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Sequential Signaling Crosstalk Regulates Endomesoderm Segregation in Sea Urchin Embryos

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The segregation of embryonic endomesoderm into separate endoderm and mesoderm fates is not well understood in deuterostomes. Using sea urchin embryos, we showed that Notch signaling initiates segregation of the endomesoderm precursor field by inhibiting expression of a key endoderm transcription factor in presumptive mesoderm. The regulatory circuit activated by this transcription factor subsequently maintains transcription of a canonical Wnt (cWnt) ligand only in endoderm precursors. This cWnt ligand reinforces the endoderm state, amplifying the distinction between emerging endoderm and mesoderm. Before gastrulation, Notch-dependent nuclear export of an essential β-catenin transcriptional coactivator from mesoderm renders it refractory to cWnt signals, insulating it against an endoderm fate. Thus, we report that endomesoderm segregation is a progressive process, requiring a succession of regulatory interactions between cWnt and Notch signaling.

Early endomesoderm induction and subsequent segregation of endoderm from mesoderm are fundamental processes in animal development. Although initial endomesoderm specification has been studied extensively (*1*, *2*), its separation in deuterostomes is poorly understood. In several deuterostomes including vertebrates such as zebrafish and *Xenopus* and echinoderms such as sea urchins and sea stars (*3–7*), Notch signaling induces the expression of endoderm- or mesoderm-specific markers within an endomesoderm field. Although Notch might regulate endomesoderm segregation, it is unknown whether it alters the early endomesoderm signaling milieu, a change that is probably required to stabilize lineage identities. Canonical Wnt (cWnt) signaling probably establishes that precursor environment, given its ancestral role in specifying early endomesoderm (*8*). Elucidating the mechanisms underlying such a Notch-cWnt interaction would substantially advance our understanding of the progressive specialization of the endomesoderm.

Like other deuterostomes, sea urchin embryos are enriched asymmetrically in the cWnt signaling effector nuclear β-catenin (nβ-catenin) (*9*), which specifies endomesoderm precursors in three ways. First, it establishes an early endoderm regulatory state in a tier of vegetal blastomeres (veg_2, Fig. 1A) at cleavage stages (*9–11*). Second, in micromere descendants located immediately adjacent to the veg_2 tier (Fig. 1A), nβ-catenin induces expression of the ligand Delta (*12–14*), which signals through the Notch receptor in veg_2 blastomeres and activates mesoderm gene expression (*5*, *6*, *13*, *15*). Third, cWnt also makes veg_2 cells competent to receive the micromere Delta signal (*12*). Thus, specification of both endoderm and mesoderm is initiated by the blastula stage throughout veg_2 blastomeres (Fig. 1A) (*6*, *9*, *11*, *16*, *17*).

By the hatching blastula (HB) stage, veg_2 progeny form outer and inner rings of cells (Fig. 1A). Only inner veg_2 daughters adjacent to Delta-expressing micromere progeny can transduce the cell contact–dependent Notch signal and continue expressing mesoderm markers (*17*). Transcripts encoded by endoderm regulatory genes, in turn, are detected in outer veg_2 daughters by this time (*11*, *17*). Notch is required for this restriction because it inhibits expression of the endoderm markers *foxa*, *blimp1b*, and *dac* in inner veg_2 daughters (*17–19*). During cleavage and HB stages, nβ-catenin is detected throughout the veg_2 endomesoderm precursor field (*9*). By the mesenchyme blastula (MB) stage, 6 to 8 hours after endoderm marker expression first clears from inner veg_2 daughters, nβ-catenin is down-regulated in these mesoderm precursors through a process requiring Notch (*9*, *20*, *21*). Thus, Notch probably plays a substantial role in endomesoderm segregation beyond merely activating mesoderm regulatory genes. However, several major questions remain unanswered. First, how does Notch restrict endoderm fate to a subset of the endomesoderm progenitor field? Second, how does Notch inhibit endomesoderm-inducing cWnt in presumptive mesoderm (*9*, *21*) 6 to 8 hours after initial endoderm marker expression has disappeared? Third, are these Notch-dependent events mechanistically linked?

