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Asymmetric developmental potential along the animal–vegetal axis in the anthozoan cnidarian, *Nematostella vectensis*, is mediated by Dishevelled

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Abstract

The relationship between egg polarity and the adult body plan is well understood in many bilaterians. However, the evolutionary origins of embryonic polarity are not known. Insight into the evolution of polarity will come from understanding the ontogeny of polarity in non-bilaterian forms, such as cnidarians. We examined how the axial properties of the starlet sea anemone, *Nematostella vectensis* (Anthozoa, Cnidaria), are established during embryogenesis. Egg-cutting experiments and sperm localization show that *Nematostella* eggs are only fertilized at the animal pole. Vital marking experiments demonstrate that the egg animal pole corresponds to the sites of first cleavage and gastrulation, and the oral pole of the adult. Embryo separation experiments demonstrate an asymmetric segregation of developmental potential along the animal–vegetal axis prior to the 8-cell stage. We demonstrate that Dishevelled (Dsh) plays an important role in mediating this asymmetric segregation of developmental fate. Although *NvDsh* mRNA is ubiquitously expressed during embryogenesis, the protein is associated with the female pronucleus at the animal pole in the unfertilized egg, becomes associated with the unipolar first cleavage furrow, and remains enriched in animal pole blastomeres. Our results suggest that at least one mechanism for Dsh enrichment at the animal pole is through its degradation at the vegetal pole. Functional studies reveal that NvDsh is required for specifying embryonic polarity and endoderm by stabilizing β-catenin in the canonical Wnt signaling pathway. The localization of Dsh to the animal pole in *Nematostella* and two other anthozoan cnidarians (scleractinian corals) provides a possible explanation for how the site of gastrulation has changed in bilaterian evolution while other axial components of development have remained the same and demonstrates that modifications of the Wnt signaling pathway have been used to pattern a wide variety of metazoan embryos.

Keywords: Axial properties; Polarity; Cnidaria; Anthozoa; *Nematostella vectensis*; Dishevelled; Wnt; β-Catenin

Introduction

Most bilaterian eggs possess polarity along the primary axis, the animal–vegetal (AV) axis, which is established during oogenesis. Egg polarity is manifest in the asymmetric distribution of maternal components, including mRNAs (Di Carlo et al., 2004; King et al., 2005; Momose and Houliston, 2007; Nishida, 2005; van Eeden and St Johnston, 1999), proteins (Vinot et al., 2004; Weitzel et al., 2004), organelles (Coffman et al., 2004; Zalokar and Sardet, 1984), and cytoskeletal elements (Egana et al., 2007; Sardet et al., 1992). The relationship between egg polarity and larval/adult axial properties is generally well conserved among bilaterians, such that the anterior of the larva/adult is derived from the region of the animal pole and the posterior from the vegetal pole (reviewed in Martindale, 2005). Despite such widespread conservation between the general axial properties of the egg and the adult, there exists in bilaterians a great degree of variability in terms of the site of gastrulation with respect to both the AV axis of the egg and the antero-posterior (AP) axis of the adult (reviewed in Martindale, 2005). The axial properties of the early embryo determine the site of gastrulation, and gastrulation plays a central role in organizing the axial properties of the adult. It is clear, therefore, that

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understanding the cellular and molecular aspects establishing embryonic polarity and the determination of cell fates in diverse organisms will ultimately provide insight to the evolution of body plan organization.

One of the central and conserved molecular signaling pathways involved in regulating both axis formation and germ-layer segregation in bilaterians is the Wnt/β-catenin, or canonical Wnt, signaling pathway. In several deuterostome embryos, egg polarity is translated into the axial polarity of the adult body plan as asymmetrically localized maternal factors activate Wnt/β-catenin signaling in a restricted region of the cleavage-stage embryo. This results in the stabilization and nuclearization of β-catenin in cells at one region of the embryo and the activation of target genes involved in establishing axial properties (reviewed in Croce and McClay, 2006; Marikawa, 2006). Accumulation of nuclear β-catenin in blastomeres at one pole of the embryo marks the site of gastrulation and specifies the dorsoventral (DV) axis in vertebrates (Larabell et al., 1997; Moon and Kimelman, 1998; Schneider et al., 1996), mediates establishment of the AV axis in lower chordates (Imai et al., 2000; reviewed in Nishida, 2005), and patterns the AV axis in echinoderms (Logan et al., 1999; Wikramanayake et al., 1998). Nuclear localization of β-catenin is also involved in the formation of endomesodermal precursors and the process of germ-layer segregation through the activation of *Wnt* target genes in the endomesodermal gene regulatory network in deuterostomes (Imai et al., 2000; reviewed in Loose and Patient, 2004; Oliveri and Davidson, 2004). Despite all that is known about the downstream effects of canonical Wnt signaling on axis establishment and germ-layer formation, the upstream factors that activate Wnt/β-catenin signaling in the early embryo are not as well characterized. Most recently, a maternally supplied Wnt has been shown to dorsally activate Wnt/β-catenin signaling in *Xenopus* (Tao et al., 2005), however, maternal Wnt ligands that regulate β-catenin nuclearization during early embryogenesis have not been identified in other organisms. In sea urchins, an upstream component in the Wnt/β-catenin pathway, Dishevelled (Dsh), is enriched at the vegetal cortex of unfertilized eggs and early embryos and is necessary for the accumulation of nuclear β-catenin in vegetal blastomeres, axial patterning, and endomesoderm formation (Kumburegama and Wikramanayake, 2007; Weitzel et al., 2004).

The Cnidaria is a phylogenetically important outgroup, separating from other metazoans prior to the origin of the bilaterian assemblage (Collins et al., 2006; Medina et al., 2001). Cnidarians share many critical developmental regulatory genes with bilaterians (Broun et al., 2005; Finnerty et al., 2004; Galliot, 2000; Kortschak et al., 2003; Kusserow et al., 2005; Magie et al., 2005; Matus et al., 2007; Ryan et al., 2007; Scholz and Technau, 2003; Technau et al., 2005), despite important differences in symmetry, axial properties, and germ-layer composition. Early work in hydrozoan cnidarians (Tessier, 1931) suggested that the axial properties of the embryo and adult are specified by a maternally established egg polarity, as in most bilaterians. Recent work in the hydrozoans *Podocoryne carnea* and *Clytia hemisphaerica* (formerly the genus *Phialidium*) supports the notion of localized determinants in the egg being responsible for axis polarity and germ-layer formation (Momose and Houliston, 2007; Momose and Schmid, 2006; Yanze et al., 2001). Conflicting experimental evidence, however, indicates that the single body axis of hydrozoan cnidarians, the oral–aboral (OA) axis, is not specified until the time of first cleavage by the location of the first unipolar cleavage furrow (Freeman, 1980; Freeman, 1981). Regardless of when the OA axis is established, in all hydrozoans examined the site of cleavage initiation becomes the oral pole of the planula. Under normal developmental conditions, the site of first cleavage coincides with the animal pole of the egg. Therefore, as in bilaterians, there is a consistent correlation between the animal pole of the egg and the oral end of the adult (Freeman, 1980; Freeman, 1983; Momose and Houliston, 2007; Momose and Schmid, 2006; Schlawny and Pfannenstiel, 1991; Tessier, 1931). Whether the axial properties found in hydrozoans, a highly diverse class of cnidarians with complex life cycles, are general features shared by all cnidarians is not known as other cnidarian classes have not been studied.

There is increasing evidence that, as with deuterostomes, Wnt/β-catenin signaling is crucial in establishing and maintaining polarity in cnidarian embryos. Past studies have shown that β-catenin is nuclearized in oral blastomeres in early embryos of the anthozoan *Nematostella vectensis*, and signaling by this protein in the canonical Wnt pathway is required for germ-layer segregation (Wikramanayake et al., 2003). More recent studies in two species of hydrozoans have also shown a role for canonical Wnt signaling in axial development. Messenger RNAs for both a Wnt ligand and a Wnt signaling component, Tcf, are localized to the first cleavage furrow in *Hydractinia* and are involved in oral development (Plickert et al., 2006). Additionally, in the eggs of *C. hemisphaerica*, mRNAs for two Wnt receptors, Frizzled 1 (Fz1) and Frizzled 3 (Fz3), are localized to the oral and aboral poles, respectively, and regulate both oral and aboral development, including nuclearization of β-catenin in prospective endoderm during embryogenesis (Momose and Houliston, 2007).

A universal role of the Wnt pathway in axial development and germ-layer segregation during cnidarian development is far from clear however. No Wnt signal has been detected in cleavage stages of *Nematostella* (Kusserow et al., 2005; Lee et al., 2006) or *Clytia* (Momose and Houliston, 2007), and although mRNA for Tcf in *Nematostella* becomes gradually localized to presumptive endoderm, along with nuclear β-catenin (Wikramanayake et al., 2003), it is not asymmetrically localized at first cleavage (Lee et al., 2006). Furthermore, several *Fz* genes are present in the *Nematostella* genome, including a *Fz1* ortholog, but none of them are asymmetrically expressed in the egg (N. Wijesena and A. Wikramanayake, unpublished observations), raising the possibility that asymmetric *Fz* localization is a derived feature of hydrozoan eggs. It is also not clear how the Wnt pathway may be involved in reorganizing the oral–aboral axis in *Clytia* by changing the site of first cleavage (Freeman, 1980; Freeman, 1981).

These studies in hydrozoans have further highlighted the importance of anthozoans for providing insight into the ancestral mechanisms underlying axial organization in developing

embryos. This study is the first to investigate how embryonic axial properties are established in anthozoan cnidarians, the sister-group to the more complex medusozoan clade, which includes hydrozoans (Bridge et al., 1995; Collins et al., 2006; Medina et al., 2001). We used *Nematostella*, to examine the relationship between egg polarity and adult polarity, the time at which axial polarity is established, and potential maternal determinants involved in axis specification. We show that the animal pole of the egg gives rise to the oral pole of the adult, that cell fate specification occurs before the 8-cell stage, and that Wnt/β-catenin signaling mediates the establishment of the oral–aboral axis and endoderm specification through the maternal localization of Dsh at the animal pole of the egg. We discuss the significance of this for body plan evolution in the Metazoa.

Materials and methods

In vitro fertilization experiments

Nematostella egg masses were de-jellied in 4% cysteine (pH 7.4–7.6) in 1/3× filtered sea water (FSW) for 10–15 min, then washed 3× in 1/3× FSW (Fritzenwanker and Technau, 2002). Batches of unfertilized eggs in 1/3× FSW were placed in separate wells of a 24-well tissue culture dish. Eggs were fertilized with 1 ml of sperm water (seawater from spawned bowls) at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h post-spawning (h.p.f.). Embryos developed at 24–25 °C and the number of embryos at cleavage, gastrula, planula, and polyp stages was scored for each fertilization time in two independent trials. Control experiments consisted of (1) naturally fertilized eggs that had been de-jellied prior to first cleavage, (2) naturally fertilized eggs developed in the egg jelly, and (3) eggs fertilized *in vitro* while in the egg jelly.

Fertilization experiments

Unfertilized, de-jellied eggs placed in 0.2% gelatin-coated Petri dishes were cut with glass needles into animal and vegetal halves along an equatorial plane defined by the polar position of the female pronucleus. Four independent trials were performed. In one trial, each animal and vegetal half was maintained in a separate well of a 24-well tissue culture plate, with egg pairs in adjacent wells. In subsequent experiments, all animal egg halves and all vegetal egg halves were pooled into two separate wells. In all experiments, egg halves were allowed to heal and round up before fertilization (5 min). Eggs were fertilized for 1, 5, 10, or 15 min. Fertilized egg halves were washed in 1/3× FSW and assayed for successful fertilization by noting which halves cleaved. Whole, unfertilized eggs were set aside as controls to ensure that fertilization had not occurred prior to the experiments. As a second control, whole eggs were fertilized simultaneously with experimental treatments to ensure sperm and eggs remained capable of fertilization and cleavage after the time required for the operations.

