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# Wnt6 activates endoderm in the sea urchin gene regulatory network

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## SUMMARY

In the sea urchin, entry of β-catenin into the nuclei of the vegetal cells at 4th and 5th cleavages is necessary for activation of the endomesoderm gene regulatory network. Beyond that, little is known about how the embryo uses maternal information to initiate specification. Here, experiments establish that of the three maternal Wnts in the egg, Wnt6 is necessary for activation of endodermal genes in the endomesoderm GRN. A small region of the vegetal cortex is shown to be necessary for activation of the endomesoderm GRN. If that cortical region of the egg is removed, addition of Wnt6 rescues endoderm. At a molecular level, the vegetal cortex region contains a localized concentration of Dishevelled (Dsh) protein, a transducer of the canonical Wnt pathway; however, Wnt6 mRNA is not similarly localized. Ectopic activation of the Wnt pathway, through the expression of an activated form of β-catenin, of a dominant-negative variant of GSK-3β or of Dsh itself, rescues endomesoderm specification in eggs depleted of the vegetal cortex. Knockdown experiments in whole embryos show that absence of Wnt6 produces embryos that lack endoderm, but those embryos continue to express a number of mesoderm markers. Thus, maternal Wnt6 plus a localized vegetal cortical molecule, possibly Dsh, is necessary for endoderm specification; this has been verified in two species of sea urchin. The data also show that Wnt6 is only one of what are likely to be multiple components that are necessary for activation of the entire endomesoderm gene regulatory network.

**KEY WORDS: Sea urchin, Wnt pathway, Gene regulatory networks, Wnt6, Dishevelled, β-Catenin, Maternal determinants, Endomesoderm**

## INTRODUCTION

The discovery that β-catenin is an early activator of endomesoderm specification provided a key observation that led to the assembly of a model endomesoderm gene regulatory network (GRN) in the sea urchin (Wikramanayake et al., 1998; Logan et al., 1999; Davidson et al., 2002; Oliveri et al., 2002). Many experiments expanded that early network model by adding a number of transcription factors and signals. Cis-regulatory analyses plus new mechanistic insights continue to update causal information on how embryonic sea urchin cells are specified and diversify (Davidson, 2006; Oliveri et al., 2006; Smith et al., 2007; Croce and McClay, 2010). Those early network models also stimulated interest in networks more broadly in other systems (Sandmann et al., 2007; Zeitlinger et al., 2007; Owraghi et al., 2009). A remaining gap in the sea urchin network is the issue of how the developmental program is initiated in the first place.

Boveri (Boveri, 1901) was among the first to observe that the endomesoderm originates from the vegetal half of the embryo, and studies with egg fragments experimentally confirmed that observation (Horstadius, 1927; Horstadius, 1928). More recent studies in the starfish showed that a relatively small region of the vegetal hemisphere contained the material necessary for endoderm specification (Kuraishi and Osanai, 1994). If that small vegetal region was removed, an archenteron failed to appear (Kiyomoto and Shirai, 1993). Those studies, and others like them, extending to vertebrates, strongly suggested that information near the vegetal pole was important for activating endomesoderm specification, but the identity of the active principle in the vegetal region remained unknown.

Experiments with β-catenin first suggested that the canonical Wnt pathway is involved in activation of the endomesoderm GRN (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999). Exploring this further, Weitzel et al. (Weitzel et al., 2004) showed that expressed β-catenin-GFP protein initially was found throughout the zygote, but over a short time, beginning at the 60-cell stage, was degraded in animal blastomeres and stabilized in vegetal blastomeres. The stabilization required another member of the Wnt pathway, the cytoplasmic effector Dishevelled (Dsh) (Weitzel et al., 2004). Though Dsh mRNA was ubiquitously distributed during cleavage, it was observed that an expressed Dsh-GFP protein reporter accumulated at the vegetal cortex of the egg, and further studies showed that a specific peptide in the Dsh molecule was necessary for the polarized protein accumulation (Leonard and Ettensohn, 2007). These data suggested a possible mechanism for localized activation of the endomesoderm gene regulatory network involving localized components of the Wnt pathway in the vegetal cortex of the egg. The present study was undertaken to establish the role of the vegetal cortex in activating the endomesoderm GRN and to identify the maternal determinants necessary for this process. Here, we show that the vegetal cortex, possibly exclusively through the canonical Wnt pathway, provides a localized center for endomesoderm specification, and that maternal Wnt6 is essential to trigger endoderm specification.

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## MATERIALS AND METHODS

### Animals and treatments

Adult *Paracentrotus lividus* sea urchins were collected by Laurent Gilletta and David Luquet from the bay of Villefranche-sur-mer (France). *P. lividus* eggs and embryos were cultured at 18°C in filtered sea water. Adult *Strongylocentrotus purpuratus* sea urchins were obtained from Pat Leahy, Kerkhoff Marine Laboratory, Caltech. *S. purpuratus* eggs and embryos were maintained at 15°C in filtered artificial seawater.

### Immunofluorescence and in situ hybridization

Whole-mount is situ hybridization was performed as described by Croce et al. (Croce et al., 2003) using an antisense probe specific to each gene examined, with sense controls providing no signal. Whole-mount immunofluorescent analysis was conducted as described by Sherwood and McClay (Sherwood and McClay, 1997). Antibodies used were against Endo I (endoderm antibody) (Wessel and McClay, 1985), Meso1 (PMC marker) (Hardin et al., 1992), SoxB1 (stains all nuclei) (Kenny et al., 1999), Dsh (see Fig. S1 in the supplementary material) and NgCAM (see Fig. S1 in the supplementary material). Epifluorescence images were acquired with a Zeiss axiovision upright fluorescent microscope, a Leica confocal laser microscope or a Zeiss 510 confocal microscope. The confocal images shown are snapshots of entire *z*-stack collections taken at 3-4 μm intervals.

### Gene perturbations

In vitro transcribed mRNAs were synthesized using the mMessage mMachine kit (Ambion) and Morpholinos (MASO) were obtained from Gene Tools. Morpholinos include: SpWnt6-1 (+1 to +25), CAATAGCTAAACGTGTCCACTCCAT; SpWnt6-2 (–32 to –6), AATACTCCAATAAATATCGTCCAGC; SpWnt16-1 (+1 to +25), CAAAACATCGGTAGCTTAAATCCAT; SpWnt16-2 (–57 to –33), GTCTTTCTTTATTGCTACACTCCAT; SpWnt7-1 (–63 to –38), CCTCGATACAGATTCTTCAACGATC; SpWnt8-1 (–10 to +15), CGTAAAGACATCCATGATGTACACT; SpWnt8-2 (–6 to +19), GTAGGTTTTGAGGAACCATCTTGAA.

