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Evolution of Developmental Control Mechanisms

Ectopic activation of the canonical wnt signaling pathway affects ectodermal patterning along the primary axis during larval development in the anthozoan *Nematostella vectensis*

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ABSTRACT

The primary axis of cnidarians runs from the oral pole to the apical tuft and defines the major body axis of both the planula larva and adult polyp. In the anthozoan cnidarian *Nematostella vectensis*, the primary oral–aboral (O–Ab) axis first develops during the early embryonic stage. Here, we present evidence that pharmaceutical activators of canonical wnt signaling affect molecular patterning along the primary axis of *Nematostella*. Although not overtly morphologically complex, molecular investigations in *Nematostella* reveal that the O–Ab axis is demarcated by the expression of differentially localized signaling molecules and transcription factors that may serve roles in establishing distinct ectodermal domains. We have further characterized the larval epithelium by determining the position of a nested set of molecular boundaries, utilizing several newly characterized as well as previously reported epithelial markers along the primary axis. We have assayed shifts in their position in control embryos and in embryos treated with the pharmacological agents alsterpaullone and azakenpaullone, Gsk3β inhibitors that act as canonical wnt agonists, and the Wnt antagonist iCRT14, following gastrulation. Agonist drug treatments result in an absence of aboral markers, a shift in the expression boundaries of oral markers toward the aboral pole, and changes in the position of differentially localized populations of neurons in a dose-dependent manner, while antagonist treatment had the opposite effect. These experiments are consistent with canonical wnt signaling playing a role in an orally localized wnt signaling center. These findings suggest that in *Nematostella*, wnt signaling mediates O–Ab ectodermal patterning across a surprisingly complex epithelium in planula stages following gastrulation in addition to previously described roles for the wnt signaling pathway in endomesoderm specification during gastrulation and overall animal–vegetal patterning at earlier stages of anthozoan development.

Introduction

The phylum Cnidaria includes sessile polyp forms such as anthozoan anemones and reef-building corals and medusoid swimming forms commonly referred to as jellyfish. The body plans of members of the Cnidaria are relatively simple and possess derivatives of only two embryonic germ layers (endoderm and ectoderm), have a single gut opening and non-centralized nerve net. As Cnidaria forms the sister group to the bilaterian clade (Fig. 1A) (Hejnol et al., 2009), studies of cnidarian development can provide key insights into the features that may have been present in the Eubilaterian ancestor. Anthozoan cnidarians sexually reproduce to generate a swimming, ciliated planula stage (Hand and Uhlinger, 1992) (Fig. 1B). The outer ectoderm of these planula appears to be largely radially symmetrical along their oral-aboral (O–Ab) axis with few morphological indications of axial position other than the apical tuft of cilia at the aboral pole (Marlow, 2011) (Fig. 1B). The primary axis, common to all cnidarians, is called the O-Ab axis and extends from the oral opening to the apical tuft (Fig. 1B). Embryonic axes of several bilaterian animals are initially established in part through the activity of the wnt signaling pathway (Martin and Kimelman, 2009; Onai et al., 2009; Petersen and Reddien, 2009), with the wnt proteins playing particularly important roles in the establishment of neural boundaries (Kiecker and Niehrs, 2001; Kobayashi et al., 2007; Nordstrom et al., 2002; Pani et al., 2012).

Interestingly, a strikingly complex, vertebrate-like genomic wnt complement has been identified in anthozoan cnidarians (Kusserow et al., 2005; Lee et al., 2006) and indicates that

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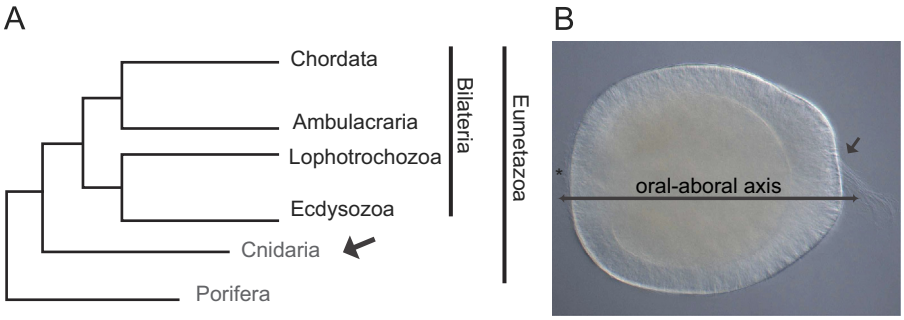

Fig. 1. (A) Phylogenetic tree depicting the outgroup relationship of cnidarians (arrow) with relation to bilaterian taxa (based on Hejnol et al. (2009)). (B) Ciliated *Nematostella vectensis* planula stage. The mouth is marked with an asterisk and the apical tuft is demarcated with an arrow.

subsequent loss of wnt components has occurred in some non-deuterostome bilaterians. However, the functional role that this nearly complete canonical wnt signaling system may play in establishing cnidarian axial identity, and what its ancestral role in Eubilateria might have been during embryogenesis remains uncertain. From studies in hydrozoan cnidarians, it has become increasingly clear that wnt signaling plays an important role in the establishment of the head organizer and overall axial polarity in embryogenesis, regeneration and morphogenesis. It has been proposed that wnt mediates the acquisition of overall axial identity for regional territories (Duffy et al., 2010; Hobmayer et al., 2000; Momose et al., 2008; Momose and Schmid, 2006; Muller et al., 2007) and it has also been hypothesized that the distribution of the wnt antagonist dickopf (dkk) may allow for wnt-free zones where neurogenesis can occur (Guder et al., 2006). Neurogenesis in anthozoan cnidarians occurs throughout the planula epithelium (Nakanishi et al., 2011), but distinct regions of wnt activity could conceivably allow for the development of specific subsets of neurons within the epithelium. These studies have provided an important link between the establishment of axial polarity and the canonical and non-canonical wnt pathway, but the role wnt signaling plays in the establishment of molecular epithelial identity and the positioning of distinct cell populations, particularly neurons, along the primary axis, remains poorly understood. Furthermore, we know very little about the role of wnt in epithelial patterning during embryogenesis in anthozoans.

Based on the phylogenetic distribution of pathway components, the ancestral bilaterian wnt signaling system is hypothesized to have encompassed twelve families of secreted ligands, several families of frizzled receptor genes, and wnt antagonists such as dkk and secreted–frizzled related proteins (sfrps) (Holstein, 2008; Kumburegama et al., 2011; Lee et al., 2006). Cnidarians, with eleven of the twelve ligands (*wnt9* is absent from all currently published cnidarian sequence data), and well-conserved representatives of the *frz*, and *dkk* gene families, possess a near complete bilaterian "wnt system" (Kumburegama et al., 2011; Kusserow et al., 2005; Lee et al., 2006). Initial expression studies of *wnt* transcript localization in planula and polyp stages in the anthozoan *Nematostella vectensis* revealed a staggered pattern of up to eight *wnt* genes expressed in both ectodermal and endodermal epithelia along the primary axis of cnidarian larvae, with several components concentrated at the future oral pole, and led to the proposal of a wnt code for cnidarian axial patterning (Kusserow et al., 2005; Miller et al., 2005).

Functional investigations of wnt pathway components during development and regeneration have implicated a role for the pathway in early embryonic polarity and subsequent acquisition and maintenance of axial identity. Early expression studies have demonstrated that wnt pathway components are asymmetrically distributed in hydrozoan cnidarians (Momose et al., 2008; Momose and Houliston, 2007; Plickert et al., 2006). Furthermore, functional experiments testing the developmental roles of these molecules in the hydrozoan *Clytia* show that both wnt ligands (wnt3) and receptors (frizzled) act as determinants of axis formation in embryogenesis (Momose et al., 2008; Momose and Schmid, 2006). It has been similarly shown that wnt3 acts as an axis determinant at later developmental stages in establishing the head organizer in regenerating adult *Hydra* (Lengfeld et al., 2009). At least some cnidarian wnts appear to harbor the capacity to act in conserved roles in the planar cell polarity (PCP) pathway in initiating changes in cell morphology and convergent extension movements during gastrulation when injected into the amphibian *Xenopus* (Rigo-Watermeier et al., 2012). The expression of two wnt ligands and a wnt receptor (frizzled) during head formation in *Hydra* in conjunction with a necessity for JNK activity during bud evagination further support a role for non-canonical wnt involvement in axial development (Philipp et al., 2009). A recent study of *N. vectensis* regeneration (Trevino et al., 2011) has also demonstrated an oralizing activity for canonical wnt signaling, similar to that observed for the hydrozoan cnidarian *Hydra* (Hobmayer et al., 2000). These studies provide hints that cnidarian and bilaterian wnt ligands may share some conserved functions during embryogenesis in cellular morphogenesis, oral identity and endomesoderm formation (Broun et al., 2005; Kumburegama et al., 2011; Lee et al., 2007; Rigo-Watermeier et al., 2012; Trevino et al., 2011; Wikramanayake et al., 2003). However, previous research has provided limited information regarding a role for wnt signaling in molecular epithelial axial patterning, particularly in anthozoan cnidarians.

