

Sandra Chevalier · Arnaud Martin · Lucas Leclère ·  
Aldine Amiel · Evelyn Houliston

## Polarised expression of FoxB and FoxQ2 genes during development of the hydrozoan *Clytia hemisphaerica*

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**Abstract** We have characterised the expression of four genes coding for Forkhead box-containing ('Fox') transcription factors identified from the hydrozoan (*Leptomedusa*) *Clytia hemisphaerica*. Phylogenetic analyses including all available non-bilaterian Fox sequences placed these genes in subfamilies B, Q2 (two genes) and O, and indicated that at least 17 Fox subfamilies were present in the common cnidarian/bilaterian ancestor, with multiple subsequent losses in cnidarian lineages. Chordate FoxB and FoxQ2A subfamily genes show polarised expression in early embryos. Correspondingly, *Clytia CheFoxB* expression was localised around the gastrulation site (future oral pole) at blastula and gastrula stages, with *CheFoxQ2a* expressed in a complementary aboral domain, maintained through larval development. Distinct later expression domains were observed for *CheFoxB* in the larval endoderm region, and in the statocyst, gonad and tentacle bulb of the medusa. A second *Clytia* FoxQ2 gene, *CheFoxQ2b*, not expressed in the embryo, larva or polyp, was detected uniquely in the gonads of the medusa. In contrast, *CheFoxO*, whose sequence indicates regulation by the PI3-Kinase/PKB signalling pathway consistent with known roles in bilaterian developmental regulation, was detected throughout the *Clytia* life cycle. *CheFoxO* expression was enhanced in regions associated with growth control including larval poles, gonad and the margin of the medusa

bell. These results support the idea that an early embryonic patterning system involving FoxB and FoxQ2 family genes has been evolutionary conserved and indicate that Fox family genes have also acquired distinct roles during other phases of the hydrozoan life cycle.

**Keywords** Forkhead · Cnidarian · Embryonic polarity · Statocyst · Hydromedusa

### Introduction

Cnidarians are well-placed to provide information concerning the evolutionary origins of embryonic patterning mechanisms. Firstly, they provide an 'outgroup' to the Bilateria, as probably the closest metazoan group not possessing dominant bilateral symmetry (Collins 1998). Secondly, many regulatory gene families in cnidarians have retained more of the ancestral complexity than highly derived bilaterian models such as *Ciona*, *Drosophila* and *Caenorhabditis* (Kortschak et al. 2003; Kusserow et al. 2005; Magie et al. 2005; Miller et al. 2005). Furthermore, studies in embryos from different cnidarian classes as well as in *Hydra* polyps have revealed many similarities in molecular patterning in relation to the gastrulation site (or oral opening) to that seen in bilateria, including Wnt pathway activation (Wikramanayake et al. 2003; Hobmayer 2000 #74) and expression of *Gooseoid*, *Forkhead* and *Brachyury* transcription factors (Martinez et al. 1997; Technau and Bode 1999; Spring et al. 2002; Fritzenwanker et al. 2004; Martindale et al. 2004). Finally, cnidarians have a simpler body plan than bilaterians, being generally considered to possess only two germ layers and radial symmetry around a single "oral-aboral" axis, although this apparent simplicity may not reflect the ancestral organisation (Ball et al. 2004; Martindale 2005; Seipel and Schmid 2005).

Cnidarians exhibit a very wide variety of morphologies, modes of development and life cycles. They are generally classified into four main groups, three of which (Cubozoa, Scyphozoa and Hydrozoa) include species with both free-swimming medusal and fixed polyp forms, while the

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S. Chevalier · A. Martin · A. Amiel · E. Houliston (✉)  
"Biologie du Développement", Observatoire Océanologique,  
UMR7009 CNRS/Université Pierre et Marie Curie (Paris VI),  
06230 Villefranche-sur-mer, France  
e-mail: houliston@obs-vlfr.fr  
Tel.: +33-493-763791

L. Leclère  
"Evolution et Développement", UMR 7138 CNRS UPMC  
MNHN IRD ENS, Université Pierre et Marie Curie (Paris VI),  
9 Quai St Bernard,  
75005 Paris, France

Anthozoa (corals and sea anemones) have only a polyp form. We have started to analyse developmental mechanisms in the cnidarian *Clytia hemisphaerica*, a hydrozoan (Leptomedusa) whose biphasic life cycle can be entirely controlled in the laboratory. In *Clytia*, sexual medusae bud from specialised polyps called gonozooids in colonies formed by vegetative growth after metamorphosis of a “planula” larva. The planula is organised with ectoderm and endoderm cell layers and a polarity defined as posterior–anterior (Bodo and Bouillon 1968), or oral–aboral (because the oral end of the planula gives rise to the oral end of the polyp upon metamorphosis (for a review, see Freeman 2005). This organisation arises during gastrulation, which proceeds essentially by ingression of cells in a restricted region at the future oral pole of the hollow blastula (Byrum 2001). The site of gastrulation, and thus of the oral pole, can be traced back to the position of the first cleavage initiation site, corresponding to the animal pole in undisturbed eggs (Freeman 1981).

“Fox” genes, coding for transcription factors containing a highly conserved winged-helix (=Forkhead) DNA-binding domain, form a large multigene family restricted to animals and fungi (Carlsson and Mahlapuu 2002). The metazoan Fox genes can be grouped into about 20 subfamilies and are involved in the regulation of a range of cellular processes including proliferation and apoptosis as well as many events involved with region and cell-type specification during embryonic development. Scanning of the draft genome sequences of the anthozoan *Nematostella vectensis* revealed 15 Fox genes, including clear members of nine bilaterian Fox gene subfamilies and six gene sequences whose bilaterian orthology was less easy to assign (Magie et al. 2005). Studies of cnidarian Fox gene family expression are so far limited to the orally expressed FoxA genes (Martinez et al. 1997; Fritzenwanker et al. 2004; Martindale et al. 2004) and to four other genes in *Nematostella* (Fritzenwanker et al. 2004; Magie et al. 2005). We have identified and characterised expression of *Clytia* genes belonging to two Fox subfamilies showing early polarised expression in chordate embryos FoxB and FoxQ2 as well as a FoxO gene. The FoxB and one of two FoxQ2 genes showed oral and aboral expression domains, respectively, before gastrulation corresponding to the expression of chordate counterparts. This first analysis of Fox gene expression in a biphasic life-cycle cnidarian species also provided evidence for adoption of novel functions for Forkhead transcription factors during the medusa phase of the hydrozoan life cycle.

## Materials and methods

### Animals

Colonies of *Clytia hemisphaerica* were established in 5-l glass beakers after metamorphosis of larvae produced by adults caught in the bay of Villefranche. Culture conditions allowing completion of successive life cycles were adapted from previous studies (Carre and Carre 2000) using natural

sea water and feeding on 3- to 6-day old artemia larvae. Medusae budded from these colonies were raised to sexual maturity and spawned by controlling the light–dark cycle, oocytes being released 2 h after light stimulus. Embryos were raised in 0.2- $\mu$ m Millipore-filtered sea water, with gastrulation occurring between approximately 10 and 17 hpf (hours post fertilisation) at 18°C.

### *Clytia* cDNA library and ESTs

Total RNA was isolated from mature medusae, embryos and larvae using the Trizol reagent (Invitrogen). A primary cDNA library in Express 1 vector was prepared by Open Biosystems (through BioCat, Heidelberg) from a mixture of approximately 50  $\mu$ g RNA from male and female adult medusae and 7  $\mu$ g RNA from mixed-stage embryos and larvae. Clones from this library were used to generate EST (Expressed Sequence Tag) sequences at the Genoscope (Ivry, France). Plasmids from four clones identified on the basis of Forkhead domain homology in the ESTs were isolated and fully sequenced (Genome Express). Accession numbers: *CheFoxB*: DQ537955; *CheFoxQ2a*: DQ537956; *CheFoxQ2b*: DQ537957; *CheFoxO*: DQ537958.

