Investigating the origins of triploblasty: 'mesodermal' gene expression in a diploblastic animal, the sea anemone *Nematostella vectensis* (phylum, Cnidaria; class, Anthozoa)

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Summary

Mesoderm played a crucial role in the radiation of the triploblastic Bilateria, permitting the evolution of larger and more complex body plans than in the diploblastic, non-bilaterian animals. The sea anemone *Nematostella* is a non-bilaterian animal, a member of the phylum Cnidaria. The phylum Cnidaria (sea anemones, corals, hydras and jellyfish) is the likely sister group of the triploblastic Bilateria. Cnidarians are generally regarded as diploblastic animals, possessing endoderm and ectoderm, but lacking mesoderm. To investigate the origin of triploblasty, we studied the developmental expression of seven genes from *Nematostella* whose bilaterian homologs are implicated in mesodermal specification and the differentiation of mesodermal cell types (*twist*, *snailA*, *snailB*, *forkhead*, *mef2*, a *GATA* transcription factor and a *LIM* transcription factor). Except for *mef2*, the expression of these genes is largely restricted to the endodermal layer, the gastrodermis. *mef2* is restricted to the ectoderm. The temporal and spatial expression of these 'mesoderm' genes suggests that they may play a role in germ layer specification. Furthermore, the predominantly endodermal expression of these genes reinforces the hypothesis that the mesoderm and endoderm of triploblastic animals could be derived from the endoderm of a diploblastic ancestor. Alternatively, we consider the possibility that the diploblastic condition of cnidarians is a secondary simplification, derived from an ancestral condition of triploblasty.

Key words: Germlayer, Evolution, *Nematostella vectensis*, Cnidaria

Introduction

The invention of mesoderm was crucial to the evolution of diverse and complex three-dimensional body plans in animals (Perez-Pomares and Munoz-Chapuli, 2002). It is generally accepted that mesoderm evolved in the bilaterian lineage. If so, then the animal kingdom can be divided between the triploblastic bilaterians, possessing three germ layers (endoderm, ectoderm and mesoderm), and the diploblastic non-bilaterians, possessing only two germ layers (endoderm and ectoderm). The evolutionary transition from diploblasty to triploblasty remains obscure. To deduce the molecular basis for the origin of mesoderm, we must compare the triploblastic Bilateria with diploblastic outgroup taxa. The phylum Cnidaria (sea anemones, corals, jellyfishes and hydras) promises to be an especially informative outgroup, as mounting molecular evidence suggests that the phylum Cnidaria is the sister group to the Bilateria (Wainright et al., 1993; Medina et al., 2001; Collins, 2002).

Cnidarians are a large and successful phylum of animals that diverged from the Bilateria perhaps 600 million years ago. Early in the evolutionary history of cnidarians, the phylum split into two major lineages (Fig. 1): the class Anthozoa (anemones and corals) and its sister group, the Medusozoa (Bridge et al., 1995; Bridge et al., 1992; Odorico and Miller, 1997; Schuchert, 1993; Collins, 2002). The Medusozoa comprises three classes: Hydrozoa (hydras and hydromedusae), Scyphozoa (true jellyfishes) and Cubozoa (box jellyfishes). Most medusozoans display a biphasic life cycle where an asexual polyp phase alternates with a sexually reproducing medusa (jellyfish) phase. The medusa phase was subsequently lost in certain lineages such as the freshwater hydras. Anthozoans possess only a polyp, and the polyp can reproduce by asexual or sexual means.

Cnidarians are widely regarded as diploblasts (Barnes et al., 2001; Brusca and Brusca, 2003; Pechenik, 2000). The outer epidermis is derived from ectoderm. The blind gut and the tentacles are lined with gastrodermis of endodermal origin (Fig. 1). The gastrodermis is a bi-functional epithelium, performing both absorptive and contractile functions, and was called 'entoderm' by early workers. The mesoglea, an extracellular matrix containing a few scattered cells, separates the endoderm and ectoderm. There is no well-organized intermediate tissue layer. Certain medusae, such as the hydrozoan jellyfish *Podocoryne*, are known to possess an additional tissue layer, the entocodon. Possible homology between the entocodon and mesoderm has been suggested (Boero et al., 1998; Muller et al., 2003; Spring et al., 2002; Spring et al., 2000).

There are no definitive muscle cells in cnidarians. However, the epithelial cells of the gastrodermis (and epidermis of

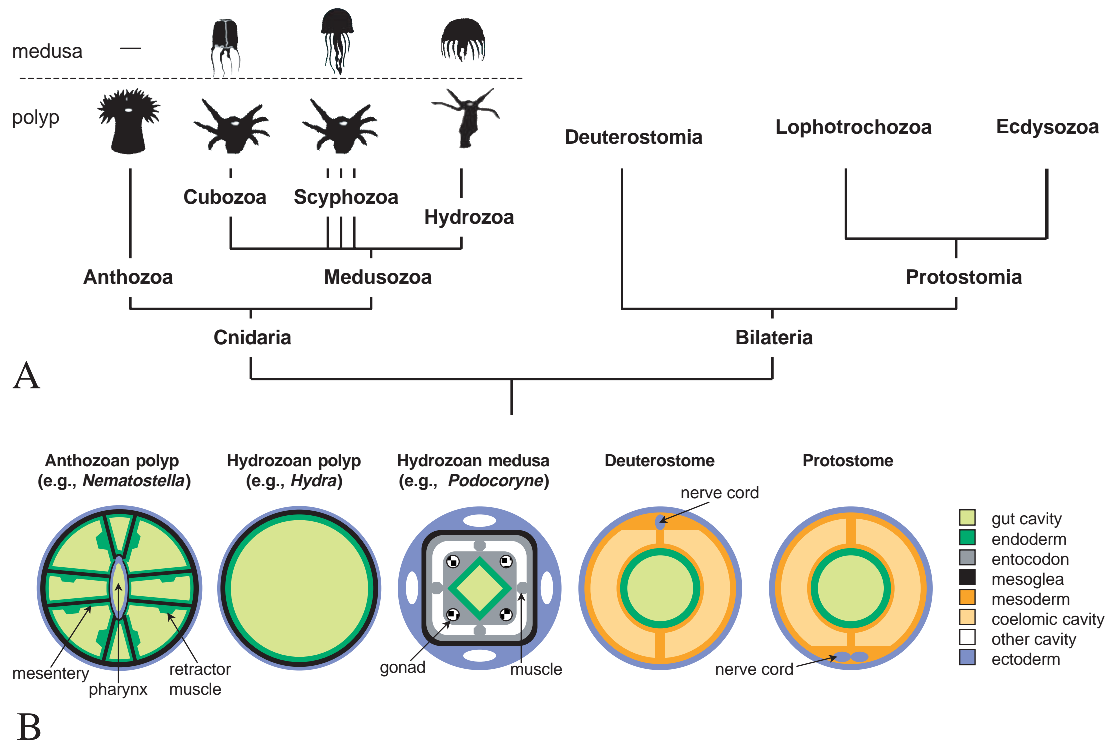

Fig. 1. Evolutionary relationships and germ layer composition of Cnidaria and Bilateria. (A) The topology of the tree summarizes the results of recent molecular phylogenetic analyses (Odorico and Miller, 1997; Schuchert, 1993). The phylum Cnidaria is an outgroup of the Bilateria, perhaps the sister group. The class Anthozoa is the sister of the Medusozoa (Odorico and Miller, 1997; Schuchert, 1993). Anthozoans exhibit only the polyp stage, while most medusozoans exhibit both polyp and medusa life stages. The Medusozoa comprises the members of the classes Hydrozoa, Cubozoa and Scyphozoa. Multiple independent scyphozoan lineages are depicted because a recent analysis suggests that the Scyphozoa may be paraphyletic (Collins, 2002). (B) The germ layer composition of representative taxa is indicated using diagrams of the cross-sectional anatomy. The anthozoan polyp is sectioned through the pharynx. Notice that the lumen of the pharynx is lined with ectoderm, while the outer surface of the pharynx is lined with endoderm. The hydrozoan polyp lacks a pharynx. Both animals exhibit two epithelial layers (endoderm and ectoderm). The hydrozoan medusa exhibits a third epithelial layer, the entocodon, that surrounds a coelom-like internal cavity, the subumbrellar cavity (Boero et al., 1998). Mesoderm lines the coelomic cavity of both protostomes and deuterostomes.

hydrozoans) have myoepithelial extensions on their basal surfaces. In anthozoan cnidarians (anemones and corals), longitudinal myoepithelial processes are concentrated on structures known as mesenteries. The mesenteries are lamellae consisting of two layers of gastrodermal epithelium separated by an intervening layer of mesoglea. The mesenteries radiate outward from the throat or pharynx, to the outer body wall. The pharynx is a tubular intrusion of the outer body wall that projects from the mouth into the spacious gastrovascular cavity, or coelenteron (Fig. 1). The mesenteries provide structural support for the pharynx, they increase the gastrodermal surface area, and they serve as the site of gamete production.

