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ORIGINAL ARTICLE

Expansion, diversification, and expression of T-box family genes in Porifera

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Abstract Sponges are among the earliest diverging lineage within the metazoan phyla. Although their adult morphology is distinctive, at several stages of development, they possess characteristics found in more complex animals. The T-box family of transcription factors is an evolutionarily ancient gene family known to be involved in the development of structures derived from all germ layers in the bilaterian animals. There is an incomplete understanding of the role that T-box transcription factors play in normal sponge development or whether developmental pathways using the T-box family share similarities between parazoan and eumetazoan animals. To address these questions, we present data that identify several important T-box genes in marine and freshwater sponges, place these genes in a phylogenetic context, and reveal patterns in how these genes are expressed in developing sponges. Phylogenetic analyses demonstrate that sponges have members of at least two of the five T-box subfamilies (*Brachyury* and *Tbx2/3/4/5*) and that the T-box genes expanded and diverged in the poriferan lineage. Our analysis of signature residues in the sponge T-box genes calls into question whether "true" *Brachyury* genes are found in the Porifera. Expression for a subset of the T-box genes was elucidated in larvae from the marine demosponge, *Halichondria bowerbanki*. Our results show that sponges regulate the timing and specificity of gene expression for T-box orthologs across larval developmental stages. In situ hybridization reveals distinct, yet sometimes overlapping expression of particular T-box genes in free-swimming larvae. Our results provide a comparative framework from which we can gain insights into the evolution of developmentally important pathways.

Keywords Porifera · T-box · Basal metazoa · *Halichondria*

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Introduction

The earliest diverging metazoans are an important group of organisms to study if we are to understand the evolutionary history and emergence of animal body plans. Though the relationships of the basal metazoan taxa (i.e., Porifera, Cnidaria, Ctenophora, and Placozoa) are still uncertain from a phylogenetic perspective (e.g., see Sperling et al. 2009), our most current understanding of metazoan phylogenomics places sponges at the base of the animal lineage (e.g. Srivastava et al. 2008; Sperling et al. 2009; Pick et al. 2010). Thus, the phylogenetic position of the Porifera as a metazoan outgroup to all other animals makes it a crucial group for exploring hypotheses regarding early animal history. Furthermore, sponges

possess important body plan features that include both shared and derived characteristics when compared to other animals. Among the shared metazoan characteristics, one finds polarized body plans in larvae and adult sponges, as well as differentiated epithelia and sensory cells. Traits found in sponges but not the rest of the Metazoa include a canal system for pumping water built with choanocytes in the choanosome. Thus, sponges provide the only opportunity for comparative studies directed at (1) understanding the genetic regulatory components that were in place prior to the advent of nerves, complex tissues, and complex body plans (e.g. Simpson 1984; Larroux et al. 2006; Nichols et al. 2006; Sempere et al. 2006) and (2) how important components of the genetic regulatory machinery were utilized in an animal lineage with simple tissues and body plans.

Recent analyses of the genetic toolkit present in sponges and its regulation of development have yielded important insights regarding the early evolution of animals (e.g. Larroux et al. 2008; Sakarya et al. 2007). An important family of genes known to play crucial roles in a wide variety of developmental processes across the animal kingdom is the T-box family. This evolutionarily ancient class of transcription factors contains a large DNA-binding domain, and T-box proteins exhibit a strong conservation of DNA binding functions among family members (Naiche et al. 2005). The eponymous T-box family member, *T (Brachyury)*, is instrumental in vertebrate mesodermal formation and notochord differentiation (Herrmann et al. 1990; Holland et al. 1995). Other members of the T-box family play roles in endodermal and ectodermal specification in vertebrates, function in extraembryonic tissues, gastrulation and patterning of embryonic mesoderm, cardiogenesis, limb patterning, craniofacial development, pituitary cell fate, and T-cell differentiation (reviewed in Naiche et al. 2005). Given the expansive roles of T-box genes, it is not surprising that the gene family is quite large with 18 members in mammals (Wilson and Conlon 2002). The biologically important roles for T-box family members have also been demonstrated in a variety of triploblastic model systems other than the vertebrates. Some genes, like *Brachyury*, have homologous functions across bilaterians in processes involving morphogenetic movements, while other family members demonstrate species-specific roles (reviewed in Wilson and Conlon 2002; Showell et al. 2004). Comparative studies indicate that the gene family includes five evolutionarily related subfamilies designated as *Brachyury/T*, *Tbx1* (including *Tbx1/10, 15/18/22, 20*), *Tbx2* (including *Tbx2/3, 4/5*), *Tbx6*, and *Tbr1* (including *Tbr1*, *Tbx21* and *Eomes/Tbr2*) (Papaioannou 2001).

T-box orthologs have been found in all of the basal metazoans including cnidarians (Technau and Bode 1999; Spring et al. 2002; Scholz and Technau 2003), ctenophores (Martinelli and Spring 2005; Yamada et al. 2007), placozoans (Martinelli and Spring 2003), and sponges (Adell et al. 2003; Manuel et al. 2004; Larroux et al. 2006; Larroux et al. 2008). In the Porifera, T-box family members have been found in all major sponge lineages including the demosponges, calcareous sponges, and hexactinellids. T-box genes were not found, however, in the genome of the choanoflagellate *Monosiga brevicollis* (King et al. 2008), a unicellular protist belonging to a lineage that shares ancestry with the animal lineage. This finding supported a long held view that T-box genes arose in the common ancestor of metazoans. Recently, however, putative T-box genes were detected in sequences from the genomes of the unicellular amoeba opisthokont, *Capsaspora owczarzaki* which is a close relative of multicellular animals and fungi (Broad Institute Sequence Database), and the mesomycetozoean *Amoebidium parasiticum* (Mikhailov et al. 2009). These findings reveal that T-box-like genes were lost in the choanoflagellate lineage and evolved early in the opisthokont lineage long before multicellularity evolved.

