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ORIGINAL ARTICLE

Embryonic development and metamorphosis of the scyphozoan *Aurelia*

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Abstract We investigated the development of *Aurelia* (Cnidaria, Scyphozoa) during embryogenesis and metamorphosis into a polyp, using antibody markers combined with confocal and transmission electron microscopy. Early embryos form actively proliferating coeloblastulae. Invagination is observed during gastrulation. In the planula, (1) the ectoderm is pseudostratified with densely packed nuclei arranged in a superficial and a deep stratum, (2) the aboral pole consists of elongated ectodermal cells with basally located nuclei forming an apical organ, which is previously only known from anthozoan planulae, (3) endodermal cells are large and highly vacuolated, and (4) FMRFamide-immunoreactive nerve cells are found exclusively in the ectoderm of the aboral region. During metamorphosis into a polyp, cells in the planula endoderm, but not in the ectoderm, become strongly caspase 3 immunoreactive, suggesting that the planula endoderm, in part or in its entirety, undergoes apoptosis during metamorphosis. The polyp endoderm seems to be derived from the planula ectoderm in *Aurelia*, implicating the occurrence of "secondary" gastrulation during early metamorphosis.

Keywords Cnidaria · Scyphozoa · Embryogenesis · Planula · Polyp

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Introduction

Cnidarians have been often considered diploblastic, radially symmetrical animals that include species of jellyfish, corals, and sea anemones (Brusca and Brusca 2003; but see Martindale 2004 for newly emerging views of "basal" metazoans). The recent molecular phylogenetic studies favor the view that cnidarians form a sister group to bilaterally symmetrical animals (i.e., bilaterians) within Metazoa (Medina et al. 2001; Wallberg et al. 2004). Consequently, understanding the evolution of development in Cnidaria is crucial in inferring the ancestral condition of development from which a diversity of bilaterian developmental patterns evolved. So far, several cnidarian developmental model systems have been established, including the anthozoan sea anemone *Nematostella vectensis* (Darling et al. 2005), the coral *Acropora millepora* (Miller and Ball 2000), the hydrozoans *Hydra* (Steele 2002), *Hydractinia echinata* (Frank et al. 2001), *Podocoryne carnea* (Galliot and Schmid 2002), and the cubozoan *Tripedalia cystophora* (Piatigorsky and Kozmik 2004). Much effort has been directed towards understanding the molecular mechanisms of early development in order to gain insights into, in particular, the origin of axial patterning in Metazoa (de Jong et al. 2006; Finnerty et al. 2004; Matus et al. 2006; Rentzsch et al. 2006; Wikramanayake et al. 2003; Yanze et al. 2001). However, establishing the ancestral condition of cnidarian–bilaterian axial patterning has proven contentious due to a high degree

of difference in expression patterns of homologous genes within Cnidaria (Ball et al. 2007; Kamm et al. 2006; Rentzsch et al. 2006). Divergence in gene expression patterns within Cnidaria is perhaps expected, as cnidarians have undergone 600+ million years of evolution since they split from the bilaterian lineage (Chen et al. 2002). This necessitates the inclusion of additional phylogenetically informative cnidarian taxa to the list of developmental model systems, in order to achieve a higher resolution in reconstructing the evolutionary history of development in Cnidaria. To date, examination of scyphozoan development by molecular tools has been limited relative to the other major cnidarian classes—Anthozoa, Hydrozoa, and Cubozoa. We thus examined the embryonic development and metamorphosis of the scyphozoan jellyfish *Aurelia*.

Cnidarians show highly variable cleavage patterns that result in two common forms of blastulae: a hollow coeloblastula (Martin et al. 1997) or a solid stereoblastula (Martin 1987). Gastrulation has been reported to take on various forms such as invagination (Magie et al. 2007), unipolar ingression (Freeman 1981), multipolar ingression (Okada 1927), delamination (Chia and Crawford 1973), or epiboly (Freeman 1983). However, at least some of the inferred heterogeneity of gastrulation mode of cnidarians may be due to misidentification of yolk cells, which move into the blastocoel and become phagocytosed by bona fide endodermal cells during gastrulation, as ingressing or delaminating endodermal cells (Marlow and Martindale 2007).

Following gastrulation, cells differentiate, and ciliated cylindrical planula larvae emerge. Planulae may be planktotrophic or lecithotrophic depending on the species (Widersten 1968, 1973). Environmental cues trigger a planula to settle on the surface of appropriate substrates at its aboral end (Leitz 1997; Muller et al. 1976). The ectoderm at the aboral end of some anthozoan planulae contains an apical organ, which consists of a cluster of long columnar (sensory receptor) cells surrounded by supporting cells with a bundle of cilia that form an apical tuft (Chia and Koss 1979). This putative sensory organ has been suspected to be the site of sensory perception of chemical signals for settlement (Chia and Koss 1979). Most planula larvae then metamorphose into polyps by formation of the mouth–anus ("oral") opening at the site of the original blastopore, followed by the development of tentacles through evagination of body wall ectoderm and endoderm around the oral opening. The aboral end develops into the foot of a polyp during metamorphosis.

Metamorphosis into a polyp is a highly complex, drastic transformation in hydrozoan cnidarians, involving cell differentiation, migration, proliferation, apoptosis, and nervous system reorganization. In *Mitrocomella polydiademata*, the metamorphosis is accompanied by the differentiation of polyp-specific cell types such as "perisarc" cells and a subset of "glandular" cells (Martin et al. 1983). Interstitial cells (or "I cells"), which are believed to be pluripotent stem cells of Cnidaria (Brusca and Brusca 2003), migrate from the endoderm to ectoderm during metamorphosis in *H. echinata* (Weis and Buss 1987). Also in metamorphosing *H. echinata*, the proliferative activity of cells, as indicated by the numbers of S-phase nuclei, increases drastically (Plickert et al. 1988). In addition, caspase-dependent apoptosis in ectodermal cells of *H. echinata* planulae is necessary for metamorphosis to occur (Seipp et al. 2006). Furthermore, hydrozoan planulae reorganize the nervous system through a loss of RFamide- and GLWamide-immunoreactive neurons during metamorphosis (Martin 2000; Plickert 1989; Schmich et al. 1998). However, as the process of metamorphosis into a polyp in nonhydrozoan cnidarians has not been investigated in comparable detail, it is unclear whether the above information represents general characteristics of cnidarian metamorphosis.

Relatively little is known about embryogenesis and metamorphosis in Scyphozoa. The scyphozoan genus *Aurelia*, commonly known as the moon jelly, includes many species that are distinguished by morphology (Gershwin 2001; Russell 1970), mitochondrial sequence, and timing of strobilation (Dawson and Jacobs 2001; Schroth et al. 2002). *Aurelia* species are reported to undergo gastrulation by multipolar ingression (Mergner 1971), invagination (Fioroni 1979), or mixture of both (Hyde 1894). No apical organ, apical tuft, or interstitial cells have so far been identified in the planula of *Aurelia* (Martin and Koss 2002; Widersten 1968). Here, we examine the embryogenesis and metamorphosis of *Aurelia* using molecular markers combined with confocal and transmission electron microscopy (TEM). The development and reorganization of the FMRFamide-immunoreactive nervous system during metamorphosis from a planula into a polyp is reported in an accompanying paper (Nakanishi et al. 2008, this issue). Our analysis provides a framework for future gene expression and experimental studies in scyphozoans.

Materials and methods

Animals

The embryos and planula larvae were collected from oral arms of adult *Aurelia sp.1* (*sensu* Dawson and Jacobs 2001; *Aurelia aurita sensu* Schroth et al. 2002) medusae at the Cabrillo aquarium in San Pedro, CA, USA. We note that *Aurelia sp.1* medusae have been housed with *Aurelia labiata* medusae, and it may be possible that some of the specimens are F1 hybrids between *A. sp.1* and *A. labiata*, although we have found no evidence to support this. Planulae recovered from the oral arms of *A. sp.1* were allowed to settle and

develop in a bottle dish for a week in order to collect metamorphosing planulae and primary polyps.