The starlet sea anemone, *Nematostella vectensis*, has recently been developed as a model system to investigate cnidarian development. *Nematostella* is a small, solitary, burrowing sea anemone found in coastal estuaries along the Atlantic and Pacific coasts of North America and the southeast coast of England (Hand and Uhlinger, 1994). *Nematostella* has many practical advantages as a developmental model, including a simple body plan and a simple life history. It is a hardy species, easy to culture (Hand and Uhlinger, 1992) and will spawn readily throughout the year under laboratory conditions (Fritzenwanker and Technau, 2002; Hand and Uhlinger, 1992). Sexes are separate and fertilized embryos develop rapidly to juvenile adults bearing four tentacles. After fertilization, cleavage generates a hollow blastula (Fig. 2A). Gastrulation occurs by invagination and ingression (Fig. 2B) (C. Byrum and M.Q.M., unpublished). The blastopore becomes the ‘mouth’, the sole opening into the gastrovascular cavity. After gastrulation, the swimming planula larva assumes a teardrop shape (Fig. 2). At this point, the larva consists of a ciliated epidermis surrounding a solid core of presumptive mesendodermal cells. In the planula larva, the lumen of the gut begins to resolve itself as the first two of eight mesenteries arise from the thickened epidermis of the pharynx (Fig. 2C). The planula settles after a few days and generates a primary polyp with four tentacles (Fig. 2D). The mature adult polyp measures 1-6 cm in length and possesses 12-16 tentacles (Fig. 2E).

Recent reviews suggest an ancient origin for mesendodermal patterning (Baylies and Michelson, 2001; Davidson et al., 2002; Ransick et al., 2002; Rodaway and Patient, 2001). Conserved gene expression patterns in deuterostomes and protostomes provide evidence for the homology of mesoderm across the Bilateria. Genes involved during mesendodermal specification in protostomes and deuterostomes include various GATA transcription factors, LIM transcription factors, *twist*, *snail* and *forkhead* (Essex et al., 1993; Hukriede et al., 2003; Leptin, 1991; Lespinet et al., 2002; Olsen and Jeffery, 1997; Olsen et al., 1999; Perez Sanchez et al., 2002; Thisse et al., 1988; Wolf et al., 1991). Several mesoderm-associated genes have already been isolated in hydrozoan cnidarians (Groger et al., 1999; Muller et al., 1999; Spring et al., 2002; Spring et al., 2000; Technau and Bode, 1999), including *brachyury*, *mef2*,

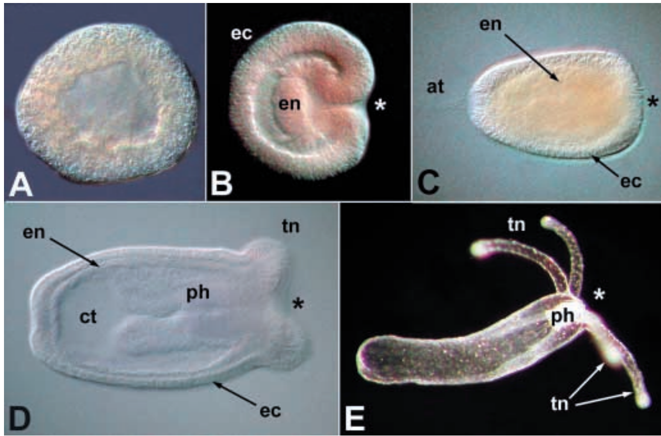

Fig. 2. *Nematostella* development. In *Nematostella*, the sexes are separate and adults generate either eggs or sperm. The cleavage program is chaotic with no two embryos developing identically. (A) A hollow blastula is formed. Yolk fragments break off from the apical regions of cells and remain trapped in the blastocoel. (B) Gastrulation proceeds by the invagination of the future oral end of the embryo (*). Cells entering the blastocoel from the prospective oral pole will give rise to endoderm (en) surrounded by outer ectoderm (ec). (C) The planula 'larva' is initially shaped like a teardrop and swims with its apical sensory tuft (at) directed forward. The endoderm (en) forms a solid ball of cells at the planula stage. (D) The coelenteron (gut cavity) has formed by the hollowing out of the solid planula endoderm (ct). Tentacle buds (tn) begin to form around the oral opening (*) in the swimming larva prior to settlement. Note that the pharynx (ph) extends into the coelenteron. (E) The polyp forms ~7 days after fertilization. When it first settles onto the substrate, it has four tentacles and eight endodermal mesenteries. Endoderm lines the hollow tentacles. The thickened pharyngeal wall connects the mouth/anus to the coelenteron.

snail and *twist*. *brachyury* has also been isolated from *Nematostella* (Scholz and Technau, 2003). We report the developmental expression of seven genes in *Nematostella* whose bilaterian homologs are associated with mesendoderm formation or differentiation of mesodermal derivatives (*Nv-snailA*, *Nv-snailB*, *Nv-twist*, *Nv-GATA*, *Nv-mef2*, *Nv-forkhead* and *Nv-muscle LIM*).

In Bilateria, genes associated with mesoderm specification are predominantly expressed in the mesoderm or presumptive mesoderm. By characterizing the developmental expression of cnidarian homologs, we may gain insights into the earliest stages of mesoderm developmental evolution. For each cnidarian gene: (1) expression may occur predominantly in the endoderm; (2) expression may occur predominantly in the ectoderm; or (3) expression may not exhibit a germ-layer bias. Six of the seven genes described here exhibit endodermally restricted expression during early development. Only *mef2* is expressed exclusively in ectodermal derivatives. The germ layer restricted expression of these genes suggests that they may play a role in germ-layer specification. Furthermore, the overwhelming predominance of endodermal expression supports the hypothesis that both the endoderm and mesoderm of triploblasts evolved from the endoderm of diploblasts. However, a plausible evolutionary argument can be made that the diploblastic condition of cnidarians is a secondary simplification of a triploblastic ancestor.

Materials and methods

Gene isolation

Nv-muscle LIM was recovered from a preliminary EST screen of a late larval cDNA library (J.R.F., unpublished). Each clone was fully sequenced from both directions at the Automated DNA Sequencing Facility in the Biology Department at Boston University (Accession number, AY465177). Degenerate primers were designed to the most highly conserved regions of bilaterian orthologs for *snail*, *twist*, *GATA*, *mef2* and *forkhead*. Gene fragments were obtained by PCR amplification from either genomic DNA (*Nv-snailA*, *Nv-snailB* and *Nv-twist*) or embryonic cDNA (*Nv-GATA*, *Nv-mef2*, *Nv-forkhead*). PCR fragments were cloned in the pGEM-T easy plasmid vector (Promega) and sequenced at the University of Hawaii Biotechnology Center. Sequences from authentic clones were used to design nested sets of non-degenerate primers with annealing temperatures between 68-70°C for RACE (rapid amplification of cDNA ends). Both 3′-RACE and 5′-RACE were performed using the Smart Race cDNA amplification kit (BD Biosciences Clontech). Overlapping 3′ and 5′ RACE fragments for each gene were conceptually spliced and submitted to GenBank as composite transcripts (Accession numbers: *Nv-GATA*, AY465174; *Nv-forkhead*, AY465175; *Nv-mef2*, AY465176; *Nv-snailA*, AY465178; *Nv-snailB*, AY465179; *Nv-twist*, AY465180).

