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β-Catenin specifies the endomesoderm and defines the posterior organizer of the hemichordate *Saccoglossus kowalevskii*

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SUMMARY

The canonical Wnt/β-catenin pathway is a key regulator of body plan organization and axis formation in metazoans, being involved in germ layer specification, posterior growth and patterning of the anteroposterior axis. Results from animals spanning a wide phylogenetic range suggest that a unifying function of β-catenin in metazoans is to define the posterior/vegetal part of the embryo. Although the specification of vegetal territories (endoderm) by β-catenin has been demonstrated in distantly related animals (cnidarians, a protostome, echinoderms and ascidians), the definition of the posterior part of the embryo is well supported only for vertebrates and planarians. To gain insights into β-catenin functions during deuterostome evolution, we have studied the early development of the direct developing hemichordate *Saccoglossus kowalevskii*. We show that the zygote is polarized after fertilization along the animal-vegetal axis by cytoplasmic rearrangements resembling the ascidian vegetal contraction. This early asymmetry is translated into nuclear accumulation of β-catenin at the vegetal pole, which is necessary and sufficient to specify endomesoderm. We show that endomesoderm specification is crucial for anteroposterior axis establishment in the ectoderm. The endomesoderm secretes as yet unidentified signals that posteriorize the ectoderm, which would otherwise adopt an anterior fate. Our results point to a conserved function at the base of deuterostomes for β-catenin in germ layer specification and to a causal link in the definition of the posterior part of the embryonic ectoderm by way of activating posteriorizing endomesodermal factors. Consequently, the definition of the vegetal and the posterior regions of the embryo by β-catenin should be distinguished and carefully re-examined.

KEY WORDS: β-Catenin, Endomesoderm, Wnt, Hemichordate, Deuterostome, Anteroposterior axis, Germ layers

INTRODUCTION

The Wnt pathway regulates many aspects of metazoan embryonic development, such as germ layer specification, determination of embryonic polarity, binary cell fate decision through asymmetric cell division, anteroposterior axis patterning and posterior growth (Guder et al., 2006a; Kusserow et al., 2005; Manuel, 2009; Martin and Kimelman, 2009; Niehrs, 2010; Petersen and Reddien, 2009). The animal-vegetal axis is the primary polarity of the oocyte and is defined by the site of polar body formation at the animal pole during meiosis. Asymmetries along this primary axis result from a variety of factors during oogenesis and are often evident by either the position of the female pronucleus, or the deposition of yolk platelets and pigment granules. Broad comparative studies have highlighted an ancient function of the Wnt pathway to position germ layers along this primary axis. In various metazoans, activation of the Wnt pathway at one pole of the embryo leads to endomesoderm specification. This was first described in a deuterostome, the sea urchin, in which β-catenin protein, the key downstream effector of the canonical Wnt pathway, becomes nuclear in the vegetal blastomeres at late cleavage stages (Logan et al., 1999; Wikramanayake et al., 1998). The regulated stabilisation of β-catenin is required to specify endomesodermal fate and leads to activation of a specific transcriptional regulatory network that is responsible for fate specification and differentiation. A very similar situation has been described in other deuterostomes (other echinoderms and in ascidians that belong to chordates) (Imai et al., 2000; Kawai et al., 2007; Miyawaki et al., 2003). In bilaterians outside of deuterostomes, the function of the Wnt/β-catenin pathway has been deciphered in great detail in the ecdysozoan model systems *C. elegans* and *D. melanogaster*, in which it is involved in different aspects of anteroposterior polarity. Although the Wnt pathway participates in the specification of the endoderm lineage in the nematode, this is part of its more general function in fate decisions during asymmetric cell divisions along the anteroposterior axis during embryonic and larval life (Mizumoto and Sawa, 2007). In fly, *wingless* controls parasegment polarity (Gonsalves and DasGupta, 2008). β-Catenin has been functionally implicated in germ layer specification during the development of only one protostome species, the nemertean *Cerebratulus lacteus*, which belongs to the still understudied lophotrochozoans (Henry et al., 2008). Although phylogenetic sampling for this specific function of the Wnt pathway is still sparse, the experimental observations described above raise the possibility of an ancient role of β-catenin in germ layer formation at the base of the bilaterians. This

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hypothesis is further supported by comparative data from cnidarians suggesting that this function for the Wnt pathway even predates the emergence of bilaterians. It has been experimentally established in embryos of two cnidarian species (*Nematostella vectensis* and *Clytia hemisphaerica*) that nuclear β-catenin accumulates on the animal side of the blastula and is necessary and sufficient to specify the endoderm (or entoderm) at this location (Momose et al., 2008; Momose and Houliston, 2007; Wikramanayake et al., 2003). These observations date the use of β-catenin in germ layers specification at least back to the cnidarian/bilaterian ancestor.

Specification of the endomesoderm is crucial for subsequent development of the embryonic axes. Positioning endomesoderm defines the site of gastrulation and blastopore formation, which is of great importance for morphogenesis and building of the body plan. Furthermore, the endomesoderm and its derivatives are often involved in organizing embryonic axes. For example, in vertebrates the organizer has a key role in controlling both patterning and morphogenesis of the anteroposterior and dorsoventral axes of all three germ layers (De Robertis et al., 2000). In ascidians, signals from the endomesoderm induce neural tissue and pattern the overlying ectoderm (Nishida, 2005). A large set of signaling molecules has been implicated in the organizing activities of the endomesoderm, including members of the TGFβ, BMP, FGF and Wnt families. In a variety of animals representing both non-bilaterian and bilaterian phyla, Wnt ligands are expressed around the blastoporal region, whereas secreted Wnt inhibitors like members of the SFRP or Dkk families are expressed at the opposite side of the embryo (Niehrs, 2010; Petersen and Reddien, 2009). Such observations have led to speculation that the Wnt pathway plays a conserved role in regulating eumetazoan embryonic axial patterning by participating in the specification of the blastoporal side, which corresponds to the posterior region in bilaterians and the oral region of cnidarians (Niehrs, 2010; Petersen and Reddien, 2009). However, homology between anteroposterior axis of bilaterians and oral-aboral axis of cnidarians is still a subject of controversy (Manuel, 2009; Martindale and Hejnol, 2009; Niehrs, 2010; Ryan and Baxevanis, 2007).

The above proposal originates from the observation that in vertebrates there is a clear function for a gradient of Wnt activity in giving positional information along the anteroposterior axis, especially for central nervous system formation (for reviews, see Holland, 2002; Marikawa, 2006; Yamaguchi, 2001). A similar activity has been shown during regeneration and homeostasis of adult body plan in planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008) and cnidarians (*Hydra*) (Broun et al., 2005; Guder et al., 2006b; Hobmayer et al., 2000; Lengfeld et al., 2009). It is crucial to distinguish these later patterning roles of Wnts from the early function of β-catenin in endomesoderm formation. Importantly, in the models in which the late patterning function of Wnt/β-catenin has been demonstrated, the earlier function in germ layer definition is not prominent. For example, in vertebrates, endomesoderm formation does not crucially depend on β-catenin but relies more heavily on downstream effectors such as Nodal (Shen, 2007). β-Catenin has, however, been co-opted to implement organizer formation (Croce and McClay, 2006). Conversely, in ascidian, sea urchin or nemertean, in which a clear function of β-catenin in endomesoderm specification has been demonstrated, there is no obvious function for late blastoporal Wnts in patterning the anteroposterior axis of the embryo. In cnidarian embryos in which both germ layer formation and axis patterning are under the control of the Wnt pathway (Momose and Houliston, 2007; Wikramanayake et al., 2003), the mechanistic relationships between the two processes are unclear (Primus and Freeman, 2004; Primus and Freeman, 2005). It is rather thought that endoderm specification is a consequence of embryonic polarity establishment, which would be directly controlled by Wnt (Momose and Houliston, 2007).

