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The organizer in evolution–gastrulation and organizer gene expression highlight the importance of Brachyury during development of the coral, *Acropora millepora*

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ABSTRACT

Organizer activity, once thought to be restricted to vertebrates, has ancient origins. However, among non-bilaterians, it has only been subjected to detailed investigation during embryonic development of the sea anemone, *Nematostella vectensis*. As a step toward establishing the extent to which findings in *Nematostella* can be generalized across the large and diverse phylum Cnidaria, we examined the expression of some key organizer and gastrulation genes during the embryonic development of the coral *Acropora millepora*. Although anemones and corals both belong to the cnidarian class Anthozoa, the two lineages diverged during the Cambrian and the morphological development of *Acropora* differs in several important respects from that of *Nematostella*. While the expression patterns of the key genes *brachyury*, *bmp2/4*, *chordin*, *goosecoid* and *forkhead* are broadly similar, developmental differences between the two species enable novel observations, and new interpretations of their significance. Specifically, *brachyury* expression during the flattened prawnchip stage before gastrulation, a developmental peculiarity of *Acropora*, leads us to suggest that it is the key gene demarcating ectoderm from endoderm in *Acropora*, and by implication in other cnidarians, whereas previous studies in *Nematostella* proposed that *forkhead* plays this role. Other novel observations include the transient expression of *Acropora forkhead* in scattered ectodermal cells shortly after gastrulation, and in the developing mesenterial filaments, with no corresponding expression reported in *Nematostella*. In addition, the expression patterns of *goosecoid* and *bmp2/4* confirm the fundamental bilaterality of the Anthozoa.

Introduction

The ability of the dorsal lip of the amphibian blastopore to induce a second axis when transplanted led to the concept of the embryonic, or Spemann's, organizer. In vertebrates, this region is not only responsible for breaking the symmetry of the early embryo but also ultimately gives rise to the mesoderm. Vertebrate organizer activity is conferred by complex interactions involving the Wnt, TGFß and FGF signaling pathways and a large and diverse suite of transcription factors. While the organizer was originally thought to be a vertebrate or chordate innovation, it is now evident that some aspects of organizer function are evolutionarily ancient and have pre-bilaterian origins. For example, in transplantation experiments reminiscent of those of Spemann and Mangold (1924), the blastopore lip of the cnidarian *Nematostella vectensis* was shown to have organizer activity (Kraus et al., 2007). A number of genes implicated in organizer function in vertebrates are present in *Nematostella* and other non-bilaterians (see below), but many aspects of organizer origins remain unclear.

Genes of particular interest in terms of organizer origins include those coding for the transcription factors Brachyury, Goosecoid and Forkhead. Brachyury is the founding member of the T-box transcription factor family (Sebé-Pedrós et al., 2013). While it was originally studied in the context of mesoderm formation and notochord differentiation in vertebrates (e.g. Chesley, 1935; reviewed in Smith, 2004), it is an evolutionarily ancient gene. Brachyury orthologs have been identified in Porifera, Placozoa, Ctenophora and Cnidaria, as well as in the non-metazoan ichthyosporeans, filastereans, and fungi (summarized in Sebé-Pedrós et al., 2013). The ability of the *Mnemiopsis leidyi* (Ctenophora) protein to mimic Brachyury functions in *Xenopus*, as well as morpholino knockdown and expression data, support the idea of an ancient role for Brachyury proteins as regulators of genes required for cell movements (Yamada et al., 2007, 2010).

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Goosecoid (*gsc*) has been extensively studied in *Xenopus*, mouse and chick (reviewed in De Robertis, 2004), in all of which it is expressed asymmetrically in the dorsal lip of the blastopore, plays an organizer role, and is expressed in endomesoderm. Clear homologs of *goosecoid* are present in cnidarians (Broun et al., 1999; Matus et al., 2006a; Röttinger et al., 2012), but not in *Trichoplax* or the sponges and ctenophores that have been studied to date.

The Fox (forkhead box) proteins comprise a large family of transcription factors (ca 50 in humans, Jackson et al., 2010), defined by the presence of a 110 amino acid winged helix domain, the forkhead/HNF3 domain. Bilaterian Fox genes are involved in diverse developmental functions including development of the eye, lung, notochord and nerve cord (Ruiz i Altaba et al., 1995). A survey of non-bilaterian Fox genes (Shimeld et al., 2010) indicates that this is an ancient and diverse gene family. Members of the FoxA family, to which the *Acropora* gene described here belongs, have been described from *Trichoplax* and *Nematostella* but not from *Amphimedon* or ctenophores.

BMP2/4 and its antagonist Chordin are products of the vertebrate counterparts of the *Drosophila decapentaplegic* (*dpp*) and *short gastrulation* (*sog*) genes; these genes are key determinants of the dorsal/ventral (D/V) axis across the Bilateria (reviewed in Holley et al., 1995; Arendt and Nübler-Jung, 1997). We have previously described the early embryonic expression of *Ambmp2/4* in *Acropora*, establishing that its expression defined a bilateral axis and that the coral gene had dorsalizing activity in *Drosophila* (Hayward et al., 2002). Among non-bilaterians, unequivocal orthologs of *chordin* are found only in Cnidaria and *bmp2/4* only in Cnidaria and Placozoa.

For molecular genetic analyses and evolutionary comparisons, the sea anemone *Nematostella* is the cnidarian of choice, and the tractability of early development in this organism is enabling functional analyses of the organizer genes. In *Nematostella*, *brachyury* (*Nvbra*) is the least studied of the genes considered here. However, it also has the simplest expression pattern, as it is initially expressed in the ectoderm surrounding the blastopore (Fritzenwanker et al., 2004; Röttinger et al., 2012) and this expression is continued in the pharyngeal ectoderm as the blastopore becomes the mouth (Scholz and Technau, 2003; Fritzenwanker et al., 2004).

Nematostella goosecoid (*Nvgsc*) is initially weakly expressed in the endoderm at the time of gastrulation (Röttinger et al., 2012) and later appears in opposing domains in the endoderm of the cylindrical body wall of the planula, spreading to ectoderm and endoderm of the pharynx. There is also expression in the endoderm at the center of the aboral end directly below the apical tuft of sensory cilia (Matus et al., 2006a).

Martindale et al. (2004) and Fritzenwanker et al. (2004) characterized the expression of *forkhead* (*NvfoxA*) in *Nematostella*, finding that expression starts in the late blastula as a circle of spots surrounding the blastopore. These spots later link up, forming a partial or complete circle of expression and continuing to be expressed thereafter in the developing pharynx. During the course of development, expression is present in the mesenteries, but by the primary polyp stage expression has become limited to the pharyngeal ectoderm (Fritzenwanker et al., 2004). One hypothesized function of Forkhead is marking the boundary between future endoderm and ectoderm (Fritzenwanker et al., 2004; Magie et al., 2007).

The expression and interaction of *Nvbmp2/4* and *Nvchd* during embryonic development have been extensively investigated (Finnerty et al., 2004; Matus et al., 2006a, 2006b; Rentzsch et al., 2006; Saina et al., 2009). The earliest detectable expression of both genes is in spots symmetrically arranged around the blastopore (Rentzsch et al., 2006; Röttinger et al., 2012). Slightly later their expression becomes more intense, simultaneously becoming restricted to a zone of co-expression in the ectoderm on the same side of the blastopore (Rentzsch et al., 2006; Matus et al., 2006b). *Chordin* expression continues in the ectoderm while also extending aborally in the endoderm, while *bmp2/4* expression disappears from the ectoderm but is coexpressed with *chordin* in the endoderm.

