

RESEARCH ARTICLE

Dose-dependent nuclear β -catenin response segregates endomesoderm along the sea star primary axis

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ABSTRACT

In many invertebrates, the nuclearization of β -catenin at one pole of the embryo initiates endomesoderm specification. An intriguing possibility is that a gradient of nuclear β -catenin ($n\beta$ -catenin), similar to that operating in vertebrate neural tube patterning, functions to distinguish cell fates in invertebrates. To test this hypothesis, we determined the function of $n\beta$ -catenin during the early development of the sea star, which undergoes a basal deuterostomal mode of embryogenesis. We show that low levels of $n\beta$ -catenin activity initiate *bra*, which is expressed in the future posterior endoderm-fated territory; intermediate levels are required for expression of *foxa* and *gata4/5/6*, which are later restricted to the endoderm; and activation of *ets1* and *erg* in the mesoderm-fated territory requires the highest $n\beta$ -catenin activity. Transcription factors acting downstream of high $n\beta$ -catenin segregate the endoderm/mesoderm boundary, which is further reinforced by Delta/Notch signaling. Significantly, therefore, in sea stars, endomesoderm segregation arises through transcriptional responses to levels of $n\beta$ -catenin activity. Here, we describe the first empirical evidence of a dose-dependent response to a dynamic spatiotemporal $n\beta$ -catenin activity that patterns cell fates along the primary axis in an invertebrate.

KEY WORDS: Echinoderm, Endomesoderm, Gene regulatory network, Nuclear β -catenin, Patiria

INTRODUCTION

During development, animals construct a complex organism comprising multiple distinct cell types, beginning with only a limited number of maternal anisotropies. The specification of these cell types is controlled by interacting networks of regulatory genes that transduce early asymmetries into distinct combinations of genes expressed in different cell types. Global, systems-level approaches that compile these interactions into gene regulatory networks (GRNs) have been key to understand how early maternal processes establish zygotic regulatory hierarchies that direct cell specification (see e.g. Davidson, 2006; Levine and Davidson, 2005). The specification of endomesoderm, a transient bipotential territory that gives rise to both endoderm and mesoderm, is a crucial early step in animal development. Detailed GRNs explaining the genesis of this territory have been described for the invertebrates *Nematostella vectensis*, *Caenorhabditis elegans* and *Strongylocentrotus purpuratus*, and for vertebrates, such as *Xenopus laevis* and *Danio rerio* (Chan et al., 2009; Loose and Patient, 2004; Maduro, 2009; Peter and Davidson, 2011; Röttinger

et al., 2012). In these and other, less well-studied organisms (Darras et al., 2011; Henry et al., 2008; Imai et al., 2000), nuclear β -catenin ($n\beta$ -catenin) is required for endomesoderm specification, or, in vertebrates, for the specification of anterior endomesoderm (Logan and Nusse, 2004). Therefore, the polar nuclearization of β -catenin is an ancestral mechanism among the metazoa used to establish endomesoderm.

Several well-characterized model systems, most notably vertebrate species and *Drosophila*, make extensive use of molecular gradients during early development (see e.g. Green and Smith, 1991; Ip et al., 1992). These gradients are thought to be formed via diffusion of a factor from a localized source, and function to activate gene expression in a dose-dependent manner to organize territories of discrete cell types along embryonic axes. In vertebrate embryos, a Wnt-driven, posterior-to-anterior gradient of $n\beta$ -catenin functions to specify positional cell fates in the neural tube during axial patterning (see e.g. Kiecker and Niehrs, 2001). It has been suggested that endoderm and mesoderm are patterned by this gradient (In der Rieden et al., 2010; Kiecker and Niehrs, 2001; McLin et al., 2007; Schneider and Mercola, 2001). Early *Drosophila* development is also characterized by the use of gradients to pattern the embryo, although there is no known requirement for a gradient of $n\beta$ -catenin during axis specification (see e.g. Jaeger, 2011). However, as numerous studies have shown that β -catenin is nuclearized in one pole of invertebrate embryos, it has been proposed that a gradient of $n\beta$ -catenin might also act to specify fates along the primary axis during early development in many taxa, and that it might be an ancestral feature of metazoan development (see e.g. Niehrs, 2010). As yet, there is no evidence in any invertebrate for a long-range $n\beta$ -catenin gradient being used to establish different territories, although differential accumulation has been observed within restricted domains (Darras et al., 2011; Henry et al., 2008; Imai et al., 2000; Logan et al., 1999; Miyawaki et al., 2003; Röttinger et al., 2012).

In several taxa, $n\beta$ -catenin is selectively restricted to one pole of the embryo during early cleavage (Henry et al., 2008, 2010; Miyawaki et al., 2003; Wikramanayake et al., 1998, 2003). In sea urchins and the cnidarian *Nematostella vectensis*, $n\beta$ -catenin is stabilized in the vegetal pole by cortically localized Dishevelled (Weitzel et al., 2004; Wikramanayake et al., 2003). This differential stability in sea urchins establishes a gradient of $n\beta$ -catenin, such that its levels are higher in the vegetal-most cells (micromeres) and lower in the blastomeres fated to endomesoderm (Lhomond et al., 2012; Logan et al., 1999; Weitzel et al., 2004). Therefore, a mechanism to localize and generate gradients of $n\beta$ -catenin activity along one pole of invertebrate embryos might be a widespread phenomenon. However, to date there is no indication that this gradient functions to direct different cell fates within the endomesoderm. How such a gradient might regulate development, or whether different levels of $n\beta$ -catenin themselves elicit distinct transcriptional responses, is currently not known.

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Once endomesoderm is specified, it is segregated into spatially and molecularly distinct endoderm and mesoderm territories. In several organisms, this asymmetry is also controlled by a change in β -catenin localization. In *C. elegans* and ascidians, an asymmetric cell division rapidly clears β -catenin from the presumptive mesoderm (Hudson et al., 2013; Thorpe et al., 1997). In sea urchins, Delta/Notch signaling from the micromeres induces mesoderm in the overlying cells, in part by inhibiting β -catenin function (Croce and McClay, 2010; Materna and Davidson, 2012; Ransick and Davidson, 2006; Röttinger et al., 2006; Sethi et al., 2012; Sherwood and McClay, 1999; Sweet et al., 2002; Ben-Tabou de-Leon and Davidson, 2010). Therefore, restriction of β -catenin localization might be a key step in the formation of separate endoderm and mesoderm territories commonly found among animals.

The central role of β -catenin in early endomesoderm development is a highly conserved mechanism among many taxa, and might thus constitute a common connection between early, maternal anisotropies and the later, taxon-specific networks that drive subsequent endoderm and mesoderm development. It remains to be determined whether gradients of β -catenin could operate in a dose-dependent manner to segregate distinct territories along the primary axis in invertebrates. If this is the case, the use of β -catenin dose dependence in territory segregation in both invertebrates and vertebrates could be a unifying feature of their development.

To address these issues, we analyzed the early specification and segregation of endoderm and mesoderm in the sea star *Patiria miniata*. Sea stars form hollow blastulae of a few thousand cells by the onset of gastrulation. Mesoderm forms at the central-most vegetal pole, with a torus of endoderm-fated cells situated above (Kuraishi and Osanai, 1992). In a closely related sea star species, β -catenin has been shown to accumulate in the nuclei of endomesoderm-fated vegetal blastomeres, and the vegetal-most cells appear to have higher levels of β -catenin than more peripheral vegetal blastomeres (Kuraishi and Osanai, 1992; Miyawaki et al., 2003). Given the basal mode of sea star development, and the fact that β -catenin is known to accumulate in endomesoderm-fated cells, the sea star is an ideal invertebrate model for assessing the function of β -catenin in endomesoderm segregation.