It thus appears that two conceptually distinct functions of the Wnt pathway, i.e. specification of the endomesoderm and definition of the posterior (or oral) part of the embryo, are intricately coupled and have so far not been experimentally separated in a single model organism. In order to gain insights into the relationship of these two functions in deuterostomes, we chose to analyze the role of canonical Wnt signaling in embryonic development of the hemichordate *Saccoglossus kowalevskii*. Hemichordates are the sister group of echinoderms but are closely related to chordates. Understanding their development should shed light on the early evolution of deuterostomes and the emergence of chordates. It has been, for example, previously shown that the neurogenic ectoderm of *Saccoglossus* is extensively patterned at the transcriptional level along the anteroposterior axis in a manner highly similar to that observed in the vertebrate central nervous system (Aronowicz and Lowe, 2006; Lowe et al., 2003). These observations led to the hypothesis that the deuterostome ancestor had an anteroposterior axis patterned in a complex manner, a conclusion that could not have been reached by examining only non-vertebrate chordates and echinoderms. Hemichordates might thus have retained some of the ancestral features that have been greatly modified in these other branches of the deuterostomes.

By determining the expression patterns of the Wnt ligands and other regulators of the pathway and by interfering with their function, we have uncovered two temporally and functionally separable roles for the Wnt pathway. In a separate study (our unpublished results), we describe a late role for the Wnt pathway in endowing ectodermal cells with positional information along the anteroposterior axis in a similar way to what has been described in vertebrates. In the present manuscript, we show that, early during development, β-catenin protein is stabilized and accumulates in nuclei in vegetal cells fated to form endomesoderm. β-Catenin appears to be necessary and sufficient for endomesoderm fate specification. We further show that the endomesoderm defines a posterior organizer that is required to pattern the ectoderm along the anteroposterior axis. Consequently, β-catenin polarizes the embryo by defining the endomesoderm, which in turn patterns the embryo.

MATERIALS AND METHODS

Animals and embryos

Reproductively mature adults of *Saccoglossus kowalevskii* were collected at Waquoit Bay (Falmouth, MA, USA). Spawned eggs were fertilized in vitro and embryos were cultured in filtered seawater at 20-23°C as described previously (Lowe et al., 2004). Embryos were staged according to Colwin and Colwin (Colwin and Colwin, 1953).

Embryo manipulation

Vital staining of egg membrane with Nile Blue

Because Nile Blue precipitates in the presence of Cl^- ions, Cl^--free artificial seawater was used (450 mM sodium isethionate, 10 mM magnesium sulfate, 3 mM calcium sulfate, pH 7.0). At 25 minutes post-fertilization, embryos were washed a few times in Cl^--free artificial seawater before being transferred into the Nile Blue solution (20-40 μl of a Nile Blue saturated solution in 10 ml of Cl^--free artificial seawater). Staining takes a few minutes and was monitored using a dissecting scope. Stained embryos were washed in Cl^--free artificial seawater before additional washes in regular seawater. Embryos initially appear uniformly stained, but by 50 minutes post-fertilization the blue staining concentrates at the vegetal pole.

Classical embryology

The vitelline membranes that surround the embryo were chemically removed 1 hour post-fertilization using 1% thioglycolate in seawater at basic pH (four drops of 2.5 M NaOH in 7 ml of solution). Naked embryos or explants were cultured at low density in 1% agarose-coated Petri dishes containing filtered seawater (0.2 μm pore size) and gentamycin (50 μg/ml) with extreme care to avoid dislodging the loosely cohesive blastomeres at cleavage stages. Blastomeres or pieces of embryos were identified by combining landmarks described previously (Colwin and Colwin, 1953) and by Nile Blue staining (demarcating the presumptive endomesoderm territory), and were isolated using an eyelash probe. At blastula stages, ectodermal explants were produced by removing a wider region than the presumptive endomesoderm to avoid contamination.

Drug treatments

The canonical Wnt pathway was activated using the GSK3β inhibitors LiCl and 1-azakenpaullone (191500, Calbiochem). A stock solution of 0.5 M LiCl prepared in water (same osmolarity as seawater) was diluted in seawater for culturing embryos. Concentrations in the range of 25 to 250 mM were tested, and 250 mM gave the most robust and penetrant phenotypes. A 10 mM stock of 1-azakenpaullone was prepared in DMSO and stored at –20°C. Working solutions were prepared by diluting the stock into seawater (the same dilutions of DMSO in seawater were used as control). Concentrations ranging 2-20 μM were tested on embryos. Whereas 20 μM led to some toxicity, 10 μM treatments produced very robust and penetrant phenotypes (this concentration has been used for most experiments unless indicated).

To interfere with the actin network, we used cytochalasin B (228090010; Acros Organics) (stock: 10 mg/ml in DMSO; working dilutions in seawater: 5 to 20 μg/ml).

Microinjection

Injections into fertilized eggs were performed under a stereomicroscope using a simple device: a back-filled needle is connected to a glass syringe with plastic tubing. The entire system is filled up with mineral oil and the injection performed manually with the syringe under visual control [injected solution is colored by 1% Fast Green FCF (F-7252, Sigma)]. Injection was either performed into intact fertilized eggs (i.e. with the vitelline membrane on) during the period of a few minutes post-fertilization, as described previously (Lowe et al., 2006), or into naked eggs for an extended period of time during the second half of the first cell cycle. Injection success is further monitored by co-injection of 1% rhodamine dextran (D-1817, Molecular Probes).

To monitor activation of the canonical Wnt pathway, a mRNA encoding a fusion between the fluorescent protein Venus (Nagai et al., 2002) and the β-catenin protein from *S. kowalevskii* was injected. The construct was based on the design by Momose and Houliston (Momose and Houliston, 2007) and was generated by cloning the full-length β-catenin coding sequence 5′ of the Venus-coding sequence into the expression vector pCS2. mRNA was synthesized in vitro from linearized DNA using SP6 Message Machine Kit (Ambion) according to the manufacturer protocol. mRNA was injected at 0.5-2.0 μg/μl in water.

Two synthetic duplex siRNAs targeting different regions of the coding region of β-catenin mRNA were designed and purchased from Qiagen. The sequences are as follows: siRNA1 sense r(ACAUGCUGUUGUAAAU-CUUUU), antisense r(AAGAUUUACAACAGCAUGUUU); siRNA2 sense r(CCAGAAUGCUGUCCGAUUA)dTdT, antisense r(UAAUCG-GACAGCAUUCUGG)dGdC. siRNAs were resuspended at 67 mM in suspension buffer (100 mM potassium acetate, 30 mM HEPES brought to pH 7.4 with KOH). Both siRNAs yield an identical, homogeneous and penetrant phenotype when injected at 1/10 to 1/100 dilution (in water), whereas lower concentrations produce milder phenotypes.

In situ hybridization

In situ hybridization was carried out, as described previously (Lowe et al., 2004; Lowe et al., 2006; Lowe et al., 2003). Accession Numbers are GU076117 (*sfrp1/5*), GU224267 (*foxQ2-1*), EU939737 (β-*catenin*) and EU915508 (*foxA*).

RESULTS

Germ layers and anteroposterior patterning of the ectoderm

The normal development of *Saccoglossus kowalevskii* embryos was described in great detail by the Colwins in the 1950s (Colwin and Colwin, 1953). In addition, fate maps of early cleaving embryos (two- to 16-cell stages) have been established (Colwin and Colwin, 1951). The eggs laid by the female do not display any visible polarity. The first asymmetries are detected at 20 and 40 minutes post-fertilization as the first and second polar bodies emerge, and then at the eight-cell stage when the animal tier is formed by four smaller cells compared with the four larger vegetal cells (Fig. 1A). The former cells form the anterior ectoderm. At the 16-cell stage, a segregation occurs between the upper vegetal cells (veg1 tier) that will form the posterior ectoderm and the lower vegetal cells (veg2 tier) that give rise to the entire internal endomesoderm. After blastula stages, the veg2 progeny flattens to form a vegetal plate that will be internalized during gastrulation. The separation between endoderm and mesoderm occurs rather late in development (after gastrulation) with the out-pocketing of the coelomic pouches. In the present study, we will largely ignore this later event and focus on the segregation of endomesoderm and ectoderm.