To visualize the location of sperm binding, batches of unfertilized, de-jellied eggs were fertilized for 1, 2, 3, 5, or 10 min. Two treatments were applied to fertilized eggs to remove unbound sperm from the egg surface (1) washing in Ca^{2+}/Mg^{2+}-free artificial 1/3× FSW with 2 mM EDTA or (2) washing in 1/3× FSW (Deguchi et al., 2005). Control eggs were not washed. Three independent trials were performed for each treatment. Fertilized eggs were fixed and stained with DAPI as previously described (Deguchi et al., 2005). Eggs were dehydrated in 100% MeOH, cleared in BABB (1:2 benzyl alcohol:benzoyl benzoate), and scored for the number of sperm and the location of sperm relative to the position of the female pronucleus on a Zeiss AxioImager.Z1 equipped with a Hamamatsu ORCA-ER digital camera.

Polarity experiments

To determine the relationship between the site of cleavage initiation and the AV axis of the egg, unfertilized de-jellied eggs were marked near the female pronucleus at the animal pole with either Nile blue or DiI (Molecular Probes). For Nile blue staining, a micromanipulator (Narishige Scientific Instrument Lab, Tokyo, Japan) was used to touch the end of a microcapillary tube containing 1% Nile blue in a 2% agarose plug to the egg surface for about 30 s (Freeman, 1980; Freeman, 1981). For DiI labeling, a small droplet of 5% DiI dissolved in soybean oil (Martindale and Henry, 1997) was injected beneath the cell membrane near the female pronucleus using a Picospritzer microinjector (General Valve Corp., Fairfield, NJ, USA). For all polarity experiments, eggs were fertilized with sperm water and developed at 24–25 °C. The site of cleavage initiation relative to the Nile blue stain or DiI label was scored under a Zeiss Stemi SV11 dissection microscope equipped with epifluorescence.

To establish the relationship between the egg AV axis and the adult OA axis, unfertilized de-jellied eggs were stained on the cell membrane with Nile blue at the position of the female pronucleus, 90° from the female pronucleus, or 180° from the female pronucleus. The location of the Nile blue stain along the OA axis of the embryo and planula was noted at different developmental stages under a Zeiss Axio Imager.Z1 microscope.

To examine the timing of cell fate specification 8-cell, blastula, and gastrula-stage embryos were separated into two halves, either parallel to the OA axis or perpendicular to it. Since the axial properties of the embryo are not apparent until gastrulation, unfertilized eggs were marked with Nile blue either above the female pronucleus or at the opposite pole as previously described in order to orient embryos for 8-cell and blastula cuts. For gastrula cuts, unmarked embryos were oriented in relation to the site of the blastopore. Embryos were cut with a glass needle at the appropriate stage and orientation in gelatin-coated Petri dishes. Each embryo half was maintained in a separate well of a 48-well tissue culture plate at 24–25 °C.

For confocal imaging, planulae were fixed as previously described (Martindale et al., 2004; Wikramanayake et al., 2003) and then treated with RNase A (50 μg/ml) and stained with AlexaFluor488–phalloidin (2 U/ml) (Molecular Probes) and propidium iodide (1 μg/ml) in PBT (PBS, 0.2% Triton X-100) to visualize filamentous actin and cell nuclei, respectively. Stained planulae were dehydrated in isopropanol and cleared in BABB. Images were obtained on a Zeiss LSM510 laser scanning confocal microscope.

Whole mount in situ hybridizations

Whole-mount *in situ* hybridization of *Nematostella* embryos was performed as previously described (Finnerty et al., 2003; Martindale et al., 2004). Digoxigenin-labeled riboprobes for *NvExd*, *Nvsnail-A*, *Nvanthox1*, and *NvDsh* were synthesized using the MegaScript Transcription Kit (Ambion, Austin, TX). Hybridization of DIG-labeled riboprobes (1 ng/μl) was carried out at 60–65 °C. Visualization of the labeled probe was performed using NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Bioscience).

Immunohistochemistry

Nematostella and scleractinian coral embryos (*Pocillopora meandrina* and *Fungia scutaria*) at different stages were fixed as previously described (Marlow and Martindale, in press; Martindale et al., 2004; Wikramanayake et al., 2003). Affinity-purified *Nematostella* anti-Dsh (anti-NvDsh) peptide antibodies were raised against a selected amino acid region of the NvDsh protein (SLPESDRYTEIPEGEA; Bethyl Inc., Montgomery, TX). Blast searches against the *Nematostella* genome sequences showed that the SLPESDRYTEIPEGEA amino acid sequence was not present in any predicted *Nematostella* proteins other than NvDsh. Antibody incubations 1:100 were carried out in blocking buffer (PBS, 1% Triton X-100, 5% normal goat serum) at 4 °C overnight, unless otherwise noted. *P. meandrina* and *F. scutaria* embryos were incubated in anti-NvDsh peptide antibodies (1:50 and 1:100, respectively). Embryos were washed 3 times quickly and 3 times for 30 min in PBS, blocked for 30 min in blocking buffer, and incubated in either peroxidase-conjugated donkey anti-rabbit (1:500) (Jackson ImmunoResearch), AlexaFluor594 donkey anti-rabbit (1:250), or Fluorescein-conjugated goat anti-mouse (1:250) (Invitrogen) secondary antibodies for 1 h at 24–25 °C or at 4 °C overnight. For the corals, embryos were washed 3 times quickly and once for 30 min in AlexaFluor488-conjugated phalloidin (2 U/ml), then three times for 10 min in PBS. Embryos were dehydrated in isopropanol, cleared in

BABB, and examined under a Zeiss AxioImager.Z1 microscope or a Zeiss LSM510 confocal microscope.

cDNA construction, mRNA synthesis and microinjection

Nematostella Dsh cDNA constructs were cloned into the pCS2 vector using routine molecular biology techniques (details of specific constructs are available upon request). Linearized constructs of NvDsh:GFP, NvDsh-DIX:GFP, Nvβ-catenin:GFP (Wikramanayake et al., 2003), NvDsh-DIX:Flag, Xptβ-catenin:GFP (Yost et al., 1996), Lv-Δcadherin (Logan et al., 1999) and GFP were used as templates for transcription using the mMessage mMachine Kit (Ambion). *NvDsh:GFP*, *NvDsh-DIX:GFP*, and Lv-Δcadherin mRNAs were injected at a final concentration of 0.75 μg/μl, 1–1.2 μg/μl and 0.5 μg/μl respectively in 40% glycerol. Co-injection of *NvDsh-DIX:Flag*, *Nvβ-catenin:GFP* (or *Xptβ-catenin:GFP*), and rhodamine dextran was carried out at a final concentration of 1.5 μg/μl, 0.3 μg/μl, and 0.2 μg/μl respectively in 40% glycerol. Embryos developed in 1/3× seawater at 24–25 °C.

Western blot analysis

To determine the expression of endogenous NvDsh, *Nematostella* embryos were collected at different stages from unfertilized eggs to 7–10-day polyps. Nvβ-catenin:GFP, Xptβ-catenin:GFP, and NvDsh-DIX:GFP expressing embryos were collected 5 h.p.f. All embryo samples were solubilized in sample buffer (0.5 M Tris pH 6.8, glycerol, 10% w/v SDS and 0.25% bromophenol blue) and resolved by SDS-PAGE. Western analysis was carried out using affinity-purified anti-SU-DIX (1:1500) or anti-GFP (1:1000) (BD Living Colors; Clontech) antibodies and detected by peroxidase-conjugated donkey anti-rabbit antibodies (1:8000) (Jackson ImmunoResearch).

Results

Nematostella vectensis embryonic development

N. vectensis eggs are yolky and opaque with an average diameter of 227±13 μm (range 198–247 μm, $n=25$) (Fig. 1A). They are spawned through the oral opening of the adult polyp and are embedded in a gelatinous matrix (egg jelly). Male gametes are released through the oral opening directly into the water column. *Nematostella* spermatozoa have triangular-shaped heads, 3.6±0.2 μm (range 3.0–3.9 μm, $n=25$) in length and 2.4±0.2 μm (range 2.0–2.6 μm, $n=25$) in width at the base with a single, long flagellum. The site of polar body formation and the eccentric position of the female pronucleus demarcate the animal pole of the egg. Two polar bodies are released from the animal pole but are displaced soon after as the egg jelly expands since there is no vitelline membrane surrounding the egg. The female pronucleus can be seen near the egg surface in unfertilized eggs (Fig. 1A), but the zygotic nucleus is not visible in living embryos. First cleavage is unipolar and initiates approximately 1.5 h post-fertilization (h.p.f.) at 25 °C (Fig. 1B). Typically, the first two cleavage furrows are orthogonal and form simultaneously at the same pole, giving rise to a 4-cell embryo (Fig. 1C). Two-cell embryos are rarely observed, and occasionally more than four blastomeres form simultaneously. Subsequent cell divisions occur approximately every 30 min and appear synchronous at least through late cleavage stages. There is no discernable cleavage program and individual embryos are highly variable in terms of the size and position of individual blastomeres (Figs. 1D–F). Cleavage stages give rise to a ciliated coeloblastula by 4–6 h.p.f. (Fig. 1G). Oral–aboral polarity is first evident morphologically at 12–15 h.p.f. as gastrulation begins at one pole of the blastula (Fig. 1H). Gastrulation occurs by invagination (Byrum and Martindale, 2004; Kraus and Technau, 2006; Magie et al., 2007), and possibly immigration (Kraus and Technau, 2006), and takes approximately 12 h to complete. By 24 h.p.f., a bilayered planula is formed composed of an outer ectodermal epithelium, an inner endodermal layer, a developing pharynx, a pair of primary "directive" mesenteries at the oral pole, and an apical tuft at the aboral pole (Figs. 1I–J). The planula becomes pear-shaped, tapering towards the aboral pole as it continues to elongate along the oral–aboral axis and exhibits directional swimming. The planula emerges from the egg jelly at this stage.