Among the basal metazoans, roles for T-box genes have been elucidated only partially. The most in depth study of T-box function in a diploblast is from the ctenophore *Mnemiopsis leidyi*. Expression analysis of T-box genes during gastrulation and early organogenesis in this organism showed that all five ctenophore T-box family members exhibited distinct expression patterns during gastrulation and that some members are also expressed during the formation of the mouth, presumptive mesendoderm, sensory organs, and the tentacular system (Yamada et al. 2007). Further studies demonstrated that the *M. leidyi Brachyury* ortholog (*MlBra*) is expressed in ectodermal cells around the site of gastrulation and in cells derived from the blastopore; it is also involved in regulating morphogenetic movements involved with gastrulation as determined by morpholino oligonucleotide knockdown (Yamada et al. 2010). Using *Xenopus* embryos, it was shown that *Xbra* and *MlBra* are functionally interchangeable, thus showing that the primitive role of *Brachyury* is to regulate morphogenetic movements involved in the blastopore (Yamada et al. 2010). In the Hydrozoa, the Hydra *Brachyury* ortholog, *HyBra1*, is expressed in endodermal cells of the head and plays a role in head formation (Technau and Bode 1999). In the anthozoan *Nematostella vectensis*, the *Brachyury* ortholog is expressed around the blastopore (Scholz and Technau 2003), and another *Brachyury* gene is expressed at the site of ingression in early jellyfish gastrula (Spring et al. 2002). In placozoans, two T-box genes show distinct expression patterns with the *Brachyury*-like ortholog expressed in potential outgrowth zones while the *Tbx2/3* ortholog is expressed at the periphery of attached animals (Martinelli and Spring 2003).

Adult expression patterns of T-box proteins have been reported for only one species of demosponge, *Suberites domuncula* (Adell and Müller 2005). In *S. domuncula*, a

purported *Brachyury* ortholog is expressed in early sponge cell cultures ("pre-primmorphs"), when cell–cell and cell–matrix interactions are being established, and also in adherent primmorphs during a stage of tissue reorganization (Adell and Müller 2005). The latter finding may suggest a role for this *Brachyury*-like gene in morphogenetic movements through regulation of cell adhesion and migration. A second *Suberites Tbx* gene (*SdTbx2*), a likely *Tbx4/5* ortholog, is expressed during the first day of sponge cell culture and in isolated cells of the mesohyl of adult sponges suggesting possible roles in cell identity determination (Adell and Müller 2005). Expression of T-box proteins has so far not been studied during sponge embryogenesis or metamorphosis of larvae into adults, when ganstrulation-like movements may occur (Leys 2004).

The relatively limited body of data regarding the structure and function of the T-box class of transcription factors among poriferan and diploblast animals underscores why it is important that additional studies be conducted to elucidate the early evolution of this gene family. Here, we report T-box family members from degenerate PCR surveys in both a freshwater and marine sponge (*Halichondria* sp. and *Ephydatia muelleri*). We compare these sequences with T-box genes from the genomes of *Amphimedon queenslandica*, the homoscleromorph sponge *Oscarella carmela*, and other known sponge T-box genes, to illustrate that T-box gene duplication in Porifera may be more extensive than was previously believed. We show that some T-box family members appear to have been lost in certain sponge lineages. We also present in situ hybridization data on expression patterns during larval development in a marine sponge (*Halichondria* sp.). These findings show that T-box family members have distinct yet overlapping expression profiles, and they reveal patterns of expression at the anterior and posterior ends, as well as along the larval midline in swimming sponge larvae for two T-box family members.

Methods

Collection and rearing of sponges

Halichondria sponges were collected from the Chesapeake Bay at Virginia Institute of Marine Science, Gloucester Point, Virginia. In the laboratory, sponges were reared in re-circulating seawater aquaria (all water was replaced weekly) or were used immediately. For larval collection, individual sponges were placed in beakers containing filtered, sterile seawater. The mother sponges were allowed to naturally release larvae into the water column. Newly released, mature larvae of *Halichondria* were easily collected as they swim toward the surface in slow spirals and congregate at the air–water interface. Larvae were washed several times in filtered, sterile seawater and were grown in 24-well plates (≈50 larvae per well) in filtered, sterile seawater, which was replaced daily.

For the purposes of this experiment, we defined four basic developmental stages: free-swimming larvae, skating larvae, attached larvae, and settled/spreading tissue. Free-swimming larvae were periodically collected from the mother sponge over the course of a day; since no larvae were present when the mother sponge was initially placed in the beaker, we considered all larvae collected during the day to belong to the 0–8 h post-release age cohort. Before settlement, the larvae of this species stop moving, sink to the bottom of the container, lose the characteristic free-swimming morphology, and skate or crawl along the substrate. We call this stage "skating" since larvae look as if they are gliding along the bottom surface. After the skating stage, larvae form an attachment to the substrate and cannot be removed without force; we define this stage "attachment." After attachment, the larval cells begin "spreading" across the bottom of the well, differentiate, and will eventually develop into juvenile sponges (i.e., rhagons).

E. muelleri gemmules were collected and harvested from sponges collected from a dam outflow near Griswold, Connecticut, USA (41°35′4″ N, 71°55′15″ W). Gemmules were washed in 3% hydrogen peroxide and re-washed several times in sterile, cold, ×1 Strekal's (Strekal and McDiffett 1974) media, and stored at 4°C in the dark until use. Gemmules were hatched in ×1 Strekal's media and grown to developmental stages 0–6 as per Funayama et al. (2005).