Except for Wnt7, which was not pursued in detail, each morpholino experiment used two different non-overlapping morpholinos that gave identical phenotypes and identical perturbations at the molecular level. All injections were 1-2 pl per embryo. As a control for morpholino function, addition of Wnt6 mRNA rescued the Wnt6 morpholino phenotypes, confirming the specificity of this morpholino knockdown (see Fig. S2 in the supplementary material). Added Wnt16 rescued Wnt16 morpholino knockdowns (see Fig. S2 in the supplementary material). mRNA for stabilized β-catenin (Logan et al., 1999), Wnt6, Dsh and dnGsk3 were used at the optimal concentration of 0.5 μg/μl, 0.04 μg/μl, 1.4 μg/μl and 0.5 μg/μl, respectively, whereas the Wnt6 MASO was injected at 300 μM and the Wnt16 MASO at 150 μM. In *P. lividus*, Wnt6 mRNA was injected at 0.5 μg/μl and dnWnt6 at 0.2 μg/μl; a rescue of dnWnt6 at that injected concentration occurred with 0.012 μg/μl of the Wnt6 mRNA (see Fig. S3 in the supplementary material). The injected embryos were fixed for staining or photographed with a Zeiss Axioplan fluorescent microscope using DIC filters.

### RT-PCR and QPCR

cDNA was prepared from isolated total RNA using an Iscript Kit (BioRad). Standard PCR reactions were run for 30 cycles with a 55°C hybridization for 30 seconds, extension at 68°C for 1 minute and 95°C for 30 seconds. QPCR experiments were run for 40 cycles with a ubiquitin control for calculation of copies of mRNA for each probe per embryo. Preliminary PCR reactions identified all Wnts in the genome (Croce et al., 2006), using primers listed in Table S1 in the supplementary material.

Primer sets for Wnt6 included: (1) the primer set from (Stamateris et al., 2010); (2) QSpWnt6-2F (CGACCCTAATCGAATCTG) and QSpWnt6-2R (ATGAAAGAGTTGTGCATGG); (3) Wnt6-forward (GGCTCCTGTACTCTCAAG) and Wnt6-reverse (CCGACACCTGCAATT); (4) Wnt6F (ACTCTCAAGACCTGCTGGAA) and Wnt6R (GGGCTGTTTGACAGTTTCGT). Wnt 7 primer sets included: 1F (CAAGGCCAAACGAACTAGGA) and 1R (ACAGTCCCCATCGAACCATT); 2F (GTAGCGAGCCCTGATG) and 2R (GCCGCTCTCGTTACAA); 3F (TTGTAACGAGAGCGGC) and 3R (CGACACTCCTGTACGG). SpWnt 8 primer sets were from the Davidson lab website (http://www.spbase.org/SpBase/resources/methods/q-pcr.html): Wnt8F (TGTCGTTCATTCAAGCCATC) and Wnt8R (TATCACTCGCCATTCGTTCA). The Wnt16 primer set used was: W16F (TGGGAGCCTTGGACC) and W16R (GCGAGTTACGGCGTAT).

### Embryo manipulations

Cell transplantations were performed using a micromanipulator system as described previously (McClay and Logan, 1996). The vegetal cortex of *Paracentrotus lividus* was identified using a fiber optic light with a blue filter to optimize contrast of the red sub-equatorial pigment band. Two approaches were used to remove the vegetal cortex. The first method used a suction pipette to remove cortical membrane as depicted below in Fig. 4. The other approach used a glass needle to compress the egg below the pigment band to separate the vegetal material from the remaining egg. When vegetal material was removed, it immediately rounded up into a single membrane-bound fragment. For animal pole transplant experiments, a vegetal fragment was inserted into the animal region of the egg by insertion of a pipette at the vegetal pole and deposition of the fragment at the animal pole beneath the hyaline layer, all of this in the presence of normal levels of calcium. The embryos were then treated with 10% poly-ethylene glycol in sea water to promote membrane-membrane fusion. The embryos were washed out of the poly-ethylene glycol and incubated in normal filtered sea water. Cell lineage tracers used were RITC-dextran or CFDA dye, introduced at a concentration of 1:10,000 of a 1 mg/ml stock in DMSO.

## RESULTS

### Three transcripts encoding maternal Wnts are in the egg

To identify maternal Wnt ligands potentially required for the initial activation of the endomesoderm GRN, primers for the 11 Wnt genes in the *Strongylocentrotus purpuratus* genome were designed (Croce et al., 2006). RT-PCR on total RNA extracted from *S. purpuratus* eggs established that three transcripts encoding Wnt ligands are expressed maternally: Wnt6, Wnt7 and Wnt16 (Fig.

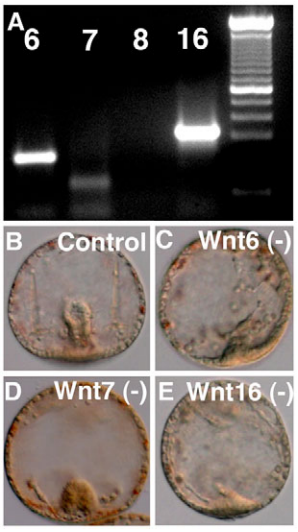


**Fig. 1. Maternal Wnts in the *S. purpuratus* egg and knockdown phenotypes.** (**A**) Wnt6, Wnt7 and Wnt16 are readily detected by RT-PCR, whereas Wnt8 is not detected in eggs. (**B**) Control embryo at late gastrula stage. (**C**) Phenotype of Wnt6 knockdown shown at the equivalent stage as the control at late gastrula stage. (**D**) Phenotype of Wnt7 knockdown at late gastrula stage. (**E**) Phenotype of Wnt16 knockdown at late gastrula stage. All embryos are ~80 μm in diameter.

1A). This finding is at variance with a recent report indicating the presence of seven maternal Wnts, not including Wnt6 mRNA in *S. purpuratus* eggs (Stamateris et al., 2010). We repeated a Wnt6 RT-PCR analysis using four different sets of primers, including the primer set used by Stamateris et al. (Stamateris et al., 2010). We found that all four sets detected maternal RNA for Wnt6 (see Fig. S4 in the supplementary material) in every batch of eggs tested. Stamateris et al. (Stamateris et al., 2010) also reported that Wnt8 was detected in eggs. However, in repeating that experiment, Wnt8 PCR products were not detected in our hands using either their primers or our primers (Fig. 1A), in agreement with previously published studies (Wikramanayake et al., 2004; Minokawa et al., 2005). The three identified Wnts were cloned and sequenced, and the sequences matched the sequences for the three Wnts in GenBank.