In *P. miniata* blastulae, three concentric territories are established in the vegetal pole in response to a changing profile of β -catenin transcriptional activity. Low levels of β -catenin activity drive *bra*

expression, which is later restricted to the *veg1*, endoderm/ectoderm boundary territory; intermediate levels drive *foxa* and *gata4/5/6* expression, which later are restricted to the endoderm-fated cells; and high levels of β -catenin are necessary for the expression of *ets1* and *erg* in the vegetal-most territory fated for mesoderm. The changing spatial distribution of β -catenin activity through time, coupled to a zygotic GRN, establishes the boundaries of endoderm and mesoderm. We also show that the expression of factors that ultimately define endoderm is cleared from the central mesoderm territory through the activation of repressive network circuitry established by high levels of β -catenin activity. The specification of endoderm is further reinforced by Delta/Notch induction and a positive-feedback circuitry between *hex* and *gata4/5/6* that is coincident with the loss of β -catenin from this territory. Thus, in this system, segregation of endoderm and mesoderm arises as the result of distinct transcriptional responses to levels of β -catenin activity. This dosage response to the dynamic spatiotemporal activity of β -catenin provides the first empirical demonstration of its use in invertebrate territory segregation, and establishes this response as a potentially widespread mechanism among metazoans.

RESULTS

Spatiotemporal regulatory states of the sea star vegetal pole

To understand the dynamics of endomesoderm territory specification, we determined the regulatory state of the sea star vegetal pole through the late blastula stage. Previous gene expression profiling identified three concentric domains, defined by distinct patterns of gene expression within the endomesoderm of hatched blastulae (Hinman and Davidson, 2003a,b, 2007; Hinman et al., 2003a; McCauley et al., 2010, 2013). However, little is known about gene expression prior to the formation of these domains, although this information is essential for understanding how these territories are established. Quantitative RT-PCR (qPCR) was used to assess the temporal profile of expression of 21 genes, namely *erg*, *ets1*, *foxn2/3*, *hesc*, *otx β b*, *tbr*, *tgif*, *wnt8*, *blimp1*, *bra*, *wnt3*, *otx β a*, *otx α* , *gata4/5/6*, *foxa*, *wnt16*, *wnt4*, *delta*, *foxg*, *gatac* and *hex*, which have been implicated in the studies mentioned above to play a role in sea star endomesoderm development. The results are summarized in Fig. 1. Two trends are evident: (1) seven transcripts (*erg*, *ets1*, *foxn2/3*, *hesc*, *otx β b*, *tbr* and *tgif*) are present maternally; and (2) an increase in gene expression is observed between 12 and 16 h post fertilization (h).

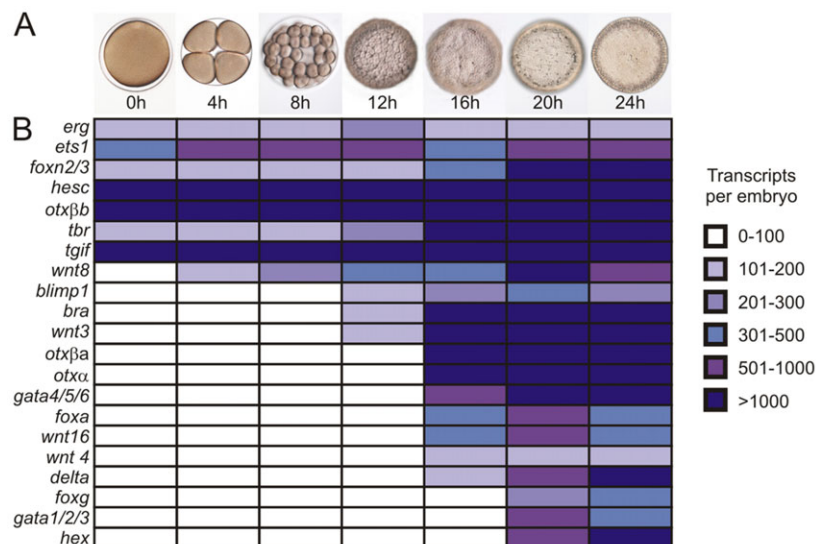


Fig. 1. Temporal gene expression in early sea star embryos. (A) Representative embryo morphology at the indicated time points. (B) Quantitation of expression levels of indicated transcripts at the listed time points. White signifies that a gene is not expressed; intensity of blue scales with increasing gene transcript abundance. Transcript levels were assessed by qPCR (to calculate transcripts per embryo). h, hours post fertilization.

As expression of most zygotic genes is initiated by the 16 h early blastula stage, we next assessed spatial patterns of gene expression in the vegetal pole, starting at this time point. Maternal factors, e.g. *erg* and *ets1*, are expressed ubiquitously (Fig. 2A,B). Conversely, genes that are first expressed after cleavage, e.g. *blimp1*, *bra*, *foxa*, (Fig. 2C–G), *wnt3* (supplementary material Fig. S1) and *gata4/5/6* (McCauley et al., 2012), are restricted to one embryonic pole. Therefore, at this stage, there is a single vegetal territory that localizes to the overlap of ubiquitously expressed maternal transcripts and the polar expression of zygotically activated regulatory genes (summarized in Fig. 2V). It is noteworthy that *bra* and *blimp1*, which are among the first genes expressed zygotically, are localized to a somewhat broader domain than that of *foxa* and *gata4/5/6*, although we cannot confirm the precise expression boundaries at this stage.

By the mid-blastula-stage (20 h), the earliest zygotically expressed factors in our study, *blimp1*, *bra* (Fig. 2H,I) and *wnt8* (supplementary material Fig. S1), are localized to a torus surrounding the central vegetal pole. Other genes, including *foxa*, *hex*, *foxn2/3* (Fig. 2J–L,N; supplementary material Fig. S1), *tbr*, *wnt3* (supplementary material Fig. S1) and *gata4/5/6* (McCauley et al., 2012), remain expressed throughout the vegetal pole. By contrast, *ets1* (Fig. 2M,N) and *erg* (supplementary material Fig. S1) expression is observed in a domain more closely restricted to the vegetal pole. Thus, at this stage, the initial restriction of gene expression to the veg1 territory is observed, which will contribute to the posterior endoderm and blastopore region of the gastrula [see Kuraishi and Osanai (1992); and schematic in Fig. 2W]. We also note the first restriction of gene expression solely to the mesoderm-fated territory. This territory invaginates first and contributes to the coeloms and mesenchyme of the gastrula [Kuraishi and Osanai (1992); and schematic in Fig. 2W]. Another suite of genes, namely

foxa, *hex*, *foxn2/3*, *tbr*, *wnt3* and *gata4/5/6*, is expressed within an endomesodermal domain that encompasses both presumptive mesoderm and endoderm.

By the late blastula stage (26 h), *foxa* and *gata4/5/6* expression is now cleared from the central domain and is thus, for the first time, specifically restricted to the torus of endoderm-fated territory. There are three distinct territories, as defined by localized patterns of regulatory gene expression: *ets1*, *erg*, *foxn3*, *delta* (Fig. 2O–S; supplementary material Fig. S1) and *gata1/2/3* (Hinman and Davidson, 2007) are expressed within the central-most domain; expression of *foxa* and *gata4/5/6* is localized to a torus surrounding this domain [Fig. 2P–R; see Hinman and Davidson (2003b)]; and *bra*, *blimp1* (Fig. 2T,U) and *wnt8* (McCauley et al., 2013) are restricted to a torus above the endoderm-fated cells (summarized in Fig. 2V). These are the mesoderm, endoderm and veg1 endoderm territories, respectively (Fig. 2W). Some factors in the vegetal pole domain remain expressed throughout both endoderm and mesoderm, namely *hex* (Fig. 2O), *tgif*, *foxn3*, *tbr*, *otxa* and *otxβ* (Hinman et al., 2003a; McCauley et al., 2010).