Determination of gene orthology

The evolutionary relationships of the *Nematostella* sequences were determined by neighbor-joining analyses based on predicted amino acid sequences. The amino acid sequences included in each analysis were selected through BLAST searches aimed at identifying homologous protein domains from potential orthologs and outgroup sequences. We included sequences from other cnidarian species where available, from the sequenced human genome (a representative deuterostome bilaterian) and from the sequenced *Drosophila* genome (a protostome bilaterian). Conserved domains were aligned using an Internet implementation of ClustalW at the website of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Default alignment parameters were used (Matrix, BLOSUM; GapOpen, 10; GapExt, 0.05; GapDist, 8). Orthology of *Nv-forkhead* was determined using an 81 amino acid alignment spanning part of the conserved forkhead domain. Orthology of *Nv-GATA* was assessed using an 80 amino acid stretch that encompasses a C2C2 zinc finger motif. The *Nv-mef2* analysis used 130 amino acid positions spanning the conserved Mef2/MADS box motif of *mef2*, *serum response factor* and *blistered*. The *Nv-muscle LIM* analysis was based on 62 amino acids spanning the LIM domain. The evolutionary relationships of *Nv-snailA* and *Nv-snailB* were inferred from 105 amino acid positions spanning four C_2H_2 zinc-finger motifs from *snail*, *scratch* and *Kruppel* genes. The *Nv-twist* analysis incorporated a conserved 56-residue region of the *twist*, *atonal* and *nautilus* genes. In each phylogenetic analysis, the support for specific clades was assessed by 2000 replications of the bootstrap (Felsenstein, 1985). Bootstrap proportions equaling or exceeding 40% are shown.

Gene expression

Embryos from various stages were fixed in fresh ice-cold 3.7% formaldehyde with 0.2% gluteraldehyde in 1/3× seawater for 90 seconds and then post-fixed in 3.7% formaldehyde in 1/3× seawater at 4°C for 1 hour. Fixed embryos were rinsed five times in Ptw (PBS buffer plus 0.1% Tween-20) and once in deionized water, and transferred to 100% methanol for storage at –20°C. Early embryos were removed from the jelly of the egg mass by treating with freshly made 2% cysteine in 1/3× seawater (pH 7.4-7.6) for 10-15 minutes. Planula and polyp stages were relaxed in 7% $MgCl_2$ in 1/3× seawater for 10 minutes prior to fixation.

In situ hybridization using 1-2 kb digoxigenin-labeled riboprobes were performed to determine the spatial and temporal distribution of transcripts as previously described (Finnerty et al., 2003). Although

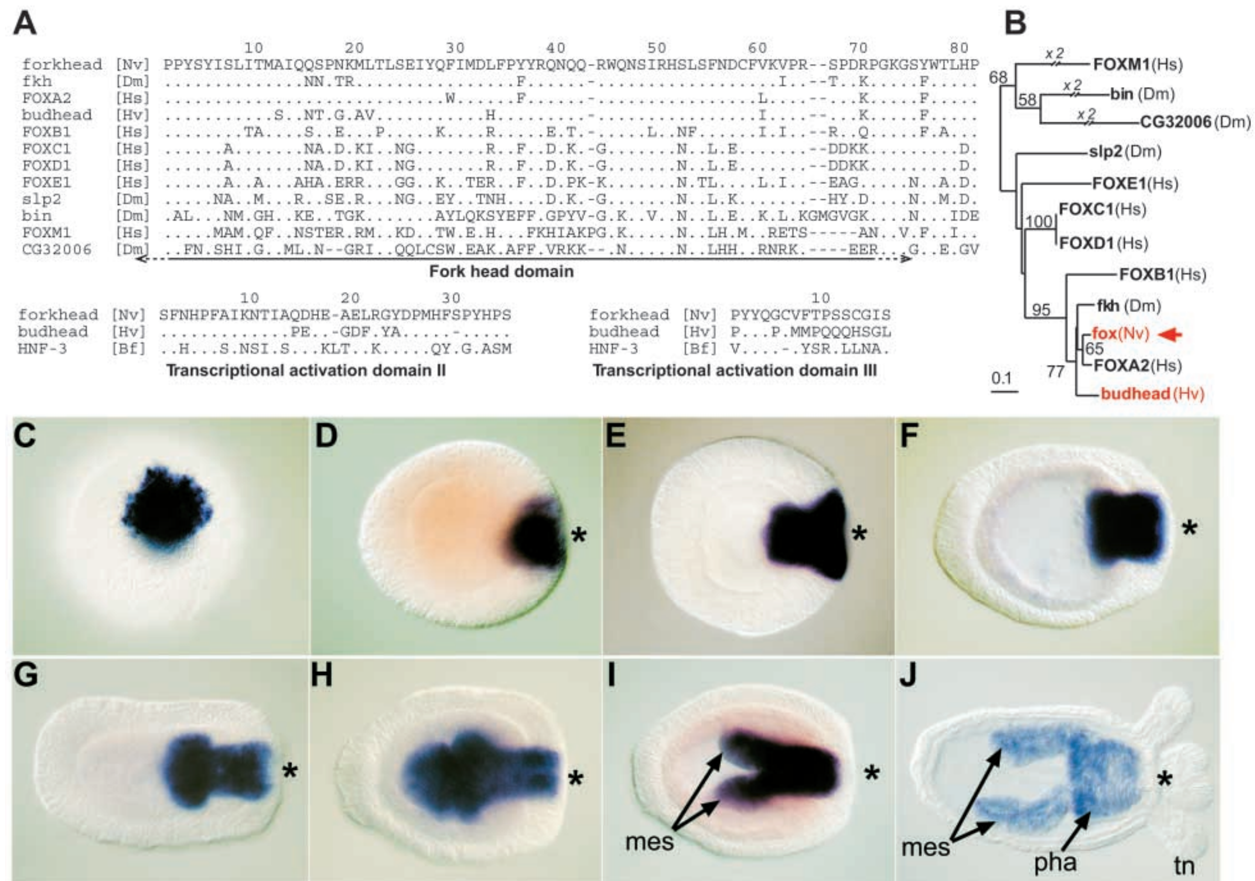

Fig. 3. *Nv-forkhead*. Phylogenetic analysis and gene expression. (A) Alignment of 81 amino acids from the conserved *forkhead* domain (top), 35 amino acids from the transcription activation domain II (bottom left) and 16 amino acids from the transcription activation domain III (bottom right) (Pani et al., 1992). (B) Neighbor-joining tree produced from the 81 amino acids of the *forkhead* domain. Branch lengths are proportional to the number of substitutions per residue. Numbers at nodes indicate bootstrap support (percentage of 2000 bootstrap replicates in which the given clade was recovered). Some branch lengths were truncated by 50% for presentation purposes (indicated by *x2*). Sequences included in the analysis are: *bin* (biniou), *Drosophila*, residues 312-391; *budhead*, *Hydra vulgaris*, residues 82-159; CG32006-PA (hypothetical protein), *Drosophila*, residues 147-220; *fkh* (fork head), *Drosophila*, residues 211-288; *FOXA2* (forkhead box A2), *Homo*, residues 160-237; *FOXB1*, *Homo*, residues 14-91; *FOXC1*, *Homo*, residues 79-157; *FOXD1*, *Homo*, residues 126-203; *FOXE1*, *Homo*, residues 54-131; *FOXM1*, *Homo*, residues 237-312; *slp2* (sloppy paired 2), *Drosophila*, residues 182-258; *fox* (fork head box protein), *Nematostella*, residues 40-117. (C) Surface view of a late blastula showing localized expression at the future site of the blastopore. (D) Lateral view of the same stage. (E) The first cells that enter the blastocoel express *Nv-forkhead*. (F-J) Only cells of the pharynx (pha) and pharyngeal mesenteries (mes) express this gene. Asterisks indicate the site of the blastopore, the future mouth.

both 5′ and 3′ RACE fragments were recovered for most genes, 3′ fragments tended to be longer and were used for probe construction (MegaScript, Ambion, Austin, TX). Longer probes generated better signal to noise ratios and shortened developing time. Probe concentration ranged from 0.05-1.00 ng/μl and hybridizations were performed at 65°C for 20-44 hours. Probe detection was achieved by incubation with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). Subsequently, the presence of alkaline phosphatase was detected by a colorimetric detection reaction using the substrate NBT-BCIP. Specimens were photographed on a Zeiss Axioplan with a Nikon Coolpix 990 digital camera. Detailed protocols are available upon request.

Results

Nv-forkhead

The *forkhead* genes encode transcription factors characterized by the possession of a 110 amino acid ‘fork head’ or ‘winged helix’ domain, which is involved in DNA binding (Kaufmann and Knochel, 1996). The *forkhead* gene family in humans consists of nearly 50 genes, and these genes are involved in a diverse range of developmental functions, including the development of the eye (Kidson et al., 1999), the lung (Whitsett and Tichelaar, 1999), the notochord and nerve cord (Ruiz i Altaba et al., 1995). The *forkhead* family in *Drosophila* is significantly smaller, comprising fewer than 20 genes, many of which have not been characterized functionally. A common feature of the *forkhead* family in *Drosophila* and vertebrates, as well as non-vertebrate deuterostomes such as echinoderms and ascidians, is involvement in gastrulation and mesendodermal patterning (Kusch and Reuter, 1999; Olsen and Jeffery, 1997; Olsen et al., 1999; Perez Sanchez et al., 2002; Weigel et al., 1989).