Recent studies describing nervous system architecture of *N. vectensis*, its cell types and development have shown that cryptic cell type diversity exists along the cnidarian primary axis, but it is largely unclear what establishes and maintains these regions along the primary axis. Molecular markers show that neurotransmitters, visual family opsins, and cnidocyte sensory cells are differentially localized within the planula and polyp (Marlow, 2011; Marlow et al., 2009). Explant experiments in developing gastrulae of *N. vectensis* and in both direct developing hydrozoans and indirect developing hydrozoans have demonstrated differential neurogenic potential between oral and aboral explants and suggest that the localization of neural elements may be the result of early developmental patterning in embryonic and larval ectodermal precursors along the oral–aboral axis (Freeman, 1983; Nakanishi et al., 2011; Thomas et al., 1987). While neurogenesis from stem cell populations in adult *Hydra* occurs through widespread cell migration, the migration of neural precursors in embryonic anthozoans has not been demonstrated (Marlow et al., 2009). An absence of migration among neurons and neural precursor cells indicates that cell differentiation occurs within locally restricted epithelial territories. Thus, positional identity along the O–Ab axis may be an important component for generating regional cell type specific identity during cnidarian

development. While cnidarian hox genes have been implicated, based on expression data, in axial patterning of the planula larva (Finnerty et al., 2004), their expression, with the exception of *anthox*1 has been described in the endoderm, indicating that additional patterning systems are likely functioning in ectodermal larval patterning.

In order to assess the role of canonical wnt signaling in early ectodermal patterning, we use molecular markers to define ectodermal territories along the oral–aboral axis and the neural apical tuft region in the anthozoan *N. vectensis*, including the spatial relationships of transcripts of three putative wnt-responsive effectors and regulators, *Nvsp5/buttonhead*, *Nviroquois* and *Nvsix3/6/optix* (Fujimura et al., 2007; Janssens et al., 2010; Lagutin et al., 2003; Sinigaglia et al., 2013) as well as the homeodomain transcription factor *NvNK*3 (Kamm and Schierwater, 2006), of which *Nvsp*5 and *NvNK*3 expression were previously undescribed in cnidarians. We show that the position of ectodermal domains change along the O–Ab axis in a dose dependent manner following treatments with the wnt signalling agonists, alsterpaullone and azakenpaullone. These phenotypes are consistent with an "oralization" of molecular territories, while the opposite effect is observed following treatment with a wnt antagonist. These data suggest that wnt signaling serves a role in maintaining spatial molecular territories in the cnidarian planula in a manner that leads to the differential spatial placement of neural cell populations.

Methods

Adult *N. vectensis* polyps were reared and spawned as previously described (Hand and Uhlinger, 1992). Embryos were de-jellied prior to first cleavage in a solution of 1/3 × filtered seawater (FSW) and 4% cysteine, maintained in glass dishes and cultured at 25 °C until fixation. In order to determine effective concentrations for drug treatments, embryos were incubated from 30 hpf (following gastrulation) to 72 hpf with 0.1 μM, 0.5 μM, 1, 5 μM and 10 μM concentrations of alsterpaullone in 0.5% DMSO in 1/3x FSW (Sigma A4847) to determine efficacy and dose-dependence. Embryos were also treated at progressively earlier time points to determine the global effects that might occur in animal–vegetal patterning and gastrulation as well as to assess what localized changes in cell division might result from wnt activation (*e.g.* selective proliferation of animal or vegetal hemispheres). These treatments were initiated at 0 hpf, 8 hpf, 14 hpf, and 24 hpf. These results were compared to the inhibitor azakenpaullone (Sigma A3734) to determine if both inhibitors produced comparable phenotypic effects.

For pharmacological treatment and determination of shifts in ectodermal territories, embryos were maintained as above until gastrulation was completed (30 hpf) and were incubated with 1 μM or 5 μM alsterpaullone in 0.5% DMSO in 1/3 × FSW until 48 hpf. Control embryos were treated in 0.5% DMSO and fixed at the same time points as alsterpaullone or azakenpaullone treated embryos. For washout treatments, embryos were reared until 30 hpf and then incubated with 1 μM or 5 μM alsterpaullone from 30 hpf to 48 hpf. Following treatment, embryos were washed 5x in 0.5% DMSO in 1/3x FSW and then incubated until 72 hpf in 0.5% DMSO in 1/3x FSW. Controls for washout experiments were reared from 30 hpf to 48 hpf in 0.5% DMSO in 1/3x FSW, then washed as with experimental treatments into fresh 0.5% DMSO and reared until 72 hpf. For all treatments, control and experimental, treatments were initiated after gastrulation. For iCRT14 experiments, embryos were raised to 30 hpf, following gastrulation, and then placed in either a 2% DMSO control treatment or a 50 μM iCRT14 in 2% DMSO treatment. Embryos were then fixed at 60 hpf for *in situ* hybridization. In some batches of fertilized embryos, a percentage of embryos from both control and experimental treatments failed to undergo normal gastrulation due to a developmental defect (which occurs before the onset of the treatments). These embryos were easily identified and excluded during subsequent analyses.

Fragments for transcripts encoding orthopedia (*Nvotp*) DQ206247.1, distaless (*Nvdlx*) DQ206283.1, *Nvwnt*2 AY725201.1, *Nvwnt*4 AY687348.1, fibroblast growth factor receptor (*NvfgfRA*) EF173463, *NvpaxD* AY730692.1, homeobrain (*Nvhbn*) HM004558.1 were isolated for previous studies (Kusserow et al., 2005; Matus et al., 2007a, 2007b; Mazza et al., 2010; Ryan et al., 2007) and were used to generate riboprobes for the present study. A sequence for *NvNK*3 was previously published but uncharacterized by *in situ* hybridization (AY339870) (Kamm and Schierwater, 2006). We cloned this gene and characterized the pattern by *in situ* hybridization in this study. Using previously deposited sequences from *Nv*sp5/*buttonhead*, *Nv*irx and *Nv*six3/6 (Nina et al., 2010; Ryan et al., 2006), extended fragments were isolated using 5′ and 3′ RACE PCR with gene specific primers on a cDNA pool generated from mixed stage RNA using the Clontech SMART RACE II cDNA synthesis kit or from an existing EST library (XP_001635002, DQ206294, DQ206288).

Fixation and *in situ* hybridization were carried out according to previously published protocols for *N. vectensis* (Martindale et al., 2004) with the following modifications; embryos were fixed in MOPS buffer (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4,.1% Tween20) rather than 1/3x FSW as a fixation buffer, proteinase k digestion times were reduced to 5 min, and incubations in *in situ* probes were conducted overnight rather than for 36 h. Following hybridization and development, embryos were cleared in 70% glyercol and imaged using a Zeiss Axioscope and photographed with a Zeiss AxioCam HRc using Axiovision image acquisition software. Images were adjusted (brightness, contrast and cropped) in Adobe Photoshop. Reflection microscopy of NBT/BCIP precipitate generated during *in situ* hybridization in apical tuft domains was performed as previously published (Jékely and Arendt, 2007) using a Leica SpE confocal laser scanning microscope. Acetylated tubulin and phospho-histone staining were performed according the antibody staining protocol previously published for *N. vectensis* (Marlow et al., 2009) embryos with a commercially available anti-acetylated tubulin antibody (Sigma T6793) and anti-phospho-histone H3 antibody (Abcam Ab5176). Measurements to determine expression domain position and size were performed in most cases on ten randomly chosen planulae for each gene from each treatment (except for the following cases: 5 planulae for *Nvhbn* and 8 larvae for *Nvdlx* and 9 larvae for *Nvrx*) according to the following method: (1) Total planulae length from oral pole to the tip of the aboral pole was recorded as total pixel number in Adobe Photoshop (2) Length from the oral tip to the start of the domain was determined in pixels (3) Length from the oral tip to the aboral edge of expression domain was determined in pixels (4) Domain size and position in pixels were normalized to the total embryo length in pixels, resulting in a fractional size and position for each domain were normalized in relation to the total embryo length of 1. To confirm the general validity of the boundaries resulting from measurements on stained embryos, we performed double *in situ* hybridization for the most robustly expressed genes with the previously published *Nematostella* two-color *in situ* hybridization protocol (Matus et al., 2006).

