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ORIGINAL ARTICLE

Developmental expression of *COE* across the Metazoa supports a conserved role in neuronal cell-type specification and mesodermal development

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Abstract The transcription factor *COE* (collier/olfactory-1/early B cell factor) is an unusual basic helix–loop–helix transcription factor as it lacks a basic domain and is maintained as a single copy gene in the genomes of all currently analysed non-vertebrate Metazoan genomes. Given the unique features of the *COE* gene, its proposed ancestral role in the specification of chemosensory neurons and the wealth of functional data from vertebrates and *Drosophila*, the evolutionary history of the *COE* gene can be readily investigated. We have examined the ways in which *COE* expression has diversified among the Metazoa by analysing its expression from representatives of four disparate invertebrate phyla: Ctenophora (*Mnemiopsis leidyi*); Mollusca (*Haliotis asinina*); Annelida (*Capitella teleta* and *Chaetopterus*) and Echinodermata (*Strongylocentrotus purpuratus*). In addition, we have studied COE function with knockdown experiments in *S. purpuratus*, which indicate that COE is likely to be involved in repressing serotonergic cell fate in the apical ganglion of dipleurula larvae. These analyses suggest that *COE* has played an important role in the evolution of ectodermally derived tissues (likely primarily nervous tissues) and mesodermally derived tissues. Our results provide a broad evolutionary foundation from which further studies aimed at the functional characterisation and evolution of *COE* can be investigated.

Keywords COE · Ectoderm · Mesoderm · Neurogenesis · Metazoa

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Introduction

Understanding the molecular events that supported the morphological expansion of metazoan body plans is a fundamental aim of evo-devo studies. With the increasing availability of whole genome datasets from a variety of metazoan taxa (Sea Urchin Genome Sequencing Consortium, 2006; Putnam et al. 2007, 2008; Chapman et al. 2010; Srivastava et al. 2010), a growing challenge is to identify common (and truly homologous) genomic outputs and to assess the similarities and differences in their functions across a phylogenetically informative spread of representatives. Such studies hold the potential to provide deep insight into the features that common ancestors were likely to have possessed, and the events that accompanied the genesis and expansion of new clades of animals in the pre- and early Cambrian (Martindale 2005; Wheeler et al. 2009). The identification of deeply conserved developmental patterning mechanisms, such as the Hox code (or at least the precursors to such genes), support the notion that the metazoan ancestor possessed a developmental toolkit of genes that has been modified in lineage-specific ways to generate disparate body plans (Carroll et al. 2001; Larroux et al. 2006; Adamska et al. 2007). Related to the notion of a conserved developmental toolkit is the suggestion that cell types have specific 'molecular fingerprints' that can be used to identify cellular homologies across taxa (Arendt 2008). Explicitly, this concept interprets the expression of orthologous genes in similar tissues at similar times as an indication of cellular homology (Arendt 2005); however, it must be pointed out that when comparing the expression of homologous genes between cells, tissues or organs, structures that share homologous gene expression are not necessarily homologous in themselves; for example, orthologs of the gene *Distal-less* pattern many non-homologous structures across the Metazoa. This important point led Claus Nielsen to coin the term 'homocracy' in order to distinguish between non-homologous structures patterned by homologous genes (Nielsen and Martinez 2003). True homology of cells, tissues or organs is more convincingly demonstrated on the molecular level by the synexpression of a demonstrable gene regulatory network (GRN), as such a network is presumably less likely to be co-opted to a non-homologous structure than an individual gene (Davidson 2006). A clear example of this was provided by Dunn et al. (2007) to demonstrate that apical sensory structures of larval urchins and gastropods are unlikely to share homology. They conducted a GRN analysis to test the hypothesis that the apical tufts of larval urchins and gastropods were homologous. In many marine invertebrates, the apical end of the larval body plan consists of what is termed the apical organ, which generally consists of two components: the ciliated apical tuft and the apical ganglion. Because the apical tufts of urchins and gastropods use different GRNs for their specification, Dunn et al. (2007) argued that they were the result of convergent evolution, at least in these two taxa, sometime in the late Precambrian (Peterson 2005).

The transcription factor COE was first isolated from vertebrate models and ascribed a role in regulating the expression of olfactory (Wang and Reed 1993) and immune cell fates (Hagman et al. 1993; Travis et al. 1993). A *Drosophila* homolog of *COE* was isolated shortly after the vertebrate gene (Crozatier et al. 1996) and has been found to play a variety of roles, including head segmentation (Crozatier et al. 1999), wing patterning (Crozatier et al. 2002), muscle specification (Crozatier and Vincent 1999) and immune cell specification (Crozatier et al. 2004). Vertebrate paralogs of COE have also been reported to play roles in limb development (Mella et al. 2004), and a recent analysis of ascidian development suggests that the last common ancestor of the Chordata may have used COE to specify pharyngeal mesoderm development (Stolfi et al. 2010). Pang et al. (2004) reported that *COE* expression is restricted to the apical organ (the ciliated and innervated structure at the aboral end of the developing planula larva) of the cnidarian *Nematostella vectensis*, suggesting that the ancestral eumetazoan function of COE was to specify chemosensory neurons. This last study provides an evo-devo foundation from which the interpretation of various studies demonstrating a role for COE in vertebrate (Wang and Reed 1993; Garel et al. 1997; Dubois et al. 1998; Pozzoli et al. 2001; Corradi et al. 2003; Garcia-Dominguez et al. 2003) and invertebrate (Prasad et al. 1998; Baumgardt et al. 2007) neurogenesis can be placed.

These previous studies, primarily focused on vertebrate models, *Drosophila* and *C. elegans*, indicate that COE is highly pleiotropic and plays important roles in the specification of cell types originating from all three embryonic germ layers (Liberg et al. 2002). Here, we have isolated the *COE* homologues from a variety of invertebrate metazoans and investigated their expression patterns with the goal of uncovering conserved and divergent features of *COE* expression. *COE* displays complex expression patterns in all taxa investigated; however, broad commonalities and differences can be identified. Our results suggest that *COE* may be part of a molecular fingerprint specific to a subset of ectodermally derived cell types, possibly chemosensory, non-serotonergic and located in the anterior ciliated structures. We stress that this proposed cell type homology neither requires nor implies homology of apical organs; apical ganglia may consist of homologous cell types, but apical tufts could be the products of convergent evolution (see "Results and discussion"). Early mesodermal expression of *COE* in ctenophores, protostomes and vertebrates suggests another shared function may relate to

the development of the haematopoietic system and/or connective tissue differentiation (Hagman et al. 1993; Akerblad et al. 2002).

Materials and methods

Gene isolation, whole mount in situ hybridization and MASO injections

For all organisms, previously published protocols for RNA extraction, gene isolation and RACE PCR were employed (for example see Jackson et al. 2005; Matus et al. 2006; Dunn et al. 2007; Thamm and Seaver 2008). Where genomic or EST sequences were available, these were first searched and used to design gene-specific primers for RACE PCR. Organism-specific methods for whole mount in situ hybridizations (WMISH) were employed for the ctenophore *Mnemiopsis leidyi* (Pang and Martindale 2008), the tropical abalone *Haliotis asinina* (Jackson et al. 2007), the annelids *Capitella teleta* (Thamm and Seaver 2008; Blake et al. 2009), previously *Capitella* sp. I (Blake et al. 2009) and *Chaetopterus* (Boyle and Seaver 2010), and the sea urchin *Strongylocentrotus purpuratus* (Dunn et al. 2007). Newly obtained *COE* sequences have been deposited in GenBank HQ529594 - HQ529597. *SpNK2.1* morpholino anti-sense oligonucleotide (MASO) and *SpCOE*-MASO injections were performed as described in Takacs et al. (2004). The sequences of the *SpCOE*-MASO and the standard control MASO are, respectively 5'-GATATGACGGTCTCCATCT TACTCC-3' and 5'-CCTCTTACCTCAGTTACAATT TATA-3'. All primer sequences are available from the authors upon request.