Immunohistochemistry and confocal microscopy

Polyps were anesthetized in 7.3% $MgCl_2$. All specimens were fixed in 4% formaldehyde for 1 h at room temperature (20–25°C). Some specimens were dehydrated in an ethanol series (30%, 50%, 80%, and 100% EtOH) and were preserved in 100% methanol. These specimens were rehydrated in an ethanol series before the following immunostaining procedure. The fixed specimens were washed in PBSTr (PBS+ 0.3% Triton-X100) for 2 h and blocked by 3% NGS in PBSTr for 1 h at room temperature (20–25°C). Primary antibodies were incubated with the specimens in PBSTr overnight at 4°C. Primary antibodies that were used for this study were antibodies against FMRFamide ("FMRF"; rabbit, 1:500 dilution, US Biological), phosphorylated histone H3 ("pH3"; rabbit, 1:1,000,), caspase 3 ("Casp"; rabbit, 1:200), GnRH (rabbit, 1:500, Sigma), acetylated tubulin ("acTub"; mouse, 1:1,000, Sigma), and tyrosinated tubulin ("tyrTub"; mouse, 1:800, Sigma). Following washes in PBSTr for 2 h nd blocking in 3% NGS in PBSTr for 1 h at room temperature (20–25°C), secondary antibodies were incubated with the specimens overnight at 4°C. Secondary antibodies that were used for this study were AlexaFluor 568 (rabbit. 1:200, Molecular Probes), AlexaFluor 488 (mouse, 1:200, Molecular Probes), Cy3 (rabbit, 1:500, Jackson Laboratory), and Cy5 (rabbit, 1:200, Jackson Laboratory). Nuclei were labeled using fluorescent dyes Sytox ("Syt"; 1:2,000, Molecular Probes); filamentous actin was labeled using rhodamine phalloidin (1:25) or phalloidin conjugated to AlexaFluor 568. Specimens were incubated with fluorescent dyes together with secondary antibodies overnight at 4°C. The specimens were washed in PBSTr for 2 h at room temperature (20–25°C) and were mounted in Vectashield (Vector Laboratories Inc.). Preparations were then viewed and their images were recorded using a Biorad Model MRC1024ES Confocal Microscope and Laser Sharp version 3.2 software. The confocal stacks were viewed using the program ImageJ.

To provide a reasonable estimate of cell numbers, we made the assumption that the embryo until the postgastrula stage has the shape of a sphere and the planula that of a cylinder. With the help of the "measure" Plug-in of ImageJ, we then counted for five specimens the number of Sytox-positive nuclei in a measured part of the embryonic or planula surface and projected the number to the entire surface.

Transmission electron microscopy

Two mature planulae were fixed for TEM in 4% paraformaldehyde and 2.5% glutaraldehyde for 24 h at 4°C. They were then stained with 1% osmium tetroxide for 1 h at 4°C. Following acetone dehydration series (50%, 70%, 96%, and 100% acetone), specimens were transitioned to increasing concentrations of epon (1:3 epon–acetone, 2:2, 3:1, followed by overnight evaporation to 100%) and then transferred to flexible plastic molds for polymerization at 60°C for 16 h. Transverse serial sections were made on one of the specimens by the Microscopic Techniques Laboratory at UCLA. Sections were examined using the JEOL 100CX transmission electron microscope at the Electron Microscope Laboratory of the UCLA Brain Research Institute.

Results

Aurelia embryos undergo equal cleavage, resulting in a coeloblastula of columnar epithelial cells surrounding a small central blastocoel (Fig. 1a–f). During the blastula stage, cells do not have cilia; ciliation becomes apparent with the onset of gastrulation. Cytoplasmic microtubules radiate outward and basally from around apically located nucleus of each blastomere, forming a conspicuous cortical cytoskeleton (Fig. 1c–e). Cell nuclei are arranged in a single superficial layer underneath the apical membrane (Fig. 1f). Cell divisions are prevalent in the blastula; as observed in proliferatively active epithelia throughout the animal kingdom, dividing cells round up at the apical surface (Fig. 1g,h). As the number of cells increases, the shape of blastomeres changes from cuboidal to columnar (Fig. 1b,e).

Gastrulation begins at a time when the blastoderm contains approximately 700–1,000 cells. Cells on one side of the embryo invaginate (Fig. 2a,c,e) and form an inner epithelial layer, the primary endoderm, that surrounds a closed cleft-like central lumen (Fig. 2g,h). The blastopore is only transient and does not form a mouth opening in the planula. Prior to gastrulation, future endodermal cells cannot be recognized by their shape, size, or yolk content from other cells (Fig. 2b–f). During the course of gastrulation, ectodermal and endodermal cells alike start to produce cilia at their apical pole (Fig. 2b,i endoderm not shown). Cell division is still frequent during and after gastrulation in both germ layers (Fig. 2j). Thus, towards the end of gastrulation, there are an estimated 2,000–3,000 cells in the ectoderm, and 250–500 in the endoderm. In an elongated, motile planula (Fig. 3f), this number has increased to over 10,000 ectodermal cells and 1,000–2,000 endodermal cells, respectively. One noteworthy morphogenetic change that takes place during gastrulation is the pseudostratification of the ectoderm and, slightly later, the endoderm. Nuclei which had all formed a single stratum in the blastula now move to different positions (Fig. 2d,f,h). One can distinguish a sharp superficial layer of nuclei from a more loosely packed deep layer. It is possible that the cells that contain the deep nuclei are still