Isolation of Tbx sequences

Halichondria bowerbanki and *E. muelleri* RNA was isolated from either larvae, reaggregated adult tissue, or hatched gemmules all of which were washed several times in sterile media before RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacture's protocol. cDNA was made using Thermoscript Reverse Transcriptase (Invitrogen) using oligo(dT) and/or random hexamer primers.

Degenerate PCRs were performed using primer pairs designed from an alignment of the conserved T-domain from a collection of T-box genes isolated in other animals. The following primer combinations were used in all possible combinations including nesting when appropriate: GRRMFP, NP(Y/F)AKAF, TAYQNE, NEMIVT or (T/N) EMI(V/I)TK, FG(S/A)HWM. cDNA from *Halichondria* sp. from pooled larval stages and from reaggregated adult tissue or cDNA from *Ephydatia* isolated from gemmules hatched and harvested across several developmental stages served as template. Annealing temperatures ranged from 40°C to 55°C. All resulting PCR products of expected sizes

were excised and cloned using the TOPO TA Cloning Kit (Invitrogen). Clones were sequenced using the SequiTherm EXCEL II kit (Epicenter) on a LiCor DNA Sequencing System or using an ABI sequencer. Sequences for each gene were extended (when possible) in the 5′ and 3′ directions using RACE (SMART RACE, Clontech). Genbank accession numbers are given in Supplemental Fig. 1 where sponge sequences are highlighted according to classes.

Phylogenetic analyses

Bilaterian species were chosen based on the availability of whole-genome protein models. Sequences from two deuterostomes, *Homo sapiens* and *Strongylocentrotus purpuratus*, and two ecdysozoans, *Caenorhabditis elegans* and *Drosophila melanogaster*, were obtained by using BLAST searching of the NCBI genome databases. Sequences from two lophotrochozoans, *Capitella telata* and *Lottia gigantea*, were obtained by using BLAST searching of the JGI genome databases (JGI, unpublished data) as was placozoan sequence from *Trichoplax adhaerans* (Srivastava et al. 2008), choanoflagellate sequence from *M. brevicollis* (King et al. 2008), and cnidarian sequence from *N. vectensis* (Putnam et al. 2007). Sequence from a second cnidarian species, *Hydra magnipapillata*, was obtained from NCBI. A single sponge genome, *A. queenslandica*, was searched using BLAST at the Trace Archive draft genome downloaded from NCBI. Four opisthokont genomes,

Fig. 1 Unrooted ML phylogenetic tree of the T-box gene family. The T-box phylogeny was evaluated with both ML and Bayesian methodologies. Bootstrap support values and posterior probabilities are shown in the numerator and denominator, respectively. The topology shown includes clades with >60% support. Major families are identified with *blue bars*; sponge genes are identified in *red*. Accession numbers for each of the entries are provided in the supplementary files

Allomyces macrogynus, *C. owczarzaki*, *Spizellomyces punctatus*, and *Proterospongia* sp., were searched using BLAST at the Broad Institute website (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html). Additional cnidarian, ctenophore, and poriferan sequences were obtained from the published literature (Bielen et al. 2007; Yamada et al. 2007; Martinelli and Spring 2005; Adell et al. 2003; Spring et al. 2002) or using the methods described above. EST sequences from *Acropora mellifera*, a cnidarian coral, were also used and were obtained by BLAST searches of a larval EST database (http://sequoia.ucmerced.edu/SymBioSys/) and genomic sequences from *O. carmela* were obtained via BLAST searches by Dr. Scott Nichols (unpublished data).

Alignments were performed using Muscle (Edgar 2004) implemented in Seaview using the default settings. Sequences were trimmed to contain only the Tbox regions. Gblocks was run under a variety of conditions and yielded subsets of the sites we used in our phylogenetic analysis (Dereeper et al. 2008). We employed both Maximum Likelihood (ML) and Bayesian approaches in our phylogenetic analyses. A model of sequence evolution was determined from aligned sequences using ProtTest (v1.2.6, Abascal et al. 2005). PhyML (Guindon and Gascuel 2003) was used for ML analysis with 500 bootstrap replicates; in PhyML, gaps are treated as ambiguous characters. All parameters were optimized based on empirical data. Bayesian analysis, as implemented in Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001), was performed until the average standard deviation of split frequencies achieved stationarity (n=2,000,000 generations). We used four independent chains in our analyses. The gamma distribution for among site substitution rates was approximated using four rate categories with a proportion of invariable sites. The first 25% of the samples were discarded as burn-in. All branches with less than 60% support were collapsed in the ML tree shown (Fig. 1).

Expression analyses

Tissues from the various developmental stages were either stored in RNAlater (Ambion) overnight and then placed at −80°C for subsequent RNA isolation or fixed for in situ hybridization. RNA was isolated using the RNAeasy Mini Kit (Qiagen) and treated on column with DNase I to limit contaminating genomic DNA. For RT reactions, 200 ng of RNA was reverse transcribed using the Thermoscript RT kit (Invitrogen) and subsequent PCRs were first carried out using Platinum Taq DNA polymerase (Invitrogen) to test gene specific primers and RT reactions. SYBR Green chemistry and the Chromo4 (BioRad) were used for qRT-PCR using cycling conditions of: 94°C for 3 min followed by 30 s 94°C, 30 s 55–61°C, 1 min 72°C for 35 cycles. Gene-specific primers were used to amplify isolated *Halichondria* Tbx genes for profiling expression by qRT-PCR across developmental stages. In each case, controls were performed to ensure expression levels were from cDNA and not contaminating genomic DNA and that each primer pair only amplified the target Tbx gene (as determined by testing each primer pair on plasmids for each Tbx gene). Actin was picked to standardize the amount of expression calculated for *Tbx* genes at each developmental stage because it has often been utilized in other systems (including cnidarians) for this purpose (McCurley and Callard 2008; Rodriguez-Lanetty et al. 2008; Yüzbaşıoğlu et al. 2010). Nonetheless, we used qRT-PCR to compare actin mRNA levels at each developmental stage compared to total RNA amounts and though some degree of variability was observed, the expression stability across larval developmental stages was high. Further, we have also used Ef1α (Siah et al. 2008; Curtis et al. 2010) as a standardization control for some of these genes as well and do not see differences in the relative expression profiles. For each gene, data from two different batches of RNA were assessed, and all PCRs were performed with two replicates.