Results

Ectodermal regional identity in early embryos

Examination of the expression patterns of eleven transcription factors and signaling pathway components in the *Nematostella* ectodermal epithelium at 30 hpf, 48 hpf and 72 hpf planula stages indicates the presence of a molecularly complex spatial topography.

While the expression of several of these factors have been previously published, the number of developmental time points available as well as precise relative delimitation of the spatial expression boundaries were not previously determined. In order to describe the spatial molecular topography of the *Nematostella* ectodermal epithelium over developmental time, we have examined the spatial relationships of ten previously published epithelial markers; *Nvotp*, *Nvwnt4*, *Nvhbn*, *Nvwnt2*, *NvpaxD*, *Nvdlx*, *Nvrx*, *Nvanthox1*, *Nvsix3/6*, and *NvfgfRA* (Finnerty et al., 2004; Kusserow et al., 2005; Matus et al., 2007a, 2007b; Mazza et al., 2010; Ryan et al., 2007; Sinigaglia et al., 2013) as well as two previously uncharacterized markers; *Nvsp*5 and *NvNk*3 (Kamm and Schierwater, 2006; Nina et al., 2010; Ryan et al., 2006) at three developmental time points (Fig. 2). As with nearly all of the patterns used to assay molecular identity of the ectodermal epithelium, expression of *NvNK3*, *Nvsp*5 and *Nvsix*3/6 initiate prior to the completion of gastrulation and are present during early planula stages at 30 hpf at 16 °C. While expression of *Nvsp*5 transitions from primarily aboral expression to strongly oral expression, it should be noted that some embryos can be found with both an oral and aboral expression domain at 48 hpf and 72 hpf (*e.g.* Fig. 4D). With the exception of *Nvsp*5, which appears to be quite dynamic in expression, the relative positions of these transcription factors are stable over developmental time with most patterns refining down to more discreet regions at later developmental time points (Fig. 2; 30 hpf, 48 hpf and 72 hpf time points).

At 48 hpf, oral-most ectodermal epithelial cells express the homeodomain transcription factor *Nvotp*, the *Nvwnt*4 ligand, the *Nvsp*5 zinc-finger transcription factor and the *Nvhbn* homeodomain transcription factor (Fig. 2A and D). More medial territories, marked by the start of the *Nvwnt*2 expression domain, also express the transcription factor *Nvhbn*, in addition to *Nvdlx*, and *Nvrx* (Fig. 2E and H). In some of these oral and medial bands of expression, *Nvotp* and *Nvrx,* are composed of near solid rings of expression as well as more scattered single cell expression at the oral and aboral boundaries (Fig. 2A and H, arrows). For the purposes of this study, the position of these solid band domains was used to characterize their expression along the oral–aboral axis. However, it should be noted that individual scattered cells outside of these solid bands have been observed in some cases to express these markers (*e.g. Nv*rx is expressed in a solid band and in another population of scattered cells) and represent a second domain for these factors. These individually expressing cells may have a unique combinatorial expression profile and may represent a second role for these transcription factors. We did not explore these cells further. Nearly the entire apical pole of developing planulae is marked by the expression of *Nvsix*3/6 (Fig. 2I). Slightly aboral to *Nvsix3/6*, lies the expression domains of *Nvanthox*1 and of *NvfgfRA*, respectively (Fig. 2J and K). *NvNK*3 shows the most restricted expression (Fig. 2L).

The animal pole of the developing embryo gives rise morphologically to the blastopore and later the oral opening, while the most aboral tip of the larva forms the apical tuft, a neural structure. Development of the blastopore has been molecularly characterized in a number of studies and has been previously linked to wnt signaling activity (Kumburegama et al., 2011; Lee et al., 2007; Röttinger et al., 2012). Less is known regarding the molecular identity of the apical region and about distinct molecular and morphological territories at the apical plate, therefore we performed a characterization of this region. The apical tuft of *N. vectensis* is marked molecularly by the expression of *NvfgfRA* (Rentzsch et al., 2008) (Fig. 3A and C) and other genes, like *Nvcoe* (Pang et al., 2004). We find that the apical tuft of cilia is demarcated by an absence of expression of the transcription factor *Nvsix3/6* (Fig. 3D). *Nvs*ix3/6 is an ortholog of the vertebrate Six3 and Six6 genes and fly Optix genes that are involved in neural and visual patterning (Jean et al., 1999; Lagutin et al., 2001; Seimiya and Gehring, 2000). While this manuscript was under revision, another study similarly identified six3/6 and irx orthologs in *Nematostella*. This study tested the functional role of six3/6 on the development of the apical plate and apical tuft and

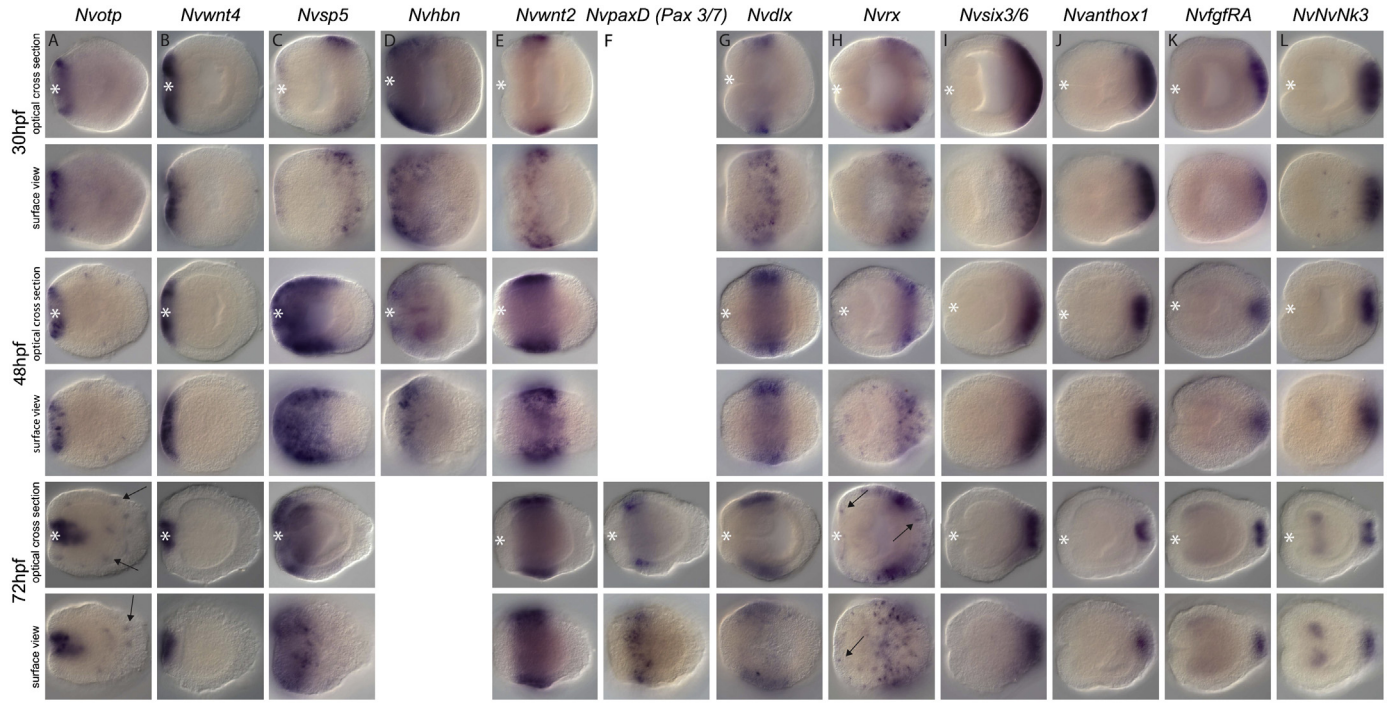

Fig. 2. Spatial expression of three signaling pathway components (*Nvwnt4*, *Nvwnt*2 and *NvfgfRA*) and eight transcription factors at three developmental time points (30 hpf, 48 hpf and 72 hpf). *Nvhbn* is shown only at 30 hpf and 48 hpf and *NvpaxD* is shown only at 72 hpf. Two views of each time point are shown for each embryo. Upper view is an optical cross section and lower view is a surface view of the larval epithelium. The oral pole (asterisk) is to the left and the apical tuft is located at the aboral tip on the right. Cells which lie outside the main band of expression of *Nvotp* and *Nvrx* are indicated with arrows.