Morpholino antisense oligonucleotides (MASOs) for the three Wnts were designed, tested with controls (see Materials and methods, and Fig. S2 in the supplementary material) and used at 150 and 300 μM. In each case the morpholino had a phenotype at gastrulation, and re-addition of mRNA to Wnt6 or Wnt16 rescued normal embryos if that RNA lacked the site complementary to the morpholino (see Fig. S2 in the supplementary material) (Wnt7 MASO was not used beyond the preliminary survey). The phenotypes indicated some disruption of endomesoderm development by the gastrula stage (Fig. 1C-E). These results suggested a role for Wnt6 and Wnt16 in endomesoderm development, and possibly a role for Wnt7, at least in mesoderm development. However, the preliminary data did not provide information about specific early activities of the maternal Wnt molecules. We therefore proceeded to analyze the Wnts using perturbations combined with molecular analyses.

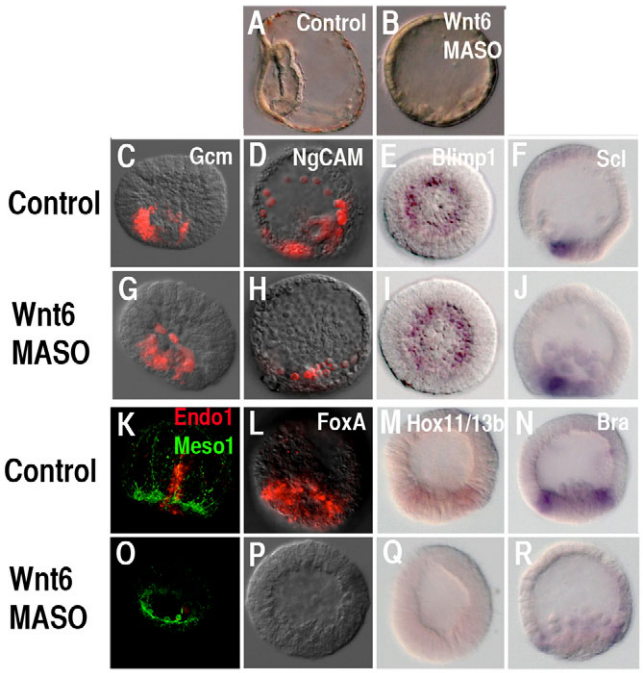


**Fig. 2. Wnt6 knockdown in *S. purpuratus*.** (**A**,**B**) Wnt6 control and morpholino (MASO) knockdown embryos at the same stage. (**C-F**) Control embryos expressing mesoderm markers. (**G-J**) Embryos injected with Wnt6 MASO express the mesoderm markers. (**K-N**) Control embryos with endoderm markers. In K, a mesoderm marker is also expressed. (**O-R**) Embryos injected with Wnt6 MASO failed to express endoderm markers but expressed the mesoderm marker in O. Embryos are ~80 μm in diameter.

## Knockdown of Wnt6 prevents activation of genes in the endoderm GRN

The knockdowns of Wnt7 and Wnt16 were complex because there were both maternal and zygotically produced activities that were complicated by activities of other Wnts. We chose to focus on Wnt6 because perturbations of this Wnt gave a relatively straightforward interpretation and the characterization of the other Wnts will follow in a later paper. Wnt6 morphants were examined at hatched blastula stage (16.5 hpf) and later. These knockdowns expressed all endomesoderm markers known to function in mesoderm (Fig. 2G,I,J), including those expressed in skeletogenic cells (Fig. 2H,O). By contrast, the Wnt6 morphants failed to express endomesoderm markers that later function in the endoderm, or markers that are expressed only in endoderm at the stage of the assay (Fig. 2O-R). The mesoderm markers that were unaffected by Wnt6 knockdown included NgCAM and Meso1 (both PMC antibody markers), Gcm (a pigment cell marker), Scl (a transcription factor that functions in the blastocoelar cells) and Blimp1 (a transcription factor that functions first in the mesoderm and then in the endoderm). Its early expression was unaffected by loss of Wnt6 (Fig. 2I), but its later expression was affected if measured at the time it normally is expressed in the endoderm (see Fig. 3 below). Of the endoderm genes, FoxA and Hox11/13b are expressed first in the endomesoderm but then function in the endoderm. Neither gene was expressed even during the endomesoderm period in macromere progeny. Brachyury is activated only in the definitive endoderm and was not expressed in the Wnt6 morphants. These data suggest that components of endoderm specification are lost when Wnt6 is knocked down. However, under those same knockdown conditions, genes known to be mesodermal markers were not affected. QPCR analyses agree with this conclusion (see Table S2 in the supplementary material). Because Wnt6 seemed to affect endoderm only, these initial observations suggested that the endomesoderm GRN must be activated by multiple maternal signals, some of which activate mesoderm while Wnt6 (and perhaps others) activates endoderm.

Morpholino knockdowns provide one way to assess function; however, to be more convincing we sought other approaches. Even though both morpholinos to Wnt6 gave the same phenotype and the same molecular knockdown information, and even though the perturbation was rescued by re-addition of Wnt6, we sought another way to test the hypothesis that Wnt6 activates endoderm in the GRN of the sea urchin embryo.

## Vegetal cortex is required for specification of endomesoderm

We turned to a different species of sea urchin to seek a positive test of Wnt6 function based on a finding of Boveri more than 100 years ago. Boveri observed that eggs of *Paracentrotus lividus* contained a subequatorial pigment band that enabled one to know where the vegetal pole of the egg is located (Boveri, 1901). Before attempting experiments directed toward the vegetal material, however, we repeated the knockdown experiments of Wnt6 on that second species, *P. lividus*, this time using dnWnt6 expression and ectopic expression of Wnt6. It has been estimated that *P. lividus* is separated from *S. purpuratus* by about 20 million years. In GRN analyses on both species performed so far, specification is very

similar, with perhaps minor differences in the timing of expression of specific genes being the only known differences. Thus, we felt the inclusion of this second species provided a legitimate comparison.

Fig. 3 shows that embryos lacking Wnt6 function uniformly failed to express endomesoderm genes that are expressed in the endoderm, and failed to express Brachyury, the earliest known gene expressed by the definitive endoderm, though the oral ectoderm patch of Brachyury expression was present (Fig. 3B). The dnWnt6 in the same experiment had no effect on expression of five genes that normally are expressed in the skeletogenic or nonskeletogenic mesoderm. Augmented expression of ectopic Wnt6 also affected endoderm and mesoderm genes differently. The expression pattern of mesoderm genes was unaffected by ectopic Wnt6 but each of the genes that function in the endoderm were expressed ectopically through all but the vegetal plate of the embryo (Fig. 3B). Thus, in two species, Wnt6 perturbations affected a group of genes that function in the endoderm but not a group of genes that function in mesodermal tissues. This suggested that the two species of sea urchin use Wnt6 to activate endoderm genes in the endomesoderm GRN. To test this hypothesis further, we moved to a system that allows a positive test of Wnt6 function.