The availability of a large collection of ESTs (Freeman et al., 2008) and extensive expression data allowed us to select a collection of markers of different regions of the ectoderm and endomesoderm. Some examples are shown in Fig. S1 in the supplementary material. *sfrp1/5* is expressed in the anterior-most region of the ectoderm from blastula stages, whereas *hox9/10* is restricted to the opposite site of the ectoderm. The selected markers are largely expressed in the same domains throughout development with some minor deviations (e.g. *foxA* is a pan-endomesodermal marker before being restricted to the endoderm proper after gastrulation, following the induction of mesoderm). By early gastrula stages, the embryo is already extensively patterned in the anterior-posterior and dorsal-ventral dimensions, but refinements continue to occur in later stages (e.g. the *engrailed* stripe is visible only from neurula stages).

The animal-vegetal axis is set up after fertilization: the vegetal contraction

As described above, an early sign of animal-vegetal polarity is observed at the eight-cell stage. However, after fertilization, the egg is subject to extensive deformations reminiscent of what happens in many other animals. Time-lapse analysis reveals a striking contraction in the vegetal hemisphere (opposite to the site of polar bodies emission) (see Movies 1 and 2 in the supplementary material). To determine whether this contraction is linked to animal-vegetal polarity, we applied the vital visible dye Nile Blue 25 minutes after fertilization (Fig. 1B), just after first polar body extrusion. The eggs first appeared uniformly stained on their entire surface. However, 30-50 minutes after fertilization the dye concentrates on the vegetal side of the egg (Fig. 1B; see Movie 3 in the supplementary material). The blue staining constitutes about one-third of the egg surface at the opposite side of the polar bodies. Interestingly, the staining is inherited by the veg2 blastomeres at the 16-cell stage and by the entire endomesoderm internalized at later stages. We termed this process ‘the vegetal contraction’. It is similar to what is observed in ascidian embryos, the so-called ‘first phase of ooplasmic segregation’ (Nishida, 2005). This latter process is an actin-based

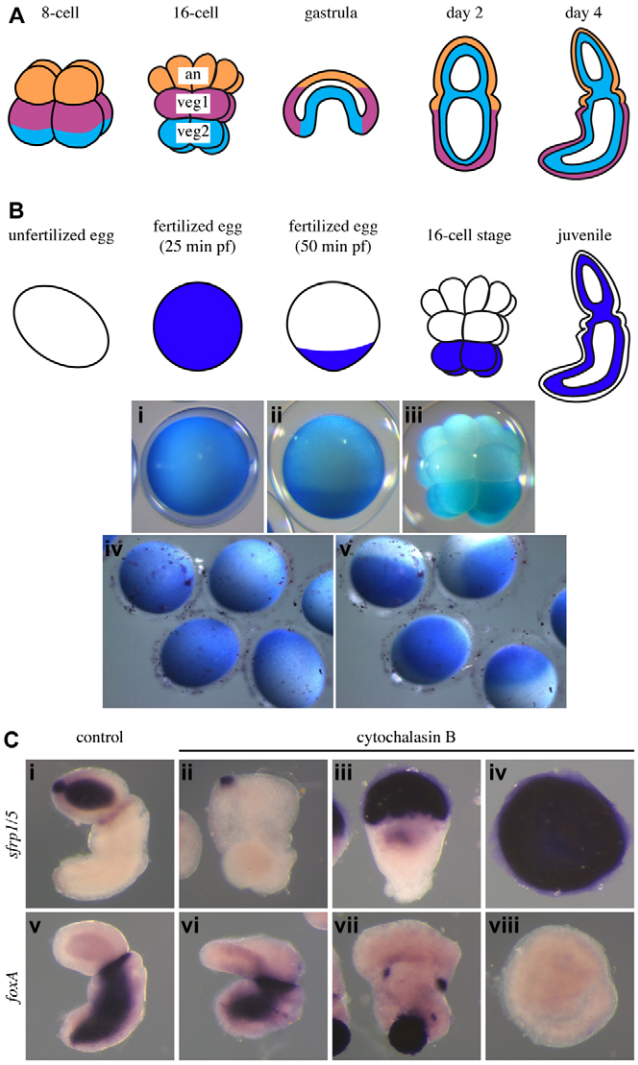

Fig. 1. The vegetal contraction controls animal-vegetal axis polarity in *Saccoglossus kowalevskii*. (**A**) Fate maps. Anterior ectoderm (orange), posterior ectoderm (purple) and endomesoderm (blue) fates are segregated by cleavage planes at the 16-cell stage. (**B**) When fertilized eggs are vitally stained with Nile Blue 25 minutes post-fertilization (i), the staining concentrates towards the vegetal pole at 50 minutes (ii) and is inherited at the 16-cell stage by the veg2 blastomeres (iii) that will eventually give rise to the entire endomesoderm. Snapshots of a time-lapse movie (see Movie 3 in the supplementary material) at the onset (iv) and at the end (v) of vegetal contraction. (**C**) Treatment with the actin polymerization inhibitor cytochalasin B during vegetal contraction leads to an expansion of the anterior ectoderm, as evidenced by the marker *sfrp1/5* (i-iv) and to a reduction in endoderm formation revealed by *foxA* expression (v-viii) at day 4 of development. Control embryos (i and v, lateral views, dorsal towards the right, anterior towards the top) and embryos treated with 20 μg/ml of cytochalasin B during vegetal contraction (ii and vi, partial animalization; iii and vii, strong animalization; iv and viii, complete animalization).

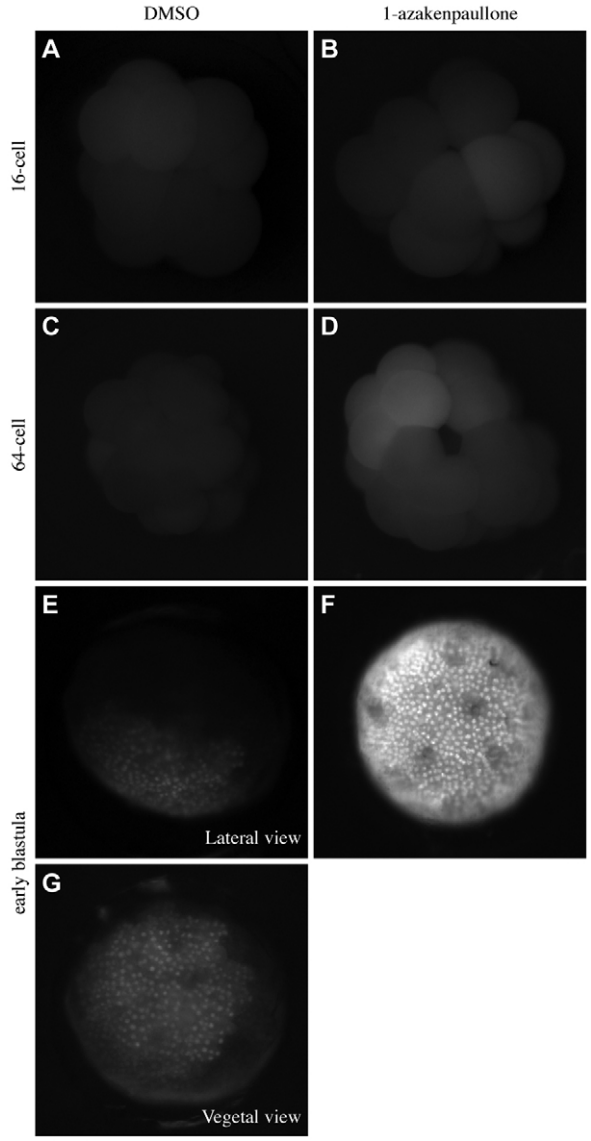

Fig. 2. The canonical Wnt pathway is active at the vegetal pole of blastulae. (**A-G**) Embryos injected with an mRNA encoding a fusion between the fluorescent protein Venus and β-catenin. (A,C,E,G) Increasing cytoplasmic fluorescent signals before both cytoplasmic and nuclear staining occurs in cells of the vegetal hemisphere at early blastula stages. (B,D,F) When GSK3β, the upstream negative regulator of β-catenin stability, is inhibited using 1-azakenpaullone, strong cytoplasmic and nuclear fluorescent signals are detected in all cells of the embryo.

rearrangement of cortical cytoplasm that concentrates, at the vegetal pole, maternal determinants required for animal-vegetal axis specification.

