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RESEARCH ARTICLE

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Gene activation of metazoan Fox transcription factors at the onset of metamorphosis in the marine demosponge *Amphimedon queenslandica*

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Abstract

Transcription factors encoded by the *Forkhead* (*Fox*) gene family have diverse, sometimes conserved, regulatory roles in eumetazoan development, immunity, and physiology. Although this gene family includes members that predate the origin of the animal kingdom, the majority of metazoan *Fox* genes evolved after the divergence of animals and choanoflagellates. Here, we characterize the composition, structure, and expression of *Fox* genes in the marine demosponge *Amphimedon queenslandica* to better understand the origin and evolution of this family. The *Fox* gene repertoire in *A. queenslandica* appears to be similar to the ancestral metazoan *Fox* gene family. All 17 *A. queenslandica Fox* genes are differentially expressed during development and in adult cell types. Remarkably, eight of these, all of which appear to be metazoan-specific, are induced within just 1 h of larval settlement and commencement of metamorphosis. Gene co-expression analyses suggest that these eight *Fox* genes regulate developmental and physiological processes similar to their roles in other animals. These findings are consistent with *Fox* genes playing deeply ancestral roles in animal development and physiology, including in response to changes in the external environment.

KEYWORDS
biphasic life cycle, gene regulation, larval settlement, pelagobenthic life cycle, Porifera

1 | INTRODUCTION

The Forkhead box (Fox) family of transcription factors appears to have evolved early in eukaryote evolution before the divergence of the two major unikont lineages, the amoebozoans and the opisthokonts (fungi, unicellular holozoans and animals) (Sebé-Pedrós et al., 2011). All *Fox* genes encode a Forkhead domain, which is a sequence-specific domain of approximately 100 amino acids that forms a winged helix-turn-helix DNA-binding structure (Carlsson & Mahlapuu, 2002; Lam et al., 2013; Weigel & Jäckle, 1990). As members of the winged helix superfamily of transcriptional regulators that are common in both bacteria and eukaryotes (Kaestner et al., 2000), *Fox* genes likely evolved from an ancestral gene that encoded a winged helix-containing domain.

The number of *Fox* genes in the genomes of eumetazoans (cnidarians, insects, vertebrates, and other bilaterians) ranges from just under 20 to over 40. The eumetazoan *Fox* genes have been classified into 25–26 subfamilies that form two clades (Kaestner et al., 2000; Kaufmann & Knöchel, 1996; Larroux et al., 2008; Pascual-Carreras

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et al., 2021; Schomburg et al., 2022; Shimeld, Boyle, et al., 2010a; Shimeld, Degnan, & Luke, 2010b). The more ancestral clade (Clade II) is comprised of both (1) subfamilies shared with non-metazoans (*FoxJ1*, *FoxJ2/3*, *FoxM*, *FoxN1/4*) and (2) metazoan-specific subfamilies (*FoxN2/3*, *FoxO*, *FoxP*, *FoxK*). In contrast, Clade I is comprised solely of metazoan genes, some that appear to be ancestral (*FoxD*, *FoxG*, *FoxL1*, and *FoxL2*) and others (*FoxA*, *FoxB*, *FoxC*, *FoxE*, *FoxQ1*, *FoxQ2*, and *FoxF*) that appear to have evolved in eumetazoans after their divergence from sponges some 700 million years ago (Erwin, 2020; Larroux et al., 2008; Sebé-Pedrós et al., 2011; Shimeld, Degnan, & Luke, 2010b).

As is the case with other metazoan transcription factor families, the specific functions of *Fox* genes have been elucidated largely from studies in *Drosophila* and vertebrate models (reviewed in Carlsson & Mahlapuu, 2002; Golson & Kaestner, 2016). Nonetheless, spatial and temporal expression in a range of eumetazoans are consistent with *Fox* genes regulating a wide range of developmental – including embryonic development, specification, and maintenance of cell state, cell proliferation, and differentiation – and metabolic processes (Carlsson & Mahlapuu, 2002; Fritzenwanker et al., 2014; Golson & Kaestner, 2016; Hannenhalli & Kaestner, 2009; Leclère et al., 2019; Magie et al., 2005; Pascual-Carreras et al., 2021; Seudre et al., 2022; Tu et al., 2006). In some cases, specific *Fox* gene subfamilies appear to have a widely conserved function, such as FoxO that appears to regulate life spans from yeast to mammals (Finlay et al., 2019; Greer et al., 2007; Jiang et al., 2019). In mammals, a range of developmental defects, metabolic and immune disorders, and diseases, such as cancer, are associated with mutations in *Fox* genes (Bach et al., 2018; Coffer & Burgering, 2004; Golson & Kaestner, 2016; Gong et al., 2020; Moparthi & Koch, 2020).