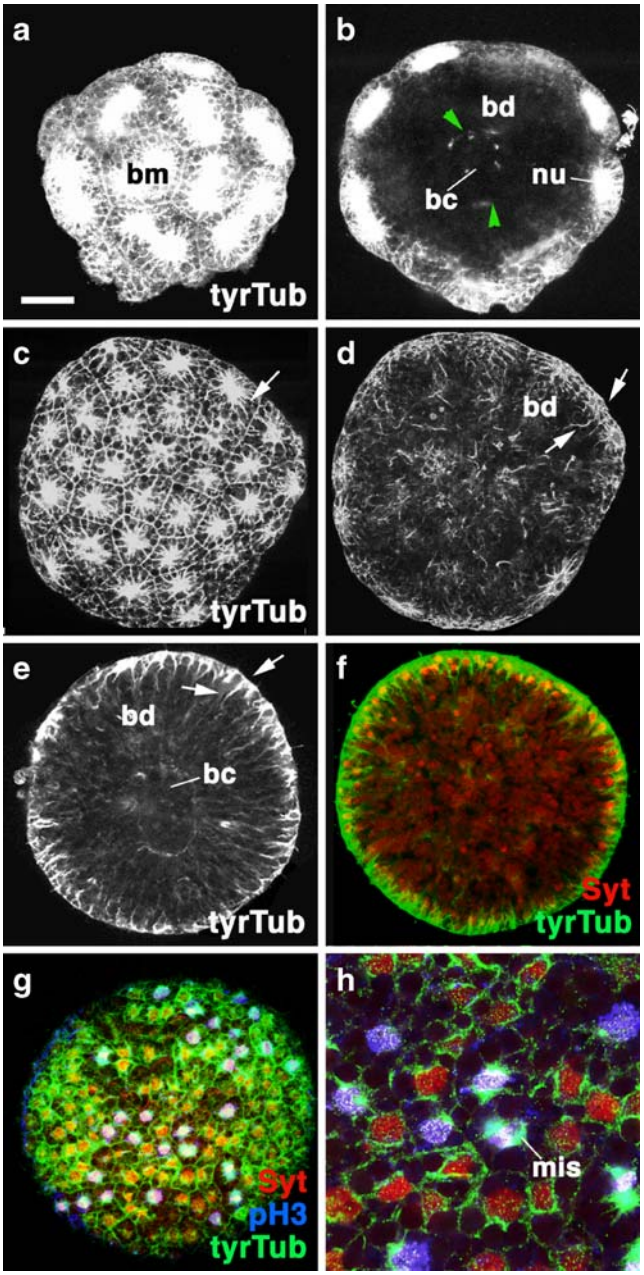


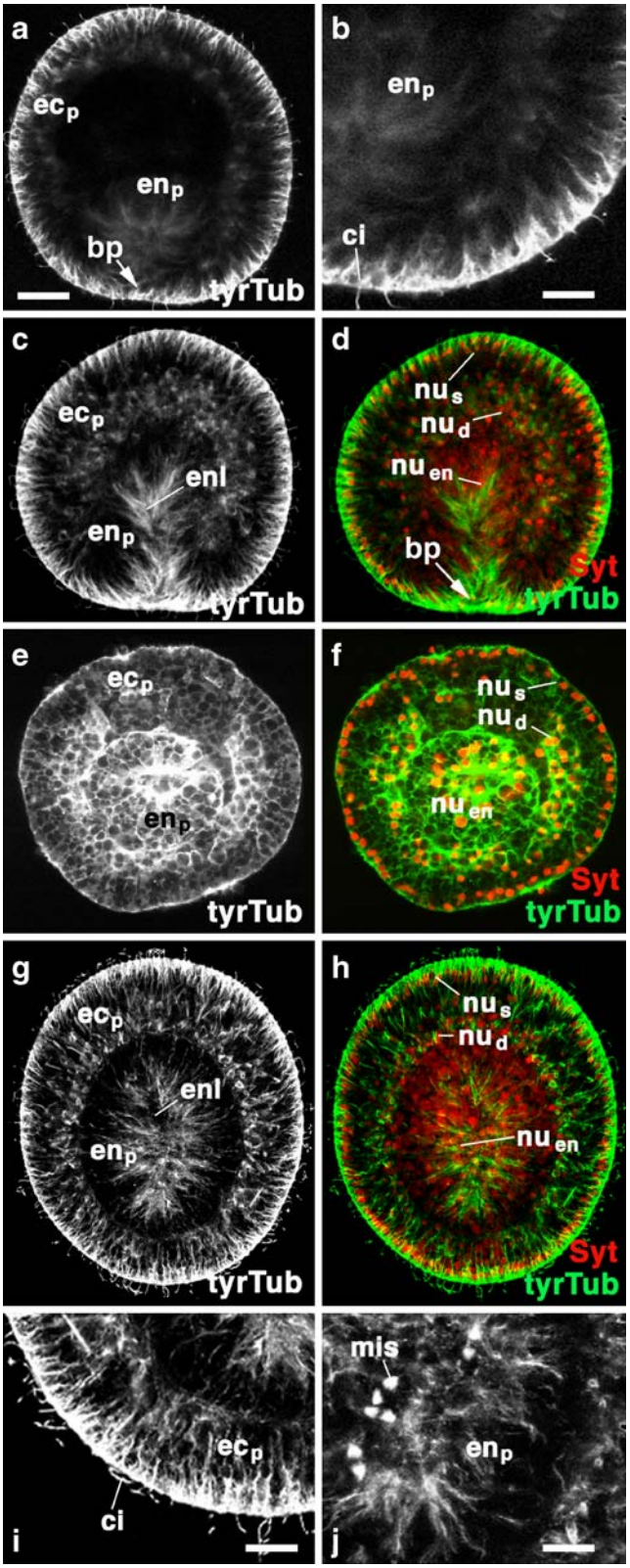
Fig. 1 Blastula stage. Confocal sections of *Aurelia* embryos labeled with the antibody against tyrosinated tubulin (*tyrTub*). In **f**–**h**, nuclei (*nu*) are labeled with Sytox (*Syt*). In **g** and **h**, mitotic nuclei are labeled with antibody against phosphorylated histone 3 (*pH3*). **a**, **b**: early blastula, surface view (**a**), equatorial section (through center of specimen; **b**); **c**, **d**: midstage blastula, surface view (**c**), equatorial section (**d**); **e**–**h**: late blastula, equatorial sections (**e**, **f**), surface view (**g**, **h**). Cytoplasmic microtubules radiate outward and basally from around apically located nucleus (*nu*) of each blastomere (*bm*), forming a conspicuous cortical cytoskeleton (*arrows* in **c**, **d**, **e**). *Green arrowheads* in **b** indicate basal membrane of blastomeres facing blastocoel (*bc*). In dividing cells, tyrTub visualizes mitotic spindle (*mis* in **h**). With increasing cell number, blastomeres change in shape from cuboidal to columnar (compare **b** and **e**). Nuclei form a single, apical layer throughout the blastula stage. Clusters of contiguous cells undergo mitosis (**g**, **h**). *Bar* 25 μm

Fig. 2 Gastrulation. Confocal sections of *Aurelia* embryos labeled with the antibody against tyrosinated tubulin (*tyrTub*; in all *panels*). In **d**, **f**, and **h**, nuclei are labeled with Sytox (*Syt*). **a**–**d** represent longitudinal sections through the center of specimens with blastopore (*bp*) at the bottom; **e**–**j** are transverse sections. **a**, **b**: early gastrula; **c**–**f**: midstage gastrula; **g**–**j**: late gastrula. **b** represents a magnified view of the embryo shown in **a**; **i** and **j** are magnified views of parts of an embryo shown in **g**. With the beginning of gastrulation cells at the oral pole invaginate through the blastopore to form the primary endoderm (*enp*). Nuclei of the ectoderm (*ecp*) have sorted out into a tightly packed superficial layer (*nus*) and a less dense deep layer (*nud*). Endodermal nuclei (*nuen*) are located apically, surrounding the lumen of the primary endoderm (*enl*). Ectodermal cells start to form cilia (*ci*). Mitotic figures (*mis*) are frequent in the endoderm (**j**) and ectoderm (not shown). *Bars* 25 μm (**a**, **c**–**h**); 10 μm (**b**, **h**, **j**)

epithelial cells with contact to the external surface, although the markers used in this study do not allow us to exclude the possibility that there exists a "middle layer" of delaminated cells in the gastrula and postgastrula embryo.

Planula stage

As in many other cnidarians, developing *Aurelia* embryos are not protected by surrounding membranes. Consequently, the transition from embryogenesis into a freely motile larval planula stage is gradual. The beginning of the planula stage can be defined as the time point at which individuals start moving unidirectionally. Early planulae are short and rounded (Fig. 3a–d); towards later stages, they become more elongated (Fig. 3e–j). The direction of movement and the apical organ, consisting of particularly elongated (sensory) ectodermal cells, define the anterior–aboral pole of the planula. The ectoderm is pseudostratified, with a superficial multilayer of densely packed nuclei and a deep layer of more widely spaced nuclei (Fig. 3f). This pattern is modified in the apical organ, where nuclei are less densely packed and are primarily basal in position (Fig. 3f). The ectoderm of the planula contains several types of specialized cells, including FMRFamide-immunoreactive neurons (Fig. 3g; Nakanishi et al. 2008, this issue) and GnRH-immunoreactive cnidocytes (Fig. 3h). The antibody against FMRFamide visualizes a subset of neurons in cnidarian nervous systems (Grimmelikhuijzen 1983). The GnRH antibody cross-reacts with an antigen present on nematocyst capsules; thus, using light and electron microscopic sections as a reference, we could unambiguously identify the GnRH-positive structures as nematocysts on the basis of their distribution, size, and shape. These cell types are not uniformly distributed. Neurons occur exclusively in the aboral half of the animal (Nakanishi et al. 2008, this issue); cnidocytes are concentrated posteriorly but are rare in the apical pole (Fig. 3h). Within a given area, neurons or cnidocytes are spaced quite regularly. For example, in regions of high density (oral pole), cnidocytes are separated from their nearest neighbor by about one ectodermal cell



diameter; in regions of low density, the distance between cnidocytes goes up to three to five ectodermal cell diameters.

The endoderm consists of large regularly stacked epithelial cells (Fig. 3i). Endodermal cells are highly vacuolated. When labeled with phalloidin (Fig. 3i), the cytoplasm of endodermal cells stands out due to their high F-actin content, and vacuoles are visible as dark (F-actin-negative) spherical areas against the bright (F-actin-positive) cytoplasm. The endodermal epithelium is ciliated at its apical surface that surrounds a closed cleft-like inner lumen (Fig. 3j).

Transmission electron microscopy adds a number of pertinent details to the structural characterization of the planula body. Cnidocytes (Fig. 4a,b) and gland cells (Fig. 4b,c) are integrated among the columnar epithelial cells of the ectoderm. Cnidocytes are easily recognized as the egg-shaped nematocysts displace the nuclei into a crescent shape in the basal portion of the cell. Most ectodermal cells have a protruding process of the apical membrane enclosing scant cytoplasm, as well as a central rootlet from which the cilium extends apically (Fig. 4d). A subset of ectodermal cells has a narrow apical neck and forms a collar-shaped protrusion around the central cilium (Fig. 4d). These cells could represent sensory neurons. All ectodermal cells, including cnidocytes, gland cells, and neurons, are surrounded by a pronounced apical junctional complex, consisting of a zonula adherens and septate junctions (Fig. 4d,e).

Basally, ectodermal cells are bounded by an electron-dense layer of extracellular matrix, the mesoglea (Fig. 4a). The mesoglea has a thickness of 20–50 nm and does not contain any cells or cellular processes. Neuronal processes (neurites) are enclosed within channels and gaps formed in between the basal necks of ectodermal cells (Fig. 4; see also Nakanishi et al. 2008, this issue). A conspicuous junctional complex forms around the basal surfaces of ectodermal cells (Fig. 4f,g). This is highly unusual for epithelial cells, which normally exhibit an apical junctional complex.

Endodermal cells are characterized by the multitude of differently shaped membrane-bound vacuoles and inclusions (Fig. 4h). Some of the inclusions could be yolk granules. Ciliary rootlets and cilia are found in the center of the endoderm, surrounding the cleft-like endodermal lumen (Fig. 4h).

Larval settlement and the onset of metamorphosis

Planulae settle by attaching themselves to the substrate with their anterior (i.e. aboral) end. During this process, the apical organ indents at its center (Fig. 5a,b). Subsequently, the solid planula body develops into a polyp, which possesses tentacles and a gastric cavity opening through a mouth at the former posterior pole of the animal.