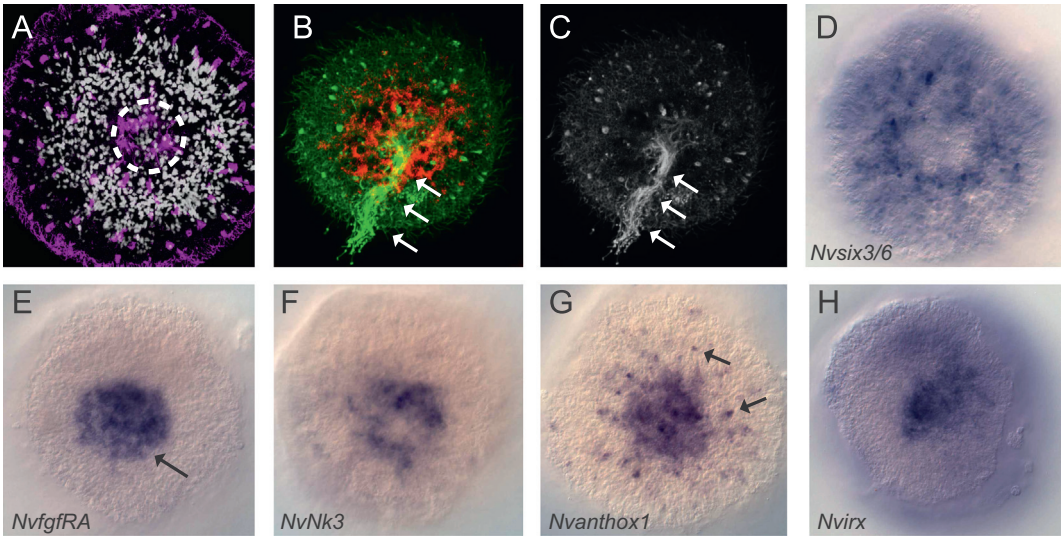

Fig. 3. The *N. vectensis* apical organ forms within a territory devoid of *Nvsix*3/6 transcript expression. (A) Cnidarian planula (apical view looking down on the apical tuft). The apical tuft, marked by acetylated tubulin staining (magenta) is outlined with a dashed circle. Nuclei are labeled with DAPI (white). (B) Cilia of the apical organ are labeled with acetylated tubulin (green) and the apical tuft marker *NvfgfRA* is shown in red. (C) A monochromatic image of the acetylated tubulin staining of the apical tuft (apical view) shown in (B). Note that cilia surrounding the apical tuft are also labeled, but that the apical tuft cilia are considerably longer and are localized to a singe apical spot. (D)–(H) Expression domains of mRNAs expressed at the apicat tuft at 48 hpf. Views are DIC images of the aboral pole centered on the position of the apical tuft following *in situ* hybridization to mRNA transcripts. (D) *Nvsix*3/6 is expressed in the aboral ectoderm surrounding the apical tuft but is not present in the apical tuft domain. (E) *NvfgfRA* is expressed in the apical tuft and surrounding cells in a continuous domain. (F) *NvNK*3 labels a narrowly restricted patch of cells at the apical tuft, with the exception of a small domain directly in the center of the apical tuft domain. (G) *Nvanthox*1 is found expressed throughout the apical tuft and expands in scattered cells beyond the apical tuft domain (arrows). (H) *Nvirx* is localized primarily to the oral pole and to the small patch of *Nvsix*3/6 negative territory at the apical tuft.

demonstrated a functional role of six3/6 in the development of the apical tuft as well as interaction with six3/6 and components of the wnt pathway (Sinigaglia et al., 2013). Examination of the aboral territory surrounding and encompassing the apical tuft structure reveals two main features. The first is that the aboral pole is molecularly heterogeneous. The *Nvsix*3/6 negative territory defining the tuft domain co-expresses a number of developmental signaling molecules and transcription factors. While these molecules, which include the previously reported *Nvanthx*1, *NvfgfRA*, and *Nvirx*, and as characterized here the transcription factor *NvNK*3, are largely overlapping, each pattern contains patches of expression-free cells (Fig. 3D and H). *NvNK*3, the most spatially restricted of the patterns examined here, is found at the apical pole but is absent from the most medial portions of the tuft region (Fig. 3E), a second *NvNK*3 expressing domain, not addressed here (Fig. 3E) is expressed in the endoderm of the pharynx. Previous phylogenetic analysis of *Nematostella NvNK*3 shows that this gene is an ortholog of NK3/Bagpipe orthologs of bilaterian animals (Kamm and Schierwater, 2006; Ryan et al., 2006). In addition to bagpipe's well-documented role in mesoderm development in both flies and vertebrates (Azpiazu and Frasch, 1993; Tanaka et al., 1998; Tribioli et al., 1997), it is also expressed in the vertebrate central nervous system (Tanaka et al., 1999). *NvIrx* is also restricted to only the tuft region of the apical plate as well as a second more orally restricted ectodermal epithelial domain, in addition to an endodermally restricted domain (not addressed here). Irx transcription factors serve a number of roles in CNS development of both flies and vertebrates, notably in sensory placode development, and have been shown to display mutually exclusive spatial distribution with *six*3 (Kobayashi et al., 2002; Schlosser, 2006). *NvfgfRA*, like *Nvirx*, is expressed across the apical tuft territory, but unlike *irx*, shows small expression-free domains (Fig. 3E and H). The heterogeneous expression profile of this territory can be explained in one of two ways. First, it is possible that some of these factors are differentially expressed temporally in progenitor populations before adopting a similar differentiated fate. The second possibility is that the apical tuft region represents a complex structure consisting of different neural and non-neural cell types. Currently, we cannot distinguish between these two possibilities. However, as is the case for *Nvanthox*1 (Fig. 3G), at least some expressing cells are located outside of the tufted region, indicating that a complex set of cell types is present at the aboral pole.

Canonical wnt signaling and the specification of oral and aboral identity

The *Nematostella* apical pole, as discussed above, is a site of expression for a number of signaling systems, signaling antagonists and transcription factors upon which the apical tuft develops. Among these apically expressed genes are *Nv*six3/6 (Fig. 3D) and *Nv*dkk (Lee et al., 2006). These factors have been demonstrated to be mediators of wnt signaling in other developmental systems (Lagutin et al., 2003; Niehrs, 2006). Surprisingly, we know very little regarding the role of wnt signaling on the development of the apical plate. As discussed above, the apical tuft develops in a small *Nvsix*3/6 negative territory at the most aboral tip of the embryo. Similarly, the oral pole is the site of expression of a number of wnt ligands and may act as a wnt signaling center in cnidarians (Kusserow et al., 2005). In order to test the role of wnt signaling on the overall development of *Nematostella* embryos, we performed pharmacological treatments with wnt antagonists and agonists initiating at 0 hpf, 8 hpf, 14 hpf and 24 hpf and continued treatment until 48 hpf, at which time embryos were fixed (Supplementary Fig. 1). Embryos in which treatment was initiated prior to 14 hpf did not consistently form and maintain a blastoporal opening (some azakenpaullone-treated embryos were found to have formed a blastopore in treatments initiated at 14 hpf) (Supplementary Fig. 1A and C). Embryos treated from 24 hpf onward consistently formed and maintained a blastopore as well as ectodermal and endodermal epithelia in all treatments (Supplementary Fig. 1D). If treatments are initiated, before gastrulation, a blastopore does not form and no clear embryonic axis is visible (Supplementary Fig. 1A). For alsterpaullone, this treatment also

results in a clear defect in cell division with many regions of the embryo undergoing little to no division which results in cell-dense regions (Supplementary Fig. 1A). As this phenotype was only observed in the earliest alsterpaullone treatment, we conclude that this is most likely an indicator of toxicity. Later treatments initiated at 8 hpf and 14 hpf did not appear to result in large scale changes in cell division patterns and cell densities as assayed with DAPI staining and phospho-histone staining (Supplementary Fig. 1B and D). Molecular markers that would normally be limited to the blastopore, *Nvfkhd* and *Nvwnt4*, are expanded to encompass the entire embryo (Supplementary Fig. 2).