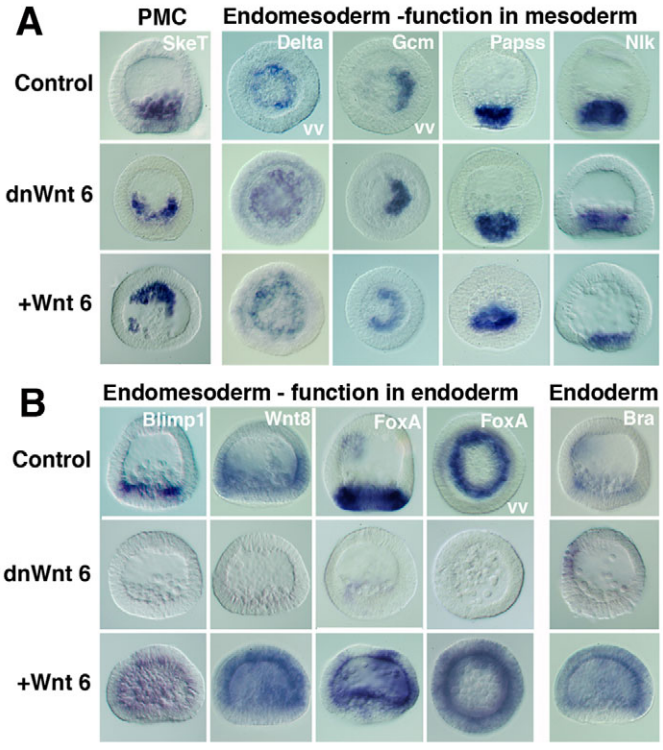


**Fig. 3. *P. lividus* endoderm gene expression was affected by Wnt6 perturbations, but mesoderm gene marker expression was not.** Eggs were injected with dnWnt6 mRNA (0.2 μg/μl) or Wnt6 mRNA (0.5 μg/μl) and grown to the mesenchyme blastula stage. Expression of mesoderm and endoderm markers in the perturbed embryos was then compared with expression of those same markers in control mesenchyme blastulae. (**A**) Knockdown or ectopic expression of Wnt6 did not affect the expression of SkeT (a PMC marker), Delta, Gcm or Nlk (NSM markers that appear prior to mesenchyme blastula), or Papss (a nonskeletogenic marker that appears at mesenchyme blastula). (**B**) Knockdown of Wnt6 eliminated expression of the markers that normally are expressed in endoderm at mesenchyme blastula stage. Ectopic Wnt6 caused each of the same markers to be expressed throughout the animal hemisphere. Embryos are ~90 μm in diameter.

The subequatorial pigment band allowed us to identify the vegetal pole to be identified prior to cleavage (Boveri, 1901; Ghiglione et al., 1996) (Fig. 4A,B). The pigment granules of that band are attached to an actin-rich meshwork in the egg cortex just beneath the plasma membrane (Sardet and Chang, 1985). By following that pigment band through development, Boveri established that the vegetal region of the egg invariably gives rise to endomesoderm (Boveri, 1901). Later, Horstadius confirmed Boveri's conclusions, and removed vegetal egg fragments with the outcome that endomesoderm was eliminated (Horstadius, 1928). Experimentally, manipulation of the egg cortex and its overlying plasma membrane can withstand damage, and will repair with a surprising retention of viability (Terasaki et al., 1997). Given this background, *P. lividus* embryos offered a way to positively test the hypothesis that Wnt6 activates endoderm through rescue experiments.

We confirmed the egg fragment experiment of Horstadius. Without the vegetal region, no endomesoderm is specified and the embryos become hollow ciliated balls. A method was devised for selectively stripping specific areas of egg cortex (Fig. 4C). Embryos missing the vegetal cortex [VC(–) embryos] developed into a hollow epithelial ball of cells that failed to gastrulate (Fig. 4G shows two identical flattened embryos partially superimposed on one another). By contrast, when an equal amount of animal pole membrane cortex was removed [AC(–) embryos, Fig. 4F], or when any other part of the egg cortex was removed, embryos developed with no noticeable adverse effects. After 36 hpf, the VC(–) embryos failed to express mesoderm or endoderm markers compared with control embryos (Fig. 4H,I).

If, instead of selectively removing the egg cortex, the suction needle removed deeper cytoplasm and removed as little cortex as possible, the embryos developed normally. The embryos were smaller but removal of up to 60-70% of the egg cytoplasm, beginning with vegetal cytoplasm, had essentially no effect except to decrease the size of larvae (Fig. 4J). So, except for scale, the small larvae were normal phenotypically. These data suggest that components necessary for endomesoderm specification are uniquely attached to the egg vegetal cortex and are not localized in the vegetal cytoplasm of the egg. In addition, we observed that VC(–) embryos cleaved equally at the 4th cleavage (Fig. 4K), as Horstadius also observed when he removed vegetal fragments of the egg (Horstadius, 1939).

## Ectopic vegetal cortex activates endomesoderm

Horstadius (Horstadius, 1939) showed that micromeres inserted at the animal pole induce a second gut and ectopic skeleton, as repeated in Fig. 5A with the result shown in Fig. 5B. This lateral view of the larva shows additional skeletal elements near the original animal pole and an additional gut. We reasoned that if the causal agent of this endomesoderm inductive capacity originates at the vegetal cortex, then transplanted vegetal cortex might also induce a second gut and additional skeleton at the animal pole. As a test of that hypothesis, isolated anucleate vegetal cortex vesicles were placed at the animal pole of a host zygote, as shown in Fig. 5C. The host zygote and donor vegetal cortex vesicle were placed into tight contact and cultured for 15 minutes in 10% polyethylene glycol in seawater to promote membrane-membrane fusion. An ectopic second gut and ectopic skeleton were produced in about 40% of the transplants, an example of which is shown in Fig. 5D, as viewed from above the original animal pole looking down on the aboral top of the larva ($n$=14/32). Controls (10 each) included sham operations,