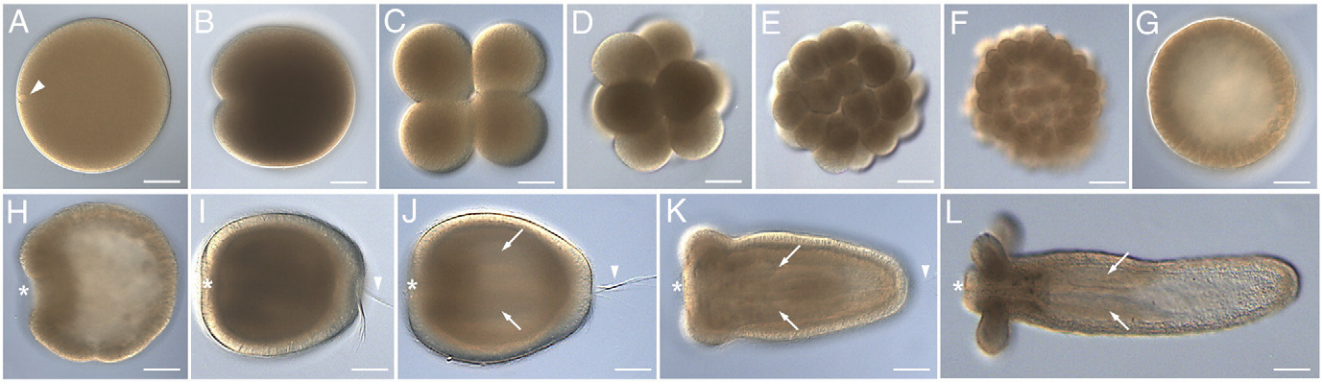

Fig. 1. Embryonic development in *Nematostella vectensis*. Various stages of the embryonic development of *Nematostella* are shown from egg to polyp. Early gastrula to polyp stages are oriented with the oral pole to the left. (A) Unfertilized egg. The female pronucleus is visible adjacent to the cell membrane at one pole of the egg (arrowhead). (B) First cleavage (1–2 h.p.f.). The first cleavage is unipolar and initiates at one pole of the egg. (C) Four-cell stage. In most cases the first two cleavages are simultaneous and give rise immediately to a 4-cell embryo. (D) Eight-cell stage. (E) Mid-cleavage stage. (F) Late cleavage stage. (G) Blastula stage (4–6 h.p.f.). Cleavage gives rise to a ciliated coeloblastula. (H) Early gastrula stage (12–15 h.p.f.). Gastrulation initiates at one pole of the coeloblastula and proceeds via invagination. (I) Early planula (~24 h.p.f.). Ectodermal and endodermal layers are well-organized and the planula exhibits directional swimming with the aboral end, distinguished by the apical tuft (arrowhead), forward. (J) Late planula. The planula elongates along the OA axis and the primary mesenteries (arrows) begin to form. The apical tuft persists (arrowhead). (K) Early tentacle-bud stage of polyp. The first four tentacle primordia develop around the mouth at the oral pole. (L) Juvenile polyp. (h.p.f., hours post-fertilization at 25 °C; *, site of gastrulation/oral pole; A–H, bar ~60 μm; I–L, bar ~90 μm).

After 4 or 5 days, the planula develops four primary tentacle buds surrounding the oral pole (Fig. 1K), and by 7 days, it develops into a four-tentacle juvenile polyp that settles to the substrate and begins feeding (Fig. 1L).

Nematostella eggs develop normally after being de-jellied and fertilized in vitro

Nematostella eggs can be fertilized *in vitro* while embedded in the egg jelly to yield large numbers of stage synchronous embryos of known age (Hand and Uhlinger, 1992). Fertilized eggs can be removed from the jelly without affecting embryonic development (Fritzenwanker and Technau, 2002). The presence of the egg jelly, however, hinders manipulation of unfertilized eggs and is problematic for experimental embryologists. To assess potential developmental effects caused by the removal of the egg jelly prior to fertilization, we de-jellied egg masses, fertilized eggs *in vitro* at different time points post-spawning, and followed their development. We found that de-jellied *Nematostella* eggs can be successfully fertilized *in vitro* and will develop normally into polyps, in contrast to what has been previously reported (Fritzenwanker and Technau, 2002). When fertilized up to 2 h after spawning, de-jellied *in vitro* fertilized eggs developed into normal blastulae, gastrulae and planulae in percentages similar to control eggs (Fig. S1, Supplementary material). After 2 h post-spawning, normal development becomes compromised in both normal and de-jellied eggs. For both de-jellied, *in vitro* fertilized eggs and control eggs the percentages of normal embryos that develop are inversely related to the time elapsed between spawning and fertilization (Fig. S1, Supplementary material), as shown previously (Fritzenwanker and Technau, 2002). The ability to successfully fertilize de-jellied eggs *in vitro* within a defined time window enabled us to manipulate and mark unfertilized eggs.

Nematostella eggs are fertilized at the animal pole

The eggs of many hydrozoans can only be fertilized at the site of polar body formation because this is the only site on the egg membrane to which sperm can fuse (Deguchi et al., 2005; Freeman and Miller, 1982; Yamashita, 1987). There may exist differences in the egg membrane at the site, including the absence of microvilli (Yamashita, 1987) and the formation of lectin-binding glycoproteins that play a role in fertilization (Freeman, 1996; Freeman and Miller, 1982). We determined where *Nematostella* eggs are fertilized through two sets of experiments. In *Nematostella*, loss of the polar bodies in the egg jelly after spawning makes identifying the site of their formation impossible. The female pronucleus at the animal pole, however, is clearly visible until fertilization. In hydrozoans, the position of the female pronucleus and the site of polar body formation are coincident (Freeman and Miller, 1982). Therefore, we used the female pronucleus as a proxy for the site of polar body formation in *Nematostella*. De-jellied, unfertilized eggs were bisected into animal and vegetal halves. Each animal half contained the female pronucleus (and site of polar body formation), each vegetal half had neither. Sperm were added to both egg halves and fertilization was determined by observing whether or not cleavage occurred. In all cases in which cleavage occurred ($n=86$), only the animal halves cleaved. None of the vegetal halves cleaved (Fig. 2A). The proportions of cleavage stage embryos and planulae developing from animal halves were similar to controls, suggesting that the vegetal region is not critical for normal development (Fig. 2A).

To determine specifically where fertilization occurs and whether sperm are able to fuse at sites in the vegetal half of the egg, the number and location of sperm on the egg surface were visualized in fixed eggs by DNA staining with DAPI following different fertilization times and wash treatments of different stringency (Deguchi et al., 2005) (Table S1, Supplementary material). In most eggs for both wash treatments and control (no wash), a single sperm was observed on the egg membrane above the female pronucleus. In some cases, a cluster of sperm (e.g., from 2–12) was located above the female pronucleus (Fig. 2B). Clusters of sperm were never observed at other locations on the egg, although single sperm were sometimes found randomly distributed across the egg surface. Decondensing sperm heads were found in association with the female pronucleus in the animal halves but were never observed elsewhere in the egg. For each wash treatment and the control, the mean number of sperm found at the female pronucleus was significantly greater than the mean number of sperm at other sites on the egg surface (Fig. 2C). These results suggest that sperm may be attracted to the site of polar body formation and/or that fertilization occurs at this site. The lowest mean number of sperm on the egg surface was found following the most stringent (Ca^{2+}/Mg^{2+}-free FSW + 2 mM EDTA) wash, indicating that many of the sperm associated with the egg after the other two treatments were only loosely associated with the membrane and that sperm–egg fusion occurs at the site of polar body formation but not elsewhere on the egg surface (Fig. 2C). Together, the results of these experiments demonstrate that fertilization occurs in the animal half of the egg at the site of polar body formation, and that the vegetal half is not normally fertilized. The possibility that vegetal halves failed to cleave because they lacked the female pronucleus, rather than failure of sperm to enter, cannot be excluded. However, experiments in hydrozoans and some bilaterian species have shown that fertilized anucleate egg fragments can cleave normally (Freeman and Miller, 1982).

Relationship between egg polarity, adult axial properties, and endoderm fate

To determine the relationship between the AV axis of the egg and the axial properties of the adult body plan, unfertilized eggs were stained at the animal pole with Nile blue, fertilized, and allowed to develop. The location of the Nile blue mark along the OA axis of the embryo was mapped at different developmental stages (Fig. 3). In all cases ($n=24$), the position of the Nile blue stain coincided with the site of cleavage initiation (Fig. 3G). Similar results were obtained when the animal pole was marked with the lipid-soluble dye, DiI (96%, $n=77$). In later stages of

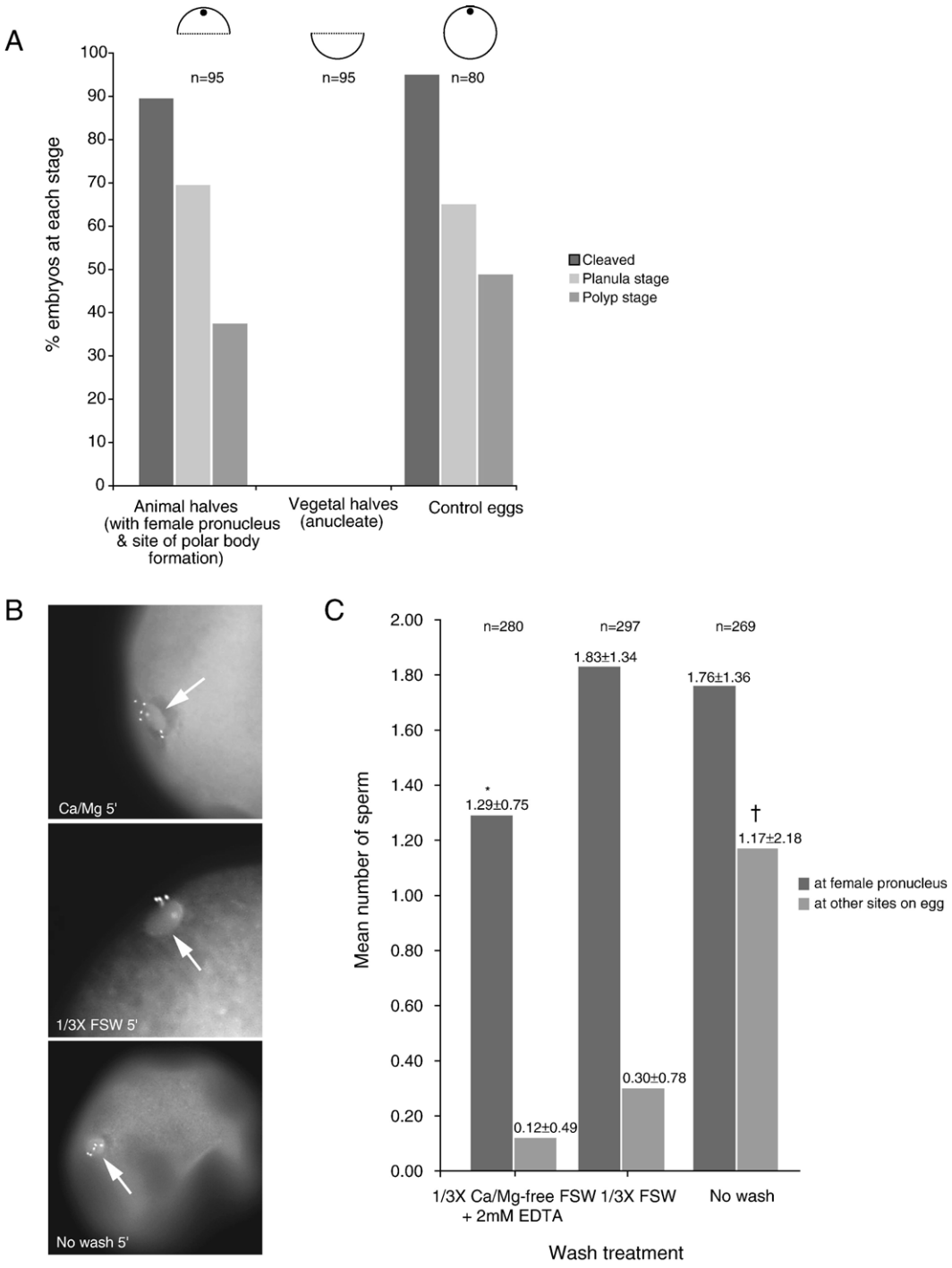

Fig. 2. *Nematostella vectensis* eggs are fertilized at the site of polar body formation. (A) *Nematostella* eggs were cut into animal and vegetal halves then fertilized. In all cases, only the animal halves of the eggs cleaved. Animal egg halves developed normally into planulae and polyps in percentages similar to uncut control eggs. Sample sizes, *n*, are shown above each category. Data represent results from four independent trials. (B) *Nematostella* sperm and egg nuclei were visualized by staining with DAPI. *Nematostella* sperm are found associated with the egg membrane at the location of the female pronucleus (arrow). In many cases, clusters of sperm were found at the egg pronucleus; such clusters were never observed elsewhere on the egg surface. Wash treatment and time for fertilization are indicated for each. (C) Mean numbers of sperm found at the female pronucleus or at other sites on the egg surface following different wash treatments. More sperm were found associated with the egg membrane at the female pronucleus than at other sites in all three treatments, suggesting that sperm may be attracted to, and fertilize, the egg at this site. The lowest mean numbers were found following the most stringent (Ca^{2+}/Mg^{2+}-free FSW + 2 mM) wash, indicating that many of the sperm associated with the egg after the other two treatments were not fused to the membrane. Means ± SD shown above each bar. The sample size, *n*, is shown above each treatment. * and † represent significant differences in mean sperm number between treatments at the female pronucleus and at other sites on the egg, respectively. Data represent results from three independent trials.