Six unique winged-helix containing transcripts were isolated from *Nematostella*. One of these transcripts, *Nv-forkhead*, is 1628 nucleotides long, and it encodes a predicted protein

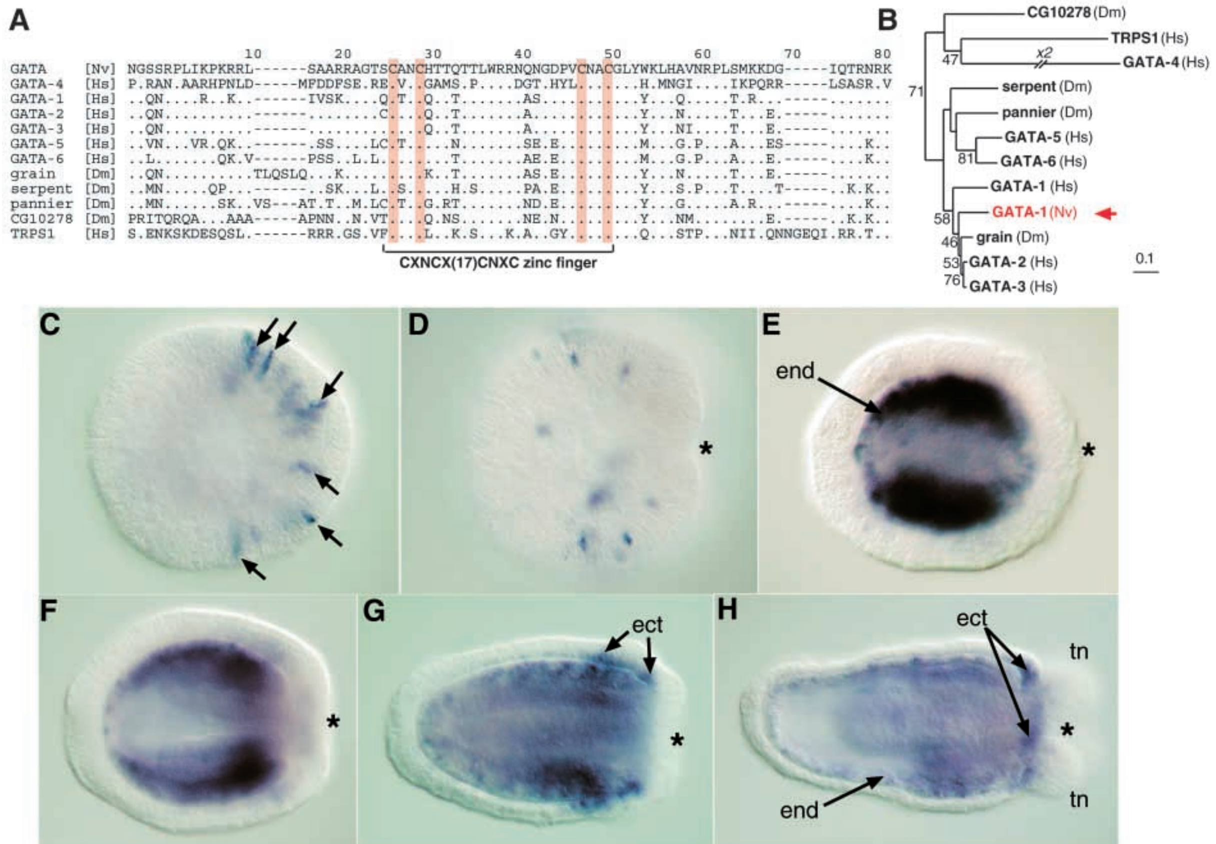

Fig. 4. Nv-GATA. Phylogenetic analysis and expression. (A) Alignment of 80 amino acids from various GATA transcription factors spanning a central C2C2 zinc finger motif. (B) Phylogeny of *GATA* sequences. The tree was constructed and labeled as in Fig. 3. Sequences included in the analysis are: CG10278-PA (hypothetical protein), *Drosophila*, residues 514-595; *GATA*, *Nematostella*, residues 248-321; *GATA-1* (GATA binding protein 1), *Homo*, residues 235-316; *GATA-2*, *Homo*, residues 326-406; *GATA-3*, *Homo*, residues 294-373; *GATA-4*, *Homo*, residues 193-273; *GATA-5*, *Homo*, residues 221-300; *GATA-6*, *Homo*, residues 274-356; *grain*, *Drosophila*, residues 292-378; *MGC2306* (hypothetical protein), *Homo*, residues 326-407; *pannier*, *Drosophila*, residues 148-224; *serpent*, *Drosophila*, residues 451-535; *TRPS1*, *Homo*, residues 873-959. (C-H) Expression of *Nv-GATA*. (C,D) Scattered cells express *Nv-GATA* at the blastula stage. *Nv-GATA*-expressing cells appear to move into the blastocoel (arrows). (E,F) At gastrulae stages expression is confined to endodermal cells. In the late planula (G) and early polyp (F) stages, ectodermal cells at the base of the developing tentacles also express *Nv-GATA*. Asterisks indicate the site of the mouth. end, endoderm; ect, ectoderm; tn, tentacles.

285 amino acids long. Within the forkhead domain, the *Nematostella* sequence is roughly 90% identical to *Drosophila* forkhead and the FOXA2 sequence of humans (Fig. 3A). Phylogenetic analysis places *Nv-forkhead* squarely within the larger *forkhead* clade, most closely related to *Drosophila forkhead*, *FOXA2*, and the *Hydra budhead* genes (Fig. 3B). Similar to *budhead* (Martinez et al., 1997), *Nematostella forkhead* exhibits several phylogenetically conserved residues within transcriptional activation domain II, and a handful of conserved residues within transcriptional activation domain III (Fig. 3A) (Pani et al., 1992).

Nv-forkhead expression is first detected in the late gastrula at the site of the blastopore (Fig. 3C). Throughout gastrulation, *Nv-forkhead* continues to be expressed at very high levels exclusively in cells that are moving into the coelenteron (Fig. 3D-H). This expression associated with the blastopore and gastrulation is similar to that reported for sea urchins (Harada et al., 1996) and ascidians (Olsen and Jeffery, 1997). In the late planula and juvenile polyp, *Nv-forkhead* is expressed exclusively in the cells of the mesenteries and pharynx (Fig. 3I,J). This expression in a terminal gut structure is reminiscent of the hindgut and foregut expression of *forkhead* in *Drosophila* (Weigel et al., 1989) and the hypostomal expression of *budhead* in *Hydra* (Martinez et al., 1997). However, *budhead* is expressed in the endoderm while *Nv-forkhead* is expressed primarily in the ectodermal lining of the pharynx. The expression of orthologous *forkhead* genes in the sea anemone pharynx and the *Hydra* hypostome suggests that these structures may be important for the development of the oral/anal opening.

Nv-GATA

The *GATA* genes constitute a family of zinc-finger transcription factors that bind the GATA motif, a widespread cis-regulatory element found in many promoters (Evans et al., 1988). GATA-binding proteins can be recognized by the presence of one or two distinctive zinc-finger motifs of the form $CXNCX_{17}CNXC$ (Fig. 4A). In vertebrates, GATA transcription factors are implicated in the development of many mesodermal cell types including red and white blood cells, smooth muscle, cardiac muscle, adipocytes and gonadal cells (Arceci et al., 1993;