To determine mRNA distribution in larvae, we used a protocol similar to that described in Hill et al. (2010) but adapted for the larvae. Briefly, sponge tissues were fixed overnight in 4% paraformaldehyde, 0.02% glutaraldehyde in sterile seawater and then transferred into ascending concentrations of methanol, and stored in 100% methanol at −80°C. Fixed tissues were rehydrated through a methanol and PTw (×1 PBS containing 0.1% Tween-20) series. Tissues were prepared for prehybridization through three washes in ×1 PTw and one wash of ×1 PTw containing Proteinase K (1 μg/mL) at 37°C (alternatively, in some cases, tissues were washed for 30 min in detergent solution (1% SDS, 0.5% Tween, 50 mM Tris–HCl pH 7.5, 1 mM EDTA, and 150 mM NaCl) followed by six washes in PTw with no differences observed). The tissues were then re-fixed in 4% paraformaldehyde in PBS, washed twice with 0.1 M TEA buffer (pH 8), and then treated once in 0.1 M TEA containing 0.25% acetic anhydride, followed by two washes in ×1 PTw. Tissue was processed to a 1:1 solution of hybridization buffer (50% formamide, ×5 SSC, 50 mg/ml heparin, 0.25% Tween-20, 1% SDS, 100 μg/ml sheared salmon sperm DNA; pH 5) and PTw. Tissue was then prehybridized in hybridization solution at 60°C for at least 3 h. All probes were labeled using the Dig RNA labeling kit (Roche). After overnight hybridization at 45–60°C, tissue was washed seven times in hybridization solution at 60°C and gradually processed to room temperature through half washes in TBST and hybridization solution. Alternatively, after overnight hybridization, tissue was washed three times in ×2 SSC, twice in ×1 SSC, and once in TBST or NTE

buffer containing 20 μg/mL RNAse A at 37°C, followed by two washes of ×0.1 SSC at 37°C. After several washes in TBST, tissue was incubated in TBST containing 1% BSA to block nonspecific binding of antibody. Anti-Dig alkaline phosphatase antibody (Roche) was diluted 1:3,000 in TBST with BSA and larvae were incubated overnight in this solution at 4°C. Larvae were washed five times in TBST and then processed for staining using NBT and BCIP in AP reaction buffer. After staining, embryos were then cleared in 80% glycerol/PBS before imaging. For further validation of the in situ staining patterns, for some genes (*TbxA* and *Tbx*), two different probes were utilized that were directed to different portions of the genes. In these cases, no differences in staining patterns were observed. For *TbxA*, one probe was labeled for position 697–928 nt which included the 3′ portion of the T-domain. The alternate *TbxA* probe was 1,527 nt in length and corresponded to the entire *TbxA* mRNA sequence including 3′ UTR. For *Tbx4/5*, one probe included a 428 nt coding region containing the majority of the T-domain and the alternate probe was a 3′ RACE product that included a 195-bp overlap with the first probe (from forward 5′ primer: GCGGTATGGGAGAAGCAGCTGAT) and extending into the 3′ end of the clone.

Results and discussion

T-box gene families in basal metazoans: divergent evolution

While the relationships between many T-box sub-families remain ambiguous, several families, along with new members of those families, have high support in our phylogenetic analysis (Fig. 1). The clear sub-families, supported by both Bayesian and Maximum Likelihood analysis, include bilaterian-specific groups, Cnidaria +Bilateria-specific groups, Porifera+Cnidaria+Bilateria-groups, and Porifera-specific groups (Fig. 1). The sponge-specific groups include a demosponge-specific *TbxPor* clade and a demosponge-specific clade within the *Tbx4/5* group (a homoscleromorph *Tbx4/5* sequence is not included in that clade). Also supported are a demosponge-specific lineage we call *TbxA* and a demosponge-specific lineage designated as *TbxC/D*. Finally, there is another group identified in Fig. 1, *TbxA/B/C/D/E*, that is supported by the ML tree, but not by the Bayesian tree. It is intriguing that this group includes protist, poriferan, and placozoan *Tbx* genes. These sequences in the *TbxA/B/C/D/E* group may represent ancestral *Tbx* genes. However, this is a region of the tree that could suffer from long-branch attraction issues (Felsenstein 2004). Additional sequences from other protists and basally branching animals would be necessary to address this issue and the hypothesis that these *TbxA/B/C/D/E* genes are ancestral and not lineage-specific duplications.

Given that some T-box family clades do not contain poriferan representatives and that the sponges appear to contain unique T-box families, we propose that the Urmetazoan had at least one T-box gene that underwent several rounds of independent duplication and divergence after the sponges split from other metazoans. While synteny studies remain to be performed, our phylogenetic analyses suggest that a proto-T-box gene evolved before the advent of multicellularity since we find three T-boxes in the genome of a unicellular opisthokont amoeba, *C. owczarzaki* (50–60% similarity to *H. sapiens* T-brain T-box). This kind of large-scale duplication and divergence has been observed repeatedly for many eumetazoan “toolkit” genes (e.g. Larroux et al. 2008; Yamada et al. 2007). However, the presence of T-box genes in the protistan genome could be the result of lateral gene transfer, though we believe common ancestry is the most parsimonious explanation of the data.