Here, we first assess the *Fox* gene complement in a new assembly of the genome of the haplosclerid demosponge *Amphimedon queenslandica* (Fernandez-Valverde et al., 2015; Srivastava et al., 2010; Xiang, 2021) and in other poriferan genomes (Francis et al., 2017; Kenny et al., 2020; Leininger et al., 2014) to determine the ancestral complement of *Fox* genes in sponges and in animals more generally. With the aim of better understanding the origin of *Fox* gene function in the animal kingdom, we then analyze expression profiles of *Fox* genes through the *A. queenslandica* pelagobenthic life cycle. In this conserved biphasic life cycle, embryogenesis leads to the formation of a ciliated swimming planktonic larva that, when competent, settles onto the benthos after contacting an inductive environmental signal (Degnan & Degnan, 2010). In the case of *A. queenslandica*, this signal can be a cue associated with coralline algae (Nakanishi et al., 2014; Say & Degnan, 2020). The initiation of metamorphosis into a feeding juvenile happens in concert with larval settlement. In *A. queenslandica*, the sessile, feeding juvenile forms 3–4 days after settlement, and grows and matures over a period of months into a reproductive adult (Degnan et al., 2015).

2 | MATERIALS AND METHODS

2.1 | Phylogenetic analysis of *Fox* genes

The origin and evolution of *Fox* genes was explored by phylogenetic analysis using *Fox* genes identified in publicly available genomes. First, we identified Forkhead domain-containing genes in the updated genome assembly of *A. queenslandica* with gene models version Aqu3.1 (Xiang, 2021). Second, we identified *Fox* genes in the genomes of three unicellular holozoans and 10 metazoans, including five other sponge genomes (Table S1), using an iterative process that included: (1) downloading the 46 human Fox protein-coding sequences from UniProt (https://www.uniprot.org/uniprot/?query=Forkhead&sort=score) to use as the local Fox database; (2) undertaking a bidirectional Blast to compare the human Fox protein database with one candidate query genome using the software TBtools with default settings (e-value: 1e−5, number of hits: 500) (Chen et al., 2020) and then saving all potential Fox sequences; and (3) screening predicted Fox proteins using NCBI-Batch-CD to identify the positions of conserved Forkhead domains and removing those sequences without a complete Forkhead domain. Although some of the Forkhead domain sequences retrieved from *Monosiga brevicollis*, *Salpingoeca rosetta*, *Nematostella vectensis*, and *Branchiostoma floridae* have been reported previously (Nakagawa et al., 2013), for consistency we used the above outlined search strategy to identify *Fox* coding sequences in all of the target species.

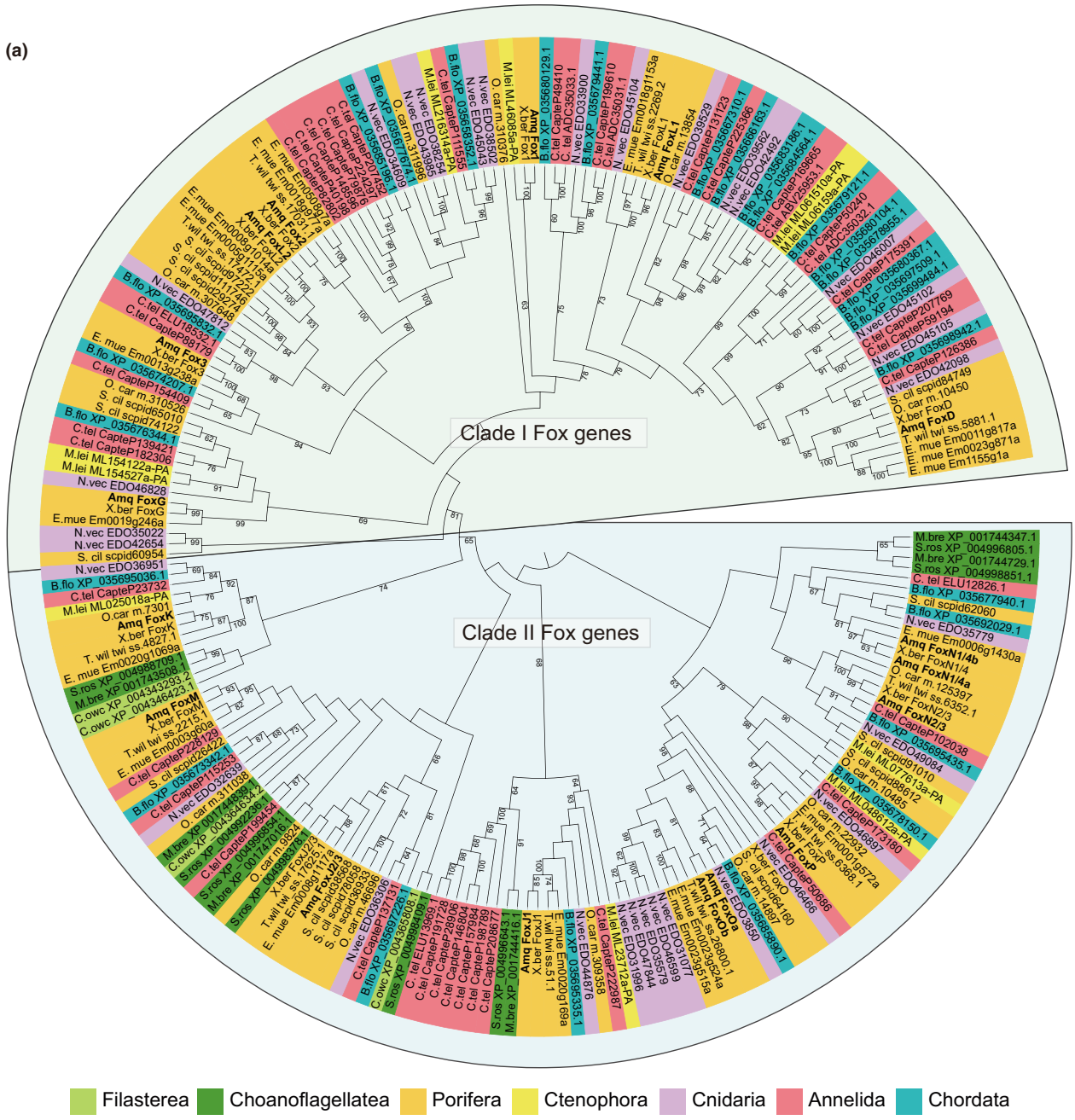
Amino acid sequences corresponding to the Forkhead domain were aligned using MAFFT with default settings (Katoh & Standley, 2013), and the alignment was manually improved using AliView (Larsson, 2014). Based on this alignment (Supplementary Information), the best-fit evolutionary model was identified using IQ-TREE2 with the ModelFinder function (Minh et al., 2020). A maximum likelihood phylogenetic tree was generated in IQ-TREE2, with ultrafast bootstrap, based on 1000 bootstrap replicates (-B 1000, --bnni, -T AUTO). The final tree was presented with mid-point rooting and annotated for visualization using the online tool iTOL (https://itol.embl.de/).