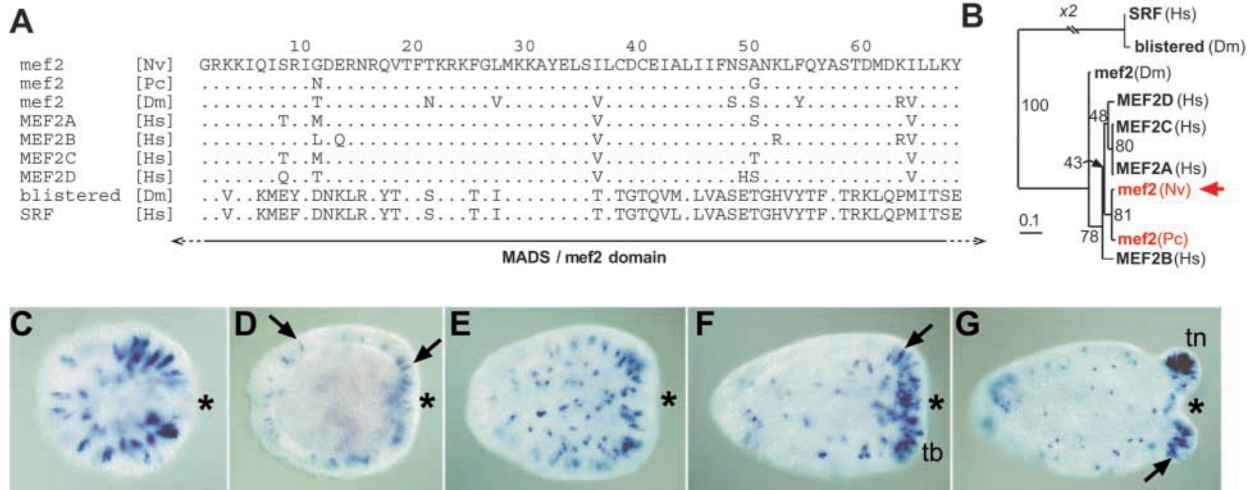

Fig. 5. *Nv-mef2*. Phylogenetic analysis and gene expression. (A) Alignment of 68 amino acids from the conserved MADS domain of various *mef2* genes plus *blistered* and *serum response factor (SRF).* (B) Phylogeny of *mef2* sequences. The tree was constructed and labeled as in Fig. 3. Phylogenetic analysis was based on the amino acid sequence of the MADS domain. Sequences included in the analysis are: *blistered*, *Drosophila*, residues 166-233; *mef2* (myocyte enhancing factor 2), *Drosophila*, residues 2-69; *mef2*, *Nematostella*, residues 2-69; *mef2*, *Podocoryne*, residues 2-69; *MEF2A* (myocyte enhancer factor 2A), *Homo*, residues 2-69; *MEF2B*, *Homo*, residues 2-69; *MEF2C*, *Homo*, residues 2-69; *MEF2D*, *Homo*, residues 2-69; *SRF* (serum response factor), *Homo*, residues 142-209. (C-G) Expression of *Nv-mef2*. (C) Expression begins in individual cells distributed broadly around the circumference of the blastula. (D,E) Throughout the development of the planula, expression remains confined to epidermal cells. (F,G) As development continues, *Nv-mef2* expression comes to be preferentially expressed at the oral pole (arrows), particularly along the surface of the developing tentacle buds (tb) and tentacles (tn).

Ketola et al., 2000; Laitinen et al., 2000; McDevitt et al., 1997; Morrisey et al., 1998; Tong et al., 2000; Tsai et al., 1994). The *Drosophila* GATA transcription factor, *grain*, has been shown to impact epithelial morphogenesis by affecting cell rearrangements (Brown and Castelli-Gair Hombria, 2000).

The *Nv-GATA* transcript is 2309 nucleotides long, and it encodes a predicted protein of 422 amino acids. *Nv-GATA* is highly similar to bilaterian *GATA* genes. For example, over the 69 amino acid stretch of the protein used for phylogenetic analysis, Nv-GATA is identical to human GATA3 at 59 positions (Fig. 4A). Phylogenetic analysis places *Nv-GATA* squarely in a clade of *GATA* genes, including *GATA1*, *GATA2* and *GATA3* from humans as well as the *Drosophila* gene *grain* (Fig. 4B).

Nv-GATA expression is first detected in individual cells around the circumference of the blastula (Fig. 4C,D). *Nv-GATA* expression appears absent from the extreme oral and aboral poles of the blastula (Fig. 4D). In the late blastula/early gastrula, *Nv-GATA*-expressing cells appear to ingress into the blastocoel (Fig. 4D) and by late gastrula/early planula stages all of the gastrodermal cells lining the coelenteron appear to express *Nv-GATA*, while the cells of the pharynx do not express *Nv-GATA* (Fig. 4E-F). In the early polyp stages (Fig. 4G-H), expression is confined to a subset of gastrodermal cells distributed all along the oral-aboral axis, with the strongest expression near the oral pole. Ectodermal staining also appears at this time, in the base of the tentacles (Fig. 4G-H), but not in the tentacles themselves.

Nv-mef2

The *mef2* genes are MADS-box transcription factors. Four *mef2* genes are known from vertebrates (MEF2A-D). *Drosophila* and *C. elegans* each possess a single *mef2* gene. In both the vertebrates and fruitfly, *mef2* genes are preferentially expressed in muscle and mesodermal tissues where they are essential for muscle development (Black and Olson, 1998; Lilly et al., 1994). However, in both vertebrates and *Drosophila*, *mef2* can also be detected in non-muscle and non-mesodermal tissues (Black and Olson, 1998; Schulz et al., 1996). Surprisingly, despite a high degree of sequence conservation, the *C. elegans mef2* gene is not essential for muscle development (Dichoso et al., 2000). In the hydrozoan jellyfish *Podocoryne*, *mef2* expression is widespread and highly dynamic (Spring et al., 2002). *mef2* can be detected throughout the complex life history of *Podocoryne* including the unfertilized egg, the blastula, the gastrula, the planula larva, the polyp, the attached medusa and the free-living medusa stage. In early developmental stages, the transcript does not appear highly localized. After gastrulation, *Podocoryne mef2* may be expressed in the endoderm or the ectoderm, or even the entocodon of the medusa, an intermediate tissue layer which is hypothesized to have homology with mesoderm (Boero et al., 1998). *Podocoryne mef2* is expressed in precursors of both muscle and non-muscle cells.

The *Nv-mef2* transcript is 2229 nucleotides encoding a predicted protein of 209 amino acids. *Nv-mef2* is over 90% identical to bilaterian *mef2* genes in the MADS domain (Fig. 5A). A neighbor-joining analysis including the MADS domain from *Nv-mef2*, plus other bilaterian and cnidarian *mef2* genes and putative outgroup genes (*blistered* and *serum response factor*), places the *Nematostella* sequence solidly within the *mef2* radiation (Fig. 5B). As expected, *Nv-mef2* appears most closely related to *mef2* from the hydrozoan jellyfish *Podocoryne* (Spring et al., 2002).