Immunofluorescence and imaging

S. purpuratus embryos were fixed in 4% paraformaldehyde in filtered sea water for 15 min at room temperature and then post-fixed for 1 min in −20°C methanol. Fixed embryos were rinsed in phosphate-buffered saline (PBS) then incubated in PBS containing 5% normal lamb serum and 0.01% Tween 20 for 30 min at room temperature to reduce non-specific binding. Specimens were incubated in primary antibody for 16 h at 4°C with rabbit anti-serotonin (Chemicon, CA) (1:500) and mouse or rat anti-synaptogamin B (1:800) according to Nakajima et al. (2004). Embryos were rinsed in PBS and incubated in goat anti-rabbit Alexa 488 (1:800) and goat anti-rat Alexa 568 (1:1500) (Molecular Probes). Preparations were examined and photographed with a Leica DM 6000 B epifluorescence microscope (Leica Microsystems) and Hamamatsu Orca-

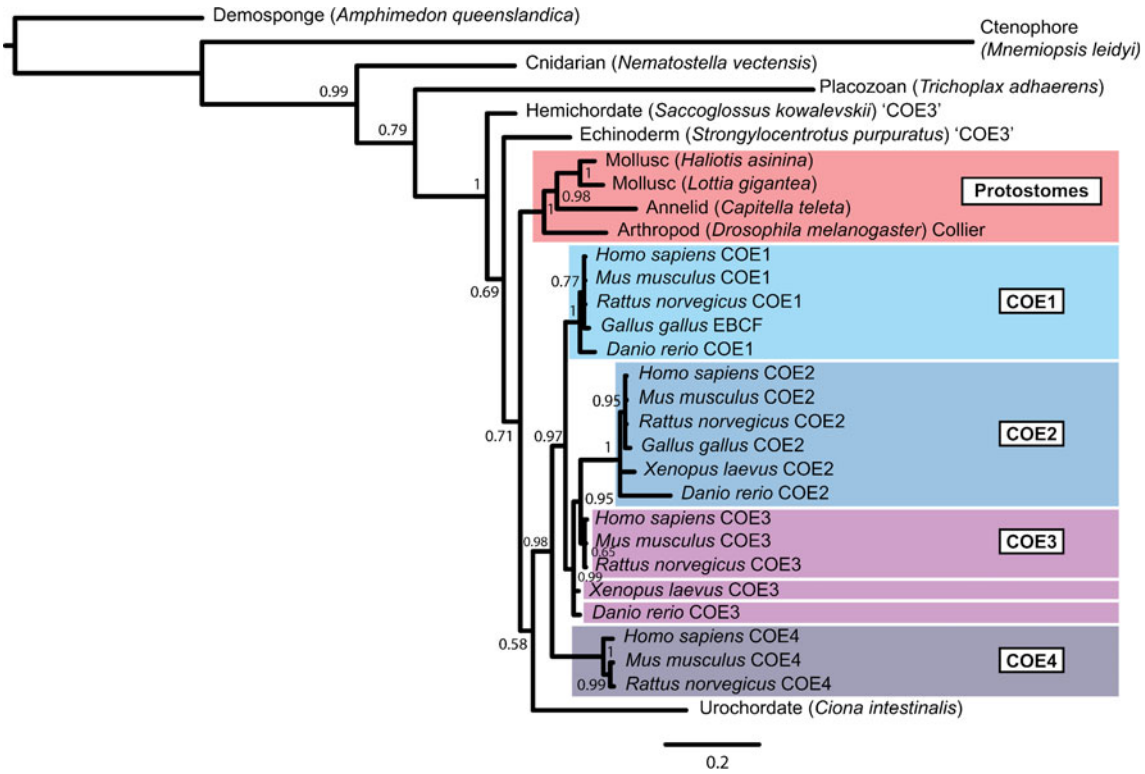

Fig. 1 Phylogenetic reconstruction of COE evolutionary history. The topology shown is a 50% majority rule tree derived from a Bayesian analysis of unambiguously aligned positions (see supplementary material for alignment). Posterior probabilities following 1.8 million generations are indicated

ER camera (Hamamatsu Photonics) using Openlab 4.0.4. To determine the number of immunoreactive cells present, a double-blinded procedure was utilised such that näive observers examined specimens on a monitor focusing through the specimen to identify all immunoreactive cells. Statistical analyses were conducted using GraphPad Prism (4.03).

Phylogenetic analyses

COE homologues from a variety of metazoan taxa were retrieved from public databases following BLAST searches against GenBank and from whole genome draft assemblies of the placozoan *Trichoplax adhaerens* (see Supplementary Online Material for all accession numbers and genomic locations). These sequences were combined with full-length fragments isolated by RACE PCR and aligned using MUSCLE (Edgar 2004). Following manual adjustment (especially of the HLH region), the alignment was used to schematically represent conserved COE domains and was then processed by GBlocks (Talavera and Castresana 2007) to retrieve highly conserved positions for phylogenetic analysis. A Bayesian analysis of the resulting dataset was carried out with the parallel version of MrBayes (MrBayes v. 3.1.2p) on a Linux cluster at the Gesellschaft für wissenschaftliche Datenverarbeitung, Göttingen. The following settings were used: prset aamodelpr=mixed nchains=4 nruns=4 temp=0.2 printfreq=1000 samplefreq=1000 ngen=1800000. A burnin value of 25% was set to exclude non-convergent tree topologies.

Results and discussion

Phylogenetic analyses

We isolated *COE* orthologs from the ctenophore *M. leidyi*, the polychaete annelid *C. teleta* the gastropod mollusc *H. asinina*, and the polychaete annelid *Chaetopterus* (HQ529594 - HQ529597). Our bayesian analysis recovered previously recognised vertebrate COE subfamilies (COE1, COE2 and COE4), although the COE3 subfamily was not recovered as a monophyletic group (Fig. 1). The branching order of these vertebrate subfamilies is concordant with a recent phylogenetic analysis of COE (Daburon et al. 2008), with COE4 apparently representing the most ancestral-like paralog of the vertebrate *COE* genes. All non-deuterostome bilaterian invertebrates (traditionally referred to as protostomes, however, see Martindale and Hejnol 2009 for an alternative view) form a monophyletic group, with spiralian taxa forming a well-supported subgroup. Early branching taxa (notably ctenophore and placozoan representatives)