While these data for *Nematostella* have provided new perspectives on the origins of the vertebrate organizer, given their developmental diversity, it is unclear how representative the sea anemone is of developmental mechanisms across the Phylum Cnidaria. To better understand the molecular bases of the diversity of developmental mechanisms in cnidarians, sequences and expression patterns were determined for the coral (*Acropora millepora*) orthologs of five key genes involved in gastrulation and axis formation in vertebrates: *brachyury* (*Ambra*), *bmp2/4* (*Ambmp2/4*), *chordin* (*Amchd*), *goosecoid* (*Amgsc*) and *forkhead* (*Amfkh*).

The embryonic development of *A. millepora* is summarized in Fig. 1. Although both *Nematostella* and *Acropora* belong to the cnidarian Class Anthozoa, Subclass Hexacorallia, their lineages diverged during the Cambrian (Shinzato et al., 2011), so it is not surprising that there are several important morphological and developmental differences between them. First, before gastrulation the *Acropora* embryo passes through a stage during which it forms an extended cellular bilayer, colloquially known as a "prawn chip". While the same term has been used for the stage containing a comparable number of cells in *Nematostella* (Fritzenwanker et al., 2007), the degree of flattening is much more extreme in *Acropora* and other complex corals (e.g. Okubo and Motokawa, 2007; Okubo et al., 2013), which may relate to the way in which gastrulation occurs. Second, *Acropora* undergoes a dramatic metamorphosis at the time of settlement, while settlement in *Nematostella* is gradual. Third, *Acropora* is colonial while *Nematostella* is solitary. Finally,

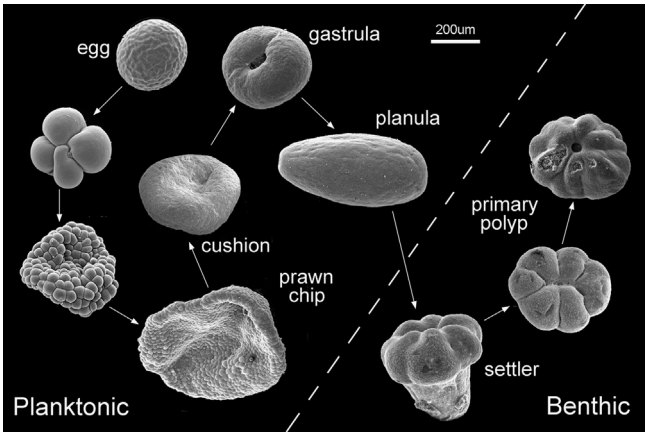

Fig. 1. Development of *A. millepora*. At spawning, buoyant egg-sperm bundles are released by polyps of the adult colony, and float to the surface, breaking up as they go, to release individual eggs and sperm. The early stages of cell division are unremarkable, except that rather than maintaining a compact organization the cells become organized into a flattened cellular bilayer, the prawn chip, before rounding up to form a gastrula. On the way they pass through a "cushion" stage, during which genes involved in controlling gastrulation, such as *brachyury* and *snail*, begin to be expressed. At the completion of gastrulation the blastopore closes, creating a spherical embryo, on the surface of which flagella begin to develop. As the sphere begins to elongate into an early planula the oral pore opens at the former site of the blastopore. Elongation continues, forming an actively swimming, torpedo-shaped planula, which can spend up to several months in the plankton before beginning settlement behavior. It then settles on its aboral end, which is resorbed and reorganized, as the oral end expands. This normally happens quickly, but sometimes fails to go to completion, as illustrated by the embryo labeled "settler". Once this process is completed the crown-shaped primary polyp attaches itself to the substratum and begins to calcify. It then begins to grow upward and the forming tentacles become apparent.

Acropora lays down a massive aragonite skeleton, starting at settlement, while *Nematostella* does not.

In contrast to the dramatic differences at the morphological level between the two organisms in both early development and metamorphosis, the overall expression patterns of *gsc*, *fkh*, *bra*, *dpp* and *chd* are surprisingly similar, although some cases of apparent "evolutionary tinkering" were also observed. During gastrulation in *Acropora*, one major difference from what has so far been described in *Nematostella* is that expression of *brachyury*, rather than *forkhead*, separates presumptive endoderm from ectoderm.

Materials and methods

cDNA clone isolation

cDNA clones were isolated from a planula cDNA library (Brower et al., 1997) using standard techniques (Sambrook et al., 1992). Clones were recovered as inserts in pBluescript SK- (Stratagene) and sequenced with internal and vector primers using Big Dye Terminator v. 3.1 (Applied BioSystems). Sequencing reactions were run on an ABI 3730 DNA Analyzer at the Biomolecular Resource Facility (JCSMR, ANU). Sequences were analyzed using MacVector 12.7.3 and Lasergene (DNASTAR).

Chordin PCR

A *chordin* sequence was identified in the *A. millepora* transcriptome database and the primers 5′ CAGCCATCCTCATCTAAACCTGG 3′ and 5′ CGTCTTTGAACTTGCTCCCACTG 3′ were used to amplify a product from first-strand oligo dT–primed cDNA prepared from planulae. The PCR conditions were: 94 °C for 30 s followed by 35 cycles of 94 °C for 5 s, 55 °C for 10 s, 72 °C for 3 min. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen) and cloned into pGem-T Easy (Promega).

In situ hybridization

Preparation of embryos and larvae for hybridization is described by Hayward et al. (2001) and production of antisense digoxygenin labeled probes from plasmid templates by Kucharski et al. (2000). Antisense fluorescein labeled probes were prepared using the fluorescein RNA labeling mix (Roche). To detect *snail* and *brachyury* expression in the same specimen, embryos were hybridized simultaneously with a digoxygenin labeled *snail* probe and a fluorescein labeled *brachury* probe. The *brachyury* expression was detected first using an anti-fluorescein alkaline phosphatase conjugated antibody (Jackson ImmunoResearch) and NBT/BCIP (Vector Labs). Development was carried out in PBS, 0.1% tween-20, 2.5% Polyvinyl Alcohol (PVA). Embryos were fixed for 10 min in 4% formaldehyde, 1X PBS, washed in PBS, 0.1% tween-20 before *snail* expression was detected using anti-digoxygenin alkaline phosphatase, Fab fragments (Roche) and INT/BCIP (Roche). Double in-situs were also carried out by labeling both *snail* and *brachyury* probes with digoxygenin and detecting both with anti-digoxygenin alkaline phosphatase, Fab fragments and NBT/BCIP. It is possible to distinguish the two expression patterns because *brachyury* is more strongly labeled.

Clearing and photography

In situ preparations were dehydrated through a graded series of glycerol solutions and mounted in 90% glycerol. Photographs were taken using a Zeiss Axioskop or a Wild Photomakroskop. Some images of *bmp2/4* expression were captured on Kodak Ektachrome 64 tungsten film and then scanned. Those of the other genes were directly captured in digital form using QImaging or Spot digital cameras. Digitized images were processed using Adobe Photoshop. In the case of *brachyury* and some other genes, backgrounds were removed for better visibility of staining or enlarged because the object of interest was too tightly framed when the image was captured.

Results

cDNA sequences

Sequences corresponding to *Acropora brachyury*, *forkhead* and *goosecoid* were identified in an *A. millepora* EST collection (Grasso et al., 2011). The *brachyury* and *goosecoid* sequences were incomplete; a planula cDNA library (Brower et al., 1997) was screened to isolate full-length cDNA clones. A *chordin* sequence was identified in the *A. millepora* transcriptome database (Moya et al., 2012) and a cDNA sequence corresponding to amino acids 39–804 was produced by PCR (see *Material and methods*). The isolation of an *Acropora bmp2/4* ortholog has been previously described (Hayward et al., 2002). The domain structures and amino acid sequence alignments of these proteins are shown in Supplementary material.