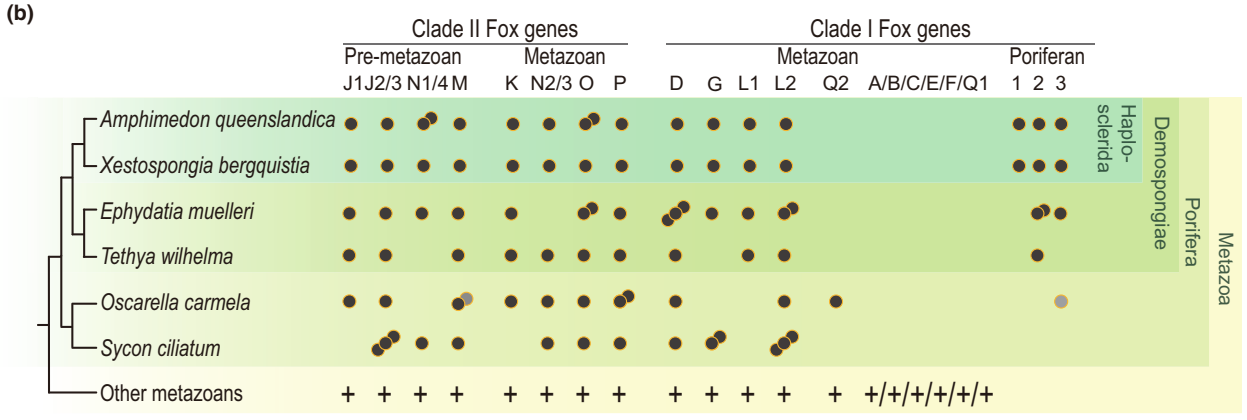
2.2 | Analysis of *Fox* gene expression in *A. queenslandica*

To gain an overview of *Fox* gene expression in *A. queenslandica*, we analyzed two existing transcriptome datasets. The first dataset is comprised of 82 CEL-Seq2 transcriptomes encompassing 17 biologically replicated developmental stages spanning the life cycle of the sponge (Anavy et al., 2014; Gaiti et al., 2015; Levin et al., 2016) (data available from NCBI under accession number PRJNA258388). The second dataset is comprised of 31 CEL-Seq2 transcriptomes generated from three *A. queenslandica* adult cell types (Sogabe et al., 2019) (data available from NCBI under accession number PRJNA412708). For each of the analyses, collapsed raw expression counts were transformed into variance stabilizing-transformed (VST) counts using the Bioconductor R package DESeq2 (Love et al., 2014), and expression heatmaps were generated using the R package pheatmap v1.0.12 (Kolde, 2012).

2.3 | Characterization of genes that are co-expressed with *Fox* genes during metamorphosis

Co-expression networks based on *Fox* gene expression during *A. queenslandica* development were constructed by conducting a


(a)
Clade I Fox genes
Clade II Fox genes
Filasterea
Choanoflagellatea
Porifera
Ctenophora
Cnidaria
Annelida
Chordata


(b)
Clade II Fox genes
Clade I Fox genes
Pre-metazoan
Metazoan
Metazoan
Poriferan
J1J2/3 N1/4 M K N2/3 O P D G L1 L2 Q2 A/B/C/E/F/Q1 1 2 3
Amphimedon queenslandica
Xestospongia bergquistia
Ephydatia muelleri
Tethya wilhelma
Oscarella carmela
Sycon ciliatum
Other metazoans
+ + + + + + + + + + + + + + +/+/+/+/+/+
Haplo-sclerida
Demospongiae
Porifera
Metazoa

weighted correlation network analysis (WGCNA version 1.61) (Langfelder & Horvath, 2008) on the VST-transformed developmental dataset using R (version 3.4.3). The "dynamic topological overlap matrix" method was used to identify modules with a minimum size of 30 genes, and a merging distance threshold of .1 was used to ensure that all co-expression modules were retained. Modules containing one or more *Fox* genes were further analyzed using Cytoscape (version 3.6.0) to identify genes with a direct network connection to a *Fox* gene. Co-expression networks were visualized using Cytoscape (Shannon et al., 2003) and Gephi (version 0.9.2) (Mathieu et al., 2009).