These data show that a largely uniform, initial vegetal endomesoderm region is segregated into three, molecularly distinct territories. Expression of genes that will be restricted to a veg1 territory occurs first, whereas the restricted zygotic expression of genes localized to the mesoderm occurs last. The early, ubiquitous phase of expression of *erg* and *ets1* can be attributed to maternally deposited transcripts. The GRN developed here, therefore, aims to explain: (1) the earliest activation of genes later expressed in the veg1 territory; (2) the initial activation of gene expression in a broad territory within the vegetal pole of the embryo; (3) the later expression of factors in the mesoderm-restricted territory; and (4) the clearing of gene expression from the central territory of the vegetal plate to form discrete mesoderm and

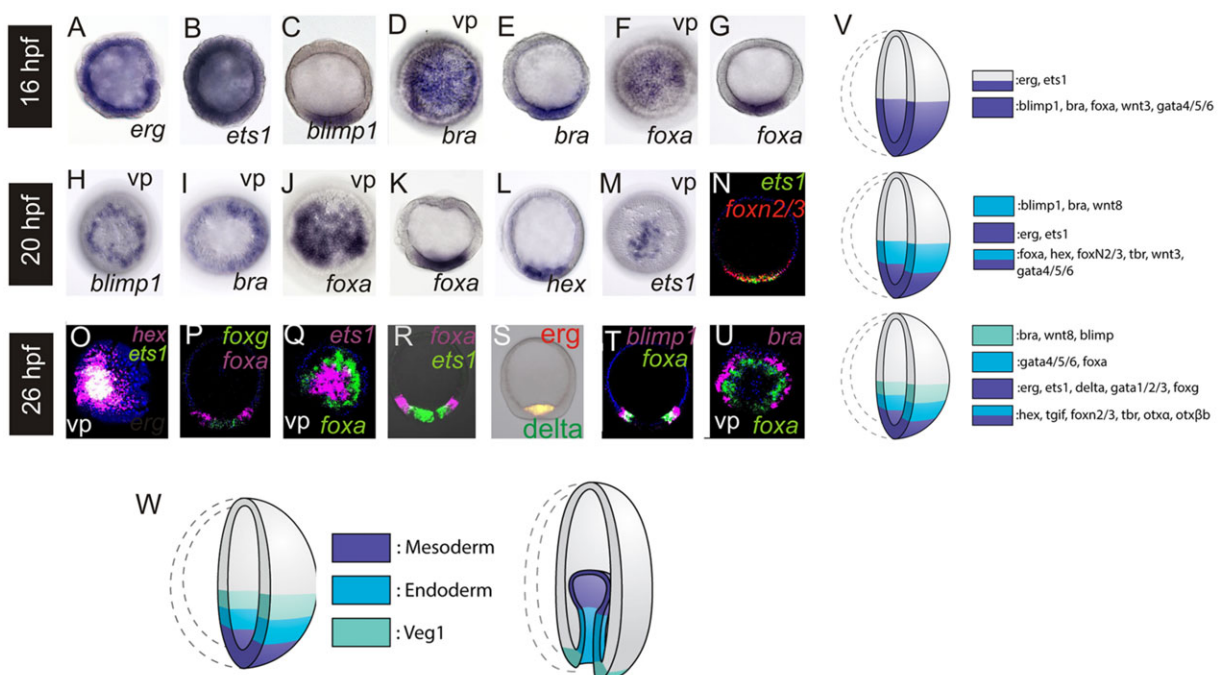


Fig. 2. Spatial gene expression in early sea star embryos. Whole-mount *in situ* hybridization (WMISH) was used to determine localization of gene expression in embryos at 16–26 h. Probe used is indicated on right. (A–G) 16-h-embryos (early blastulae); (H–N) 20-h-embryos (mid blastulae); (O–U) 26-h-embryos (late blastulae). Embryos are shown in the lateral view with vegetal towards the bottom unless indicated by vp, which is a view from the vegetal pole. N–U show two-color FISH. (V,W) Schematic depicting dynamics of gene expression in the sea star vegetal pole during blastula (V), and corresponding territories at blastula and gastrula (W) stages.

endoderm. At this time, the GRN model does not attempt to describe the mechanism that leads to the early segregation of the *bra*, *blimp1* and *wnt8* expression domain from the remaining endomesoderm.

n β -catenin is progressively restricted to the vegetal pole as its transcriptional activity increases

Given the dynamic changes in the regulatory states of the vegetal pole starting at the early blastula stage, we characterized the localization of n β -catenin during this time window and assayed its ability to activate gene expression. A β -catenin:GFP fusion construct (Logan et al., 1999) was used to visualize the localization of this protein during early development. The domain of n β -catenin becomes more restricted toward the vegetal pole between 16 h and 20 h (Fig. 3A,B; compare with Fig. 3C,D and schematic in Fig. 3E). We compared the number of β -catenin:GFP positive nuclei with the number of *ets1*- or *foxa*-positive cells as determined by WMISH at the same stage, and found that the number of β -catenin:GFP-positive nuclei at 16 h and 20 h is not significantly different from the number of *foxa*- or *ets1*-positive cells, respectively, at these same stages (Fig. 3F; two-tailed *t*-test, $P>0.05$). This indicates that, at 16 h, β -catenin is nuclearized within the *foxa*-expressing endomesoderm domain, whereas at 20 h, n β -catenin is restricted to the *ets1*-expressing, mesoderm-fated territory (schematic in Fig. 3E).

n β -catenin-driven gene expression was then quantified using the exogenous TOPFlash reporter. This construct contains six TCF-binding sites linked to a *luciferase* reporter gene (Veeman et al., 2003), expression of which is activated by n β -catenin. As assayed by qPCR, normalized *luciferase* expression increases significantly (ANOVA, $P=0.0405$) as development proceeds from 16 h to 24 h (Fig. 3G). Thus, n β -catenin-driven transcription increases during the time that the endomesoderm territories are being specified. We also used two-color fluorescent *in situ* hybridization (FISH) to show that, at 24 h, *luciferase* expression from the TOPFlash reporter co-localizes with *ets1* gene expression in 42/48 embryos, and is thus restricted to the mesoderm (Fig. 3H). This corresponds visually with the restriction of β -catenin:GFP-positive nuclei to the central-most vegetal pole at this stage (Fig. 3I). At this point, we cannot examine

the activity of n β -catenin in individual nuclei. Although there might be differential activity within each territory, our measurements for now are an average of the n β -catenin activity at each time point.

Taken together, these data (Fig. 3) show that, although gene expression driven by n β -catenin activity increases as development proceeds, the domain in which β -catenin is nuclearized becomes more restricted. At 16 h, when *foxa* is first expressed, the transcriptional activity of n β -catenin is lower than when *ets1* is first expressed within the presumptive mesoderm. An increase in n β -catenin activity over time has also been reported during early development in sea urchins (Lhomond et al., 2012; Logan et al., 1999), although whether this has a consequence for subsequent development has not been explored. As protein levels are not directly measured, we do not know whether there is an increase in β -catenin protein accumulation in the vegetal nuclei of sea star embryos or whether the increased transcriptional activity is due to its prolonged accumulation in the mesoderm-fated central-most territory compared with the endoderm-fated cells.