To understand initial Notch-dependent restriction of endoderm potential from mesoderm, we used Notch-deficient embryos to systematically assess the expression of each gene in the early endoderm gene regulatory network (GRN) (fig. S1A), which represents cWnt-induced endoderm specification until the HB stage (*10*, *11*). We found that *hox11/13b*, *brachyury*, *blimp1b*, and *foxA* transcripts accumulated ectopically in

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inner veg_2 descendants when Notch was knocked down (fig. S1, B to E and I to L). Because only part of the cWnt-activated early endoderm GRN is thus subject to Notch inhibition (fig. S1, F to H and M to O), Notch does not initially modulate upstream cWnt signaling. Instead, it probably inhibits an intermediate endoderm GRN factor(s) activated in endomesoderm precursors by the earlier cWnt input. Such a mechanism would also allow mesoderm induction in veg_2 progeny, which require cWnt for competence to transduce the Notch signal (*12*). Furthermore, because *brachyury*, *blimp1b*, and *foxA* are Hox11/13b target genes (Fig. 1, B to I) (*11*), we hypothesized that Notch could initiate endoderm restriction simply by suppressing *hox11/13b* expression in mesoderm. We confirmed this through two observations. First, *brachyury*, *foxa*, and *blimp1b* are not expressed ectopically in inner veg_2 descendants (Fig. 1, K to M versus O to Q) in Notch-deficient embryos that also lack Hox11/13b. Second, Notch-suppressed endoderm genes still accumulate ectopically in inner veg_2 descendants in double morphants that lack both Notch and any one of Brachyury, FoxA or Blimp1b (figs. S2 to S4). Thus, Notch initially suppresses mesodermal accumulation of a single endoderm transcription factor, Hox11/13b. This prevents ectopic activation of the Hox11/13b-dependent regulatory circuit, consisting of Brachyury, Blimp1b, and FoxA, despite the presence of nβ-catenin in the mesoderm (*9*).