Our data suggests that earlier treatments with the pharmacological wnt agonists alsterpaullone and azakenpaullone interfere with the formation of the blastopore and would therefore have a global effect on the development of distinct ectodermal and endodermal tissue layers (Supplementary Fig. 1). Thus, to specifically investigate the effects of canonical wnt signaling agonism on oral and aboral molecular and morphological territories, we chose timepoints following gastrulation, 30 hpf to 48 hpf, for Wnt agonist treatments. In order to determine the appropriate concentrations for pharmacological treatments, we performed dose–response experiments and assayed the effect of the drugs using *in situ* hybridization (Supplementary Fig. 3). Upon treatment with alsterpaullone, even at very low concentrations, the apical tuft at the aboral tip of the larva fails to form (Fig. 4A and B). Developing *Nematostella* larvae are uniformly ciliated throughout the ectoderm and in alsterpaullone treated embryos, these cilia still form normally, however the long, specialized apical tuft cilia do not form (Fig. 4A and B). The expression of *Nvwnt*4, which is normally orally restricted, expands aborally following alsterpaullone treatment (Fig. 4C and C″). It should also be noted that at higher alsterpaullone concentrations, *Nvwnt*4 expression is still expanded aborally, but signal intensity is less pronounced than at lower concentrations (Supplementary Fig. 3K and L). This may reflect a threshold-dependent mechanism by which *Nvwnt*4 regulates its own expression, or could also indicate that more orally localized tissues acquire a slightly distinct molecular fate at higher alsterpaullone concentrations. We are unable to determine the precise mechanism for this behavior at this time. *Nvsp*5, a mediator of wnt signaling in bilaterian animals (Takahashi et al., 2005), is similarly expanded following alsterpaullone treatment (Fig. 4D and D″). Markers of pharyngeal tissue which lie internal to the blastopore, including *NvFoxA* and *NvBrachyury* are expanded throughout the larval epithelia after activation of canonical wnt signaling. At lower concentrations of the less potent activator, azakenpaullone, the expression of oral markers is most highly upregulated at the oral and aboral pole with a smaller number of cells in the intermediate tissue and at higher concentrations is expanded throughout the ectoderm (Supplementary Fig. 4A and B). *Nvotx* expression, in contrast, is found at the margin of the blastopore in wildtype embryos at this stage but expands to a large region of embryonic epithelial cells that does not include the tissue directly surrounding the blastopore (Supplementary Fig. 4C). Conversely, *Nvsix*3/6, a marker of the aboral plate, is lost following treatment (Fig. 4E and E″). The change in spatial distribution of these molecular territories, as well as a loss of the apical tuft and apical plate marker *Nvsix*3/6 indicates that an oralization of *Nematostella* embryos has occurred as a result of wnt agonism (Fig. 4F and G).

We also tested the effect of wnt inhibition on the development of oral and aboral territories in post-gastrula stage embryos with the small molecule wnt inhibitor iCRT14 (Supplementary Fig. 5). In contrast to embryos treated with the wnt agonists alsterpaullone and azakenpaullone, these embryos displayed phenotypes consistent with an expansion of aboral fate and a restriction of oral fate as assayed by *in situ* hybridization to transcripts expressed in the epithelium. Specifically, *Nvwnt*4 mRNA expression signal, as detected by *in situ* hybridization, was greatly diminished (Supplementary Fig. 5C and D). Conversely, the apical plate marker *Nvanthox*1 dramatically spatially expanded (Supplementary Fig. 5A and B). In vertebrates, interactions between the wnt pathway, *six*3 and *irx* mediate anterior neural patterning (REF?).

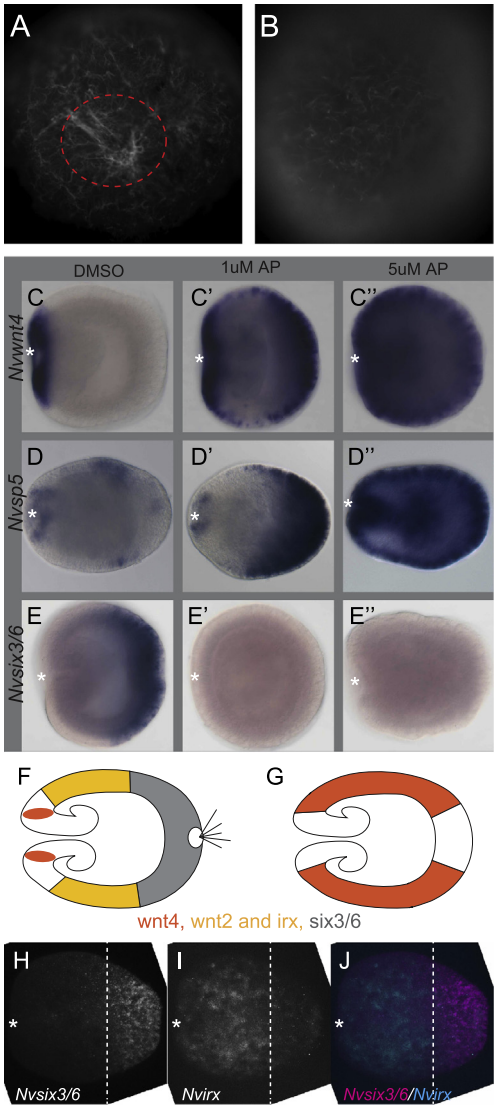

Fig. 4. (A) Aboral pole of a control embryo at 72 hpf stained with acetylated tubulin antibody in which the prominent cilia of the apical tuft (red dashed circle) are visible. (B) An embryo treated from 30 hpf to 72 hpf with 0.5 μM alsterpaullone in which the apical tuft is absent. Note the absence of prominent apical tuft cilia. (C)–(E″) *Nvwnt*4, *Nvsp*5, and *Nvsix*3/6 in control DMSO-treated embryos ((C)–(E)) and those treated from 30 hpf to 48 hpf with alsterpaullone at 1 μM (C′–E′) and 5 μM (C″–E″). (C) *Nvwnt*4 is expressed around the future site of the mouth (the blastopore) in control embryos. (C′) In embryos treated with alsterpaullone, *Nvwnt*4 expands to include an aboral expression domain in 1 μM treatments, and covers the entire epithelium in 5 μM treatments (C″). (D) *Nvsp*5 is found in an oral and aboral domain in control embryos and expands considerably in the aboral pole of 1 μM treated embryos (D′) and covers the entire epithelium in 5 μM treated embryos (D″). E. *Nvsix*3/6 is localized to the aboral pole of wildtype embryos. Treatment of embryos with alsterpaullone results in a complete loss of *Nvsix*3/6 expression at both 1 μM (E′) and 5 μM (E″). (F)–(G) Spatial relationships between *Nvwnt*2 and *Nvwnt*4 ligand expression and *Nvsix*3/6 and Nvirx expression. (H)–(J) The dashed vertical line is placed in the same position in each image to illustrate the interface between *Nvsix*3/6 and *Nvirx* staining. (H) Expression of *Nvsix*3/6 as assayed by *in situ* hybridization and imaged using fluorescent signal of FastRed substrate. (I) Expression of *Nvirx* as assayed by *in situ* hybridization and imaged using reflection microscopy. (J) Overlay of *Nvsix*3/6 and *Nvirx* fluorescent signal in an embryo subjected to double *in situ* hybridization. (C)–(E), (C′)–(E′), (C″)–(E″), and (F) and (G) are lateral views with the oral pole (asterisk) to the left and the apical tuft to the right. (D),(E), (H) and (I) are aboral views looking down on the apical tuft (arrow).

