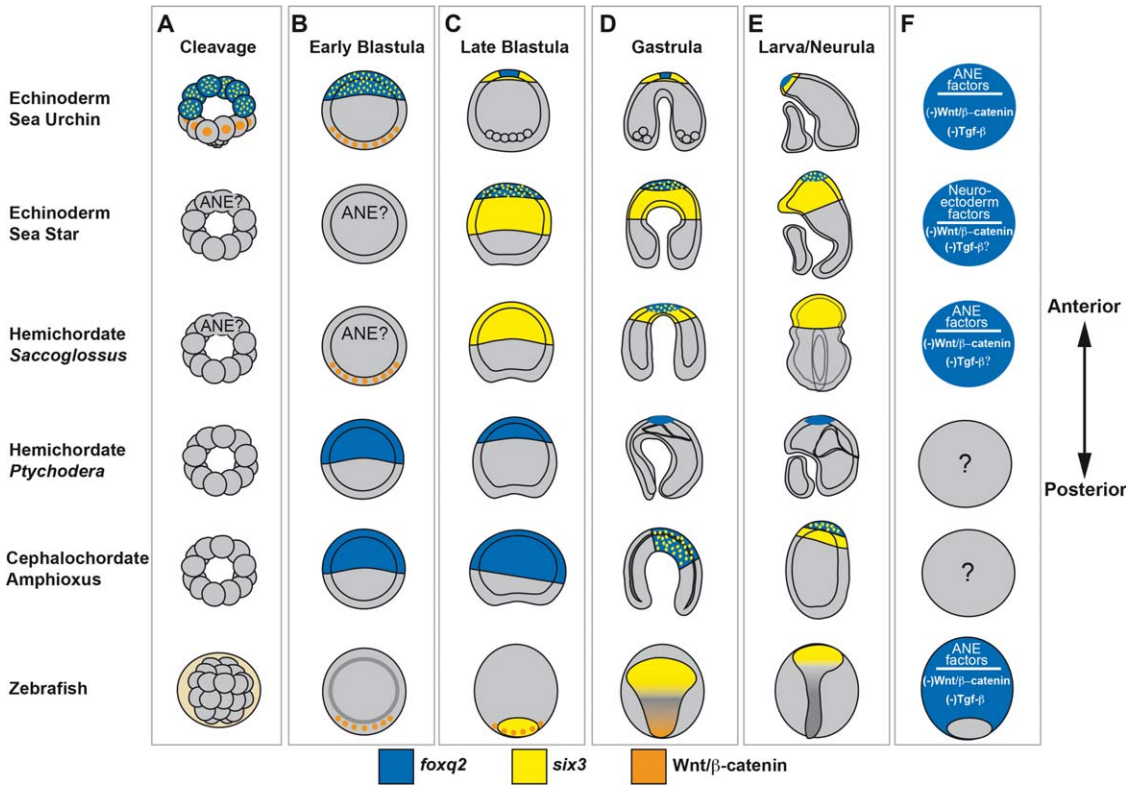

FIG. 3. Conservation of ANE restriction among deuterostome embryos. (**a**–**e**) Columns represent similar stages of development for each deuterostome model organism. The expression of the upper regulatory genes *six3* and/or *foxq2* is used as a marker for the ANE. The initial spatial/temporal expression pattern of *foxq2* and/or *six3* is not known in sea star or in *Saccoglossus* embryos. All deuterostomes express putative ANE factors broadly within the ectoderm, and then restrict these factors to the anterior pole as development progresses. Zebrafish embryos are oriented with their dorsal sides facing up from the page. Data taken from Darras *et al*. (2011), Kozmik *et al*. (2007), Lowe *et al*. (2003), Rottinger and Martindale (2011), Seo *et al*. (1998), Wei *et al*. (2009), Yaguchi *et al*. (2008), Yankura *et al*. (2013), Yankura *et al*. (2010), and Yu *et al*. (2003). (a-D) In several species, functional studies have shown that Wnt/β-catenin signaling is necessary for ANE restriction. In these columns, orange balls indicate where studies show a posterior-to-anterior Wnt/β-catenin signal during ANE restriction. Data taken from Darras *et al*. (2011), Kiecker and Niehrs (2001), Logan *et al*. (1999), Range *et al*. (2013), Varga *et al*. (2011), and Yaguchi *et al*. (2008). (**f**) The diagrams in this column illustrate what happens to sea urchin and vertebrate embryos in the absence of Wnt/β-catenin and TGF-β signaling as well as sea star and *Saccoglossus* embryos when Wnt/β-catenin is knocked down. In sea stars and hemichordates, it is not known whether TGF-β signaling persists in the absence of Wnt/β-cat, thus the "(−) *TGF-β signaling?"*. Data taken from Darras *et al*. (2011), Range *et al*. (2013), Reversade *et al*. (2005), Varga *et al*. (2011), and Yankura *et al*. (2013).