### Sequence alignments

We sampled all Forkhead-related sequences from *Homo sapiens*, *Strongylocentrotus purpuratus*, *Ciona intestinalis*, *Caenorhabditis elegans* and *Drosophila melanogaster* as well as all reported sequences from sponges (*Reniera* sp. Larroux et al. 2006; *Suberites domuncula*, Adell and Muller 2004), Ctenophora (*Mnemiopsis leidyi*, Yamada and Martindale 2002) and Cnidaria (*Nematostella vectensis*, Magie et al. 2005 #24; *Hydra vulgaris*, Martinez et al. 1997 #20). Six new sequences from *Hydra magnipapillata* (identified by BLAST at <http://www.hydrabase.com>) and the four new *Clytia hemisphaerica* sequences were also added. Fungus sequences (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Debaryomyces hansenii*, *Aspergillus fumigatus*) were included as an outgroup. Accession numbers are in Supplementary data S2. Some *Nematostella vectensis* sequences absent from the database (*NvFoxD.2*; *NvFoxL2*; *NvFoxO* (re-named *NvFoxO.1*); *NvFox2*; *NvFox3*; *NvFox4*; *NvFox5* and *NvFox6*), were recovered from the Joint Genome Institute (DOE) trace files from the *N. vectensis* genome project using the representative identifiers cited by (Magie et al. 2005). A *N. vectensis* FoxN gene, dubbed *NvFoxNx*, was also identified from these trace files (representative identifier 557686699), which did not contain the *NvFoxN* Forkhead sequence published in (Magie et al. 2005). The previously undescribed *NvFoxO.2* sequence was assembled from sequences with trace identifiers 558826953, 601164801, 572141612, 558183920 and 568600662.

Forkhead domain amino acid sequences were aligned using ClustalW (via BioEdit) with manual correction. Alignment positions with more than 90% missing data

were removed. After initial Maximum Likelihood (ML) analysis, some sequences were removed to eliminate very long branches (*C. elegans*: PES-1, Fkh-3, 4, 5, 6, 8 and 9; *D. melanogaster*: Fd3F, Fd19b, CG9571; *S. purpuratus*: Fox2, FoxX; *Homo sapiens*: FoxN5, FoxN6). The final data set analysed comprised 141 sequences and 110 characters.

### Phylogenetic analyses

ML analysis was performed using PhyML (Guindon et al. 2005) with the JTT model of amino acid substitution giving the highest likelihood value of all proposed models (log lk = -12,243.9; other models with the same analysis parameters: RtREV: -12,265.9, WAG: -12,267.9, Dayhoff: -12,331.3, DCMut: -12,332.0, MtREV: -13,044.6, MtMan: -13,452.4). A Neighbour-Joining tree was used as the input to generate the ML tree. A gamma distribution with four discrete categories was used in the ML analyses. The gamma shape parameter and the proportion of invariant sites were optimised during the ML search. Sensitivity of the ML analysis was assessed by comparison of different protein substitution models and by comparison with Bayesian analysis (Huelsenbeck and Ronquist 2001) (JTT model; gamma distribution with four discrete categories; proportion of invariable sites model combined with the gamma model; see Supplementary Figure S1).

### In situ hybridisation

DIG-labelled antisense RNA probes were synthesised from linearised Express 1 plasmid using T7 polymerase and the RNA DIG-labelling mix (Roche). Samples were fixed in ice cold 3.7% formaldehyde/0.2% glutaraldehyde/PBS for 40–60 min at 4°C, washed thoroughly in PBSt (PBS pH 7.4; 0.1% tween 20) and stored in methanol at -20°C. After stepwise rehydration to PBSt and 10-min digestion with 10 µg/ml Proteinase K /PBSt at 37°C, samples were washed in PBSt with 2 mg/ml glycine and transferred progressively to hybridisation buffer (HB: 5×SSC pH 7.4, 50% formamide, 0.1% tween 20), prehybridised for 1–3 h at 60–65°C in HB containing 0.1% DMSO, 50 µg/ml heparin and 50 µg/ml tRNA, then hybridised with DIG-labelled RNA probes overnight at 60–65°C.

Washes were in HB at 60–65°C then 50% HB/PBSt, followed by PBSt at room temperature. After 30–60 min in blocking reagent (Roche), 2-h incubation in 1/2000 anti-DIG-alkaline phosphatase (Roche) and washes in 100 mM maleic acid pH 7.5/150 mM NaCl, colour was developed using 100–225 µg/ml NitroBlue Tetrazolium, 175 µg/ml Bromochlorolindolophosphate in 100 mM Tris-HCl pH 8.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl (all steps at room temperature). Levamisole (5–10 mM) was included to reduce endogenous phosphatase activity in digestive areas of medusae (endodermal cavities of manubrium and tentacle bulbs). After washing with distilled water and several changes of PBSt and post fixation for 30–60 min in 3.7% formaldehyde/PBS, nuclei were stained using

Hoechst dye 33358 at 5 µg/ml in PBSt for 30 min followed by PBSt washes and mounting in 20–50% glycerol/PBS.

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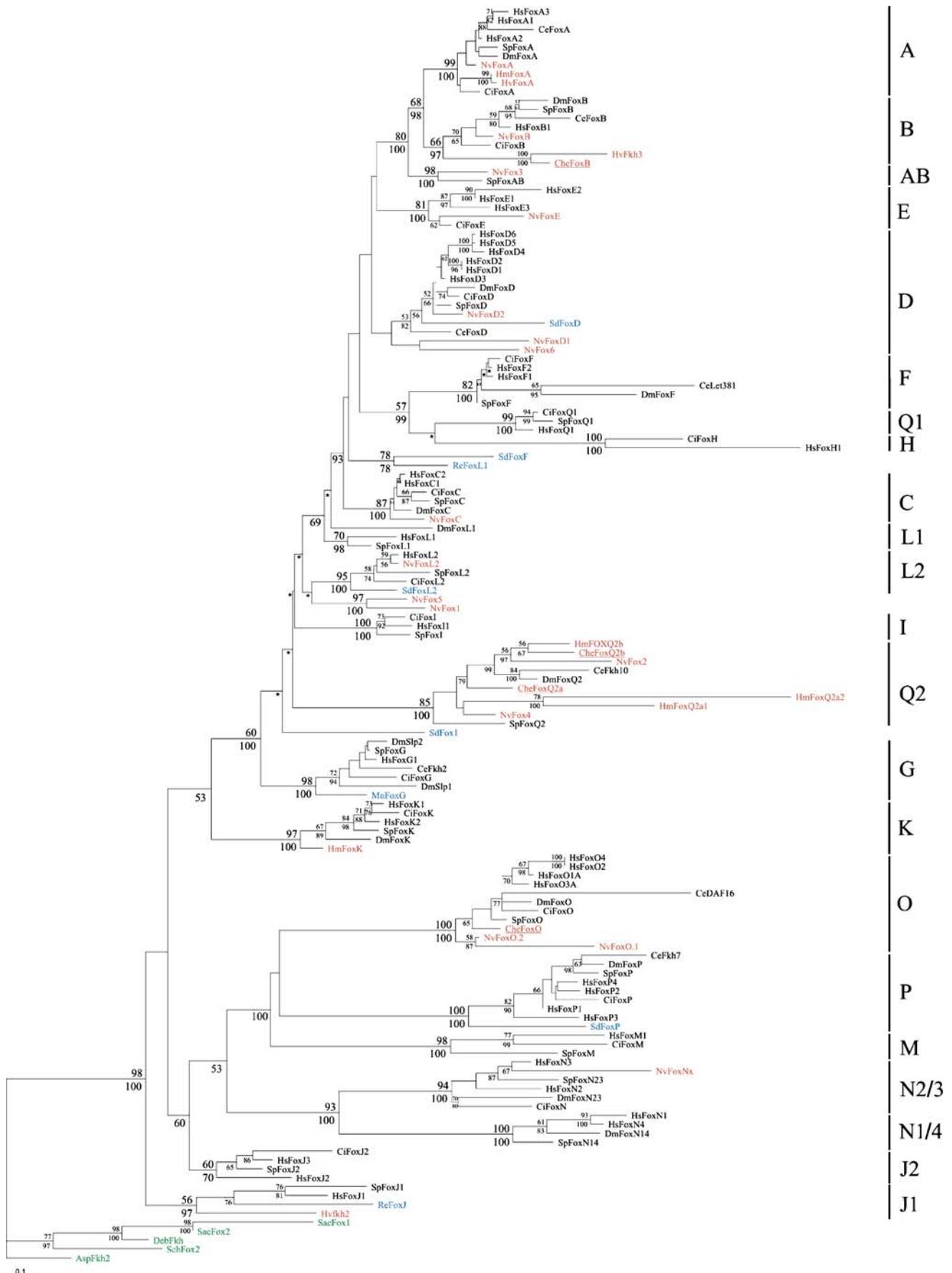
## Results