Our analysis recovered three demosponge-specific *Tbx* clades. The *TbxPor*, *TbxA*, and *TbxC/D* clades have strong support in both Bayesian and Maximum Likelihood analysis. They are comprised of T-box sequences from four, three, and two sponge species respectively, and each clade contains two of the orders in the Demospongiae (Haploclerida and Halichondrida). Additionally, our analysis indicates that there are a number of divergent T-box genes in Porifera (e.g., *AqTbxE* and several putative *O. carmela* Tbox sequences) that are not associated with any specific T-box clades. Another sponge sequence, *AqTbxB* groups with the amoeboid *C. owcsarzaki* with weak support.

While the sponge-specific groups contain only demosponge sequences (i.e., they lack representatives from the Calcarea or Hexactinellida), it is unclear whether this is due to demosponge-specific duplications or because of the extremely small amount of sampling that has been done in Porifera. However, with two poriferan genomes (*A. queenslandica* and *O. carmela* (S. Nichols, unpublished data)), and our exploration using degenerate PCR in *H. bowerbanki* and *E. muelleri*, we can make inferences about the evolution of *Tbx* genes in sponges. For example, it is clear that the non-bilaterian *Tbx* clades contain a large number of sponge members. This, and the presence of several sponge-specific groups, is suggestive of extensive duplication and divergence of *Tbx* genes specific in the sponge lineage.

Tbx1 subfamily (Tbx1/10, 15/18/22, 20)

The *Tbx1* subfamily contains bilaterian, cnidarian, and ctenophore members. This subfamily has no sponge members, making it likely that it arose after sponges split from the rest of Metazoa. The *Tbx-1/10* clade groups with

the *Tbx-15/18/20/22* clade, a clade with Bilateria+Cnidaria genes. These groups were likely present in the bilaterian/cnidarian ancestor, but not in the Urmetazoan unless Tbx1 was lost in the sponge lineage. The evolutionary history of the ctenophore (*M. leidyi*) *Tbx1* gene is unclear and since there is no ctenophore genome sequenced and the position of ctenophores relative to bilaterians and cnidarians is debated, there may be additional ctenophore *Tbx1* subfamily members. Our *Tbx1/10* group is consistent with results from previous studies (Larroux et al. 2008; Yamada et al. 2007). In those studies, sponge sequences *AvTbx1/15/20* and *AqTbx1/15/20* were placed in the *Tbx1* subfamily. In our analysis, a separate poriferan-specific clade, that we call *TbxPor*, has strong support. Whether this clade is part of the *Tbx1* subfamily or a distinct Tbx lineage will require greater resolution.

Tbx2 subfamily (Tbx2/3, Tbx4/5)

The *Tbx2/3/4/5* subfamily genes are most often known for their demonstrated roles in heart and eye development and in the evolution of developmental programs involved in appendage outgrowth and patterning across the vertebrates (reviewed in Papaioannou 2001; Horton et al. 2008). While our ML analysis (but not Bayesian) supports the grouping of Tbx2/3 with Tbx1, though with low bootstrap support, synteny analyses across Metazoa suggest that Tbx2/3 and Tbx4/5 are more closely related (see below). However, a fuller understanding of the true evolutionary history of the origins and order of appearance of these gene families await additional data from more species. By our analysis *Tbx2/3* clade has high support and comprises genes from bilaterian, cnidarian, placozoan, and ctenophore species (Fig. 1). Sponges are not represented in the *Tbx2/3* group. The Tbx-4/5 sub-family is one of only two large clades in our phylogeny with representatives from Porifera, Placozoa, Cnidaria, and Bilateria (Fig. 1). We recovered a single clade containing all known sponge *Tbx4/5* members (Fig. 1). Interestingly, while *A. queenslandica* and *O. carmela* have single *Tbx4/5* representatives, there are two *E. muelleri Tbx4/5* genes, possibly representing a duplication within the spongillid sponge lineage. The lack of a ctenophore *Tbx4/5* may be due to incomplete sampling or gene loss in this lineage (it is known that *Tbx4/5* has also been lost in two major bilaterian lineages (Horton et al. 2008)).

Close linkage of the *Tbx2/3* to the *Tbx4/5* genes has been reported across chordates (except ascidians) and cephalochordates (Horton et al. 2008), and recently, it was shown that the *Nematostella* genome contains a *Tbx2/3* and *Tbx4/5* gene in the same orientation within 20 kb of each other (Yamada et al. 2007). It has thus been proposed that a duplication of an ancestral *Tbx2/3/4/5* locus pre-dated the divergence of modern diploblasts and triploblasts (Horton et al. 2008; Yamada et al. 2007). There are two possible historical explanations for the distribution of *Tbx2* subfamily genes as indicated by our data. First, the *Tbx2/3* gene may have been lost in the poriferan lineage. This would imply that duplication of the *Tbx2/3/4/5* ancestral gene occurred before the sponges and Metazoa diverged; alternatively, that duplication event occurred after sponges and Metazoa split. The problem with this interpretation of the data is that there is strong support for sponge *Tbx4/5* genes in our phylogeny. Either convergent evolution pushed the sponge lineage toward the diploblast/triploblast *Tbx4/5*-like gene sequence, or the ancestral *Tbx2/3/4/5* gene was *Tbx4/5*-like.