A dose-dependent response to the spatiotemporal profile of n β -catenin establishes distinct regulatory territories in early endomesoderm development

The changing spatiotemporal profile of n β -catenin transcriptional activity (Fig. 3), along with the description of the regulatory state of the vegetal territories through time (Figs 1 and 2), led us to hypothesize that differential n β -catenin activity is required to specify distinct cell fates within the sea star vegetal pole. Specifically, we propose that the low transcriptional activity of n β -catenin present throughout the vegetal pole prior to 16 h is sufficient to initiate expression of the earliest-expressed factors, e.g. *bra* and *blimp*; that increasing levels of n β -catenin activity initiate expression of endoderm-restricted genes, e.g. *foxa* and *gata4/5/6*; and, finally, that the highest activity, which is only achieved when n β -catenin is restricted to the central-most mesoderm-fated territory, drives *ets1* and *erg* expression. To test this, we manipulated the levels of n β -catenin by either sequestering β -catenin to the plasma membrane, using the Δ -cadherin construct (Logan et al., 1999) or driving it into nuclei with LiCl (Poustka et al., 2007; Stambolic

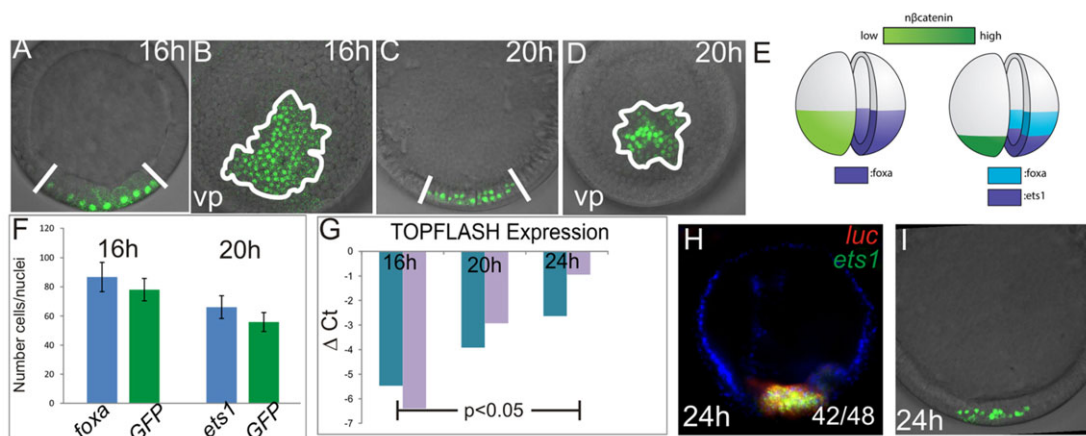


Fig. 3. Spatiotemporal gradient of n β -catenin in the vegetal pole. (A–D) Subcellular localization of β -catenin in 16-h- and 20-h-embryos assessed by confocal microscopy of GFP derived from the β -catenin:GFP fusion construct. 16-h-embryos are shown in A,B and 20-h-embryos in C,D. (A,C) Lateral views and (B,D) vegetal pole views. (E) Schematic of β -catenin nuclearization at 16 h and 20 h compared with expression of *foxa* and *ets1* at these stages. (F) Comparison of number of GFP-positive nuclei and *foxa*- or *ets1*-positive cells at time points shown. Error bars are from one s.d. of ten individuals. (G) qPCR analysis of TOPFlash reporter expression in 16-h-, 20-h- and 24-h-embryos. Increased Δ C_T (cycle threshold) values indicate increased reporter transcript abundance normalized to *lamin2 β receptor*. (H) FISH showing co-expression of *luciferase* driven by the TOPFlash reporter and the mesoderm marker *ets1* at 24 h. Co-expression of *ets1* and *luciferase* was detected in 42 of 48 embryos (expression in the presumptive endoderm was detected in five embryos). (I) Subcellular localization of the β -catenin:GFP fusion construct in 24-h-embryos for comparison with (H) at the same stage.

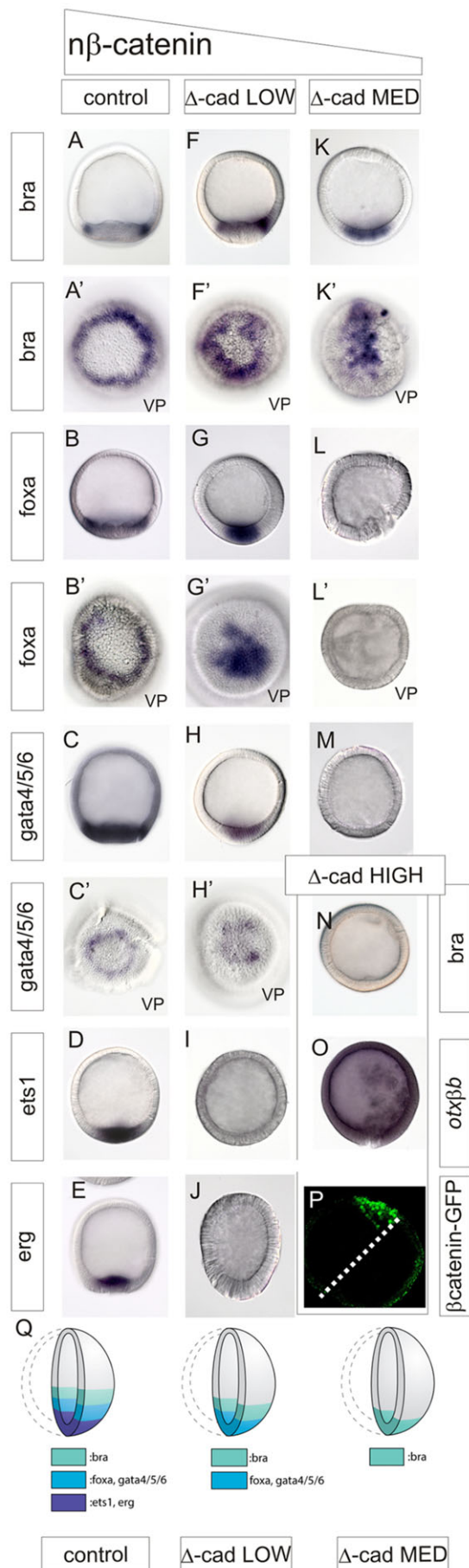


Fig. 4. Progressive loss of mesoderm, then of endoderm, with decreasing levels of n̄-catenin.

Localization of transcripts shown boxed on right or left as visualized by WMISH in 24-h-blastulae that were injected at single cell-stage, with GFP as a control (A–E), low levels of Δ-cadherin (F–J), medium levels of Δ-cadherin (K–M) or high levels of Δ-cadherin (N,O). (P) A 24-h-embryo that was injected at the one-cell stage with β-catenin:GFP and additionally in a single cell at the two-blastomere stage with high levels of Δ-cadherin. GFP is visualized at 24 h by confocal microscopy and shows vegetal n̄-catenin in one half of the embryo and complete loss of n̄-catenin in the half of the embryo that developed from the Δ-cadherin-injected blastomere. Embryos are depicted in a lateral view, unless indicated by vp for vegetal pole view. (Q) Schematic showing the effects of different levels of Δ-cadherin on vegetal territory specification.

et al., 1996). The appropriate amount of Δ-cadherin transcripts to use in these experiments was empirically determined by examining the phenotype of gastrula larvae expressing varying levels of the construct. When Δ-cadherin is injected at low levels (250 ng/μl), there is no or a greatly reduced mesodermal bulb and the archenteron is somewhat reduced (supplementary material Fig. S2). At medium levels (500 ng/μl) of introduced Δ-cadherin, there is no mesoderm, and a greatly reduced endoderm (supplementary material Fig. S2). At the highest levels of Δ-cadherin expression (750 ng/μl), we observe no gastrulation and no endomesoderm formation (Yankura et al., 2013).

