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Clustered Fox genes in lophotrochozoans and the evolution of the bilaterian Fox gene cluster

Sebastian M. Shimeld [a,*], Michael J. Boyle [b], Thibaut Brunet [a,1], Graham N. Luke [c], Elaine C. Seaver [b]

[a] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK
[b] Kewalo Marine Laboratory, Pacific Biosciences Research Center, University of Hawaii, 41 Ahui Street, Honolulu, HI 96813, USA
[c] School of Biological Sciences, Hopkins Building, University of Reading, Whiteknights, Reading, RG6 6UP, UK

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ABSTRACT

FoxC, FoxF, FoxL1 and FoxQ1 genes have been shown to be clustered in some animal genomes, with mesendodermal expression hypothesised as a selective force maintaining cluster integrity. Hypotheses are, however, constrained by a lack of data from the Lophotrochozoa. Here we characterise members of the FoxC, FoxF, FoxL1 and FoxQ1 families from the annelid *Capitella teleta* and the molluscs *Lottia gigantea* and *Patella vulgata*. We cloned *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* genes from *C. teleta*, and *FoxC*, *FoxF* and *FoxL1* genes from *P. vulgata*, and established their expression during development. We also examined their genomic organisation in *C. teleta* and *L. gigantea*, and investigated local syntenic relationships. Our results show mesodermal and anterior gut expression is a common feature of these genes in lophotrochozoans. In *L. gigantea FoxC*, *FoxF* and *FoxL1* are closely linked, while in *C. teleta Ct-foxC* and *Ct-foxL1* are closely linked, with *Ct-foxF* and *Ct-foxQ1* on different scaffolds. Adjacent to these genes there is limited evidence of local synteny. This demonstrates conservation of genomic organisation and expression of these genes can be traced in all three bilaterian Superphyla. These data are evaluated against competing theories for the long-term maintenance of gene clusters.

Introduction

Clusters of homologous genes arise through tandem duplication. Most such clusters are likely to be relatively recently evolved, however a few have been shown to predate the divergence of Phyla or Superphyla. The canonical example is the Hox genes, which form a cluster that evolved prior to the radiation of the bilaterians, and are still clustered in the genomes of many living species. The collinear relationship between chromosomal gene order and spatiotemporal activation of expression provides a possible explanation of a selective force that could have kept these genes clustered for so long (Monteiro and Ferrier, 2006).

Hox genes are not the only example of such ancient gene clusters. Several other groups of ANTP-class homeobox genes show evidence of ancient clustering, for example the Parahox and Nkx genes (Brooke et al., 1998; Luke et al., 2003), and there is also evidence for an ancient cluster of Wnt genes (Nusse, 2001). Another type of gene that shows evidence of ancient clustering is the Fox gene class (Mazet et al., 2006). The Fox genes encode forkhead box transcription factors, and have been classified into 23 subclasses (also called families) in vertebrates (Kaestner et al., 2000). Most of these families have orthologues in protostome invertebrates and cnidarians, thus, they evolved prior to the radiation of the Bilateria (Larroux et al., 2008; Magie et al., 2005; Mazet et al., 2003). Comparative genomics have shown that four of these Fox gene families, FoxF, FoxC, FoxL1 and FoxQ1, are clustered in the amphioxus and vertebrate genomes (Carlsson and Mahlapuu, 2002; Mazet et al., 2006; Wotton and Shimeld, 2006; Yu et al., 2008). These genes are not clustered in the *Drosophila melanogaster* or *Caenorhabditis elegans* genomes, but are to some extent in the bee *Apis mellifera* (Mazet et al., 2006). The differences are that: (1) the *FoxQ1* gene is missing from all these species, and indeed has not been described from any protostome to date, and (2) the *A. mellifera* cluster is more dispersed than that in chordates, with the *FoxL1* gene separated from *FoxF* and *FoxC* by many intervening genes (Larroux et al., 2008; Mazet et al., 2006; Mazet et al., 2003).

The expression of these Fox genes has been described from several chordates and for all but FoxQ1 in *D. melanogaster*. The chordate data, which include expression studies in amphioxus, *Ciona intestinalis*, dogfish, zebrafish, trout, *Xenopus*, chick and mouse, have been recently reviewed in detail (Wotton et al., 2008). There is considerable variation in expression between individual species, but also an underlying consistency that probably reflects primitive roles: in chordates the *FoxF* genes are consistently expressed in the visceral mesoderm, while *FoxC* genes are expressed in paraxial mesoderm.

* Corresponding author. Fax: +44 1865 310447.
E-mail address: sebastian.shimeld@zoo.ox.ac.uk (S.M. Shimeld).
[1] Present address: Département de Biologie, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France.

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FoxL1 is also consistently expressed in the mesoderm, and can overlap with both *FoxF* and *FoxC* genes. Finally, *FoxQ1* is expressed in the pharyngeal endoderm. Parallels between these expression domains can be drawn to *D. melanogaster*. The *D. melanogaster FoxF* gene, also known as *biniou*, is expressed in the visceral mesoderm (Perez Sanchez et al., 2002; Zaffran et al., 2001). The *FoxC* and *FoxL1* genes (also known as *crocodile* and *fd2* respectively) are expressed in the somatic mesoderm (Hacker et al., 1992; Hacker et al., 1995).

Several studies have addressed the function of these Fox genes in *D. melanogaster* and in vertebrates. In *D. melanogaster*, a mutation in the *FoxC*/*crocodile* gene results in head defects that probably relate to very early anterior embryonic expression (Hacker et al., 1995). Later expression in the mesoderm has not been experimentally investigated. *D. melanogaster FoxF*/*biniou* has been shown to be necessary for maintaining distinct visceral and somatic mesoderm precursors, as well as for correct differentiation of visceral mesoderm derivatives (Perez Sanchez et al., 2002; Zaffran et al., 2001).

In vertebrates, mouse genetics has been used to dissect the functions of these genes, with some additional studies on other vertebrates. Duplications of *FoxC* and *FoxF* genes in the vertebrate lineage have led to paralogues with redundant functions. Removal of all *FoxC* paralogue function results in loss of somite formation in both mice and zebrafish, and to expansion of intermediate mesoderm characters into paraxial territory (Kume et al., 2001; Topczewska et al., 2001; Wilm et al., 2004). In an analogous fashion, loss of *Foxf1* function in mice results in defects in the separation of splanchnic and somatic mesoderm and expansion of somatic character into the splanchnic mesoderm (Mahlapuu et al., 2001), as well as later developmental abnormalities principally involving internal organ development (Kalinichenko et al., 2001; Tseng et al., 2004).

Foxl1 and *Foxq1* are found as single copies in mice, although additional *FoxQ1* paralogues are present in some fish genomes (Wotton and Shimeld, 2006). Mouse *Foxl1* has been extensively studied, and its deletion results in a variety of gastrointestinal defects primarily resulting from its lack of expression in the mesoderm surrounding the gut (Kaestner et al., 1997; Katz et al., 2004; Perreault et al., 2005; Sackett et al., 2009). The phenotype of *Foxq1* mutant mice is more subtle, manifesting as the *satin* mouse strain with an unusually silky coat derived from abnormal hair shaft development probably related to the expression of *Foxq1* in hair follicles (Hong et al., 2001). This may be specific to mammals since, as summarised above, in anamniote vertebrates *FoxQ1* expression marks the pharyngeal endoderm (Choi et al., 2006; Wotton et al., 2008), and *Foxq1* knockout mice have also been shown to have defects in stomach development (Goering et al., 2008). These parallels suggest that expression patterns and functions are conserved, in that they have evolved from a common ancestor of chordates and *D. melanogaster* which had *FoxF*, *FoxC* and *FoxL1* expression in specific compartments of the mesoderm.

D. melanogaster and chordates belong to two of the three major superphyletic assemblages of the Bilateria, the Ecdysozoa and Deuterostomia respectively. The third, commonly called the Lophotrochozoa, includes familiar lineages such as the annelids and molluscs, but is far less studied. Here we present an analysis of these four Fox gene families in three lophotrochozoans, the annelid *Capitella teleta*, formerly *Capitella* sp. I (Blake et al., 2009), and the molluscs *Lottia gigantea* and *Patella vulgata*, both limpets. We show that *C. teleta* has members of all four Fox families, making it the only protostome so far shown to have a full complement of these Fox genes. We also describe *FoxF*, *FoxC* and *FoxL1* genes from both limpet species. In the *Lottia* genome, all three genes are clustered within about 150 kb, while in the *C. teleta* genome *FoxC* and *FoxL1* lie within 20 kb of each other, with *FoxF* and *FoxQ1* on different scaffolds.

We also establish the expression of these Fox genes in developing mollusc and annelid embryos and larvae. Comparison between these phyla shows conserved mesodermal expression for *FoxF* and *FoxC*, confirming the ancestry of these genes in the subdivision of visceral and somatic mesoderm. *FoxQ1* marks the oesophagus in *C. teleta* and chordates, demonstrating anterior gut to be an ancient site of expression. *FoxL1* expression varies between taxa, but shows overlap with *FoxC* and *FoxF* in most animal lineages. Integrating expression and genomic organisation data leads us to an evolutionary model for this gene cluster, depicting an ancestral four gene cluster that evolved prior to the separation of the cnidarian and bilaterian lineages, with gene expression marking the mesodermal cell types associated with gut and body wall musculature. Cluster breakup and/or gene loss has occurred in several lineages, and is discussed.

Methods

Capitella teleta Fox genes

The draft assembly of the *C. teleta* genome (assembly version 1.0; http://genome.jgi-psf.org/Capca1/Capca1.home.html) was reiteratively searched to identify all members of the Fox gene family as previously described for *Branchiostoma floridae* (Yu et al., 2008). In brief, first the predicted gene models were searched with a selection of Fox sequences representing the known diversity of Fox genes using BLASTP. Next, the genome was searched using the same set of Fox sequences and the TBLASTN algorithm. A preliminary molecular phylogenetic analysis was then used to assign the resultant *C. teleta* Fox dataset to specific Fox families (not shown). PCR primer pairs for *C. teleta FoxC*, *F*, *L1* and *Q1* genes were designed from the relevant gene models and gene fragments were isolated and amplified by PCR from a cDNA template. PCR products were cloned, verified by sequencing and used to synthesize riboprobes for in situ hybridisation. Details of gene models, primers, clone sizes and GenBank accession numbers can be found in Table S1.

Lottia gigantea and Patella vulgata Fox genes

L. gigantea Fox genes were surveyed (assembly version 1.0; http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html) as for *C. teleta*, identifying putative *FoxC*, *FoxF* and *FoxL1* genes but no *FoxQ1* gene. Degenerate primers to putative *FoxC*, *FoxF* and *FoxL1* genes were used to amplify bands from *P. vulgata* genomic DNA. Bands of the correct predicted size were cloned and sequenced to confirm their identity. Details of gene models, primers, clone sizes and GenBank accession numbers can be found in Table S1.

Molecular phylogenetics

Predicted amino acid sequences were aligned using ClustalX then trimmed to include only the fork head domain. Alignments were imported into MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) and analyses conducted under the Poisson model with two chains and default priors. Analyses were run for one million generations and checked for chain convergence, with the first 25% of trees discarded when compiling summary statistics and consensus trees. Trees were visualized in TREEVIEW (Page, 1996) and transferred to Powerpoint for labelling.

Local synteny analysis

Predicted amino acid sequences for gene models adjacent to the *L. gigantea* and *C. teleta* Fox genes were reciprocally searched between the two genomes and the scaffold with the highest hit recorded. In many cases a single scaffold was hit with high probability, with a clear gap between this and other scaffold hits, if any. In some instances multiple scaffolds were hit with similar *e* values. In such cases all were recorded if the number of hits was 9 or less, or the results were recorded as the top hit plus additional hits $>X$, where X is a multiple of 10. Both data sets were also searched against the human genome in

the same fashion, except that chromosome arm was recorded instead of scaffold.