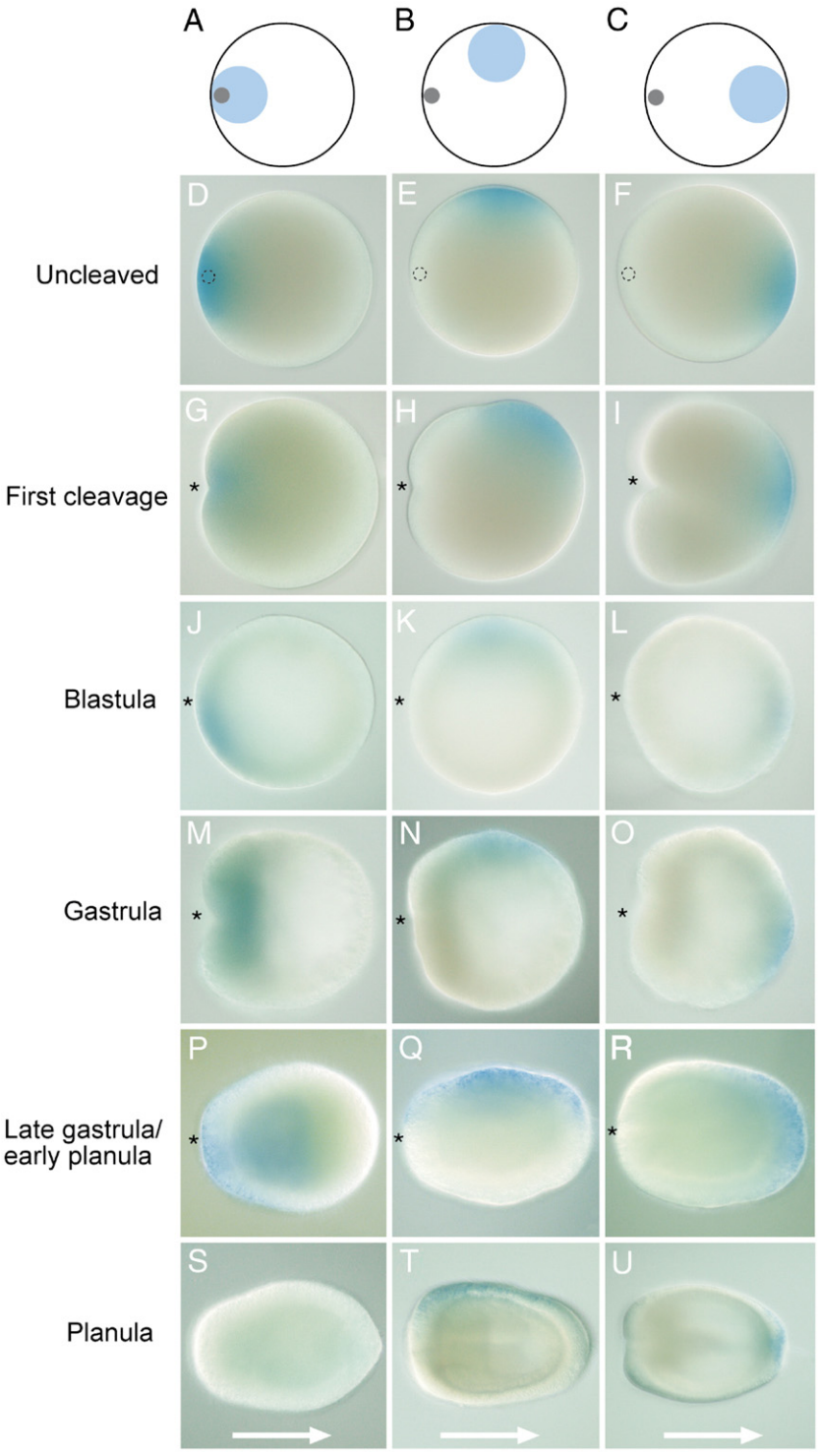

Fig. 3. The animal pole of the egg becomes the site of cleavage initiation, the site of gastrulation, and the oral pole of the planula in *Nematostella*. (A–C) Schematic figures of unfertilized *Nematostella* eggs indicating the location of the female pronucleus (gray circle) and the Nile blue mark (blue circle) at (A) the site of the female pronucleus, (B) 90° from the site of the female pronucleus, and (C) 180° from the site of the female pronucleus. Nile blue marks on live embryos of different stages are shown below each schematic. (D–F) Uncleaved zygotes (original site of female pronucleus indicated with dashed circle). (G–I) Unipolar first cleavage. (J–L) Blastula stage embryos. (M–O) Gastrula stage embryos. (P–R) Late gastrula/early planula stage. Note that the endoderm in panels P and S is marked with Nile blue. (S–U) Planula stage. These marking experiments demonstrate the first cleavage furrow forms at the animal pole of the egg, corresponds to the site of gastrulation, and becomes the posterior pole of the planula/oral pole of the adult. Eggs marked at the animal pole give rise to planulae with Nile blue-stained endoderm, indicating the prospective endoderm territory is located at the animal pole of the egg. *, Future oral pole; arrows, direction of swimming.

development, the Nile blue stain was observed at the site of gastrulation, at the posterior pole of the planula, and at the oral end of the polyp (Fig. 3) (Table S2, Supplementary material). The endoderm, and in some cases part of the oral ectoderm, of planulae from eggs marked at the animal pole was stained with Nile blue (Figs. 3M and P). These observations demonstrate that first cleavage initiates at the animal pole, and that the animal pole of the egg gives rise to the oral end of the embryo. The marking results also indicate that gastrulation in *Nematostella* initiates at the animal pole and endoderm is derived from the animal region of the egg.

To confirm that labeling the eggs did not entrain polarity, unfertilized eggs were marked 90° or 180° from the animal pole and scored for the position of the dye along the OA axis at different stages. Eggs stained midway between the animal and vegetal poles developed into gastrulae, planulae, and polyps marked in lateral regions (Fig. 3) (Table S2, Supplementary material). When eggs were marked at the vegetal pole, the resulting embryos were labeled opposite the site of cleavage initiation, opposite the blastopore, and at the aboral pole of the planula (Fig. 3) (Table S2, Supplementary material). These results demonstrate that the AV axis of the egg is directly translated to the OA axis of the embryo. In embryos from both the 90° and 180° labeling experiments none of the endoderm was labeled demonstrating that the prospective endoderm territory is restricted to the animal pole of the egg.

The results from the labeling experiments indicate a consistent relationship between the AV axis of the egg, the site of cleavage initiation, and the OA axis of the embryo in which first cleavage at the animal pole marks the site of gastrulation and the future oral pole of the adult. It is not clear from these experiments, however, if in *Nematostella* the axis is initially established in the egg during oogenesis or whether the cleavage initiation site specifies the OA axis, as in hydrozoans (e.g., *Clytia*) (Freeman, 1980; Freeman, 1981). Experimental manipulations to move the site of first cleavage relative to the AV axis of the egg would be required to show a causative role of cleavage initiation in axis specification.

The oral pole is required for axial polarity and endoderm formation

We examined when the axial properties are established by isolating regions of *Nematostella* embryos at different stages and observing their developmental potential. Eight-cell, blastula, and early gastrula stage embryos were cut parallel to the OA axis into two lateral isolates (Isolate 1 and Isolate 2), each containing cells descended from animal and vegetal regions of the egg. For all three developmental stages, both isolates developed into normal planulae by 24 h.p.f. (83%, $n=154$), although in many cases the endoderm was less organized than in control embryos as visualized by fluorescently labeled phalloidin and propidium iodide staining (Fig. S2A and Table S3, Supplementary material). These results demonstrate that there is no segregation of developmental potential parallel to the OA axis in *Nematostella* embryos.

We next tested for differences in developmental potential along the AV axis of the egg by cutting 8-cell, blastula, and gastrula stage embryos equatorially into oral and aboral isolates. For 8-cell cuts, oral isolates developed into normal planulae by 24 h.p.f. (74%, $n=27$). The endodermal organization in many of these planulae was slightly abnormal as in the lateral isolates (Fig. 4A) and likely resulted from the procedure since it was observed in most planulae regardless of the orientation or stage of separation. In striking contrast to the oral isolates, aboral isolates failed to gastrulate and formed ciliated coeloblastulae that did not elongate or show any signs of polarity (96%, $n=27$) (Fig. 4A) (Table S3, Supplementary material). In most cases, the coeloblastulae were hollow, although in a few the blastocoel contained anucleate material as seen secreted from the ectodermal epithelium in normal gastrulae (Magie et al., 2007). The coeloblastulae formed cilia but did not show directional swimming.

We assessed the axial properties and germ-layer composition of isolates from 8-cell stage embryos by whole-mount in situ hybridization using regional and germ-layer-specific molecular markers: *Nvsnail-A*, a *snail* ortholog and pan-endodermal marker with expression beginning at the onset of gastrulation (Fig. 4B) (Martindale et al., 2004); *NvExd*, an ortholog of *extradenticle*, which has distinct spatial patterns of expression in oral ectoderm, aboral ectoderm, and endoderm (Fig. 4B) (Matus et al., 2006); and *Nvanthox1*, a posterior *Hox* ortholog, which is only expressed in aboral ectoderm throughout embryogenesis (Fig. 4B) (Finnerty et al., 2004). Planulae from oral halves and lateral isolates expressed *Nvsnail-A*, *NvExd*, and *Nvanthox1* in patterns similar to controls (Fig. 4B and Fig. S2B, Supplementary material; Table S4, supplementary material). None of the aboral isolates expressed *Nvsnail-A* (Fig. 4B) confirming a failure to specify endoderm. All aboral isolates showed only the aboral ectodermal *NvExd* expression domain, and most had expression throughout the ectoderm (Fig. 4B). Similarly, most aboral isolates expressed *Nvanthox1* throughout the ectoderm of the coeloblastula, reflecting an absence of polarity (Fig. 4B). Results from embryo separation experiments in *Podocoryne* suggested that the default cell fate is aboral, and that it may be inhibited by factors in the oral region (Momose and Schmid, 2006). Ectodermal expression of *NvExd* and *Nvanthox1* throughout the aboral isolates in *Nematostella* may reflect an expansion of aboral cell fates in the absence of such negative regulation by the oral half. In some cases, *NvExd* (42%, $n=43$) and *Nvanthox1* (20%, $n=45$) expression was localized to one pole of the coeloblastula (Fig. 4B) (Table S4, Supplementary material), possibly reflecting variability in the distribution of potential factors regulating oral identity. These results demonstrate an early segregation of developmental potential along the prospective OA axis in *Nematostella* embryos and confirm that endoderm cell fate is restricted to the oral pole of the developing embryo.