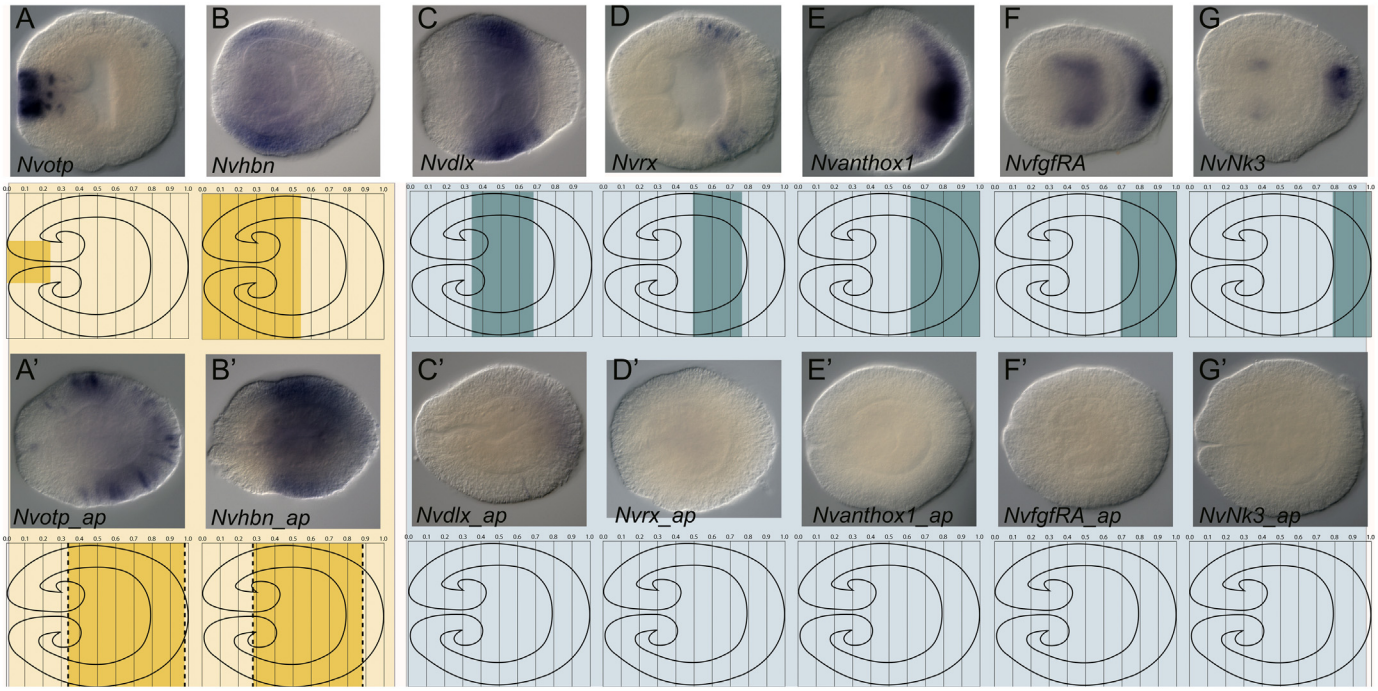

Fig. 5. Transformation of epithelial identity following activation of canonical wnt signaling with alsterpaullone. (A) and (B) Markers of oral ectoderm, *Nvotp* and *Nvhbn*, in DMSO treated embryos (upper panels) and in alsterpaullone treated embryos (lower panels). Graphs of *N. vectensis* embryos below photos indicate the average expression domain boundaries calculated from 10 measured embryos. (A′) and (B′). *Nvotp* and *Nvhbn* are greatly expanded toward aboral territories in alsterpaullone treated embryos. (C)–(G) Aboral territories marked by expression of *Nvdlx*, *Nvrx*, *Nvanthox1*, *NvfgfrA* and *NvNK*3 in DMSO treated control embryos. (D)–(G′). Markers of aboral epithelial molecular identity are lost following alsterpaullone treatment.

While expression of *Nvsp*5 is highly dynamic, expression of *Nvsix*3/6 and *Nvirx* are stable across embryonic development in *N. vectensis*. We also show by *in situ* hybridization that the more oral territory of *Nvirx* expression is directly abutting, but not overlapping with *Nvsix*3/6 expression at the aboral pole (Fig. 4H and J). This spatial relationship further suggests that conserved interactions between *Nvsix*3/6 and *Nvirx* may also control boundary formation in *N. vectensis*. Further work will be needed to test this scenario.

As both oral and aboral gene expression were affected by the manipulation of wnt signaling, we endeavored to assay the effect of wnt signaling of the global specification of the larval epithelium along the entirety of the larval axis. Utilizing the epidermal molecular identities described above as a proxy for epithelial identity along the oral–aboral axis, we assessed the role of canonical wnt signaling in epithelial specification in developing planula larvae. We quantified the spatial expression of mRNA as assayed by *in situ* hybridization detection of these markers in control treatments (0.5% DMSO) at 48 hpf, which allowed us to precisely define the molecular topography of these epidermal territories (Supplementary Fig. 6). We further confirmed the spatial relationships derived from the measurements of epithelial territories for a subset of these markers in planula stages (Supplementary Fig. 7). After defining the molecular topography of the epithelium, we tested the effect of alsterpaullone treatment on the molecular patterning of the epithelium. We find that pharmacological activation of canonical wnt signaling using alsterpaullone results in the absence of aboral gene expression and the expansion of oral markers across the larval epithelium (Fig. 5). Both 1 and 5 μM concentrations of alsterpaullone produced the most marked changes in epidermal patterning and were used for subsequent manipulations (Supplementary Fig. 3), with lower concentrations producing less severe shifts in molecular boundaries in a dose-dependent fashion. Azakenpaullone produced nearly identical phenotypes in drug treated embryos, but required higher concentrations for similar effects to be observed than with alsterpaullone treatments (Supplementary Fig. 3). The *Nvotp* and *Nvhbn* transcription factors shift toward the oral pole, consistent with an oralization of ectodermal territories (Fig. 5A and B). Specifically, we find an expansion of oral ectodermal domains (Fig. 5A and B) that originate in epithelium oral to the *Nvwnt*2 expression boundary, and a complete loss of aboral and medial molecular domains (Fig. 5C and G). We find that the shift in expression domain is dose-dependent, with higher concentrations of inhibitors producing more pronounced changes in molecular topography (Supplementary Fig. 3). Changes in epithelial molecular identity of these regions, as assayed by *in situ* hybridization to mRNA transcripts, are reversible by washout of the alsterpaullone treatment when embryos are allowed to recover for 24 h in the absence of the drug (Fig. 6). By comparing embryos that have undergone alsterpaullone treatment from 30 hpf to 72 hpf, *versus* those which have undergone treatment from 30 h to 48 h and which were then washed out of the alsterpaullone treatment, we see that expression of oral markers are completely expanded, while aboral ones are lost. However, only in washouts is a wildtype pattern of expression recovered. It should also be noted that additional incubation time in the inhibitor does not result in a more severe molecular topography shift, as embryos treated from 30 h to 48 h only show a similar loss of aboral markers and expansion of oral markers (refer to Fig. 5). We detect no change in overall embryo length suggesting that these changes in expression represent a transformation of ectodermal identity. These changes are also not likely to represent developmental delay as we find these domains to begin expression of these markers early in development before the initiation of alsterpaullone treatment (prior to 30 hpf) and to remain relatively constant over the course of planula development as shown above (refer to Fig. 2).

To determine the consequences of canonical wnt activation via the GSK3β antagonist alsterpaullone and the associated shift in molecular epithelial identity on the complement of differentiated cell types in planula larvae we treated embryos with alsterpaullone following gastrulation up until 72 h of development. These embryos were fixed,