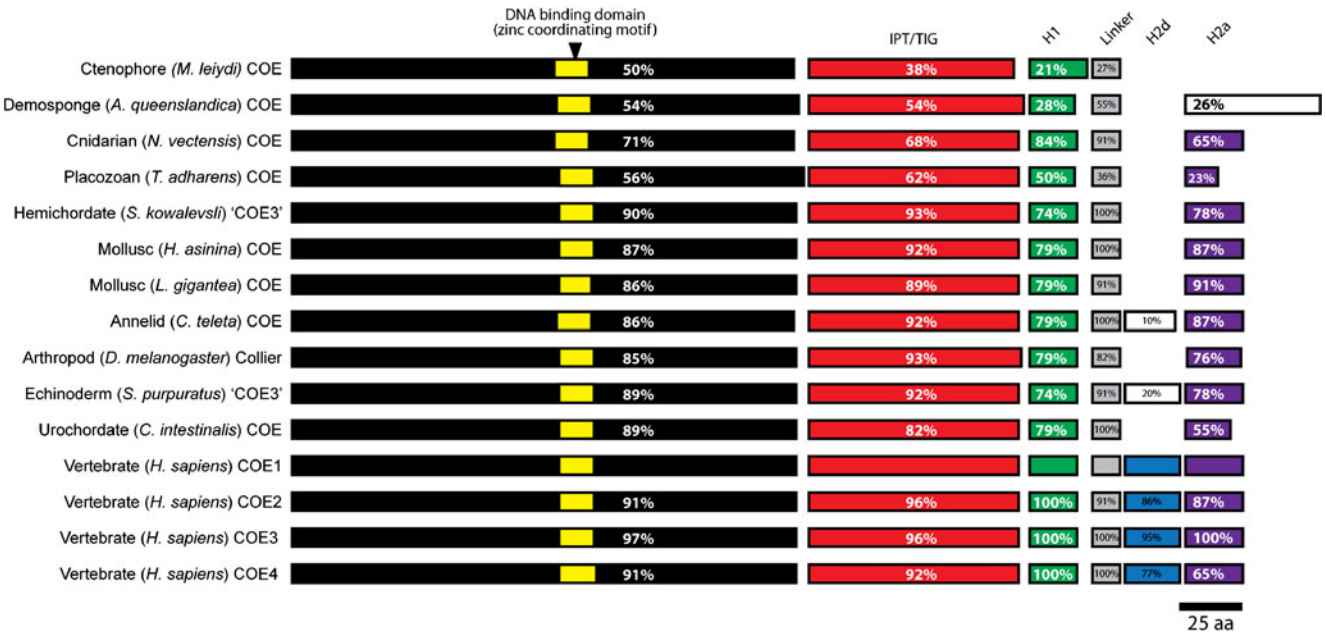

Fig. 2 Schematic representation of metazoan COE sequence architectures. The highly conserved nature of the DNA-binding domain (with the embedded zinc coordination motif), the IPT/TIG (immunoglobulin-like, plexins, transcription factors/transcription factor immunoglobulin) domain and the helix–loop–helix domains are indicated. Daburon et al. (2008) proposed that the second helix domain (H2d) is derived from the carboxyl-most helix domain (H2a), and we have followed this terminology here. Pairwise distances are indicated and were calculated using PAUP with human COE1 protein as the reference sequence. Only highly conserved alignable regions are shown, amino and carboxy regions are not included. All domain lengths are represented to scale

display conspicuously long branches. This trend was also revealed by calculating pairwise distances for each conserved domain using the human COE1 protein as a reference (Fig. 2). In agreement with Daburon et al. (2008), our alignments, which include a wider representation of early branching taxa, also support the hypothesis that in the vertebrate lineage one of the alpha helices, H2d, was derived from the carboxyl-most helix H2a (Fig. 2 and sequence alignment in Supplementary Online Material).

COE spatial expression

Ctenophore expression *MlCOE* expression begins at gastrulation; it is transiently expressed in the endomesodermal macromeres and in ectodermal micromeres associated with the blastopore (Fig. 3a, b). *MlCOE* expression persists in the macromeres and their daughters, the oral micromeres, which enter the blastocoel and give rise to future mesoderm (Fig. 3c, d). At later stages of gastrulation, *MlCOE* expression is maintained in these mesodermal cells as they migrate to a position underlying the apical ectoderm within the forming tentacle bulbs (Fig. 3e, g). At these stages, *MlCOE* transcripts are also transiently detected in invaginating pharyngeal cells at the oral pole (Fig. 3e–h). *MlCOE* was not detected in the apical organ at any stage of development, including the free-swimming (cydippid) stages.

Lophotrochozoan expression *COE* expression in lophotrochozoan larvae is more complex, although there are several shared expression domains in *H. asinina*, *Chaetopterus* and *C. teleta* (compare Figs. 4, 5, 6, 7, see detailed descriptions below). In contrast to *Nematostella vectensis* (Pang et al. 2004) and *M. leidyi*, all three lophotrochozoans express *COE* across a broad developmental time window. The three main domains of *COE* expression are in (1) subsets of mesoderm, (2) the developing nervous system and (3) small patches of ectoderm, including cells associated with the stomodeum and apical ectodermal cells. In all three lophotrochozoans, *COE* is expressed in a subpopulation of mesodermal cells (labelled as me in Figs. 4, 5, 6, 7). In *C. teleta*, *COE* is initially expressed in the mesodermal stem cells and their progeny (Fig. 4a, b), and soon becomes largely restricted to the mesodermal stem cells (Fig. 4e, f). In addition, there is a transient anterior–posterior wave of *COE* expression through the segmental trunk in the lateral mesodermal cell clusters (arrowheads, Fig. 5a–c). At any time point, there are one to two *COE*+ cell clusters visible on each side of the animal and one cluster/segment. In the other two lophotrochozoans (*H. asinina* and *Chaetopterus*), development of the mesoderm is less well characterised, and at this time, we do not know to what differentiated structures the *COE*+ populations of mesodermal cells contribute. In *H. asinina*, mesodermal expression of *COE* is evident in what are likely to be descendents of 4d (Hejnol et al. 2007), the mesodermal bands (Fig. 6a, b, i, j). In *Chaetopterus*, *COE* also is expressed in distinct subsets of trunk mesoderm (Fig. 7a, b, e, f, i, j). Shared mesodermal expression in the three lophotrochozoans examined is intriguing considering the mesodermal expression of *MlCOE* in the ctenophore *M. leidyi* (Fig. 3e) and the

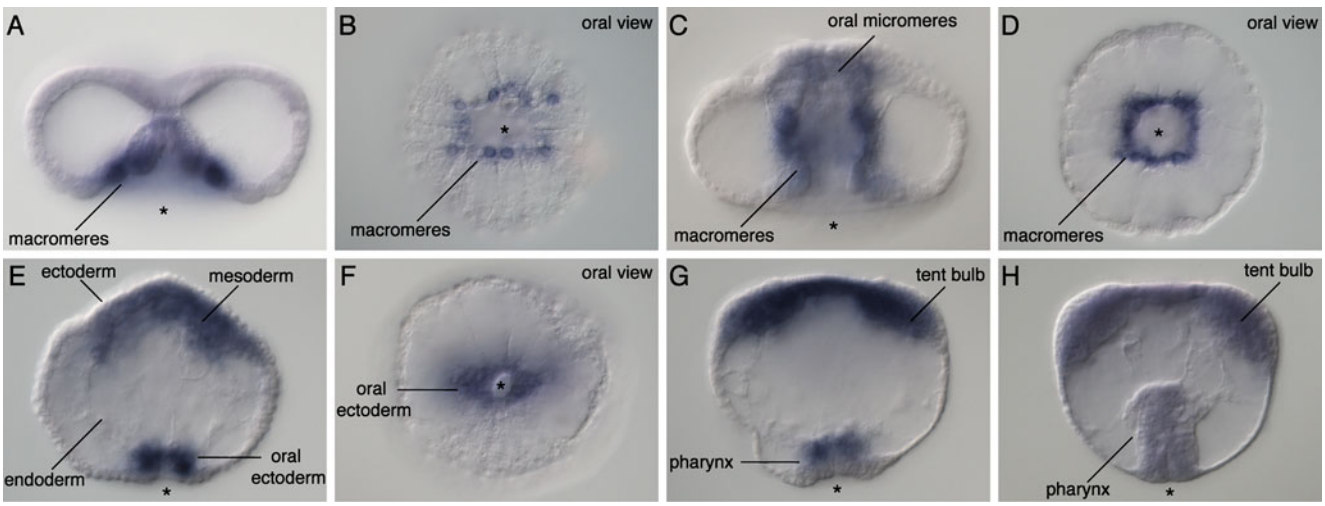

Fig. 3 Developmental expression of *MlCOE* in the ctenophore, *M. leidyi*. **a**, **c**, **e**, **g**, **h** Lateral view with the *asterisk* denoting the blastopore. **b**, **d**, **f** Oral view. **a**, **b** At 3 hpf, *MlCOE* is expressed in the macromeres (endoderm). **c**, **d** At 4 hpf, the *MlCOE*$^{+}$ macromeres have completely gastrulated and expression is present in the oral micromeres (mesoderm) which have entered the blastocoel. **e**, **f** At 6 hpf, *MlCOE* expression in the macromeres has decreased while expression remains in the descendents of the oral micromeres which line the aboral part of the blastocoel, as well as in oral ectoderm around the blastopore. **g** Expression is similar at 8 hpf in the mesoderm of the forming tentacle bulb and near the blastopore, which forms part of the pharynx. **h** At 9 hpf, when the comb plates have fully formed, *MlCOE* expression becomes more diffuse in the pharynx and tentacle bulb. In subsequent stages, we do not detect *MlCOE* expression