Nv-mef2 expression first becomes apparent in isolated cells

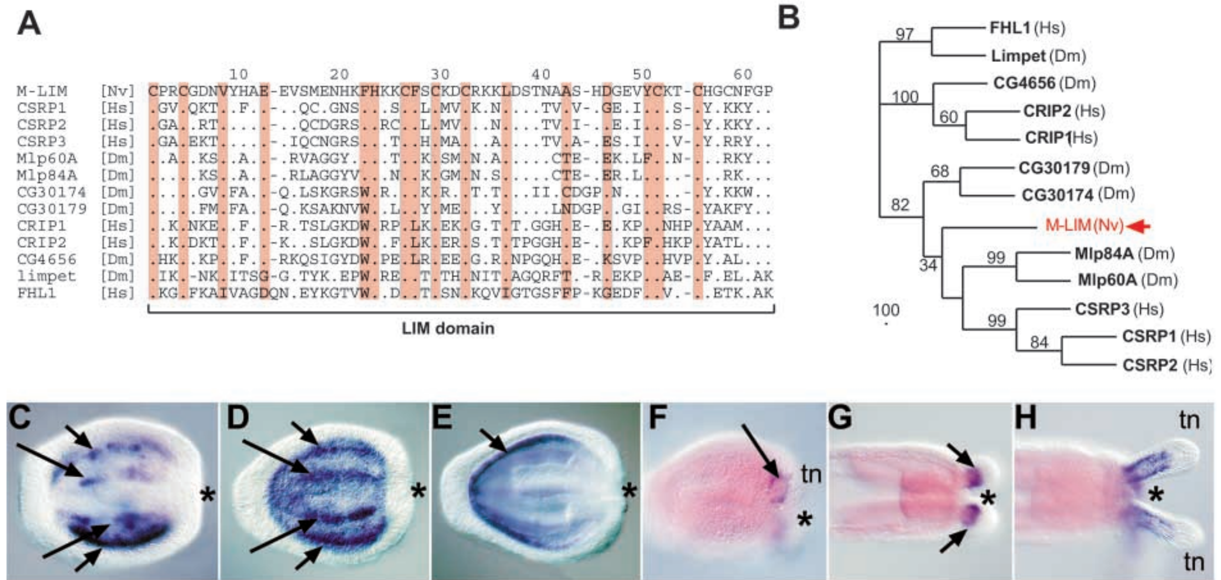

Fig. 6. *Nv-muscle LIM*. Phylogenetic analysis and gene expression. (A) Alignment of 62 amino acid residues from the conserved LIM domain. Conserved residues are indicated in red (Conserved Domain Database at NCBI: pfam00412.8, LIM). (B) Phylogeny of LIM sequences based on neighbor-joining analysis. The tree was constructed and labeled as in Fig. 3. Sequences included in the analysis are: CG4656-PA (hypothetical protein), *Drosophila*, residues 4-63; CG30174-PA (hypothetical protein), *Drosophila*, residues 93-152; CG30179-PA (hypothetical protein), *Drosophila*, residues 104-163; *CRIP1* (cysteine-rich protein 1), *Homo*, residues 4-63; *CSRP1* (cysteine and glycine-rich protein 1), *Homo*, residues 10-68; *CSRP2* (smooth muscle LIM protein), *Homo*, residues 10-68; *CSRP3* (cysteine and glycine-rich protein 3, cardiac LIM protein), *Homo*, residues 10-68; *FHL1* (four and a half LIM domains 1), *Homo*, residues 101-160; *limpet*, *Drosophila*, residues 220-277; *M-LIM* (muscle LIM protein), *Nematostella*, residues 4-62; *Mlp60A* (muscle LIM protein at 60A), *Drosophila*, residues 11-69; *Mlp84B* (muscle LIM protein at 84B), *Drosophila*, residues 12-70. The tree is drawn as though rooted between the Limpet/CRIP clade and the muscle LIM/CSRP clade. (C-H) Expression of *Nv-muscle LIM*. (C,D) Expression begins at mid-late planula stages in the precursors of the eight endodermal mesenteries (arrows). (E) Mid level optical section of a late planula, indicating expression in the gastrodermal lining of the coelenteron. The endodermal mesenteries are not in the plane of focus. Note that the pharynx and outer epidermis do not express *Nv-muscle LIM*. (F-H) In early polyp stages, the highest expression is in the endodermal lining (arrows) of the developing tentacles (tn). (G,H) Lateral views. In F, the mouth is bent towards the viewer to reveal the tip of the developing tentacle (arrow). The asterisk indicates the site of the mouth.

of the blastula (Fig. 5C), similar to the early expression of *Nv-GATA* (Fig. 4C). However, *Nv-mef2*-expressing cells are initially more widely distributed over the surface of the blastula than *Nv-GATA*-expressing cells (compare Fig. 4C,D with Fig. 5C,D). In the gastrula and planula larva, *Nv-mef2* is expressed in columnar cells in the ectodermal epidermis, but *Nv-mef2* expression does not appear in the gastrodermis (Fig. 5E,F). By late larval and polyp stages, *Nv-mef2* becomes preferentially expressed at the oral pole, particularly in the tentacular epithelium (Fig. 5F,G). Many of the *Nv-mef2*-expressing cells appear similar to precursors of the stinging cells, or nematocytes. In the polyp, nematocysts are present at higher densities in the tentacles than in the body column. However, the precursors of many nematocytes in the body column do not express this gene, and other cells expressing *Nv-mef2* in the epithelium that do not look like nematocytes based on their morphology. Their more intense basal staining and thin projections towards the apical surface of the epithelium suggests that the *Nv-mef2*-expressing population of cells may constitute a specialized subset of neurons, or perhaps multipotent stem cell precursors for multiple adult cell types. It is not known if other *mef2* genes exist in *Nematostella*, but the expression of this particular member of the *mef2* family suggests a role in the differentiation of ectodermal cell types.

Nv-muscle LIM

LIM proteins are characterized by the presence of one to five LIM domains (Stronach et al., 1996), a double zinc-finger motif known to be involved in protein dimerization (Feuerstein et al., 1994). Many LIM proteins also possess a DNA-binding homeodomain adjacent to the LIM domains. Members of the cysteine-rich protein family of LIM proteins can associate with the actin cytoskeleton when expressed in rat fibroblast cells (Louis et al., 1997; Stronach et al., 1996). These proteins are known to be involved with muscle differentiation in *Drosophila* (Stronach et al., 1996) and vertebrates (Louis et al., 1997).

The *Nv-muscle LIM* transcript is 523 nucleotides long, and it encodes a predicted protein of 73 amino acids. This short peptide is very similar in scale and sequence to the peptides encoded by the vertebrate cysteine-rich protein 1 [*CRIP1*, 77 amino acids (Tsui et al., 1994)] and the *Drosophila* muscle LIM protein *Mlp60A* [92 amino acids (Stronach et al., 1996)]. *Nv-muscle LIM* encodes a single canonical LIM domain with two zinc fingers connected by a short linker ([$CX_2CX_{17}HX_2C$]-X_2-[$CX_2CX_{17}CX_2C$]), but it does not encode a homeodomain (Fig. 6A). The neighbor-joining analysis (Fig. 6B) places *Nv-muscle LIM* as the sister group to a clade containing *Drosophila* muscle *LIM* proteins (*Mlp60A* and *Mlp84A*) and human cysteine rich proteins (*CSRP1-3*).