Brachyury expression in Acropora

Acropora brachyury (*Ambra*) is first expressed in the late prawn chip (pre-gastrula) stage (Fig. 2A) as the cells of what was previously a flattened bilayer are elongating and separating to form a cushion-like structure (Fig. 1), on the surface of which the areas of expression gradually become more connected (Fig. 2B–D). Within the circle of *Ambra*-expressing cells, a depression then forms (Fig. 2E). As this depression (the blastopore) deepens the *Ambra*-expressing cells move inward, forming a smaller circle of expression (Fig. 2F). As invagination continues, the blastopore closes (Fig. 2G), ultimately leaving just a spot of expressing cells marking its former site (Fig. 2H). Then, as the spherical embryo begins to elongate to become pear-shaped, *Ambra* expression encircles the newly forming oral pore (Fig. 2I). Because a spot of stain can still be seen when the blastopore is apparently fully closed and because there is never more than one spot in the absence of obvious injury, we conclude that the position of the oral pore corresponds to that of the blastopore. As the planula larva elongates, the ectodermal tissue surrounding the oral pore continues to express *Ambra*, while the aboral ectoderm thickens as the larva becomes competent to undergo settlement (Fig. 2J–L). Oral expression continues throughout settlement and metamorphosis (Fig. 2M and N), gradually weakening as the polyp develops (Fig. 2O and P).

Comparison of the pre-gastrula expression pattern of *Ambra* (Figs. 2B–D and 3A) with that of *Amsnail* (Hayward et al., 2004; Fig. 3B) suggested that the zone of *Ambra* expression might delimit the margins of *Amsnail* expression and thus of the presumptive endoderm. Results of double ISH conducted with probes for both *Ambra* and *Amsnail* (Fig. 3C–F) are consistent with this hypothesis. Thus, the zone of *Ambra* expression separates the future endoderm, which arises within it, from the future ectoderm, which lies outside it.

Expression of bmp2/4 (dpp)

We have previously reported the expression pattern of *Acropora bmp2/4* up to the point of blastopore closure (Hayward et al., 2002). The micrographs of early stages shown here (Fig. 4A–C) are consistent with the earlier findings, and Fig. 4D–K follows

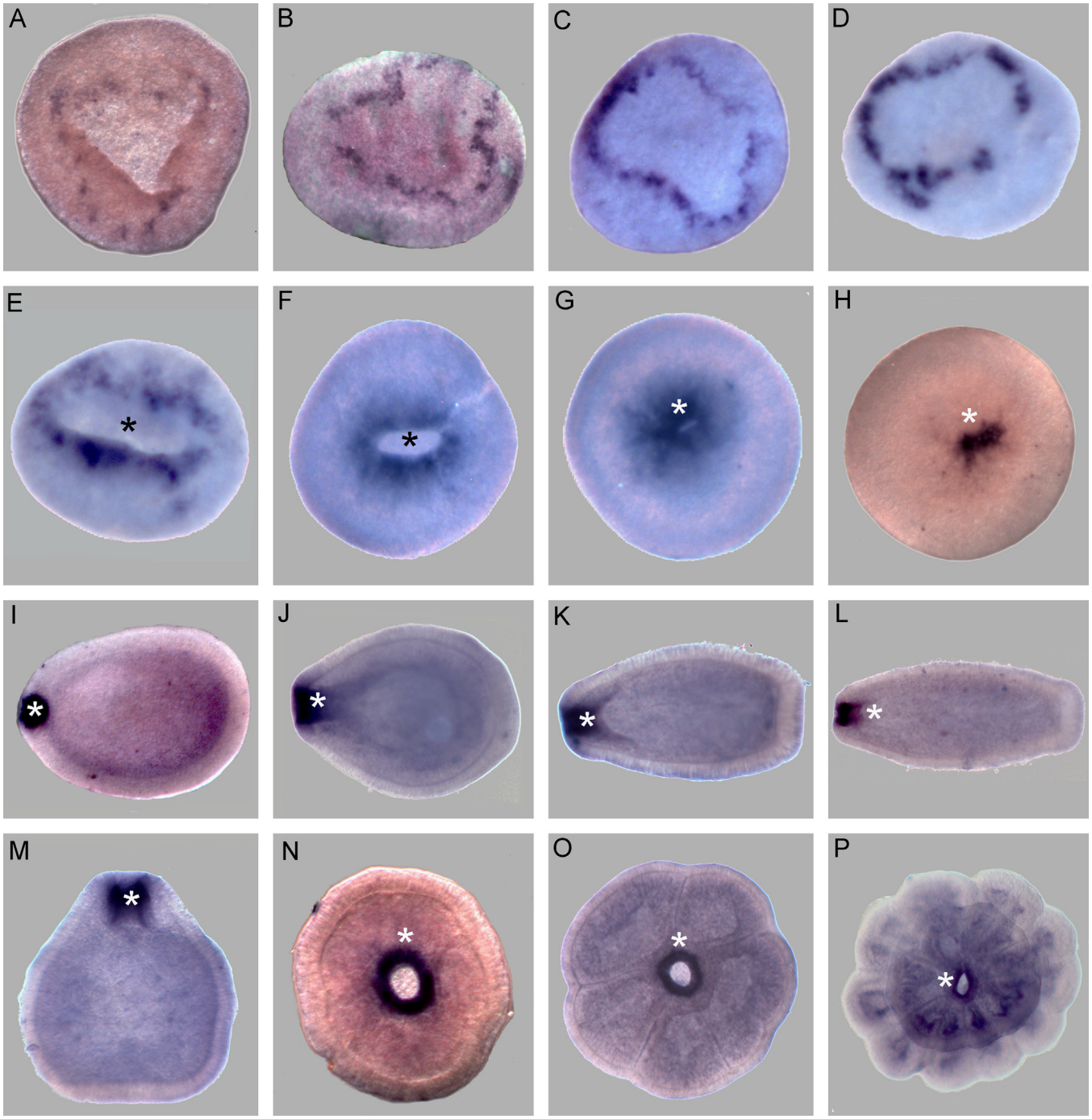

Fig. 2. Expression of *Ambra* during *Acropora* development. (A) Toward the end of the prawn chip stage expression begins as a series of spots surrounding the developing blastopore. (B–G) The former islands of expression join up as expression increases, forming an almost complete ring, which then shrinks in diameter as the expressing cells move into the deepening cavity. (H) At the gastrula stage the embryo is spherical and the blastopore has closed, leaving only a small spot of expression marking its former position. (I–L) The embryo begins to elongate, first taking on a pear shape and then gradually elongating to a spindle shape. It appears that the oral pore opens in the same position as the blastopore has closed, as two spots of expression are never seen simultaneously. Expression continues in the ectoderm of the developing pharynx as the embryo elongates. (M–P) Expression continues in the pharyngeal ectoderm across settlement and metamorphosis, gradually weakening as the primary polyp develops. Embryos are not stained a uniform shade of blue because of the use of two different detection systems; BM-Purple (Roche) and BCIP/NBT (Vector Labs) and have been placed on a uniform gray background due to the diverse backgrounds on which they were originally photographed. Asterisks indicate the position of the blastopore (E–H) or oral pore (I–P).

expression through settlement and into the (post-settlement) primary polyp. After the initial expression in the epithelium, adjacent to the blastopore (Fig. 4A), expression spreads to the neighboring endoderm (Fig. 4B and C), initially at the periphery (Fig. 4B) and then spreading more widely (Fig. 4C). This more general staining then gradually fades to leave only a single stripe of endodermal expression that runs the length of the cylindrical planula (Fig. 4D, D′, E, and E′) and may mark the site of a future septum. Some variation is apparent in the early oral expression pattern; whereas in some cases expression in this region had faded by settlement, in others expression around the oral pore was maintained into the early post-settlement phase (Fig. 4F). In contrast with the apparent variation in oral expression, at the time of settlement *Ambmp2/4* is consistently expressed by ectodermal