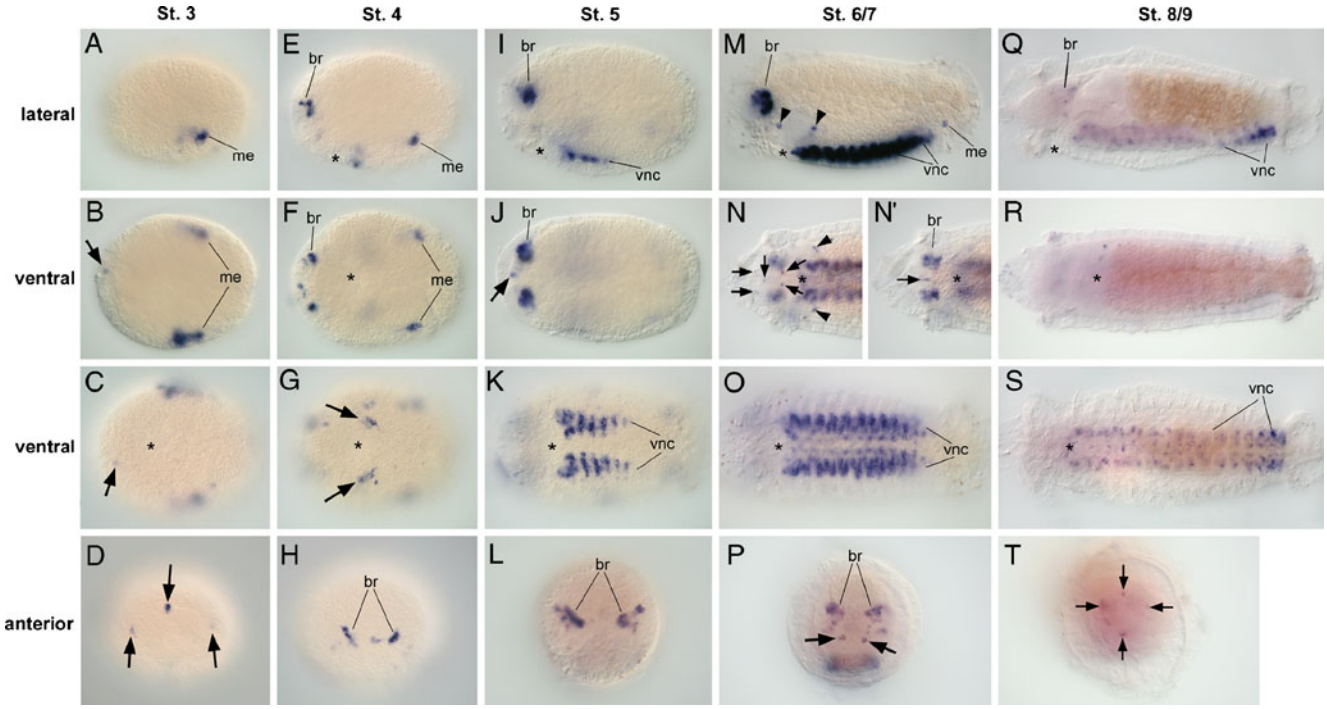

Fig. 4 Developmental expression of *CtCOE* in the polychaete *Capitella teleta*. **a**, **e**, **i**, **m**, **q** Lateral; **b**, **c**, **f**, **g**, **j**, **k**, **n**, **n'**, **o**, **r**, **s** ventral; and **d**, **h**, **l**, **p**, **t** anterior views. Anterior is to the left in all panels except **d**, **h**, **l**, **p**, **t**. In **d**, **h**, **l**, **p**, **t**, dorsal is up. **a**–**d** In St. 3 embryos, *CtCOE* is expressed in the trunk mesoderm and a small number of ectodermal cells in the head (*arrows*). **e**–**h** St. 4 larvae show expression in a subset of cells in the forming brain (**e**, **f**, **h**); ectodermal cells in the head (**f**, **h**); a mesodermal cell cluster (**e**, **f**) and cells in the presumptive subesophageal ganglion (**g**, *arrows*). **i**–**l** In St. 5 larvae, *CtCOE* is expressed in a subset of cells in the brain (*br* in **i**, **j**, **l**), in the forming ventral nerve cord (*vnc* in **i**, **k**) and subsurface anterior cells (**j**, *arrow*). **m**–**p** In St. 6 larvae, *CtCOE* is prominently expressed in the brain (**m**, **p**) and VNC (**m**, **o**). In addition, there are small *CtCOE*-expressing clusters associated with the foregut (**m**, **n**, *arrowheads*) and head (**n**, **n'** and **p**, *arrows*). **n** (more ventral) and **n'** (more dorsal) are different focal planes of the same specimen. **q**–**t** In late larval stages (St. 8, 9), *CtCOE* is expressed in a small subset of VNC cells (**q**, **s**, compare with the broad VNC expression during St. 6). Expression in the brain at this stage is largely undetectable (**q**, **r**) except in a small number of posterior cells (**q**, **t**, *arrows*). Abbreviations are as follows: *br* (brain), *me* (mesoderm), *vnc* (ventral nerve cord). The position of the mouth is demarcated with a *black asterisk*

conserved function of COE in both vertebrate and invertebrate hematopoiesis (Hartenstein 2006). Detailed analysis of COE activity during fruit fly development indicates that *collier* (the fruit fly ortholog of *COE*) is also involved in specification of muscle subtypes (Crozatier and Vincent 1999; Baumgardt et al. 2007; Dubois et al. 2007). This suggests that COE may have had an ancestral role in specifying mesodermal derivatives.

Besides mesodermal expression in *H. asinina*, *C. teleta* and *Chaetopterus*, *COE* transcripts also are detected in the developing central nervous system and in other putative neural elements. In the mollusc *H. asinina*, *COE* is expressed in the supra- and subesophageal ganglia (labelled as spg and sbg in Fig. 6c, d, g, h, k, l) while in *Chaetopterus* and *C. teleta*, *COE* is expressed in the developing brain (labelled as br in Figs. 5, 6 and 7) and ventral nerve cord (labelled as vnc in Figs. 5, 6 and 7). These results indicate that COE likely plays a role in neural specification in these animals. Outside of the developing central nervous system, *COE* is detected in the apical tuft of *H. asinina* (labelled as at in Fig. 6). In *Chaetopterus*, *ChCOE* is expressed in dorso–apical ectoderm (labelled as de in Fig. 7) but not in apical tuft cells (labelled with a red asterisk in Fig. 7). In *C. teleta*, *CtCOE* is expressed in a small number of cells in the central, anterior ectoderm (Fig. 4h, arrows in Fig. 4j, n, n', p). Among these three lophotrochozoans, *C. teleta* is the only larva that lacks an apical tuft. In addition to apical ectodermal expression, strong *COE* expression is detected in the posterior ectodermal cells of *H. asinina* (pc in Fig. 6a, e, m–o) and *Chaetopterus* (Fig. 7a–h, m–o), but not in *C. teleta* (Fig. 4a–j, r). Interestingly, in both *H. asinina* (Hinman et al. 2003) and *Chaetopterus* (NPM, unpublished data), but not in *C. teleta* (NPM, unpublished data), neurons are present in this posterior region. Thus, the posterior ectodermal domain of *COE* expression may also be involved in neural specification.

COE^{+} cells are also associated with the stomodeum of *H. asinina* (Fig. 6f) and foregut of *C. teleta* (Fig. 4g, m, n). In *C. teleta*, this labelling initially appears as two clusters, each one lateral to the stomodeum (arrows in Fig. 4g). At