In situ hybridisation

A colony of *C. teleta* was maintained in the laboratory according to the culture methods originally developed by Grassle (Grassle and Grassle, 1976). Preparation of culture media, handling of adults, and the collection of larvae were performed as described previously (Seaver et al., 2005). Whole-mount in situ hybridisation of *C. teleta* larvae followed a published protocol (Seaver and Kaneshige, 2006) with the following modifications: all specimens were hybridized at 65 °C for 72 h. Antisense digoxigenin-labeled riboprobes were synthesized with a T7 (*Ct-foxC*, *Ct-foxF*, *Ct-foxL1*) or SP6 (*Ct-foxQ1*) MEGAscript kit (Ambion Inc, Austin, TX). Stage 5–9 larvae were treated with antisense *Ct-foxC*, *Ct-foxF*, *Ct-foxL1*, and *Ct-foxQ1* riboprobes at working concentrations of 0.5 ng/µl, 0.5 ng/µl, 1.0 ng/µl and 1.0 ng/µl, respectively. For each riboprobe, in situ hybridization was repeated two times with negative and positive control treatments. Riboprobes were detected by incubation with alkaline phosphatase-conjugated anti-digoxigenin Fab frements (1:5000; Roche Diagnostics, USA) in 1× blocking buffer (Roche blocking powder in 100 mM maleic acid) overnight at 4 °C. Visualization of in situ hybridization was conducted by incubation in NBT/BCIP (US Biological, Swampscott, MA). Post-hybridization specimens were 'cleaned' in hybridization buffer and washed into PTw (1× phosphate-buffered saline (PBS), 0.1% Tween-20), then equilibrated in glycerol (80% glycerol/10% 10× PBS/10% diH20), and mounted on Rainex®-coated slides. Microscopic analyses were performed with DIC optics on a Zeiss Axioskop 2 compound, light microscope. Micrographs were captured with a stem-mounted, 4.0 megapixel Nikon Coolpix 4500 digital camera. A detailed in situ hybridization protocol is available upon request.

P. vulgata adults were collected from Tinside, Plymouth in October and November 2007 and maintained in recirculating aquaria at 12 °C. Gametes were liberated by dissection, fertilised in vitro as previously described (Hodgson et al., 2007) and cultured in filtered sea water at 16 °C. Staged embryos were fixed overnight in 4% paraformaldehyde in MOPS buffer (0.1 M MOPS, 0.5 M NaCl, 5 mM $MgSO_4$, 10 mM EGTA, pH 7.5), then dehydrated in 100% ethanol and stored at −20 °C. For in situ hybridisation, all washes are for 5 min at room temperature unless otherwise stated. Embryos were rehydrated with three washes in phosphate-buffered saline with 0.1% Tween 80 (PBT) and then digested with PBT plus 4 µg ml^{-1} proteinase K for 5 min at room temperature. Next they were fixed for 20 min in 4% paraformaldehyde in MOPS buffer, and washed 3 times in PBT, twice in hybridisation solution (50% formamide, 5× SSC, 100 µg ml^{-1} yeast RNA, 50 µg ml^{-1} heparin, 0.1% Tween 80) before incubation in hybridisation solution at 65 °C for at least 2 h. Hybridisation was static overnight at 65 °C. Probes were synthesized from the cloned DNA fragments originally amplified using the primers detailed in Table S2, and the optimum concentration of probe was determined experimentally for each probe. The following day embryos were washed 6 times for 20 min each at 65 °C in wash solution (50% formamide, 5× SSC, 0.1% Tween 80), 3 times in PBT and blocked for at least 2 h at 4 °C in 20% heat-treated sheep serum in PBT. Alkaline phosphatase-conjugated Anti-digoxigenin antibody (Roche) was pre-absorbed for at least 2 h on ice in 20% heat-treated sheep serum in PBT at a dilution of 1:3000. Embryos were then incubated with antibody overnight at 4 °C. The following day, embryos were washed 3 times with PBT, three times with APT (100 mM NaCl, 100 mM Tris.Hcl pH 9.0, 50 mM $MgCl_2$, 0.1% Tween 80) and incubated in staining solution (APT + 20 µl BCIP/NBP mix (Roche) per ml). For photography embryos were mounted in 100% glycerol, or in BABB (a 1:2 (v:v) mix of benzyl alcohol:benzyl benzoate). For histology, embryos were fixed in 4% paraformaldehyde, stained with Poinceau S and embedded in LR White medium resin before sectioning at 3.5 µm.

Results

Identification and sequence analysis of polychaete and mollusc FoxC, FoxF, FoxL1 and FoxQ1 genes

BLAST searches of the draft *C. teleta* and *L. gigantea* genomes identified a number of predicted genes encoding forkhead domains, including putative *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* orthologues from *C. teleta* and putative *FoxC*, *FoxL1* and *FoxF* orthologues from *L. gigantea*. No *FoxQ1* orthologue was identified in *L. gigantea*, and molecular phylogenetic analyses of all identified *L. gigantea* Fox sequences confirmed this absence from current sequence data (not shown). To confirm BLAST predictions of orthology, we carried out a more focused molecular phylogenetic analysis of putative clustered Fox orthologues with a selection of clustered Fox sequences from other species plus the full mouse and *D. melanogaster* Fox gene complements. The results show these genes fall robustly into the respective Fox family groups, supporting the prediction of orthology (Fig. 1).

To confirm the *C. teleta* sequences and obtain clones for in situ hybridisation, we used RT-PCR to amplify a fragment of each gene (Table S1). *L. gigantea* is a limpet, and as such relatively closely related to *P. vulgata*, a limpet previously used for embryological and developmental studies (Damen and Dictus, 1994; Lartillot et al., 2002b). We used the *L. gigantea* sequences as starting points for primer design and hence amplified fragments of all three genes from *P. vulgata* genomic DNA. Sequence comparisons (see Supplementary data) confirm that the *P. vulgata* genes, like their *L. gigantea* orthologues, appear to lack introns in the amplified regions. Molecular phylogenetic analysis confirmed the identity of the *P. vulgata* sequences (Fig. 1). In naming genes we have elected to follow previous convention for *C. teleta*; this species was formally known as *Capitella sp. I* (Blake et al., 2009) and genes prefixed with *CapI-*. Thus with the new name genes are now prefixed *Ct-*. For *P. vulgata* and *L. gigantea* the genes have no prefix, as previously different authors have used different prefixes. Where groups of genes are referred to, no prefix is employed.

Linkage, clusters and syntenic relationships of FoxC, FoxL1, FoxF and FoxQ1 in polychaete and mollusc genomes

To examine the extent of clustering of Fox genes we examined the *L. gigantea* and *C. teleta* genome assemblies and recorded the scaffolds on which the clustered Fox genes were localised. We also examined the location of the *GMDS* gene, which encodes an enzyme (GDP-mannose 4,6-dehydratase) involved in the production of GDP-fucose (Sullivan et al., 1998), and which is closely linked to the Fox genes in chordate genomes. In the *L. gigantea* genome, all three Fox genes and *GMDS* were located within a 150 kb region of scaffold 26. Intergenic distances were comparable to those in amphioxus and human genomes, and a small number of predicted genes were found between and *GMDS* (Fig. 2A). In *C. teleta*, we found *Ct-foxC*, *Ct-foxL1* and *Ct-GMDS* all present within 26 kb on scaffold 34. This scaffold is about 800 kb long, and the three genes were located within 30 kb of one end (Fig. 2A). *Ct-foxF* was located within 5 kb of one end of scaffold 87, which is about 600 kb long. Finally *Ct-foxQ1* was located about 100 kb from one end of scaffold 64, which is also about 600 kb long. These results show that in *L. gigantea* the cluster is preserved (although *FoxQ1* has probably been lost from this lineage). In *C. teleta*, a minimum clustering of *Ct-foxC*, *Ct-foxL1* and *Ct-GMDS* is preserved. It is possible that either *Ct-foxF* or *Ct-foxQ1* are in fact closely linked to these genes, or that *Ct-foxF* and *Ct-foxQ1* are closely linked to each other, if some of these scaffolds are contiguous in the genome.

To examine the Fox loci more closely, we constructed synteny maps comparing these regions of the *L. gigantea* and *C. teleta* genomes to each other and to the human genome (selected as the best annotated and assembled chordate genome). Note that the vertebrate lineage is ancestrally tetraploid (Putnam et al., 2008) and hence a syntenic gene

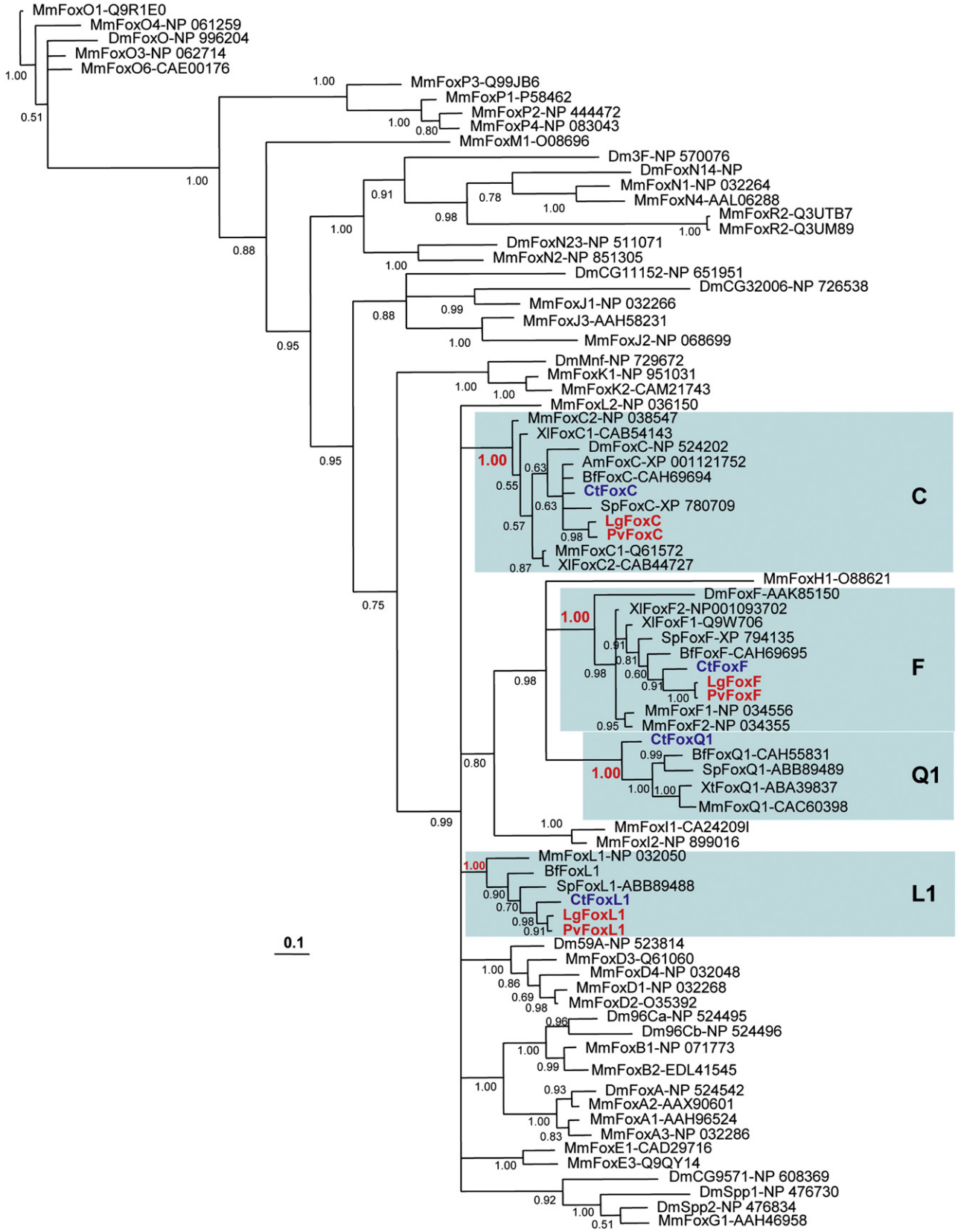

Fig. 1. Molecular phylogenetic analysis of *C. teleta* (Ct), *L. gigantea* (Lg) and *P. vulgata* (Pv) *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* genes. Mouse (Mm) and *Drosophila* (Dm) sequences reflect the diversity of other Fox families and are used as outgroups. This is a Bayesian analysis and the numbers adjacent to nodes represent posterior probabilities. Additional taxa are included for the *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* genes; *Apis mellifera* (AM), *Branchiostoma floridae* (Bf), *Strongylocentrotus purpuratus* (Sp), *Xenopus laevis* (Xl), *Xenopus tropicalis* (Xt). Accession numbers are given after the sequence name, and the four gene families are boxed with the relevant supporting value shown in bold.