The gastric cavity and mouth form prior to the outgrowth of tentacles through morphogenetic cell rearrangements that

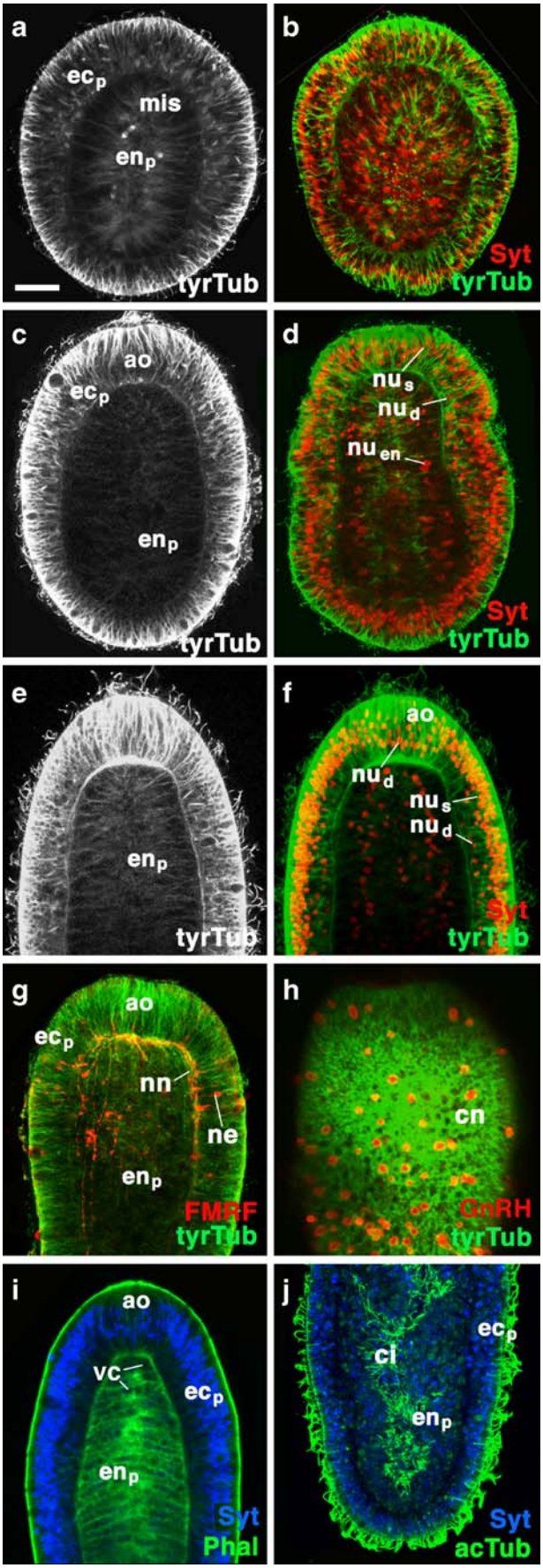


Fig. 3 Planula. Confocal sections of *Aurelia* planulae labeled with the antibody against tyrosinated tubulin (*tyrTub*). In **b**, **d**, **f**, **i**, and **j**, nuclei are labeled with Sytox (*Syt*). In **g**, a subset of neurons is labeled with the anti-FMRFamide antibody; in **h**, cnidocytes (*cn*) are labeled by the anti-GnRH antibody; in **i**, actin cytoskeleton is labeled with phalloidin; in **j**, cilia (*ci*) are labeled by the antibody against acetylated tubulin (*acTub*). All *panels* represent longitudinal sections through the center of the specimen. **a**, **b**: early (ovoid) planula; **c**, **d**: midstage (ellipsoid) planula; **e**–**j**: late planula. Abbreviations: *ao* apical organ; *ci* cilia; *ecp* primary ectoderm; *enp* primary endoderm; *ne* neuron; *nn* nerve net; *nud* deep ectodermal nuclei; *nuen* endodermal nuclei; *nus* superficial ectodermal nuclei; *vc* vacuole. *Bar* 25 μm

involve both ectoderm and endoderm. The settled planula adopts a bell shape, shortening along the oral–aboral axis and widening in the transverse axis (Fig. 5i–l). A densely packed layer of endodermal cells appears in the oral region (Fig. 5f,g, j,k). This layer, called secondary endoderm in the following, spreads from the oral to the aboral pole. A lumen, lined by the ciliated apical surfaces of secondary endodermal cells, forms the gastric cavity and mouth (Fig. 5h,l).

Secondary endodermal cells appear to be derived from the ectoderm, rather than the primary endoderm, of the planula. This surprising finding will need to be substantiated by cell lineage tracing studies which are in progress. The following observations, based on serial confocal imaging of fixed specimens, support the ectodermal origin of the secondary endoderm.

1. The primary endoderm can be followed throughout early metamorphosis as a solid mass of cells that are demarcated from the emerging secondary endoderm by a cell-poor boundary layer (indicated by arrowheads in Fig. 5j). As the secondary endoderm spreads, the primary endoderm becomes restricted to the aboral pole of the settled planula. At the same time, a high level of immunoreactivity to caspase 3, the apoptotic protease, is evident in the primary endoderm (Fig. 6e,f), suggesting that the vast majority, if not all, of the primary endodermal cells degenerate. Cell death is also suggested by the fact that the Sytox-labeled nuclei become pyknotic and disintegrate into small fragments (Fig. 6g,h). We note, however, that activity of caspase 3 alone does not prove apoptosis since this enzyme can also function in cellular differentiation (cf. Fernando et al. 2002; Szymczyk et al. 2006). Therefore, further verification of the hypothesis by other methods for detecting apoptosis such as the terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay would be beneficial.
2. Secondary endodermal cells are very numerous; we estimated a number exceeding 10,000 for several specimens fixed at a stage shortly prior to tentacle formation. By contrast, the number of primary endodermal cells in the planula is much lower than that (see above). Cell proliferation, visualized with an antibody