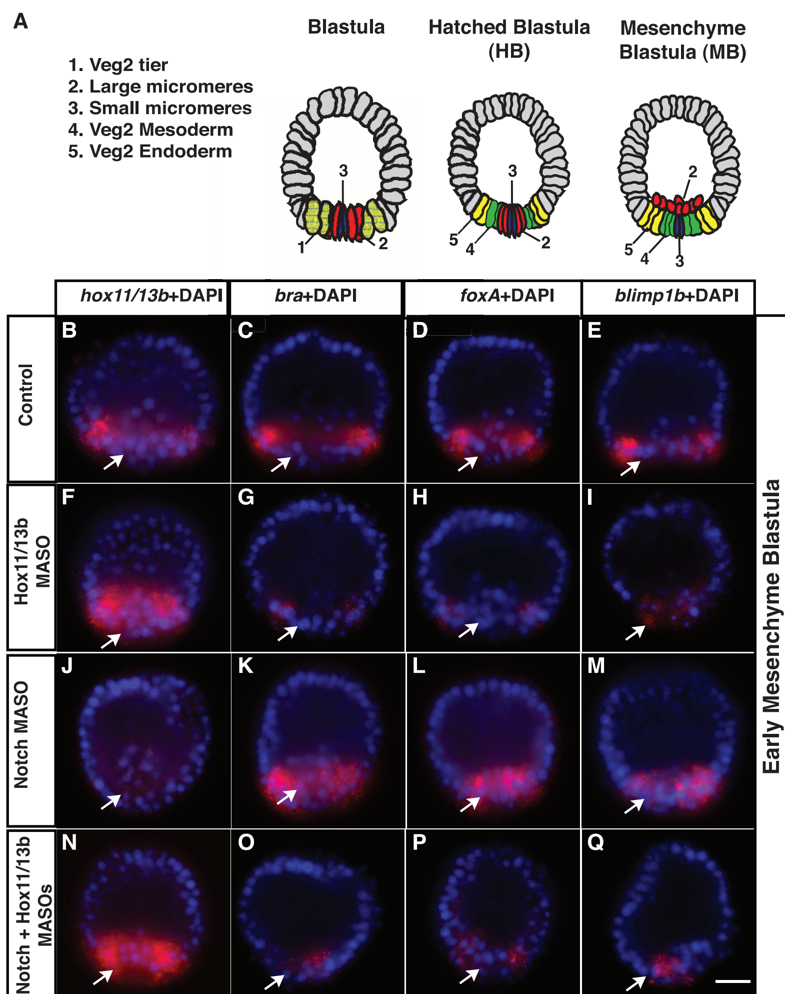

Fig. 1. Notch inhibits a Hox11/13b-dependent regulatory circuit in mesoderm. (**A**) Diagram of endomesoderm blastomeres: Large (red) micromere progeny are adjacent to veg_2 cells in which early cWnt activates endoderm and mesoderm specification (hatched light green) in early blastulae. By the HB stage, veg_2 descendants form outer (presumptive endoderm, yellow) and inner (presumptive mesoderm, green) rings. Large micromere progeny (red) ingress by the MB stage. (**C** to **E**) versus (**G** to **I**) Endodermal *brachyury (bra)*, *foxa*, and *blimp1b* transcripts are down-regulated in Hox11/13b morphants (MASO). Arrows indicate inner veg_2 cells (presumptive mesoderm). DAPI, 4´,6-diamidino-2-phenylindole stain. (**B**), (**F**), and (**N**) *hox11/13b* mRNA is up-regulated in Hox11/13b morphants because Hox11/13b represses its own transcription (*11*). (**K**) to (**M**) Ectopic expression of *brachyury (bra)*, *foxa*, and *blimp1b* mRNA occurs in inner veg_2 cells of Notch morphants. (**J**) *hox11/13b* mRNA concentration is low through autorepression by ectopic Hox11/13b protein (*11*). (**O**) to (**Q**) In Notch+Hox11/13b double morphants, ectopic expression of Hox11/13b target genes in inner veg_2 cells (arrows) is significantly lower (Q). Scale bar, 20 μm.

We hypothesized that Notch clears nβ-catenin from mesoderm by the MB stage (*9*, *21*) by inhibiting expression of a cWnt ligand in this lineage, and we identified *wnt1* as a candidate through two observations: First, like nβ-catenin, *wnt1* mRNA is detected throughout veg_2 endomesoderm (fig. S5) between the HB and MB stages, and is down-regulated through Notch in mesoderm by the MB stage (Fig. 2, A to D) (*9*, *21*). Second, at the MB stage, Wnt1 stimulates nβ-catenin activity, as revealed by cWnt-dependent TOPFLASH (*22*) luciferase reporter assays (Fig. 2E). Because *wnt1* expression is restricted to the endoderm 6 to 8 hours after Notch confines the expression of Hox11/13b-activated regulatory genes to this lineage, we assessed the potential linkage between these events. Both endodermal *wnt1* expression in normal embryos at the MB stage and its persistence in mesoderm in the absence of Notch require Hox11/13b and its target, Brachyury (Fig. 2, F to K). These *wnt1* regulators are also essential for normal levels of cWnt activity at this time (Fig. 2E). Thus, by inhibiting mesodermal *hox11/13b* expression at the HB stage, Notch indirectly restricts *wnt1* accumulation to the endoderm, where it probably contributes to maintaining cWnt activity.