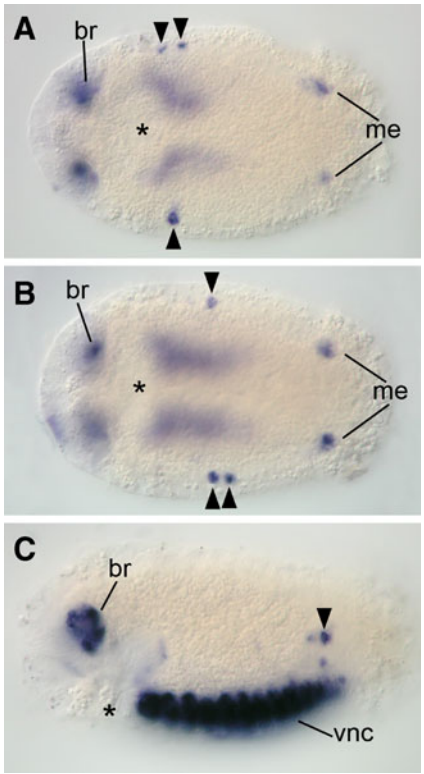

Fig. 5 Transient expression of *CtCOE* in lateral cell clusters and mesodermal cells. Anterior is to the left in all panels **a**, **b** ventral views, **c** lateral view. As development proceeds, *CtCOE* is sequentially expressed in one to two lateral subsurface clusters (*arrowheads*) in anterior (**a**, early St. 5), middle (**b**, mid-stage 5) and posterior (**c**, stage 6) segments. Abbreviations are as follows: *br* (brain), *me* (mesoderm), *vnc* (ventral nerve cord). The position of the mouth is demarcated with a *black asterisk*

later stages, COE^{+} cells are in two clusters of one to two cells each, closely associated with, but outside the foregut epithelium. The position and appearance of these small COE^{+} clusters is most consistent with them being neural elements. COE is also transiently expressed in the foregut of *M. leidyi* (Fig. 3e–h). In contrast, *COE* is not detected near the region of the future mouth of cnidarian (Pang et al. 2004) or sea urchin (Figs. 8a; 12) representatives. As *COE* has not yet been implicated in the specification of the larval or adult mouth in any metazoan taxa, we suggest the stomodeal/foregut *COE* expression we observe in some trochophore larvae may be acting downstream of 'mouth specifying' genes.

Sea urchin expression In the sea urchin, *SpCOE* is first detected at around 24 h post-fertilisation with transcripts diffusely localised to the apical end of the embryo (data not shown). By 44 h, *SpCOE* is expressed in three clear loci in the apical organ (Fig. 8a). *SpCOE* expression is not detected around the blastopore (Fig. 8a) as in *M. leidyi*, nor is it detected in mesodermal cells as in *M. leidyi* and the lophotrochozoans analysed.

In summary, these and previously reported results demonstrate both similarities and differences in *COE* expression patterns from various metazoan taxa. We detected a clear *COE* expression in the mesoderm of the ctenophore (Fig. 3a–g) and all three lophotrochozoan species: *H. asinina* (Fig. 6a, b, j); *Chaetopterus* (Fig. 7a, b, e, f, i, j); *C. teleta* (Fig. 4a, b, e, f) but not in *S. purpuratus* (Fig. 8a). Furthermore, *COE* is expressed in the developing central nervous system of all three lophotrochozoan species examined (Fig. 4, 5, 6 and 7) and in putative neural elements in the apical organs of both *H. asinina* and *S. purpuratus* (Figs. 6a–c; 8a) but not *Chaetopterus* (Fig. 7a–g, k, l).

Functional characterization of COE in *S. purpuratus*

To determine whether *Sp*COE functions in neuronal and/or ciliary tuft specification in the apical ganglion of sea urchin larvae, we knocked down *SpCOE* translation with morpholino anti-sense oligonucleotides (MASOs) and assayed the resulting phenotypes by WMISH for *SpCOE*, *SpNK2.1*, and the ciliary marker *Sptektin3* and by immunofluorescence labelling of serotonin and synaptogamin B. Previously, Dunn et al. (2007) showed that *Sp*NK2.1 is upstream of several ciliary markers and that abrogation of *Sp*NK2.1 function resulted in the absence of ciliary marker transcripts (*α2tubulin*, *radial spoke 3*, *tektin3*, *RSH p63* and *dynein p33*) and the apical tuft itself. Here, we show that injection of an *Sp*COE-MASO had no affect on its own expression (Fig. 8a; cf. 8b). This suggests that *SpCOE* is not autoregulated in the sea urchin apical plate, unlike *Drosophila-COE* which autoregulates after gastrulation in cells of the posterior intercalary and anterior mandibular segments (Crozatier et al. 1999). Furthermore, like *Sp*NK2.1 (Fig. 8f; cf. 8d), *Sp*COE does not regulate *SpNK2.1* expression (Fig. 8e; cf. 8d). Conversely, injection of an *Sp*NK2.1-MASO resulted in the abrogation of *SpCOE* transcripts (Fig. 8a; cf. 8c) suggesting that *Sp*COE acts downstream of *Sp*NK2.1. Unlike the *Sp*NK2.1-MASO phenotype (Dunn et al. 2007), knockdown of COE resulted in the apparently normal expression of the cilia marker *Sptektin3* (Fig. 8h; cf. 8g), the presence of an apparently normal apical tuft and larvae with normal swimming behaviour.

Because these data suggest that *Sp*COE is not involved in fate specification of apical tuft ciliary cells, we next asked if *Sp*COE is involved in specification of apical ganglion cells. The exact number of serotonergic neurons present in the apical ganglion of dipleurula larvae is stage dependent. Thus, we used carefully matched cohorts of embryos to determine the effects of *Sp*COE-MASO injection. In 72-h embryos, control MASO-injected larvae had a mean of 2.3±0.28 (n=18) serotonergic neurons in the apical ganglion, which was not significantly different from