Putative functions of genes co-expressed with one or more *Fox* genes were explored by Gene Ontology (GO) annotation and enrichment analysis. The enrichment analysis was performed using the Bioconductor R package clusterProfiler based on a custom annotation for all *A. queenslandica* genome genes (script: https://github.com/hfyuanuq/thesis.scripts.git) and an FDR-adjusted *P*-value cut-off of .05 (Yu et al., 2012). The online tool REVIGO was used to summarize and remove redundant GO terms (http://revigo.irb.hr/Results.aspx?jobid=449024484) with the SimRel measure method and redundancy > .7 (Supek et al., 2011). Based on the script generated by REVIGO, the final treemaps were plotted in R.

3 | RESULTS

3.1 | *Fox* gene evolution

Phylogenetic analysis of Forkhead domain sequences from 13 holozoans (Table S1) – one filasterean, two choanoflagellates, six poriferans, one ctenophore, one cnidarian, one annelid, and one chordate – is consistent with previous classifications of this family into two clades and 25–26 subfamilies (Figure 1) (Kaestner et al., 2000; Kaufmann & Knöchel, 1996; Larroux et al., 2008; Pascual-Carreras et al., 2021; Schomburg et al., 2022; Shimeld, Boyle, et al., 2010a; Shimeld, Degnan, & Luke, 2010b). Clade I *Fox* genes are nested within Clade II. Based on this phylogenetic analysis, we also find that all non-metazoans have only Clade II genes, while all metazoans have genes from both clades. Clade II *Fox* genes are also distinguished from Clade I genes by the presence of one or more introns in the Forkhead domain (Figure 2).

In most cases, our phylogenetic analysis supports the classification of genes into particular Fox subfamilies and allows for the assignment of sponge genes into these subfamilies. However, we note that some previously identified Fox subfamilies are not well supported in our analysis. This is similar to previous studies that have generated phylogenetic trees with varying topologies (e.g., Kaestner et al., 2000; Kaufmann & Knöchel, 1996; Larroux et al., 2008; Pascual-Carreras et al., 2021; Schomburg et al., 2022; Shimeld, Boyle, et al., 2010a). For instance, our phylogeny supports *FoxN1/4* and *FoxN2/3* diverging from an ancestral holozoan *FoxN* gene, in contrast to previous analyses that support *FoxN1/4* being an ancestral premetazoan gene and *FoxN2/3* evolving after metazoans diverged from choanoflagellates (Larroux et al., 2008; Shimeld, Degnan, & Luke, 2010b). Likewise, although *Fox1* and *Fox3* have previously been assigned as sponge-specific genes, we find that they are nested amongst other metazoan *Fox* genes in our study.

Our analysis identified 17 Forkhead domain-containing genes in the *A. queenslandica* version Aqu3.1 gene models, 16 of which have previously been reported (Larroux et al., 2008). We identified here for the first time a *FoxM* gene in *A. queenslandica*, *AmqFoxM*. Orthologs of *AmqFoxM* are present in four out of the five other sponges surveyed (Figure 1a,b), and the FoxM subfamily appears to have originated before the divergence of animals and choanoflagellates (Sebé-Pedrós et al., 2011). The *A. queenslandica Fox* genes can be broadly classified based on their clade and time of origin: (1) Clade II genes that have a premetazoan origin (*AmqFoxJ1*, *AmqFoxJ2/3*, *AmqFoxN1/4* and *AmqFoxM*); (2) Clade II genes that originated after animals and choanoflagellates diverged (*AmqFoxK*, *AmqFoxN2/3*, *AmqFoxO*, and *AmqFoxP*, although we note in our tree that metazoan *Fox* genes are sister to a clade of non-metazoan holozoan *Fox* genes that may indeed be *FoxK* orthologs; Figure 1a); (3) Clade I genes that also originated after animals and choanoflagellates diverged (*AmqFoxD*, *AmqFoxG*, *AmqFoxL1*, and *AmqFoxL2*); and (4) Clade I genes that appear to have evolved sometime after sponges diverged from other animals (*AmqFox1*, *AmqFox2*, and *AmqFox3*).