gastrula stage. At this time they begin to generate more vertebrate-like structures, including the notochord and the dorsal hollow nerve chord (Bertrand and Escriva, 2011; Kowalevsky, 1867). The fact that *foxq2* and *six3* are initially broadly activated throughout the presumptive ectoderm during the early blastula stages in ambulacrarians and cephalochordates (Kozmik *et al*., 2007; Yu *et al*., 2003; Fig. 3, Column B) suggests that, in addition to the similarities in their early developmental morphologies, they share similar molecular mechanism for neuroectoderm specification. Remarkably, this early broad activation of ANE factors is not confined to the invertebrate deuterostomes: Several studies in vertebrates show that the first genes to be activated during the initial specification of the presumptive neuroectoderm encode ANE factors, including Six3, which are expressed broadly throughout this territory (Foley and Stern, 2001; Seo *et al*., 1998; Fig. 3, Column C). Taken together these studies suggest that broad, early expression of ANE factors at the initiation of neuroectoderm specification is a common feature in deuterostome embryos.

Early, Broadly Active Regulatory Networks Activate Specification of Deuterostome ANEs

In vertebrates and sea urchin embryos tumor growth factor β (TGF-β) and Wnt/β-catenin signaling pathways inhibit the activation of neuroectoderm specification, resulting in proper neuroectoderm patterning along the dorsal–ventral (DV) and AP axis, respectively (see subsequently and Lapraz *et al*., 2009; Niehrs, 2010; Yaguchi *et al*., 2010). A remarkable phenotype arises when Wnt/β-catenin and TGF-β functions are eliminated in the sea urchin: From the very beginning of neuroectoderm initiation and throughout embryogenesis the

entire embryo expresses ANE factors. Moreover, these embryos eventually develop into a ball of ectoderm that differentiates neurons normally restricted to the ANE territory (Fig. 3; Range *et al*., 2013). This study shows that the entire sea urchin embryo has the potential to be fated as neuroectoderm. Interestingly, perturbing Wnt/β-catenin signaling also causes the ANE factors Six3 and sFrp1/5 in hemichordate and general neuroectoderm factors in sea star to be expressed throughout the early embryo (Fig. 3; Darras *et al*., 2011; Yankura *et al*., 2013). Taken together, the available functional studies suggest that a maternally supplied GRN active throughout the early ambulacrarian embryo initially drives ANE gene expression.

Several studies provide evidence that chordates and ambulacrarians may share common mechanisms for the initiation of neuroectoderm specification. For example, in ascidians, a *ubiquitous*, maternally expressed transcription factor, GATAa (vertebrate GATA4/5/6), is necessary for ectoderm specification, including the ANE, in the animal half of the embryo (Bertrand *et al*., 2003). If Wnt/β-catenin signaling is perturbed, GATAa is able to activate ectoderm throughout the embryo (Rothbacher *et al*., 2007). Moreover, recent studies in *Xenopus* and zebrafish show that blocking both Wnt/β-catenin-mediated organizer formation and the expression of bone morphogenetic protein (BMP) 2, 4, and 7 converts these embryos almost entirely into neuroectoderm that expresses primarily ANE factors (Fig. 3; Reversade *et al*., 2005; Varga *et al*., 2011). Finally, a similar phenotype is produced in mouse and human embryonic stem cells when they are deprived of exogenous growth factors, most importantly Wnts and BMP ligands. In this environment, they develop almost entirely into ANE cells (Eiraku *et al*., 2008; Nakano *et al*., 2012). Collectively, these results imply that early, broadly active regulatory networks are also used to activate ANE specification in many chordate embryos.

Progressive Restriction of ANE Factors

After the presumptive ANE is established in deuterostome embryos, the next step that appears to be conserved is the progressive downregulation of ANE factors in posterior ectoderm, which will hereafter be termed "ANE restriction". In sea urchin embryos the broad, radial expression of upper level genes in the ANE GRN hierarchy, such as *six3* and *foxq2*, is downregulated in posterior ectoderm during the early cleavage and blastula stages until it is confined to a small region around the anterior pole of the embryo (Range *et al*., 2013; Wei *et al*., 2009; Yaguchi *et al*., 2008). These same factors have a remarkably similar developmental expression profile in the ambulacrarian sea star and hemichordate embryos as well in the cephalochordates; however, the timing and extent of ANE downregulation in the posterior ectoderm differ for each species (Fig. 3). Similarly, in vertebrates, Six3 and other early ANE factors are progressively restricted from the posterior neuroectoderm until they are positioned around the anterior pole where they establish the forebrain and eye field (Fig. 3).