We tested whether the vegetal contraction in *Saccoglossus* could have a similar role in axis specification. We postulated that, like its ascidian counterpart, vegetal contraction might involve actin filaments. We thus treated eggs with the actin polymerization inhibitor cytochalasin B before the onset of vegetal contraction (25 minutes post-fertilization) until the end of this process (55 minutes post-fertilization). Cytochalasin B effect was reversible, as treated embryos cleaved normally despite a slight delay, whereas embryos kept in the drug did not cleave. The control embryos developed normally (91% normal, n=80). By contrast, most treated embryos developed with a severely affected body axis (Fig. 1C). Although the extent of the phenotype was variable among treated embryos, we observed that the anterior part was expanded, as revealed by enlargement of the apical tuft (the sensory cells at the anterior tip of the proboscis

Table 1. Cytochalasin B treatment leads to animalization

Cytochalasin B concentration (μg/ml)	Treatment time post-fertilisation (minutes)	Percentage normal	Percentage partial animalization	Percentage strong animalization	Percentage complete animalization	Percentage with non-specific abnormalities	*n*
0	–	91	0	0	0	9	80
10	5-25	79	4	1	0	16	100
20	5-25	60	12	2	0	26	42
10	25-55	34	15	25	0	25	59
20	25-55	7	17	59	5	12	41

characterized by very long stiff cilia, not shown) and the posterior part was strongly reduced (in 40-81% of the treated embryos; Fig. 1C, Table 1). These results were confirmed when specific markers were analyzed. The apical tuft marker *sfrp1/5* was expanded, whereas the endodermal marker *foxA* was strongly downregulated in the treated embryos. Importantly, when the eggs were exposed to cytochalasin B prior to the vegetal contraction and the drug was washed out, most embryos developed normally and only a minority were animalized (Table 1). Interference with the vegetal contraction leads to embryos that are both animalized (decrease of endomesoderm) and anteriorized (increase of anterior ectoderm). These results indicate that key events take place after fertilization for the specification of the animal-vegetal axis and the embryonic anteroposterior axis.

The Wnt/β-catenin pathway is activated at the vegetal pole

As knockdown of β-catenin leads to animalized embryos in both ascidians and sea urchins (Imai et al., 2000; Kawai et al., 2007; Logan et al., 1999; Wikramanayake et al., 1998), we tested whether this pathway had a similar function in *S. kowalevskii*. When the Wnt/β-catenin pathway is activated, β-catenin protein becomes stabilized and translocates to the nucleus where it activates transcriptional targets. To visualize Wnt pathway activity, we have injected into fertilized eggs a mRNA encoding a fusion protein between the fluorescent protein Venus and *S. kowalevskii* β-catenin. Weak fluorescent levels (above background) were observed in the cytoplasm of 16-cell stage embryos (Fig. 2A,B). As β-catenin might be subject to active degradation, we treated a subset of these embryos with the GSK3β inhibitor 1-azakenpaullone (Kunick et al., 2004). The treated embryos showed a gradual increase in fluorescence, first in the cytoplasm and then with a clear nuclear accumulation in all blastula cells (Fig. 2D,F). By contrast, non-treated embryos harbored very reduced levels of fluorescence, suggesting a tight control of β-catenin stability at cleavage stages. By blastula stages, the fluorescent protein was observed in the nuclei of vegetal cells (roughly the vegetal-most third of the embryo), whereas animal cells were almost completely devoid of fluorescence (Fig. 2E,G). At gastrula stages, no fluorescence could be detected (data not shown), which was probably due to degradation of the β-catenin-Venus protein, preventing description of Wnt/β-catenin pathway activity at later stages using this method. These observations show that β-catenin protein levels are tightly controlled, and that the vegetal cells undergo active Wnt/β-catenin signaling and nuclear accumulation.

The β-catenin/Wnt pathway is responsible for endomesoderm specification and anteroposterior patterning of the ectoderm

In order to evaluate whether β-catenin plays a role in axis formation, we performed knock-down experiments by injecting double-stranded siRNA against β-catenin into fertilized eggs. Two siRNAs targeting different regions of the coding region of β-catenin mRNA were tested and gave identical phenotypes (Table 2). Results obtained with siRNA1 are shown here. As shown in Fig. 3A, injected embryos failed to gastrulate and resembled flattened late blastulae. Later during development, the appearance of these embryos did not change significantly, but eventually they developed very long ectopic cilia, similar to the ones found in the apical tuft, demonstrating that these embryos do differentiate and are likely to comprise only anterior-most ectoderm (not shown). These embryos were tested for the expression of various markers by in situ hybridization, and the molecular analysis supports the conclusions drawn by the morphological observations (Fig. 3, Table 2). First, endomesoderm appears to be absent, as the β-catenin siRNA-injected embryos did not express the endomesodermal marker *foxA*. Second, the ectoderm is anteriorized, as only the anterior ectodermal markers *six3*, *sfrp1/5* and *foxQ2-1* were expressed in all cells of the embryo, whereas the expression of the posterior ectodermal marker *hox9/10* was completely abolished (Fig. 3, Table 2; data not shown). Importantly, these effects could be detected as early as the onset of gastrulation (Fig. 3B), arguing that the role of β-catenin on endomesodermal germ layer specification is direct rather than caused by the failure to gastrulate.

Altogether, these results show that embryos depleted of β-catenin are composed of anterior-most ectoderm only (complete lack of endomesoderm and non-anterior ectoderm). These phenotypes are similar to the ones caused by interference with the vegetal contraction, suggesting that this latter process controls in some way activation of the β-catenin pathway.

Table 2. Effects of β-catenin siRNA injection: expression of markers analyzed by in situ hybridization

	Gastrula				Day 2				
	foxA	*hox9/10*	*foxQ2-1*	*six3*	*foxA*	*hox9/10*	*sfrp1/5*	*foxQ2-1*	*barH**
Control	34/34	12/12	12/12	20/20	45/45	20/20	20/20	37/37	16/16
β-Catenin siRNA1	1/19†	0/6	14/14‡	6/6‡	1/27†	0/11	14/14‡	18/18‡	
β-Catenin siRNA2	0/4				0/9	0/8		8/8‡	0/7

**barH* is expressed as an ectodermal ring in an intermediate domain fated to form the anterior collar.
†Expression found only in a small group of cells.
‡Expression detected throughout the embryo.

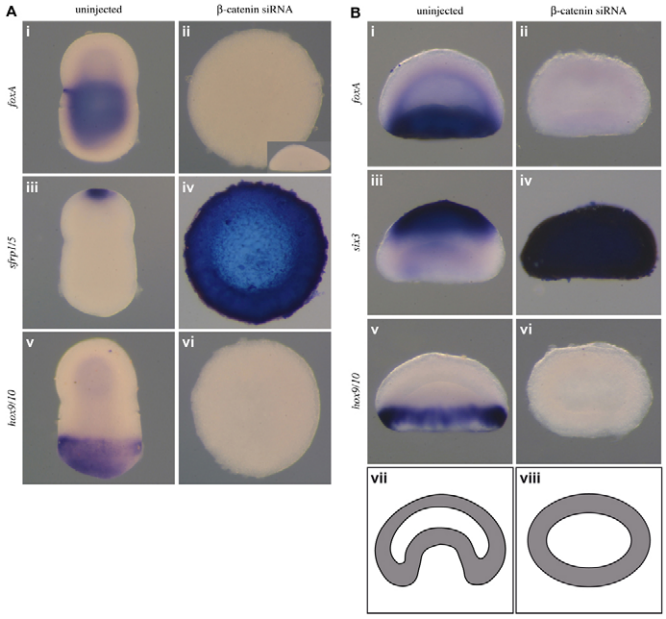

Fig. 3. β-Catenin is required for endomesoderm and posterior ectoderm formation. Embryos were injected with a siRNA targeting β-catenin and analyzed by in situ hybridization at (**A**) day 2 of development and (**B**) gastrula stages. The expression of the endomesoderm marker *foxA* and of the posterior ectoderm marker *hox9/10* is completely abolished (A, parts ii, vi; B, parts ii, vi), whereas the anterior ectoderm markers *sfrp1/5* (A, part iv) and *six3* (B, part iv) are expressed throughout the embryo. Control embryos (A, parts i, iii, v; B, parts i, iii, v) are shown in a lateral view with anterior towards the top. β-Catenin siRNA-injected embryos (A, parts ii, iv, vi; B, parts ii, iv, vi). Schematic cross-section of control (B, part vii) or β-catenin siRNA-injected embryo (B, part viii) at gastrula stages.