Because the endoderm GRN depends on cWnt (*11*, *18*, *23*), we hypothesized that ligands activating cWnt signaling stabilize the endoderm regulatory state and amplify its distinction from mesoderm. Consistent with this idea, two different Wnt1 morpholinos interfere with endodermal expression of *hox11/13b*, *brachyury*, *foxa*, and *blimp1b* (Fig. 2, L to S, and fig. S6) at the MB stage and substantially delay gastrulation (fig. S7). Additionally, Notch-mediated *wnt1* down-regulation could help deplete nβ-catenin in the mesoderm (*9*), leading to repression of cWnt-sensitive endoderm genes through its transcriptional binding partner T cell factor (TCF) (*24*). This would further reinforce endomesoderm segregation, because TCF represses at least one endoderm factor, FoxA, in the mesoderm at the MB stage (*18*). Other Wnt ligands, including Wnt6 and Wnt8, which are required for endoderm specification (*25*–*27*), might also contribute to endomesoderm separation by increasing cWnt signaling. Consistent with the timing of its restriction to endoderm, Wnt1 reinforces endoderm segregation only at the MB stage. It does not affect its initial restriction at the HB stage because Notch-deficient embryos lacking Wnt1 protein still show ectopic expression of Hox11/13b target genes in inner veg_2 descendants (fig. S8).

Delta-Notch signaling can also inhibit cWnt activity in sea urchin embryos by activating Nemo-like kinase (NLK) expression in veg_2 descendants (*28*). In other systems, NLK, a MAP kinase, modulates cWnt signaling by phosphorylating β-catenin's essential transcriptional coactivator, TCF, and promoting its nuclear export (*29*). The

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loss of TCF could eliminate cWnt signaling via any Wnt ligand in the mesoderm. We detected TCF protein in all nuclei until the late MB stage (fig. S9, A to C). However, just before gastrulation begins, 4 hours after the MB stage, TCF clears specifically from mesoderm nuclei (fig. S9) in a Notch- and NLK-dependent manner (Fig. 3, A to I), which strongly antagonizes cWnt in these cells.

In this study, we elucidated sequential Notch-dependent mechanisms that precisely regulate endomesoderm segregation (steps 1 to 3 in Fig. 3J). Notch initially restricts a transcription factor(s) to the endodermal daughters of endomesoderm precursors, where it subsequently activates cWnt. Such a mechanism could be shared with other deuterostomes, because Notch affects expression of *brachyury* and *foxA* during endomesoderm segregation in both echinoderm and vertebrate embryos (*3*, *4*, *7*, *17*–*19*). The regulatory device through which Notch indirectly modifies

Fig. 2. Through the Hox11/13b circuit, Notch indirectly restricts *wnt1* to endoderm, reinforcing its differentiation. (**A** to **D**) In Notch morphants, ectopic *wnt1* mRNA accumulates in inner veg_2 descendants (arrows). (**E**) cWnt signaling activity (TOPFLASH, materials and methods in supporting online material) is strongly reduced in Wnt1 (MASO or MASO2), Hox11/13b, or Brachyury morphants [error bars indicate ±SD from the mean (n = 3)]. At MB stage, Hox11/13b and Brachyury are necessary for (**F** to **H**) normal *wnt1* mRNA expression in endoderm and (**I** to **K**) its ectopic accumulation in inner veg_2 cells (arrows) of Notch morphants. (**L** to **S**) Endodermal MB expression of *hox11/13b*, *brachyury (bra)*, *foxa*, and *blimp1b* mRNA is reduced in Wnt1 morphants.