Nv-muscle LIM expression commences in the early planula

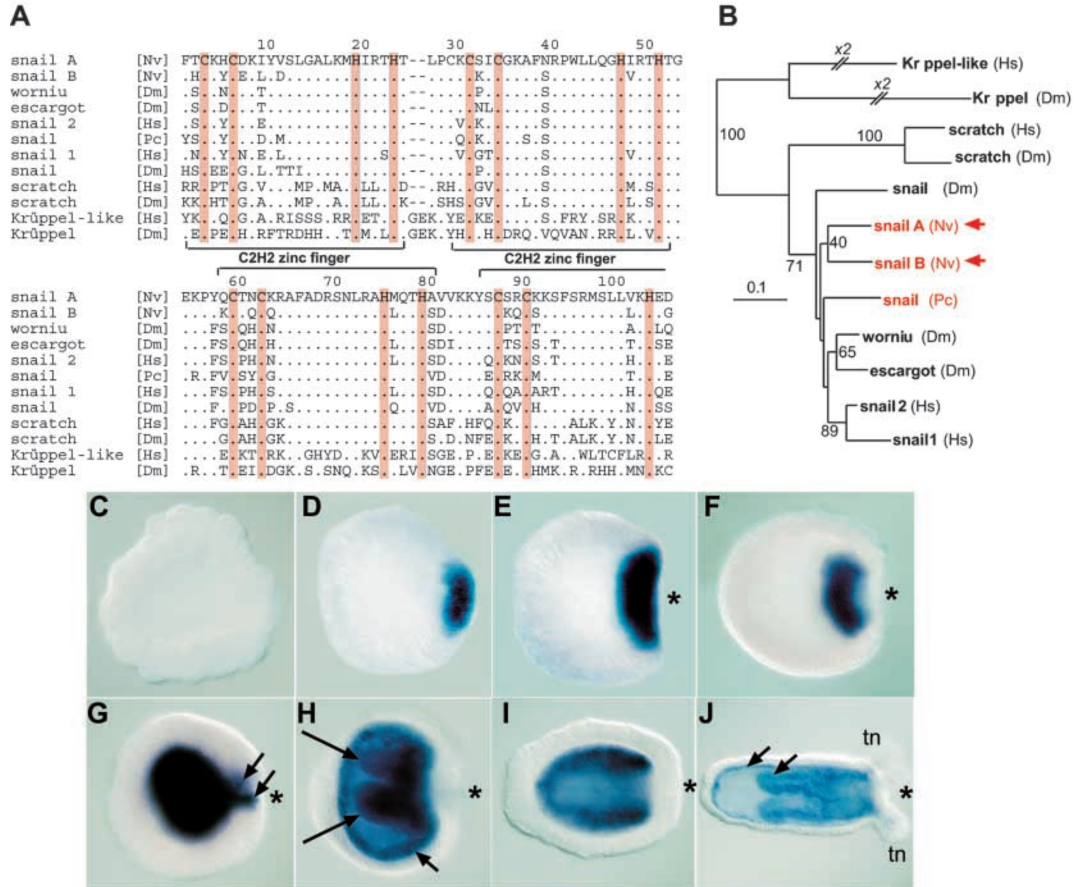

Fig. 7. *Nv-snailA* and *Nv-snailB*. Phylogenetic analysis and gene expression. (A) Alignment of 105 amino acids containing repeated C_2H_2 zinc-finger motifs. (B) Phylogeny of *snail* sequences. The tree was constructed and labeled as in Fig. 3. Sequences included in the analysis are: *escargot*, *Drosophila*, residues 344-446; *Kruppel*, *Drosophila*, residues 250-354; *Kruppel-like protein* (zinc finger protein 443), *Homo*, residues 449-477 plus 505-580; *scratch*, *Drosophila*, 498-600; *scratch1*, *Homo*, 222-324; *snail*, *Drosophila*, residues 280-383; *snail*, *Podocoryne*, residues 226-238; *snail 1*, *Homo*, residues 154-257; *snail 2*, *Homo*, residues 159-261; *snail A*, *Nematostella*, residues 156-258; *snail B*, *Nematostella*, residues 154-256. The tree is drawn as though rooted using the *Kruppel* clade as an outgroup. (C-J) Expression of *Nv-snailA* through development. (C) *Nv-snailA* transcript is not detectable at early cleavage stages. (D) Expression becomes visible in the late blastula at the site of the blastopore. (E) The embryo initiates gastrulation at the site of *Nv-snailA* expression. (F) The first cells that invaginate into the blastocoel express *Nv-snailA*. (G) Expression continues throughout the process of gastrulation as cells move in to the blastocoel. (H-J) All of the cells of the gastrodermis, but none of the cells of the epidermis express *Nv-snailA* up through the polyp stages. The asterisk indicates the site of the mouth. The expression of *Nv-snailB* is indistinguishable from *Nv-snailA*. Arrows in G,H,J indicate endoderm.

in the presumptive mesenteries (Fig. 6C,D) and the gastrodermis lining the coelenteron (Fig. 6E). In the polyp, the transcript appears most abundant in the endodermal lining of the developing tentacles (Fig. 6F-H). *Nv-muscle LIM* was not detected at all in the ectodermal epidermis, pharynx or mesenteries.

Nv-snailA and *Nv-snailB*

Proteins in the *Snail* family possess 4-6 C_2H_2 zinc fingers (Hemavathy et al., 2000). In addition, several *snail* family members in the fruitfly genome possess a short conserved motif at the amino terminus called the NT box (Hemavathy et al., 2000). Vertebrate *snail* proteins display a different conserved motif at the N terminus known as the SNAG domain. Although the function of the NT box is unknown, the SNAG domain is implicated in nuclear localization and transcriptional repression (Grimes et al., 1996). Snail proteins play a phylogenetically widespread role in the development of mesoderm (Hemavathy et al., 2000). *Snail* is expressed in the blastoderm at the time of mesoderm specification in phylogenetically diverse bilaterians, including *Drosophila* (Kosman et al., 1991; Leptin, 1991), non-vertebrate deuterostomes (Wada and Saiga, 1999), and vertebrates (Essex et al., 1993). *Snail* proteins are specifically implicated as regulators of mesodermal invagination (reviewed by Hemavathy et al., 2000). Later in development, *snail* plays a role in neurectodermal differentiation in both *Drosophila* and vertebrates (Essex et al., 1993; Kosman et al., 1991; Leptin, 1991).

The *Nv-snailA* gene encodes a transcript 1565 nucleotides long and the predicted protein spans 265 amino acids. The *Nv-snailB* gene encodes a transcript is 1729 nucleotides long and the predicted protein spans 272 amino acids. Both proteins possess four consecutive C_2H_2 zinc fingers (Fig. 7A). In the zinc-finger region, the two *Nematostella* proteins are highly similar to each other (82% identical) and other *snail* family members, specifically *worniu* from *Drosophila* (82-83% identical) and *snail-2* from human (74-82% identical). Both *Nv-snailA* and *Nv-snailB* possess a conserved SNAG domain at the N terminus, sharing eight out of nine and seven out of nine residues with vertebrate *snail-1*, respectively. Neighbor-joining analysis based on the zinc-finger region groups *Nv-snailA* and *Nv-snailB* together in a larger clade comprising the snail gene of *Podocoryne* (Spring et al., 2002), *snail-1* and *snail-2* of human, and *worniu* and *escargot* of *Drosophila* (Fig. 7B).

The expression profiles of *Nv-snailA* and *Nv-snailB* are

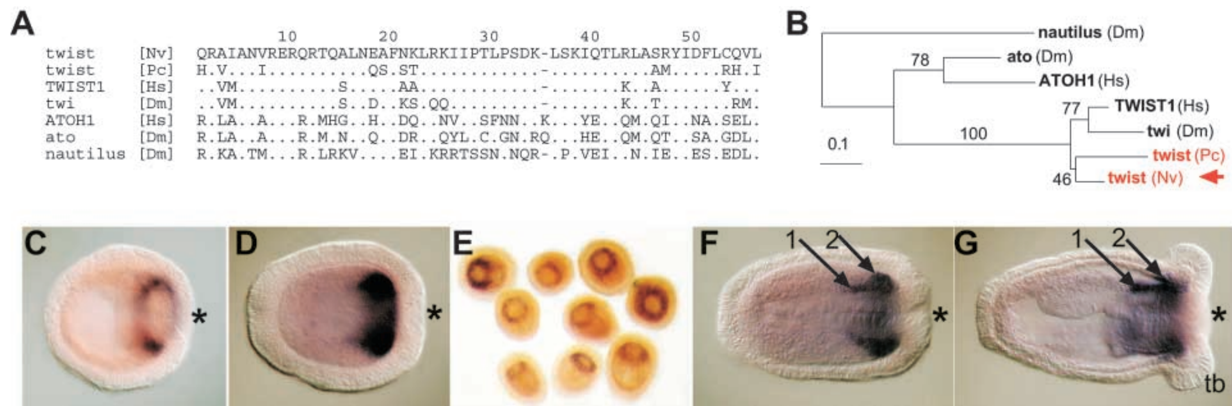

Fig. 8. *Nv-twist*. Phylogenetic analysis and gene expression. (A) Alignment of 56 amino acids from *twist*, *atonal* and *nautilus* proteins. (B) Phylogeny of *twist* sequences. The tree was constructed and labeled as in Fig. 3. Sequences included in the analysis are: ato (atonal), *Drosophila*, residues 256-311; ATOH1 (atonal homolog), *Homo*, residues 160-215; nautilus, *Drosophila*, residues 162-216; twi (twist), *Drosophila*, residues 363-417; twist, *Nematostella*, residues 37-92; twist, *Podocoryne*, residues 52-106; TWIST1, *Homo*, residues 109-163. (C-G) Developmental expression of *Nv-twist*. (C) Expression begins after gastrulation has started in a ring of endodermal cells encircling the presumptive mouth. (D) Expression continues in endodermal cells surrounding the future mouth. (E) Low magnification image of early planula stage embryos seen from the oral pole showing the ring of expression surrounding the future mouth. (F,G) As development proceeds, expression remains confined to a circum-oral ring, in the endodermal epithelium of the pharynx facing (arrow 1) and external gastrodermal wall under the tentacles (arrow 2).

practically identical, although *Nv-snailB* appears to be expressed at lower levels as evidenced by the relatively lengthy colorimetric substrate detection reactions that are required to visualize the expression pattern. *Nv-snail* transcripts first appear at the blastula stage in a small group of cells located at the future site of gastrulation (Fig. 7D,E). Cells that migrate into the interior of the blastocoel continue to express *Nv-snail* (Fig. 7F-H). After gastrulation, *Nv-snail* expression persists in all of the derivatives of the gastrodermis and pharynx, including the pharyngeal mesenteries (Fig. 7I,J). Expression was not detected in the epidermis at any stage of development.

Nv-twist

Twist is a basic helix-loop-helix (bHLH) transcription factor that is required for mesoderm specification in *Drosophila* and vertebrates (Castanon and Baylies, 2002; Thisse et al., 1988; Wolf et al., 1991). The *Nv-twist* transcript is 1182 nucleotides long, and it encodes a predicted protein of 129 amino acids. Nv-twist displays two domains that are highly conserved relative to other twist proteins. In the 53-residue bHLH domain (positions 36 through 87), Nv-twist is identical to human *TWIST1* at 44 positions and *Drosophila twist* at 43 positions (Fig. 8A). *Nv-twist* is also identical to human *TWIST1* and *Podocoryne* twist at all 14 residues of a conserved C-terminal motif known as the WR motif (ERLSYAFSVWRMEG) (Spring et al., 2000). Phylogenetic analysis strongly supports the orthology of *Nv-twist* to *twist* genes of *Podocoryne*, human, and fruitfly (Fig. 8B).