Early activation of β-catenin induces endomesoderm

We then tested whether β-catenin activity was sufficient for endomesoderm specification. In order to activate the β-catenin pathway in a time-controlled manner, we made use of two pharmacological inhibitors of the upstream negative regulator of β-catenin GSK3β: LiCl and 1-azakenpaullone (Kunick et al., 2004). The two inhibitors gave similar results. However, because LiCl appeared to produce some side and/or toxic effects (such as delay in development), only results obtained with 1-azakenpaullone (thought to be a more specific inhibitor) are shown here. We first applied 1-azakenpaullone at different concentrations from the eight-cell stage and analyzed the effects after 3 days of development when control embryos exhibited tripartite body organization. Whereas a concentration of 20 μM had toxic effects, concentrations ranging from 2 to 10 μM led to a range of phenotypes. The most affected embryos (10 μM) developed very penetrantly as spheres showing folds but no elongation (Fig. 4A). Lower concentrations led to a more heterogeneous population of embryos, each usually containing two parts. As treated embryos did not show clear morphological signs of polarity, we analyzed the expression of several ectodermal markers and of the endodermal marker *foxA*. Very clearly, embryos treated with 10 μM inhibitor were only composed of endodermal cells, and the expression of all ectodermal markers tested was abolished (Fig. 4A). The milder phenotypes correspond to embryos comprising one part of endomesoderm and one part of posterior ectoderm. Anterior ectoderm was almost always absent. We also fixed embryos at the beginning of gastrulation, before major morphogenetic movements occur (Fig. 4B). We first observed that treated embryos kept a round blastula-like morphology and did not show signs of vegetal plate flattening and onset of gastrulation. The two markers we used that stain the presumptive endomesoderm (vegetal most third of the embryo) at this stage (*foxA* and *otx*) showed clearly that all cells of the embryos were converted into endomesoderm at the highest dose of the drug. At intermediate doses, both markers displayed a clear displacement towards the animal pole. Interestingly, the posterior ectodermal markers *caudal* and *hox9/10* did track the new position of the endomesoderm/ectoderm boundary and were expressed as a ring, whereas anterior ectoderm markers were not expressed (Fig. 4B; data not shown).

Because, in the most extreme cases, all cells of the embryos are converted into endomesoderm, it suggests that all cells are competent to make endomesoderm upon β-catenin pathway activation. We wanted to further test this idea by activating the pathway in isolated ectoderm precursors. Whereas anterior ectoderm precursors (animal tier blastomeres) do not form endomesoderm upon isolation at the eight-cell stage, treatment with 10 μM of 1-azakenpaullone is sufficient to convert the entire explant into endomesoderm expressing *foxA* and *otx* (Fig. 5A). This result suggests that indeed all cells of the embryos are competent and likely respond directly to β-catenin.

To estimate when the β-catenin pathway is active in vivo, we applied 10 μM of 1-azakenpaullone at various time points. As shown in Fig. 5B,C, embryos treated from 4 or 6 hpf (hours post fertilization) (four- and eight-cell stages) exhibited a complete conversion into endomesoderm. Treatments from 8 or 10 hpf (32- and 256-cell stages) led to a partial ectopic formation of endomesoderm, whereas treatments after 12 hpf (early blastula stages) did not induce ectopic endomesoderm.

Together with the previous section, these results indicate that β-catenin pathway activity at early cleavage stages, before 12 hpf, is necessary and sufficient for endomesoderm specification.

The ectoderm is patterned by signals from the endomesoderm

β-Catenin-depleted embryos are not only devoid of endomesoderm but they also lack anteroposterior polarity. It is possible that this patterning defect was due to the lack of β-catenin or the lack of endomesoderm in those embryos. To address directly the potential role of the endomesoderm in ectoderm patterning, we performed a series of blastomere isolations at different times.

Endomesoderm precursors always differentiated into endomesoderm expressing *foxA*, regardless of the isolation stage (data not shown), suggesting that the specification of this fate does not rely on the presence of ectoderm, in agreement with the proposed role of β-catenin as a determinant.

When blastomeres were isolated at the eight-cell stage, two drastically different phenotypes were observed. The vegetal tier, normally fated to form endomesoderm and posterior ectoderm, was made of two parts: an endomesoderm-like region with obvious signs of gastrulation; and an ectodermal region (Fig. 6A). The most striking observation is that these explants were actively swimming showing the presence of ciliated band-like tissue (posterior ectoderm). These morphological phenotypes were confirmed with molecular markers. By contrast, the animal tier explants formed ball- or pancake-like structures, suggesting they were made of simple epithelia. These explants were also marked by the presence of very long apical tuft-like cilia and were very similar to β-catenin-depleted embryos. Anterior-most ectodermal markers