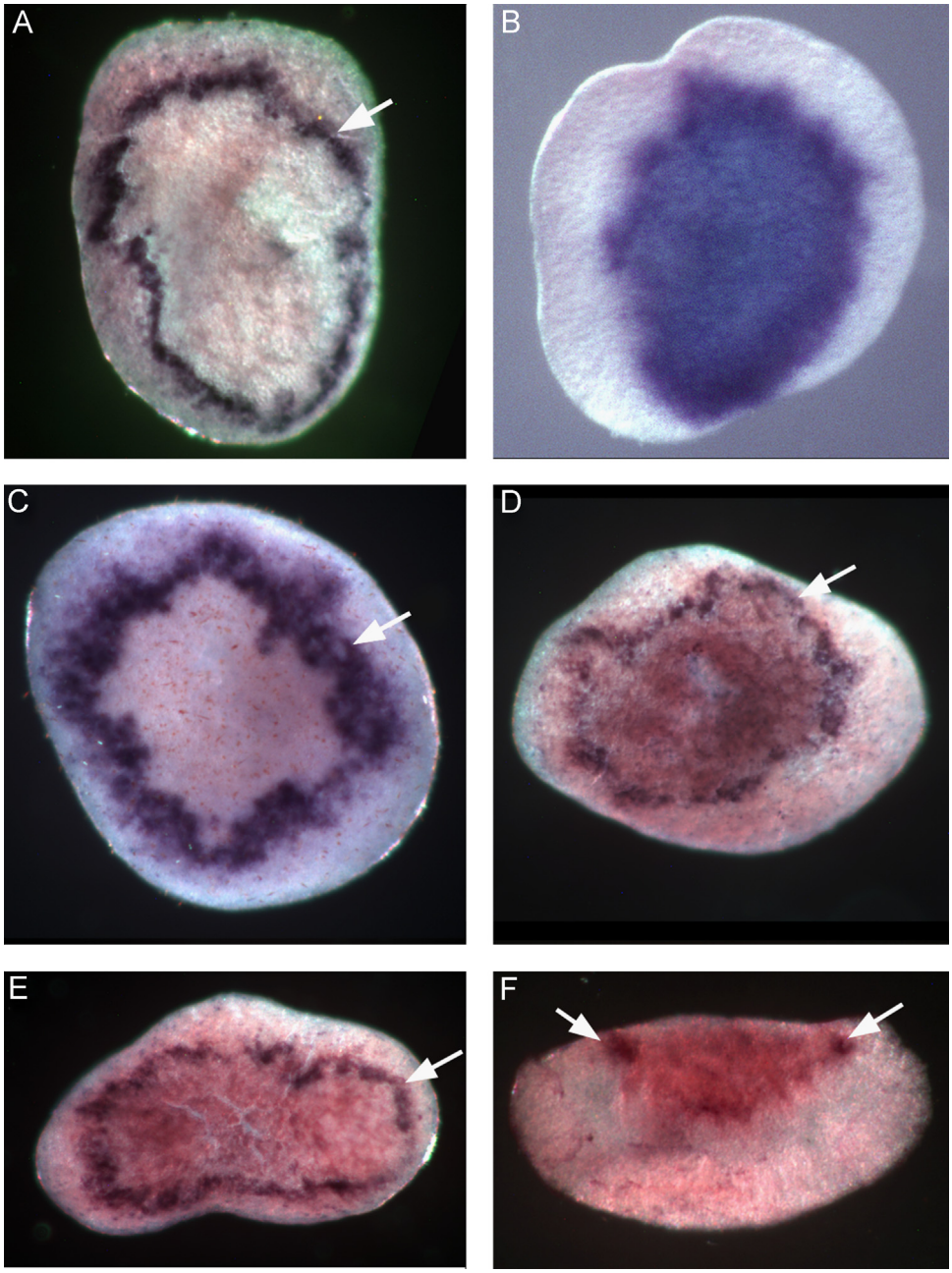

Fig. 3. In situ hybridization of *Amsnail* and *Ambra* singly and together reveal that *Ambra* demarcates the region of *Amsnail* expression. (A) Expression of *Ambra* on a pre-gastrulation embryo. (B) Expression of *Amsnail* on an embryo of similar size and shape. (C) A two color double in situ reveals the relationship between the two expression patterns. *Brachyury* expression is purple, *snail* expression is orange. The orange particles are the result of PVA intensification. (D and E) Single color, double in situs reveal the relationship between expression of the two genes more clearly. (F) Vertical section of an embryo like those shown in D and E reveals darker ectodermal spots of *brachyury* expression framing the *snail*-expressing, invaginating endodermal tissue. Arrows indicate *brachyury* expression.

cells on the central base of the polyp (Fig. 4G and H), where calcification is beginning, and then later along the septa, which are also sites of calcification (Fig. 4I–K).

Chordin expression in Acropora

Amchd expression was only detected from immediately prior to blastopore closure until the early planula stage; thus this gene has a narrow window of developmental expression in comparison with the other genes considered here. The earliest expression detected was in cells or groups of cells scattered asymmetrically around the blastopore (Fig. 5A). As development continues the expressing cells become restricted to one side of the blastopore and their number rises, forming a nearly solid zone of expression (Fig. 5B and C) that is clearly restricted to the ectoderm (Fig. 5D). Later in development, expression becomes stronger and the expression domain expands until it covers approximately one third of the surface of the oral half of the planula (Fig. 5E–H). At this point (early planula), expression is still tightly restricted to the ectoderm (Fig. 5I). Thereafter expression fades rapidly to below detection limits by the late planula stage.

Goosecoid expression in Acropora

Goosecoid expression first becomes clearly apparent after blastopore closure, as the spherical gastrula begins to elongate