### Identification of four *C. hemisphaerica* Fox genes

Four *C. hemisphaerica* Fox genes were identified from a collection of 10,000 5' ESTs generated from a mixed-stage cDNA library (see [Materials and methods](#)) on the basis of sequence homology in the Forkhead domain by BLAST searching. Full sequencing of the corresponding cDNA clones yielded a complete open reading frame sequence for each one. To position the four genes within the Forkhead gene family, we aligned the amino acid sequences with bilaterian representatives from each of the established Forkhead subfamilies as well as all previously described Fox sequences from non-bilaterians (see [Materials and methods](#) for species and references; alignments available as Supplementary data S3). Maximum Likelihood or Bayesian analyses, either limited to the part of Forkhead domain conserved between all families (Fig. 1; Supplementary data S1) or extended to other domains alignable within subsets of sequences (not shown), clearly placed all four *Clytia* genes in existing subfamilies. They were named correspondingly as *CheFoxB*, *CheFoxO* and *CheFoxQ2a* and *CheFoxQ2b*.

Testing of tree topology by varying the dataset and applying different amino acid substitution models and comparison methods confirmed the monophyly of the metazoan Forkhead family and of most subfamilies (A, B, C, D, E, F, G, H, I, J1, J2, K, L2, M, N1/4, N2/3, K, O, P, Q1 and Q2). As suspected by Mazet et al. (2003), the subfamily J appeared polyphyletic in all analyses, divided into two well-supported clades J1 and J2. Some disruption of L1 subfamily was observed, presumably due to long branch attraction artefacts, and the sponge genes assigned to the L1 and F groups along with *SdFox1* did not adopt consistent relationships when analysis parameters were varied. The closely related *Nematostella* sequences *NvFox1* and *NvFox5* lie in this problematic part of the tree, accounting for the difficulty in assigning their orthology. We were, however, able to assign orthology to *NvFox2* and *NvFox4*, which are clearly FoxQ2 subfamily members, and to *NvFox6*, likely a FoxD. *NvFox3* remains enigmatic and may, along with the echinoderm *SpFoxAB*, be an extant member of an additional subfamily derived from an earlier duplication of the AB clade.

After these readjustments of Fox gene affiliation, nine of the 20 unambiguous Fox subfamilies contained representatives in the *Nematostella* genome. This is more than previously thought, but it remains true that many subfamilies lack *Nematostella* members (assuming that none were overlooked in the genome trace archive; Magie et al. 2005). In theory, this situation could result from increased diversification of the Fox family in bilaterians (as suggested previously: Magie et al. 2005) and/or from extensive loss in the cnidarians of genes present in a



**Fig. 1** Phylogenetic position of cnidarian *Fox* genes. Phylogenetic analysis of Forkhead domains using the Maximum Likelihood (ML) method. The tree was calculated from an amino acid alignment of Forkhead domains (see [Materials and methods](#) and [Supplementary data S3](#)). It was rooted using five fungi sequences. Bootstrap values (500 replicates) are indicated above the branches when >50%. The posterior probability obtained by Bayesian analysis of each node on the ML tree is indicated below the node (when >50%). Stars indicate disagreement between ML and Bayesian tree topology. *Ce Caenorhabditis elegans*, *Ce Clytia hemisphaerica*, *Ci Ciona intestinalis*, *Dm Drosophila melanogaster*, *Hm Hydra magnipapillata*, *Hs Homo sapiens*, *Hv Hyra vulgaris*, *Mn Mnemiopsis leidyi*, *Nv Nematostella vectensis*, *Re Reniera sp.*, *Sd Suberites domuncula*, *Sp Strongylocentrotus purpuratus*. Colour code: Ctenophores and sponges—blue; cnidarians—red; bilaterians—black; fungi—green. Vertical bars indicate subfamilies defined by previous studies (Kaestner et al. 2000; Mazet et al. 2003; Adell and Muller 2004)

Polarised expression of a *Clytia* FoxB gene

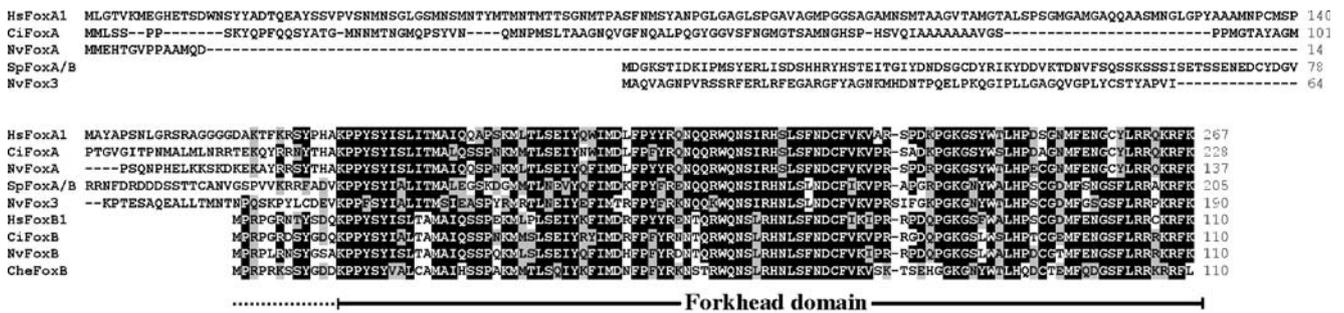
Sequence comparisons revealed strong similarity of the *CheFoxB* Forkhead domain with the *HvFkh3* fragment obtained previously from *Hydra* by degenerate PCR (Martinez et al. 1997), indicating that the two corresponding hydrozoan genes are orthologues. Preliminary Neighbor-Joining analysis (not shown) placed *HvFkh3* and *CheFoxB* as a sister group of FoxA and FoxB clades as found previously (Adell and Muller 2004); however, ML and Bayesian analyses (Fig. 1, S1) placed them in the FoxB subfamily with relatively high confidence values. This latter conclusion was confirmed by detailed analysis of the entire *CheFoxB* ORF. The *CheFoxB* amino acid sequence showed strong similarity with other FoxB sequences within a characteristic 11 amino acid N-terminal sequence immediately preceding the DNA-binding domain (Fig. 2), and no obvious similarity with FoxA (or FoxAB) amino acid sequences outside the Forkhead domain (not shown).

common ancestor. Our analyses favour predominantly the latter hypothesis. Firstly, the position of the *Nematostella* genome set with respect to tree topology indicates that major diversification of Fox gene family predated the separation of the cnidarian and bilaterian lineages. This conclusion does not depend on the precise relationships between some of the deeper tree branches, which could not be determined reliably. Secondly, the presence of a sponge gene, but no cnidarian gene in the FoxP subfamily argues strongly for loss of an ancestral cnidarian FoxP gene. Likewise, the presence of a ctenophore gene in group G implies cnidarian gene loss, assuming that the ctenophores are confirmed as sister phylum to the cnidarian/bilateria (Collins 1998). Finally, two additional Fox subfamilies (J1 and K) contained *Hydra* but not *Nematostella* members, implying that ancestral cnidarian genes in these families were lost selectively in the *Nematostella* lineage.