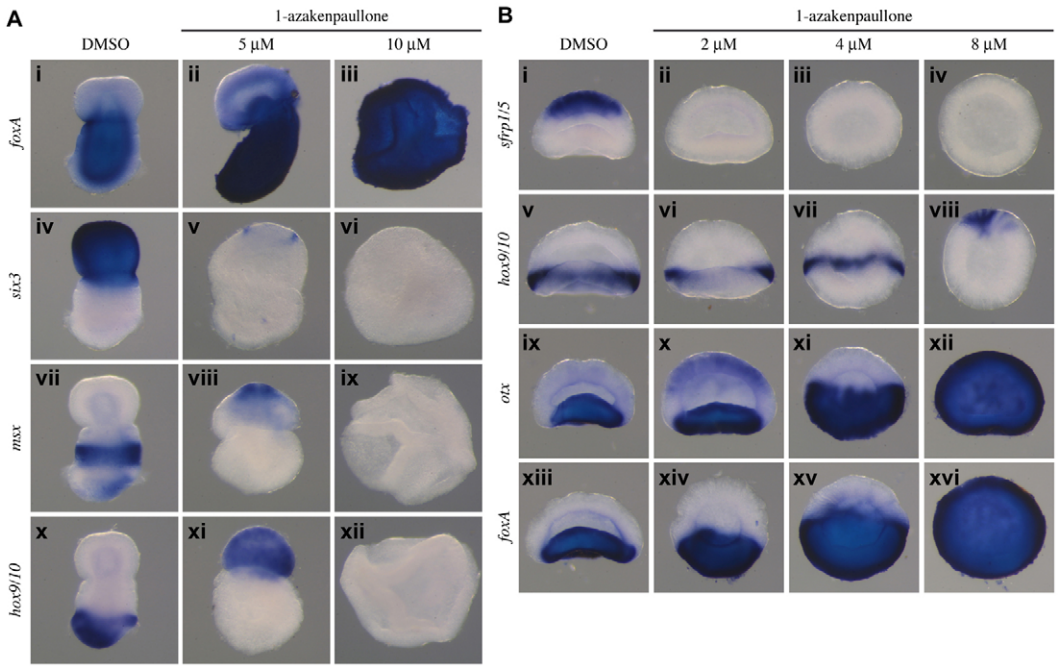

Fig. 4. Activation of the canonical Wnt pathway leads to ectopic endomesoderm formation. (A,B) Blocking the negative regulator of β-catenin GSK3β leads to ectopic endomesoderm formation in a dose-dependent manner examined at day 3 of development (A) and gastrula stages (B). (**A**) At day 3, the embryos treated with 5 μM of 1-azakenpaullone are composed of endoderm stained by *foxA* (A, part ii) and posterior ectoderm (A, parts v, viii and xi), whereas the embryos treated with 10 μM are only made of endoderm (A, parts iii, vi, ix and xii). (**B**) Treatments with sub-optimal doses of 1-azakenpaullone lead to a progressive shift of the endomesodermal markers *otx* and *foxA* at gastrula stages towards the animal pole as the dose increases (B, parts x-xii and xiv-xvi). The posterior ectodermal marker *hox9/10* follows the new endomesoderm/ectoderm boundary (B, parts vi-viii), whereas the anterior ectodermal expression of *sfrp1/5* is lost (B, parts ii-iv). Control embryos at day 3 of development are oriented with anterior towards the top and dorsal towards the right (A, parts i, iv, vii and x). Gastrulae have been bisected after in situ hybridization; animal side towards the top (B). Control embryos at gastrula stages are shown in lateral view with anterior towards the top (B, parts i, v, ix and xiii).

(*sfrp1/5* and *foxQ2-1*) were expressed at high levels in all cells of these explants, whereas more posterior markers were not expressed (Fig. 6A, Table 3; data not shown). This experiment suggests that animal cells normally receive signals from the vegetal cells to restrict very anterior identity only to the anterior-most progeny.

To determine whether this apparent anterior 'default fate' of the animal cells extend to all ectodermal precursors, we separated the three tiers of 16-cell stage embryos: an, veg1 and veg2 (Fig. 6A). As expected, the an tier (progeny of the animal blastomeres at the eight-cell stage) developed into an apical tuft expressing *sfrp1/5*. Interestingly, the veg1 tier, fated to form the posterior ectoderm and the progeny of which does not express *sfrp1/5* in intact embryos, developed like the an tier after isolation and did express *sfrp1/5* at high levels in all cells. The veg2 tier developed into an archenteron-like explant and did not express *sfrp1/5*. This experiment shows that ectoderm precursors, which are fated to adopt posterior identities in the intact embryo, adopt an anterior character in the absence of signals from endomesoderm precursors.

In order to determine when vegetal posteriorizing signals are sent, we isolated ectoderm precursors at different time points (Fig. 6B, Table 3). As expected from the above experiments, the ectoderm isolated at early cleavage stages developed into an apical tuft-like explant expressing *sfrp1/5* and *foxQ2-1* in all cells. However, when the ectoderm was isolated at mid-blastula stages, the expression of anterior markers was restricted to a subdomain, whereas the intermediate marker *engrailed* and the posterior marker *hox9/10* were activated as circumferential rings, similar to the ones observed in intact embryos.

The above experiments show that: (1) the endomesoderm and ectoderm germ layers may form autonomously; (2) the ectoderm has an anterior-most 'default' fate; and (3) signals emitted by the vegetal cells between cleavage and blastula stages posteriorize the ectoderm.

DISCUSSION

Zygote polarization

By blastomere isolation experiments, we have shown that, in *Saccoglossus* embryos, the third cleavage (eight-cell stage), which is the first that occurs perpendicular to the animal-vegetal axis, separates blastomeres with different developmental potentials. The polarization of the embryo is likely to be a result of the vegetal contraction that occurs during the first cell cycle. Although the identification of the precise cellular and molecular players involved will require further investigation, zygote polarization leads to differential stabilisation/degradation of β-catenin protein by cleavage/blastula stages allowing activation of the Wnt pathway in the vegetal hemisphere. These data are very similar to the ascidian 'first phase of ooplasmic segregation' in which an actin-based contraction of the cortical cytoplasm towards the vegetal pole leads to an enrichment in a still elusive endoderm maternal determinant (Kumano and Nishida, 2007). By contrast, the sea urchin zygote is already polarized before fertilization, with vegetal enrichment of Dishevelled protein at the cortex (reviewed by Croce and McClay, 2006). One could speculate that these differences stem from the fact that ascidian and hemichordate eggs are arrested in meiotic metaphase I, whereas in sea urchin eggs, meiosis is complete at the