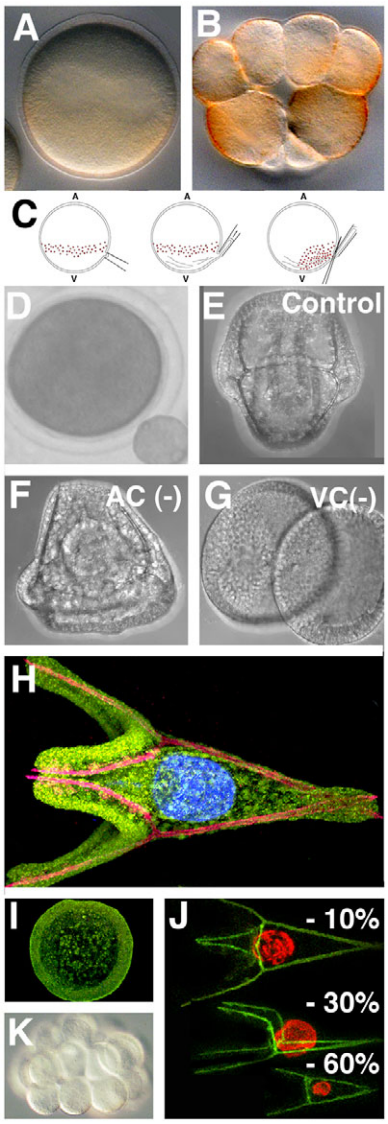


**Fig. 4. Removal of the vegetal cortex of *P. lividus* eggs eliminates endomesoderm.** (**A**) An egg with the orange pigment band visible below the equator. (**B**) At 16-cell stage, the pigment band is concentrated in membranes about one-third up from the bottom of the macromeres and is absent from micromeres. (**C**) The technique used for eliminating the vegetal cortex. A pipette begins suction of membrane just beneath the pigment band. The egg is turned relative to the pipette and suction applied so that most of the material suctioned is vegetal cortex. A glass needle severs the membrane from the egg. (**D**) VC(–) embryo is shown next to an insert of the cortical vesicle fragment removed from that egg. (**E**) Control early pluteus viewed from the aboral side. (**F**) Embryo missing animal cortex viewed from the oral side. (**G**) Two identical VC(–) embryos partially superimposed to show the uniform simple flattened hollow ball phenotype. (**H**) Control pluteus larva viewed from above the aboral side. An endoderm marker, Endo 1 (blue), stains the midgut; a skeletogenic cell marker (red) stains the skeleton; an antibody to SoxB1 (green) is used to show the shape of the larva. (**I**) VC(–) embryo at the same stage as H failed to stain with either endoderm or mesoderm markers. (**J**) Removal of about 10%, 30% or 60% of the cytoplasm, while retaining cortex, resulted in normal, but progressively smaller, pluteus larvae. In this series, endoderm is stained with a red marker and the skeleton with a green marker. (**K**) VC(–) embryo at the 16-cell stage cleaved equally.

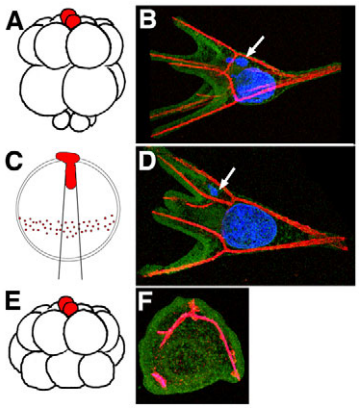


**Fig. 5. Induction of endomesoderm at the animal pole and failure to rescue VC(–) embryos with control micromeres.** (**A**) Two normal micromeres transplanted to the animal pole of a 16-cell stage embryo led to (**B**), at 48 hours, a pluteus larva (side view) with an induced second gut (endoderm stained blue, arrow) and ectopic skeletal elements (immediately beneath the tip of the arrow). SoxB1 nuclear staining (green) outlines the embryo. (**C**) Vegetal cortex is inserted at the animal hemisphere as shown. (**D**) Embryo with vegetal cortex fused to animal pole at 48 hours (viewed from above aboral top). The arrow indicates an induced gut; the skeletal elements contain an extra rod (the rod just below the ectopic gut). (**E**) Control micromeres transplanted to a VC(–) embryo at the 16-cell stage. (**F**) At 48 hours, a VC(–) embryo contained skeletal rods produced by the donated micromeres, but no induced endoderm or mesoderm.

fertilized eggs without vegetal cortex transplants in polyethylene glycol alone, animal cortex transplanted and fused, and vegetal cortex transplanted but without the polyethylene glycol fusion step. These control manipulations produced normal embryos. Thus, the vegetal cortex contains information sufficient to induce activation of the endomesoderm-GRN locally when that cortex is at the animal pole. The active area of vegetal cortex is restricted to an area below the pigment band, because isolated cortex from other regions of the egg had no activity in this assay.

We tested the ability of VC(–) embryos to respond to micromere induction in a Horstadius-type experiment similar to that in Fig. 5A. The 16-cell stage VC(–) embryos received micromeres from sister control embryos, either at the vegetal pole or at the animal pole [as cells in these VC(–) embryos divide equally it was not apparent which pole was which, so we assume that roughly 50% of the transplants were to the original animal pole; Fig. 5E]. Most of the VC(–) embryos with micromeres added back developed a skeleton (Fig. 5F) (42/50). This was expected, as normal micromeres develop autonomously and produce skeleton even if isolated in culture. However, the added micromeres, even though presumably producing their normal complement of signals, including Delta, failed to induce endoderm and non-skeletogenic mesoderm in the VC(–) embryos (0/50) (Fig. 5F). As there was no induction at the vegetal or animal pole, we conclude that, normally, information localized to the vegetal cortex has a specification impact throughout the entire egg. Removal of the vegetal cortex eliminates that responsiveness when micromeres are placed at the animal (or vegetal) pole. Thus, perhaps surprisingly, a vegetal influence driven by the vegetal cortex must provide the entire embryo with a competence to respond to transplanted micromeres. This provision probably occurs prior to third cleavage (the first equatorial cleavage).

## Wnt pathway members rescue missing vegetal cortex

Previous experiments showed several Wnt pathway molecules to be distributed asymmetrically by 4th cleavage, and each is involved in the asymmetrical activation of endomesoderm (Chuang et al., 1996; Kenny et al., 1999; Logan et al., 1999; Wikramanayake et al., 2004; Minokawa et al., 2005; Smith and Davidson, 2009). As mentioned in the Introduction, a Dishevelled-GFP (Dsh-GFP) reporter protein concentrated to one pole of the egg, suggesting perhaps that endogenous Dsh protein also localizes to the vegetal cortex (Weitzel et al., 2004; Leonard and Ettensohn, 2007). To visualize the endogenous distribution of Dsh protein in the egg, an antibody was produced against sea urchin Dsh (see controls in Fig. S1 in the supplementary material). As shown in Fig. 6A, Dsh is present in puncta throughout the egg, but is concentrated to a region of the egg cortex. To verify that the Dsh-rich region is located at the vegetal pole, a pipette pulled out a 'pucker' at the vegetal pole of the *P. lividus* eggs, using the pigment band as a reference, and the embryos were fixed before the pucker was reincorporated into the egg. Fig. 6B shows that the cortical concentration of Dsh is centered at the site of the pucker, confirming that Dsh is at the vegetal pole. After removal of the vegetal cortex from eggs of *P. lividus*, the Dsh cortical concentration was missing (Fig. 6C) and it failed to reappear later during cleavage stages. Thus, the VC(–) embryos lack the vegetal cortical concentration of Dsh.