In situ hybridisation indicated that *CheFoxB* mRNA is present uniformly in eggs and cleavage-stage embryos (Fig. 3a,b). In blastula-stage embryos, RNA was detected at higher levels and showed an asymmetric distribution (Fig. 3c). At the early gastrula stage, this expression could be seen to involve both superficial and ingressing cells at the site of gastrulation (Fig. 3d). Cells from this region give rise to most of the endodermal region of the planula as well as oral ectoderm (Byrum 2001). The in situ hybridization signal decreased during gastrulation (Fig. 3e), such that only weak expression was detectable in the endodermal region of 1-day planulae (Fig. 3f). Later, during larval development, expression increased within this region to give a strong signal in 3-day planulae, often appearing most intense in the aboral half. Some cells extending into the aboral ectoderm were also stained (Fig. 3g), which may correspond to developing nematocytes or ganglion cells that originate from the endodermal region (Thomas et al. 1987), although mature nematocytes were not labelled.

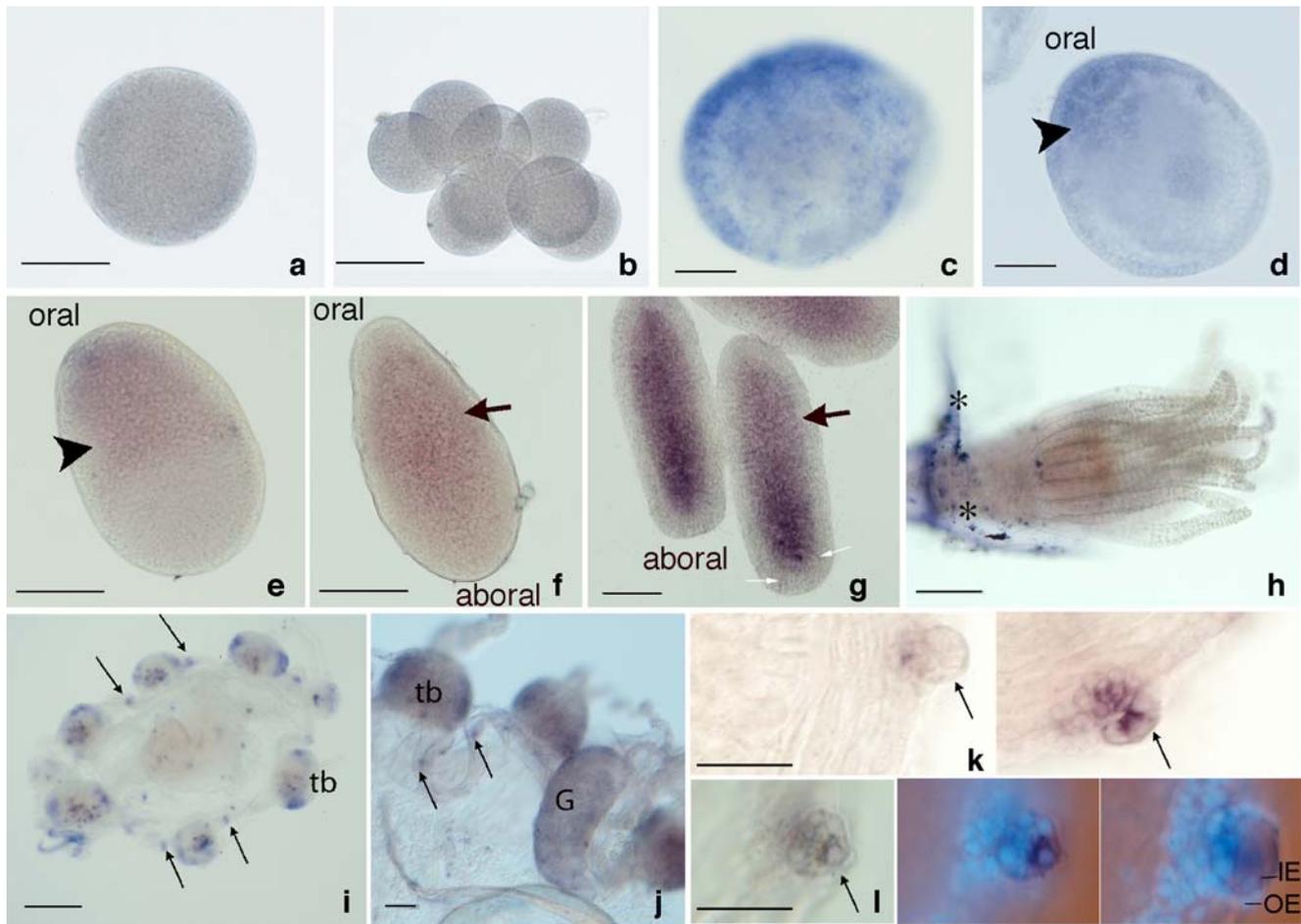
Combining these arguments, we can conclude that the relatively poor representation of Fox gene families in *Nematostella* is due to 8–11 losses: J1 and K (which have been retained in the *Hydra* lineage), G, N1/4, I, P and at least once in the F/Q1/H and J2/M groups. Thus, cnidarian ancestors probably possessed almost all of these Fox subfamilies (at least 17), with the only clear possibility for a bilaterian-specific expansion concerning the F/Q1/H clade. Given the poor resolution of tree topology in the J2/M/N/P/O region, we cannot rule out the possibility of bilaterian expansion in a potential J2/M clade.

No localised *CheFoxB* expression was detected in polyps (Fig. 3h); however, expression was detected at three distinct sites in medusae (Fig. 3i–l). Strong expression was detected as a band running around each tentacle bulb in the epithelial layer (Fig. 3i,j). Expression was also



**Fig. 2** *CheFoxB* as a member of the FoxB subfamily. Alignment of the amino acid sequence of the forkhead domain (solid line) and 5' flanking region of *CheFoxB* compared to *FoxA*, *B* and “*AB*” genes.