Wnt/β-catenin signaling is a critical mechanism many deuterostome embryos use to restrict the broad ANE potential toward the anterior pole. Sea urchin and hemichordate embryos both have a posterior-to-anterior gradient of Wnt/β-catenin signaling during early specification of the ANE (Fig. 3, Column B), and in both species ANE restriction fails in the absence of Wnt/β-catenin signaling (Darras *et al*., 2011; Range *et al*., 2013; Yaguchi *et al*., 2008). It is somewhat difficult to compare ascidians with other deuterostomes during early patterning events because they rely more on mosaic development rather the regulative development strategy used by the other phyla. Yet, even in these embryos, which primarily use short-range inductive interactions, vegetal Wnt/β-catenin signaling progressively restricts the ectoderm and some aspects of neuroectoderm to the animal pole (Hudson *et al*., 2013; Imai *et al*., 2000; Rothbacher *et al*., 2007). In vertebrates, zygotic Wnt/β-catenin also forms a posterior-to-anterior gradient within the presumptive neuroectoderm (Fig. 3, Columns B and C). This zygotic Wnt/β-catenin signaling activity is necessary to downregulate ANE factors and activate posterior neuroectoderm factors during the early blastula and gastrula stages (Kiecker and Niehrs, 2001; Nordstrom *et al*., 2002). The similarities in ANE specification and restriction among these deuterostome embryos raise the possibility that they might share many common aspects of the AP ANE restriction mechanism. Until recently, the only Wnt signaling pathway to have been shown to be necessary for ANE restriction in any deuterostome embryo was the Wnt/β-catenin pathway. However, it was shown that the sea urchin uses much more than the Wnt/β-catenin pathway to pattern the neuroectoderm along the AP axis. In fact, the sea urchin uses an interconnected network of Wnt signaling that includes at least three Wnt signaling branches—Wnt/β-catenin, Wnt/JNK, and Wnt/PKC (Range *et al*., 2013).

WNT SIGNALING NETWORKS IN DEVELOPMENT

Wnt signaling is complex which allows it to take on a multitude of roles in developing embryos and adult tissue homeostasis. To a large degree, this complexity comes from the staggering number of possible ligand/receptor/coreceptor combinations. For example, there are 19 Wnt ligands and 10 Frizzled (Fzl) receptors in vertebrates, which means there are 190 potential pairwise combinations of these proteins alone. Another level of complexity comes from the fact that each of

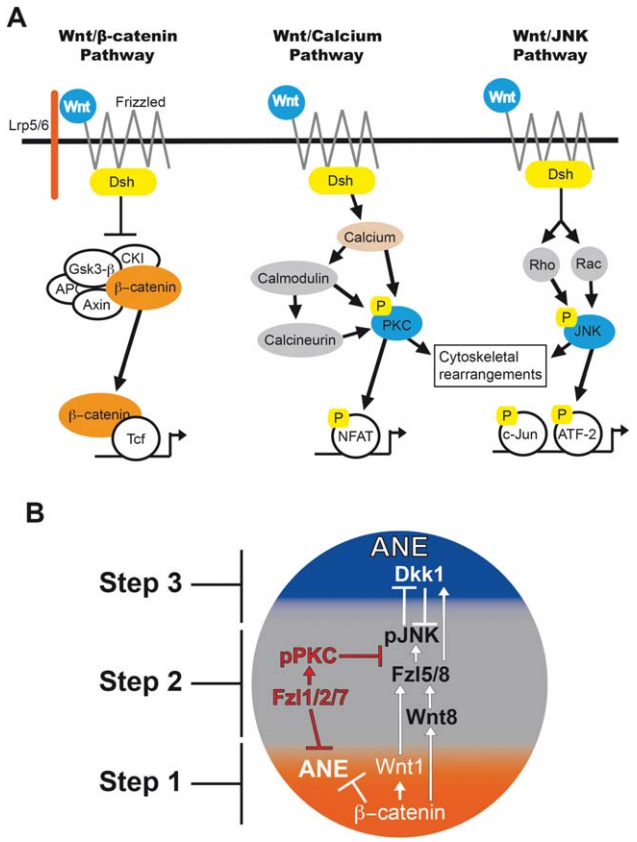

FIG. 4. Wnt signaling pathways and the sea urchin Wnt network. (**a**) Wnt signaling employs at least three pathways, Wnt/β-catenin, Wnt/JNK, and Wnt/Calcium. For simplicity these are represented as linear, but in reality they often are connected through extracellular and intracellular components, many of which are not diagramed here (van Amerongen and Nusse, 2009). (**b**) In sea urchin early development, the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC pathways all converge on the same developmental process: ANE restriction. This diagram shows the model for ANE restriction in the sea urchin detailed in the text.

these receptor/ligand combinations can potentially activate any of the three different Wnt branches (Kestler and Kuhl, 2008; van Amerongen and Nusse, 2009).