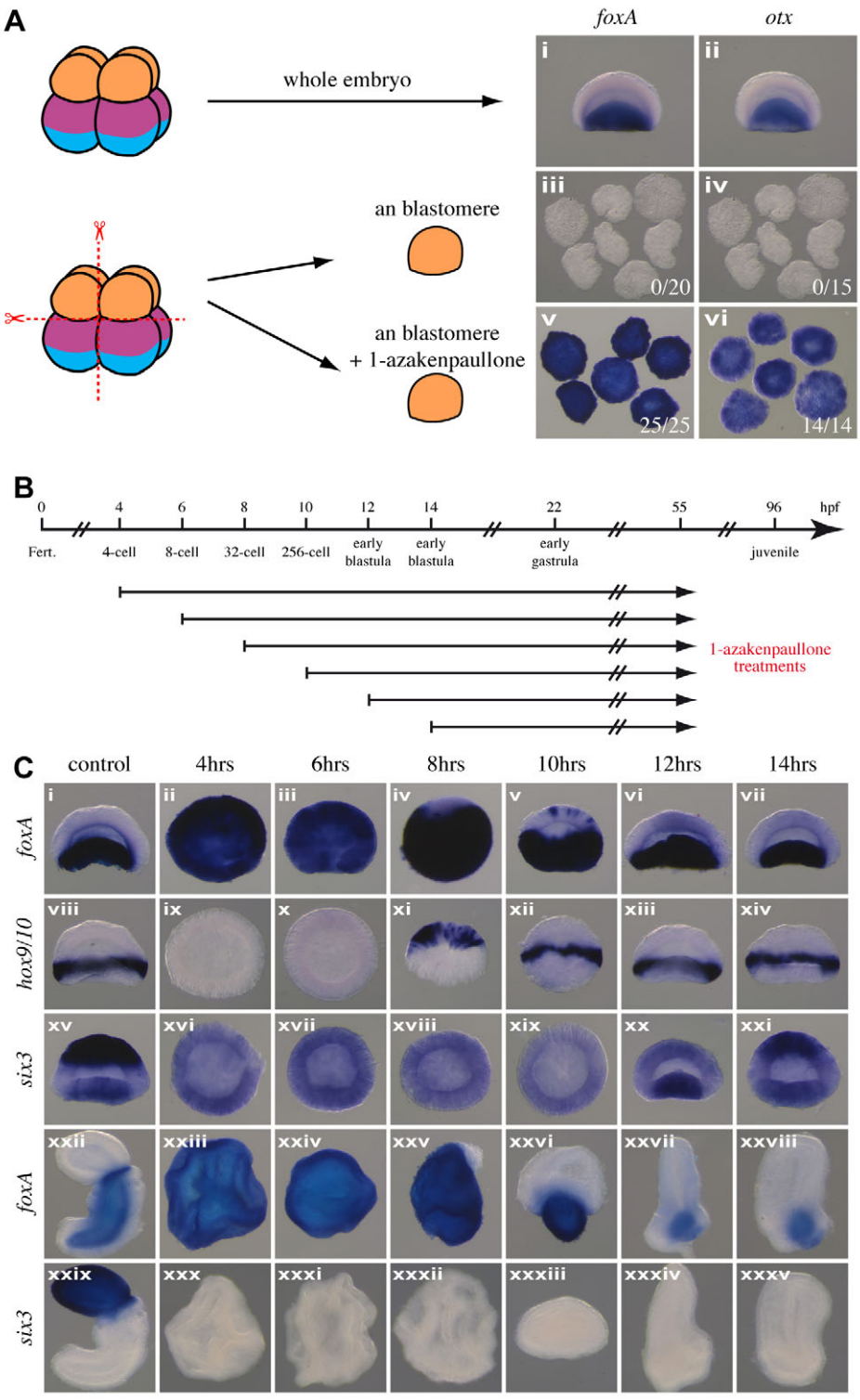

Fig. 5. Activation of the canonical Wnt pathway is sufficient to induce endomesoderm at cleavage stages. (**A**) Activating the Wnt pathway is sufficient to convert isolated ectoderm precursors into endomesoderm. Animal blastomeres isolated at the eight-cell stage do not form endomesoderm (A, parts iii, iv) and treatment with 10 μM of 1-azakenpaullone converts them into endomesoderm expressing *foxA* and *otx* (A, parts v, vi). Control embryos at gastrula stages, animal towards the top (A, parts i, ii). Numbers indicate positively stained explants over analyzed explants. (**B**,**C**) The Wnt pathway must be activated before blastula stages to induce endomesoderm. (B) Experimental design. (C) In situ results at gastrula stages (parts i-xxi) and day 4 of development (parts xxii-xxxv). Complete vegetalization is observed for treatments starting at early cleavage stages (4 and 6 hpf), as revealed by the expression of *foxA* in all cells of the treated embryos (C, parts ii, iii, xxiii and xxiv) and the loss of expression of all ectodermal markers examined (C, parts ix, x, xvi, xvii, xxx and xxxi). Partial vegetalization occurs for treatments during late cleavage stages (C, parts iv, v, xxv and xxvi), posterior ectoderm forms at the new endomesoderm/ectoderm boundary (C, parts xi, xii), but anterior ectoderm expressing *six3* does not form (C, parts xviii, xix, xxxii and xxxiii). Treatments at early blastula stages do not induce ectopic expression of *foxA*, marking the endomesoderm at gastrula stages (C, parts vi, vii) but lead to a reduction of endoderm at day 4 (C, parts xxvi, xxvii). Posterior ectoderm expression of *hox9/10* is unaffected (C, parts xiii, xiv), but expression of *six3* in anterior ectoderm is lost (C, parts xx, xxi, xxxiv and xxxv). Gastrulae have been bisected after in situ hybridization, animal pole towards the top (C, parts i-xxi). Embryos at 4 days of development are shown with anterior towards the top and dorsal towards the right (C, parts xxii-xxxv). hpf, hours post-fertilization.

time of fertilization. Cytoplasmic rearrangements that polarize the eggs along the animal-vegetal axis might rather be linked to meiosis progression than to fertilization.

The upstream factors regulating β-catenin stabilization that have been identified to date can be diverse. In frog, the ligand Wnt11 is the signal initiating organizer formation (Tao et al., 2005), whereas in the cnidarian *Clytia hemisphaerica*, both Frizzled receptors and the ligand Wnt3 control axial patterning (Momose et al., 2008; Momose and Houliston, 2007). In sea urchin, Dishevelled protein is enriched at the vegetal pole and is required for endoderm formation (Weitzel et al., 2004), whereas in ascidians it does not exhibit localized enrichment and is largely dispensable (Kawai et al., 2007). We have identified four Wnt ligands that are maternally supplied in *Saccoglossus*, two of which are expressed at relatively high levels (our unpublished results). β-Catenin protein itself could be enriched at the vegetal pole following vegetal contraction, but further work will be needed to identify which factors lead to nuclear β-catenin accumulation in endomesoderm precursors.

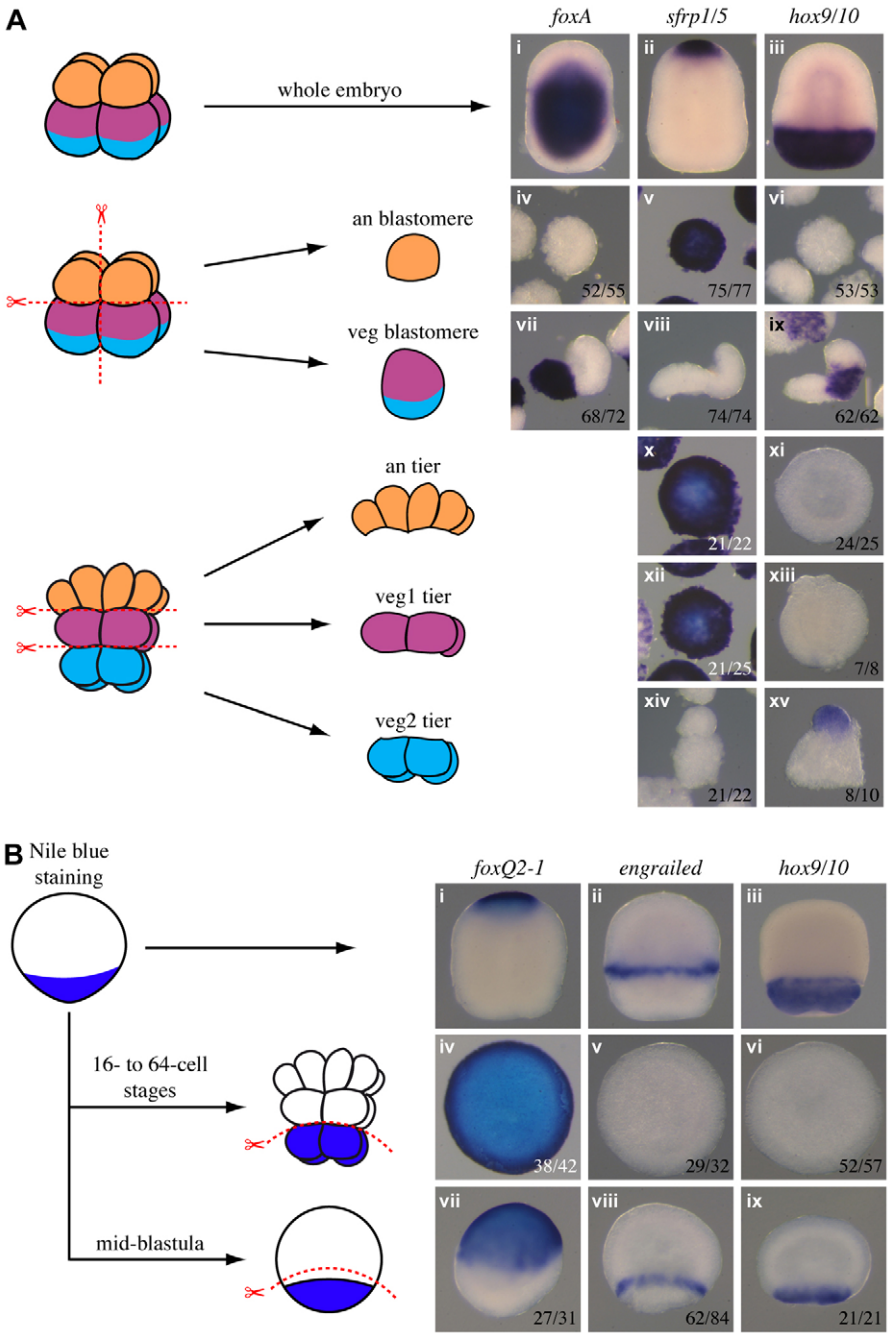

Fig. 6. Endomesoderm posteriorizes ectoderm that would otherwise develop anterior character. (**A**) Ectoderm precursors develop into anterior ectoderm upon isolation. Vegetal explants derived from blastomeres isolated at the eight-cell stage comprise endomesoderm (A, part vii) and posterior ectoderm (A, parts viii, ix), whereas all cells of animal explants express the anterior ectodermal marker *sfrp1/5* (A, parts iv-vi). When the veg1 tier, which is fated to form posterior ectoderm, is isolated at the 16-cell stage, it gives rise to anterior-most ectoderm (A, parts xii, xiii) like the an tier explant (A, parts x, xi). Veg2 tier explants comprise endomesoderm and a small portion of posterior ectoderm expressing *hox9/10* (A, parts xiv and xv). (**B**) Endomesoderm precursors send posteriorizing signals between the 16-cell and blastula stages. When endomesoderm is removed at early cleavage stages, the ectodermal explants are entirely stained by the anterior marker *foxQ2-1* (B, parts iv-vi). However, the ectodermal explants obtained from removal of endomesoderm precursors at mid-blastula stages display an anteroposterior pattern (B, parts vii-ix). Embryos are shown at day 2 of development with anterior towards the top. Numbers indicate the proportion of explants that exhibited the expression depicted.