Species prefixes as in Fig. 1. The 12 aa N-terminal sequence preceding the Forkhead domain (dashed line) is highly similar in all the FoxB sequences, including *CheFoxB*



**Fig. 3** *CheFoxB* expression. Whole mount in situ detection of *CheFoxB* RNA at different stages of the *Clytia* life cycle. **a** egg; **b** 8-cell stage; **c** blastula (7 hpf); **d** early gastrula (11 hpf); **e** mid-gastrula; **f** 1-day planula; **g** 3-day planula (white arrows indicate expression extending into the anterior ectoderm); **h** gastrozooid (asterisks denote non-specific colour precipitation on remnants of the acellular theca); **i** young medusa; **j** part of adult medusa; **k** two developing statocysts from the same medusa illustrating the location of the expressing cells at the junction with the bell and on the inner surface of the statocyst pocket; **l** staining of a fully developed

statocyst, focussed the proximal region between the statolith and the bell (left and centre) and on a lower focal plane (right). Visualisation of nuclei (Hoechst staining overlaid in blue in centre and right panels) confirms that the stained cells are in the inner epithelium (IE) rather than the outer epithelium (OE). *G* Gonad, *tb* tentacle bulb. Arrowheads indicate ingressing presumptive endoderm in gastrulae. Thick arrows delimit the endodermal region in planulae. Thin black arrows in **i**, **j**, **k** and **l** point to statocysts. All gastrulae and planulae oriented with oral pole at top, medusae with manubria facing up. Bars 100  $\mu$  in **a-i**; 20  $\mu$  in **j-l**

detected in statocysts (Fig. 3i-l), sensory organs positioned on the bell rim (Singla 1975). In larger statocysts of older medusae, *CheFoxB* expression was distinguished predominantly in cells of the inner statocyst epithelium situated between the mineralised statolith and the bell margin (Fig. 3k,l). Given the position of these cells, they probably correspond to ciliated mechanosensory cells, whose basal projections run to the subumbrellar nerve ring (Singla 1975). In mature medusae, *CheFoxB* expression persisted in these regions and was also detected in the gonad, most strongly in the epithelial envelope surrounding it (Fig. 3j).

#### Two *Clytia* FoxQ2 genes with distinct expression profiles

The *CheFoxQ2a* and *CheFoxQ2b* sequences grouped clearly with the bilaterian FoxQ2 subfamily sequences (Fig. 1). We also found that three unpublished sequences

from *Hydra magnipapillata* (retrieved from <http://www.hydrabase.com>) and the *Nematostella* sequences *NvFox2* and *NvFox4* group with this subfamily. These *Nematostella* genes were previously not thought to be orthologues of known bilaterian Fox family genes due to omission of FoxQ2 members from the analysis (Magie et al. 2005). FoxQ2 genes have not been identified in mammalian genomes; however, they have now been identified in fish (Yu et al. 2003), with distinct FoxQ2 genes identifiable in the *Tetraodon nigroviridis* (identifiers CAF90529 and CAF98414) and *Takifugu rubripes* (identifiers gw2.25.375.1 and gw2.2.202.1) genomes as well as in zebrafish and chicken.

We were unable to determine clearly the phylogenetic relationships between the different *Clytia*, *Hydra* and *Nematostella* FoxQ2 genes irrespective of the methods of analysis and models applied. It was clear, however, that two of the three *Hydra* FoxQ2 genes (dubbed *HmFoxQ2a1*

and *HmFoxQ2a2*) are closely related and presumably derived from a duplication in the *Hydra* lineage. Furthermore, we found that (*CheFoxQ2b*, *HmFoxQ2b*, *NvFox2*) and (*HmFoxQ2a1/a2*, *NvFox4*) formed two separate clades in ML analysis (Fig. 1). This observation suggests that an ancestral FoxQ2 gene duplicated before diversification of the cnidarian groups rather than separately within the Anthozoa and Medusozoa groups. If this scenario is correct, *CheFoxQ2a* could be orthologous to *NvFox4* and *HmFoxQ2a1/a2*, with phylogenetic reconstruction artefacts disrupting this grouping in our analyses.

In situ hybridization revealed entirely distinct temporal and spatial expression profiles for the two *Clytia* FoxQ2 subfamily members (Fig. 4). *CheFoxQ2a* expression was detected during larval development but not in polyps or medusae, while *CheFoxQ2b* was detectable only in medusae. *CheFoxQ2a* expression was first detectable in blastulae, being initially uniform and then progressively developing a polarised distribution (Fig. 4a,b). In embryos fixed at the onset of gastrulation, it was clear that the expression domain was restricted to about 1/3 to 1/2 of the embryo, opposite to the site of cellular ingression (Fig. 4c). This *CheFoxQ2a* expression corresponds to the future aboral pole and is complementary to the expression domain of *CheFoxB* (Fig. 3d). In contrast to the transient polarised expression of *CheFoxB*, polarised expression of *CheFoxQ2a* was maintained throughout larval development. In 1-day planulae, the aboral *CheFoxQ2a* domain included cells in both endoderm and ectoderm regions (Fig. 4d), whereas in older larvae it appeared to be restricted to ectodermal cells (Fig. 4e). No localised *CheFoxQ2a* RNA was detected in polyps or medusae (Fig. 4f–h).

*CheFoxQ2b* expression was essentially confined to the gonad of the medusa (Fig. 4i–p). Staining was detectable before morphological appearance of the gonad in newly budded medusae. In mature adults, it was concentrated in the peripheral gametogenic zone (Roosen-Runge and Szollosi 1965) and detected less strongly in fully grown oocytes (Fig. 4o,p). A low uniform signal, presumably from residual maternal RNA, was detected in eggs and cleavage-stage embryos.

A FoxO subfamily gene expressed dynamically in the planula, polyp and medusa

Sequence analysis placed the fourth *Clytia* Forkhead box-containing cDNA identified in this study firmly in the FoxO subfamily (Fig. 1). An incomplete FoxO Forkhead domain was recently identified from the trace archive of the *N. vectensis* genome (Magie et al. 2005). BLAST searching of this archive with the *CheFoxO* sequence led us to identify a distinct genomic sequence with strong amino acid identity spanning the entire Forkhead domain. The coding sequence was interrupted by a 159-nt intron corresponding exactly in position to that reported for the mammalian FoxO6 genes (Anderson et al. 1998) (Fig. 5). A complete amino acid sequence for this *Nematostella* FoxO gene, dubbed *NvFoxO.2*, was derived from over-

lapping genome sequences (see [Materials and methods](#)). The previously described *NvFoxO* sequence, which we refer to as *NvFoxO.1*, corresponds to a distinct first exon and part of the following intron, as it loses similarity with the sequence 3 of the splice site. The *NvFoxO.1* sequence is much more divergent than *NvFoxO.2* from other FoxO genes, both within the Forkhead domain and elsewhere (Fig. 5), including positions showing strong identity between all other species, and may be non-functional.