The Wnt/β-catenin pathway, also known as the "canonical" Wnt pathway, is the best characterized of these three pathways. In this pathway Wnt ligands bind to a Fzl receptor and the coreceptor LRP5/6, which then interact with the scaffolding protein Disheveled (Dsh). This complex leads to a signal that causes the disassembly of the intracellular β-catenin destruction complex (APC, Axin, and GSK3-β), which facilitates the proteolysis of cytoplasmic β-catenin in the absence of Wnt signaling. After disassembly of this complex, stabilized β-catenin accumulates in the cytoplasm and subsequently enters nuclei where it interacts with the TCF transcription factor, resulting in the activation of downstream gene transcription (Fig. 4a; Macdonald *et al.*, 2007). The Wnt/JNK and Wnt/Ca^{2+} pathways, also referred to as the "noncanonical", or "alternative", Wnt pathways are not as well characterized (Reviewed in van Amerongen, 2012), but there is agreement on some aspects of these signaling pathways. Both pathways use Wnt ligands, Frizzled receptors, and Dsh, but the resulting signals are transduced by different intracellular messengers. The Wnt/Ca^{2+} pathway uses various Ca^{2+}-sensitive proteins, such as PKC, calmodulin, and calcineurin. Subsequently, these factors often cause cytoskeletal changes and they also have been shown to activate transcription factors, such as NFAT, by phosphorylation, leading to gene transcription (Fig. 4a; Semenov *et al.*, 2007). In general the Wnt/JNK pathways use a different suite of intracellular messengers including the small GTPases Rho and Rac that activate JNK, also leading to cytoskeletal rearrangements and activation of transcription factors such as c-Jun and ATF-2 (Fig. 4a; Semenov *et al.*, 2007). In the past these pathways were thought to run independently from one another. However, recent studies indicate that while many Wnt ligand/receptor/coreceptor combinations favor one downstream pathway over the other, most are associated with all three pathways. Moreover, many of the intracellular pathway members (e.g., JNK and PKC) are not limited to a particular Wnt pathway. These studies strongly suggest that Wnt signaling works as an interconnected network in which information from two or more pathways is necessary to specify cell fates (Kestler and Kuhl, 2008; van Amerongen and Nusse, 2009).

A Sea Urchin Wnt Signaling Network

The sea urchin embryo integrates information from the Wnt/β-catenin, Wnt/JNK, and Wnt/PKC signaling pathways during ANE restriction (Range *et al.*, 2013). Currently, this mechanism can be separated into three interconnected, sequential steps. The first phase of ANE restriction begins by the 32–60-cell stage, when posterior Wnt/β-catenin signaling prevents ANE activation in the posterior blastomeres by the initially ubiquitous, maternal regulatory state that can activate ANE throughout the embryo. The result is that the earliest "master ANE regulators", *six3* and *foxq2*, are expressed only in anterior blastomeres (Fig. 4b). Posterior Wnt/β-catenin signaling also activates expression of two Wnt ligands, Wnt1 and Wnt8, during the 32–60-cell stage, and these ligands initiate the second phase of ANE downregulation. Beginning around the 60-cell stage, Wnt1 and Wnt8 signal through the Fzl5/8 receptor to more anterior blastomeres (posterior ectoderm). This receptor activates the Wnt/JNK signaling pathway, resulting by the late blastula stage in downregulation of ANE factors in all the ectoderm except the anteriormost cells (Fig. 4b). In an unexpected finding, another Frizzled receptor, Fzl1/2/7, which activates a third Wnt pathway involving PKC, attenuates both the Wnt/β-catenin and the Wnt/Fzl5/8/JNK signaling pathways,

preventing them from eliminating ANE specification at the anterior pole during the first two phases (Fig. 4b). Finally, in the last phase of ANE positioning, Fzl5/8 signaling around the anterior pole activates the expression of the secreted Wnt antagonist, Dkk1, near the end of ANE restriction. This step is crucial since Dkk1, acting via a negative feedback mechanism, prevents Fzl5/8 signaling from downregulating ANE factors at the anterior pole thereby defining the perimeter of the ANE territory (Fig. 4b). The data from the sea urchin demonstrate for the first time in any deuterostome embryo that *three different*, *but interconnected*, *Wnt signaling pathways* are used to provide precise spatiotemporal control of ANE positioning along the AP axis. These are exciting results in keeping with the new idea that Wnt signaling networks are essential for cell fate specification during development.

CONSERVATION OF A WNT SIGNALING NETWORK IN DEUTEROSTOMES?