With VC(–) embryos serving as the controls and incapable of producing endomesoderm gene expression on their own, the next set of experiments asked whether expression of Wnt pathway molecules would rescue endomesoderm in the VC(–) background. As above, neither endoderm nor mesoderm antibody markers stained the control VC(–) embryos (Fig. 7B). Addition of mRNA expressing stabilized β-catenin rescued endomesoderm specification in VC(–) embryos (Fig. 7C). Similarly, although at a lesser extent, dominant-negative GSK3 (Fig. 7D) and overexpression of Dsh (Fig. 7E) rescued endomesoderm, thus indicating that ectopic activation of the canonical Wnt pathway is able to restore endomesoderm specification following vegetal cortex removal (although most embryos displayed little recognizable pattern).

To determine whether the rescue occurred early, we examined expression of *ets1*, a transcription factor expressed by hatched blastula stage in the micromere GRN (Rottinger et al., 2004; Rizzo et al., 2006) (Fig. 7F). If the Wnt pathway activates the earliest components of the skeletogenic mesoderm GRN in VC(–) embryos, there should be a rescue at the beginning of the specification sequence. Accordingly, VC(–) embryos that lack *ets1* expression (Fig. 7H), regain expression of *ets1* if they also express activated β-catenin (Fig. 7I). However, unlike the normal specification pattern of micromeres, the *ets1* expression caused by ubiquitous activation of the Wnt pathway occurred more randomly in blastomeres (Fig. 7I). Thus, augmented β-catenin expression in VC(–) embryos rescues expression of a transcription factor necessary for early micromere specification.

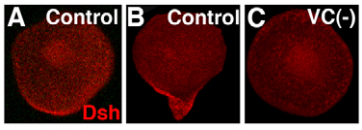


**Fig. 6. Eggs of *P. lividus* stained with an antibody to Dishevelled.** (**A**) Control egg with a concentration of Dsh in the cortex. (**B**) The vegetal cortex was pulled into a pipette using the sub-equatorial pigment band as a reference. The concentration of Dsh is localized to that vegetal cortex. (**C**) VC(–) embryo lacking the cortical concentration of Dsh.

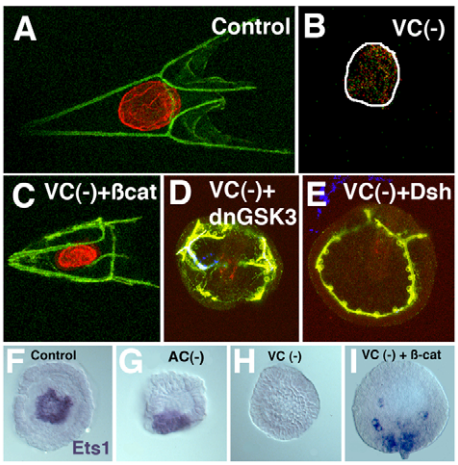


**Fig. 7. β-Catenin and other cytoplasmic components of canonical Wnt pathway rescue the removal of vegetal cortex.** (**A**) Control larva at 48 hours stained with an endoderm marker (red) and a mesoderm marker (green) viewed from above the larva. (**B**) A VC(–) embryo failed to stain for mesoderm and endoderm markers (image gain increased to show that an embryo was present). The white line outlines the VC(–) embryo (*n*=0/40). (**C**) VC(–) embryos expressing stabilized β-catenin produced endomesoderm (*n*=39/40). (**D**) VC(–) embryos expressing dn-GSK3 produced endomesoderm (*n*=13/14). (**E**) VC(–) embryos expressing Dsh produce endomesoderm (*n*=3/11, 9/16 in two trials). (**F-I**) In situ analysis of *ets1*, a gene expressed early in micromere specification. (F) *ets1* was expressed in micromeres in controls (vegetal view). (G) Removal of animal cortex had no effect on *ets1* expression (lateral view) (*n*=10/10). (H) VC(–) embryos failed to express *ets1* (*n*=10/10). (I). *ets1* expression was rescued in VC(–) embryos when stabilized β-catenin was expressed (*n*=10/10 embryos).

## Endoderm development is rescued in VC(–) embryos by expression of Wnt6

As activation of the canonical Wnt pathway rescues endoderm and mesoderm development in the absence of the vegetal cortex, we returned to the question of maternal Wnt6. Ectopic expression of Wnt6 in control embryos caused a variety of vegetalized phenotypes, including (at high frequency) extra endoderm and ectopic guts (Fig. 8B), plus axial aberrations. This set up the positive test for addition of Wnt6 to VC(–) embryos. Wnt6 mRNA was injected into eggs, the eggs were fertilized and the vegetal cortex removed. Endoderm was produced by all VC(–)+Wnt6 embryos scored initially by confocal microscopy (32/32), but PMCs were observed only occasionally in the embryos scored (3/32) (Fig. 8D,E). Thus, in the VC(–) embryos and in normal embryos the most consistent outcome of added Wnt6 was rescue of or augmented endoderm specification.