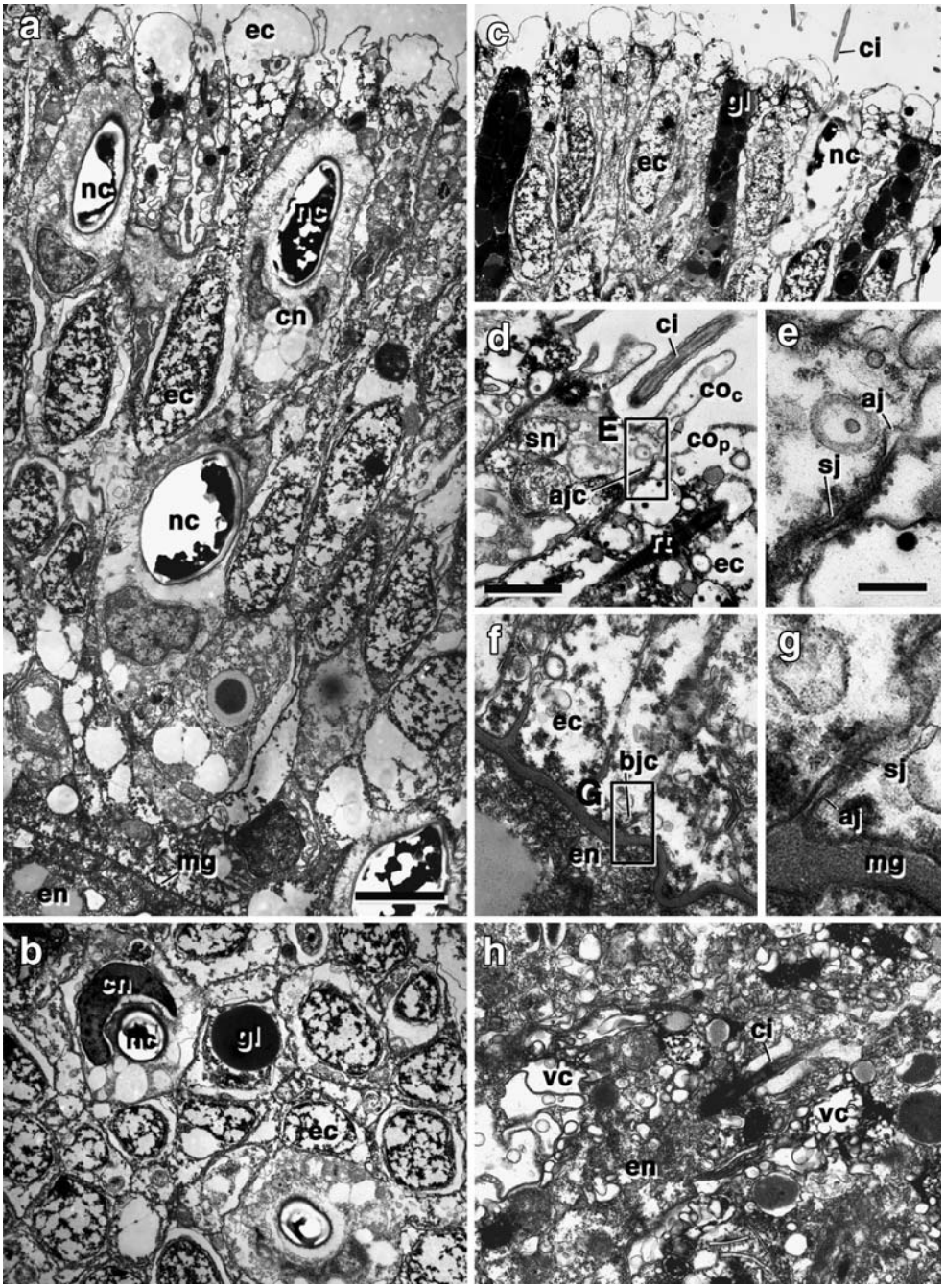


Fig. 4 Electron-microscopic sections of the late planula. **a**: Ectoderm, longitudinal section. Apical surface at the *top*, basal surface at the *bottom*. Note multilayered arrangement of ectodermal nuclei (*ec*) and cnidocytes (*cn*) containing nematocysts (*nc*). The mesoglea (*mg*) represents a thin layer of electron-dense extracellular material separating ectoderm from endoderm (*en*). **b**: Ectodermal cells, tangential section at superficial level, showing cross-sectioned nuclei of ectoderm (*ec*) and cnidocytes (*cn*), as well as nematocysts (*nc*) and gland cells (*gl*). **c**–**e**: Sections of apical pole of ectodermal cells (*ec*), showing apical necks of gland cells (*gl*), nematocysts (*nc*), and cilia (*ci*). **d** shows specialized ectodermal cell, probably sensory neuron (*sn*), with large cilium surrounded by central and peripheral collar (*coc*, *cop*). A magnified view of the apical junctional complex (*ajc*) consisting of the adherens junction (*aj*) and septate junction (*sj*) is shown in **e**. **f**, **g**: Basal domain of ectoderm facing mesoglea (*mg*) and endoderm (*en*). Note prominent basal junctional complex (*bjc*), consisting of adherens junction and septate junction. **h**: endodermal cell (*en*) with vacuoles (*vc*) and cilium (*ci*). *Bars* 2 μm (**a**–**c**); 0.5 μm (**d**, **f**, **h**); 0.1 μm (**e**, **g**)

against phosphorylated histone H3, seems to be low or absent in the primary endoderm of the planula before and during settlement, whereas it is high in the secondary endoderm during metamorphosis (see below). These numerical considerations make it highly unlikely that the primary endoderm is the source of the secondary endoderm.

3. A similar argument can be made when analyzing the morphogenetic changes taking place in the ectoderm during settlement and early metamorphosis. As described above, the planula ectoderm is a pseudostratified epithelium containing in the order of 10,000–15,000 cells. Nuclei are densely packed into four to five strata (Fig. 3f). After settlement, the ectoderm decreases in diameter; nuclei are less densely packed and form one to two layers (Fig. 7d,e). In other words, the ectoderm of the early polyp contains a significantly lower number of cells than the planula per given area, even though the entire surface area of the early polyp (at or before tentacle bud stage) is approximately the same as that of a planula (compare Fig. 3f,i with Fig. 7d,g). Thus, during early metamorphosis when the secondary

endoderm appears, the number of (primary) ectodermal cells seen in the planula decreases. Since in the ectoderm no evidence of cell death was observed (Fig. 6e,f) and cell proliferation appears high during metamorphosis (Fig. 6a,d), we argue that cells of ectodermal origin most likely contribute to the formation of the secondary endoderm.

Late metamorphosis: the formation of tentacles and gastric pouches

During the development of scyphozoan polyps, tentacles do not appear all at once but sequentially in a regular spatiotemporal pattern. In *Aurelia*, the sequence is four, eight, 16, and 32. The appearance of the first four tentacles, which are arranged in a regular quadrant, is coordinated with the formation of gastric longitudinal septa, or tanioles, and is initiated in the secondary endoderm. Viewed from the oral pole (Figs. 5m–s; 7a–c), the settled larva prior to the appearance of tentacles has a square-like shape. At this stage, the secondary endoderm shows condensations that foreshadow the appearance of gastric septa and tentacles. Primordia of the gastric septa form four spikes, arranged in the shape of a cross, which radiate out from the endoderm surrounding the mouth opening (Figs. 5o, 7b). These septa persist and subdivide the periphery of the gastric cavity into four "alcoves," the gastric pouches (Fig. 7b). At locations where they contact the perioral ectoderm, the gastric pouches produce small hemispherical evaginations for which we propose the term "tentacle papillae." The ectoderm overlying the papillae evaginates and forms the tentacles; the papillae themselves are incorporated into the tentacle lumen and develop into the tentacle endoderm (Fig. 7a,c).

Growth of tentacles involves cell proliferation. Thus, labeling with antiphosphohistone reveals a belt of cell proliferation that surrounds the oral pole (Fig. 6a) and coincides with the tentacle anlagen and, at a slightly later stage, the tentacle buds (Fig. 6c). Proliferation is also observed in the endodermal tentacle papillae that elongate to form solid rod-shaped cell clusters in the lumen of the tentacles (Fig. 6b).

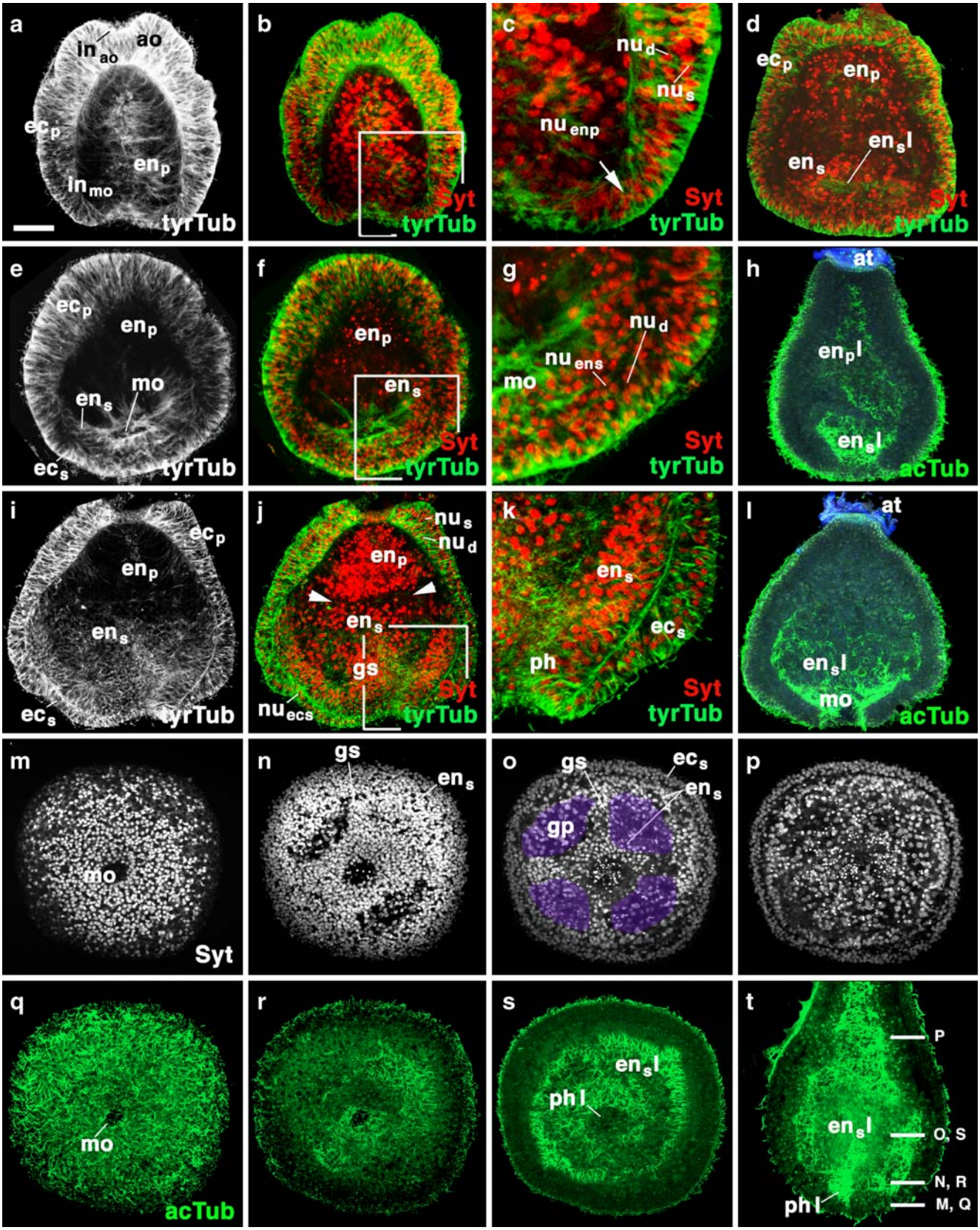
At a stage when the first four tentacles have reached a length corresponding to approximately twice their diameter, the next four tentacles appear at positions in between the existing tentacles (Fig. 7g–i). This process repeats itself, resulting in a final number of 32 tentacles (Fig. 7j–l). Tentacles of mature primary polyps consist of a monolayered cuboidal epithelium enclosing a solid endoderm formed by large vacuolated cells aligned in a single row (Fig. 7l).