Next, we assessed the effects of low and medium levels of Δ-cadherin on gene expression in 24-h-blastulae. Strikingly, when a low dose of Δ-cadherin is injected, *bra* expression is contracted towards the vegetal pole, although expression remains in a torus (Fig. 4F,F'; compare with Fig. 4A,A'). *foxa* and *gata4/5/6* remain expressed but are now localized to a domain that encompasses the central vegetal pole (Fig. 4G–H'; compare with Fig. 4B–C'). Expression of the mesodermal markers *ets1* and *erg* is entirely lost (Fig. 4I,J; compare with Fig. 4D,E). Thus, at a low dose of Δ-cadherin, there is a loss of mesodermal markers, and a vegetal retraction of the endoderm and veg1 territory markers (summarized in Fig. 4Q).

When Δ-cadherin is injected at medium levels, the expression of the veg1 marker *bra* is localized within the vegetal pole region of the embryo (Fig. 4K,K'; compare with Fig. 4A,A'), and expression of *foxa* and *gata4/5/6* is lost entirely (Fig. 4L–M; compare with Fig. 4B–C; summarized in Fig. 4Q). At the highest levels of Δ-cadherin injected, expression of *bra* is also lost (Fig. 4N; compare with Fig. 4A) and the ectodermal markers *onecut* and *otxβb* are now expressed throughout the resultant blastula (Hinman et al., 2003a; Yankura et al., 2013) (Fig. 4O). We visually inspected n̄-catenin:GFP in embryos overexpressing Δ-cadherin to confirm that the domain of nuclearization was also reduced (Fig. 4P). These patterns of gene expression changes are largely maintained through to the gastrula stage, reflecting the continued loss of these expression domains (supplementary material Fig. S3).

As blocking the nuclearization of β-catenin inhibited endomesoderm development in a dose-dependent manner, we wanted to determine whether increased n̄-catenin also has a graded effect on sea star endomesoderm development. Embryos were bathed in high (50 mM) or medium (30–40 mM) concentrations of LiCl, and the expression of *ets1*, *foxa* and *bra* was assessed at 24 h. Levels of LiCl used were also determined empirically by observing morphological effects on gastrulae (supplementary material Figs S4 and S5). At medium levels of LiCl, *bra* expression extends throughout the animal ectoderm, whereas *foxa* is expressed in a torus abutting this (Fig. 5C; compare with Fig. 5A). Both *bra* and *foxa* are expressed in concentric domains that extend further from the vegetal pole than in controls. The *ets1* expression domain remains associated with the vegetal pole but is

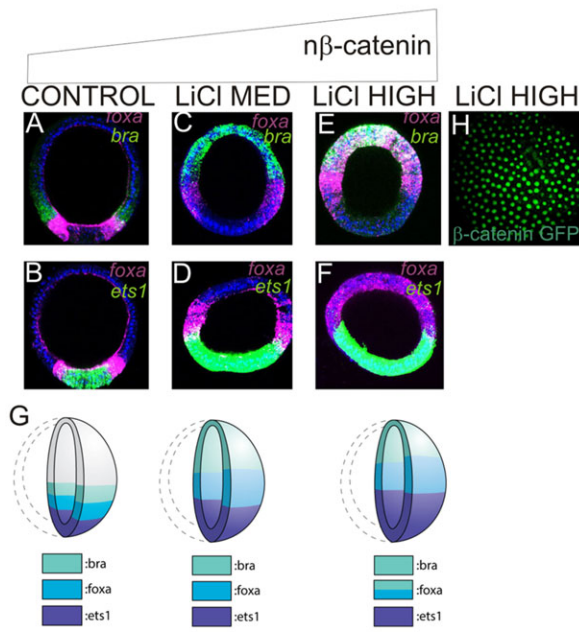


Fig. 5. LiCl dose-dependent expansion of endoderm and mesoderm territories. FISH of pairs of transcripts shown in top right in NaCl (control)-treated (A,B), and in embryos treated with medium (C,D) or high (E,F) doses of LiCl. (G) Gene expression patterns in late blastulae treated with increasing doses of LiCl. (H) Confocal image of β -catenin:GFP in high LiCl-treated late blastula showing extensive expansion of nuclear β -catenin. All embryos are shown at the late blastula stage in the lateral view.

expanded further toward the animal pole than in untreated embryos (Fig. 5D; compare with Fig. 5B). When embryos were treated with high levels of LiCl, the domains of *bra* and *foxa* expression were expanded even further toward the animal pole and, notably, are observed to overlap in the animal-most region of the embryo, although expression of *foxa* extends further toward the vegetal pole than that of *bra* (Fig. 5E). *ets1* expression remains within the vegetal pole, but this domain is even broader than at medium LiCl treatments (Fig. 5F; compare with Fig. 5D). We further confirmed that these changes in gene expression are maintained during gastrulation (supplementary material Fig. S5). In each of these perturbations, the *ets1*- and *foxa*-expressing territories remain distinct, which suggests that a robust mechanism functions to segregate mesoderm and endoderm cell fates. This is consistent with our later observation that an Ets1-driven GRN sub-circuit establishes the mesoderm/endoderm boundary (see below). The results are summarized in Fig. 5G. We also confirmed that the domain of β -catenin, as visualized using the β -catenin:GFP fusion construct, is greatly expanded in LiCl-treated embryos (Fig. 5H). Taken together, the results summarized in Fig. 4Q and Fig. 5G suggest that the increase in β -catenin transcriptional activity through time leads first to the expression of at least some genes that will later be restricted to the presumptive veg1 and endoderm territories, and last to those expressed in the presumptive mesoderm.

FoxA repressive circuitry distinguishes mesoderm and endoderm territories

So far, our model explains the establishment of overlapping *foxa*-expressing endoderm and *ets1*-expressing mesoderm territories through the 20-h mid-blastula stage. However, *foxa* and *gata4/5/6* are subsequently cleared from the central vegetal pole territory. Previous work has shown that FoxA represses its own expression within the mesoderm, as *foxa* transcripts are not cleared from the

mesoderm-fated territory in FoxA morphants (Hinman et al., 2003b). We are now able to extend this model to explain why this auto-repression only occurs in the mesoderm. At least two mesodermally restricted factors, *gata1/2/3* and *foxg*, both of which are first expressed between 16 h and 20 h, require normal FoxA function for their expression (Fig. 6A,D; compare with Fig. 6C,F). Ets1 function is additionally required for the expression of both these genes (Fig. 6A,D; compare with Fig. 6B,E), so that the combinatorial regulation drives their expression solely in the mesoderm-fated territory. That is, *foxg* and *gata1/2/3* require an input from FoxA at a time when it is expressed broadly through the vegetal pole, but are restricted to the mesoderm due to an additional requirement for an input from Ets1. We envision that Gata1/2/3 or FoxG then function to repress *foxa* and, possibly, *gata4/5/6* expression specifically from the presumptive mesoderm. This clears the endodermal factors from the mesoderm-fated territory. According to this model, the expansion of *foxa* into the central vegetal pole territory in FoxA morphants is due to the loss of *gata1/2/3* or *foxg*, as the product of one of these genes is what represses *foxa* expression. As these scenarios have not been explicitly tested, the gene that represses *foxa* expression in the mesoderm is referred to as *geneX* in our model, and it might also be another factor that is regulated similarly to *gata1/2/3* and *foxg*. These data are summarized in the GRN circuit shown in Fig. 6G.