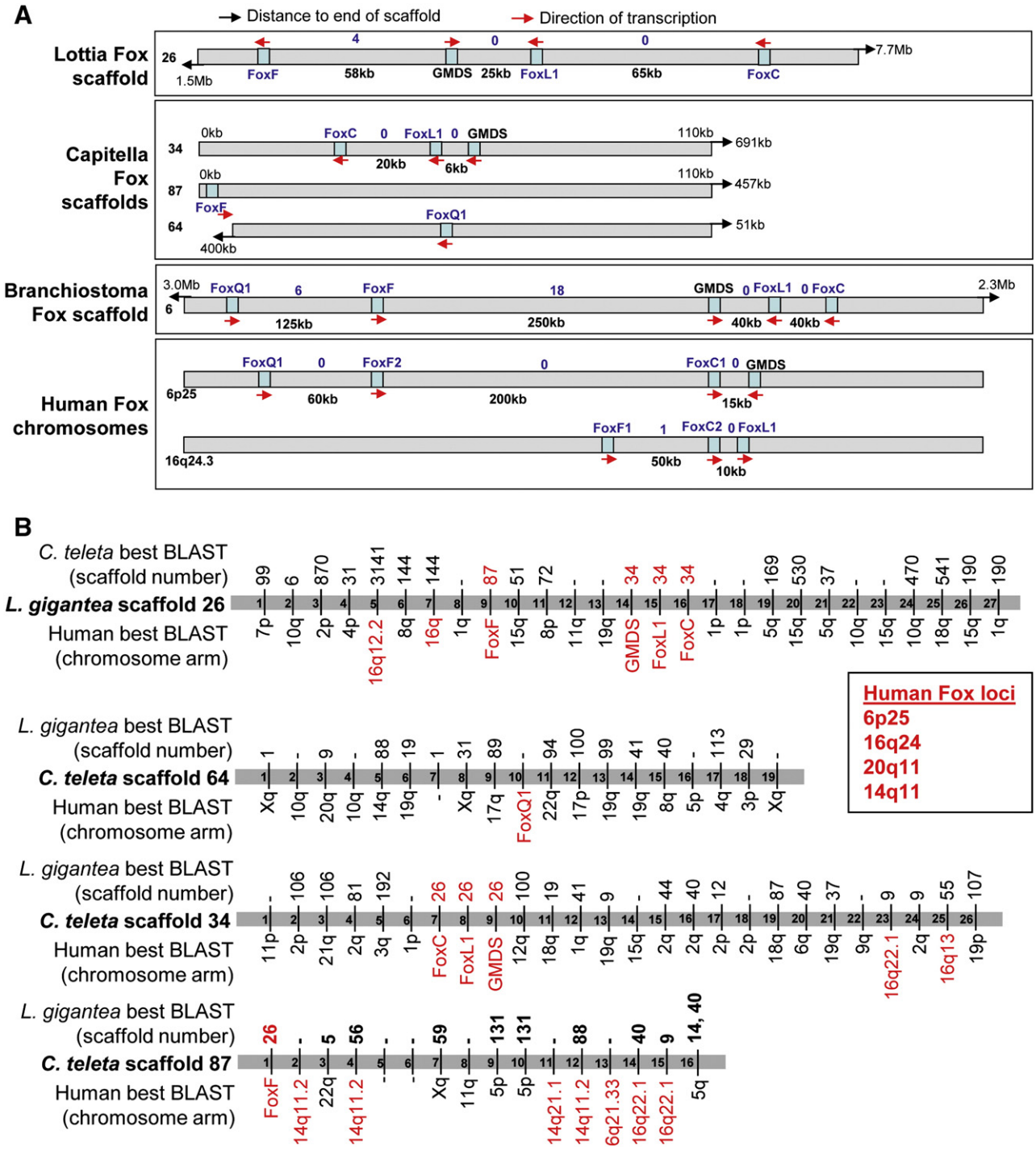

Fig. 2. A. Schematic maps of the *L. gigantea* and *C. teleta* scaffolds on which the *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* genes are located. Also shown is *GMDS*, which is typically linked to the cluster. Underneath are similar representations of the amphioxus (*B. floridae*) cluster and the two human clusters. Numbers below each scaffold show intergenic distances in kb. Numbers above indicate the number of genes lying between each pair of named genes (based upon gene model predictions). Arrows and numbers at the end of each scaffold indicate the distance to the end of the scaffold. Direction of transcription of named genes is shown by red arrows. B. Analysis of local synteny between *L. gigantea*, *C. teleta* and the human genome. Each *L. gigantea* or *C. teleta* scaffold is represented as a grey bar and the scaffold number and species name are in bold. Predicted genes within each segment shown are numbered sequentially from 1, with the numbers shown within the grey bar. The location of the best BLAST match in the other two genomes is shown above and below each bar. Dashes indicate where no hit was seen. BLAST hits that are potentially syntenic are shown in red; a small number show synteny to the human genome. No synteny was found between *L. gigantea* and *C. teleta* genomes other than the Fox genes and GMDS. For more details, see Supplementary Tables 2 to 5.

may map to any of four paralagous human genome regions; for the Fox clusters these are chromosomes 6p25, 16q24, 20q11 and 14q11 (Wotton and Shimeld, 2006). The results of this analysis are shown in Fig. 2B. For *L. gigantea* scaffold 26, two genes close to *FoxF* mapped to the relevant human genomic region, but other nearby genes did not. Comparison of *C. teleta* scaffolds to the human genome showed no evidence of synteny of scaffold 64 (*Ct-foxQ1*) to the human Fox cluster regions. Scaffold 34 (*Ct-foxC*/*foxL1*/*GMDS*) has two neighbouring genes which map to human 16q. On scaffold 87 *Ct-foxF* was adjacent to several genes that mapped to human 14q11 or 16q22. We also compared *L. gigantea* to *C. teleta*. None of the analysed genes on *L. gigantea* scaffold 26 mapped to any of the three relevant *C. teleta*

scaffolds. Similarly, the genes on these three *C. teleta* scaffolds did not map to *L. gigantea* scaffold 26 (Fig. 2B). This included the genes from both species which did map to human Fox cluster regions.

In summary, we found evidence for limited synteny beyond the Fox clusters to the human genome, though this was primarily confined to genes adjacent to *FoxF* in both species. We found no evidence for preserved synteny between the two lophotrochozoan genomes beyond the Fox genes and *GMDS*.

Expression of Ct-foxC, Ct-foxF, Ct-foxL1 and Ct-foxQ1 in C. teleta

C. teleta is a marine, polychaete annelid that is considered to be a non-selective subsurface deposit feeder, although morphological and 'non-selective' generalizations have been reconsidered in light of recent detailed studies on foregut development and anatomy (Boyle and Seaver, 2008, 2009). From anterior to posterior, the gut tube includes a mouth, foregut (buccal cavity, pharynx, and oesophagus), midgut, hindgut and terminal anus. Along the length of the gut tube, the foregut is the most diverse morphologically, and distinct subregions become apparent during larval development (Fig. 3). The anterior foregut develops an eversible proboscis, which is primarily composed of a highly muscularized dorsal pharynx invested with ciliated columnar epithelia, pharyngeal nerve fibres, glandular tissues and bilateral sets of proboscis retractor muscles (Boyle and Seaver, 2009). We follow an established developmental staging system for *C. teleta* and describe expression patterns during larval development (Fig. 3; (Seaver et al., 2005)).

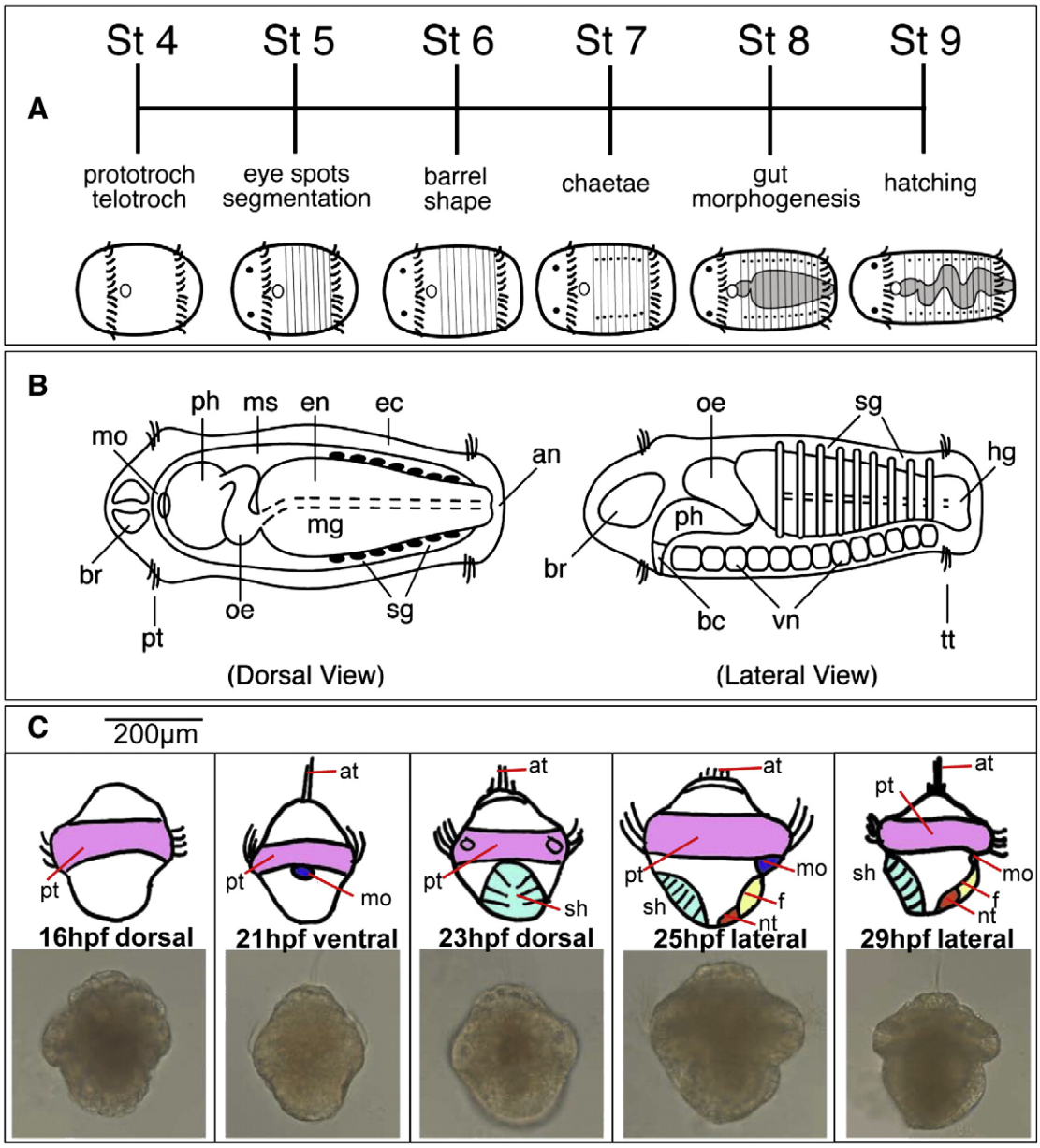

Fig. 3. Summary of the development of *C. teleta* and *P. vulgata* embryos. A and B show a developmental staging chart and generalized anatomy for the larvae of *C. teleta*. A. Diagrammatic larval stages 4–9 in ventral view with anterior to the left. Developmental timeline represents approximately five days (adapted from (Seaver et al., 2005)). B. Schematic of a stage 8 larvae in dorsal and lateral views with anterior to the left. Schematic views highlight the major organ systems and germ layers (adapted from (Boyle and Seaver, 2008)). C. Generalized developmental series through trochophore larval development for *P. vulgata*. A staging series for *P. vulgata* is yet to be established, hence we use hours post fertilisation (hpf) at 16 °C to indicate age. Below are DIC micrographs of fixed *P. vulgata* embryos mounted in 100% glycerol. All stages have a high yolk content making visualization of internal structures difficult. Above each micrograph is a sketch diagram indicating the major external structures, which are also colour coded: the prototroch (pt) is in pink, mouth (mo) in dark blue, shell gland (sh) in light blue, foot field (f) in yellow and neurotroch (nt) in red. dorsal, ventral and lateral indicate the orientation of the embryo and sketch. Other abbreviations are: an, anus; at. apical tuft; bc, buccal cavity; br, brain; ec, ectoderm; en, endoderm; hg, hindgut; mg, midgut; ms, mesoderm; oe, oesophagus; ph, pharynx; pt, prototroch; sg, segmental mesoderm; tt, telotroch; vn, ventral nerve cord.