Brachyury/T subfamily

The *Brachyury* clade is the only large group with representatives from all phyla sampled, including an amoeba sequence from *C. owczarzaki.* As *Brachyury* is typically associated with mesoderm development, gastruation, and morphogenic movements, we were surprised to recover *Brachyury*-like sequences from a unicellular organism. Placozoan, ctenophore, and cnidarian sequences also fell into this clade as well as sequences from three sponges—*Oopsacas minuta* (*OmBra*—a hexactinellid), *S. domuncula* (*SdBra*—a demosponge), and *Sycon raphanus* (SyBra—calcareous sponge). Of particular relevance to this point, it should be noted that neither the *A. queenslandica* genome or the *O. carmela* genome possess a gene in the *Brachyury* clade, nor did we recover a *Brachyury*-like gene from *Halichondria sp.* or *E. muelleri* despite numerous attempts with *Tbx* and *Brachyury*-specific primers. Several explanations of this pattern are possible. It is possible that the *Brachyury*-like gene was lost in some sponge lineages. Alternatively, sponges may lack a true *Brachyury* gene, and those sponges that do fall in this group may have converged on similar signature sequences.

To further distinguish between these possibilities, we examined key residues in the T-box regions of proteins falling within the *Brachyury* clade (See Supplemental Figure 2). While the placozoan, ctenophore, and cnidarian sequences appear to represent true *Brachyury* proteins, inspection of the sequence alignments reveals that sponge and protist sequences are likely not true *Brachyurys*. *Brachyury* is the most well studied Tbx gene family and, as such, functionally and phylogenetically important residues have been elucidated. Among putative DNA and protein binding residues (~30 total (Müller and Herrmann 1997)), there are two *Bra*-specific residues that differentiate *Brachyury* genes from other closely related T-box family members (except *S. purpuratus*). These are a diagnostic Lys106, involved in DNA-binding specificity (rev. in Wilson and Conlon 2002), and Met-42, potentially

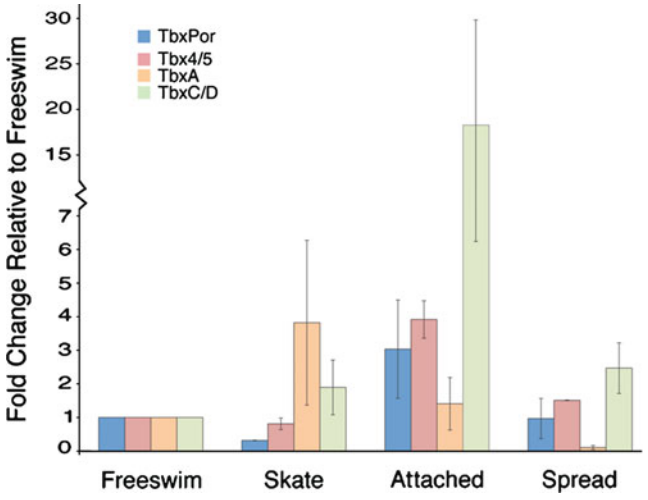

Fig. 2 qRT-PCR analysis of T-box gene expression during larval developmental stages of *Halichondria*. Gene expression levels are plotted relative to free-swimming larvae and normalized to actin expression levels at each developmental stage. Y-axis denotes relative levels of expression

involved in dimerization (Müller and Herrmann 1997) (all numbering is based on the *Drosophila Byn* T-box protein sequence starting at LDDRELW). A third residue, Asn-85, is found in all canonical *Brachyury* and *Eomes/Tbx* genes recovered in our analysis, excepting *S. purpuratus*. This residue is a potential synapomorphy of the Brachyury + Eomes clade and is not found in protist or *Sy-Bra*.

Both the protist and *SyBra* lack Lys106 and have the canonical T-box Arginine at this position (note that the Eomes group has an Asparagine at this position, unlike most other Tbox groups). Of the two sponges included in the *Brachyury* clade we recovered (Fig. 1), the known *OmBra* sequence is extremely short and lacks much of the T-box domain. Of 30 residues directly involved in DNA binding and protein–protein interactions, 11 have not yet been elucidated from the *OmBra* sequence. The third sponge sequence, *SdBra* does not have the Met-42. Interestingly, the functionally important Alanine at position 171 in *Brachyury* genes is also found in the *TbxPor* group. This residue is known to be involved in DNA-binding (Müller and Herrmann 1997) and is typically replaced with a Guanine in other T-box genes. Since *Brachyury* and *TbxPor* are not sister groups, it seems likely that they converged on this Alanine, especially due to the fact that it has functional significance. The potential convergence in T-box sequence is likely due to strong selective pressures on these sites given the constraints of DNA-binding and dimerization. This may also be responsible for the difficulties encountered in resolving branching orders among the T-box families (i.e., the high degree of polytomy) and the presence of non-*Bra* genes in the *Brachyury* sub-family. The presence of T-box genes but absence of a *Brachyury* homolog in some sponges may be important to our understanding how sponges form tissues without undergoing the typical or "conventional" morphogenetic movements seen in gastrulation in other animals (e.g., Stern 2004). Whereas sponge embryos show cellular differentiation and form layers made of distinct cell types during early development, a feeding epithelium (equivalent of a gut) is only formed at metamorphosis (Leys 2004). T-box's may, therefore, be involved in directing morphogenetic events involved in the formation of polarity during larval development and at metamorphosis.

Distinct larval expression profiles of Poriferan T-box genes

We used real time RT-PCR to assess relative levels of expression for four *Halichondria Tbx* genes. For each gene, we determined expression levels during settlement and attachment relative to free-swimming larvae, across four larval developmental stages (Fig. 2). The first stage examined was free-swimming larvae, which consisted of a pool of larvae that were collected from the top of the water column between 0 and 24 h post-larval release. Though these larvae are positively phototactic throughout the free-swimming period (which typically lasts 48 h but can continue past 72 h), they enter a stage where they swim at or near the bottom of the dish where they may temporarily settle. This behavior has been described in a variety of sponges (see Simpson 1984) as "creeping," "crawling," or "preattachment" and may involve cilia cell-substratum interactions. We call this stage "skating" since the larval behavior looks more like a gliding movement than a creep or a crawl in *Halichondria*. In fact, the larvae are often observed to be spinning on their axis as they glide along the bottom of the dish. During the skating stage, larvae may resume swimming near the bottom of the dish if a pulse of water current is applied near their site of contact, but they resume skating quickly after the disturbance. The next stage we collected at included larvae that were attached to the dish and clearly had basopinacocyte formation that adhered to the larvae to the substrate. Finally, we collected larvae that had begun the metamorphosis process with proliferative (archaeocyte) cells that were "spreading" across the surface of the dish. In this study, we did not follow development through metamorphosis to the juvenile rhagon stage because of a parasite that preys on the juveniles that we could not eliminate from the cultures without also compromising the sponge's development. Future studies using another species of demosponge (*E. muelleri*) will be aimed at examining the role of orthologs to these *Tbx* genes during metamorphosis and adult sponge function.