Fig. 5 Settling larva. Confocal sections of *Aurelia* larvae attached to the substrate. Embryos are labeled as indicated in the *right corner* of each *image* with Sytox (*Syt*) and/or antibody against tyrosinated tubulin (*tyrTub*) or antibody against acetylated tubulin (*acTub*). *Panels* **a**–**l** and **t** represent longitudinal sections through the center of specimens; **a**–**d**: early settled larva (ovoid shape; prior to formation of mouth opening); **e**–**h**: midstage settled larva (ovoid shape; mouth opening has formed); **i**–**l**: late settled larva (bell-shaped; septated gastric cavity has formed). **c**, **g**, and **k** show parts of panels to the *left* of them at twice the magnification than the panels to their *left* (**b**, **f**, and **j**, respectively). **m**–**s** are transverse sections at different levels along the oral–aboral axis of a late, bell-shaped settled larva; levels are indicated by *lines* in *panel* **t**. **m**, **q**: level of mouth opening; **n**, **r**: level basal of mouth opening ("pharynx" or proboscis); **o**, **s**: level of septated gastric cavity; **p**: level of distal part of gastric cavity. At the onset of settlement, the apical organ (*ao* in **a**) forms an invagination (*inao*) and becomes the attachment site (in **h** and **l**; blue color labels secreted material connecting larva to the substrate). At an early stage of settlement, the primary endoderm (*enp*) still fills the entire interior of the larva (**a**, **b**, **c**). A shallow invagination of the ectoderm at the oral pole (*inmo*) marks the future position of the mouth opening (**a**). The larval ectoderm becomes thinner towards the oral pole, whereas the aboral ectoderm (primary ectoderm, *ecp*, in **a**–**c**, **e**–**g**) still shows superficial and deep layers of nuclei (*nus* and *nud* in **c**); ectoderm near the oral pole has only one layer of nuclei (*arrow* in **c**). In some specimens of settled larvae prior to mouth formation (*panel* **d**), densely packed cells underneath oral ectoderm demarcate the first appearance of secondary endoderm (*ens*) surrounding a small lumen (*ensl*). At the stage at which the mouth opening forms (*mo* in **e**–**l**), secondary endoderm has become a prominent feature of the larva. The primary endoderm becomes displaced towards the aboral pole, with a sharp boundary between primary and secondary endoderm evident (*arrowhead* in **j**). Secondary endoderm forms ciliated cells that enclose an inner lumen (*ensl*) that is divided by four gastric septa (*gs*) into the gastric pouches (*gp*; *shaded violet* in *panel* **o**). By the late settled larva stage, most of the ectoderm has thinned out (*ecs* in K), presumably due to the fact that many cells have invaginated–delaminated from it to form the secondary endoderm. The cuboidal cells remaining at the surface form the secondary ectoderm of the polyp. Other abbreviations: *ph* pharynx; *phl* lumen of pharynx. *Bar* 25 μm

Discussion

Gastrulation

Berrill (1949) stated that the mode of gastrulation in scyphozoans and stauromedusae depends on the size of the eggs; gastrulation occurs by ingression in smaller eggs (0.06–0.15 mm) and by invagination in larger eggs (>0.15 mm). Thus, mixed modes of gastrulation, involving invagination and ingression, documented in *Aurelia* by Hyde (1894) have been attributed to a high degree of variation in the egg size of *Aurelia* (Berrill 1949). However, our morphological study suggests that invagination may be the sole mode of gastrulation in our *Aurelia* embryos (Fig. 8). This was also found to be the case in *Aurelia* embryos collected from the adult female medusae found in Tokyo Bay, Japan, despite variation in egg sizes (0.15–0.23 mm; Ishii and Takagi 2003).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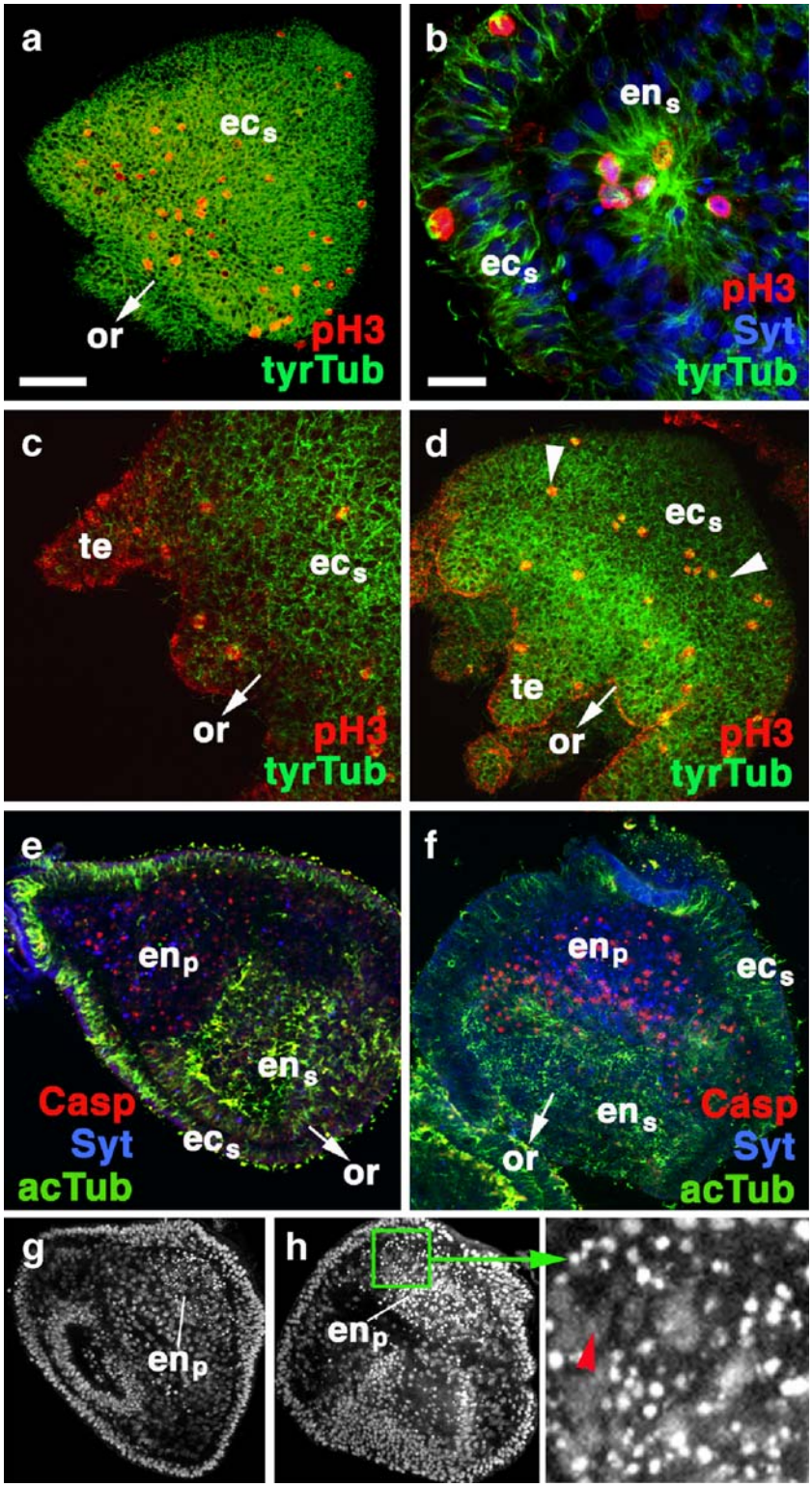

Fig. 6 Proliferation and apoptosis during early metamorphosis. Confocal sections of settled larvae (**a**, **b**, **e**, **f**, **g**, **h**) and early polyps (**c**, **d**) labeled with the antibody against tyrosinated tubulin (*tyrTub*). In **b**, **e**, **f**, **g**, and **h**, nuclei are labeled with Sytox (*Syt*). In **a**–**d**, mitotic cells are labeled with antiphosphohistone 3 (*pH3*). In **e** and **f**, apoptotic cells are labeled by anti-caspase-3 (*Casp*). *Arrows* point at oral pole (*or*). In settled larva (**a**, **b**), proliferating cells are concentrated in a belt-shaped region of secondary ectoderm (*ecs*) and secondary endoderm (*ens*) surrounding the oral pole. During polyp stage (**c**, **d**), proliferation is still found in the oral belt (*arrowhead*) that now has come to lie at the base of the tentacles (*te*). Scattered proliferation is also found in the ectoderm of the tentacles themselves. Cells of the primary endoderm (*enp*) undergo apoptosis at the settled larva stage (**e**, **f**). Apoptosis is also indicated by fragmentation of cells in the primary endoderm (**g**, **h**). Note in *inset* of panel **h** small, dense bodies which represent fragments of nuclei. *Red arrowhead* indicates size of regular endodermal nucleus. *Bar* 25 μm (**a**, **c**–**f**); 10 μm (**b**)