For blastula- and gastrula-stage cuts, oral isolates developed into normal planulae (82%, $n=56$ and 83%, $n=66$, respectively) (Fig. 4A) (Table S3, Supplementary material). Many blastula aboral isolates (46%, $n=56$) and most gastrula aboral isolates (77%, $n=66$) developed into ciliated coeloblastulae. In contrast

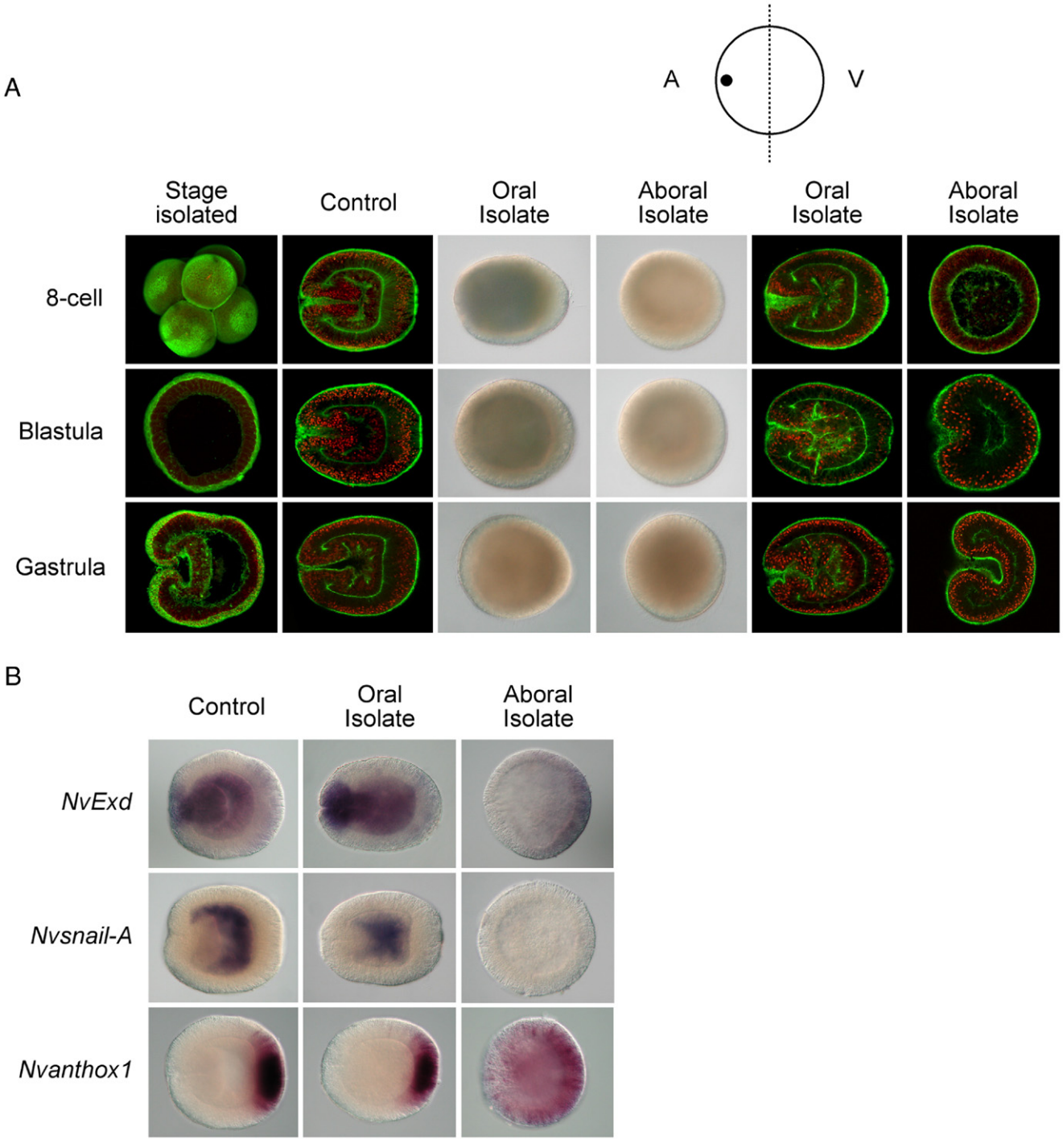

Fig. 4. Embryo separation experiments demonstrate segregation of developmental potential along the oral–aboral axis as early as the 8-cell stage. Eight-cell, blastula, and gastrula-stage embryos were separated perpendicular to the OA axis into oral and aboral isolates. (A) Confocal and DIC images of fixed and live embryo isolates approximately 24 h.p.f. Oral isolates developed into normal planula with axial properties and endoderm. Aboral isolates failed to develop endoderm and formed ciliated coeloblastula. For 8-cell and blastula stages, unfertilized eggs were marked at the animal pole with Nile blue prior to the separation experiment. In oral isolates the endoderm of the developing planulae was stained blue. Embryos for confocal imaging were stained to visualize actin filaments (green) and cell nuclei (red). (B) Gene expression studies in embryo isolates indicate animal pole determinants are necessary for axial properties and endoderm specification. Expression of *NvExd*, *Nvsnail-A*, and *Nvanthox1* was examined by *in situ* hybridization. *NvExd* expression in oral isolates was similar to controls. *NvExd* expression in aboral isolates was uniform in the ectoderm, but in some cases *NvExd* expression was restricted to one pole of the coeloblastula. *Nvsnail-A* expression in oral isolates was similar to controls but reflected the slightly disorganized endoderm observed by confocal microscopy. Aboral isolates did not express *Nvsnail-A*. Oral isolates expressed *Nvanthox1* in the aboral ectoderm in the same pattern as controls. Aboral isolates expressed *Nvanthox1* throughout the ectoderm in most cases. In a few cases, *Nvanthox1* expression was polarized. All images are oriented with the oral pole to the left.

to results obtained from 8-cell embryos, a significant number of blastula aboral isolates (51%, $n=56$) and a small percentage of gastrula aboral isolates (22%, $n=66$) appeared to form a blastopore and, in some cases, an apical tuft. In addition, these isolates were capable of swimming behavior although it appeared erratic and abnormal. Marking experiments at the vegetal pole of the egg prior to the operation revealed that in these cases, the "blastopore" formed in the oral-most region of the aboral half (84%, $n=25$) indicating that the original embryonic polarity had been retained, even in isolated embryo parts. Confocal microscopy revealed in most of these cases epithelial folding and initiation of invagination, but a failure to segregate germ layers (Fig. 4A). In *Podocoryne* experiments, aboral halves from blastula stage embryos similarly developed into one of two forms, a coeloblastula or a "collapsed coeloblastula", in which the ectoderm was folded into the blastocoel (Momose and Schmid, 2006). In *Nematostella*, aboral isolates that initiated gastrulation movements may have resulted from variability in either the orientation of separation relative to the OA axis or in the position of separation along the OA axis, resulting in aboral halves that included cells derived from more animal regions of the egg. In addition, blastula and gastrula separations were performed after the time at which β-catenin is nuclearized in animal-derived blastomeres (Wikramanayake et al., 2003), and aboral isolates containing cells derived from the animal-half may have had levels of nuclear β-catenin sufficient to initiate gastrulation (at the original animal pole), but not to specify endoderm. A small percentage of blastula aboral isolates (19%, $n=56$) developed into polyps indicating endoderm formation in these cases, similar to results seen in *Podocoryne* (Momose and Schmid, 2006). Taken together, the embryo separation results demonstrate that factors localized to the animal pole of the egg during early development are responsible for the establishment of axial polarity and specification of endoderm in *Nematostella*.

The Dishevelled protein, a component of the Wnt signaling pathway, is asymmetrically localized to the animal pole of the Nematostella egg

We have previously shown that β-catenin is differentially stabilized in *Nematostella* embryos as early as the 16-cell stage (Wikramanayake et al., 2003). However, our embryo isolation experiments show that the down-regulation of β-catenin in the vegetal half of the embryo occurs after the stage at which embryonic polarity is established. Work in *Xenopus* and sea urchins has shown that Dsh is a critical upstream regulator of β-catenin nuclearization in early embryos of these species (Miller et al., 1999; Weitzel et al., 2004). Thus, we hypothesized that the Dsh protein may also play a conserved role in the asymmetric nuclearization of β-catenin in early *Nematostella* embryos. We cloned and sequenced a *Nematostella* ortholog of Dsh, *NvDsh* (Fig. S3A, Supplementary material), and confirmed the orthology of *NvDsh* with other metazoan *Dsh* genes using phylogenetic analyses (Fig. S3B, Supplementary material).

To examine *NvDsh* expression, whole-mount *in situ* hybridization was performed on unfertilized eggs and embryos from different developmental stages. *NvDsh* expression was uniform in all stages from the unfertilized egg to the gastrula stage embryo (Fig. S4, Supplementary material). Affinity-purified *Nematostella* anti-NvDsh peptide antibodies were used to determine the spatial and temporal expression of the protein during development. In contrast to the distribution of *NvDsh* transcripts, NvDsh protein was observed to be enriched at one pole of the unfertilized and fertilized egg (Fig. 5A). Similarly, an enrichment of NvDsh at one pole of the developing embryo was also observed during early cleavage stages, the blastula stage, and at the oral pole of the gastrula (Figs. 5B–E). This staining pattern was further confirmed by a second affinity-purified anti-Dsh antibody (anti-SU-DIX) generated against the conserved DIX domain of sea urchin Dsh (data not shown). The specificity of the antibodies was demonstrated by pre-adsorption experiments, where pre-incubation of the antibodies with the peptide used as the antigen (10-fold molar excess of the antigen) eliminated the staining pattern in eggs and embryos (data not shown). Western blotting of embryo lysates using the anti-SU-DIX antibodies showed that NvDsh is expressed throughout development and that Dsh levels decrease by the polyp stage (~7 days post-fertilization) (Fig. 5O). Pre-adsorption of the antibodies with a 10-fold excess of the fusion protein used to generate and affinity purify the antibodies eliminated the staining of both bands seen on the Western blots. Additionally, Western blot analysis of embryos overexpressing Dsh-Flag using anti-SU-DIX antibodies showed specific interaction of the antibodies with the overexpressed protein (data not shown).

To determine the relationship between the asymmetric expression of NvDsh and the AV axis of the egg, unfertilized eggs were double immunostained for NvDsh and the female pronucleus using anti-NvDsh and anti-Histone antibodies. NvDsh was concentrated at the cortex and the female pronucleus and a gradient of NvDsh was observed in the form of cytoplasmic puncta concentrated at the animal pole (Fig. 5F). This experiment showed that NvDsh protein is enriched specifically at the animal pole of the unfertilized egg. The asymmetric pattern of NvDsh persists in the zygote where the protein localizes around the newly formed zygotic nucleus (Fig. 5G), and in the first cleavage furrow at the animal pole (Fig. 5H). Dsh remains asymmetrically distributed to one side of the embryo through the blastula stage (Fig. 5I). We also used anti-NvDsh antibodies to examine Dsh expression in eggs of two other anthozoan cnidarians, the scleractinian corals *F. scutaria* and *P. meandrina*. In both species, immunostaining with the cross-reactive anti-NvDsh antibodies showed the localization of Dsh at the animal pole associated with the zygotic nucleus (Fig. 5J) and the cell cortex at the first cleavage furrow (Fig. 5K), similar to the results in *Nematostella*.