Delta/Notch signaling maintains endoderm specification in late blastulae

The expression of *ets1* and *erg* in the mesoderm, and of *foxa* and *gata4/5/6* in the endoderm, are a consequence of the spatiotemporal dynamics of β -catenin nuclearization and its activity level-dependent gene regulation. A central tenet of this model is that *ets1* and *erg* expression are confined to only the central-most vegetal pole territory. This is due to the spatial restriction of β -catenin to only this region of the embryo by the time sufficient β -catenin activity is achieved to drive expression of these genes. The endoderm-fated territory consequently becomes an β -catenin-free domain shortly after its specification. This suggests that, at around the 20-h blastula stage, there is a transition from maternally initiated β -catenin-mediated regulation to a zygotically driven regulatory program in the endoderm. Expression of three genes in this study – *foxg*, *gata1/2/3* and *hex* – is first detected at 20 h (Fig. 1); of these genes, the first two are expressed in the presumptive mesoderm, whereas *hex* is expressed throughout the endomesoderm (Figs 2 and 7; Hinman and Davidson, 2007). We therefore questioned how *hex*, which is initially expressed at 20 h, could be expressed in both the endoderm and mesoderm at a time when β -catenin is only present in the mesoderm-fated territory.

To determine how *hex* expression might be regulated by β -catenin, we first assessed how *hex* expression responds to Δ -cadherin perturbations. Strikingly, *hex* expression is lost throughout the endomesoderm territory in embryos expressing low levels of Δ -cadherin (Fig. 7A–D). Thus, *hex* does not respond to Δ -cadherin in the manner that we describe for *foxa* and *gata4/5/6* (Fig. 4), as expression of these factors is first lost in medium levels of Δ -cadherin expression. An explanation for the dependence of *hex* on high β -catenin activity is that its expression requires the correct formation of the *ets1*- and *erg*-expressing mesoderm domain. We therefore examined more carefully the regulation and function of *hex* to understand which other regulatory events might be directing the formation of endoderm and mesoderm in 20-h embryos.

hex expression is lost throughout the endomesoderm in Ets1, Erg and Gata4/5/6 morphants, but not in FoxA morphants (Fig. 7E–H).

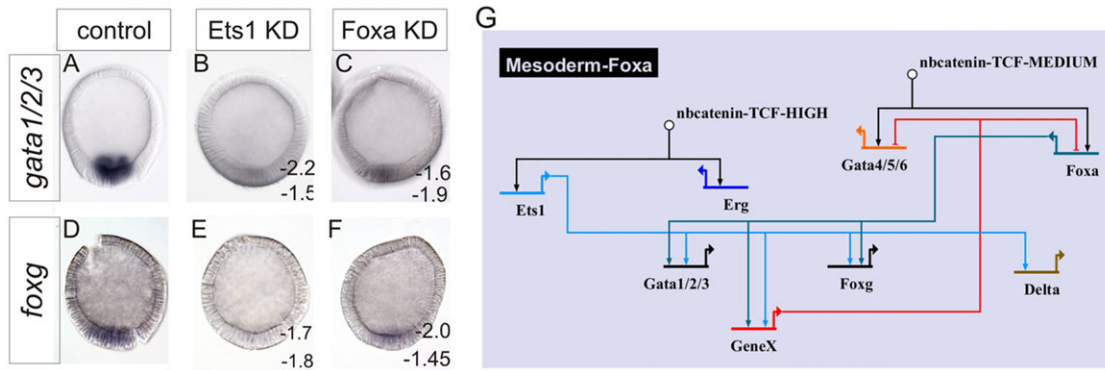


Fig. 6. Mesoderm and endoderm segregation is achieved downstream of FoxA dual-positive/repressive circuitry. WMISH visualization of *gata1/2/3* (A-C) or *foxg* (D-F) expression in 24-h-blastula in control MASO- (A,D), Ets1 MASO- (B,E) or FoxA MASO-injected embryos (C,F). (G) BioTapestry (Longabaugh, 2012) schematic detailing the GRN representation of these data. Numbers are ΔC_T measurements from two independent qPCR analyses of control minus perturbation, as indicated in the figure. A twofold difference, i.e. $\Delta C_T > \pm 1.6$, is considered significant.

At the time of *hex* activation (20 h), *ets1* and *erg* expression is already restricted to the mesoderm, whereas *gata4/5/6* remains expressed throughout the endomesoderm (Fig. 2; supplementary material Fig. S1); (McCauley et al., 2012). The requirement for Gata4/5/6 in particular, which is first expressed between 12 h and 16 h, can explain the later activation of *hex* and its expression throughout the endomesoderm. The loss of *hex* expression throughout

the endomesoderm in Ets1 and Erg morphants, however, further requires that our model incorporates a mesoderm-derived signal for correct *hex* expression. Previous work has shown that a Delta/Notch signal is needed for the expression of *gata4/5/6* and, to a lesser extent, *foxa* in the endoderm (Hinman and Davidson, 2007). Therefore, we tested whether *hex* expression in the endoderm requires a Delta/Notch input. Embryos bathed in the γ -secretase

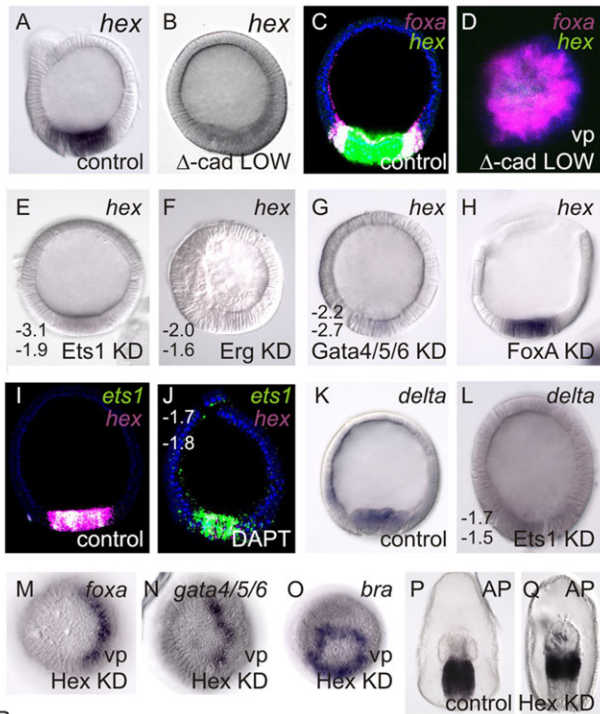
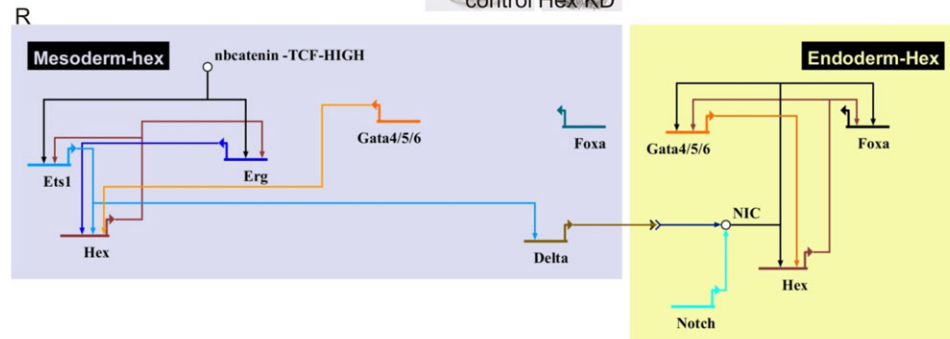


Fig. 7. Delta/Notch regulation of *hex* is required to maintain endoderm specification. (A-O) WMISH localization of transcripts shown in the top right; C, D, I and J show FISH. Perturbation type or control is shown in bottom right. Numbers are ΔC_T measurements from two independent qPCR analyses of control minus perturbation, as indicated in the figure. A twofold difference, i.e. $\Delta C_T > \pm 1.6$, is considered significant. M and N show half-morphant blastulae with the morphant half to the left. (P,Q) Staining of endogenous alkaline phosphatase, which is a marker of endoderm specification (Hinman et al., 2003a). All embryos are late blastulae, shown in a lateral view unless indicated by vp for vegetal pole view. KD, antisense morpholino oligonucleotide-based knockdown. (R) Results are summarized in a BioTapestry GRN wiring diagram.



inhibitor DAPT, which prevents activation of Notch, showed a significant reduction in *hex* expression in the endoderm and mesoderm (Fig. 7I,J). *delta* expression, in turn, is significantly reduced in *Ets1* morphants (Fig. 7K,L). Together, these data explain the loss of *hex* expression in *Ets1* morphants and low Δ -cadherin-expressing embryos. A functional *ets1*-expressing mesoderm is required for the expression of *delta*, and Delta/Notch signaling is in turn required for the expression of *hex* in the endoderm. As Hex is required for mesoderm development (McCauley et al., 2010), these data also suggest that Delta signaling has a role in mesoderm formation through the regulation of *hex*.