The *TbxA* gene showed the highest expression levels during the "skating" stage relative to free-swimming and had very low expression in spreading larvae. *TbxC/D*, *Tbx4/5*, and *TbxPor* all showed the greatest expression

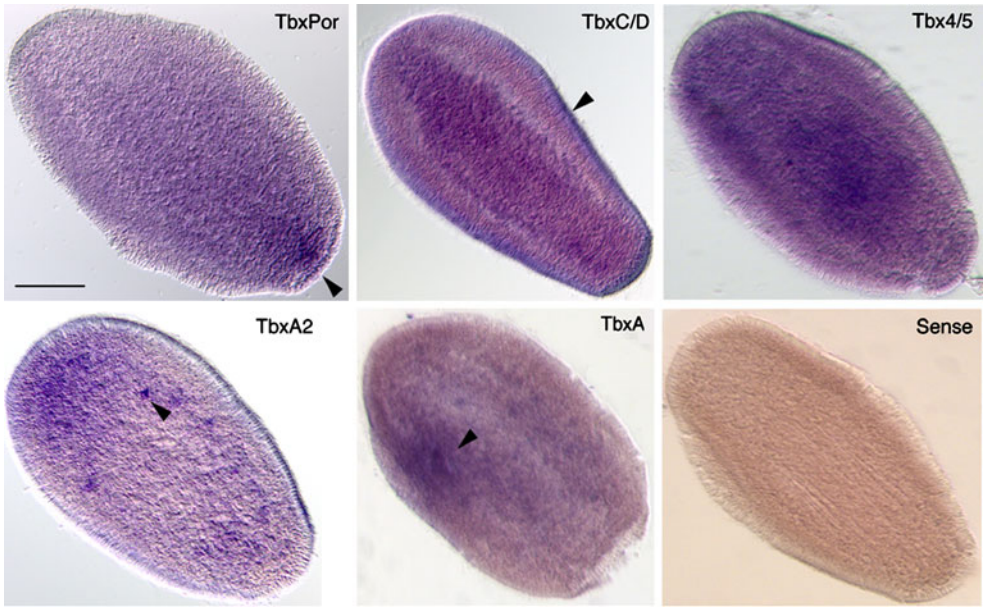

Fig. 3 Expression of T-box genes in early stage free-swimming *Halichondria* larvae with whole-mount in situ hybridization. Anterior poles of larva are oriented at the *top left* of each panel. The sense probe for *HbTbx 4/5* is shown, other sense probes also exhibited no staining. *HbTbxPor* is highly expressed at the poster pole as indicated by *black arrow*. *HbTbxC/D* is expressed in the columnar epithelial layer (CE, *black arrow*) as well as throughout the subepithelial layer (SE) that is directly beneath the CE and also in the ICM. *HbTbx4/5* is expressed throughout the ICM and SE, but not in the CE. *HbTbxA2* is expressed at the anterior end of the larvae and in cells around the inner cell mass (*black arrow*). *HbTbxA* reveals an asymmetric expression pattern on one side of the larvae as indicated by *black arrow*

levels at larval attachment to the substrate. For *Tbx4/5*, expression during larval attachment is more than two-fold higher than at the spreading stage and is more than three-fold higher than free-swimming or skating stages. *TbxC/D* expression at attachment is up to six-fold higher than all other developmental stages. The overall expression profile for *TbxPor* and *Tbx4/5* are similar with attachment as the highest expression level, spreading as the next highest, followed by free-swimming larval expression and skating as the lowest level of expression. We did not assay expression levels of the *TbxA2* gene since it was discovered much later in our study (during RACE of TbxA), and we would not have been able to compare qRT-PCR data directly with the other genes since each gene was analyzed from multiple matched sets of RNA/cDNA at each developmental stage. Nonetheless, it is clear from the expression profiles for *TbxA*, *TbxC/D*, *Tbx4/5*, and *TbxPor* that there has been some level of functional divergence of these *Tbx* genes. The different levels of expression over several developmental stages