In addition to the three potential sponge-specific genes, *AmqFox1*, *AmqFox2*, and *AmqFox3*, *A. queenslandica* has two sets of *Fox* paralogs, *FoxO* (*AmqFoxOa* and *AmqFoxOb*) and *FoxN1/4* (*AmqFoxN1/4a* and *AmqFoxN1/4b*). Analysis of the genome of another haplosclerid demosponge, *Xestospongia bergquistia* (unpublished, data available upon request), revealed that this closely related sponge has the same conserved Fox subfamilies as *A. queenslandica* (Figure 1a,b). It also has orthologs of *AmqFox1*, *AmqFox2*, and *AmqFox3*, indicating that all these genes were present in the last common ancestor of

FIGURE 1 Analysis of holozoan and sponge *Fox* genes. (a) A midpoint-rooted maximum-likelihood (ML) phylogenetic tree of 222 Forkhead domains from 13 holozoan taxa (Table S1) – one filasterean, two choanoflagellates, six poriferans, one ctenophore, one cnidarian, one annelid, and one chordate – revealing relationships of clade I and clade II *Fox* genes. C.owc: *Capsaspora owczarzaki*; M.bre: *Monosiga brevicollis*; S.ros: *Salpingoeca rosetta*; Amq: *Amphimedon queenslandica*; X.ber: *Xestospongia bergquistia*; E.mue: *Ephydatia muelleri*; T.wil: *Tethya wilhelma*; S.cil: *Sycon ciliatum*; O.car: *Oscarella carmela*; M.lei: *Mnemiopsis leidyi*; N.vec: *Nematostella vectensis*; C.tel: *Capitella teleta*; B.flo: *Branchiostoma floridae*. Gene naming follows previous publications, except for *X. bergquistia*, which follows *A. queenslandica* naming, and numbers on branches indicate bootstrap values. (b) *Fox* genes present in sponge genomes. Indication of relationships between the six sponge species is to the left. Black dots indicate that a member of a given *Fox* subfamily is present. Multiple clustered dots indicate the number of paralogs detected in the genome. Gray dots indicate members with weak affinity to a particular subfamily. Plus signs along the bottom row indicate subfamilies present in bilaterians, cnidarians, and/or ctenophores.

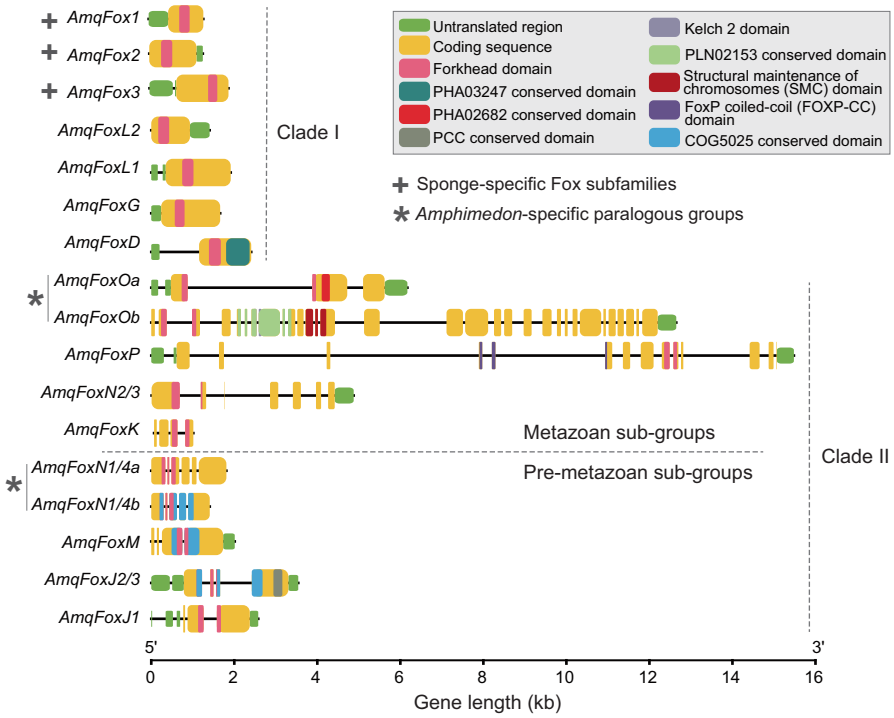


FIGURE 2 The structure of *Amphimedon queenslandica Fox* genes. Seven clade I *Fox* genes do not have an intron in the Forkhead domain, while all 10 clade II *Fox* genes do have an intron. Although they likely exist for all genes, not all models have both 5′ and 3′ untranslated regions (UTRs). Open reading frames/coding sequences are shown as large mustard boxes; UTRs are shown as thinner green boxes; introns are shown as thin lines. Protein domains, including the Forkhead domain, are shown in the coding sequence (see legend box in figure for domain details).

these two demosponges. In contrast, we detected only one copy of *FoxO* and *FoxN1/4* in *X. bergquistia*, suggesting that these two genes duplicated after the *Amphimedon* and *Xestospongia* genera diverged from each other.