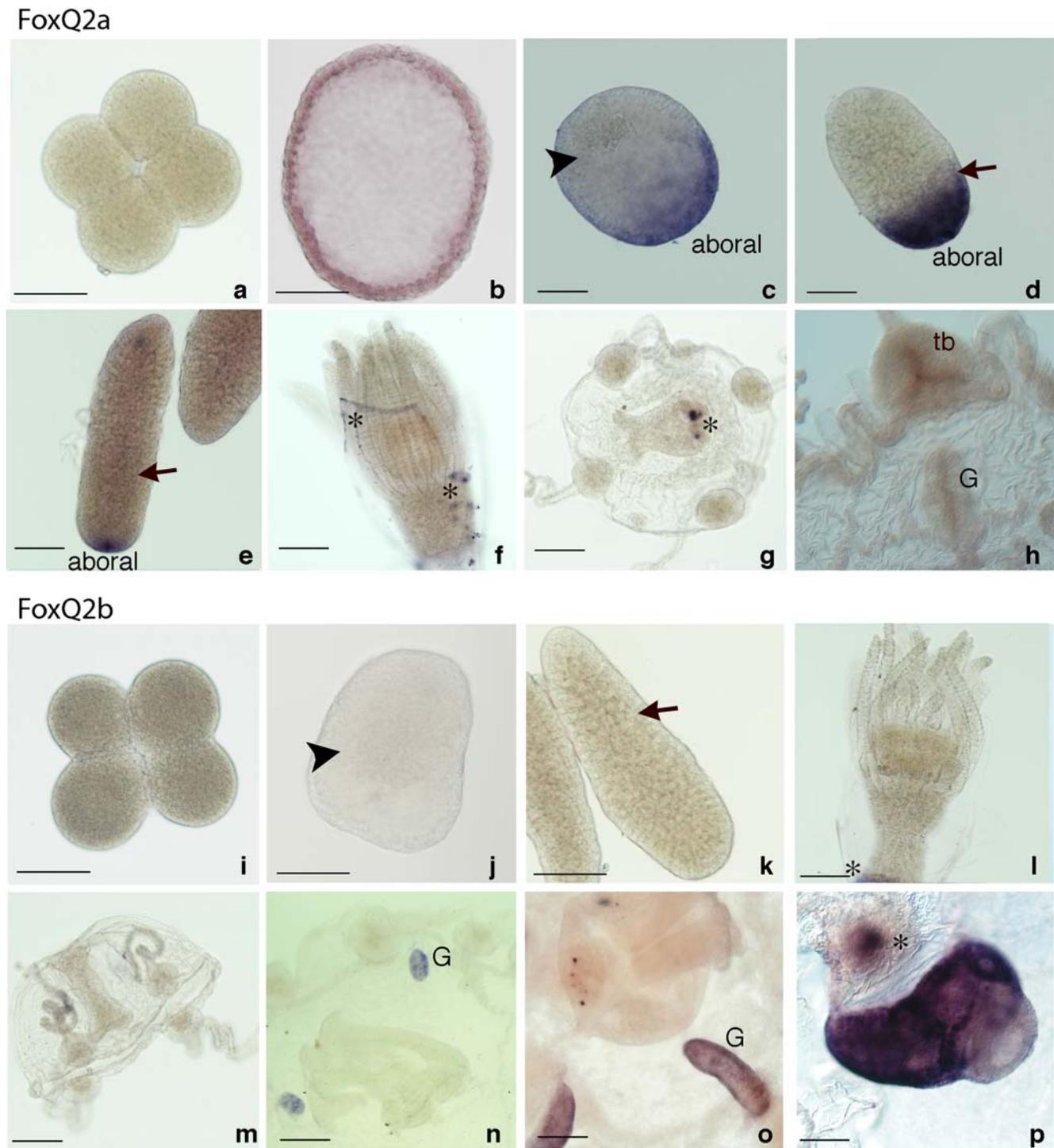
Both the *CheFoxO* and *NvFoxO.2* sequences were found to share features of bilaterian FoxO genes outside the forkhead domain (Fig. 5), including three potential AKT/PKB phosphorylation sites highly conserved amongst FoxO proteins. Phosphorylation at these sites promotes nuclear export and thus allows down-regulation of activity in response to signalling by cytokines or growth factors such as insulin (Burgering and Kops 2002). Spaced clusters of basic amino acids typical of a bipartite Nuclear Localisation Signal (NLS) were found overlapping with the Forkhead domain, in the region corresponding to the mammalian FoxO NLS.

In situ hybridisation revealed *CheFoxO* RNA at all stages of the *Clytia* life cycle; however, the levels of *CheFoxO* expression were modulated in different cell populations as development progressed. A low level of maternal RNA was detected (Fig. 6a,b; see also expression in oocytes in Fig. 6l), then asymmetric expression at the blastula stage (Fig. 6c). Expression during gastrulation was reduced in ingressing presumptive endoderm and the overlying presumptive oral ectoderm compared with the rest of the embryo (Fig. 6d,e). In planulae, elevated expression was detected at both poles, the aboral in situ staining becoming more intense at later stages (Fig. 6f). In polyps, *CheFoxO* expression was generally uniform; however, during their formation it was enhanced in elongating tentacles (Fig. 6g). In medusae, the RNA was detected strongly in a ring of superficial cells positioned at the bell margin as well as scattered cells in the tentacle bulb epithelium, but not in the statocysts (Fig. 6h–j). The staining at the bell margin persisted during medusal growth, although the density of the expressing cells diminished (compare Fig. 6h and k). *CheFoxO* was also expressed strongly within the germinal tissue of the gonads, being more concentrated in smaller oocytes than in larger ones (Fig. 6l).

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## Discussion

Our study of the expression of FoxB and FoxQ2 subfamily genes from the hydrozoan *Clytia hemisphaerica* supports the idea that embryonic patterning in vertebrates and cnidarians involves conserved molecular components associated with both an oral (gastrulation site) domain and a complementary aboral domain. Our data also indicate that Fox transcription factors have retained other ancestral regulatory functions in cnidarians and bilaterians while adopting specific functions during cnidarian diversification.



**Fig. 4** Expression of *CheFoxQ2a* and *CheFoxQ2b*. Whole mount in situ detection of *CheFoxQ2a* (a–h) and *CheFoxQ2b* (i–p) RNA. a, i 4-cell stage; b blastula (7 hpf); c early gastrula (11 hpf), d 1-day planula; e, k 3-day planula; f, l gastrozooid; g, m newly budded medusae; h, n, o parts of adult medusae; j mid-gastrula; p female gonad undergoing oocyte release. Arrowheads in gastrulae indicate

ingressing presumptive endoderm and thick arrows in planulae the endodermal layer. Asterisks indicate non-specific colour precipitate on the theca in gastrozooids, and in digestive areas (stomach and tentacle bulb endoderm) in medusae. G gonad, tb tentacle bulb. All gastrulae and planulae oriented with oral pole at top. Bars 100  $\mu$ m

#### Conserved gene expression domains in the early embryo

In early gastrula embryos of diverse species, FoxA subfamily (Forkhead) genes, along with *Brachyury*, *Snail* and *Gooseoid* homologues, are expressed in territories

adjacent to the initial site of cell ingression corresponding to the future oral pole in cnidarians and the dorsal organiser region in vertebrates (see [Introduction](#)). FoxB genes are also found expressed on the dorsal side of the early gastrula in vertebrates (Odenthal and Nusslein-Volhard 1998;

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      AKT1
MmFoxO1 .. (19) RQRSC*TWPLRP*... (152) RRNAWGNLSYADLITKAI*ESSAEKRLT*LSQIYEW*MKSV*PYFKDKGDS
CheFoxO .. (21) RPRSRT*WPI*LP*... (76) RKN*AWGNLSYADLITRAI*QSSPE*ORL*TL*SQIYEW*VRNIP*YFKDKGDS
NvFoxO.2 .. (16) RPRSNT*WPL*PEP*... (59) RKN*AWGNLSYADLITQAI*QSSPE*EKRLT*LSQIYD*W*V*NSP*VYFRDKGDS
NvFoxO.1 ..... (166) TQNPW*GSE*SYSEMISKAIM*SC*PSQEATLHTIYEW*IV*NNV*SYFADKADY

      <<
MmFoxO1 NSSAGWKNSIRHNLSLH*SKFIRVQNEGTGKSSW*WMLNPEGGKSGKSP*RRRRAASMDNNSKFAKRGRAAKK*
CheFoxO TSSAGWKNSIRHNLSLH*RFMRVQNDNNGKSS*Y*WV*INPDAKP-GKSNRRRSGSIDGQPKDGKRR--TKK*
NvFoxO.2 NSSAGWKNSIRHNLSLH*SKFV*RVQNEGN*GKSSW*WV*LN*PDAKG-AKTS*RRRSGSLDSAQKSTEKRRGRSKK*
NvFoxO.1 PSTHGWK