Cell types of the planula

In the *Aurelia* planula, we have identified gland cells, cnidocytes, and FMRFamide-immunoreactive neurons in the ectoderm and actin-rich highly vacuolated cells in the endoderm. Cnidocytes are enriched in the posterior–oral end of the planula, as in other cnidarians (Groger and Schmid 2001). FMRFamide-immunoreactive neurons and their neurites are enriched in the anterior–aboral half of the planula (Fig. 8; Nakanishi et al. 2008), similar in the spatial

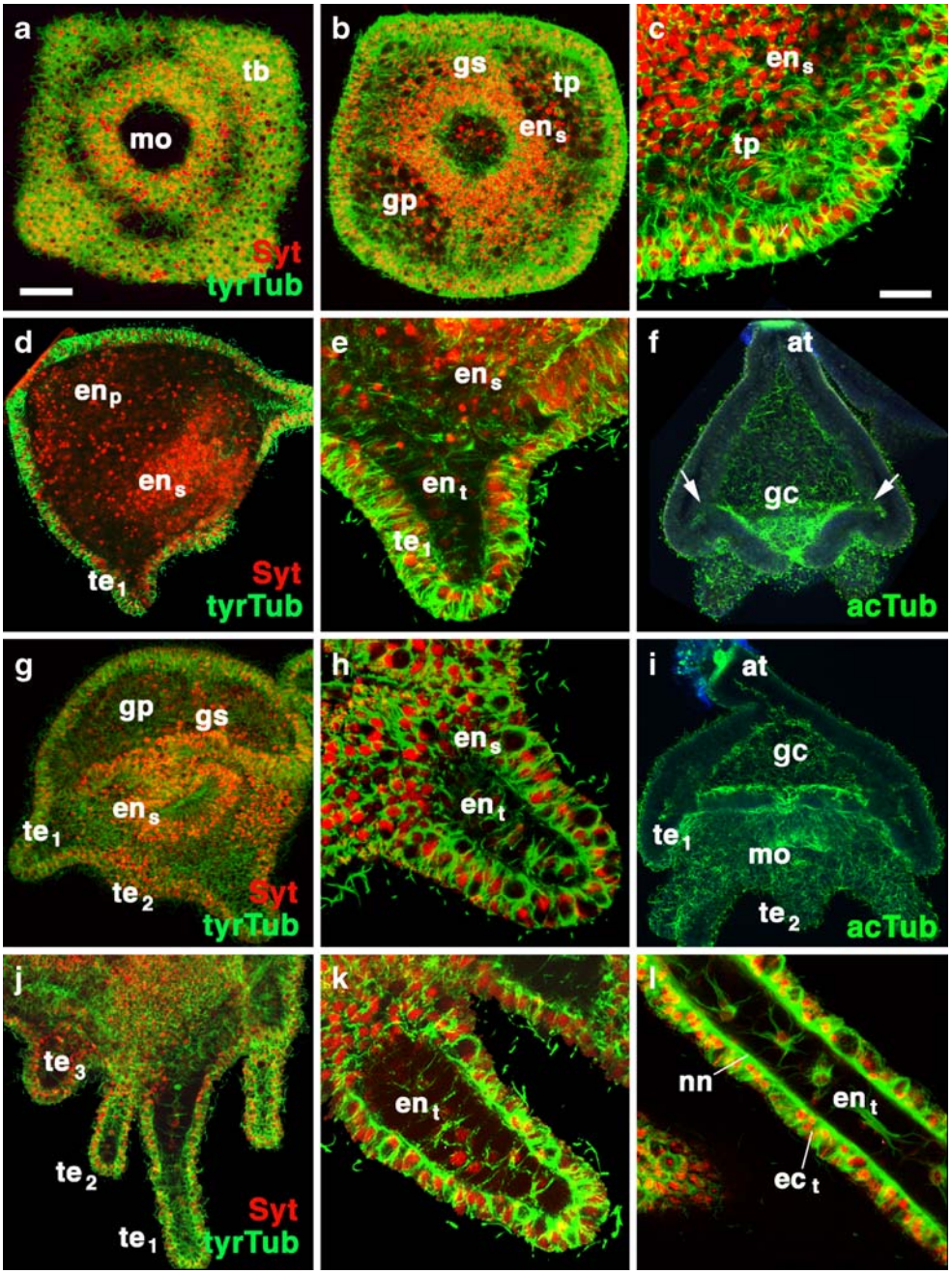

Fig. 7 Primary polyp. Confocal sections of *Aurelia* polyps, labeled with the antibody against tyrosinated tubulin (*tyrTub*). In **a**–**c**, **d**, **e**, **g**, **g**, and **j**–**l**, nuclei are labeled with Sytox (*Syt*). *Panels* **a**–**c** are transverse sections; **d**–**f** are longitudinal sections; **g**–**i** are sections cut at plane tilted between horizontal and longitudinal; **J**–**l** are longitudinal sections of tentacles. **a**–**c**: Tentacle bud stage. **d**–**f**: Four-tentacle stage. **g**–**i**: Eight-tentacle stage. **j**–**l**: 16-tentacle stage. Tentacle buds (*tb* in **a**–**c**) consist of regularly spaced condensations of ectoderm and underlying secondary endoderm. The tentacle endoderm ("tentacle papillae", *tp* in **b** and **c**) appears at positions within the secondary endoderm (*ens*) that alternate with the position of gastric septa (*gs*). As the tentacle ectoderm grows out, the endoderm filling the interior of each tentacle (*ent*) initially has a small lumen that communicates with the gastric cavity (*gc*; *arrows* in F). At later stages, the tentacle endoderm forms solid rods of large, vacuolated cells (**j**–**l**). Other abbreviations: at attachment site of polyp; *ect* tentacle ectoderm; *enp* primary endoderm; *ens* secondary endoderm; *gp* gastric pouch; mouth; *te1*: first quartet of tentacles; *te2* second quartet of tentacles; *te3* third octet of tentacles. *Bars* 25 μm (**a**, **b**, **d**, **f**, **g**, **i**, **j**); 10 μm (**c**, **e**, **h**, **k**, **l**)

distribution to GLWamide-immunoreactive neurons in the *H. echinata* planula (Leitz and Lay 1995).

Interstitial cells

Interstitial cells, also called "I cells," are small basophilic round cells measuring 7.5 μm diameter with large centrally located nuclei and one or more nucleoli (Martin 1991). They are known to be self-renewing multipotent stem cells capable of differentiating into multiple cell types such as cnidoblasts (Slautterback and Fawcett 1959) and gametes (Burnett et al. 1966). The presence of I cells has been documented in many hydrozoans, as well as in a scyphozoan *Cassiopeia xamachana* (Martin and Chia 1982) and in a staurozoan *Haliclystus salpinx* (Otto 1978). Therefore, I cells have been considered a general cnidarian character that is important in asexual reproduction and regeneration (Brusca and Brusca 2003). Yet, our method using light as well as electron microscopy failed to reveal any cells with the above morphological characteristics in the scyphozoan *Aurelia*. This does not, however, hinder the ability of regeneration in *Aurelia*, as an ectodermal fragment of

Aurelia devoid of I cells can regenerate the entire polyp (Steinberg 1963). Also, regeneration of the endoderm from the ectoderm does not require the presence of I cells in the ectoderm of a hydrozoan *Cordylophora* (Zwilling 1963). These observations suggest that I cells may not be absolutely required for regeneration in cnidarians.

Apical organ

Apical organs are presumptive sensory structures ubiquitous in invertebrate larvae, located in the anterior pole with a distinct cluster of apical cells and neurons (Lacalli 1994). The most aboral region of the ectoderm in the *Aurelia* planula is clearly distinct from the rest of the ectoderm in containing a cluster of elongated cells with basal nuclei as well as cells immunoreactive with anti-FMRFamide and antitaurine antibodies, called "apical neurons" (Fig. 8; Nakanishi et al. 2008, this issue). Prior to this study, formal apical organs were only known from some anthozoan planulae in Cnidaria, but similarities between the apical organ of the planula of *Anthopleura elegantissima*, an anthozoan studied by Chia and Koss (1979), and the aboral ectodermal structure of *Aurelia* planulae are evident: both structures are located in the ectoderm at the aboral end of the planula larvae, where substrate attachment occurs during settlement; both structures are enriched with cells that are elongated and have basal nuclei; cnidocytes are rare or absent; sensory cells are enriched, beneath which lie fibers of the nerve plexus. These sensory cells likely perceive external chemical cues for settlement and/or metamorphosis (Chia and Bickell 1978; Nakanishi et al. 2008, this issue). These positional, structural, and presumptive functional similarities strongly suggest that the apical organ of an anthozoan planula shares an evolutionary origin with the aboral ectodermal structure of *Aurelia*. Therefore, we regard this specialized region of the aboral ectoderm in the *Aurelia* planula as the apical organ.