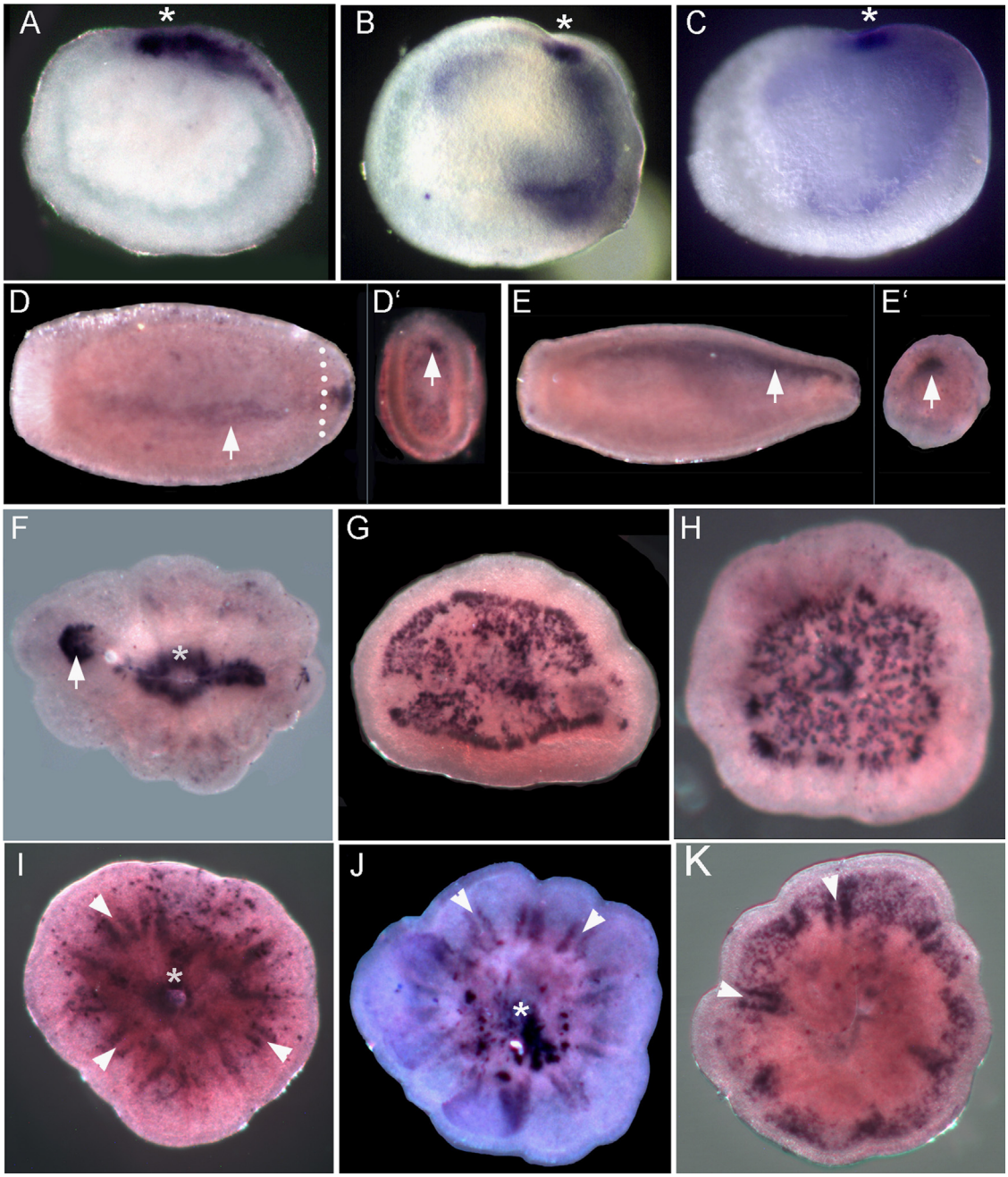

Fig. 4. Expression of *Ambmp2/4* during *Acropora* development. As previously reported (Hayward et al., 2002), expression begins as ectodermal spots limited to one side of the blastopore. The spots then expand and coalesce, forming a zone of continuous expression adjacent to the blastopore, as shown in (A). Expression then starts to appear in the endoderm as well (B and C), partly mediated by movement of expressing cells, but perhaps also by upregulation there. (D and E) Later, as the planula develops, expression becomes limited to a single endodermal stripe running the length of the larva (arrows). (D′ and E′) Transverse sections of the corresponding embryos, sectioned in the plane of the white dots in D, and viewed end-on. (F) This early primary polyp retains expression around the mouth as well as in the peripheral endoderm (arrow). (G and H) These primary polyps are viewed from the aboral end (i.e. from the surface that normally faces the substratum). The central portion of the base of each shows *Ambmp2/4* expression, perhaps associated with the beginning of calcification there. (I) Primary polyp, viewed from the oral side, with paired lines of expression marking the sides of each of the calcifying septa (arrowheads) and a minor area of expression around the mouth. (J) A polyp, viewed from the oral side, and lit with transmitted light, more clearly shows the paired stripes of expression (arrowheads). (K) Another polyp, viewed aborally, clearly shows that *Ambmp2/4* expression associated with the septa does not extend to the periphery of the primary polyp; there is an expression-free zone of ectoderm at the periphery. Asterisks indicate the position of the blastopore (A–C) or oral pore (F, I and J).

into a pear-shaped early planula larva. At that time expression is restricted to the endoderm and is strongest at the aboral end (Fig. 6A). As the planula continues to elongate, expression continues on that side of the embryo, and becomes limited to a well-defined stripe while also forming a second less-complete stripe on the side diametrically opposite (Fig. 6B–E). In some embryos there also appears to be a thin zone of endodermal expression lining the cylindrical planula; in these embryos the two stripes stand out as thicker, more strongly expressing areas (Fig. 6D). When viewed end-on these two stripes consistently differed from each other (Fig. 6F), one forming a much broader zone (Fig. 6D and F–I). This expression continues post-settlement and the limits of expression coincide with segmental boundaries in the post-settlement polyp (Fig. 6H and I).

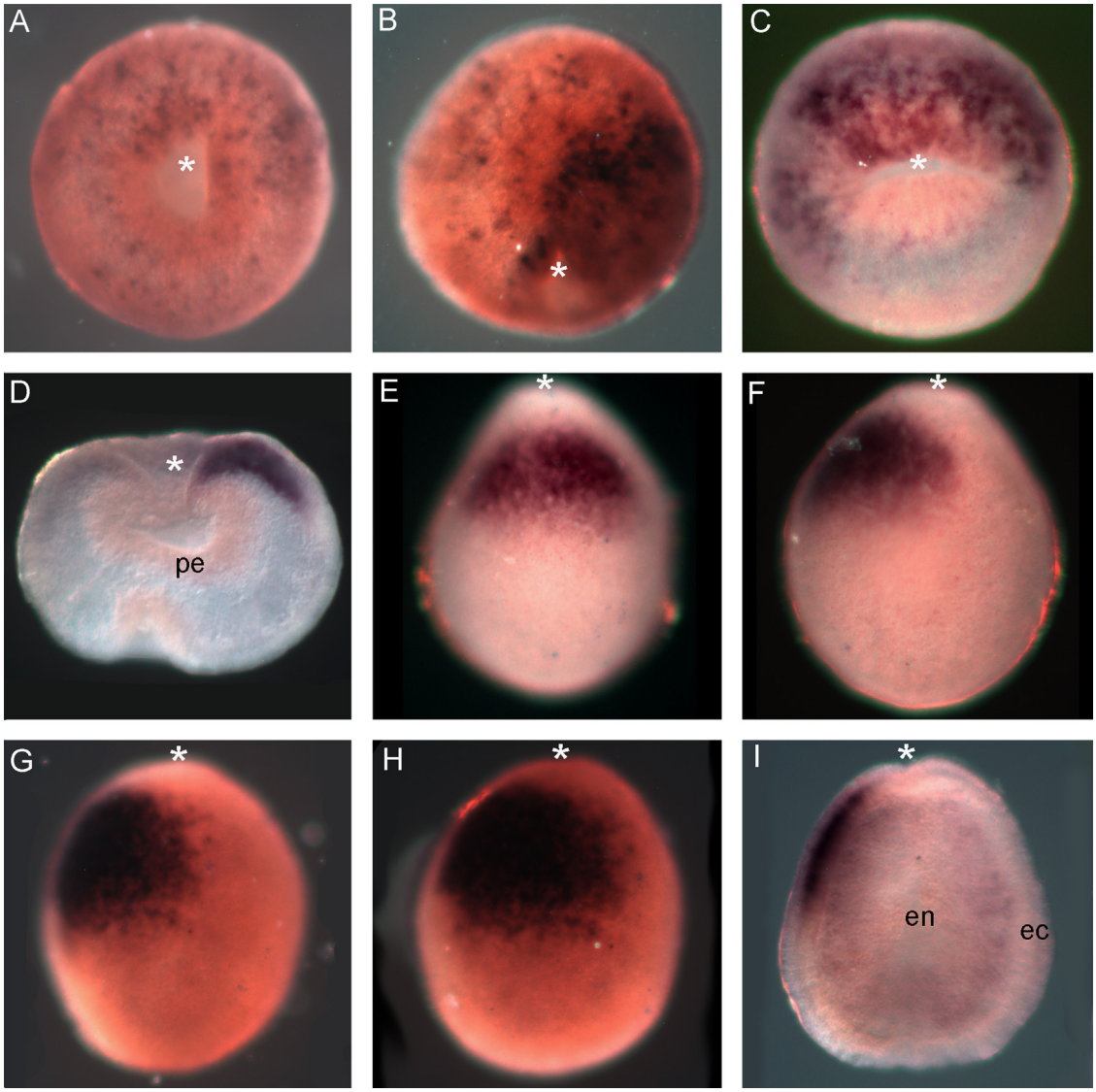

Fig. 5. Expression of *Amchd* during *Acropora* development. (A) Patchy, relatively weak, expression begins as the blastopore is closing, concentrated on one side of the blastopore. (B and C) Expression increases and becomes relatively contiguous on one side of the blastopore. (D) This expression is clearly ectodermal. (E–H) Expression continues, increasing in area and intensity, as the formerly spherical embryo elongates along the oral–aboral axis. (I) Expression remains limited to the ectoderm, and then as the larva continues to elongate, fades away. Abbreviations: ec-ectoderm, en-endoderm, pe-presumptive endoderm. The asterisk marks the blastopore in A–D, which closes at the gastrula stage and then reopens as the oral pore (asterisk in E–I) as the embryo elongates.