The spatial discrepancy between the localization of *NvDsh* transcripts and the protein in *Nematostella* suggested that the Dsh protein is unstable in vegetal blastomeres. In order to test if NvDsh is differentially stabilized along the AV axis, we overexpressed NvDsh:GFP by mRNA injection into fertilized

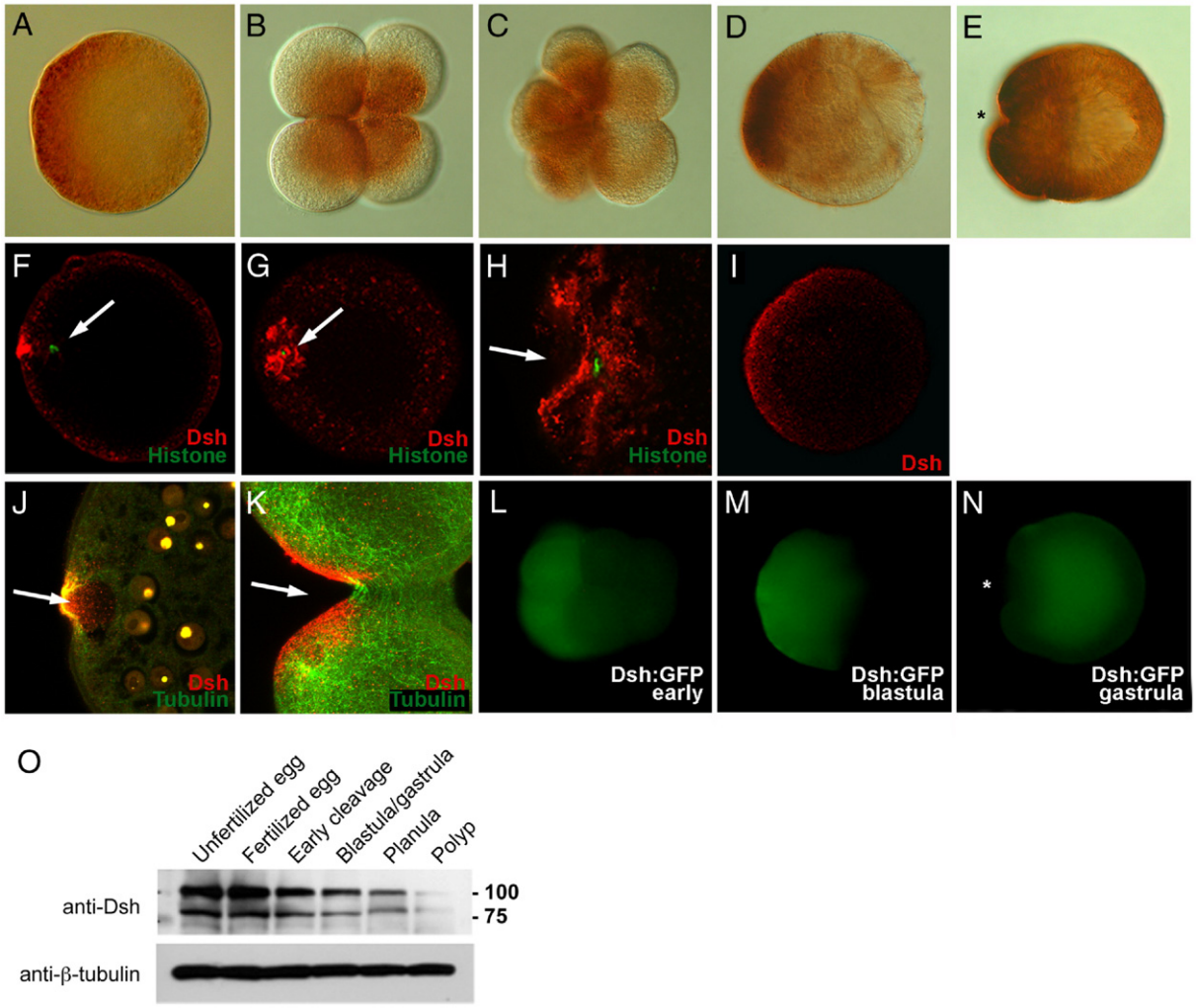

Fig. 5. NvDsh is asymmetrically localized to the animal pole in *Nematostella* eggs and embryos. (A–E) Visualization of NvDsh using an anti-NvDsh antibody. NvDsh protein is enriched at one pole of the egg (A), at one pole of the 4-cell embryo (B), through early cleavage stages (C), blastula stage (D), and in presumptive endoderm of the gastrula (E). (F–I) Confocal images of an unfertilized egg, fertilized egg, first cleavage, and blastula-stage embryo stained with an anti-NvDsh (red) and an anti-histone (green) antibody show that NvDsh is localized to the animal pole of the unfertilized egg (F) and the fertilized egg (G), and to the cleavage furrow (H) and one pole of the blastula (I). Arrows point to the female pronucleus (F), the zygotic nucleus (G), and the cleavage furrow (H). The images shown represent a few medial confocal stacks that makes the Dsh staining appear to be more localized than in the immunohistochemical staining of the egg (A). (J) Dsh localization at the zygotic nucleus (arrow) at the animal pole of a *Pocillopora* egg. Yellow spots in the cytoplasm are autofluorescence from symbiotic zooxanthellae. (K) Dsh localization to the cortex of the first cleavage furrow and surrounding the nuclei in a *Fungia* zygote. (L–N) Overexpression of NvDsh:GFP reveals asymmetric localization of the fusion protein starting around the 8-cell stage (L), which is maintained through the blastula stage (M). By the gastrula stage (N) it is observed throughout the endoderm. (O) Western blot analysis shows that NvDsh is expressed maternally and throughout early development. NvDsh migrates at the expected molecular weight of ~80 kDa. The higher molecular weight form at ~100 kDa is likely due to post-translational modification of Dsh. β-Tubulin is used as a loading control. Asterisks mark the blastopore in panels E and N.

eggs. Expression of the fusion protein was first observed at the 8- or 16-cell stage (approximately 2 h post-injection). Similar to β-catenin, NvDsh:GFP expression becomes restricted to one pole of the early embryo (Fig. 5L) and blastula (Fig. 5M). Interestingly, by the gastrula stage NvDsh:GFP was solely expressed in the endoderm of the embryo (Fig. 5N). This distribution of NvDsh:GFP is likely due to selective degradation of full-length NvDsh in the future ectoderm as overexpression of a truncated, dominant-negative form of NvDsh (NvDsh-DIX:GFP) results in uniform distribution of the protein throughout the embryo (data not shown). The asymmetric localization of endogenous NvDsh described above reveals a primordial polarity that is already present in the unfertilized *Nematostella* egg, and the results from NvDsh:GFP expression suggest that this polarity is at least partly established and maintained by the asymmetric stabilization of NvDsh along the AV axis during development.

Dishevelled is required for endoderm specification in Nematostella embryos

The spatial and temporal patterns of NvDsh expression identify it as a potential candidate for regulating the nuclear localization of β-catenin in early embryos. Here, we tested the functional role of Dsh in regulating nuclear β-catenin in the early *Nematostella* embryo. We have previously shown that

blocking nuclear β-catenin signaling in *Nematostella* by overexpression of either cadherin or a β-catenin-engrailed repressor domain fusion protein blocked gastrulation (Wikramanayake et al., 2003). In these studies, the similar phenotype produced by the two different methods used to block nuclear β-catenin signaling indicated that the effects on gastrulation were not due to the potential effects of cadherin overexpression on cell adhesion (Wikramanayake et al., 2003). In the current study, we confirmed that overexpression of cadherin in *Nematostella* blocked gastrulation, and in addition, we showed that loss of nuclear β-catenin led to the inhibition of endoderm specification. Embryos overexpressing cadherin failed to gastrulate (Fig. 6E) and failed to express the endodermal marker *Nvsnail-A* (Fig. 6F). We hypothesized that if NvDsh regulates the asymmetric expression of nuclear β-catenin in the early *Nematostella* embryo, then blocking Dsh signaling in the canonical Wnt pathway would produce a phenotype similar to that seen when cadherin is overexpressed. To determine if NvDsh has a role in regulating canonical Wnt signaling in *Nematostella* embryos, we used a dominant-negative form of Dsh tagged with GFP (NvDsh-DIX:GFP) to inhibit Wnt/β-catenin signaling. Activation of the Wnt/β-catenin pathway by Dsh requires its homodimerization through the DIX domain (Axelrod et al., 1998; Rothbacher et al., 2000; Weitzel et al., 2004). Overexpression of the Dsh-DIX domain has been shown

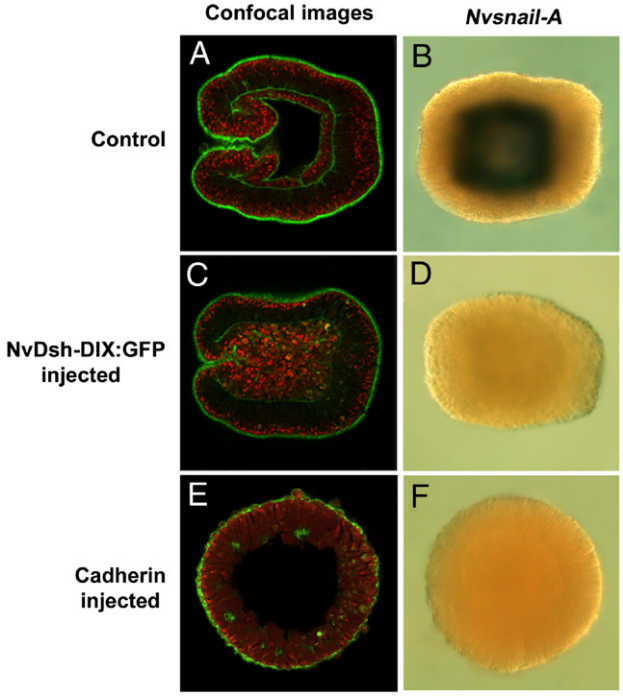

Fig. 6. NvDsh function through the Wnt/β-catenin pathway is essential for *Nematostella* endoderm specification. (A, C, E) Confocal images of late gastrula embryos (20–22 h.p.f.) showing actin (green) and cell nuclei (red). (B, D, F) Whole-mount *in situ* hybridization for the endodermal marker *Nvsnail-A* on late gastrula embryos (20–22 h.p.f.). Uninjected- or GFP-injected (A) late gastrula embryos have a well-defined endoderm lining the gut, which expresses *Nvsnail-A* (B). In contrast, in *NvDsh-DIX:GFP*-injected embryos (C) the gut is absent, even though the blastopore is visible in many of these embryos. Furthermore, these embryos fail to express *Nvsnail-A* (D). Cadherin-injected embryos at the same stage also lack a gut (E) and do not express *Nvsnail-A* (F).

to specifically inhibit the Wnt/β-catenin pathway in bilaterians such as *Xenopus*, *Drosophila* and sea urchins most likely by interacting with endogenous Dsh (Axelrod et al., 1998; Rothbacher et al., 2000; Weitzel et al., 2004). *In vitro* synthesized *NvDsh-DIX:GFP* mRNA was injected into fertilized *Nematostella* eggs, and embryos expressing the fusion protein were collected when controls were at late gastrula (20–22 h.p.f.) stages. Compared to uninjected or GFP-injected control embryos (Fig. 6A), *NvDsh-DIX:GFP* mRNA-injected embryos lacked a gut when control embryos had completed gastrulation, although the blastopore was visible in most of these embryos (Fig. 6C). To determine if NvDsh-DIX also blocked endoderm specification, whole-mount *in situ* hybridization was carried out using *Nvsnail-A*, an early marker for endoderm (Martindale et al., 2004). In normal embryos *Nvsnail-A* expression is first observed in the blastula at the site of gastrulation and, subsequently, only in the endodermal tissue (Fig. 6B). *NvDsh-DIX:GFP* mRNA-injected late gastrula stage embryos failed to express the *Nvsnail-A* marker (Fig. 6D). The presence of a blastopore in many NvDsh-DIX overexpressing embryos is puzzling since we never observed this in cadherin-overexpressing embryos. It is possible that NvDsh-DIX does not completely block nuclear β-catenin and low levels of canonical Wnt signaling are sufficient to induce a blastopore, but not to specify endoderm. Incomplete dominant-negative effects of DIX overexpression have also been observed in sea urchins (AHW, personal observations). This idea is supported by Western blot results that show that overexpressed β-catenin is not completely eliminated by NvDsh-DIX (see below). Together these results demonstrate that the function of NvDsh through the Wnt/β-catenin pathway is critical for endoderm specification in *Nematostella* embryos.