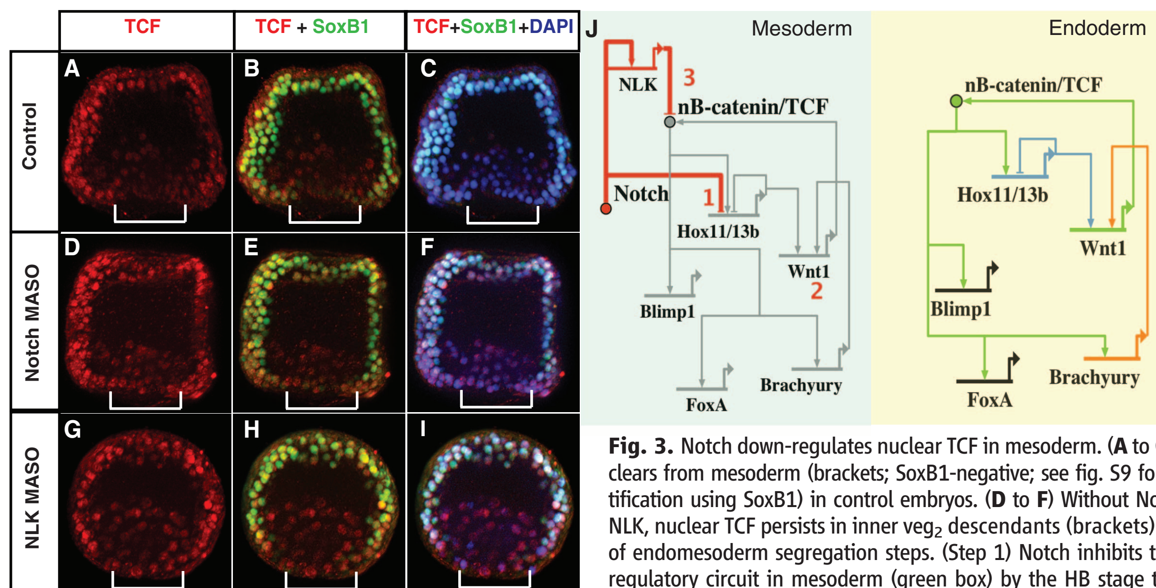

Fig. 3. Notch down-regulates nuclear TCF in mesoderm. (**A** to **C**) Nuclear TCF clears from mesoderm (brackets; SoxB1-negative; see fig. S9 for lineage identification using SoxB1) in control embryos. (**D** to **F**) Without Notch or (**G** to **I**) NLK, nuclear TCF persists in inner veg_2 descendants (brackets). (**J**) Summary of endomesoderm segregation steps. (Step 1) Notch inhibits the Hox11/13b regulatory circuit in mesoderm (green box) by the HB stage to initiate segregation. The Hox11/13b circuit stays active in endoderm (yellow). (Step 2) Consequently, by the MB stage, Hox11/13b- and Brachyury-dependent expression of *wnt1* is restricted to endoderm, where it reinforces cWnt signaling and endoderm fate. (Step 3) By the pre-gastrula stage, a Notch-NLK pathway clears nuclear TCF from mesoderm, making it unresponsive to cWnt. The endoderm retains nuclear TCF and cWnt sensitivity.

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the early endomesoderm cWnt signaling state could also be conserved, because Brachyury maintains transcription of cWnt ligands in both zebrafish and sea urchin embryos (*30*). Restricting Brachyury expression to either endoderm or mesoderm would also confine cWnt signaling. This could, in turn, reinforce lineage segregation, as seen with the Brachyury→Wnt1→endoderm and the Brachyury→cWnt→posterior mesoderm pathways in sea urchins and zebrafish, respectively (*30*). More generally, similar Notch-dependent mechanisms could modulate additional pathways such as Nodal/transforming growth factor–β that induce endomesoderm in vertebrate embryos (*31*). Finally, it is unknown whether Notch also insulates mesoderm or endoderm from incident cWnt signals through NLK activity in vertebrate embryos. We thus have uncovered a remarkable timing buffer that uses a cell contact–dependent signal to separate regulatory states within a broadly induced endomesoderm field without immediately altering its signaling milieu. This preserves the competence of each lineage and correctly institutes its specification. Individual lineage choices are then reinforced and cemented through successive signaling state changes.