Ct-foxC is expressed in stage 5–7 larvae in two anterior, subsurface domains: a medial patch of muscle cells on the ventral–anterior side of the brain (Figs. 4B, D, F, H), and in both left and right lateral margins of the foregut (Figs. 4A, C, E, H, J). During stage 6, a third expression domain appears in ventro-lateral cells on each side of posterior trunk adjacent to the midgut (Fig. 4F). In the foregut region of stages 6–7, *Ct-foxC* is predominantly transcribed in cells that surround the developing pharynx and oesophagus (Figs. 4E–H), and at very low levels in their epithelia. This *Ct-foxC* foregut expression is distinct from the expression of *Ct-foxA* (Fig. 4I; Boyle and Seaver, 2008), which is strongly expressed in epithelial cells throughout both the pharynx and oesophagus. In the trunk of stages 7–8, *Ct-foxC* is expressed in discrete groups of mesoderm cells near segmental boundaries of the five abdominal segments (Figs. 4H, L, M), posterior to the thoracic–abdominal transition.

Ct-foxF shows a bilateral expression pattern in three domains during larval development: the brain, foregut and posterior trunk (Fig. 5). Transcription of *Ct-foxF* in the brain is broad in early larval stages (Figs. 5A, B), and localised to cells surrounding the neuropile in stage 7 and stage 8 larvae (Figs. 5C, I). In the foregut of stage 5–7 larvae, *Ct-foxF* is expressed on the dorsal–posterior side of both left and right lateral margins (Figs. 5A, E–H). After the foregut is more fully developed in stage 8 larvae (Fig. 5I), *Ct-foxF* expression is concentrated in cells between the brain and oesophagus, and between the oesophagus and pharynx at its medial-posterior end, external to the pharyngeal epithelium. *Ct-foxF* expression in the posterior trunk during stage 5–7 larvae spans the ventro-lateral mesoderm of the five abdominal segments (Figs. 5A–D). This expression is adjacent to the midgut and does not appear to be segmental.

Ct-foxL1 is localised predominantly in foregut tissue in larval stages 5–9 (Fig. 6). In stages 5–6, *Ct-foxL1* is expressed in surface cells on the posterior face of the mouth (Figs. 6A, C, D, F, I), and is not expressed in the mouth during later stages. *Ct-foxL1* also is expressed in a bilateral pair of patches on the dorsal–posterior side of the foregut during stages 5–7 (Figs. 6B, C, E, G–I, K, N). These expression foci are adjacent to, but not continuous with, the midgut. There is a third small domain of *Ct-foxL1* expression bordering the left and right lateral–anterior rims of the pharynx pad throughout larval development (Figs. 6H, K). In stages 8–9 (not shown) this expression marks two separate lateral patches of cells on the dorsal–anterior side of the pharynx, outside of the pharyngeal epithelium. There are very low levels of *Ct-foxL1* expression in the brain and pharynx regions of stages 7–8 larvae (not shown).

Ct-foxQ1 is expressed in a single domain, the oesophagus, during larval development. In stages 5–7, the expression pattern within the presumptive foregut is highly asymmetric between left and right sides of the animal (Figs. 7A, C). *Ct-foxQ1* is initially transcribed in dorsal–anterior, subsurface cells of the stomodeum (Figs. 7B, D), and shows comparatively higher levels of expression on the left side. During morphogenesis of the oesophageal epithelium in stages 8 and 9, *Ct-*

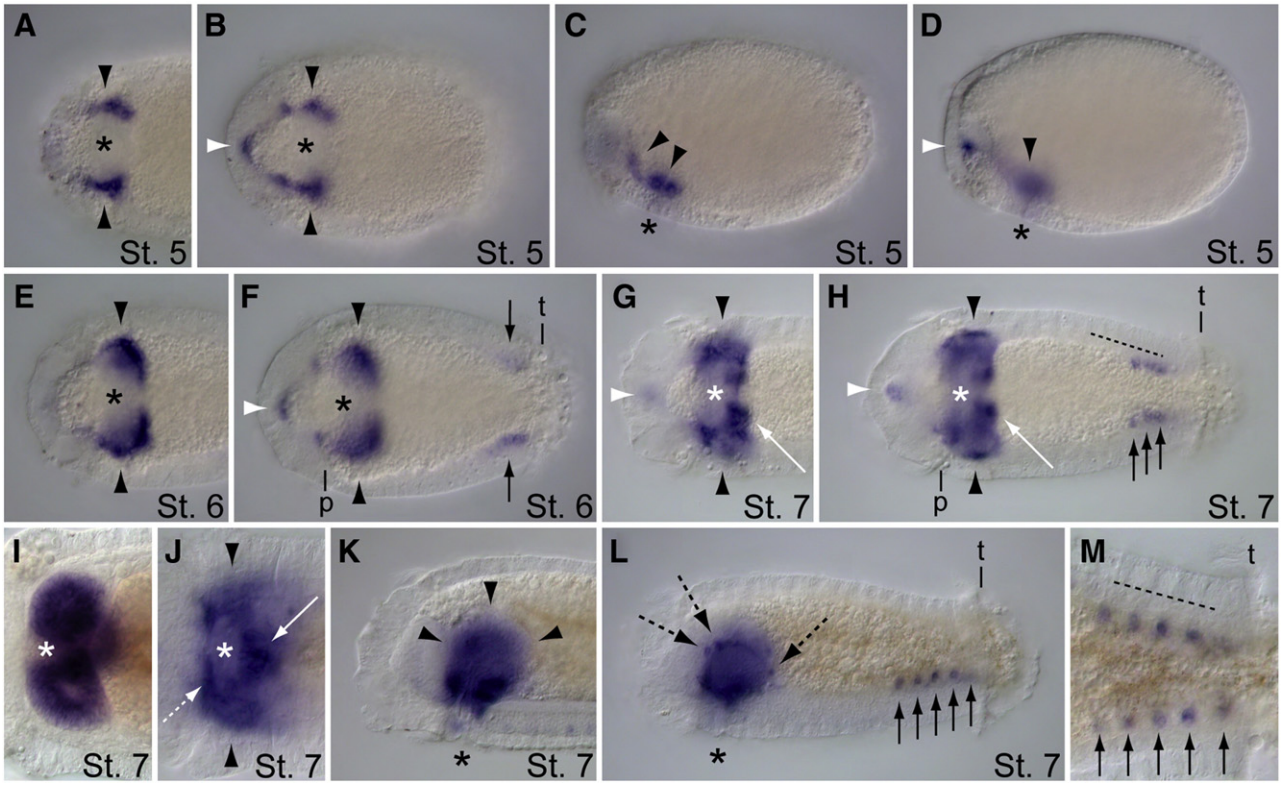

Fig. 4. Expression of the *foxC* gene during larval development in *C. teleta*. Blue staining in each panel shows *Ct-foxC* expression. Larval stages are indicated in the lower right corner of panels. A, B, E, F G, H, I, J and M are ventral views with anterior to the left. C, D, K and L are lateral views with ventral down and anterior to the left. A–D are the same stage 5 larva. A, B. Two ventral focal planes showing bilateral *Ct-foxC* expression flanking the stomodeum (black arrowheads) and in a medial–anterior patch of subsurface cells (white arrowhead). C, D. Mid-lateral and medial focal planes. Both the stomodeal (black arrowheads) and anterior expression domains (white arrowhead) are subsurface. E, F. Two ventral focal planes of the same stage 6 larva showing *Ct-foxC* expression in three domains: lateral margins of the developing foregut (black arrowheads), a medial–anterior patch of cells (white arrowhead) and posterior ventro-lateral tissue (black arrows) adjacent to the developing midgut. G, H. Two ventral focal planes of an early stage 7 larva. *Ct-foxC* expression is associated with the lateral (black arrowheads) and posterior sides (white arrows) of the foregut. The anterior patch is still detectable (white arrowhead) and the posterior expression is in discrete subgroups of mesodermal cells (black arrows). I. *Ct-foxA* expression in epithelial tissues of the developing pharynx. J. Close-up ventral view of *Ct-foxC* expression in anterior (dashed white arrow) lateral (black arrowheads) and posterior tissues (white arrow) of the developing pharynx. K, L. Lateral views of the same late stage 7 larva as in J. *Ct-foxC* is both broadly expressed (black arrowheads) and restricted to particular marginal cells (dashed black arrows) in the foregut. The posterior ventro-lateral expression (black arrows) is in discrete, segmental cell clusters. (M) close-up ventral view of the same larva as in J–L. The segmental expression of *Ct-foxC* is in mesoderm cells (black arrows) adjacent to the midgut endoderm. Dashed line marks the trunk mesoderm–ectoderm boundary. An asterisk marks the position of the stomodeum. p, prototroch; t, telotroch.