Apoptosis

We observed the morphological hallmarks of apoptosis (nuclear pyknosis and fragmentation) as well as a high level of immunoreactivity to caspase 3, a marker for apoptotic cell death, in the compressed endoderm near the aboral pole of metamorphosing *Aurelia* planula larvae, suggesting that the planula endoderm degenerates during metamorphosis (Fig. 8). It has been reported that caspase-3-specific inhibitors prevent metamorphosis into a polyp in a hydrozoan *H. echinata*, indicating that caspase-3-dependent apoptosis is necessary for metamorphosis in *H. echinata* (Seipp et al. 2006). This suggests that endoderm degeneration in *Aurelia* might also be important for completion of metamorphosis.

If, as we observed, the *Aurelia* planula endoderm degenerates, then where does the polyp or the "secondary" endoderm originate? Our observations support the argument that the polyp endoderm is at least partially derived, after settling of the planula, from the planula ectoderm, a process that we called "secondary gastrulation" (see "Results"); the term "delayed gastrulation" would be equally appropriate. The exact morphogenetic process by which the polyp endoderm is formed during secondary gastrulation remains unclear. Our data show that a mass of secondary endoderm can be first detected in the oral pole and it expands to the aboral pole in the metamorphosing planula (Fig. 8), which indicates that an involution-like process may be in action. In *Aurelia* polyps, ectodermal fragments devoid of endoderm can regenerate the entire animal, by ingression of nondividing ameboid cells from the ectoderm (Steinberg 1963). This suggests that ingression may also generate the secondary endoderm during metamorphosis. Identification of the exact morphogenetic mechanism of secondary endoderm formation, as well as determination of the extent of ectodermal contribution to the formation of this polyp endoderm, will require further investigation with more direct methods such as dye injection experiments.

Secondary gastrulation and implications on the interpretation of comparative gene expression data in the Cnidaria

Gastrulation is usually portrayed as a relatively short, temporally well-defined event in animal development. In many instances, this may be misleading because in reality the process of internalizing endoderm and mesoderm from the blastoderm can be long and complex. For example, formation of the head mesoderm in *Drosophila* extends several hours past the short interval usually defined as gastrulation (De Velasco et al. 2004). This also implies that the surface epithelium forming the head of the *Drosophila* embryo, usually referred to as "head ectoderm," is not entirely ectodermal but represents a "mixed" germ layer since it still contains cells with mesodermal fate. Our observation on *Aurelia* development suggests a similar scenario in which the planula "ectoderm" is a mixed germ layer that contains cells that will internalize during metamorphosis to form the polyp endoderm. A secondary gastrulation may occur in hydrozoans as well, although the ectoderm–endoderm relationship seems reversed. In the hydrozoan *H. echinata*, the majority of the ectoderm undergoes apoptosis during metamorphosis into a polyp (Seipp et al. 2001). At least some of the polyp ectoderm probably derive from the planula endoderm, as cell proliferation primarily occurs in the endoderm and I cells migrate from endoderm to ectoderm during metamorphosis in *H. echinata* (Plickert et al. 1988; Weis and Buss 1987).

The occurrence of a secondary gastrulation creates potential difficulties in comparing gene expression patterns

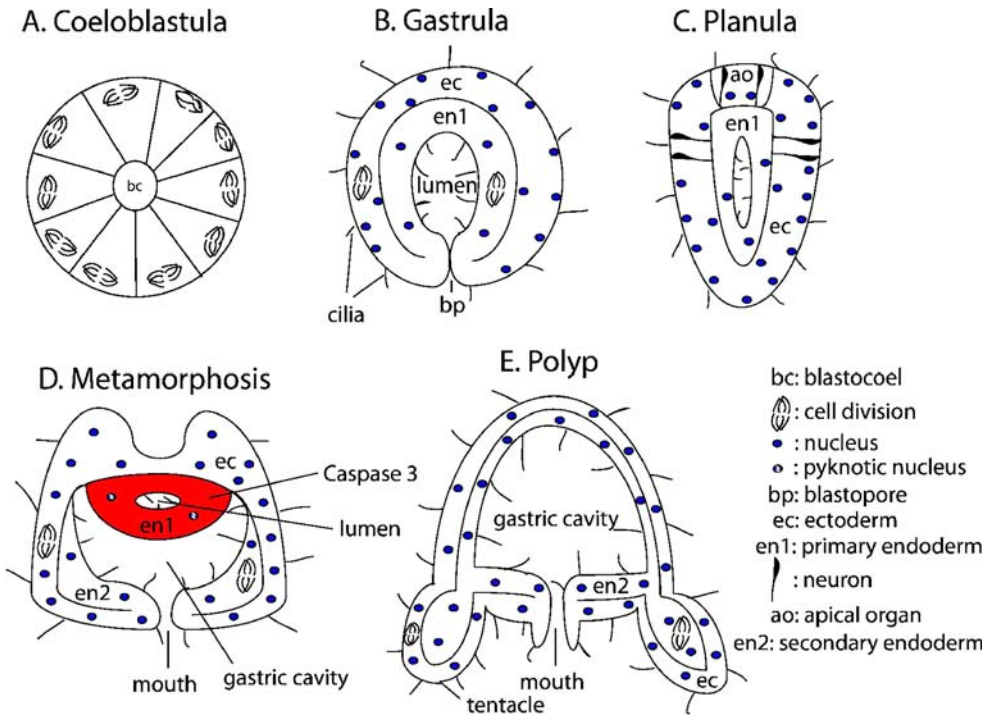

Fig. 8 Schematic representation of *Aurelia* embryogenesis and metamorphosis. Aboral side at the *top*, oral side at the *bottom*. **b**–**e** represent longitudinal sections through the center of the embryo–larva. **a** An early *Aurelia* embryo forms a coeloblastula, in which cells actively divide. **b** Invagination occurs, forming a ciliated gastrula with (primary) endoderm and ectoderm. Nuclei in the ectoderm become separated into superficial and deep layers. **c** Neurons and an apical-organ-like structure develop in a planula. **d** During metamorphosis into a polyp, primary (planula) endoderm becomes compressed near the aboral pole, which is strongly caspase 3 immunoreactive and contains pyknotic nuclei, suggesting apoptosis. Secondary (polyp) endoderm begins to form near the oral pole and expands aborally. **e** Budding and growth of tentacles, which involves cell proliferation, complete the metamorphosis

among planulae of different species. The above observations indicate that the planula ectoderm in *Aurelia* contains the future polyp ectoderm as well as progenitors to the polyp endoderm. In contrast, the planula endoderm in hydrozoans may contain the future polyp endoderm as well as progenitors to the polyp ectoderm. Thus, *the planula* "*ectoderm*" *and* "*endoderm*" *of one species cannot be assumed developmentally equivalent to the planula* "*ectoderm*" *and* "*endoderm*" *of another species in cnidarians*. If this is the case, expression patterns of genes, particularly those likely involved in the patterning of polyps such as the Hox genes, may be expected to differ in planulae of species with different modes of "gastrulation." Hox genes are known to encode positional information along the anterior–posterior axis of bilaterians (cf. Slack et al. 1993). Although the relationship of cnidarian Hox-like genes to bilaterian Hox genes has been highly controversial (Chourrout et al. 2006; Kamm et al. 2006; Ryan et al. 2007), the authors appear to agree on the presence of cnidarian orthologs to the bilaterian "anterior" Hox gene. However, its expression pattern in the planula through metamorphosis into the polyp differs among hydrozoans and an anthozoan. In the hydrozoans, *P. carnea* and *Eleutheria dichotoma*, the homologs of the bilaterian "anterior" Hox gene, named Cnox-1pc and Cnox-5ed, respectively, are initially expressed in the aboral end of the planula but their expression extends to the oral end during metamorphosis (Kamm et al. 2006; Yanze et al. 2001). In contrast, anthox6, the ortholog to Cnox-1pc and Cnox-5ed occurs in the oral endoderm in the planula and persists through the juvenile polyp in the anthozoan *N. vectensis* (Finnerty et al. 2004). This high level of divergence in expression patterns may indicate a high level of divergence in the function of the gene, but it could also be due to a high level of divergence in cellular dynamics that generate the polyp body plan. Both the hydrozoan *E. dichotoma* and the anthozoan *N. vectensis* share the expression of Cnox-5ed/anthox6 in the oral endoderm of the polyp. However, no oral (endodermal) expression occurs at an earlier planula stage in *E. dichotoma*, whereas such expression is clear in *N. vectensis*. Could it be that in hydrozoan planulae, the oral (polyp) endoderm is not yet specified at the planula stage, whereas in the *N. vectensis* planula it is? This would explain why committed oral endodermal cells express a Hox gene in *N. vectensis* but not in *E. dichotoma*; such cells do not exist yet but are specified during secondary gastrulation in *E. dichotoma*. Thus, caution must be used when gene expression patterns in planulae of distantly related cnidarian species are compared. A range of additional work would be merited to confirm aspects of the work reported here as well as to

generate comparable data from other cnidarian taxa. Such studies might include expression studies complemented by gene knockdown experiments, detailed investigations of gastrulation–secondary gastrulation movements using specific markers, as well as cell-labeling experiments. Such work may prove critical to generating an integrated picture of the genetic mechanism controlling early patterning in cnidarians.

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