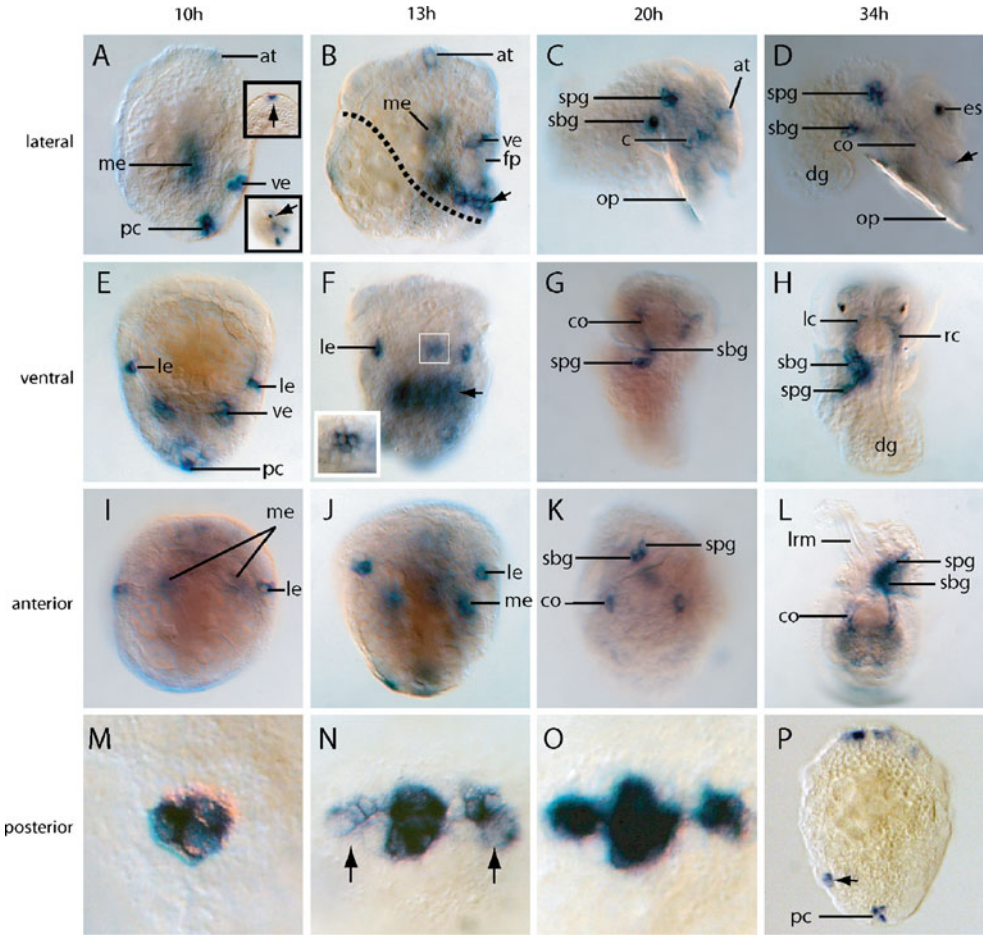

Fig. 6 Developmental expression of *HasCOE* and *HasElav* in the tropical abalone *Haliotis asinina*. Orientations are **a**–**d** lateral; **e**, **f**, **p** ventral; **g** and **h** dorsal; **i**–**l** apical/anterior; **m**–**o** posterior. **a**, **e**, **i** *HasCOE* expression in a hatched (10 hpf) trochophore larva, with expression detected in a group of posterior cells (*pc*), paraxial mesodermal bands (*me*), a pair of ventral ectodermal cells (*ve*) and within the apical tuft (*at*). Upper inset in (**a**) shows expression in the apical tuft (*vertical arrow*), lower inset shows an individual cell associated with the lateral ectoderm (*black arrow*, the *white arrow* in this inset indicates the COE^{+} paraxial mesodermal bands in a lower focal plane). **b**, **f**, **j** 13 hpf trochophore larva prior to torsion. A band of expression marking the division between the foot primordia (*fp*) and the expanding shell field (approximately indicated by the *dashed line*) has developed (*arrow*). The boxed region in F is expanded in the inset and highlights a triplet of COE^{+} cell within the vicinity of the stomodeum. **c**, **g**, **k** A 20-hpf larva (post-torsion) with *HasCOE* expression within the apical tuft and the presumed supraesophageal (*spg*) and subesophageal ganglia (*sbg*). Cells that will later form connectives (*co*) between the esophageal and pedal ganglia are also visible. The refractive operculum (*op*) can also be seen. **d**, **h**, **l** A 34-hpf veliger with a well-developed eyespot (**e**), operculum and digestive gland (*dg*). The supra- and subesophageal ganglia maintain expression of *HasCOE* and faint expression is also detected within the anterior of the foot (*arrow*). The fibres of the larval retractor muscle (*lrm*) are also visible. **m**–**o** Representative variation in the spatial expression of $HasCOE^{+}$ posterior cells (labelled *pc* in **a**) viewed posteriorly between individual 10-h-old larvae. Expression ranges from (**m**) a distinct triplet of closely associated cells to (**n**) a group of cells with an additional lateral population of COE^{+} cells (*arrows*), through to (**o**) strong expression of *HasCOE* in the lateral and central groups. **p** A ventral view of a 10-hpf trochophore larva expressing *HasElav* in apical ectodermal cells associated with the apical tuft, a pair of lateral ectodermal cells (*arrow*) and a set of ectodermal posterior cells (*pc*)

uninjected larvae (mean=2.4±0.22, n=19) (Figs. 9 and 10). In both sets of control larvae (MASO control injected and uninjected), serotonergic cells were distributed with bilateral symmetry and projected neurites toward the midline, contributing to the apical organ neuropil (Fig. 9). Larvae derived from eggs injected with *Sp*NK2.1-MASO had a significantly higher mean number of serotonergic neurons in the apical ganglion (mean=3.6±0.15, n=20; $p<0.001$). The serotonergic neurons in these larvae had bifurcating neurites with terminal growth cones that projected in random directions and lacked an obvious bilateral distribution (Fig. 9c). Larvae derived from eggs injected with *Sp*COE-MASO had a mean of 4.5±0.15 serotonergic neurons (n=18), which also differed significantly from controls ($p<0.001$) and from *Sp*NK2.1-MASO-injected larvae ($p<0.05$). In these embryos, the neurons were tightly clustered within the apical ganglion and contributed neurites to the neuropil (Fig. 9d). These data suggest that