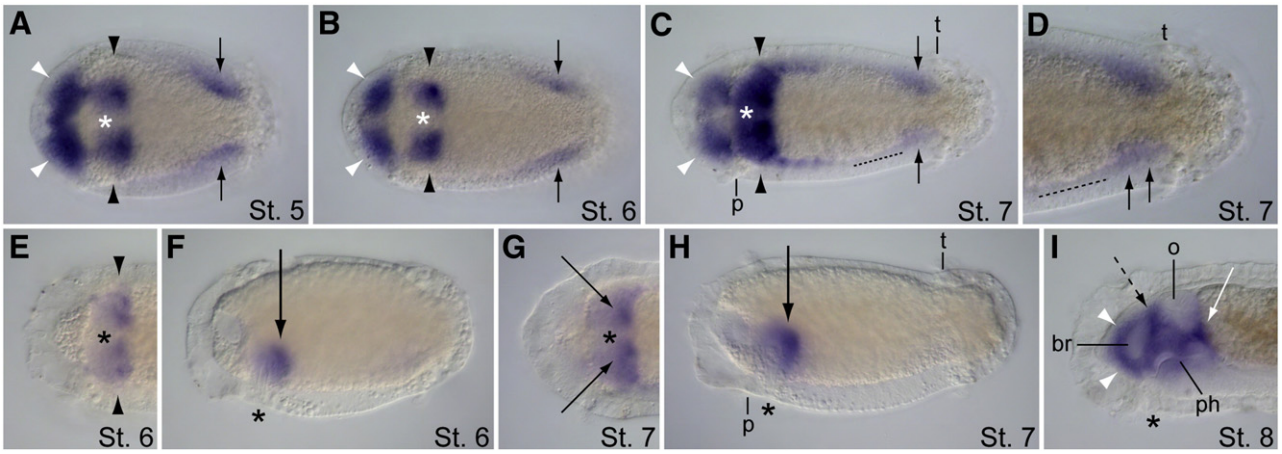

Fig. 5. Expression of the *Ct-foxF* gene during larval development in *C. teleta*. Blue staining in each panel shows *Ct-foxF* expression. Larval stages are indicated in the lower right corner of panels. A, B, C, D, E, and G are ventral views with anterior to the left. F, H and I are lateral views with ventral down and anterior to the left. A. Stage 5 larvae with *Ct-foxF* bilaterally expressed in three domains: the brain (white arrowheads), foregut (black arrowheads) and posterior ventro-lateral mesoderm (black arrows) adjacent to the developing midgut. B, C. *Ct-foxF* is expressed in the same three domains in stages 6 and 7. D. Enlarged posterior region of the same larva as in C. *Ct-foxF* expression (black arrows) is on the mesodermal side of the mesoderm–ectoderm boundary (dashed line) and does not extend posteriorly beyond the telotroch (t). E–H colour reactions were stopped early to examine foregut expression in detail. E. Stage 6 larva with *Ct-foxF* expression (black arrowheads) across the width of the developing foregut. F. Lateral view of the same stage 6 larva as in E. *Ct-foxF* is strongly expressed (arrow) on the dorsal posterior side of the stomodeum. G, H. Ventral and lateral views, respectively, of a stage 7 larva with *Ct-foxF* expression (black arrows) predominantly in dorsal–posterior foregut tissue. I. Lateral view of the anterior of a stage 8 larva. The expression of *Ct-foxF* is concentrated around the brain (white arrowheads), between the brain and oesophagus (dashed black arrow) and posteriorly between the oesophagus and pharynx (white arrow). An asterisk marks the position of the stomodeum. br, brain; o, oesophagus; ph, pharynx, p, prototroch; t, telotroch.

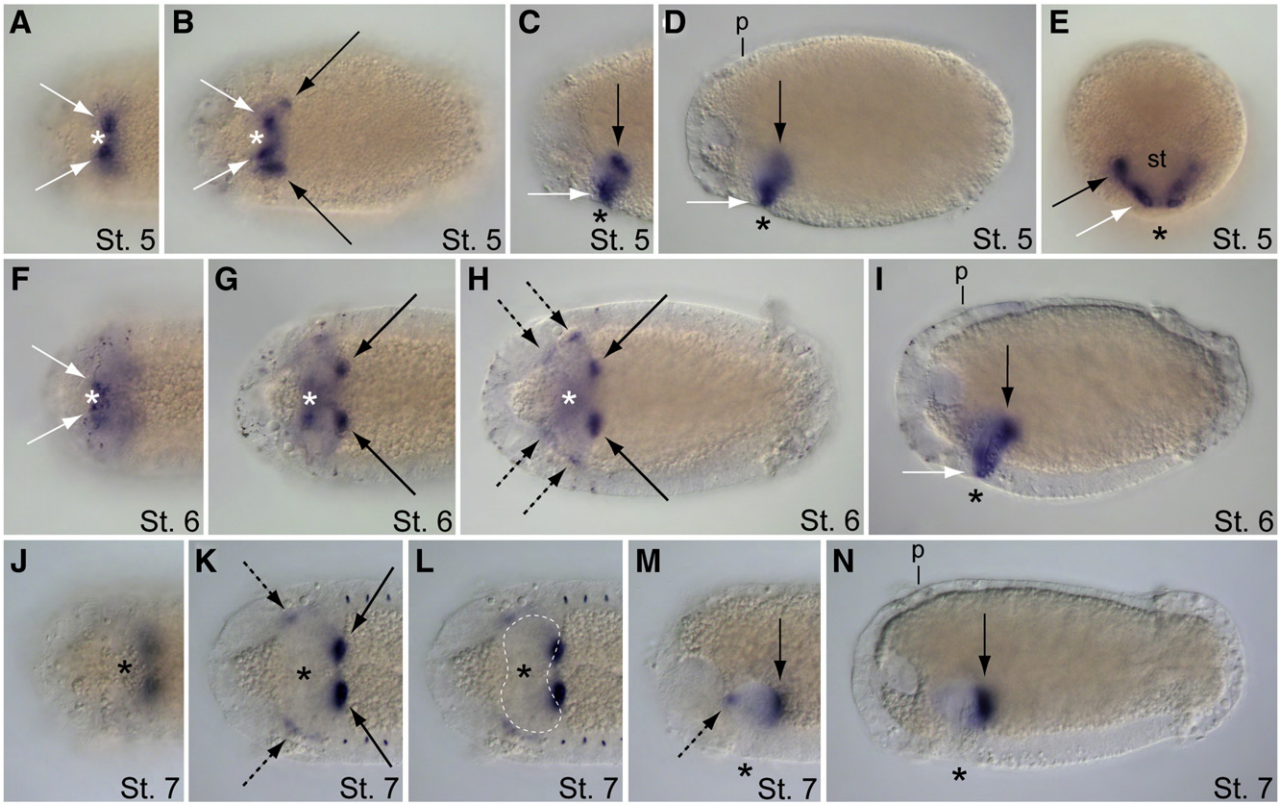

Fig. 6. Expression of the *Ct-foxL1* gene during larval development in *C. teleta*. Blue staining in each panel shows *Ct-foxL1* expression. Larval stages are indicated in the lower right corner of panels. A, B, F, G, H, J, K, and L are ventral views with anterior to the left. C, D, I, M and N are lateral views with ventral down and anterior to the left. E is an anterior view with ventral down. A–E, same stage 5 larva; F–I, same stage 6 larva; J–N, same stage 7 larva. A, B. Two ventral focal planes showing *Ct-foxL1* expression in cells on the posterior face of the mouth (white arrows) and within lateral margins (black arrows) of the stomodeum. C, D. Mid-lateral and medial focal planes showing both surface (white arrows) and subsurface (black arrows) *Ct-foxL1* expression. E. Anterior view showing the relationship of surface (white arrow) and internal (black arrow) expression along both sides of the stomodeum (st). F–H. Series of ventral focal planes through the stomodeum. *Ct-foxL1* is expressed symmetrically in surface cells on the posterior side of the mouth (white arrows), in two medial patches (black arrows) on the posterior side of the foregut, and along lateral–anterior foregut margins (dashed black arrows). I. Stage 6 lateral view showing *Ct-foxL1* extending from the surface (white arrow) to the dorsal–posterior side (black arrow) of the stomodeum. J–K. Surface and midlevel ventral focal planes of the stage 7 foregut. *Ct-foxL1* is no longer expressed at the mouth surface (asterisk on J) but remains in the medial foregut patches (black arrows) and lateral margins (dashed black arrows). L. Exact same image as in K with an outline of the developing pharyngeal epithelium (dotted white line) overlaid onto the *Ct-foxL1* expression pattern. M, N. Lateral focal planes of the same larva as in J and K. *Ct-foxL1* is expressed in lateral–anterior (dashed black arrow) and posterior foregut cells. An asterisk marks the position of the stomodeum. p, prototroch; st, stomodeum; t, telotroch.

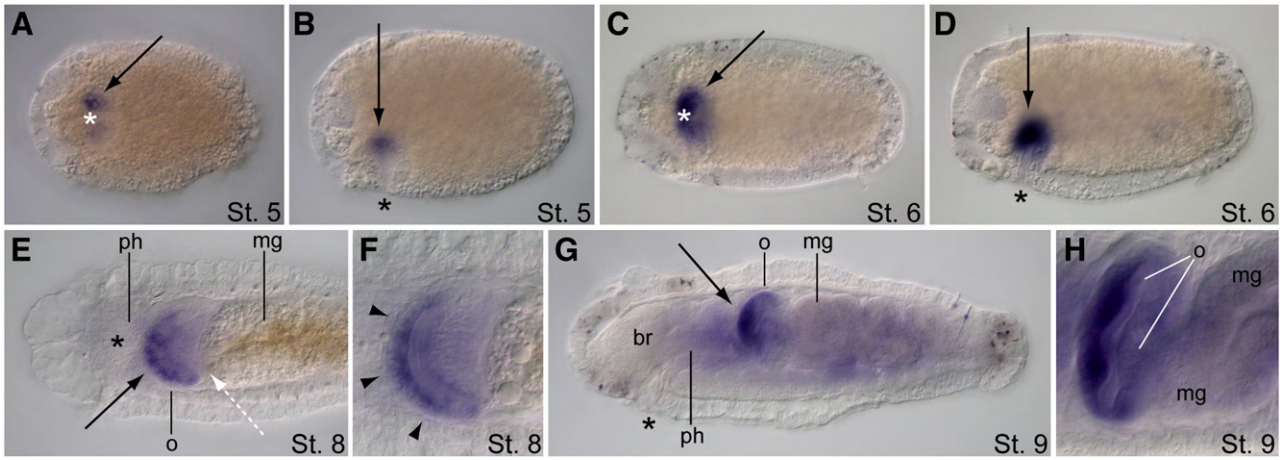

Fig. 7. Expression of the *Ct-foxQ1* gene during larval development in *C. teleta*. Blue staining in each panel shows *Ct-foxQ1* expression. Larval stages are indicated in the lower right corner of panels. A and C are ventral views with anterior to the left. B, D, and G are lateral views with ventral down and anterior to the left. E, F, and H are dorsal views with anterior to the left. A. Stage 5 larva with asymmetric *Ct-foxQ1* expression in the stomodeum. Expression is stronger on the left side (black arrow). B. Lateral view of same stage 5 larva as in A. *Ct-foxQ1* is expressed at the dorsal–anterior end (black arrow) of the stomodeum. C. The expression of *Ct-foxQ1* is stronger on the left side (black arrow) of the stomodeum. D. The same stage 6 larva as in C with expression at the dorsal–anterior end (black arrow) of the stomodeum. E. In stage 8 larvae, *Ct-foxQ1* expression (black arrow) is restricted to the foregut, primarily within the oesophagus (o). The expression terminates posteriorly at the foregut–midgut junction (dashed white arrow). F. Close-up dorsal view of the oesophagus in E showing *Ct-foxQ1* expression (black arrowheads) in epithelial cells. G. lateral view of a stage 9 larva showing strong expression in the oesophagus (black arrow). H. Close-up dorsal view of the same stage 9 larva in G showing *Ct-foxQ1* expression in the oesophagus. *Ct-foxC* expression is more localised to the anterior oesophageal epithelium (also see G). An asterisk marks the position of the stomodeum. mg, midgut; o, oesophagus; ph, pharynx.

foxQ1 is expressed throughout the oesophagus (Figs. 7E–H); however, the highest levels of expression are primarily within epithelial cells on its anterior side (Figs. 7F, H). *Ct-foxQ1* also is expressed at low levels in posterior pharynx tissue, but is not expressed within midgut endoderm.

FoxC, FoxF and FoxL1 expression in P. vulgata

The expression of all three genes was examined in staged *P. vulgata* embryos ranging from fertilised eggs to 29 h post fertilisation (hpf), by which time the trochophore larva had a well defined foot field, shell gland and neurotroch (a summary of *P. vulgata* development is shown in Fig. 3). The gut is relatively simple at this stage, with the mouth open but the anus yet to break through; this happens after torsion, with the gut later becoming considerably more complex during metamorphosis (Smith, 1935). *FoxC* expression is first detected in trochophore larvae at 21hpf (Figs. 8A–D). Expression is localised to the developing mantle cavities (Figs. 8A, B), to a bilateral pair of mesodermal cells posterior to the prototroch (Fig. 8B) and to a number of ventral mesodermal cells adjacent to the mouth and oesophagus (Figs. 8C, D). This pattern is maintained through the period of development studied, with sections confirming the location of *FoxC* expressing cells (Figs. 8E–J). Specifically we note *FoxC* expression in anterior mesoderm associated with the oesophagus (Figs. 8E, F, G, I) and in paired ventral stripes of posterior mesoderm lying either side of the foot field (Figs. 8E, J).