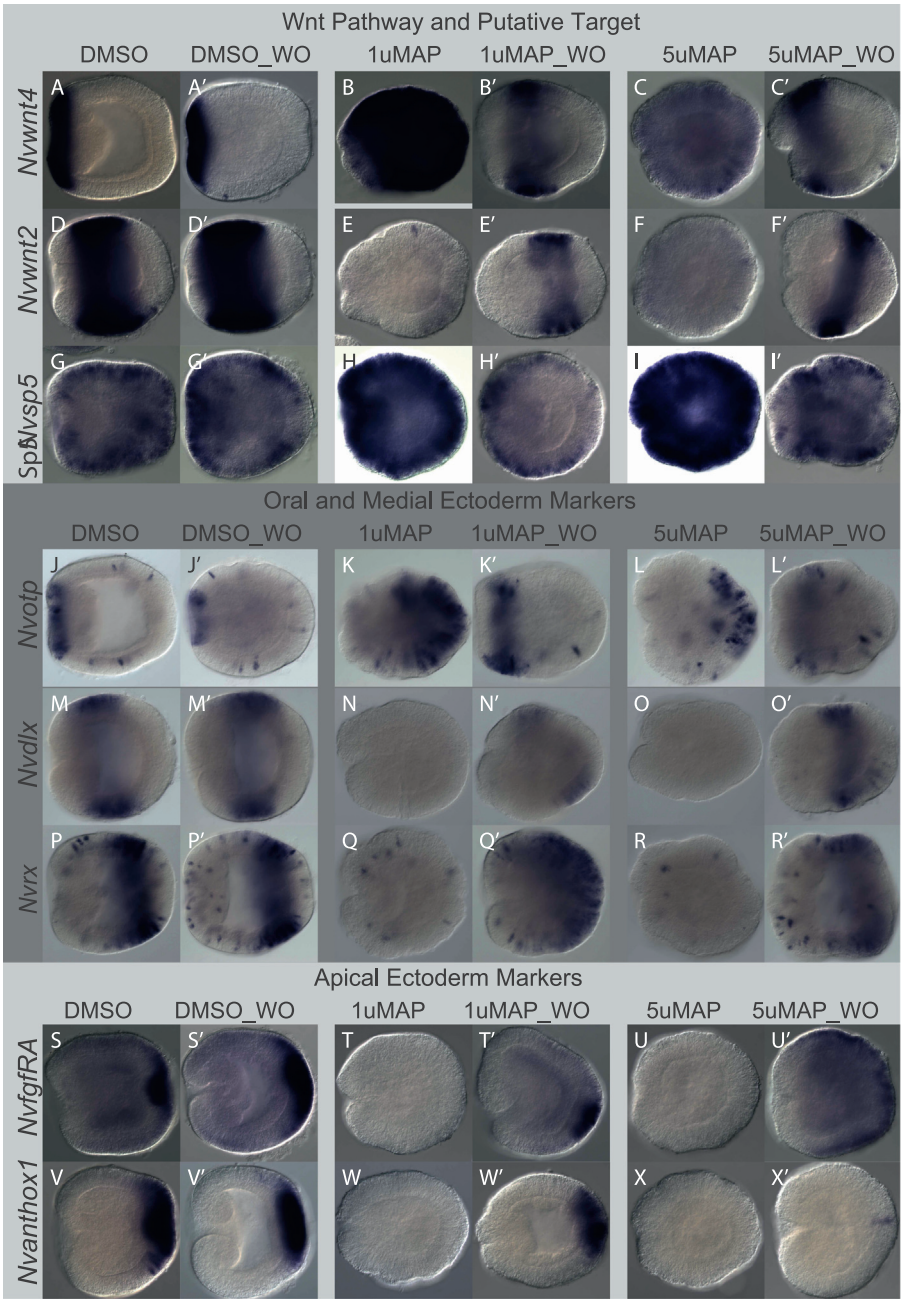

Fig. 6. Recovery of epithelial mRNA transcript expression in alsterpaullone-treated embryos following washout, as scored by *in situ* hybridization. All views are lateral, optical cross-sections with the oral pole to the left. (A)–(C′) Expression of *Nvwnt4*. (D)–(F′)– Expression of *Nvwnt2*. (G)–(I′–. Expression of *Nvsp5*. (J)–(L′– Expression of *Nvotp*. (M)–(O′– Expression of *Nvdlx*. (P)–(R′) Expression of *Nvrx*. (S)–(U′)– Expression of *NvfgfRA*. (V)–(X′) Expression of *Nvanthox1*. (A), (D), (G), (J), (M), (P), (S), and (V) DMSO-treated control embryos incubated in 0.5% DMSO from 30 hpf to 72 hpf. (A′), (D′), (G′), (J′), (M′), (P′), (S′) and (V′). Embryos treated from 30 hpf to 48 hpf in 0.5% DMSO and then washed into a new DMSO control treatment until 72 hpf (washout control). (B), (E), (H), (K), (N), (Q), (T) and (W). Embryos treated with 1 μM alsterpaullone from 30 hpf to 72 hpf. (B′), (E′) , (H′) , (K′) , (N′) , (Q′) , (T′) and (W′a. Embryos treated from 30 hpf to 48 hpf in 1 μM alsterpaullone and then washed out into DMSO only until 72 hpf. (C), (F), (I), (L), (O), (R), (U), and (X) Embryos treated with 5 μM alsterpaullone from hpf 30 to 72 hpf. (C′) (F′). (I′) (L′) (O′) (R′), (U′), and (X′) Embryos treated from 30 hpf to 48 hpf in 5 μM alsterpaullone and then washed out into DMSO only until 72 hpf.

and two different transcripts indicative of differentiated neurons were detected by *in situ* hybridization, *NvAntho-rfamide* and an opsin, *Nv-op*85309. In wildtype embryos, opsin-expressing cells are primarily localized to the aboral pole of the larva (Fig. 7A), while *Nvantho-rfamide* is expressed in epithelial neurons in the planula (Marlow et al., 2009) which are primarily localized to the oral pole by 72 hpf (Fig. 7B). Following treatment with alsterpaullone, we observed an aboral shift in expression of *Nv-op*85309, with nearly all opsin expressing cells being lost in 5 μM alsterpaullone treatments (Fig. 7A). We also saw a dose dependent expansion in the expression of *Nvantho-rfamide* in the planula epithelium, a marker found throughout the larval epithelium with a bias toward expression at the oral pole (Fig. 7B). These results demonstrate that a change in the ectodermal cell complement expressing these transcripts indicative of distinct subsets of differentiated neurons is altered following alsterpaullone treatment.

Washout experiments in which embryos were allowed to recover from 48 hpf to 72 hpf show that a primarily wildtype differentiated cell complement returns at 1 μM concentrations and to a slightly lesser extent at 5 μM alsterpaullone treatments. These washouts indicate that just as maintenance of epithelial identity is