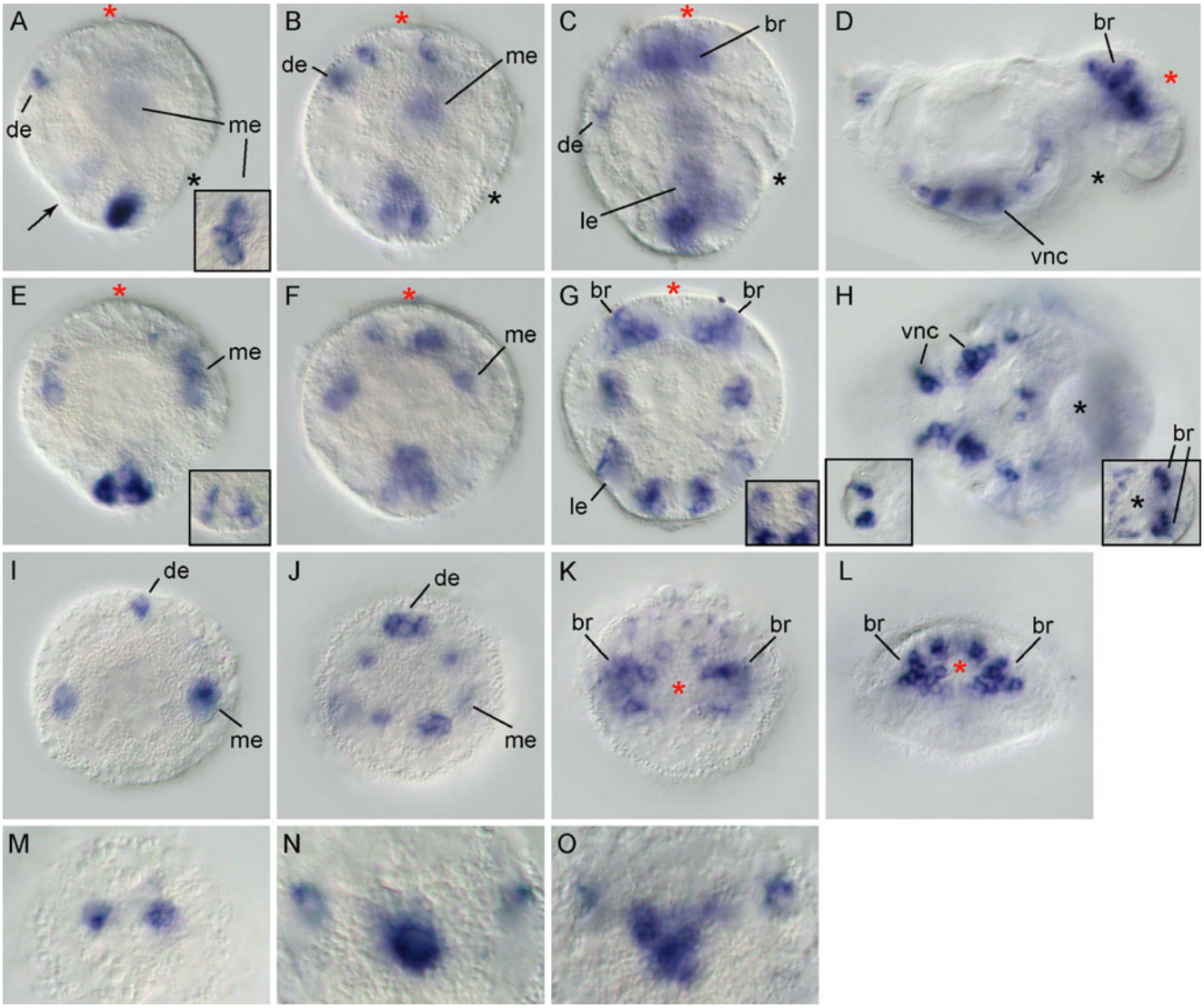

Fig. 7 Developmental expression of *ChCOE* in the polychaete *Chaetopterus* **a**–**d** lateral; **e**, **f**, **h** ventral; **g** dorsal; **i**–**l** anterior; **m**–**o** posterior views. Anterior is up in **a**–**c** and **e**–**g** and to the right in **d** and **h**. Dorsal is up in **i**–**o**. **a**, **e**, **i**, **m** In 11 hpf trochophore larvae, *ChCOE* transcripts are detected in two posterior groups of cells (**e**, **m**); two lateral groups of cells presumed to be mesoderm (*me* in **a**, **e** and **i**); and a dorsal, anterior ectodermal cell (*de* in **a** and **i**). The inset in **a** is a more superficial focal plane of the same animal showing the *ChCOE*$^+$ mesodermal cells which vary in number (one to three) from left to right. The animal shown in the inset in **e** and in panel **m** is of a different animal for which the colour reaction was not incubated as long. The posterior group of cells also can be variable in number. At this stage, there are occasional *ChCOE*$^+$ ectodermal cells in the region of the forming brain but not the apical tuft (position is marked with a *red asterisk*). *ChCOE* is also expressed in posterior, dorsolateral ectodermal patches (*arrow* in **a**). **b**, **f**, **j**, **o** 15 hpf trochophore larvae have similar clusters of *ChCOE*$^+$ cells as the 11 hpf animals. By this stage, the dorsal ectodermal cluster (*de*) has expanded to three to five cells (**j**), and the *ChCOE*$^+$ cells in the region of the forming brain are more visible but are still not localised around the apical tuft (**b**, **f**, **j**). The posterior region of *ChCOE* expression consists of a central group of cells and two *ChCOE*$^+$ cells positioned just anterior and dorsal to the central posterior patch (**o**). This is similar to the posterior pattern of expression at 13 hpf (**n**), although the central, posterior *ChCOE*$^+$ patch is just one cell at this stage. **c**, **g**, **k** At 19 hpf, *ChCOE* is expressed in clusters of ectodermal cells that appear to prefigure the central nervous system. *ChCOE* is expressed in the developing brain (**c**, **g**, **k**), in a dorsal ectodermal patch of cells (*de* in **c**) and in clusters of cells in the lateral (*le*) and posterior ectoderm (**c**, **d**). There are also two clusters of *ChCOE*$^+$ cells on either side of the mouth, which may be neural or mesodermal. In addition, there are two small clusters of *ChCOE*$^+$ cells in the ventral ectoderm (inset in **g**, anterior cluster of *ChCOE*$^+$ cells). The animal in the inset in **g** is a different animal for which the colour reaction was carried out longer. The two posterior clusters of *ChCOE*$^+$ cells correspond to the posterior ectodermal *ChCOE*$^+$ cell clusters in **g**. **d**, **h**, **l** In L2–L3 animals (**d**, 46 hpf; **h** and **l**, 72 hpf), *ChCOE* is expressed in subsets of the forming central nervous system, including in the developing brain (*br* in **d**, *right inset* in **h** and **l**), the forming ventral nerve cord (*vnc* in **d**, **h**) and in clusters of posterior neural cells in the pygidium (*left inset* in **h**). There is a patch of *ChCOE*$^+$ cells in the ventral ectoderm just posterior to the mouth (**h**) as well as single *ChCOE*$^+$ cells around the posterior edge of the mouth (*right inset* in **h**), which spatially correspond to the subesophageal connective. *br*, brain; *de*, dorsal ectodermal cluster; *le*, lateral ectoderm; *me*, mesoderm; *vnc*, ventral nerve cord. The position of the mouth is demarcated with a *black asterisk* and the apical tuft with a *red asterisk*

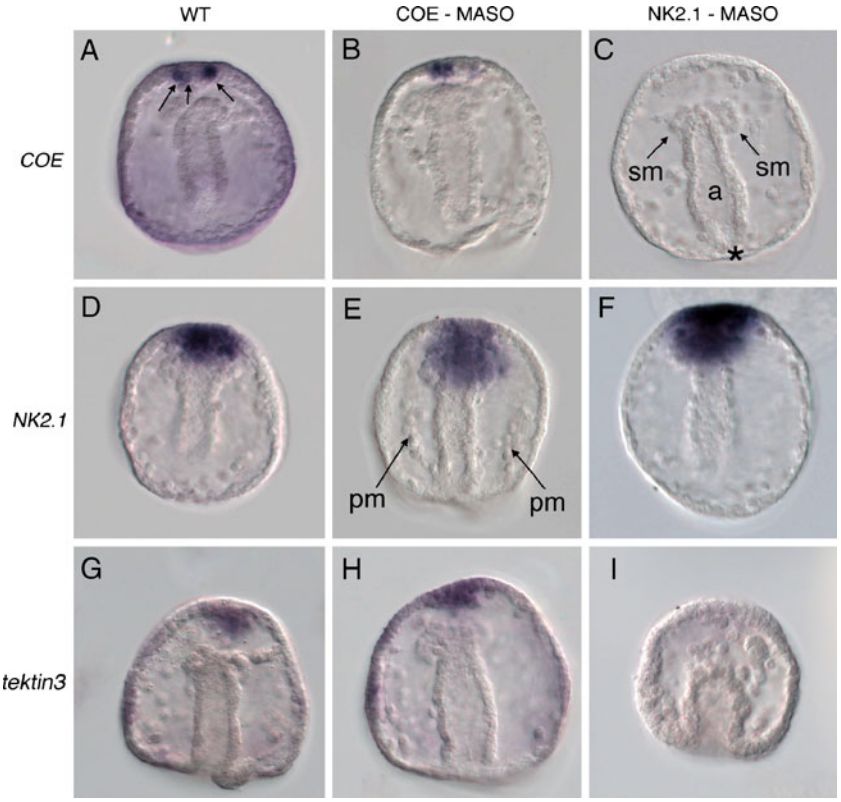

Fig. 8 Expression of *SpCOE*, *SpNK2.1* and *Sptektin3* in normal and MASO-injected embryos. **a** Expression of *SpCOE* in wild type (*WT*) embryo. Expression is clearly seen in three distinct loci at the apical end of the embryo (*arrows*). *SpCOE* expression is never detected in either the primary (*pm*) or secondary mesenchyme (*sm*) cells. **b**, **c** Injection of the *SpCOE*-MASO (**b**), unlike the *SpNK2.1* MASO (**c**), has no affect on *SpCOE* expression, suggesting that *SpCOE* is downstream of *Sp*NK2.1, but does not autoregulate. **d** Expression of *SpNK2.1* in WT embryo in the apical domain (Takacs et al. 2004). **e**, **f** *SpNK2.1* expression in embryo injected with *SpCOE* (**e**) and *SpNK2.1* (**f**) MASO is unchanged suggesting that *SpNK2.1* is not downstream of *Sp*COE, and does not autoregulate. **g** Expression of the cilia gene *tektin3* in WT embryo in apical domain. **h**, **i** Injection of embryos with *SpCOE*-MASO does not alter *tektin3* expression (**h**), whereas expression is abrogated upon introduction of the *SpNK2.1* MASO (**i**) suggesting that *Sp*COE does not regulate apical tuft development. Abbreviations: *a*, archenteron; *pm*, primary mesenchyme; *sm*, secondary mesenchyme. The position of the blastopore is indicated with a *black asterisk*

*Sp*COE and *Sp*NK2.1 (possibly via *Sp*COE) are involved in apical neuronal specification and differentiation. Specifically, *SpCOE* appears to suppress serotonergic differentiation in the apical tuft of dipleurula larvae.

The expression data from lophotrochozoans and the functional results in *S. purpuratus* suggest that COE may have functioned ancestrally to repress the specification of serotonergic neurons. In *H. asinina*, serotonin-positive cells are absent from the posterior end of the larvae (Hinman et al. 2003) where strong *COE* expression is detected. $FMRF^{+}$ cells are present in this region of *Aplysia californica* (Dickinson et al. 2000) *Ilyanassa obsoleta* (Dickinson and Croll 2003) and *H. asinina* (Cummins, Croll and Degnan unpublished data) trochophores. The expression of the pan-neuronal marker *HasElav* (Perrone-Bizzozero and Bolognani 2002; Benito-Gutiérrez et al. 2005; Marlow et al. 2009) in this region of *H. asinina* trochophores (Fig. 6p) further supports the hypothesis that non-serotonergic neurons will be born here. In *Chaetopterus*, a single, large 'horn-shaped' posterior serotonergic neuron is visible by the end of gastrulation (~9 hpf); however, there are likely other, non-serotonergic neurons that form in this region at similar times (NPM, unpublished data). *C. teleta* larvae do not posses posterior serotonergic neurons and do not express *COE* posteriorly. It will be interesting to functionally determine whether COE represses serotonergic cell fate in annelids and molluscs as it does in *S. purpuratus*.