We also analyzed the *Fox* gene complement of two more distantly related demosponges, *Ephydatia muelleri* and *Tethya wilhelma*, and of the even further distantly related *Sycon ciliatum* (calcareous sponge) and *Oscarella carmela* (homoscleromorph) (Francis et al., 2017; Kenny et al., 2020; Leininger et al., 2014). This revealed that metazoan *Fox* subfamilies are highly conserved across the phylum Porifera, with a few lineage-specific losses and expansions (Figure 1b). *Fox* genes reported previously as unique to *A. queenslandica* – *AmqFox1*, *AmqFox2*, and *AmqFox3* (Larroux et al., 2008) – appear to have different evolutionary histories, with *AmqFox1* having an ortholog in *X. bergquistia*, but clustering with a closely related ctenophore sequence in our tree (Figure 1a). *AmqFox2* has orthologs only in demosponges, although this demosponge clade is sister to a clade comprised of other animal *Fox* genes, including an *O. carmela* gene (*O. car m.310376*) (Figure 1a). *AmqFox3* has orthologs in *X. bergquistia*, *E. muelleri*, and *O. carmela* (Figure 1b), but this Fox3 clade also includes two bilaterian *Fox* genes (Figure 1a). We also note that two *O. carmela* genes (*O.car m.13854* and *O.car m.310376*) and one *S. ciliatum* gene (*S.cil scpid60954*) do not group with any *A. queenslandica* subfamily clade with strong support (Figure 1a). Although we have assigned these *Fox* genes to sponge-specific subfamilies (Figure 1b), more detailed phylogenetic analyses are needed to determine if these genuinely are sponge-specific *Fox* genes or members of larger metazoan subfamilies.

Analysis of the exon–intron structure and encoded domain architecture of the revised *A. queenslandica* (Aqu3.1 version) *Fox* genes confirms the original Aqu1 gene models (Larroux et al., 2008; Srivastava et al., 2010). As previously noted, all *A. queenslandica* Clade I *Fox* genes (*AmqFoxD*, *AmqFoxG*, *AmqFoxL1*, *AmqFoxL2*, *AmqFox1*, *AmqFox2*, and *AmqFox3*) lack introns in the coding sequence, as observed in other metazoan Clade I genes (Figure 2). In contrast, *A. queenslandica* Clade II *Fox* genes all have multiple introns in the coding sequence (Figure 2), including between positions 48 and 49 of the Forkhead domain in most genes. *AmqFoxOb*, which appears to be a unique *A. queenslandica* gene, has a unique domain architecture that includes Structural Maintenance of Chromosome (SMC) and PLN02153 (which includes a Kelch2 motif) domains. These domains may be involved in mediating interactions with other proteins, including chromatin (Laflamme et al., 2014). The duplicated *FoxO* gene in *E. muelleri* (Figure 1a,b; Table S1) does not possess these domains and thus most likely evolved independently.

3.2 | Developmental expression of *Fox* genes

Using CEL-Seq2 transcriptomes from 82 individual *A. queenslandica* embryos, larvae, postlarvae, juveniles, and adult biopsies comprising 16 developmental stages and adults (Gaiti et al., 2015; Levin et al., 2016; Wong et al., 2020), we assessed the expression profiles of the 17 *Fox* genes (Figure 3; Table S2). Although CEL-Seq2 transcriptomes from embryonic and larval stages have been previously studied in detail (Levin et al., 2016), postlarval and juvenile transcriptomes have not.

There is relatively little *Fox* gene expression during embryogenesis, with *AmqFoxN1/4a*, *AmqFoxN1/4b*, and *AmqFox2* expressed only in the earliest stages of embryogenesis and *AmqFoxJ1*, *AmqFoxJ2/3*,