We examined embryos more closely by in situ hybridization to determine which early GRN components are rescued by the addition of Wnt6 to VC(–) embryos. Without addition of Wnt6 mRNA, none of the VC(–) expressed any of the endomesoderm markers tested (Fig. 9B,E,H,K,N). When Wnt6 mRNA was added to VC(–) embryos, neither skeletogenic (Tbr) nor non-skeletogenic mesoderm markers (Gcm) were expressed (Fig. 9C,F). Endoderm

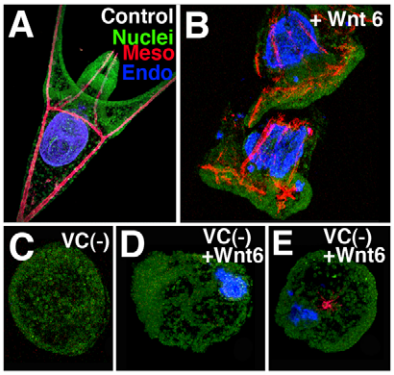


**Fig. 8. Wnt6 rescued endoderm in the VC(–) embryos.** (**A**) Control pluteus larva viewed from below the anus. (**B**) Two embryos at the same stage as A overexpressing Wnt6. Each has enlarged and ectopic guts (blue) and mispatterned skeletal elements (red). (**C**) VC(–) control expressed no endomesoderm. (**D**) VC(–) embryos injected with Wnt6 mRNA expressed endoderm (*n*=32/32) or occasionally (**E**) endoderm plus one or two PMCs (*n*=3/32). SoxB1 staining (green) outlines the embryo.

markers at the stage tested (Wnt8, Blimp1 and FoxA) were expressed (Fig. 9I,L,O). We conclude that Wnt6 has a role in activating the endoderm GRN in *P. lividus*. It did not rescue skeletogenic or pigment cell mesoderm in VC(–) embryos.

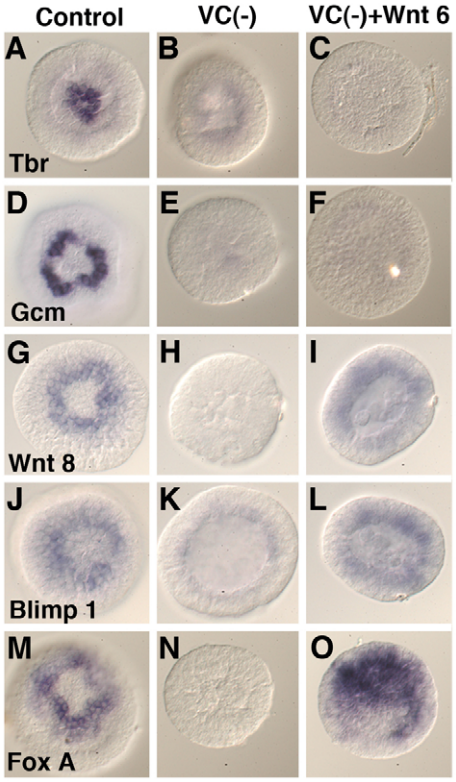


**Fig. 9. VC(–) embryos plus Wnt6 expressed endoderm but not mesoderm markers.** (**A**,**D**,**G**,**J**,**M**) Control patterns of expression of the indicated genes at hatched blastula stage of *P. lividus* (vegetal views). (**B**,**E**,**H**,**K**,**N**) VC(–) embryos without added Wnt6 failed to express any of the endomesoderm genes tested (*n*=0/10 in each case). (**C**,**F**,**I**,**L**,**O**) VC(–) embryos with added Wnt6. Markers normally expressed in endoderm at this stage were expressed (I,L,O), whereas those normally expressed in mesoderm at this stage were not expressed (mesoderm markers, *n*=0/10 for each marker; endoderm markers, *n*=10/10 for each marker).

## DISCUSSION

### Wnt6 restoration of endoderm in the absence of vegetal cortex

In two species, we observed that morpholino knockdown of Wnt6 prevented specification of endoderm, as established by the failure of early or later endoderm markers to appear. Under the same knockdowns, early and later mesoderm markers were unaffected. Reciprocally, if the vegetal cortex was removed from eggs, added Wnt6 rescued expression of endodermal genes but failed to rescue the mesoderm markers tested. The rescue experiment was limited because Delta is necessary for skeletogenic mesoderm and pigment cell specification (Sherwood and McClay, 1999; Sweet et al., 2002), although when Delta was provided to VC(–) embryos by re-addition of micromeres there was no rescue of mesoderm. Loss of Wnt6 in whole embryos had no effect on genes involved in skeletogenic specification, pigment cell specification or blastocoelar cell specification. Therefore Wnt6 activates endoderm in absence of the vegetal cortex and loss of Wnt6 prevents endoderm genes from being activated. We were unable to observe Wnt6 perturbation effects on any mesoderm tissue tested, although it remains possible that Wnt6 is necessary for coelomic pouch and/or muscle mesoderm.

The earliest genes activated by Wnt6 include Wnt8, Hox11/13 and FoxA, genes activated in the early endomesoderm. β-Catenin enters nuclei of macromeres at the 5th cleavage and Wnt8 is first activated in those cells by the 6th to 7th cleavage where it appears in all macromeres and their progeny (Logan et al., 1999; Wikramanayake et al., 2004). Hox11/13 and FoxA appear shortly thereafter (Oliveri et al., 2006; Croce and McClay, 2010; Peter and Davidson, 2010). The precise mechanism of Wnt6 signal transduction that leads directly or indirectly to activation of these genes and the network that follows is currently not established; however, in VC(–) embryos, cells throughout the embryo respond to Wnt6 addition and, endogenously, in situ evidence suggests that maternal Wnt6 is distributed ubiquitously in the egg (see Fig. S5 in the supplementary material). To date, only Dsh is shown to be anisotropic in the egg, so how could the asymmetric activation work? The high concentration of Dsh at the vegetal cortex may offer a local sensitized area for Wnt6 activation, and the ubiquitous distribution of Dsh at lower concentrations offers a mechanism to explain how added Wnt6 can activate early endoderm genes without pattern when expressed in the VC(–) embryos (assuming the appropriate Frizzled receptor is also expressed beyond the vegetal cortex).

Though Wnt6 rescued endoderm in the VC(–) embryos, the experiments also showed that components of the canonical Wnt pathway activated both mesoderm and endoderm in virtually all VC(–) embryos injected either with β-catenin mRNA or dnGSK3. These data suggest two possibilities for selective activation of endoderm by Wnt6. One possibility is that macromeres fated to be endoderm uniquely contain the Frizzled receptor for Wnt6. A search for that receptor is under way, though it is hard to imagine how the receptor alone directs endoderm specification and not mesoderm specification as both cell types derive from macromeres.