Comparative data from a broad sampling of metazoan embryos indicates that posterior Wnt signaling and anterior Wnt antagonism mediated by Dkk1 is a mechanism used to pattern several tissues along the primary axis in diverse metazoan animals, suggesting that this is an ancient mechanism that unifies the formation of the embryonic body plan in most animals (Niehrs, 2010; Petersen and Reddien, 2009). In vertebrates Dkk1 is expressed at the anterior end of the embryo where it helps protect the presumptive ANE from Wnt/β-catenin downregulation and establish the ANE territory (Glinka *et al*., 1998). Range *et al*. (2013) showed for the first time that the anterior Dkk1–posterior Wnt/β-catenin neuroectoderm patterning mechanism observed in vertebrates exists in a nonchordate deuterostome. The significance of this observation is that it implies that the ancient Dkk1-Wnt/β-catenin AP patterning mechanism present in extant prebilaterian and protostome embryos was likely co-opted to provide AP polarity to the neuroectoderm in the deuterostome ancestor. If this is true, then it is possible that aspects of the multistep mechanism that restricts the ANE regulatory state around the anterior pole in the sea urchin embryo could be widely shared among deuterostome embryos.

Echinodermata/Sea Star

The sea star and sea urchin separated ~500 million years ago (Hinman *et al*., 2003) and yet the expression patterns of many of the main players involved in sea urchin AP ANE positioning are similar in both species. For example, maternally provided *fzl5/8* and *fzl1/2/7* are both in the right place at the right time to influence ANE positioning during early stages of ANE restriction in the sea star. At the same time *wnt8* is expressed in the endomesoderm of sea stars as it is in sea urchin embryos (Fig. 5; McCauley *et al*., 2013; Yankura *et al*., 2013). As development progresses in the sea star, ANE factors and *fzl5/8* are progressively restricted to the anterior of the embryo. Simultaneously, *wnt8* expression moves into the posterior ectodermal region, again remarkably similar to what is observed in sea urchin embryos (Fig. 5; Yankura *et al*., 2013). Data are lacking on the exact location of Wnt/β-catenin activity in sea stars, but the gene regulatory architecture necessary for posterior endomesoderm specification is conserved (Hinman *et al*., 2003), suggesting that it may function in this region of the embryo. Taken together, these data combined with the fact that the ANE is no longer

FIG. 5. Conservation of sea urchin Wnt network orthologs in deuterostomes during ANE restriction. The diagram of each embryo is colored to indicate the general ANE regulatory network (blue) and the expression of *wnt8* (green). The spatial expression patterns of ANE factor orthologs in the neuroectoderm along the AP axis are indicated to the left and to the right of each diagram. No expression data are available for the genes shaded light gray. (**a**) Expression of sea urchin ANE factors overlaps with that of *fzl5/8* and *fzl1/2/7* during early ANE restriction while Wnt8, and to a lesser extent Wnt1, are expressed more posteriorly. Later *flz5/8* expression is restricted to the ANE, whereas *fzl1/2/7* is expressed in the posterior ectoderm. The Wnt modulators *dkk1*, *sfrp1/5*, and *dkk3* are expressed in the sea urchin ANE. Functional tests indicate that these factors are necessary for ANE specification and patterning as they are in vertebrates (Range *et al*., 2013; Range lab, unpublished results). (**b**) As in sea urchin embryos, in the sea star *wnt8* is expressed just posterior to the ANE during early ANE restriction and then moves into the territory where ANE factors are downregulated. Moreover, expression of *fzl1/2/7* and *fzl5/8* overlaps early and then segregates, as do the sea urchin orthologs (McCauley *et al*., 2013; Yankura *et al*., 2013). (**c** and **d**) *fzl5/8* is expressed broadly throughout the anterior half of *Ptychodera* embryos during the initial stages of ANE specification and in both *Ptychodera* and *Saccoglossus* it is subsequently restricted to the anterior pole in the putative ANE (Pani *et al*., 2012; Rottinger and Martindale, 2011). Recent functional data from Pani *et al*. (2012) show that a role for Fzl5/8 in ANE restriction is conserved in ambulacrarians. In addition, the expression pattern of the Wnt modulator *sfrp1/5* is similar to that of the sea urchin ortholog. (**e**) In early gastrula stage *Amphioxus* embryos *fzl1/2/7* and *fzl5/8* are expressed within the broad ANE territory and again *wnt8* is expressed just posterior to this territory throughout the vegetal plate. By the late gastrula stage the ANE and the putative Wnt modulators *dkk1* and *dkk3* are restricted to the anterior-dorsal side of the embryo, along with *flz5/8*. Once again, *wnt8* is activated in the posterior ectoderm territory where ANE factors are downregulated (Holland *et al*., 2000; Onai *et al*., 2012; Qian *et al*., 2013; Yu *et al*., 2007). (**f**) ANE factors are initially expressed throughout the presumptive zebrafish neuroectoderm during late blastula/early gastrula stages. *fzl7a, fzl7b*, *fzl8a*, and *wnt8* expression overlaps in the presumptive neuroectoderm at the same time. As gastrulation continues (right hand diagram) ANE factors and *fzl8a* are downregulated from posterior neuroectoderm and *wnt8* as well as *wnt1* are expressed in this posterior neuroectoderm adjacent to the ANE territory. The Wnt modulators *dkk1, sfrp1*, and *dkk3* are also expressed in the ANE territory at this time. Expression studies show that *fzl1, fzl2, fzl7a, fzl7b*, and *fzl5* are all expressed in the neuroectoderm during ANE restriction. Functional studies show that Fzl8a, Wnt1, Wnt8 are necessary for ANE restriction and that Dkk1 and sFrp1 are necessary to protect the ANE from downregulation by the ANE restriction mechanism (Hsu *et al*., 2010; Kim *et al*., 1998, 2002; Lagutin *et al*., 2003; Lekven *et al*., 2001; Nikaido *et al*., 2013; Shinya *et al*., 2000; Sumanas *et al*., 2000; Tendeng and Houart, 2006).