β-Catenin as an endomesoderm determinant

We have shown that nuclear β-catenin at the vegetal pole of the early *Saccoglossus* embryo is necessary and sufficient to specify the endomesoderm (Fig. 7). Because the same function has been found in other deuterostomes (echinoderms and ascidians) (Imai et al., 2000; Kawai et al., 2007; Logan et al., 1999; Wikramanayake et al., 1998), our study reinforces the idea that this function was present at the base of this group of bilaterians. Work in amphioxus suggests that this function might have been lost in this early branch of the chordates (Holland et al., 2005; Yasui et al., 2002). Although the reported pattern of β-catenin localization does not support such a function, it is worth noting that early activation of the pathway leads to exogastrulation, which might result from a defect in germ layer specification. However, endomesoderm fates have not been carefully assessed in these studies, and additional work, particularly loss-of-function approaches, is needed to clarify this issue. In vertebrates, the maternal β-catenin pathway is primarily used to set up the dorsoventral axis and is only a minor player in germ layer specification (for a review, see Marikawa, 2006).

Studies in cnidarians have suggested that this early function of β-catenin is an ancestral one (Wikramanayake et al., 2003). An alternative non-contradictory hypothesis proposes that the ancestral function of β-catenin in metazoans is to control binary cell fate specification of sister cells rising from divisions along the animal-vegetal axis (Schneider and Bowerman, 2007). This function has been described in two distantly related protostomes, the nematode *Caenorhabditis elegans* and the annelid *Platynereis dumerilii* (Mizumoto and Sawa, 2007; Schneider and Bowerman, 2007). The endoderm specification by β-catenin has, however, been described in only one protostome species, the nemertean *Cerebratulus lacteus*

Table 3. Analysis of ectodermal explants isolated at different stages by in situ hybridization

	Anterior markers				Posterior markers			
Isolation stage		Percentage of explants entirely stained	Percentage with partial expression	*n*		Percentage with no expression	Percentage with a ring of expression	*n*
Eight-cell stage, An tier	*sfrp1/5*	97	3	77	*engrailed*	93	7	27
	foxQ2-1	100		20	*barH*	100		7
					msx	100		8
					caudal	100		8
					hox9/10	100		53
Sixteen-cell stage, An tier	*sfrp1/5*	95	5	22	*hox9/10*	96	4	25
	foxQ2-1	100		16				
Sixteen-cell stage, Veg1 tier	*sfrp1/5*	84	16	25	*hox9/10*	87	12	8
	foxQ2-1	100		13				
Sixteen- to 64-cell stages, entire ectoderm	*sfrp1/5*	85	15	60	*engrailed*	91	9	32
	foxQ2-1	90	10	42	*msx*	89	11	36
					hox9/10	91	9	57
Early blastula, entire ectoderm	*sfrp1/5*	36	64	25	*engrailed*	75	25	16
	foxQ2-1	50	50	30	*msx*	50	50	14
					hox9/10	50	50	34
Mid-blastula, entire ectoderm	*sfrp1/5*		100	45	*engrailed*	26	74	84
	foxQ2-1	13	87	31	*msx*	41	59	17
					hox9/10		100	21

(Henry et al., 2008). In order to test the relevance of these hypotheses and to understand the evolution of the function of β-catenin in metazoans, it will be important to characterize its function more broadly in protostomes, with particular attention to the lophotrochozoans that have been poorly sampled.

The gene regulatory network downstream of maternal β-catenin controlling endomesoderm specification has been described in great detail in sea urchins (Davidson et al., 2002). Comparisons have been extended to asteroids (Hinman et al., 2003). Given the existence of a common function in three branches of the deuterostomes (tunicates, echinoderms and hemichordates), it would be very informative to decipher the gene regulatory networks in both tunicates and hemichordates in order to probe the level of conservation at the network level. Similar approaches could be performed at a broader phylogenetic range, namely extended to protostomes and cnidarians.

Wnt pathway function in endomesoderm specification and posterior identity definition

The nuclear β-catenin domain defines the endomesoderm in the vegetal hemisphere and the β-catenin-free domain defines the ectoderm. This early germ layer establishment polarizes the embryo and consequently prefigures the future anteroposterior axis, which is co-linear to the animal-vegetal axis in *Saccoglossus*. In the absence of endomesoderm, ectodermal precursors are not polarized along the anteroposterior axis and remain with an anterior default fate. We have shown that endomesodermal precursors secrete signals that posteriorize the ectoderm (Fig. 7). Interestingly, both the anterior default fate and the gradual posteriorization (or repression of anterior identity) is reflected in the dynamic expression pattern of the anterior ectoderm markers *sfrp1/5* and *foxQ2-1*. Both genes are initially expressed in a broad domain at blastula stages (possibly the entire ectoderm for *sfrp1/5*) before being gradually restricted to a narrower domain that will eventually correspond to the future apical tuft (see Fig. S2 in the supplementary material). In sea urchin, ectoderm is also patterned by signals from the endomesoderm. In the absence of endomesoderm or active Wnt signaling, the animal plate, the animal-most ectodermal territory, is dramatically expanded. The nature of the signals emitted by endomesodermal cells is unknown, but Wnt antagonism in the ectoderm seems to play a role in animal plate definition (Angerer and Angerer, 2003; Wei et al., 2009; Yaguchi et al., 2006).

By specifying the endomesoderm, β-catenin defines the posterior part of the *Saccoglossus* embryo. The factors triggering this activity must be β-catenin downstream genes expressed in the presumptive endomesoderm at blastula stages and coding for secreted molecules capable of posteriorizing the ectoderm. Based on what is known in vertebrates, obvious candidates include members of the FGF, BMP, Nodal and Wnt pathways. The Wnt pathway is unlikely to be a major component of the signaling activity secreted by the endomesoderm (our unpublished results). We have not found Wnt ligands expressed early in the endomesoderm. Furthermore, abolishing β-catenin activity in the ectoderm is not sufficient to anteriorize the entire ectoderm, as would be predicted if the reception of endomesodermal Wnt signals were blocked. However, the Wnt pathway is crucial in the ectoderm at later stages to refine the initial patterning triggered by the endomesoderm and

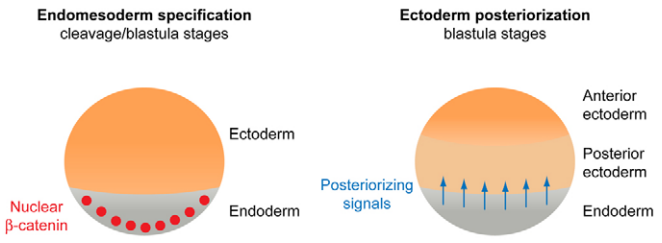

Fig. 7. Schematic representation of the function of β-catenin in *Saccoglossus kowalevskii*. During cleavage and early blastula stages, β-catenin accumulates in the nuclei of vegetal blastomeres where it is necessary and sufficient for endomesoderm specification. Endomesoderm precursors are then involved in the definition of the posterior ectoderm by sending posteriorizing signals to the overlying ectoderm that would otherwise be of anterior character.

particularly to subdivide the anterior region of the embryo. The role of other candidate pathways will be the subject of future investigations. Identification of these players should allow us to further probe the level of conservation of the developmental mechanisms controlling posterior identity in deuterostomes and also possibly outside of deuterostomes. In conclusion, the definition of the posterior part of the ectoderm of the embryo is thus most probably only indirectly controlled by the Wnt/β-catenin pathway, through the specification of endomesoderm and activation of posteriorizing signals (Fig. 7).

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Competing interests statement
The authors declare no competing financial interests.

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