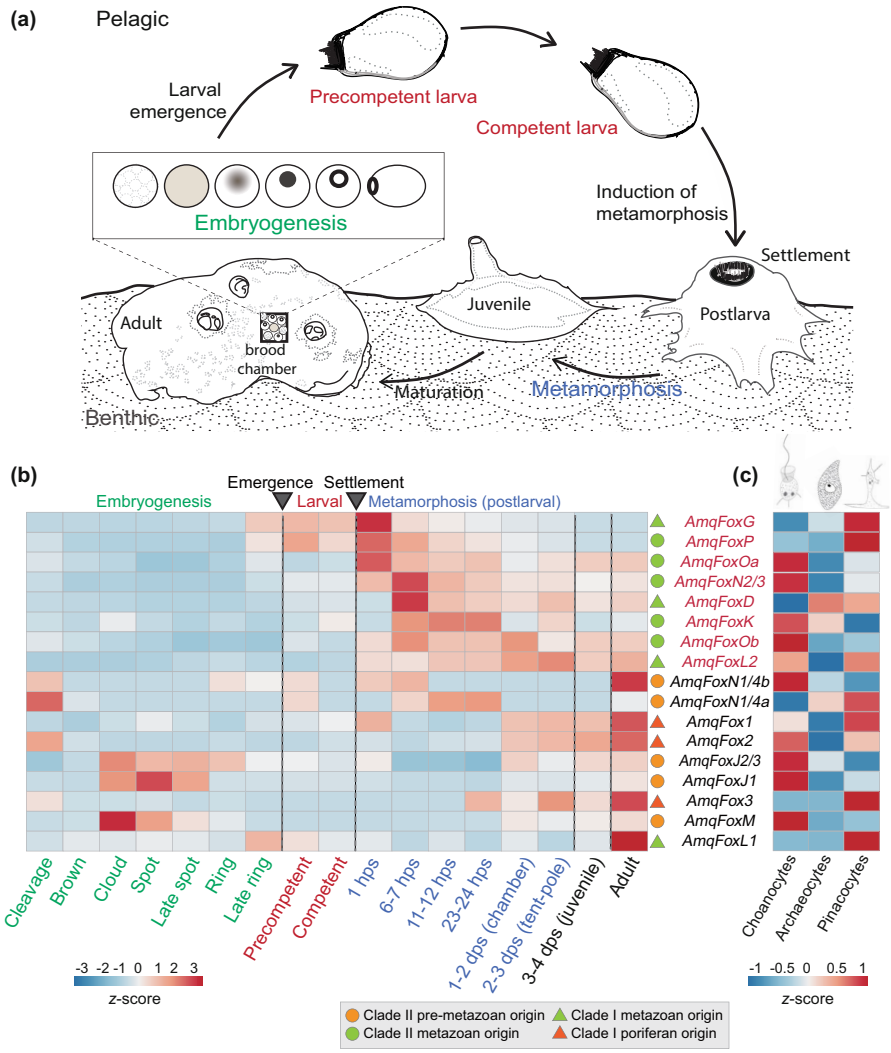


FIGURE 3 *Fox* gene expression through the *Amphimedon queenslandica* life cycle. (a) Diagram of the pelagobenthic life cycle of *A. queenslandica*. Embryos develop in a brood chamber within the adult (stages depicted in the box follow a developmental progression: cleavage, brown cloud, spot, late spot, ring, late ring). Precompetent larvae emerge from the adult and typically swim in the water column for 4–6 h before developing competence to settle and initiate metamorphosis. Competent larvae are induced to settle onto the benthos by signals associated with particular species of coralline algae. Settlement is followed by rapid morphogenetic changes over the first 24 h that include cell differentiation, transdifferentation, proliferation and migration, and apoptosis. Choanocyte chambers appear in postlarvae at 1–2 dps (chamber stage). Postlarvae then expand above the benthic surface at 2–3 dps (tentpole stage) and form a functional aquiferous system that enables filter-feeding at 3–4 dps (juvenile stage) (Degnan et al., 2015). (b) Heatmap of relative expression levels of *Fox* genes through embryogenesis (green text), larval development (red), metamorphosis (blue), and in the juvenile and adult (black text; separated by dashed lines). Times at which larvae emerge from the adult and settle onto the benthos are marked by arrowheads and vertical dashed lines. Stages of embryogenesis, larval development, and metamorphosis are shown below the heatmap. hps, hours postsettlement; dps, days postsettlement. *Fox* genes induced at metamorphosis are shown in red text. The clade and evolutionary origin of the *Fox* genes are marked with a symbol (see legend box in figure). Heatmap illustrates scaled (z-score) expression levels based on collapsed variance stabilizing-transformed (VST) from seven transcriptomes from each embryonic stage (except late spot, which has six transcriptomes); five precompetent and six competent larval transcriptomes; and three transcriptomes from each postlarval, juvenile, and adult stage (Table S2; Levin et al., 2016; Wong et al., 2020). (c) Heatmap of relative expression levels of *Fox* genes in the three primary cell types in adults: choanocytes, archaeocytes, and pinacocytes. Heatmap illustrates scaled (z-score) expression levels based on collapsed VST from 10 choanocyte, 15 archaeocyte, and six pinacocyte transcriptomes (Table S2; Sogabe et al., 2019).

and *AmqFoxM* expressed from the cloud through to the ring stage, when the embryo is establishing an anterior–posterior axis and first forming internal and external layers (Figure 3a). In contrast, a diverse suite of *Fox* genes is upregulated when larvae settle on a coralline alga and start metamorphosis. The majority of these *Fox* genes appear to be metazoan-specific (Figure 3a). One group of three genes – *AmqFoxG*, *AmqFoxP*, and *AmqFoxOa* – is highly activated within just 1 h of settling and initiating metamorphosis (1 h postsettlement [hps]).