Conserved mesodermal expression of *COE*

A principle feature of the *COE* expression patterns we have described here is expression in mesodermal tissues in ctenophore, annelid and molluscan representatives. While the fates of these tissues are not yet known, it is of note that one of the first two papers to isolate and characterise the function of COE (from a vertebrate) was to describe its role in B cell specification, a mesoderm derivative and a type of immune cell primarily responsible for antibody production (Hagman et al. 1993). Recently, a role for COE

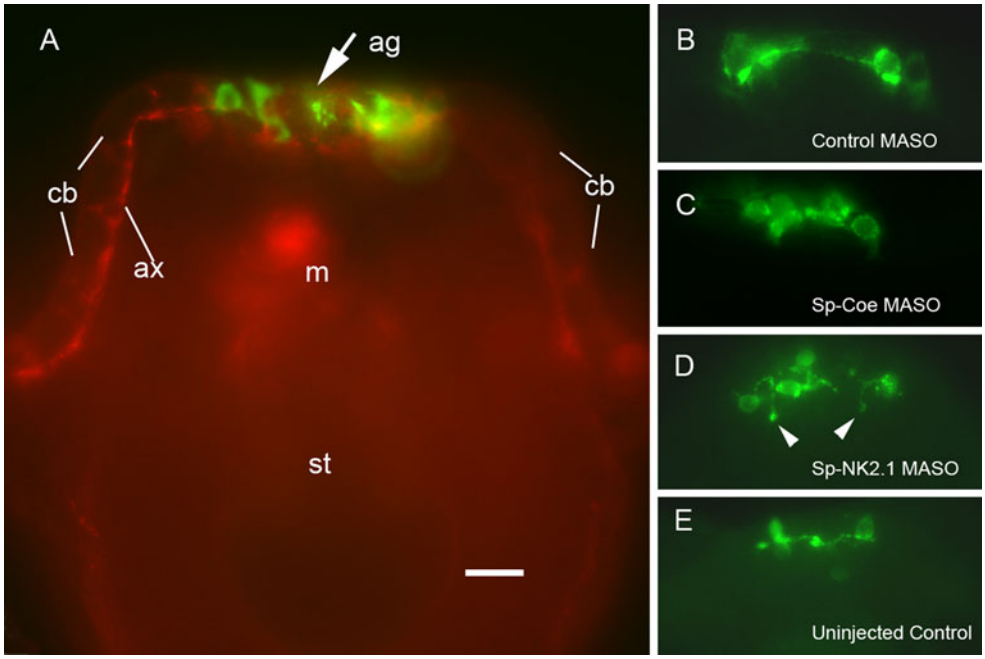

Fig. 9 Immunofluorescent images of morpholino injected larvae 72 h post-fertilisation. **a** Image of an uninjected larva combining the anti-serotonergic (*green*) and anti-synaptogamin (*red*) signals showing the location of the apical ganglion (*arrow*, *ag*). **b** Example of a control MASO-injected embryo that was used to quantify the number of serotonergic cells in the apical organs of 72-h larvae. **c** An example of an *Sp*COE-MASO-injected embryo in which there are supernumerary serotonergic neurons. **d** *Sp*NK 2.1-MASO-injected embryos had more serotonergic cells than controls and projections with terminal growth cones (*arrowheads*) were not restricted to the neuropil of the apical ganglion. **e** Example of an apical organ in an uninjected control from the stage-matched set of embryos used to quantify cells in the apical organ. *ag*, apical organ; *ax*, axonal tracts of ciliary bands; *cb*, ciliary bands; *m*, mouth; *st*, stomach. *Scale bar* in **a**=10 μM

in blood cell homeostasis was also reported for *Drosophila* where it is involved in the differentiation of immune cells (lamellocytes) following parasitisation (Crozatier et al. 2004; Krzemien et al. 2007). Activity in haematopoietic precursors is therefore likely to be a deeply conserved

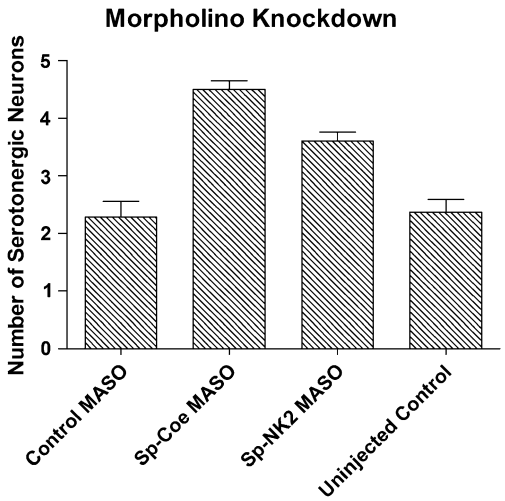

Fig. 10 Effect of *SpCOE*-MASO knockdown on the number of serotonergic neurons in the apical organ. Carefully staged embryos were prepared for immunofluorescence and the number of serotonergic neurons counted. Abrogation of *Sp*COE and *Sp*NK2.1 translation results in a significantly higher number of serotonergic neurons in the apical organ compared to wild type and control larvae

feature of *COE*, with the possibility of it having had a specific role in the evolution of the metazoan immune system (Hartenstein 2006). If *COE* also specifies haematopoietic cells in spiralians, then this function likely arose shortly after the split of cnidarians from the bilateria. Alternatively, *COE* may function in distinct mesodermal cell types in spiralians. Interpreting the expression of *COE* in early mesodermal derivatives of *M. leidyi* is challenging because the phylogenetic position of ctenophores is currently unstable (Dunn et al. 2008; Pick et al. 2010), and ctenophores do not have a circulatory system (Brusca and Brusca 2002).

A *COE* cell type molecular fingerprint in ectodermally derived apical ciliated structures

Most marine invertebrates develop via a planktonic larval period (here defined as indirect development) during which either active (by means of swimming) or passive (current driven) dispersal is achieved (Brusca and Brusca 2002). Prior to settlement and metamorphosis, many species must identify a suitable substrate upon which to attach (Jackson et al. 2002). It is believed that apical organs are involved in recognising metamorphosis-inducing cues via chemoreception (Hadfield et al. 2000; Leise et al. 2001; Thavaradhara and Leise 2001). Eluci-

dating the evolutionary origins of these structures across a variety of metazoan phyla has the potential to address questions of larval homology and more broadly whether the ancestor of the Metazoa developed indirectly or directly. Previously, Dunn et al. (2007) conducted a comparative GRN analysis of two transcription factors (NK2.1 and HNF6) and their regulation of genes required for apical tuft ciliation in the sea urchin *S. purpuratus* and the abalone *Haliotis rufescens*. They found significant differences in the synexpression of these genes and concluded that the apical tufts of urchins and abalone are specified in different ways and are therefore unlikely to have the same evolutionary origins. Given its clear ecological role and restriction to primary marine larvae, the apical tuft is an appropriate structure to evaluate the homology of the apical organ; however, a difficulty with the Dunn et al. (2007) study is that although the dissimilarity between the trochophore (*Haliotis*) and dipleurula (*S. purpuratus*) was clear, the primary gene under investigation, *NK2.1*, does not have a clear homolog in cnidarians (Ryan et al. 2006), and hence evolutionary polarity could not be unambiguously established. Expression of *COE* in the apical organ of cnidarians could suggest potential homology and indeed a parsimonious interpretation of our *COE* data per se would be that the apical ganglia of urchins, abalone and cnidarians are homologous; however an alternative hypothesis that *COE* functions in the apical ganglion to specify chemosensory neurons and that this cell type was independently recruited to the apical region in multiple metazoan lineages, must also be considered. Such a scenario would fall under Nielsen's definition of homocracy—expression of homologous genes in non-homologous structures. While our *COE* data alone cannot distinguish between these hypotheses, our functional data clearly indicate that at least in the sea urchin it is not involved in apical tuft specification, but in the specification of proper neuronal cell fate within the ganglion itself.

Conclusion

We anticipate that more detailed analyses of *COE* regulation and function and the inclusion of more metazoan representatives that posses larval apical tufts, will significantly contribute to the resolution of the larval homology problem. Homologous genomic outputs such as *COE*, which represent the genetic material upon which evolution acted to diversify cell types during the early history of metazoan evolution, are well suited for such studies. Our data has generated testable hypotheses and will serve as a platform for future studies aimed at resolving the evolutionary histories of larval and adult metazoan body plans. Our findings also highlight the need for functional assays to be developed for a wider range of metazoan model systems, which will complement the flood of sequence data the field of evo-devo is now experiencing.

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Ethical standards All experiments described here comply with the current laws and regulations of the respective countries they were conducted in.

Conflict of interest The authors declare no conflicts of interest.

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