To better understand the role of Delta/Notch signaling in endoderm development, we next examined the function of Hex in this process. Although *hex* is the last gene activated in the endomesoderm that was analyzed in this study, Hex is required for expression of *ets1* and *erg* in the mesoderm (McCauley et al., 2010) and, as we show now, for the expression of *gata4/5/6* and *foxa* in the endoderm (Fig. 7M,N). Hex, however, does not regulate expression of the *veg1*-restricted *bra* (Fig. 7O). This is consistent with the phenotype of Hex morphant gastrulae, which show impaired endoderm differentiation (as assayed by alkaline phosphatase staining) and a reduction in mesoderm, but maintenance of posterior endoderm (Fig. 7P,Q). Therefore, Hex is engaged in positive feedback between *ets1* and *erg* in the mesoderm (McCauley et al., 2010) and *gata4/5/6* in the endoderm; that is, *Ets1*, *Erg* and *Gata4/5/6* regulate *hex* expression and, in turn, are regulated by Hex. In this model, Delta/Notch, signaling through the activation of *hex*, does not function to induce *gata4/5/6* in the endoderm, but instead to maintain its expression during the period in which β -catenin is being lost from the presumptive endoderm.

DISCUSSION

The data presented here are compiled into a GRN (Fig. 8). Although incomplete, this model explains the dynamic regulatory states that we have described for endomesoderm development in the sea star

blastula. A dose-dependent response to the changing spatiotemporal transcriptional activity of β -catenin functions to segregate territories within the endomesoderm. Low levels of β -catenin activity initiate the expression of *bra*; intermediate levels that of *gata4/5/6* and *foxa*; whereas the highest levels, which are only achieved when β -catenin is restricted to the central-most vegetal plate, drive zygotic expression of *ets1* and *erg*. These data provide a mechanistic explanation for the embryonic manipulations which showed that a loss of endomesoderm occurs when the vegetal pole of sea star eggs is depleted. Furthermore, defects in mesoderm development occurred at smaller depletion volumes than are required to abrogate endomesoderm formation (Kuraishi and Osanai, 1994). We hypothesize that a vegetally localized maternal determinant is required for the nuclearization of β -catenin. Depletion of a small proportion of the vegetal pole reduces the amount of this determinant, such that only low levels of β -catenin accumulate, resulting in defects in mesoderm development. However, when most of the determinant is removed, along with a larger volume of the vegetal pole, β -catenin is not nuclearized at sufficiently high levels to initiate endoderm development. In sea urchins and cnidarians, a cortical restriction of Dishevelled mediates the stabilization of β -catenin in the vegetal pole during early cleavage (Weitzel et al., 2004; Wikramanayake et al., 2003). Our data are also consistent with this model, and we hypothesize that Dishevelled or another component of the canonical Wnt signaling pathway act as this maternal determinant in sea stars, and might represent a conserved mechanism among metazoans.

This work presents the first empirical demonstration of a changing spatiotemporal profile of β -catenin transcriptional activity that functions in a dose-dependent manner to segregate territories along the primary axis of an invertebrate. It has been proposed that a gradient of β -catenin is the ancestral mechanism used to pattern the primary axis (Niehrs, 2010). However, no evidence for a long-range gradient of β -catenin has been found in any invertebrate (Darras et al., 2011; Röttinger et al., 2012).

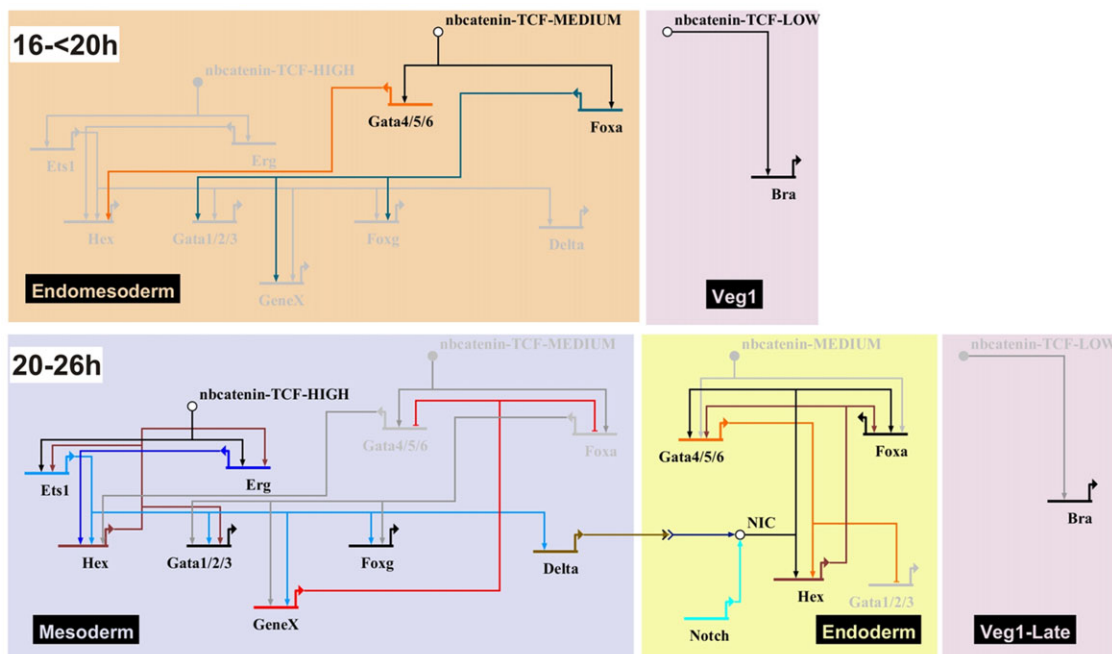


Fig. 8. The GRN for endomesoderm specification in the sea star *P. miniata*. A GRN model developed in BioTapestry, depicting regulation of gene expression in the endomesoderm. Arrowheads represent positive regulation; bars represent repression. Genes are depicted on colored background according to their expression domains. Gray genes and lines are not active at the time shown. Gene names are as described in text. NIC, Notch intracellular domain.

Although we demonstrate a dose-dependent response to β -catenin activity in territory specification in sea star embryos, this does not arise through a response to a long-range spatial gradient. Therefore, the formation of a long range β -catenin gradient in axial patterning might be unique to vertebrate embryos. Given the widespread occurrence of β -catenin localization to one pole in early invertebrate embryogenesis (Darras et al., 2011; Henry et al., 2008; Hudson et al., 2013; Logan et al., 1999; Röttinger et al., 2012; Thorpe et al., 1997), and especially in light of the increased transcriptional activity of β -catenin over time as seen in sea stars (Fig. 3) and sea urchins (Lhomond et al., 2012), we suggest that a dose-response to changing β -catenin activity is a broad phenomenon among invertebrates.