positioned around the anterior pole in the sea star in absence of Wnt/β-catenin signaling (Yankura *et al*., 2013) suggest that the posterior-to-anterior Wnt/β-catenin-to-Wnt/JNK signaling relay mechanism seen in the sea urchin may also exist in the sea star. Further strengthening this idea, anteriorly expressed Six3 appears to protect the ANE by antagonizing a posterior Wnt signaling mechanism, as is the case in the sea urchin. This posterior Wnt mechanism may rely on a Wnt8, since in the absence of Six3 there is a marked reduction in ANE while posterior *wnt8* expression expands in sea star embryos, (Yankura *et al*., 2013).

Hemichordata

Comparisons of more distantly related groups suggest that the ANE restriction mechanism is also similar in

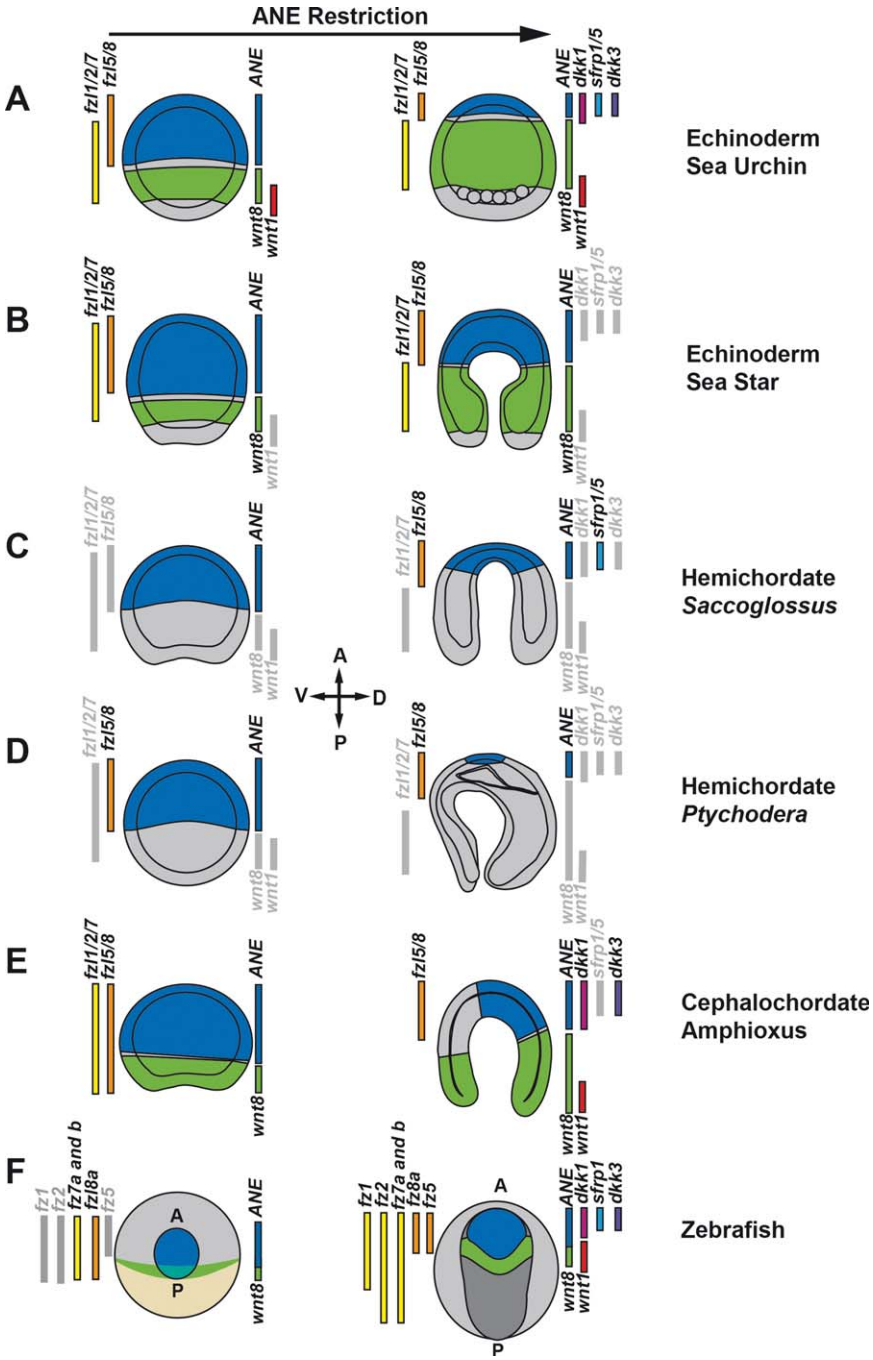

FIG. 5.

hemichordates, which diverged from echinoderms ~550 million years ago (Swalla and Smith, 2008). To date, expression data for many Wnt ligands, modulators, and receptors have not been described, but the available data place Wnt/β-catenin signaling and putative Wnt antagonists at the posterior and anterior of the embryo, respectively (Fig. 5; Darras *et al*., 2011; Pani *et al*., 2012). Although studies have not focused on early ANE specification and restriction in hemichordates, it is clear from the studies mentioned above that Wnt/β-catenin is necessary to prevent or downregulate the expression of ANE genes in the posterior half of the embryo in a similar fashion to what is seen in the sea urchin. Moreover, it suggests that an additional signaling mechanism is necessary to restrict ANE factors in anterior blastomeres since there is no evidence for Wnt/β-catenin activity there (Darras *et al*., 2011). Consistent with this hypothesis, the Wnt receptor *fzl5/8*, which activates the Wnt/JNK pathway during ANE restriction in the sea urchin, is initially broadly expressed, overlapping with *foxq2* and *six3*, then progressively restricted to the anterior pole with these factors (Fig. 5). Importantly, perturbation of Fzl5/8 function appears to block the ANE restriction mechanism in the anterior half of the embryo (Pani *et al*., 2012), which is remarkably similar to the mechanism in sea urchin embryos.