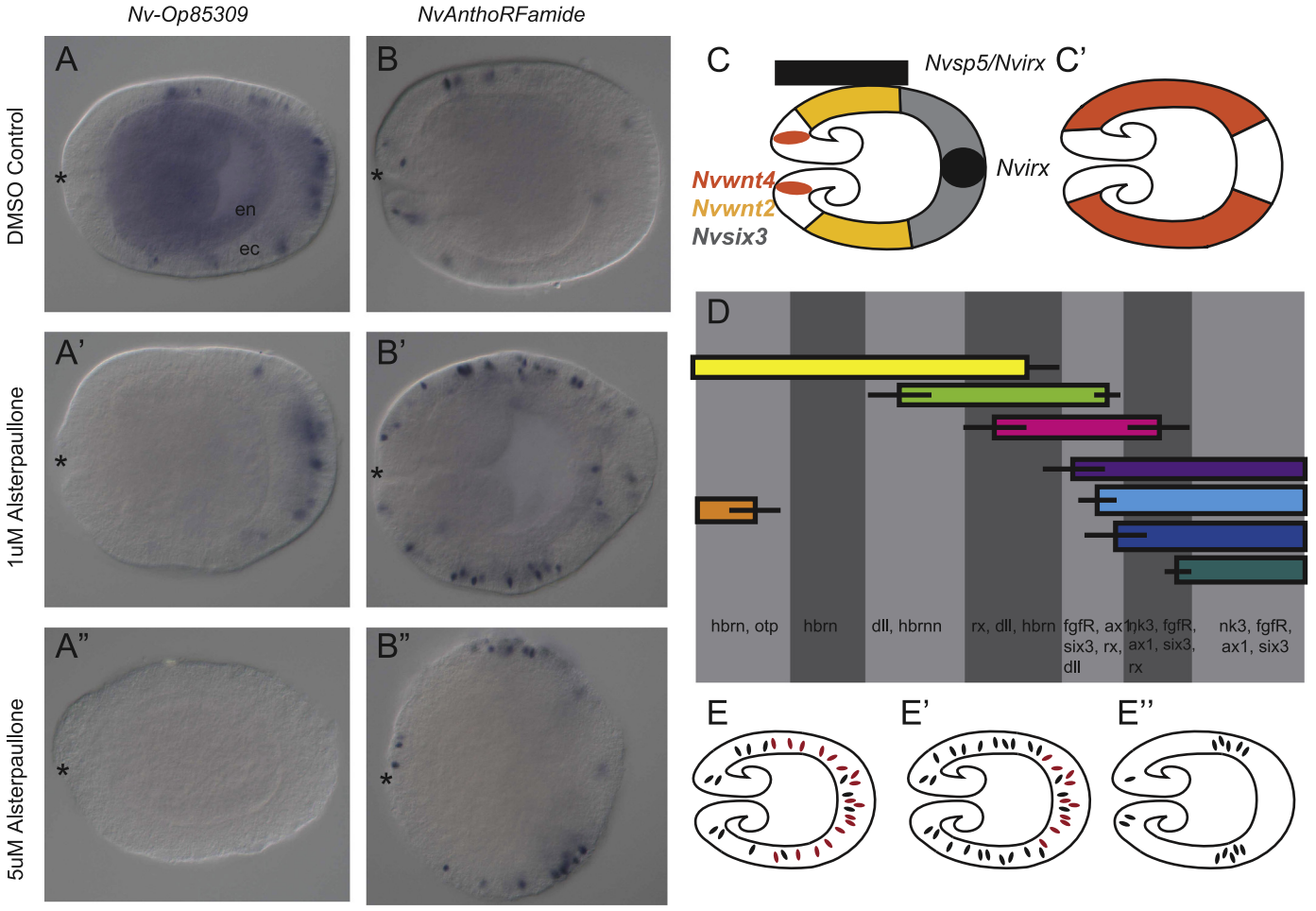

Fig. 7. Effects of canonical wnt activation via alsterpaullone treatment on epithelial patterning and later formation of neural cell types. (A)–(A′) *Nv-op85309* expression restricts to the aboral pole after incubation with low alsterpaullone concentrations (A′) compared to DMSO-treated controls (A) and is absent in high alsterpaullone concentrations (A″). Light staining in the endoderm (en) of the DMSO control embryo is background staining. Darkly staining cells in the ectoderm (ec) is specific signal. (B) *NvanthoRFamide* is expressed in oral neurons and a few scattered aboral neurons. (B′e Following treatment with alsterpaullone, *anthoRFamide* neurons are expressed throughout the larval epithelium in an approximately even distribution. (B″) At high concentrations, *NvanthoRFamide* neurons are found primarily at the aboral pole. (C)–(C′)– Summary of wnt pathway components expressed in wildtype planula larvae (C) and those treated with alsterpaullone (C′). The ectodermally expressed wnt ligands, *Nvwnt4* (orange) and *Nvwnt2* (yellow), the transcription factors *Nvsix*3/6 and *Nvirx* are indicated. (D) Epithelial patterning from the oral pole to the apical tuft of 48 hpf embryos. Genes are indicated by colored bars and standard deviations of measured embryos are indicated with black lines. Regions indicating predicted unique identity based on novel combinatorial overlap of expression of molecular markers assayed by *in situ* hybridization are indicated by alternating light and dark gray bars. See methods for description of measurements. (E)–(E″)– A summary of the distribution of opsin-bearing (red) and *anthoRFamide*-bearing (black) cells in control embryos and (E) and embryos treated with 1 uM (E′) as well as 5 uM (E″) concentrtions of alsterpaullone at 72 hpf. Views of all embryos are lateral with the oral pole to the left.

dynamically maintained by wnt signaling, the differentiation of cell types is also subject to active regulation by wnt signaling (Supplementary Fig. 8).

Discussion

These findings, in combination with previous studies which have centered on the role of stabilized β-catenin at the blastopore in early *N. vectensis* embryos (Wikramanayake et al., 2003) indicate at least two ancient roles for the canonical wnt signaling pathway in the establishment of early embryonic territories in anthozoan cnidarians. First, an early embryonic polarity and gastrulation role of wnt and β-catenin have been demonstrated in both hydrozoan and anthozoan developmental systems. A maternally localized wnt ligand has not been identified in anthozoan cnidarians, however, an early developmental role of wnt in hydrozoan cnidarians has been described in the establishment of the embryonic axis through the localization of maternally deposited wnt signaling pathway components, in the hydrozoans *Clytia* and *Hydractinia* (Lengfeld et al., 2009; Momose et al., 2008; Momose and Houliston, 2007). In anthozoans, blastoporal β-catenin stabilization specifies future embryonic endoderm and establishes global embryonic polarity along the animal–vegetal (oral–aboral) axis (Kumburegama et al., 2011; Wikramanayake et al., 2003).

As we provide evidence for here, the wnt signaling pathway also appears to have a second role throughout planula development in anthozoan cnidarians in the establishment of an oral signaling center which acts to specify the identity of planula body wall epithelia. The establishment of wnt-responsive ectodermal boundaries, which may be mediated by *Nvsp*5 and *Nvirx* transcription factors at the oral pole that abut expression of *Nvsix3/6*, a vertebrate wnt antagonist (Lagutin et al., 2003), at the aboral pole may help define these oral and aboral ectodermal identities. In support of this hypothesis, activation of the canonical wnt signaling pathway leads to an expansion of at least one wnt expression domain (*Nvwnt4*) (4C–C″), an expansion in *Nvsp*5 expression (4D–D″) and a reduction of *Nvsix*3/6 expression (Fig. 4E–E″). Additionally, washout experiments presented here demonstrate that nearly

wildtype expression patterns can be regained by the washout of the alsterpaullone treatment (Fig. 6). This indicates that wnt molecules themselves, putative wnt targets, and the molecular identity of the epithelium are dynamically influenced by wnt ligand levels. Two putative *Nematostella* wnt receptors, *Nvfz*5 and *Nvfz*10 have recently been described (Kumburegama et al., 2011). *Nvfz5*, which shows broad expression across the apical plate is a candidate receptor of the wnt signal responsible for mediating epithelial patterning. Furthermore, co-expression of *Nvwnt4*, *Nvirx*, and *Nvsp*5 to the spatial exclusion of *Nvsix*3/6 are similar to observations in both *Drosophila* and vertebrate anterior CNS structures and could indicate a conserved set of regulatory interactions in neural epithelial patterning. Recently, a functional study tested the effect of knockdown of *Nvsix*3/6 on *Nvwnt*2 and found that a knockdown of *Nvsix*3/6 resulted in an expansion of *Nvwnt*2 and a change in fate of the newly *Nvwnt*2 expressing cell population to oral identity (Sinigaglia et al., 2013). Additional work on the role of other wnt ligands, such as *Nvwnt4*, in conferring aboral and oral identity as well as the role of specific wnts, including *Nvwnt2*, on the regulation of the transcription factors defining oral–aboral position and cell fate need to be conducted in *Nematostella. N. vectensis* oral–aboral epithelial identity is established early in development, as assayed through *in situ* hybridization to many developmental transcription factors. These factors demarcate distinct molecular territories, defined by molecular co-expression domains, and additional markers might reveal even more complex patterning along this axis (Fig. 7D).

An apical tuft structure forms at the aboral pole, opposite the blastopore, in the developing planula larva (Rentzsch et al., 2008). In *Nematostella*, we and others have shown that the apical tuft region is molecularly heterogeneous (Sinigaglia et al., 2013). The apical tuft is a prominent structure with a large number of cells (approximately 50 to 100) and it is conceivable that some cells within the tuft have varying functions. The morphological formation of the ciliary tuft and the maintenance of the expression of localized transcripts that define the epithelia of the apical plate are mediated by activity of the canonical wnt singaling pathway, as determined here by alterations in wnt signaling achieved via alsterpaullone treatments. The molecular heterogeneity observed in the apical tuft might be related to the later development of different classes of sensory neurons.

The oral–aboral axis of *Nematostella* is specifically demarcated by discrete, nested sets of transcription factor transcript expression from the oral opening to the aboral tip of the larva, the apical tuft (Marlow et al., 2009)(Fig. 7D). Using these regional molecular markers as a proxy for epidermal identity we show that ectopic activation of wnt signaling through the addition of the pharmacological agonists alsterpaullone and azakenpaullone results in a significant oralization of the planula epithelium, while wnt antagonists have the opposite effect. Blastoporal markers are strongly upregulated to cover the entire larval epithelium while oral neural markers are expanded aborally, with the exception of the most blastoporal region of the epithelium. This indicates that the majority of ectodermal epitheial tissues take on a blastoporal identity, while the most aboral regions take on an identity coreesponding to both neural and blastoporal (Fig. 2 and Supplementary Fig. 4). This is consistent with previous reports of oralizing wnt activity during *Nematostella* regeneration (Trevino et al., 2011) and the hypothesis that an oral wnt signaling effects epithelial identity during early development. Additionally, by employing markers of differentiated neurons to assay the phenotypic output of this epithelial patterning, we show that ectopic activation of the canonical wnt signaling pathway using the pharmacological GSK3β inhibitors alsterpaullone and azakenpaullone has the capacity to interfere with the later development of cellular identity of the epithelium (Fig. 7E–E″). Given the widespread role of wnt signaling in patterning vertebrate and invertebrate neural elements and its effect on *N. vectensis* neural transcription factor expression and neural epithelial fate, we hypothesize a role for wnt signaling in neural epithelial patterning in the Eumetazoan ancestor.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.05.022.

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