GRN subcircuits established by β -catenin segregate endoderm and mesoderm

In early sea star embryos, low levels of β -catenin transcriptional activity establish a vegetal pole domain in which the complement of genes that will later be exclusively expressed in endoderm or veg1 are expressed. *foxa*, which is initially expressed in this broad domain, and *ets1*, which is zygotically expressed specifically in the mesoderm, coordinately activate mesodermally restricted factors. We hypothesize that these, in turn, repress *foxa* expression in the mesoderm. Thus, a positive/-negative-feedback circuit operates to clear *foxa*, and, probably, *gata4/5/6*, from the mesoderm. Our model also predicts that *hex* expression is regulated by Delta/Notch signaling. In addition to its previously characterized role in mesoderm maintenance, *hex* is engaged in a positive-feedback loop in the endoderm, i.e. *hex* regulates and is regulated by *gata4/5/6*. Positive feedback in developmental GRNs has been proposed to function as a mechanism to ‘lock down’ or stabilize regulatory states (see e.g. Davidson and Erwin, 2006; Hinman and Cheate Jarvela, 2014; Hinman et al., 2009). These types of regulatory interactions are therefore predicted to be especially prevalent downstream of transient signaling events (Davidson and Erwin, 2006). The time frame of this function of Hex coincides with the loss of β -catenin from the endoderm-fated territory. We therefore propose that, as predicted by these theories of GRN control, positive feedback between *hex* and *gata4/5/6* in the endoderm functions to maintain *gata4/5/6* and *foxa* expression, as the regulation of this territory changes from maternally driven β -catenin-dependent to a zygotic GRN.

A change in β -catenin localization or activity is implicated in endomesoderm segregation in several taxa. In sea urchins, a Delta signal from the micromeres promotes nuclear export of TCF, the binding partner of β -catenin, in adjacent cells that will be fated to mesoderm (Röttinger et al., 2006). Loss of TCF extinguishes *foxa* expression and thereby suppresses the activation of the endoderm GRN in this territory (Oliveri et al., 2006; Sethi et al., 2012; Ben-Tabou de-Leon and Davidson, 2010). In ascidians, β -catenin is present in blastomeres fated to endomesoderm at the 16-cell stage, but are restricted to the presumptive endoderm one cell division later. The loss of β -catenin from the presumptive mesoderm is required for the development of this territory (Hudson et al., 2013). This has been proposed to function as an ‘ON/OFF’ type mechanism of β -catenin activity, whereby endoderm segregates from mesoderm due to this binary change β -catenin. *Platynereis dumerilii* also has a reiterative binary ‘ON/OFF’ pattern in β -catenin accumulation in vegetal blastomere cell fate choices, although this is not related to endomesoderm segregation (Schneider and Bowerman, 2007). The lophotrochozoan nemertean worm *Cerebratulus lacteus* also shows a pattern in which β -catenin is present in the endomesoderm-fated

blastomeres, but is retained only in the endoderm progenitors one cell division later (Henry et al., 2008). Common to ascidians, sea urchins and nemerteans, therefore, is that loss of β -catenin occurs in the mesoderm lineage, whereas continued β -catenin nuclearization is a feature of the presumptive endoderm. This is distinctly different from what we report for the sea star, *P. miniata*, in which β -catenin is clearly lost from the presumptive endoderm and maintained in the mesoderm-fated cells. β -catenin is shown to be localized to the nuclei of mesoderm progenitors in blastula and even maintained during gastrulation in a closely related sea star, *Asterina pectinifera* (Miyawaki et al., 2003). Our data are consistent with a model of ‘ON/OFF’ β -catenin function operating in the endoderm, whereas an ‘ON/ON’ mechanism operates in the mesoderm. In this scenario, we hypothesize that the maintained ‘ON’ state in the mesoderm causes the increase in transcriptional activity measured by our luciferase assays, therefore generating a spatiotemporal gradient of activity that then functions to segregate territories.

Thus, changing the localization of β -catenin is a common mechanism for segregation of endoderm and mesoderm in at least nemerteans, echinoderms and ascidians, although the territory from which it is lost in each organism is different. This implies that β -catenin might have a common role in fate choices that distinguish these two territories, although whether the presence or absence of β -catenin drives endoderm or mesoderm is evolutionarily labile. Our results extend the use of β -catenin dose responses to establish territories along the primary axis from vertebrates to invertebrates, although the means of forming this gradient are not necessarily conserved and many of its other features are also different.

MATERIALS AND METHODS

Embryo culture

Embryos were cultured and injected following the protocol by Cheate Jarvela and Hinman (2014).

Time-course quantitative RT-PCR (qPCR) analysis

RNA was extracted, cDNA synthesized and qPCR performed as previously described (Hinman et al., 2003b), except that the number of transcripts was normalized to *lamin2 β receptor* (GenBank ID: 1728045; McCauley et al., 2013). See supplementary material for primer sequences. The transcript number was calculated as described (Wang et al., 1995) and genes were considered as expressed if >100 transcripts were present per embryo.

Whole-mount *in situ* hybridization (WMISH) and two-color fluorescent *in situ* hybridization (FISH)

Embryos were fixed and WMISH was performed as previously described (McCauley et al., 2010). Two-color FISH was performed as described (Yankura et al., 2010).

Preparation of mRNA constructs

mRNA was synthesized with the mMessage mMachine kit (Life Technologies) using the pBluescript- Δ -cadherin plasmid (Logan et al., 1999) and the *pCS2+ β -catenin:GFP* fusion construct (Weitzel et al., 2004) as templates.

Perturbation of gene expression

Zygotes were injected with mRNA or morpholino antisense oligonucleotides (MASOs; GeneTools) following the protocol by Cheate Jarvela and Hinman (2014). In some cases, a single blastomere was injected following the first embryonic cleavage. As the first cleavage plane maps to the plane of bilateral symmetry, this will generate half-normal/half-perturbed embryos. See supplementary material for MASO sequences (Hinman and Davidson, 2007; Hinman et al., 2003b; McCauley et al., 2010). For all MASOs, the

GeneTools standard control MASO was injected into sibling embryos. For Δ -cadherin mRNA injections, sibling embryos were injected with eGFP mRNA as a control. Li^+ perturbations were achieved by bathing embryos in 20–50 mM LiCl or 20–50 mM NaCl as a control from the two-cell stage. Notch perturbations were achieved by bathing embryos in 64 μM DAPT (Materna and Davidson, 2012) or DMSO as a control from the two-cell stage. WMISH was performed on at least three independent sets of perturbed embryos. At least ten embryos were assessed in each replicate and phenotypes were highly consistent, with >90% showing the reported phenotype. Quantitative measures of perturbation were achieved by performing qPCR on perturbed compared with control siblings. Each assay was performed on at least two qPCR replicates in each of two biological replicates.

Analysis of β -catenin nuclearization

β -catenin:GFP mRNA was injected into embryos and subcellular localization was assayed by confocal microscopy. The TOPFlash β -catenin-inducible luciferase reporter (Addgene plasmid 12456) (Veeman et al., 2003) was linearized and injected into embryos. *luciferase* abundance was determined using qPCR and normalized to endogenous expression levels of the *lamin2 β receptor*. Whereas significant *luciferase* expression was detected, expression was always lower than that of highly abundant *lamin2 β receptor* and is therefore reported as a negative number. Increasing ΔC_T values indicate higher *luciferase* expression. TOPFlash-injected embryos were co-injected with eGFP to control for batch variation in injection size bolus, and it was confirmed that GFP levels were equivalent across batches using qPCR on these samples.

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Competing interests

The authors declare no competing financial interests.

Author contributions

B.S.M. and V.F.H. designed research; B.S.M., V.F.H., E.A. and H.R.S. performed research; and B.S.M. and V.F.H. analyzed data and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.113043/-DC1>

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