Amphioxus

Wnt ligands and Wnt modulators are expressed along the AP axis during blastula and gastrula stages in Amphioxus embryos, suggesting that Wnt signaling plays an important early role in the restriction of the ANE around the anterior pole in these embryos as well (Fig. 5; Qian *et al*., 2013; Yu *et al*., 2007). Involvement of Wnt/β-catenin signaling in AP patterning has been suggested because treatment of Amphioxus embryos with LiCl (Holland *et al*., 2000), which among other things, elevates Wnt/β-catenin signaling and posteriorizes embryos. However, no specific perturbations of Wnt signaling have yet been carried out in this system. Similar to ambulacrarians *fzl5/8* and *fzl1/2/7* are expressed throughout the ectoderm, and *wnt8* is expressed in the endomesoderm coincident with the broad expression of *foxq2* and subsequently *six3* during blastula stages (Kozmik *et al*., 2007; Qian *et al*., 2013; Yu *et al*., 2003, 2007). Then, during gastrulation Wnt8 expression moves into the posterior ectoderm as the presumptive ANE factors, including *fzl5/8*, become restricted around the anterior pole of the embryo. Concurrently, expression of *dkk1* and other Wnt modulators begins at the anterior pole (Fig. 5; Qian *et al*., 2013; Yu *et al*., 2007). Interestingly, LiCl treatment completely abolishes *foxq2* expression from the embryo, suggesting positioning of the ANE requires Wnt signaling in these embryos as well (Holland *et al*., 2000). One study does suggest that protection from Wnt signaling is necessary for ANE specification because when Dkk3 is knocked down, expression of ANE factors is lost (Onai *et al*., 2012). These studies indicate that the basic posterior Wnt signaling-anterior Wnt antagonism mechanism exists in cephalochordates and that they may share aspects of AP ANE positioning mechanism with the ambulacrarians.