FoxF expression is first detected at 18hpf in the early trochophore larva in two bilateral pairs of cells, one level with the mouth and one

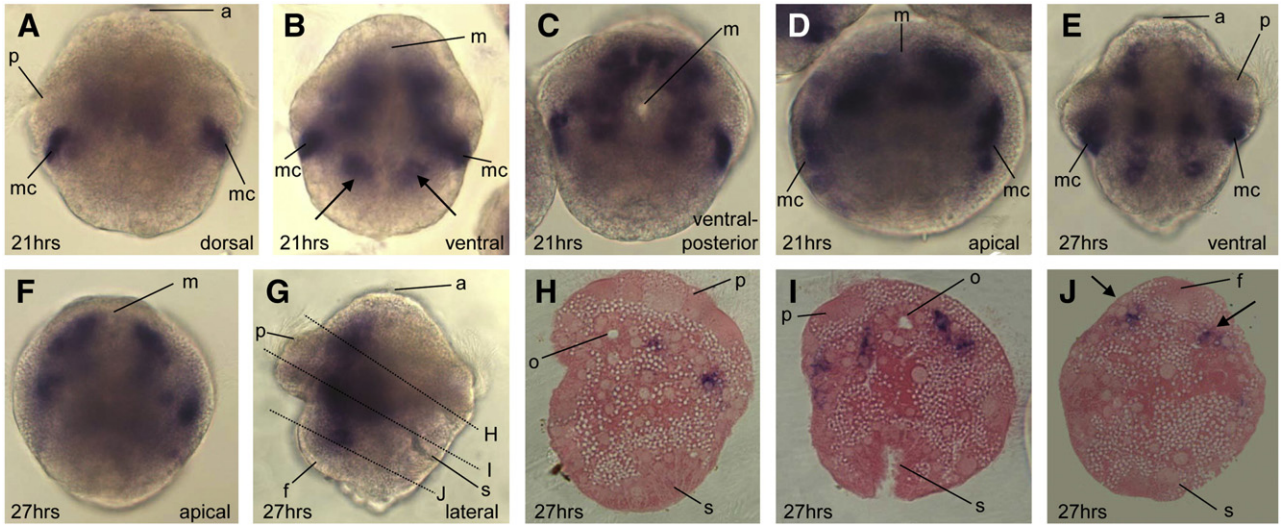

Fig. 8. *FoxC* expression during *P. vulgata* development. A–D. 21 h embryos. A shows a dorsal view, with expressing cells visible in the mantle cavities (mc). B shows a ventral view, with a bilateral pair of posterior mesoderm cells (arrows) stained. C shows an embryo tilted into a ventral–posterior perspective, revealing a set of expressing cells lying adjacent and anterior to the mouth (m). These cells lie under the ectoderm, as revealed by the apical view shown in D. E–G show 27 h embryos in ventral, apical and lateral views. Expression is localised in the mantle cavities, and in mesodermal cells posterior to the prototroch and adjacent to the foot, and anterior to the prototroch and adjacent to the oesophagus. These locations are confirmed by sections shown in H–J, the plane of which are indicated on G. a, apical tuft; f, foot field; p, prototroch; s, shell gland.

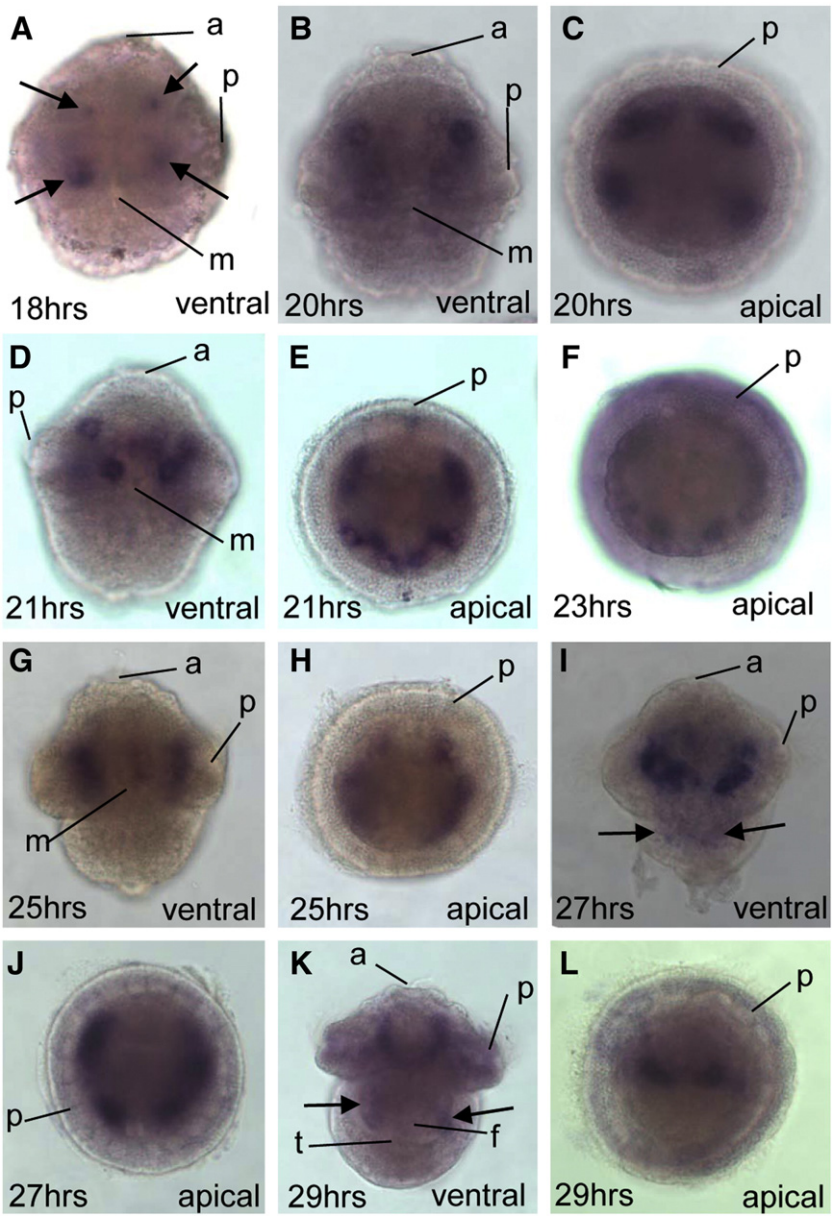

Fig. 9. *FoxF* expression during *P. vulgata* development. A. *FoxF* expression was first detected at 18 h, in four internal cells anterior to the prototroch (arrows). Note the nuclear localisation of the anterior cells. B, C. Ventral and apical views of 20 h embryos showing expression in mesodermal cells anterior to the prototroch. D, E. Ventral and apical views of 21 h embryos showing mesodermal expression of FoxF anterior to the prototroch. F. Apical view of a 23 h embryo. G, H. Ventral and apical views of 25 h embryos. I, J. Ventral and apical views of 27 h embryos. K. Ventral view of a 29 h embryo. Expression can still be seen anterior to the prototroch, and two more domains (arrows) are visible adjacent to the foot field (f). L. Apical view of a 29 h embryo. a, apical tuft; m, mouth; t, telotroch.

anterior to the prototroch (Fig. 9A). Between 18 and 27hpf additional cells are seen to express *FoxF* (Figs. 9B–J). The position of these cells suggests they may be the descendents of the original 4 cells identified at 18hpf. Apical views (Figs. 9C, E, F, H, J) show these are cells lying lateral to the endoderm. At 29hpf a second domain of expression is noted in the developing foot field (Fig. 9K, arrows).

Transient *FoxL1* expression is observed in 4 micromere cells of the 32 cell embryo at 8hpf (Fig. 10A), one in each embryonic quadrant. Expression is re-initiated in trochophore larvae in a bilateral pair of mesoderm cells adjacent to the mouth in 20–21 hpf larvae (Figs. 10B–E). By 23hpf, two small expression domains are visible on either side of the mouth (Fig. 10F), and this pattern persisted until the latest stage examined, 29hpf (Figs. 10G–I). Sectioning confirmed the location of these cells as lying under the ectoderm and adjacent to the epithelium of the oesophagus (Fig. 10I).

Discussion

The comparative genomics of FoxC, FoxF, FoxL1 and FoxQ1

Previous studies have shown that *FoxC*, *FoxF*, *FoxL1* and *FoxQ1* are ancient gene families that diverged prior to the radiation of the Bilateria (Larroux et al., 2008). They also show evidence of clustered organisation in some deuterostome genomes, notably vertebrates and amphioxus (Mazet et al., 2006; Wotton and Shimeld, 2006; Yu et al., 2008), though they are not clustered in the genomes of either the urochordate *C. intestinalis* or the sea urchin *Strongylocentrotus purpuratus* (Tu et al., 2006; Yagi et al., 2003). Only limited study of Fox gene clusters has been undertaken in protostomes, uncovering evidence of gene loss and degenerate clusters in *C. elegans* and *D. melanogaster*, and degenerating clusters in mosquito and bee

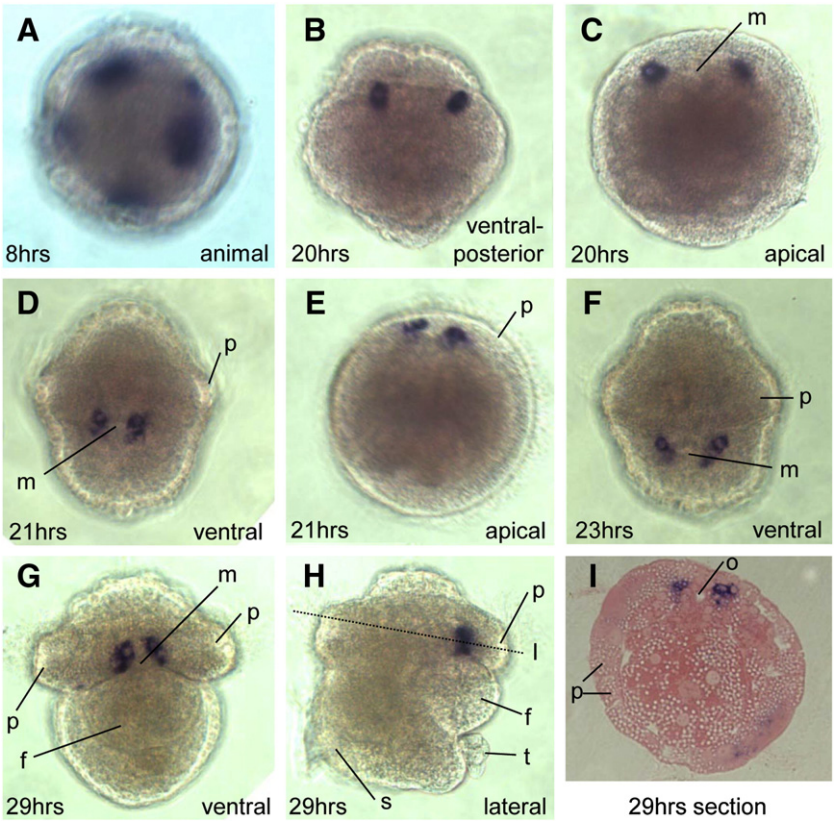

Fig. 10. *FoxL1* expression during *P. vulgata* development. A. Transient expression in four blastomeres was detected at 8 h of development, in embryos with 32 cells (animal view). Note that the small dark spot in the top right of the embryo is not gene expression but derives from debris that lies out of the plane of focus. B, C. At 20 h two cells activated FoxL1 expression. These cells are adjacent to the mouth (m), lying underneath the surface ectoderm. D, E. At 21 h a pair of expressing cells was observed either side of the mouth. F. Ventral view of a 23 h embryo showing paired expressing cells either side of the mouth. G, H. Ventral and lateral views of 29 h embryos. The foot field, shell gland and telotroch have become well defined, with the *FoxL1*-expressing cells located either side of the oesophagus, just internal to the mouth. Their location is illustrated by the section shown in I, the plane of which is shown by the dotted line in panel H. f, foot field; p, prototroch; s, shell gland; t, telotroch.

genomes (Mazet et al., 2006). These studies are limited to ecdysozoan taxa and hence exclude a large array of protostome phyla. They are also hampered by the absence of *FoxQ1* from protostome genomes studied to date.