Forkhead expression in Acropora

The earliest detectable expression of *forkhead* (*Amfkh*) was seen to surround the oral pore as it opens immediately after gastrulation (Fig. 7A). There is a short time window, as the spherical gastrula is starting to elongate, during which dispersed ectodermal cells express *Amfkh* (Fig. 7B and C arrowheads). Expression associated with the oral pore continues as the planula elongates (Fig. 7D–F). As shown in Fig. 7G–J, the oral expression of *Amfkh* is associated with the pharyngeal ectoderm up until settlement. Although expression remains strictly ectodermal (ec), the expressing tissue is intimately associated with the developing septa, which are endodermal (Fig. 7I and J; en). Expression continues in the pharyngeal ectoderm post-settlement (Fig. 7K–N). The expressing structures curving outward from the pharynx post-settlement are the developing mesenterial filaments, structures that are extensions of the pharyngeal ectoderm and grow downward (aborally) into the gastrovascular cavity. These filaments are believed to play a role in subduing and digesting prey and, in polyps on the adult colony, extend down the canal within which each polyp is located.

Discussion

Although the molecular genetics of gastrulation has been extensively investigated in a range of bilaterians, and some regulatory mechanisms appear to be widely conserved, the extent to which these principles and mechanisms can be extrapolated to non-bilaterians is unclear. To date, the most thoroughly investigated non-bilaterian is the sea anemone, *Nematostella*, the only anthozoan for which an extensive dataset is available. Like *Nematostella*, *Acropora* is a hexacorallian anthozoan but their evolutionary divergence (approximately 500MYA) is comparable to that of fish and humans (Shinzato et al., 2011). Thus, perhaps the most surprising aspect of the data presented here is the extent to which the expression patterns seen in *Acropora* for key developmental regulators resemble those that have previously been

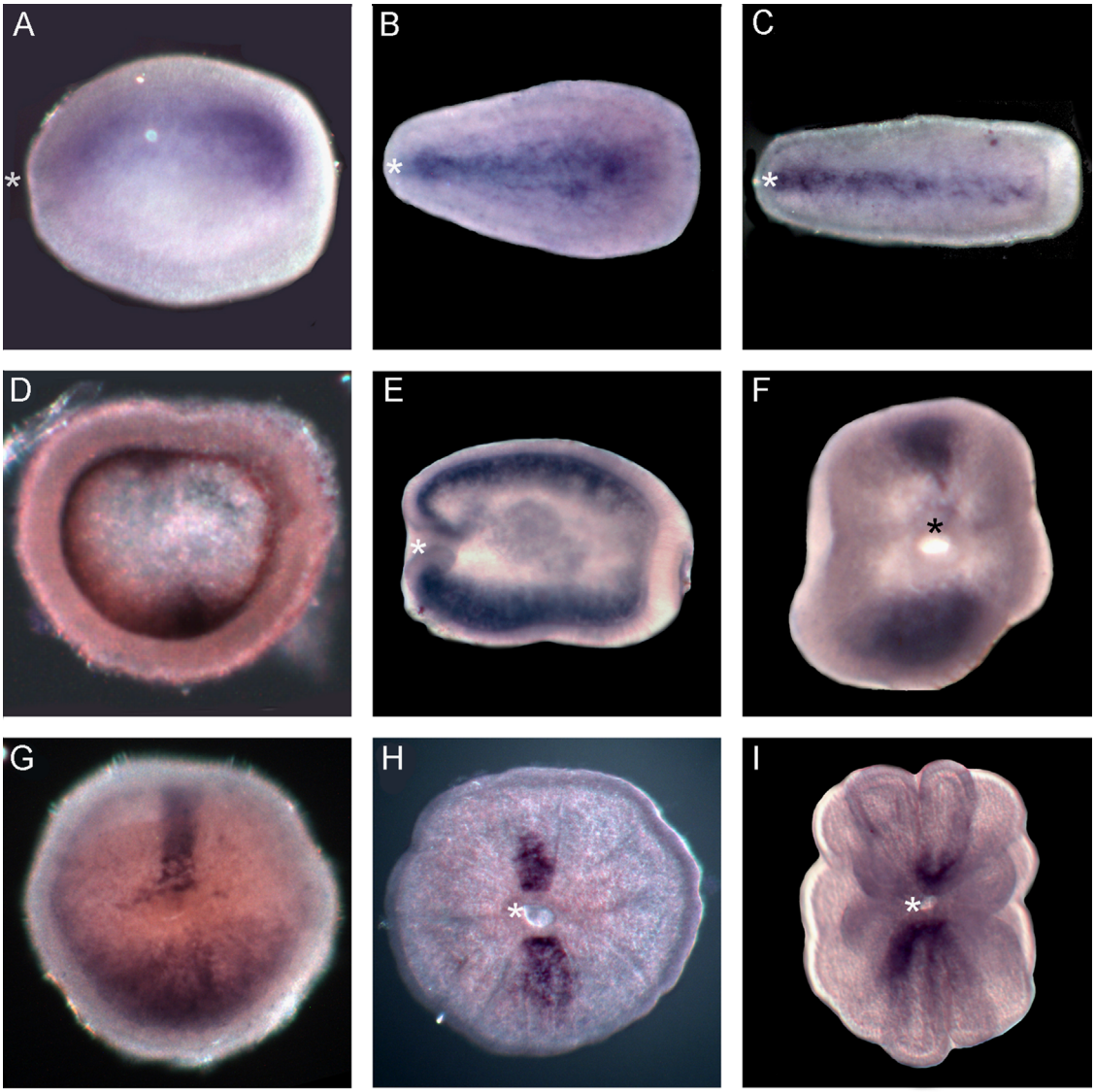

Fig. 6. Expression of *Amgsc* during *Acropora* development. (A) *Amgsc* expression is first apparent in the endoderm along one side of the early planula as it begins to elongate. Expression is strongest at the aboral end. The oral pore is marked with an asterisk in this and the following panels. (B and C) With further development and elongation expression increases, with the strongest expression now at the oral end of the stripe. (D–G) In the late planula a second stripe appears, opposite the first. (D) Cross-section of a late planula, clearly showing that expression is endodermal and the two stripes lie opposite each other. In this specimen there appears to be a narrow zone of expression in the endoderm lining the entire embryo inside of the mesoglea. (E) Strongly expressing stripes of endoderm are apparent in this longitudinal section of a planula larva. (F and G) Planula larvae viewed from the intact oral end, showing the differing size and shape of the two endodermal stripes. (H–I) Successively older primary polyps, showing that the differences between the shapes of the two areas of expression are maintained post-settlement, although expression gradually fades from the periphery until the only remaining expression is adjacent to the oral pore.

reported in *Nematostella*, despite marked developmental and morphological differences. Apparent differences in gene expression patterns between the two species are summarized in Fig. 8.