NvDsh regulates β-catenin nuclearization

To confirm that the effects of NvDsh-DIX on endoderm specification were caused by the inhibition of Wnt/β-catenin signaling, we determined if NvDsh-DIX affected the stability of β-catenin. *In vitro* synthesized *Nvβ-catenin:GFP* mRNA was injected into fertilized *Nematostella* eggs either alone or with *NvDsh-DIX:Flag* mRNA. Cleavage-stage embryos injected with *Nvβ-catenin:GFP* mRNA showed the nuclear accumulation of β-catenin in animal pole blastomeres and in the future endoderm (Figs. 7A–D), as previously described (Wikramanayake et al., 2003). When *Nvβ-catenin:GFP* and *NvDsh-DIX:Flag* were co-expressed by mRNA injection, these embryos failed to express detectable levels of Nvβ-catenin:GFP (Figs. 7F–H).

To further determine if NvDsh-DIX affected the stability of β-catenin by interacting with the "destruction complex" formed by glycogen synthase kinase-3β (GSK-3β), axin and adenomatous polyposis coli (APC) (Liu et al., 2002), NvDsh-DIX was co-expressed with a stable form of *Xenopus* β-catenin fused to GFP (Xptβ-catenin:GFP) (Wikramanayake et al., 2003; Yost et al., 1996). This fusion protein is not targeted for proteosome-mediated degradation due to the absence of GSK-3β/casein kinase I phosphorylation sites at its amino terminus

(Yost et al., 1996). Fertilized eggs were co-injected with *Xptβ-catenin:GFP* and *NvDsh-DIX:Flag* mRNA at the same concentrations as the above co-injected mRNA. These embryos were able to express the fusion protein even in the presence of NvDsh-DIX (Figs. 7I–L). Furthermore, unlike the *Nvβ-catenin:GFP* mRNA-injected embryos, those co-injected with *Xptβ-catenin:GFP* and *NvDsh-DIX:Flag* mRNA showed early uniform expression of cytoplasmic Xptβ-catenin:GFP (Fig. 7J) and by the blastula stage nuclear localization of the fusion protein was seen in both animal and vegetal blastomeres (Fig. 7L). These results were further confirmed by Western blot analysis (Fig. 7M). The results of these experiments demonstrate that inhibition of NvDsh function in the Wnt/β-catenin pathway leads to the degradation of β-catenin through GSK-3β-mediated phosphorylation.

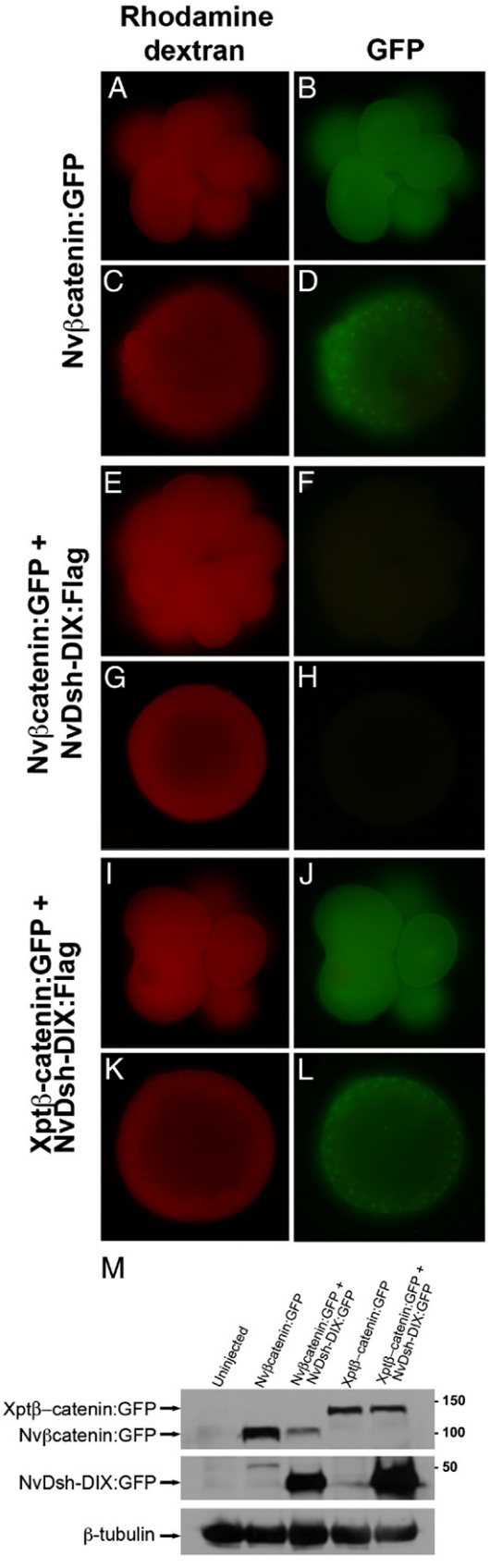

Fig. 7. NvDsh is necessary for the nuclearization of β-catenin. (A–D) Rhodamine–dextran (red), and Nvβ-catenin:GFP (green)-injected embryos. Initially Nvβ-catenin:GFP is uniformly expressed (B) in all blastomeres. By the blastula stage the fusion protein is restricted to the animal half of the embryo where enters the nuclei of the blastomeres at this pole (D). (E–H) Embryos co-injected with Nvβ-catenin:GFP, NvDsh-DIX:Flag and rhodamine–dextran. These embryos do not express Nvβ-catenin:GFP in early (F) or blastula stage (H) embryos. (I–L) But when a stable form of β-catenin (Xptβ-catenin:GFP) is co-injected with NvDsh-DIX:Flag and rhodamine–dextran, this form of β-catenin is stably expressed (J) and nuclearized in both animal and vegetal blastomeres by the blastula stage (L). (M) Western blot analysis of uninjected, Nvβ-catenin:GFP, Nvβ-catenin:GFP+NvDsh-DIX:GFP, Xptβ-catenin:GFP, or Xptβ-catenin:GFP+NvDsh-DIX:GFP expressing embryos. Fertilized eggs were injected with *Nvβ-catenin:GFP*, *Nvβ-catenin:GFP+NvDsh-DIX:GFP*, or *Nvβ-catenin:GFP+Xptβ-catenin:GFP* mRNA. This enabled us to visualize both Nvβ-catenin (100 kDa), Xβcat69 (140 kDa) and NvDsh-DIX (45 kDa) using an anti-GFP antibody. Note the marked reduction in the level of Nvβ-catenin:GFP in embryos co-injected with *Nvβ-catenin:GFP+NvDsh-DIX:GFP* mRNA when compared to the expression of the stable form of β-catenin in the presence of the same dominant-negative Dsh construct. β-Tubulin is shown as a loading control.

Discussion

Cnidarians occupy a key phylogenetic position to provide insight to the evolution of the Bilateria. *N. vectensis* is proving to be a very useful cnidarian model system for developmental and evolutionary studies, and this utility will grow with the recently annotated genome (http://www.jgi.doe.gov/). However, basic embryological information on this animal has heretofore been incomplete. In this report we present the most comprehensive embryological analysis to date on the cellular and molecular ontogeny of polarity in *Nematostella* embryos and show that Dsh signaling in the canonical Wnt pathway plays an important role in mediating the asymmetric developmental potential along the AV axis. Our results also highlight a striking similarity in the molecular mechanisms regulating early pattern formation between cnidarians and basal deuterostomes and suggest that the evolution of mechanisms to asymmetrically activate canonical Wnt signaling in the common ancestor of cnidarians and bilaterians played a crucial role in the evolution of embryonic polarity (Figs. 8A and B).

Polarity in Nematostella eggs and embryos

There are many indicators that some primordial AV polarity exists in cnidarian eggs, including the position of the pronucleus and site of polar body formation (Freeman and Miller, 1982);

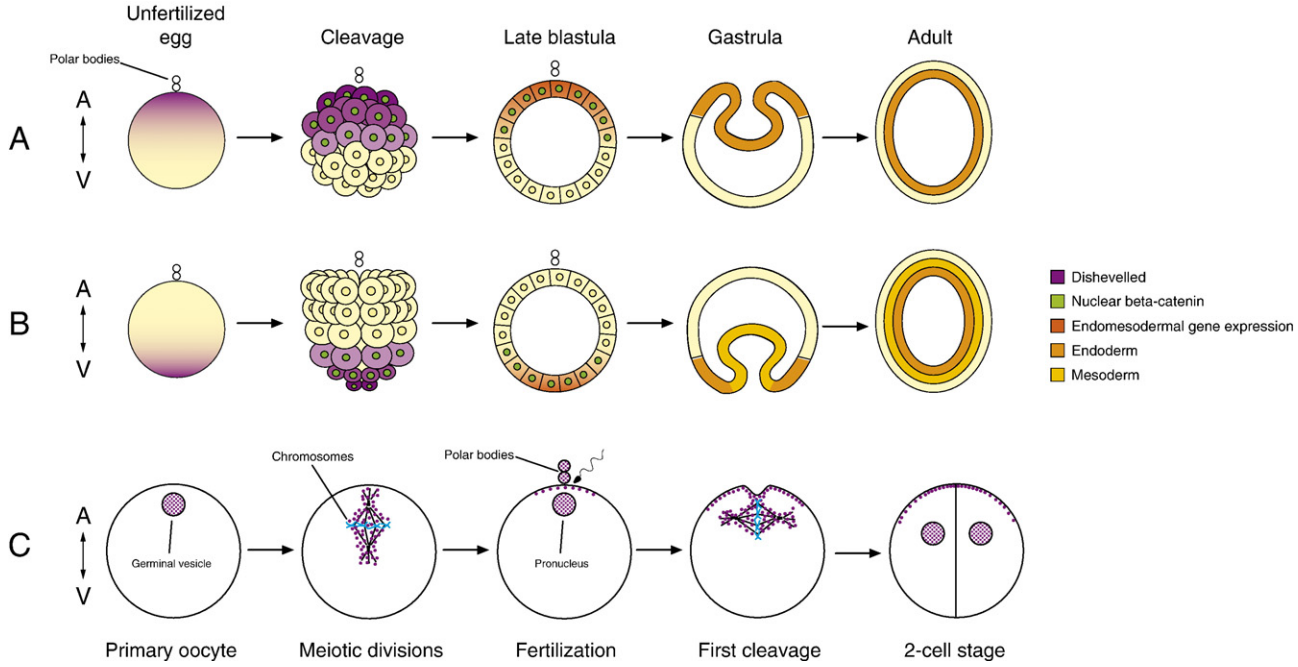

Fig. 8. The change in Dsh localization is correlated with the change in the site of gastrulation between the cnidarian sea anemone, *Nematostella*, and the deuterostome sea urchin, *Lytechinus*. (A) Dsh localization at the animal pole in *Nematostella* eggs stabilizes β-catenin in blastomeres derived from the animal half of the egg. β-Catenin enters the nuclei of animal-half blastomeres where it activates the transcription of *Wnt* target genes involved in endoderm specification. Gastrulation occurs at the animal pole and becomes the oral pole of the adult. (B) In the sea urchin, *Lytechinus variegatus*, Dsh is localized to the cortex of the vegetal pole of the egg and is responsible for the stabilization of β-catenin in vegetal blastomeres that give rise to endomesoderm (Weitzel et al., 2004). The site of gastrulation is located at the vegetal pole and becomes the posterior pole of the adult. (C) Schematic diagram representing a model of Dsh localization during the establishment of oocyte polarity in anthozoan cnidarians. Dsh is associated with the oocyte nucleus. During oocyte maturation and polar body formation, some Dsh protein is translocated from the nucleus to the egg cortex at the site where the polar bodies are extruded. Dsh can then activate canonical Wnt signaling at the animal pole to establish OA polarity, determine the site of gastrulation, and specify the cells that will give rise to endoderm. (A, animal pole; V, vegetal pole).