Our study shows that protostomes did originally have a *FoxQ1* gene, as we were able to identify a definitive member of this family in the annelid *C. teleta*. However *FoxQ1* appears to be absent from the *L. gigantea* genome. These observations provide evidence that the *FoxQ1* genes did originate as a discrete family of genes prior to the divergence of the Bilateria, but also suggest that *FoxQ1* may be lost relatively easily as loss has happened independently at least twice; once in lophotrochozoans in the mollusc lineage, and once in the ecdysozoan lineage, possibly early in ecdysozoan evolution as nematodes and arthropods studied to date lack a *FoxQ1* gene.

The two lophotrochozoan genomes studied here also show evidence of clustering of Fox genes, though in both cases clusters are derived compared to the inferred ancestral condition. In *L. gigantea*, *FoxC*, *FoxL1* and *FoxF* are closely linked in the genome. *GMDS* is also closely linked to these genes. In *C. teleta*, *Ct-foxC*, *Ct-foxL1* and *Ct-GMDS* are closely linked. It is possible either *Ct-foxF* or *Ct-foxQ1* are also closely linked to these genes (or to each other), though the positions of the genes in their scaffolds preclude tight clustering of all four Fox genes, as seen in amphioxus (Mazet et al., 2006; Yu et al., 2008).

Gene clusters probably arise by tandem duplication; the alternative, assembly of a cluster by translocation with selection then favouring the new arrangement, is possible but unlikely. Following the formation of a cluster, its maintenance over extensive evolutionary time could occur by chance or result from selection. Two hypotheses put forward to explain such selection are: (1) shared regulation and (2) genomic regulatory blocks. The former postulates that linked genes might share regulatory elements and are hence functionally required to remain in close proximity, the latter that regulatory elements and functionally-unrelated genes are interspersed such that chromosome rearrangements separating genes also separate genes from necessary enhancers (Kikuta et al., 2007; Spitz et al., 2003). A third more general force operating to maintain genes in the same genomic vicinity might also derive from a requirement for co-expressed genes to reside in a specifically-regulated region of chromatin, though this would not necessarily maintain tight linkages characteristic of gene clusters.

Comparison of annelid and mollusc Fox clusters provides two lines of informative evidence. First we note that synteny comparisons only identified extensive synteny adjacent to the *Ct-FoxF* gene. Other possible indications of synteny adjacent to *P. vulgata FoxF* and *Ct-GMDS* should be treated with caution as few genes are involved. Lack of consistent synteny suggests these regions are not tied in large genomic regulatory blocks. We also note, however, that the *GMDS*, *FoxL1* and *FoxC* genes are closely linked in both genomes with no predicted intervening genes. This is also the case in the amphioxus genome, though transcriptional orientations do differ (Fig. 2A). We regard these three genes as good candidates for strong selection on gene organisation.

These data suggest multiple factors may underlie Fox cluster evolution. *FoxQ1* seems to be easily lost, and in *C. teleta* though present is embedded in non-syntenic genes on a separate scaffold. Preservation of *FoxQ1* in the cluster in chordates may be due to chance. *FoxF* is

usually linked to the other two Fox genes; however there are multiple intervening genes in some species. FoxF is also adjacent to the strongest conserved synteny, at least in human–*C. teleta* comparisons. Finally, we note the consistent tight linkage of *FoxC*, *FoxL1* and *GMDS* in multiple taxa. This could reflect a degree of co-regulation, or a small genomic regulatory block. Gene expression data are also informative in this respect, and are discussed below.

FoxC, FoxF, FoxL1 and FoxQ1 gene expression in the Lophotrochozoa

Here we report the first description of *FoxC*, *FoxF* and *FoxL1* expression from a lophotrochozoan, and the first *FoxQ1* expression outside the deuterostomes. Comparison of expression domains between *P. vulgata* and *C. teleta* helps identify primitive characters for the Lophotrochozoa, while comparison to other Phyla helps establish the basal bilaterian condition. To help compare the expression domains within and between the two lophotrochozoans, we have constructed a summary diagram depicting the relative expression domains of each gene at a specific developmental stage (Fig. 11).

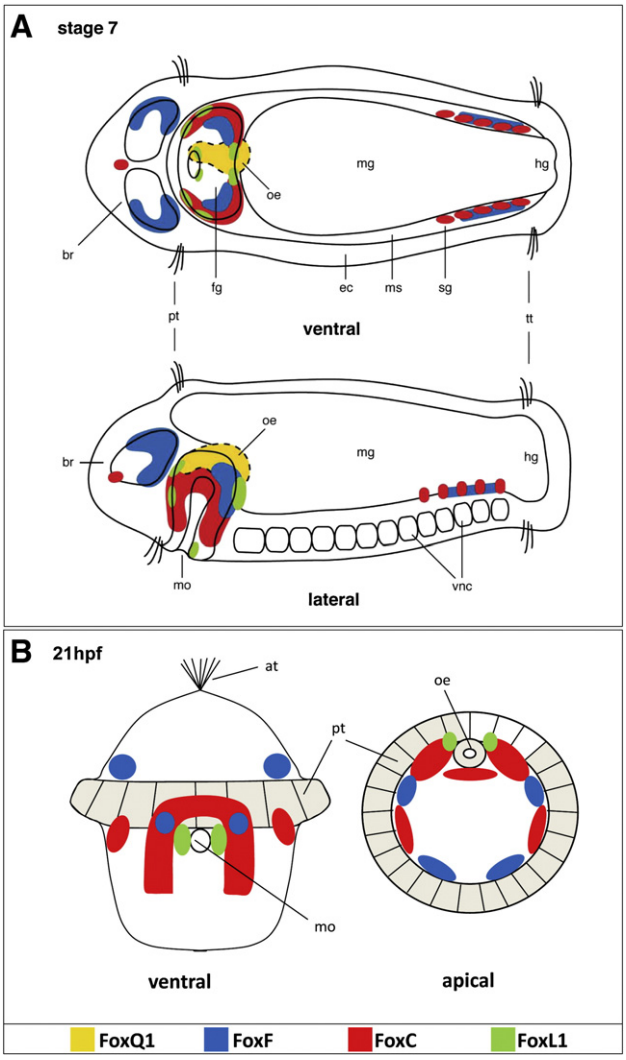

Fig. 11. Schematic comparison of Fox gene expression at selected stages in *C. teleta* and *P. vulgata*. A. *C. teleta* stage 7 larva in ventral (above) and lateral (below) views. Note overlap of *Ct-foxC* (red) and *Ct-foxF* (blue) in the posterior mesoderm, and association of all four genes with the foregut. B. *P. vulgata* 21 h post fertilisation in ventral (left) and apical (right) views. Expression of all three genes is identified in anterior mesoderm. at, apical tuft; br, brain; ec, ectoderm; hg, hindgut; mg, midgut; mo, mouth; ms, mesoderm; oe, oesophagus; pt, prototroch; sg, segmental mesoderm; tt, telotroch; vnc, ventral nerve cord.

Conservation of anterior expression of FoxF, FoxC and FoxL1

Our study of *P. vulgata* Fox genes shows some overlap of expression in the anterior of the embryo. *FoxC* and *FoxL1* overlap in cells adjacent but external to the oesophagus (compare Figs. 8I and 10I; Fig. 11). *FoxF* also appears to mark neighbouring cells (Fig. 9J; Fig. 11). Dictus and Damen conducted the most thorough examination of *P. vulgata* cell lineage published to date (Dictus and Damen, 1997). Their study shows that the 3a and 3b micromeres generate internal cells in a bilaterally symmetrical pattern on the ventral side of the trochophore, surrounding the mouth and the developing foot field. Based on this they postulate that these are ectomesodermal cells, and it is likely that the cells expressing the three Fox genes derive from this lineage. Technically challenging late embryonic lineage tracing experiments would, however, be needed to confirm this.

Ct-foxC, *Ct-foxF* and *Ct-foxL1* show complex patterns of anterior expression in *C. teleta* larvae. *Ct-foxC* is expressed in the head muscle, and also in muscle surrounding the pharynx. Expression of *Ct-foxF* is detected in the brain, and in tissue surrounding the foregut epithelium. *Ct-foxL1* was found in surface cells adjacent to the mouth, and in discrete domains of mesoderm closely apposed to the pharyngeal epithelium. Thus, all three are expressed in mesoderm surrounding the pharynx, an eversible and highly muscularized region of the foregut (Boyle and Seaver, 2009). Although their expression patterns may overlap to some degree (compare Figs. 4H, 5C and 6L for example; Fig. 11), it is also possible that each gene marks distinct mesodermal subdomains. The mesoderm surrounding the pharynx arises from the mesodermal bands that extend through the trunk (Meyer et al., in preparation). Comparison between the foregut of *P. vulgata* and *C. teleta* is complicated by their respective indirect and direct modes of development, such that *C. teleta* develops precocious adult structures seen only after metamorphosis in indirect developers such as *P. vulgata*. Nevertheless, we suggest that in both species, overlapping *FoxC*, *FoxF* and *FoxL1* expression marks the mesoderm that lines the external surface of the anterior gut.

Conservation of FoxQ1 in the anterior gut in C. teleta and chordates

In contrast to the bilateral expression patterns of *Ct-foxC*, *Ct-foxF* and *Ct-foxL1* in the foregut, *Ct-foxQ1* is asymmetrically localised in the presumptive foregut in young *C. teleta* larvae, followed by strong expression that becomes restricted to the oesophagus. Interestingly, the oesophagus has an obvious developmental left–right asymmetry in this worm (Boyle and Seaver, 2009), and the early asymmetric expression of *Ct-foxQ1* precedes this morphological asymmetry. Although the transition from ectoderm to endoderm varies in its position among the Bilateria, there is evidence from cell-lineage data that the foregut, including the oesophagus, is ectodermally derived in *C. teleta* (Meyer et al., in preparation). In chordates, *FoxQ1* expression has been identified in embryonic anterior endoderm in amphioxus, dogfish and *Xenopus*. In amphioxus it marks the endostyle and associated peripharyngeal bands, while in dogfish it is expressed by the developing gill buds and in *Xenopus* is localised to the pharyngeal pouches and anterior gut (Choi et al., 2006; Mazet et al., 2005; Wotton et al., 2008). This suggests that expression of *FoxQ1* in the anterior gut region, regardless of germ layer, was a character of the ancestor of protostomes and deuterostomes.

The developmental relevance of the loss of *FoxQ1* genes discussed above is, however, unclear; *FoxQ1* function has only been studied in mice, where *Foxq1* mutant embryos have subtle defects in stomach development and adults have defects in stomach acid secretion and hair development (Goering et al., 2008; Hong et al., 2001). Based on these studies it may be that *FoxQ1* has a role in the specification of particular tissues and organs as opposed to the patterning roles of *FoxC* and *FoxF*, but this remains speculative and further functional study is required.

Anterior expression of FoxC, FoxF, FoxL and FoxQ1 in other taxa

Anterior expression associated with the gut is seen for all of these lophotrochozoan Fox genes (Fig. 11). In chordates, anterior

expression for several of these genes has also been noted. *FoxQ1* expression in the anterior gut is discussed above. *FoxC* and *FoxL1* genes are expressed in anterior mesodermal structures in vertebrates and amphioxus. In vertebrates this includes the unsegmented head mesoderm and some of the tissues into which it develops (for example eye musculature), while in amphioxus it includes anterior segmented mesoderm and some head muscles, including those associated with the mouth and gill slits (Mazet et al., 2006; F. Mazet and SMS, unpublished). *FoxF* genes are also found in relatively anterior mesoderm though of a more ventral character, for example that from which the heart develops and, in amphioxus, including that adjacent to the anterior gut (Beh et al., 2007; Mazet et al., 2006; Wotton et al., 2008).