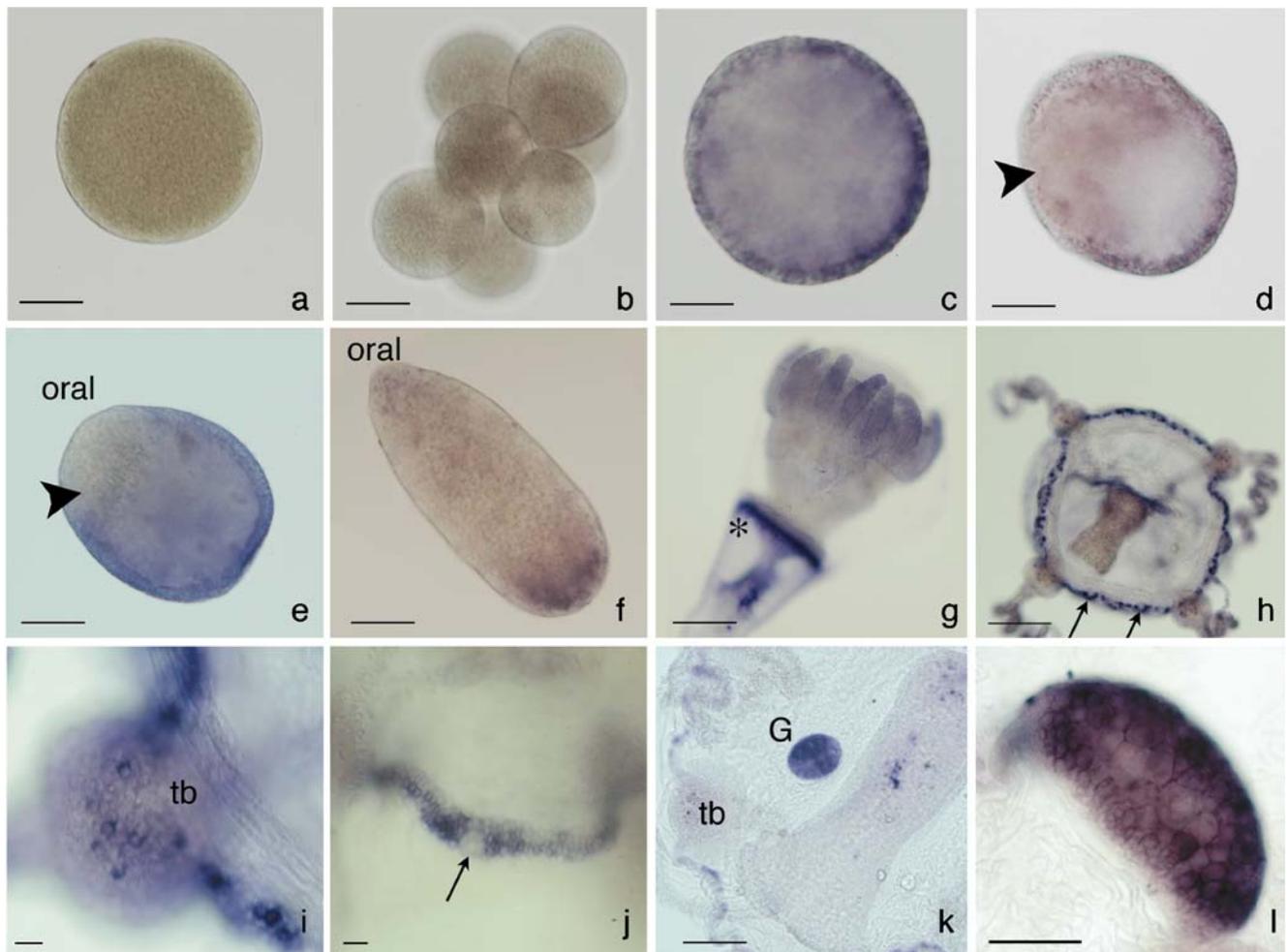
      AKT2
      * *
      * *

      AKT3
MmFoxO1 ..... (309) TFR*PR*RTSS*NASTIS ..... 542
CheFoxO ..... (254) FGR*PR*STSS*NVS*NVS ..... 508
NvFoxO.2 ..... (237) FGR*PR*RTSS*NASTVS ..... 486

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**Fig. 5** Conserved regulatory motifs in cnidarian *FoxO* genes. Alignment of cnidarian *FoxO* amino acid sequences (CheFoxO, NvFoxO.1 and NvFoxO.2) with Mouse FoxO1. The NvFoxO.1 sequence derived from Genome trace identifier 557608368 (Magic et al. 2005) corresponds to a divergent exon (see text). The splice position is marked by <<. Within the forkhead domain (black bar),

amino acids identical with the mouse FoxO1 sequence are shaded. The region of the bipartite NLS (grey outline) is characterised by separated clusters basic amino acids (grey shading). Black boxes outline the three identified AKT/PKB phosphorylation sites showing conserved RxRxT/S motifs



**Fig. 6** CheFoxO expression. Whole mount in situ detection of CheFoxO RNA. **a** Egg; **b** 8-cell stage; **c** blastula (7 hpf.); **d** early gastrula (11 hpf); **e** mid-gastrula (13 hpf); **f** 1-day planula; **g** developing gastrozooid polyp (asterisk indicates non-specific colour precipitate on the acellular theca); **h** newly budded medusa; **i** tentacle bulb of newly budded medusa **j** bell rim of newly budded

medusa; **k** gonad; **l** part of mature medusa. *G* female gonad; *tb* tentacle bulb. Arrowheads indicate ingressing presumptive endoderm. Thin arrows point to statocysts. All gastrulae and planulae are oriented with oral pole at top, medusae with manubria facing up. Scale bars 100  $\mu$ m

Gamse and Sive 2001) and around the gastrulation site in *Clytia* (this study) and *Nematostella* (Magie et al. 2005). The FoxA and FoxB subfamilies are closely related and form a monophyletic group (Fig. 1; Kaestner et al. 2000; Mazet et al. 2003; Magie et al. 2005), and so may derive from a common FoxA/B ancestor expressed in the organiser region of the last eumetazoan common ancestor.

If this scenario is correct, the putative ancestral axial patterning role of FoxB has been lost in the two protostomes for which data are available: the *Drosophila* FoxB genes *FD4* and *FD5* are implicated only in neural development, and the *Caenorhabditis* FoxB gene *Lin-31* in vulva development (Hacker et al. 1992; Tan et al. 1998). In cnidarians, as in deuterostomes, gastrula-stage FoxA and FoxB expression territories on the oral/dorsal side are distinct. In *Nematostella*, FoxA is expressed in involuting cells (Fritzenwanker et al. 2004; Martindale et al. 2004), while FoxB expression remains mainly restricted to the involution boundary (Magie et al. 2005). FoxB expression in sea urchin, like that of *CheFoxB*, is detected only transiently in involuting primary mesenchyme cells (Minokawa et al. 2004), while FoxA expression is endodermal. A comparable pattern is seen in vertebrates, where FoxB gene expression is mainly ectodermal, but FoxA genes remain expressed in different regions of involuted axial mesoderm and endoderm (Ang et al. 1993; Odenthal and Nusslein-Volhard 1998; Pohl et al. 2002). To determine to what extent do the apparent similarities in these FoxB expression patterns reflect conserved developmental functions requires more comparative data and understanding of gene function and regulation.

Relatively few patterning genes have so far been found expressed aborally (i.e. opposite the gastrulation site) in cnidarians. At the blastula/gastrula stages, only *CheFoxQ2a* (this study), *NvFoxD.1* (Magie et al. 2005) and the Hox gene *Anthox1* (Finnerty et al. 2004) have been described to have exclusively aboral expression, while *NvSoxB1* shows expression at both poles (Magie et al. 2005). The aboral expression of *CheFoxQ2a* from the blastula stage through gastrulation and larval development is intriguing, as it closely mirrors that of both amphioxus *AmphiFoxQ2* and the *Drosophila* FoxQ2 gene *fd102C* (CG11152; Mazet et al. 2003). Amphioxus is the only deuterostome for which a FoxQ2 expression profile has been reported. *AmphiFoxQ2* expression starts in animal cells (opposite the gastrulation site) at the blastula and gastrula stages and is maintained in an aboral/anterior pole territory throughout larval development (Yu et al. 2003). Likewise, *Drosophila fd102C* is expressed in a small anterior domain starting during the syncytial blastoderm stage, and in the pharyngeal and anterior neurectoderm structures that derive from it (Lee and Frasch 2004). It is tempting to speculate that an ancestral FoxQ2 gene participated in defining aboral/anterior territories during embryonic development from the blastula stage. Data on FoxQ2 gene expression from other species are clearly required. The only other report to date concerns *fkh-10* in *Caenorhabditis*, which is expressed in neurons associated with the larval and adult pharynx (Hope et al. 2003).