the distribution of pigment granules (Tessier, 1931); the polar localization of symbiotic dinoflagellates in corals and sea anemones (Davy and Turner, 2003; Hirose et al., 2000; Marlow and Martindale, in press); and the asymmetric distribution of protein, such as Dsh in *Nematostella* (this study), or mRNAs such as *Wnt* and *Tcf* in *Hydractinia* (Plickert et al., 2006), *Cnox4-Pc* and *Brachyury* in *Podocoryne* (Spring et al., 2002; Yanze et al., 2001), and *CheFz1* and *CheFz3* in *Clytia* (Momose and Houliston, 2007). Of these asymmetrically localized factors in cnidarian eggs, only *CheFz1* and *CheFz3* in *Clytia* have been shown to be causally involved in patterning along the oral aboral axis. However, localized *Fz* mRNA does not appear to be conserved in the anthozoan cnidarian *Nematostella*, suggesting that this character is a derived feature in the hydrozoans. Our results in *Nematostella*, a member of the basal anthozoan class of cnidarians, indicate that egg polarity is initially established maternally and that the asymmetric localization of Dsh in the egg plays an important role in mediating this polarity. Asymmetric localization of Dsh in eggs of two other anthozoans suggests that this may be a conserved feature in this basal cnidarian group.

The results from the current study also help to explain the role of cleavage in establishing the OA axis in hydrozoan cnidarians (Freeman, 1980; Freeman, 1981). In the hydrozoan *Clytia*, the OA axis can be re-entrained by centrifuging the zygote nucleus to a new cortical location. This moves the site of unipolar cleavage, the site of gastrulation, and the future oral pole of the adult to the new position of the zygote nucleus (Freeman, 1980). In addition, inhibition of first cytokinesis by cytochalasin B can produce zygotes with two sites of cleavage initiation that give rise to planulae with two oral poles (Freeman, 1981). These observations have raised the possibility that some critical "determinant" was being carried along with the zygote nucleus (or nuclei in the case of the cytochalasin experiments) (Primus and Freeman, 2004) and that this factor specified a new site of endoderm formation and gastrulation. Our observations in *Nematostella* indicate that Dsh is associated with the female pronucleus and later with the zygote nucleus where it then becomes associated with the first cleavage furrow (Fig. 8C). We suggest, therefore, that in the Freeman (1980, 1981) experiments, centrifugation to move the zygote nucleus (or nuclei) resulted in the movement of the associated Dsh protein to the new location(s), and this protein could now activate canonical Wnt signaling in the ectopic site(s) and establish a new oral–aboral axis and/or site of gastrulation.

Regulation of endoderm specification and gastrulation in Nematostella by Dishevelled

Translocation of β-catenin into cell nuclei is causally required for endoderm specification and gastrulation in *Nematostella* (Wikramanayake et al., 2003; this study). β-Catenin becomes selectively stabilized in animal half-derived blastomeres that give rise to endoderm starting around the 32-cell

stage. Furthermore, transcripts of *NvTcf*, the binding partner of nuclear β-catenin, are similarly asymmetrically localized in animal-half blastomeres (Lee et al., 2006). These observations suggest that a localized factor selectively activates canonical Wnt signaling in animal pole-derived blastomeres. An obvious candidate for this localized factor is a Wnt ligand, either localized in the egg or selectively activated in animal-half blastomeres during early development. In the hydrozoan *Hydractinia*, it has been shown that *Wnt-1* mRNA is localized in the egg and that downstream components of the Wnt signaling pathway play a role in patterning the primary axis during embryogenesis (Plickert et al., 2006). However, none of the *Wnt* genes (representing 11 of the 12 families found in vertebrates) recovered from *Nematostella* is expressed maternally or during the early cleavage stages when β-catenin becomes nuclearized in the future endoderm (Kusserow et al., 2005; Lee et al., 2006). Since no additional *Wnt* genes have been identified in the *Nematostella* genome (http://www.jgi.doe.gov/) our data imply that stabilization of β-catenin in the endoderm of this embryo occurs independently of a Wnt ligand. In the hydrozoan *C. hemisphaerica*, *CheF1* mRNA is localized to the animal pole and anti-sense morpholino-mediated knockdown of *CheF1* translation resulted in loss of β-catenin nuclearization in animal-pole blastomeres and the inhibition of gastrulation (Momose and Houliston, 2007). While we do not know the function of *NvFz1* in *Nematostella* embryos, the maternal mRNA is uniformly expressed in the unfertilized egg, unlike *CheF1* in *Clytia* (Momose and Houliston, 2007). These observations therefore indicate that localized *Fz* mRNA in the egg does not appear to be a conserved feature even among the cnidarians.

Several lines of evidence presented in the current study indicate that Dsh is an important localized factor that asymmetrically activates the canonical Wnt signaling pathway in *Nematostella*. NvDsh protein is localized to the animal pole of unfertilized eggs, and it remains enriched in animal pole-derived blastomeres during embryogenesis. NvDsh is, therefore, present in the blastomeres that stabilize β-catenin during the early cleavage stages. Blocking NvDsh signaling in the canonical pathway using a dominant-negative approach demonstrated that this protein is causally involved in gastrulation and endoderm specification. Embryos overexpressing the dominant-negative NvDsh-DIX construct failed to express early endodermal markers, suggesting that they cannot specify endoderm. Furthermore, our data suggest that NvDsh-DIX blocks these processes by destabilizing β-catenin. Together, these data provide strong evidence that NvDsh is one important localized factor that mediates endoderm specification and gastrulation in *Nematostella* through the canonical Wnt signaling pathway. While we have not carried out functional studies in the two coral species, the localization of Dsh to the animal pole in *F. scutaria* and *P. meandrina* suggests that Dsh has a similar function in these anthozoans.

Regulation of Dishevelled stability

The observation that *NvDsh* mRNA is expressed ubiquitously, but that the endogenous protein is expressed asymmetrically suggests that NvDsh is destabilized at the vegetal pole of the embryo. While it is possible that NvDsh is not expressed in vegetal blastomeres due to translational repression, we favor the destabilization model for the following reasons. When the full-length NvDsh:GFP fusion protein is expressed by mRNA injection it is asymmetrically expressed in the animal-half blastomeres and later in the endoderm. This asymmetric localization is likely due to degradation in the vegetal-half blastomeres since the NvDsh-DIX:GFP fusion protein and other NvDsh:GFP deletion constructs are uniformly expressed (S.K., unpublished observations). These observations indicate that full-length Dsh sequences are required for the degradation mechanism to function. In fact, an emerging theme in Dsh regulation is that this protein is destabilized and targeted for degradation via ubiquitylation and the proteosome pathway (Angers et al., 2006; Simons et al., 2005). Dsh destabilization can be mediated by different proteins, but in each case degradation is through the proteosome (Angers et al., 2006; Simons et al., 2005). In vertebrate cells, degradation of Dsh by the KLHL12-Cullin-3 ubiquitin ligase complex requires the carboxy-terminal end of the protein, but not the DIX, PDZ, or DEP domains of Dsh (Angers et al., 2006; Simons et al., 2005). It would be interesting to determine if this particular degradation pathway is conserved in cnidarians and if so, how its activity is spatially restricted during *Nematostella* development.

Canonical Wnt signaling and the evolution of polarity

Over the past several years it has become apparent that the canonical Wnt pathway plays a critical role in establishing early polarity during animal development. In sea urchins, Dsh is enriched in vesicular structures at the vegetal pole of the egg where it mediates patterning along the AV axis by regulating β-catenin stability in vegetal blastomeres (Kumburegama and Wikramanayake, 2007; Weitzel et al., 2004). The current study and our previous studies have revealed some interesting similarities in the use of the canonical Wnt pathway to establish polarity and segregate germ layers between sea urchin and *Nematostella* embryos (Figs. 8A and B). In both embryos, there is selective stabilization of nuclear β-catenin in the prospective endomesoderm/endoderm and this stabilization is regulated by localized Dsh. These observations raise the possibility that Dsh was localized in eggs of the common ancestor of cnidarians and bilaterians, and that this protein was used to stabilize β-catenin at one pole of the embryo. However, in sea urchins localization of Dsh and the stabilization of β-catenin take place at the vegetal pole, whereas in *Nematostella* these events occur at the animal pole. This leads to the segregation of endomesoderm and gastrulation at the vegetal pole in sea urchins and segregation of endoderm and gastrulation at the animal pole in *Nematostella*. This change in the position of gastrulation has profound implications for the evolution of metazoan body plans. Sea urchins are basal deuterostomes, and like other echinoderms, hemichordates, and chordates the site of gastrulation occurs at the posterior (vegetal) pole that becomes the larval/adult anus. In contrast, anthozoan cnidarians and ctenophores are protostomes, and the site of gastrulation occurs at the animal pole

and forms the mouth (Freeman, 1977). Thus, the adult axial organization of both protostomes and deuterostomes remains essentially the same with respect to the primary egg axis, with the anterior region derived from the animal pole and the posterior region from the vegetal pole.

Embryological and molecular observations suggest an evolutionary scenario for the transition in the site of gastrulation from the animal pole to the vegetal pole in the bilaterian lineage. In sea urchins, the AV polarity is fixed and polarity cannot be re-entrained by centrifugation (Horstadius, 1973). In this basal deuterostome, Dsh appears to be fixed at the vegetal cortex where it regulates endomesoderm segregation and gastrulation. We speculate that the shift in the site of gastrulation to the vegetal pole in sea urchins may have involved two distinct steps. First, there was a dissociation of Dsh from the female pronuclear-associated structures in animals like *Nematostella*, followed by a mechanism to localize and tether Dsh to membrane vesicles at the vegetal pole. Interestingly, Dsh has domains that allow it to interact with microfilaments, microtubules, and phospholipid vesicles (Capelluto et al., 2002; Krylova et al., 2000), which allow this molecule to be localized to distinct positions in a variety of different ways. Because the site of gastrulation can change radically in different bilaterian lineages (Martindale, 2005) understanding the role of Dsh, and other components of the Wnt signaling pathway (Momose and Houliston, 2007; Plickert et al., 2006), may provide insight into the positioning of endomesoderm fates in diverse metazoan lineages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.05.040.

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