Vertebrates

As mentioned above, the only Wnt pathway known to be involved in early ANE restriction in vertebrate embryos is Wnt/β-catenin. However, expression and, more importantly, functional studies in vertebrate embryos suggest that they share more than the fundamental Dkk1–posterior Wnt/β-catenin neuroectoderm patterning mechanism with their invertebrate deuterostome cousins. The vertebrate orthologs (*fz1*, *fz2*, *fz5*, *fz*7, and *fz8*) of sea urchin *fzl5/8* and *fzl1/2/7* are all expressed within the presumptive neuroectoderm at some time during the downregulation of ANE factors from the posterior neuroectoderm (Fig. 5; Kim *et al*., 1998; Nikaido *et al*., 2013; Sumanas *et al*., 2000). Moreover, both *wnt1* and *wnt8* overlap with these Frizzled receptors during the restriction process in several vertebrate model systems. For example, *wnt8* and *fz8* overlap with *six3* at the beginning of ANE restriction during the late blastula/early gastrula stage in zebrafish. Then as gastrulation proceeds, *fzl8* is downregulated along with *six3*/ANE factors in the posterior neuroectoderm, whereas *wnt8* is upregulated in this territory, similar to what is observed in sea urchins (Fig. 5). Furthermore, blocking Fzl8, Wnt1, or Wnt8 prevents ANE factor downregulation in the posterior neuroectoderm (Kim *et al*., 1998, 2002; Lagutin *et al*., 2003; Lekven *et al*., 2001). All of these results are remarkably similar to what is seen in the sea urchin embryo. However, because of the complexity of early signaling events and complex morphogenetic movements of gastrulation that occur during ANE restriction in vertebrates (Foley and Stern, 2001), it is unclear if Wnt1, Wnt8, or Fz8 directly impacts ANE downregulation from the posterior neuroectoderm. In addition, it is also unclear whether or not the Wnt/JNK and Wnt/Ca^{2+} pathways are involved in regulating gene expression in the presumptive neuroectoderm during the restriction process. Therefore, it will be of interest to look more closely at the roles of the various Frizzled receptors (Fzl1, Fz2, Fz7, Fz5, and Fz8) and Wnt ligands during the early ANE restriction in vertebrates. Given the similarities between the sea urchin and vertebrate mechanisms, it is interesting to speculate that a Wnt network involving the alternative Wnt signaling pathways may be involved in the vertebrate restriction process.

PERSPECTIVES

The studies reviewed here illustrate how comparisons among the molecular mechanisms used to generate embryonic body plans in a broad sampling of related organisms can reveal similarities that morphology obscures. For instance, due to the absence of a centralized nervous system in the penta-radial adult organism, echinoderms are often excluded from comparative studies of neuroectodermal evolution. However, recent studies in sea urchin larvae show that TGF-β signaling is necessary to pattern the neuroectoderm along the DV axis (Angerer *et al*., 2011; Molina *et al*., 2013; Yaguchi *et al*., 2010) as in most deuterostomes and protostomes (Niehrs, 2010). As shown in this review, the sea urchin ANE is specified by a GRN activated by Six3 that is remarkably similar to that required for vertebrate ANE specification (Wei *et al*., 2009). Moreover, comparing the available functional studies in vertebrate, sea urchin, sea star and hemichordate embryos, reveals that many of the factors necessary to restrict the initially broad ANE potential along the AP axis are conserved in several deuterostome embryos. Together, these studies suggest that aspects of the anterior–posterior ANE positioning mechanism are widely shared among the deuterostomes.

This review also demonstrates the importance of studying developmental mechanisms in diverse sets of model organisms since each has unique properties that make it better suited for studying a particular question. Before the sea urchin study on Wnt signaling during ANE restriction, the integrated activity of at least three different Wnt pathways during any developmental process had not been shown in a deuterostome embryo. Now, based on this work and the striking similarities in the various components known to be used or expressed during ANE restriction, it is interesting to speculate that a similar Wnt network may play a role during ANE restriction in any deuterostome embryo, including vertebrates. It will be a difficult task to study how these Wnt networks influence development in vivo. Thus, an ideal model system would have a simple morphology and genomic structure (e.g., fewer Wnt ligands, Fzl receptors, and coreceptors), like the sea urchin embryo. This embryo has many advantages over other developmental model systems for attacking this question since the three different Wnt pathways converge on the same developmental process, ANE restriction, which occurs within a relatively short window of ~17 h in a single-cell thick epithelium of nonmotile cells. Moreover, the GRNs governing early development up until the end of the ANE restriction process are well established in these embryos (Davidson *et al*., 2002), aiding our understanding of the context of the cell/territory receiving the Wnt signal. Finally, the output of the Wnt/JNK and Wnt/PKC pathways appears to be transcriptional (Range *et al*., 2013) making readout assays for these two alternative pathways feasible, a problem in other model systems and in cell culture because activation of these pathways generally results in cytoskeletal changes (van Amerongen, 2012). These capabilities, combined with ease of manipulation and access to the genome, suggest the sea urchin embryo has the potential to become a powerful model system for understanding Wnt networks in an in vivo developmental context**.**

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