Other evolutionarily conserved roles for the Fox genes?

The expression patterns of both *CheFoxB* and *CheFoxO* support the idea that these genes have retained certain roles from a bilaterian/cnidarian ancestor. The observed expression of *CheFoxB* in the developing statocyst, a sensory organ linked to the neural coordination system of hydromedusae, is particularly intriguing due to the strong implication of bilaterian FoxB genes in nervous system development. In chordates, many FoxB genes are involved in patterning the neurogenic ectoderm (Odenthal and Nusslein-Volhard 1998; Gamse and Sive 2001; Mazet and Shimeld 2002), while *Drosophila* *FD4* and *FD5* are expressed in the developing nervous system (Hacker et al. 1992) and *Caenorhabditis Lin-31* is expressed in tail neurons as well as having a role in vulval development (Tan et al. 1998).

The other observed domains of *CheFoxB* expression in *Clytia* larvae and medusae are also potentially involved with the development of subsets of neural or other sensory cell types. Nematocysts (mechanosensory stinging cells) and neuronal ganglion cells are thought to originate from the endodermal region of the planula (Bodo and Bouillon 1968; Thomas et al. 1987), neural development proceeding in an aboral–oral progression (Groger and Schmid 2001), while the tentacle bulb ectoderm is also a site of intense nematogenesis (Bouillon 1993). The gonad is known to contain photosensory cells, as light stimulae provoke gamete release in isolated gonads (Honegger et al. 1980). Confirmation of the identity of the FoxB-expressing cells in these regions of the planula and medusa awaits a more complete characterisation of sensorial cells and their development in *Clytia* at cellular and molecular levels.

FoxO transcription factors have widely conserved functions in the regulation of proliferation and growth. In yeast as well as in bilaterian metazoans, they participate in controlling cell-cycle progression, apoptosis and differentiation as well as protection from oxidative stress, DNA repair, metabolism and longevity (Burgering and Kops 2002; Carlsson and Mahlapuu 2002; Accili and Arden 2004). It is thus not surprising that they are implicated in processes of tissue modelling during morphogenesis. For instance, mouse FoxO1 is required in the embryo for angiogenesis and FoxO3 for controlling ovarian follicle development (Hosaka et al. 2004). Consistent with this picture, *CheFoxO* RNA was detected at all stages of the *Clytia* life cycle, with a dynamic expression pattern reinforced in areas associated with growth and cell proliferation. The striking distribution of *CheFoxO* expressing cells at the rim of the bell may be related to cell addition in peripheral regions during medusa growth (Schmid et al. 1974), while *CheFoxO* expression in proliferative regions of the gonad may be related to the control of gamete production in relation to spawning and food availability. It will be interesting to determine which signalling pathways are involved in regulating FoxO transcription and post-translational regulation in these regions. FoxO proteins have been implicated in integrating growth factor signals from multiple ligands (e.g. insulin,

VEGF and TGF $\beta$ s) and other external cues to regulate development of a variety of tissues (Accili and Arden 2004; Seoane et al. 2004).

### Complex evolutionary history of Fox genes within the Cnidaria

A survey of the Fox genes in the draft genome of the anthozoan *Nematostella vectensis* revealed relatively poor representation of the subfamilies (Magie et al. 2005), supporting the suggestion that Fox family diversification has been driven by increasing morphological complexity (Carlsson and Mahlapuu 2002). Our phylogenetic analyses clearly indicate that the Fox gene family had already achieved high complexity (a minimum of 17 subfamilies) before the cnidarian/bilaterian split, conforming to the situation described for other multigene families such as the Sox (Magie et al. 2005; Jager et al. 2006) and Wnt (Kusserow et al. 2005) families, and imply that many Fox subfamilies have been lost in *Nematostella*. Cnidarian species show a wide variety of morphologies, life cycles and modes of development. Anthozoans are often considered to represent an ancestral form, but ‘simple’ characteristics like the absence of a medusal stage may result at least in part from loss of structures and genes from a more complex ancestor (Kortschak et al. 2003; Kusserow et al. 2005; Collins et al. 2006). Thus, it should not be surprising to find some members of developmental regulatory gene families absent in the *Nematostella* genome but represented in the Medusozoa group, as is the case for FoxJ [*Hydra vulgaris* *Hvfk2*: (Martinez et al. 1997)], FoxK (*Hydra magnipapillata*: *HmFoxK*; see Fig. 1), and apparently a posterior group Hox gene (Kamm et al. 2006). It remains to be seen whether the six to nine other predicted Fox gene losses in *Nematostella* have also been retained in other cnidarian lineages.

The *Clytia* FoxB and FoxQ2 genes offer examples of diversification of function within the cnidarian phylum. *CheFoxB*, whose apparent involvement in nervous system development may be evolutionarily conserved (see above), shows expression in a neurosensory structure particular to Medusozoa, the statocyst. In Leptomedusae such as *Clytia*, statocysts are described as being of ectodermal origin, while in other medusae, including other hydrozoans, they are of mixed ecto- and endodermal origin (Singla 1975). Moreover, Anthomedusae and Siphonophores, which are closely related to the Leptomedusae, lack statocysts. This suggests a complex evolutionary history for statocysts, perhaps involving an origin within the Medusozoa branch, then loss in the hydrozoan clade and re-invention in the Leptomedusae, or even several convergent acquisitions within the hydrozoans (Collins et al. 2006). Further examination of the expression of FoxB genes in different cnidarian classes may help clarify this issue.

We identified two members of the FoxQ2 subfamily in the cnidarians *Nematostella* and *Clytia* and three members in *Hydra*. Sequence comparisons suggest that *CheFoxQ2b*, *NvFox2* and *HmFoxQ2b* are orthologues, while *CheFox-*

*Q2a*, *NvFox4* and *HmFoxQ2a1/a2* may be divergent orthologues. The two *Clytia* FoxQ2 genes show completely distinct expression profiles in time and space: *CheFoxQ2a* RNA detectable only in the aboral territory of embryos and larvae and *CheFoxQ2b* exclusively in the gonad. It will be instructive to determine whether *NvFox2*, like *CheFoxQ2b*, is expressed in the adult (polyp) gonad and whether either of the *Nematostella* FoxQ2 genes are expressed in aboral domains in the embryo and larva. Single FoxQ2 genes have been identified in *Drosophila*, *Anopheles*, *Ciona* and *Danio* genomes and none in mammalian genomes, again suggesting a complex evolutionary history of losses and duplications. If FoxQ2a genes have indeed a conserved function in patterning the aboral pole of the early embryo, the mammalian loss of FoxQ2 genes might reflect the extreme modification of pregastrula patterning mechanisms associated with viviparity.

The study of developmental regulatory genes in cnidarians will undoubtedly increase our understanding of the evolution of fundamental mechanisms of metazoan development, but much more information is required concerning gene representation, expression, regulation and function. *Clytia hemisphaerica* is well suited to make a valuable contribution to this effort through the ease of manipulating embryos and of controlling all the life-cycle transitions in the laboratory.

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