These comparisons suggest that all four of these Fox genes had ancient roles in the patterning of anterior gut, involving endodermal, mesodermal and ectodermal components. *FoxQ1* marks anterior gut (be it of endodermal or ectodermal origin), *FoxC* and *FoxF* may mark anterior dorsal and ventral mesoderm respectively (in parallel to their expression in the posterior, see below), while *FoxL1* appears more variable between taxa. We also note that in *P. vulgata* anterior mesoderm expression overlaps with the expression of *twist*, and possibly also *FoxA* (Lartillot et al., 2002a; Nederbragt et al., 2002). Although *Ct-foxA* expression in *C. teleta* is restricted to ectodermal components of the gut (Boyle and Seaver, 2008), *Ct-twist* is expressed in anterior mesoderm where the pattern is reminiscent of *Ct-foxC* (Dill et al., 2007). Both Twist and FoxA genes mark subpopulations of the mesoderm in chordates, suggesting some commonality of genetic mechanisms patterning the mesoderm in chordates and lophotrochozoans.

Expression of FoxF and FoxC in posterior mesoderm

In both *D. melanogaster* and chordates *FoxF* and *FoxC* mark the posterior visceral and somatic mesoderm respectively, and are required for their correct determination. This link to mesoderm patterning is also found in other taxa, for example with the *S. purpuratus FoxF* and *FoxC* genes expressed in the larval coelomic pouches (Tu et al., 2006). Both *Ct-foxF* and *Ct-foxC* expression were identified in posterior mesoderm cells adjacent to the gut and in the growth zone, with the former lining the gut and appearing non-segmented, and the latter distinctly segmented. Thus, *Ct-foxF* and *Ct-foxC* are likely expressed in distinct mesodermal subpopulations, probably both visceral because of their internally-positioned expression domains within the mesoderm. *P. vulgata* also shows evidence of expression of these two genes in posterior mesoderm. *FoxC* is expressed in bilateral stripes of mesoderm posterior to the prototroch

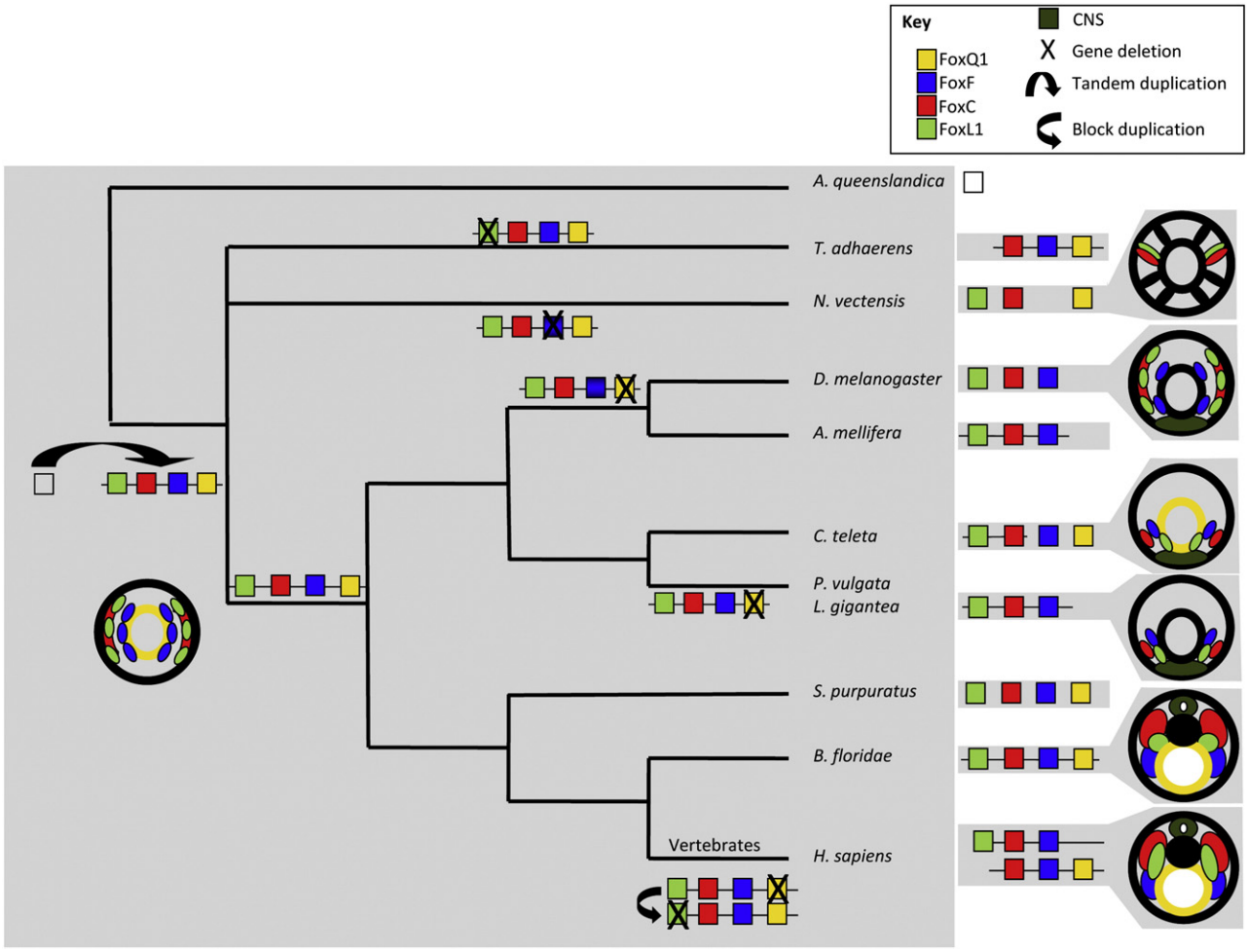

Fig. 12. Summary of known Fox clusters and expression domains coupled with predicted ancestral states mapped on to a phylogenetic tree. The colour coding for genes is the same as in Fig. 11 and is shown in the key. The tree shows only those taxa for which we have substantial data, and its topology is based on a recent phylogenomic study (Philippe et al., 2009). Uncertainty over the relative placement of *T. adhaerens* and *N. vectensis* leads us to depict them as equally related to the Bilateria. On the right side of the figure are schematic diagrams of Fox cluster organisation in the relevant taxa, with lines between genes indicating genomic linkage. These are connected to schematic transverse sections illustrating Fox gene expression in the anterior gut and mesoderm. Cluster diagrams adjacent to tree branches indicate inferred ancestral states and relevant genomic changes, namely gene duplication and gene loss. The inferred ancestral expression of the genes in a hypothetical common ancestor is shown towards the left of the picture. This ancestral state is well supported for the Bilateria, and may also reflect a deeper common ancestor (for example Bilateria + Cnidaria) if *N. vectensis* and/or *T. adhaerens* have indeed lost Fox genes and a degree of mesendodermal complexity (see text for details).

from 27hpf, while *FoxF* is expressed by bilateral patches of cells posterior to the prototroch from the same stage, which we suggest are mesodermal from their position. Without tracing the lineage of *P. vulgata* cells through metamorphosis, however, we cannot be certain these represent distinct populations of cells with different mesodermal fates. This shows that, although clear division in expression of these two genes into visceral and somatic mesoderm is seen in Ecdysozoa and Deuterostomia, such a distinction is not clear in *C. teleta* and, while possible in *P. vulgata*, is not proven.

In both *D. melanogaster* and vertebrates, *FoxL1* is co-expressed with *FoxC* and *FoxF* in the developing posterior mesoderm (Hacker et al., 1992; Kaestner et al., 1996; Kaestner et al., 1997; Wotton et al., 2008). In neither *P. vulgata* nor *C. teleta* did we observe similar overlap, though overlap among these genes may occur in the foregut in *C. teleta* (see above; Fig. 11) and between *P. vulgata FoxC* and *FoxL1* in the cells external to the oesophagus epithelium (Fig. 11). Thus, while our data demonstrate a conserved role for *FoxF* and *FoxC* in posterior mesoderm patterning, a conserved role for *FoxL1* is not supported. This contrasts with the expression of *FoxL1* associated with the foregut of both *P. vulgata* and *C. teleta*.

Correlating gene organisation, expression and function; an integrated view of Fox cluster evolution

Fig. 12 illustrates our current understanding of Fox cluster organisation and gene expression across multiple taxa, integrated with animal phylogeny and predicted ancestral states. Linkage of Fox genes is seen in all three bilaterian Superphyla (Ecdysozoa, Lophotrochozoa, and Deuterostomia), as are examples of both cluster breakup (for example *D. melanogaster*, S. *purpuratus*) and gene loss. There is consistent expression of *FoxF* and *FoxC* in mesoderm of all three Superphyla, regardless of whether the genes are linked or not. Typically *FoxF* marks visceral mesoderm, and *FoxC* somatic mesoderm. *FoxL1* overlaps with *FoxC* in *D. melanogaster* and in chordates, though not in a simple way in the two lophotrochozoans. Finally, where present, *FoxQ1* is expressed in the anterior gut. Based on this we have inferred ancestral expression patterns for the common ancestor of the Bilateria, with *FoxC*/*FoxL1*, *FoxF* and *FoxQ1* marking somatic mesoderm, visceral mesoderm and anterior gut respectively (Fig. 12).

Three taxa that provide a window on how this arrangement has evolved are the sea anemone *Nematostella vectensis*, the placozoan *Trichoplax adhaerens* and the sponge *Amphimedon queenslandica* (Fig. 12). There is some uncertainty regarding the relative placement of these three taxa (for example; (Hejnol et al., 2009; Philippe et al., 2009). We show a conservative consensus, with the sponge sited as basal and the placozoan and cnidarian lineages equally placed. The *A. queenslandica* genome has one gene with some similarity to *FoxL1* (though this is not a well-supported orthology) and no *FoxF*, *FoxC* or *FoxQ1* gene (Larroux et al., 2008). The *N. vectensis* genome has clear *FoxC* and *FoxQ1* orthologues and a possible *FoxL1* orthologue, but no *FoxF* orthologue (Larroux et al., 2008; Magie et al., 2005). *T. adhaerens*, has linked *FoxF*, *FoxC* and *FoxQ1*orthologues but no *FoxL1* (Shimeld et al., in press; Srivastava et al., 2008). We thus show the four gene Fox cluster as having evolved by tandem duplication after the divergence of the *A. queenslandica* lineage, with loss of *FoxF* in *N. vectensis* and of *FoxL1* in T. *adhaerens* (Fig. 12). Depending on the relative placement of *N. vectensis* and *T. adhaerens*, one of these lineages might however have diverged at an intermediate point with a three gene cluster. Improved phylogenetic resolution of basal animals would help resolve this.

In *N. vectensis FoxC* and *FoxL1* are co-expressed in a subset of the mesenteries, endodermal structures that share characteristics with bilaterian mesoderm (Magie et al., 2005; Martindale et al., 2004). *FoxQ1* expression has not been examined. Our predicted ancestral state of gene expression may therefore predate the divergence of cnidarians and bilaterians. This in turn suggests that the loss of *FoxF* by the *N. vectensis* lineage is congruent with secondary loss of mesodermal complexity in this lineage, an hypothesis previously discussed with respect to other genes characteristic of bilaterian mesoderm (Martindale et al., 2004). Currently this remains debatable, as *T. adhaerens* has a clear *FoxF* but no obvious mesoderm. Further characterisation of *Fox* genes from *T. adhaerens*, ctenophores and additional sponge and cnidarian classes will help resolve this.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.015.

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