Nv-twist transcript is first detected well after gastrulation has commenced in a ring of endodermal cells surrounding the future mouth (Fig. 8C). On completion of gastrulation, *Nv-twist* is expressed exclusively in endodermal cells surrounding the mouth (Fig. 8D-E). In the polyp, expression remains confined to a thin group of gastrodermal cells on the coelenteron side of the pharynx and a small group of cells between the tentacles around the mouth (Fig. 8F-G). Expression is never seen in the ectodermal epidermis or in the pharynx proper. The expression pattern of *Nv-twist* is almost indistinguishable from the expression of two muscle-specific homeobox containing *Mox* orthologs in *Nematostella* (P. Burton, K. Pang, J.R.F. and M.Q.M., unpublished).

Discussion

It is widely believed that the triploblastic bilaterians are derived from a diploblastic ancestor. Modern day cnidarians are thought to reflect this hypothetical ancestral condition. If cnidarians are primitively diploblastic, then it may seem surprising that cnidarians possess most, if not all of the genes that are implicated in mesoderm development in bilaterians, such as *brachyury*, *snail*, *twist*, *mef2* and *MyoD*-like transcription factors (this study) (Boero et al., 1998; Muller et al., 2003; Spring et al., 2002; Spring et al., 2000). The co-occurrence of these 'mesodermal genes' in Cnidaria and Bilateria suggests three scenarios. In scenario one, the mesodermal genes originated long before the origin of mesoderm. In the cnidarian-bilaterian ancestor, these genes played no role in germ-layer specification. Subsequently, they were co-opted into the mesoderm developmental pathway within the bilaterian lineage. In scenario two, the mesodermal genes originated prior to the origin of mesoderm, but they played a role in germ layer specification (endoderm versus ectoderm) in the cnidarian-bilaterian ancestor. As mesoderm was invented and became spatially segregated from the endoderm, these genes became predominantly associated with the new germ layer, mesoderm. In scenario three, the cnidarian-bilaterian ancestor was a triploblastic animal. The same genes involved in mesoderm formation in modern-day bilaterians were deployed during mesoderm development in the cnidarian-bilaterian ancestor (e.g. *twist*, *snail*, etc.). Diploblasty evolved within the Cnidaria as a secondary reduction in the number of body layers. As the ancestral mesodermal and endodermal layers fused into the derived endoderm of cnidarians, the

mesodermal genes came to be expressed in this new composite germ layer.

The predominantly endodermal expression of six ‘mesodermal’ genes studied here suggests that these genes are playing a role in endoderm specification. Therefore, it is most parsimonious to infer that these genes were involved in germ layer specification in the cnidarian-bilaterian ancestor, and thus we can rule out scenario one in favor of scenario two or three. We currently favor scenario two, where these genes were involved specification of the endoderm in diploblasts, and that as mesoderm evolved from the primordial endoderm, their expression became associated with the presumptive mesoderm. A growing body of evidence, both developmental (e.g. Henry et al., 2000; Martindale and Henry, 1999) and molecular (Maduro and Rothman, 2002; Rodaway and Patient, 2001; Stainier, 2002) supports the conclusion that mesoderm evolved from endoderm. Cell lineage studies in two basal metazoans, the non-bilaterian ctenophore *Mnemiopsis leidyi* (Martindale and Henry, 1999) and the acoel flatworm *Neochildia fusca* (Henry et al., 2000), a putative basal bilaterian (Ruiz-Trillo et al., 2002; Ruiz-Trillo et al., 1999; Telford et al., 2003), reveal that mesodermal tissues arise exclusively from endodermal precursors. In addition to the gene expression patterns reported here, other genes implicated in mesodermal patterning and differentiation in bilaterians are localized to the endoderm of *Nematostella*. These include *mox*, *bagpipe*, *tinman* and muscle-specific *tropomyosin* (J.R.F., M.Q.M. and K.P., unpublished). The widespread expression of mesodermal genes in the gastrodermis of *Nematostella* is certainly consistent with an endodermal origin for mesoderm.

However, the data presented here do not rule out the hypothesis that triploblasty predated the divergence of Bilateria and Cnidaria (scenario three). Cnidarian hydromedusae appear triploblastic as they possess a third tissue layer that is independent of either the endoderm or the ectoderm – the entocodon. The entocodon arises from polyp ectoderm, not endoderm, and it has been hypothesized that the entocodon is homologous to the mesoderm of bilaterians (Boero et al., 1998; Muller et al., 2003; Spring et al., 2002; Spring et al., 2000). If so, then the cnidarian-bilaterian ancestor was a triploblast, and diploblasty evolved within the Cnidaria by loss of the mesoderm. The observed expression of mesodermal genes in *Podocoryne*, however, is not generally supportive of this hypothesis. If the entocodon is homologous to the mesoderm, then we should expect mesodermal genes to be expressed in this tissue layer. However, none of the mesodermal genes whose expression has been studied in *Podocoryne* are predominantly expressed in the entocodon. For example, in *Podocoryne*, *twist* is barely detectable by RT-PCR during embryonic, planula or polyp stages, and appears only in the ‘striated muscle’ (an epithelial sheet lining the subumbrellar plate) of the medusa (Boero et al., 1998; Muller et al., 2003; Spring et al., 2002; Spring et al., 2000). *Podocoryne snail* (Spring et al., 2002) is expressed weakly in the endoderm of early planula larvae and in the oral ectoderm and tentacle endoderm of polyps. *mef2* (Boero et al., 1998; Muller et al., 2003; Spring et al., 2002; Spring et al., 2000) is expressed at low levels at all life stages in the endoderm but never in any localized fashion until the late planula stage where it is expressed in aboral ectoderm. *mef2* is also expressed in the entocodon of the *Podocoryne* medusa, but as it is expressed in both endoderm and ectoderm, it cannot be said to exhibit a preference for the entocodon. Overall, the expression of ‘mesodermal genes’ in *Podocoryne* appears less rigidly restricted by germ layer, so the gene expression patterns do not provide an unambiguous signal that the entocodon is homologous to the mesoderm of bilaterians. Even at equivalent developmental stages, the expression patterns of mesodermal genes in *Nematostella* are quite distinct from those of *Podocoryne*.

Despite these differences between *Nematostella* and *Podocoryne*, some genes have similar expression patterns in hydrozoans and anthozoans. The expression of *brachyury* has been studied in both *Nematostella* (Scholz and Technau, 2002) and *Podocoryne* (Spring et al., 2002). The early expression of *brachyury* in *Nematostella* and *Podocoryne* appears to be similar (around the blastopore), although expression diverges dramatically at later stages of development. The *forkhead* gene *hnf3/budhead* has been studied in a freshwater *Hydra* (Martinez et al., 1997). It is expressed in a band around lower half of the hypostome, the ring of tissue lying between the tentacles and the prospective mouth. In *Nematostella*, *forkhead* is expressed early in gastrulation and is a robust marker for the pharynx and pharyngeal mesenteries. Such similarities in gene expression between the hypostome of *Hydra* and the pharynx of *Nematostella* could argue for the homology of these structures, although there appear to be differences in the germ layer of expression. It is simple to envision how the cone of tissue that protrudes above the tentacle ring of *Hydra* intrudes into the gut cavity of *Nematostella*.

Considering the antiquity of the last common ancestor of Anthozoa and Hydrozoa, and the pronounced differences in life history between *Nematostella* and *Podocoryne*, it is not entirely surprising to find differences in gene expression. Hydrozoans and anthozoans probably diverged well over 500 million years ago and the ancestral hydrozoan life history is much more complex than the ancestral anthozoan life history. In most Hydrozoa, the benthic polyp (which looks like an adult anthozoan) gives rise to one or more pelagic medusae (‘jellyfish’) by fission or budding. The medusa is the sexually reproducing phase of the life cycle. Hydrozoans are widely regarded as the most derived group of Cnidaria with complex life histories, colony specialization and morphological novelty. Therefore, we may expect that the co-option of developmental genes for novel functions is likely to be more common in Hydrozoa than in Anthozoa. However, although the Hydrozoa may be uniquely derived with respect to some features, every evolutionary lineage is a mosaic of primitive and derived traits. For this reason, it will be necessary to obtain data from both anthozoans and medusozoans if we hope to have any confidence in reconstructing the ancestral cnidarian condition, which, in turn, is crucial for reconstructing the ancestral bilaterian condition and understanding the origin of key bilaterian innovations such as mesoderm.

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