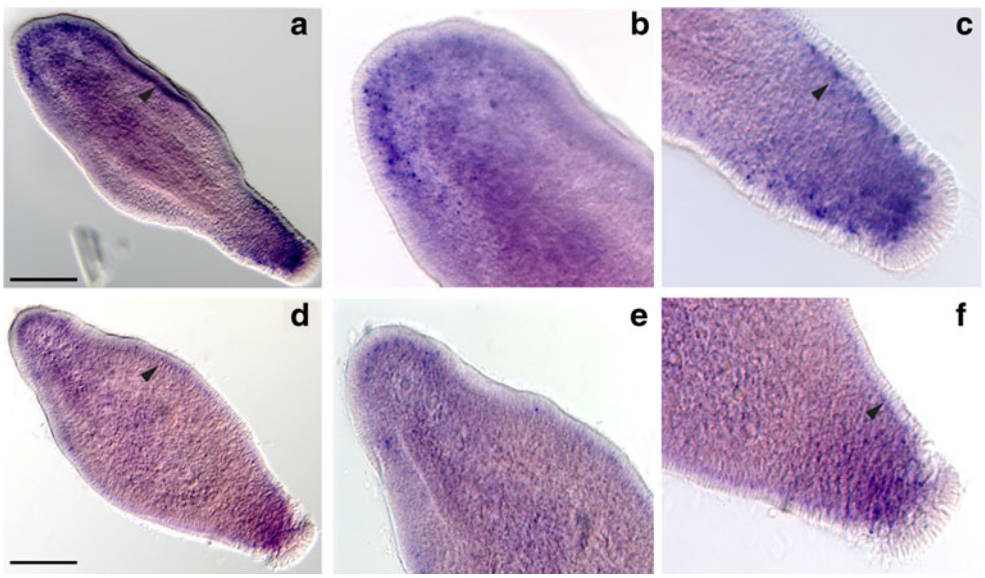

Fig. 4 Expression of *HbTbx4/5* and *HbTbxPor* in late stage free-swimming *Halichondria* larvae by whole-mount in situ hybridization. Anterior poles of larvae are oriented at the *top left*. **b** and **e** are higher magnifications of the anterior poles and **c** and **f** are higher magnifications of the posterior poles. **a**–**c** *HbTbxPor* expression is most concentrated at the anterior and posterior pole of the larvae with expression extending along the midline, mostly in the sub-epithelial cell layer (*black arrow*). **d**–**f** *HbTbx4/5* expression is also seen at the anterior and posterior poles with expression extending along the midline in the sub-epithelial layer (*black arrow*)

suggests that the sponge T-box genes have undergone divergence in their cis-regulatory regions, at least. This is likely tied to some functional divergence, which might be as simple as a split in the timing of deployment or as complex as a completely novel function for one of the duplicates.

To identify patterns of expression and possibly suggest functions, the expression of sponge T-box genes were also assayed by whole mount in situ hybridization to newly released sponge free-swimming larvae (Fig. 3). These larvae are about 250–300 μm long, and have a ciliated columnar epithelium (CE). Immediately inside the CE is a sub-epithelial layer of cells that surrounds a large spicule-containing inner cell mass (ICM). At maturity, the posterior pole of the larvae will have longer cilia (~36 μm in length compared to ~12 μm around the rest of the surface (see Fell and Jacob 1979)). Since all five *Halichondria* T-box genes were expressed in 0–24 h free-swimming larvae, we chose this stage for initial analysis. Given that expression profiles for genes in this species of larvae have not been reported, we include a supplemental figure (Supp. Fig. 2) showing positive controls for expression of actin which hybridizes in all cells and the *BarBsh* gene which has previously been reported to be expressed in the inner cell mass of *A. queenslandica* larvae (Larroux et al. 2006). We also observe expression of *BarBsh* exclusively in the inner cell mass of *Halichondria* larvae thus illustrating that the expression patterns we observe in this study have been validated with positive and negative controls (Supp. Fig. 2).

Each T-box gene exhibited a distinct pattern of expression, though some of the expression patterns (e.g., *Tbx4/5* and *TbxC/D*) were less pronounced. The *TbxPor* gene seemed to be most concentrated at the posterior end and spread toward the anterior within the inner cell mass. *Tbx4/5* staining is nearly ubiquitous throughout the inner cell mass and subepithelial layer at this stage; however, it does not seem to be expressed in the columnar epithelium. *TbxC/D* staining is evident in all cells (though most concentrated in the inner cell mass), including the ciliated columnar epithelium and it is the only T-box gene identified that is evidently expressed in these cells (see arrow). The *TbxA* gene has an interesting expression domain that is concentrated on one lateral side of the larvae (see arrow, Fig. 3). This apparently asymmetrical pattern of expression in symmetrical larvae is enigmatic. Finally, *TbxA2* has an expression pattern that includes a concentration of staining at the anterior end with several small foci (see arrow) of cells around the outside of the inner cell mass (Fig. 3). These data support the hypothesis that the T-box genes have diverged in the poriferan lineage and may perform sub-functionalized their roles in larval development.

To further investigate the potential polarity of expression observed for the *TbxPor* gene, we examined expression in later stage free-swimming larvae. These larvae have a pronounced ciliated posterior pole and can often be seen making connections with the substrate at this end. Interestingly, we see distinct expression of *TbxPor* (Fig. 4a–c) at both the anterior and posterior poles of the larvae. The expression at the posterior pole is most concentrated at the far posterior and less concentrated in more anterior cells as was seen in the earlier larvae (Fig. 3). At the anterior pole, there are distinct cells, mostly along the subepithelial layer that express *TbxPor*. The expression in the subepithelial layer extends around the lateral sides of the larvae as well. We also examined expression for *Tbx4/5* in late free-swimming larvae. Like *TbxPor*, expression is most concentrated at both the posterior highly ciliated pole and also at the anterior pole. Furthermore, expression is also observed around the lateral sides of the larvae in the subepithelial layer. These interesting patterns of expression in free-swimming larvae may suggest some involvement of *TbxPor* and *Tbx4/5* in establishing or maintaining axial polarity in these early metazoans. Additionally, the location of staining along the lateral sides of the larvae is in cells that look quite similar to the "flask" cells observed in the subepithelial layer of *A. queenslandica* larvae. These cells have been shown to express a variety of post-synaptic orthologs and have been suggested to be evolutionary intermediates to neurons (Sakarya et al. 2007). We are currently developing gain and loss of function methods in our lab that will help us test whether or not either of these *Tbx* genes play roles in larval axis formation, settlement, metamorphosis, or other aspects of development. Given the conserved roles that some T-box family members have played over the course of evolution, investigating the roles of poriferan T-box genes during early development and during metamorphosis or formation of the adult body plan is particularly relevant.

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