The other possibility is that macromere progeny fated to be endoderm inherit factors asymmetrically. Two known candidates for this asymmetry are maternal Otx (Chuang et al., 1996) and

SoxB1 (Kenny et al., 1999). SoxB1 protein is eliminated from the micromere nuclei at the 4th cleavage, and from lower Veg2 progeny by hatched blastula stage, but not from the upper Veg2 progeny of the macromeres until later; those upper Veg2 cells are specified to be endoderm in the presence of SoxB1 protein (Kenny et al., 2003; Croce and McClay, 2010). Knockdown of SoxB1 either with a morpholino or by expression of the DNA-binding domain of SoxB1 blocks gastrulation (Kenny et al., 2003). In that study, only the gastrulation phenotype was scored, because, at the time, early endoderm genes were not yet known. Thus, the known differences between the micromere GRN and the endomesoderm GRN at the time Wnt6 first activates endoderm genes are the absence versus presence of SoxB1 and the concentration of Otx, which is high in micromeres and lower in macromere progeny (Chuang et al., 1996). In the VC(–) egg fragment experiments, depletion of the vegetal cortex is followed by an equal 4th cleavage, thereby eliminating the proposed mechanism for initial loss of SoxB1 from micromeres (Kenny et al., 1999). If that hypothesis is correct, and extends to the observed loss of SoxB1 from Veg2 cells fated to non-skeletogenic mesoderm, failure to eliminate SoxB1 would block mesoderm specification in the presence of Wnt6. Because high SoxB1 is apparently normal when endoderm specification is initiated, the conditions for Wnt6 activation are at least permissive in the VC(–) experiments.

A biological difference in the experiments was also a likely contributor to the reason β-catenin was able to activate mesoderm specification while Wnt6 addition was not. Addition of augmented stabilized β-catenin or dnGSK3 addition to embryos can achieve high levels of nuclear β-catenin, whereas the effect of added Wnt6 mRNA is receptor-concentration limited. Even with very high concentrations of expressed ectopic Wnt6, the transduction that resulted could not exceed Frizzled receptor-binding saturation. Thus, it is likely that the Wnt6 rescue occurred at much lower levels of β-catenin nuclear accumulation than had accumulated after addition of stabilized β-catenin. This, combined with the high level of SoxB1 in all cells of the VC(–) embryos apparently supported a condition that allowed initiation of endoderm specification upon Wnt6 addition, but mesoderm was specified only when very high levels of nuclear β-catenin are achieved, perhaps at levels that overwhelmed the known mesoderm inhibitory effects of SoxB1.

## Wnt activation of development is not unique to sea urchin

Heasman and colleagues (Tao et al., 2005; Cha et al., 2008; Cha et al., 2009) showed in *Xenopus* that Wnt11 and Wnt5 are present maternally and activate axial components of development, perhaps as a complex. Maternal Wnts also contribute to asymmetric signaling in other embryos in the animal kingdom (Thorpe et al., 1997; Nasevicius et al., 1998; Momose and Houliston, 2007). Thus, it was not surprising that maternal Wnt signaling was involved in activation of sea urchin eggs. In most cases, maternal Wnts participate in early axial specification (Nasevicius et al., 1998; Tao et al., 2005; Momose and Houliston, 2007; Cha et al., 2008; Cha et al., 2009), though in *C. elegans* Wnt signaling provides an input enabling early blastomeres to distinguish endoderm from mesoderm (Thorpe et al., 1997). Prior to activation, the GRN is kept silent by strong TCF repression mediated by a Groucho-TCF complex (Vonica et al., 2000). When the Wnt pathway is activated and β-catenin enters nuclei, Groucho is released (Range et al., 2005), β-catenin binds to TCF and the target gene rapidly switches from strong repression to activation. This rapid switching mechanism accounts for Wnt8 expression that is detected within 20 minutes of β-catenin entering macromere nuclei (Logan et al., 1999; Wikramanayake et al., 2004). Thus, the GRN is quickly activated in response to the Wnt signal, leading to rapid onset of specification through the endomesoderm network.

## Vegetal cortex supplies the cleavage stage embryo with the capacity to respond to induction

VC(–) embryos at the 16-cell stage were unresponsive to added control micromeres that normally deliver signals capable of inducing animal cells to initiate endoderm and mesoderm specification. Receptors to Notch are ubiquitous during early cleavage (Sherwood and McClay, 1997), although when Delta was provided to the VC(–) embryos (via addition of control micromeres) the VC(–) cells were unable to respond. This indicates that Delta-Notch signaling does not operate alone in activation of non-skeletogenic mesoderm, i.e. the responding cells need additional inputs. Wnt6 does not seem to be that additional input, as removal of Wnt6 but presence of Delta did not affect mesoderm expression. Micromeres are known to release other signals to the macromere progeny, but it is unknown whether receptors for those signals are present in the VC(–) embryos. We show here that even the animal pole requires a vegetal cortex-dependent input to be responsive to ectopic micromere inductions. The activity present in the vegetal cortex is specific as neither the animal cortex nor cortex from any other area of the egg provides an endomesoderm inductive responsiveness.

## Allocation of vegetal cortex information is not a graded signal release

When the vegetal cortex was removed the most common phenotype observed is shown in Fig. 4 as a disc-like non-specified dauer blastula (in greater than 90% of the more than 10,000 embryos scored during these experiments). Experimental removal of vegetal cortex occurred consistently, with about a 10% error rate of embryos that did not show the VC(–) phenotype. Surprisingly, most of the 'mistakes' became normal larva. We had expected to observe a graded series of embryos with increasing severity of abnormal structures. To confirm this observation, an experiment was designed where we purposely removed only part of the vegetal cortex, leaving roughly half of the localized Dsh. Normal embryos resulted in almost every case. We did not observe a graded response that might be expected if the vegetal cortex contributed quantitatively to endomesoderm activation. This observation suggests involvement of the vegetal cortex is qualitative rather than quantitative in providing a signal release. A graded distribution of maternal determinants was proposed by Horstadius to explain his data and he suggested a double gradient of diffusible information to explain animal-vegetal specification (Horstadius, 1939). The data here simply do not support the existence of such graded distributions of individual vegetal components, at least those that would be necessary for activating the endomesoderm GRN. This is further supported by earlier experiments in which one micromere was sufficient to support normal specification and allocation of non-skeletogenic mesoderm (McClay et al., 2000), and by other arguments made earlier (Davidson, 1989).

Much remains to be learned, however, about the vegetal cortex, the activation of endomesoderm and the contribution of the unequal cleavages. Clearly, asymmetric information needed for endomesoderm specification is attached to the vegetal cortex and

that information is necessary to couple with the ubiquitous expression of Wnt6 to produce patterned endoderm. Removal of up to 70% of the deeper cytoplasm had no effect on specification or patterning, only on reducing the size of the resulting larva, further indicating that the vegetal cortex is crucial for patterning. The receptors for the several Wnts may play important spatiotemporal roles in early activation though: because Wnt6 can rescue VC(–) embryos, its receptor must exist outside the vegetal cortex. As seen in these experiments, the vegetal cortex contains components that control the later unequal cleavages, components that segregate the skeletal mesenchyme from the remaining endomesoderm and components involved in patterning the embryo. Thus, the discovery of Wnt6 and localized Dsh are just the beginning of an effort to identify the several activities required for initiation of endomesoderm specification.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
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