Although the gene expression profiles are generally very similar, a developmental peculiarity of *Acropora* and some other complex corals, formation of the prawn chip, may clarify the probable roles of *brachyury* and *forkhead* in cnidarians. During gastrulation in *Nematostella*, *forkhead* is expressed at the ectodermal margin that is immediately adjacent to the presumptive endoderm, the latter being marked by *snail* expression (Fritzenwanker et al., 2004; Martindale et al., 2004). On this basis, and on the basis of *NvsnailA/NvFoxA* double in situs (Magie et al., 2007) *forkhead* has been hypothesized to functionally demarcate the ectoderm/endoderm boundary in *Nematostella*. In *Acropora*, however, *forkhead* is expressed only at blastopore closure/oral pore opening (Fig. 7A), well after germ layer demarcation has occurred. In *Nematostella*, gastrulation occurs from a near spherical blastula. By contrast, the extended surface undergoing gastrulation in the case of the equivalent stage in *Acropora* (the "prawn chip" pre-gastrula) and the protracted nature of the process, greatly facilitate observing the detail of *Ambra* expression, and it is this gene that appears to define the ectoderm/endoderm boundary (Fig. 3). This observation is consistent with the idea that the ancestral function of Brachyury is as a regulator of morphogenetic cell movements—a role that is also supported by functional data for the *Mnemiopsis brachyury* gene (Yamada et al., 2010). Expression data do not rule out such a role for *Nvbra*, which is co-expressed with *Nvfkh* around the blastopore. Indeed, as pointed out by Fritzenwanker et al. (2004), *brachyury* and *forkhead* are "an evolutionarily ancient synexpression group in Eumetazoa" with both genes involved in formation of dorsal mesoderm in *Xenopus*. The early expression of *Nvbra* does not rule out a role similar to that hypothesized here for *Ambra*. Given all of the other similarities in gene expression pattern (and presumed function)

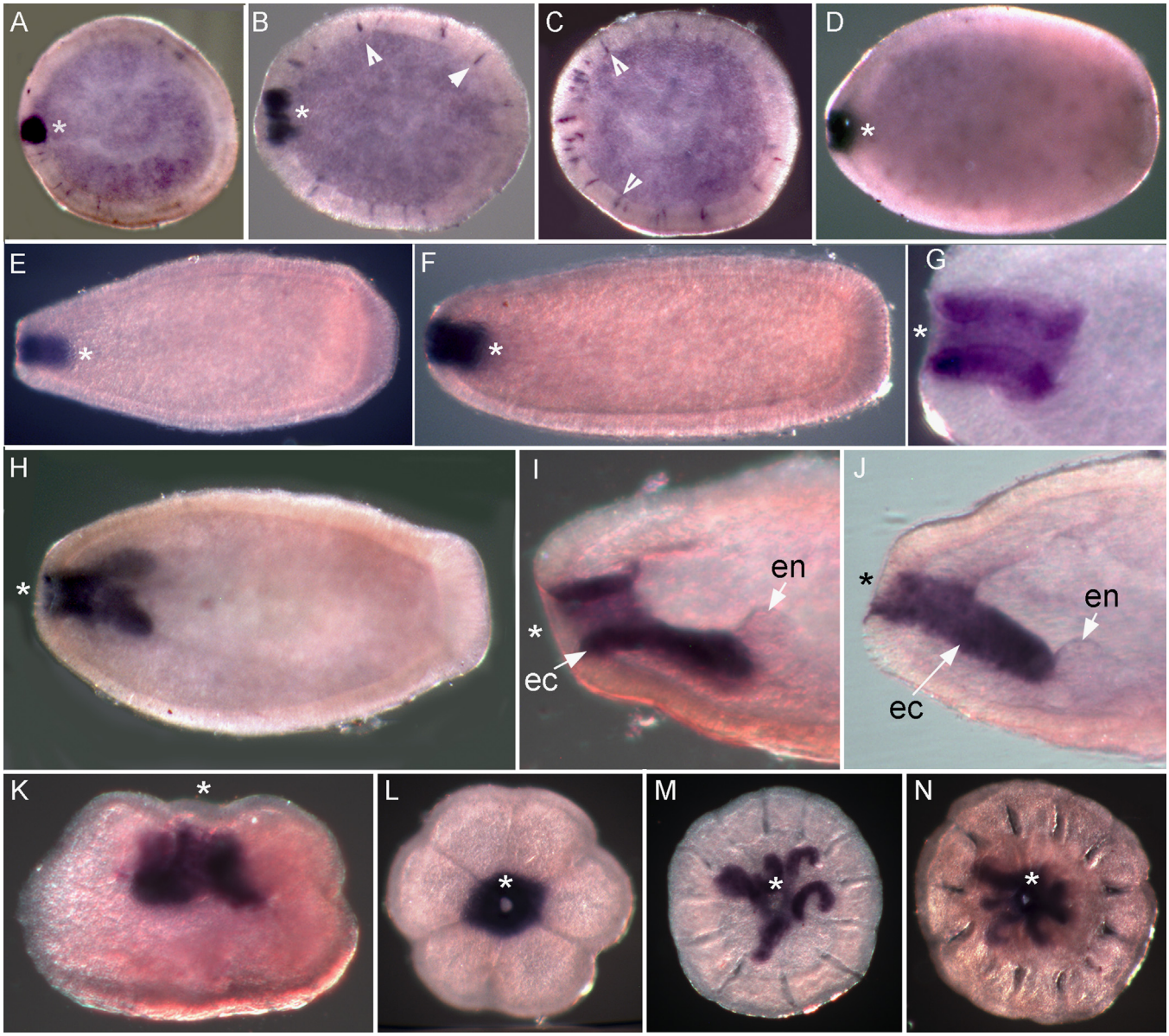

Fig. 7. Expression of *Amfkh* during *Acropora* development. (A) The earliest *Amfkh* expression is in the pharyngeal ectoderm of the oral pore as it starts to open. (B and C) Soon after the oral pore expression other, isolated, expressing cells appear in the ectoderm (arrowheads). (D–F) Expression in the pharyngeal ectoderm continues as the planula elongates. (G) This higher magnification view, passing through the axis of the hollow cylinder formed by the pharynx, confirms that expression is limited to the pharyngeal ectoderm. The strongly expressing stripes on either side are the cut walls of the cylinder, while the less strongly-expressing central region is the uncut far wall of the cylinder. (H–J) As growth continues, the inner end of the pharynx expands and elongates, held against the outer walls of the planula by the developing septa, to which it is connected. (I and J) The strongly-expressing ectoderm (ec) of the pharynx is clearly demarcated from the unstaining endoderm (en) of the developing septa. (K and L) Expression in the oral ectoderm continues, apparently unchanged, in the primary polyp after settlement. (M and N) After settlement and metamorphosis the pharyngeal ectoderm continues to grow downward (aborally) and outward, extending a filament along the edge of each of the septa. Asterisks indicate the position of the oral pore.

reported here, the role of *Nvbra* in embryonic development merits more detailed functional investigation.