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Growth of Western Australian Corals in the Anthropocene

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Anthropogenic increases of atmospheric carbon dioxide lead to warmer sea surface temperatures and altered ocean chemistry. Experimental evidence suggests that coral calcification decreases as aragonite saturation drops but increases as temperatures rise toward thresholds optimal for coral growth. In situ studies have documented alarming recent declines in calcification rates on several tropical coral reef ecosystems. We show there is no widespread pattern of consistent decline in calcification rates of massive *Porites* during the 20th century on reefs spanning an 11° latitudinal range in the southeast Indian Ocean off Western Australia. Increasing calcification rates on the high-latitude reefs contrast with the downward trajectory reported for corals on Australia's Great Barrier Reef and provide additional evidence that recent changes in coral calcification are responses to temperature rather than ocean acidification.

Coral growth is measurably influenced by the physical and chemical properties of the marine environment (*1*), which are changing rapidly owing to human interference in the global climate system (*2–4*). Emissions of CO_2 into the atmosphere from the combustion of fossil fuels, deforestation, and altered land use have resulted in current-day atmospheric CO_2 levels of around 390 parts per million (ppm), an increase of about 40% since preindustrial times. Increased concentrations of atmospheric CO_2 (along with other greenhouse gases) are associated with positive radiative forcing, which leads to a warming of the global climate system (*5*); about one-third of this extra CO_2 is taken up by the world's oceans (*6*). Oceanic uptake of anthropogenic CO_2 alters the seawater carbonate equilibrium by reducing both the pH and carbonate saturation states in the upper ocean layers (*2*, *7*) in a process known as ocean acidification (*8*). Reduced carbonate saturation state is expected to have profound effects on the calcification rates of a diverse range of marine calcifers, including reef-building corals (*4*, *9–11*). Warming of the tropical oceans is predicted to increase the frequency and severity of mass coral-bleaching events (*3*). Such changes in the marine environment are, therefore, likely to compromise coral calcification (facilitated by the coral-algal symbiosis), which forms the backbone of tropical coral reef ecosystems (*4*).

Annual density banding in certain massive corals allows retrospective analysis of historical calcification rates and inferences to be made about past environmental conditions and growth responses, including those before instrumental observations (*12*). Our study focused on coral reefs spanning an 11° latitudinal range in the southeast Indian Ocean to learn whether there have been any significant changes during the past 110 years in calcification rates on Australia's western coral reefs and how any observed changes relate to known changes in sea surface temperature (SST).

Twenty-seven long cores were collected, between October 2008 and September 2010, from massive *Porites* sp. colonies at six locations covering about 1000 km off the coast of Western Australia. Although some cores extend back to the 18th century, we focused on the period from 1900 to 2010, which was common to the majority (70%) of cores, to provide sufficient replication at each location and overlap with instrumental SST observations. The sampling locations included two reefs in the Rowley Shoals, Clerke Reef (17°16'S, 119°22'E) and Imperieuse Reef (17°31'S, 118°58'E); three locations within the Ningaloo Reef Tract, Bundegi (21°50'S, 114°11'E), Tantabiddi (21°54'S, 113°58'E), and Coral Bay (23°2'S, 113°49'E); and the Houtman Abrolhos Islands (28°28'S, 113°46'E) (Fig. 1). All sampled colonies were ≥2 m in height and selected from the leeward side of the reef or island at depths < 6 m below the lowest astronomical tide. Spatial and temporal variations in three annual coral growth parameters—annual extension (linear distance between adjacent density minima, cm $year^{-1}$), skeletal density (g cm^{-3}), and calcification

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Sequential Signaling Crosstalk Regulates Endomesoderm Segregation in Sea Urchin Embryos

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Lineage Identity
Segregation of endoderm and mesoderm lineages is a fundamental event in early embryogenesis. Using sea urchin embryos, **Sethi *et al.*** (p. 590) found that Notch and canonical Wnt pathways crosstalk progressively partitions the endomesoderm to discrete endoderm and mesoderm fates. This three-step signaling exchange demonstrates how the timing of signaling crosstalk is regulated in vivo through temporally and spatially staggered genetic circuits that intersect only at specific developmental stages.

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