Other differences in expression patterns of orthologous genes between *Nematostella* and *Acropora* are summarized in Fig. 8. One interesting morphological difference between the two species is the absence of the apical tuft of sensory cilia from the aboral end of the latter, presumably as an evolutionary consequence of the extensive modifications required to enable this end of the planula to begin secreting the calcium carbonate skeleton immediately post-settlement. Correlating with the absence of an apical tuft, the spot of *goosecoid* expression seen in the *Nematostella* endoderm immediately under the apical tuft is also missing in *Acropora*. Although the *Acropora* and *Nematostella forkhead* genes are expressed around the oral pore in similar patterns, two differences were observed between the two species with respect to expression elsewhere. First, shortly after blastopore closure in *Acropora* there is a transient period of expression in scattered ectodermal cells (Fig. 7A–C), but no corresponding pattern has been reported in *Nematostella*. A second major difference is the striking "spider's legs" pattern of expression in the mesenterial filaments seen extending outward from the mouth in the post-settlement polyp of *Acropora* (Fig. 7M). *Nvfkh* expression has been followed into the primary polyp of *Nematostella* where it is expressed in the pharyngeal ectoderm, which is sharply demarcated from the endoderm of the septa to which it is attached (Fritzenwanker et al., 2004), just as it is in the late planula of *Acropora* (Fig. 7I and J). Perhaps if expression had been followed later in *Nematostella* the situation would be very similar to that seen in *Acropora* (Fig. 7M and N) with the mesenterial filaments continuing to express *forkhead* as they elongate.

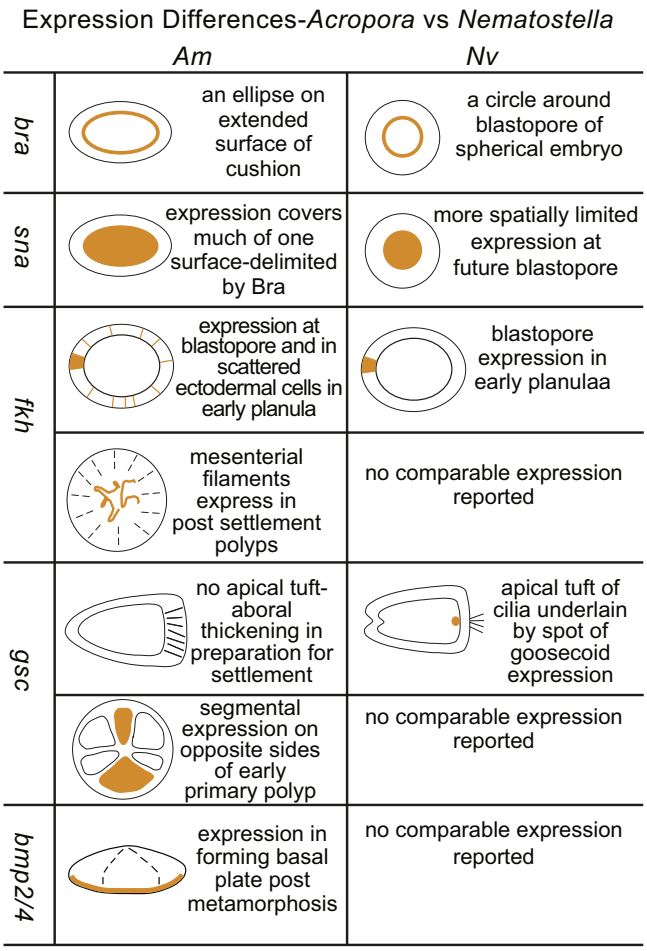

Fig. 8. Summary of the key differences and points of contrast between the expression patterns in *Acropora millepora* (*Am*) and *Nematostella vectensis* (*Nv*) of the genes considered in this paper. These are based on our observations (*Am*) and the currently available literature (*Nv*), so some of the differences, e.g. the apparent absence of isolated *Nvfkh*-expressing ectodermal cells, may be due to an absence of observation at the critical time. Developmental stages referred to here, e.g. "cushion", are shown in Fig. 1.

Notwithstanding the differences described above, in most respects developmental expression of the genes considered here is strikingly similar between *Acropora* and *Nematostella*. Although *Acropora* lacks an apical tuft, and the correlated *gsc* expression, the overall pattern of *gsc* expression is similar to that described here for *Acropora*, i.e. two endodermal stripes, running the length of the planula, on opposite sides of the directive axis (Matus et al., 2006a). In *Nematostella*, *gsc* expression has only been described through the late planula, but in *Acropora* these two stripes maintain their character post-settlement for a considerable time, with the limits of their expression corresponding to developing septa.

The early expression patterns of *bmp2/4* and *chordin* are very similar in *Nematostella* and *Acropora*, although we have not yet confirmed that these transcripts are co-localized in the coral (as they are in the sea anemone). As Chordin is an antagonist of BMP2/4, and the genes are expressed on opposite sides of the dorsal/ventral axis in *Drosophila* and chordates, co-localized expression is counter-intuitive. However, the sea urchin *Paracentrotus lividus* provides a precedent for what has been observed in *Nematostella*, in that BMP2/4 is able to act as a morphogen along the D/V axis despite being co-expressed with Chordin (Lapraz et al., 2009), as it is in *Nematostella* (Rentzsch et al., 2006; Matus et al., 2006b). In the sea urchin, *bmp2/4* and *chordin* are both expressed ventrally, but the former triggers signaling only on the dorsal side (Lapraz et al., 2009), ventral signaling activity presumably being inhibited by Chordin. A similar mechanism may exist in *Nematostella*.

Finnerty et al. (2004) show *bmp2/4* expression in the planula larva of *Nematostella* viewed laterally and end-on, with expression in all eight septa. This contrasts with the situation in *Acropora*, where expression in the late planula is limited to a single endodermal stripe running the length of the body (Fig. 4D, D′ E, and E′), while expression along all septa, as seen in the *Nematostella* planula, only appears post-settlement (Fig. 4H). There is another possible difference in *bmp2/4* expression relating to the more dramatic metamorphosis of *Acropora* and the beginning of calcification immediately thereafter, which is the expression of *bmp2/4* by cells on the aboral side of the pedal disc. This is the area where calcification begins, with the laying down of a basal plate on which calcified septa are then erected. The calicoblastic tissue overlying the developing calcified septa of *Acropora* also expresses *Ambmp2/4*, but the septa of noncalcifying *Nematostella* express *Nvbmp2–4* as well, so the significance of this expression is yet to be established. However, Zoccola et al. (2009) have localized BMP2/4 in adult *Stylophora pistillata* specifically to the calicoblastic ectoderm, so the possible involvement of BMP2/4 in coral calcification is certainly worthy of further study.

Although in corals dramatic changes in morphology occur between the elongate swimming planula and the settled polyp, comparisons between *Acropora* and *Nematostella* of the expression patterns of the genes considered here suggests that the reorganization associated with the dramatic metamorphosis of *Acropora* may not be extensive, particularly at the oral end. Several expression patterns, and sequences of patterns, seem to continue essentially uninterrupted across *Acropora* settlement and metamorphosis. These include expression around the oral pore/mouth for *Ambra*, *Amgsc*, and *Amfkh* and the *Ambmp2/4* septal expression that appears post-settlement in *Acropora*, but in the planula of *Nematostella*.

Röttinger et al. (2012) have suggested the existence of an ancestral endomesoderm kernel to the gastrulation Gene Regulatory Network (GRN) in *Nematostella*, consisting of OtxA, OtxB, OtxC, Bra and FoxA. Yasuoka et al. (2009) presented evidence that Nvlxh1 is also part of the *Nematostella* GRN. We have examined the expression of *AmotxA* (de Jong et al., 2006), which is an ortholog of *NvotxA*, *AmotxB* (unpublished) a possible ortholog of *NvotxC*, *Amfkh* and *AmBra* (both described here) and the expression patterns of all are consistent with their orthologs as described by Röttinger et al. (2012) and other papers cited above.

Finally, the expression patterns described here, particularly for *Ambmp2/4* and *Amgsc*, demonstrate once again the fundamental bilaterality of the Anthozoa. However, despite apparent similarities between expression patterns of some homologous genes, correspondence between the cnidarian axes and those of the Bilateria remains equivocal.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.01.006.

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