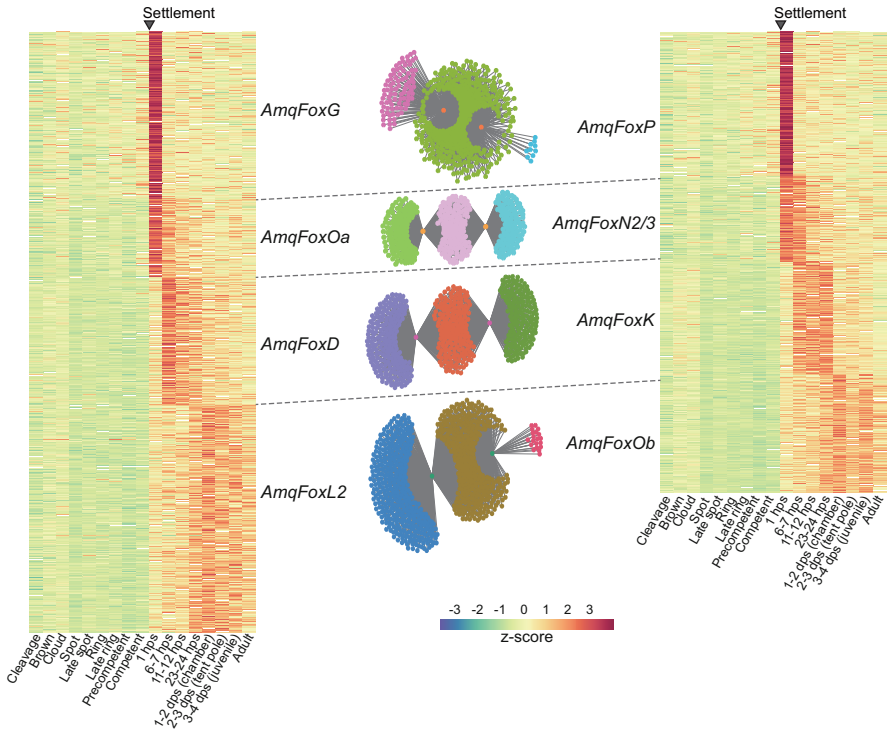


FIGURE 4 Developmental expression profiles of genes co-expressed with *Fox* genes activated at metamorphosis. Left and right heatmaps show scaled (z-score) developmental expression levels of co-expressed genes with the listed *Fox* genes (Table S3). Larval settlement is marked by triangle; see Figure 3 for details on other developmental stages. The four pairs of co-expressed *Fox* genes and their co-expressed genes are separated by dashed lines. The central panel shows overlapping co-expression networks of the *Fox* genes listed to the left and right. The eight *AmqFox* genes are shown in red in the networks. In the network of *AmqFoxG* and *AmqFoxP*, the co-expressed genes that overlap between the two *Fox* genes in the pair are labeled green, while *AmqFoxG*- and *AmqFoxP*-specific genes are pink and blue, respectively. The other three pairs of *Fox* gene networks follow the same rules using different color schemes.

A second group of four genes – *AmqFoxOb*, *AmqFoxN2/3*, *AmqFoxD*, and *AmqFoxK* – has highest relative expression at 6–7 hps. Distinct from all others, *AmqFoxL2* has high expression from settlement through to the adult stage.

AmqFoxN1/4b, *AmqFoxJ1*, *AmqFox1*, *AmqFox2*, *AmqFox3*, and *AmqFoxL1* have their highest relative expression in the adult stage. Comparison of relative expression levels of the *Fox* genes in three adult cell types – (1) epithelial pinacocytes that line external surface and internal canals, (2) choanocytes that are internal epithelial feeding cells, and (3) pluripotent archaeocytes that reside in the middle of the sponge (Sogabe et al., 2019) – reveals that all, except *AmqFoxD*, are most highly expressed in epithelial pinacocytes and/or choanocytes (Figure 3b; Table S2). Both of these cell types interact with sea water either on the outside of the sponge (pinacocytes) or when the seawater is pumped through the internal aquiferous system (pinacocytes and choanocytes).

3.3 | Co-activation of *Fox* and other genes at the initiation of metamorphosis

Four pairs of *Fox* genes – (1) *AmqFoxP* and *AmqFoxG*, (2) *AmqFoxOa* and *AmqFoxN2/3*, (3) *AmqFoxD* and *AmqFoxK*, and (4) *AmqFoxL2* and *AmqFoxOb* – are all tightly co-expressed with each other through metamorphosis (Figures 3 and 4). Although all eight are upregulated within 1 h of the larva settling, the transcript levels of *AmqFoxP*, *AmqFoxG*, *AmqFoxOa*, and *AmqFoxN2/3* increase much more markedly. At this early stage of metamorphosis, the anterior–posterior axis of the larva is being dissolved, and larval cells have begun to migrate, transdifferentiate, and undergo apoptosis (Nakanishi et al., 2014; Sogabe et al., 2016).

Strikingly, *AmqFoxP* and *AmqFoxG* expression levels drop rapidly by 6–7 hps, while *AmqFoxOa* and *AmqFoxN2/3* levels decrease more slowly over the first 24 h of metamorphosis (Figure 4). *AmqFoxD* and *AmqFoxK* transcript abundances markedly increase further by 6–7 hps and maintain a high level of expression until about 24 hps, before reducing over the next 2–3 days of metamorphosis, until the feeding juvenile forms at 3–4 days postsettlement (dps). Even by the end of the first day of metamorphosis, the first choanocyte chambers are forming. These are comprised of transdifferentiated larval epithelial and newly proliferating cells (Sogabe et al., 2016). *AmqFoxL2* and *AmqFoxO* are also activated at the start of metamorphosis, but their expression increases approximately 1 day after metamorphosis commences and they maintain high levels of expression until the juvenile is formed (Figure 4). During this period there is extensive cell proliferation and differentiation, and patterning of the juvenile/adult body plan (Degnan et al., 2015; Nakanishi et al., 2014; Sogabe et al., 2016).

Using a WGCNA co-expression analysis with a connection weight value over .1, we identified genes with expression profiles matching these eight *Fox* genes (Figure 4; Table S3). This analysis revealed that *AmqFoxG* and *AmqFoxP* are co-expressed with each other and that 566 and 489 other genes also are transiently activated at 1 hps and co-expressed with these *Fox* genes, respectively. Of these, 478 genes (82.8%) are part of the co-expression networks of both *Fox* genes (Figure 4). GO enrichment analysis of the co-expressed genes indicates that they are involved in calcium transport, GTPase signaling, and the degradation of glycine (Figure 5; Table S4).

Similarly, we found that *AmqFoxOa* and *AmqFoxN2/3* are co-expressed with each other, and with 268 and 291 genes, respectively, that are also activated by 1 hps and remain more highly expressed over the first 24 h of metamorphosis; 151 (37.0%) of these are shared

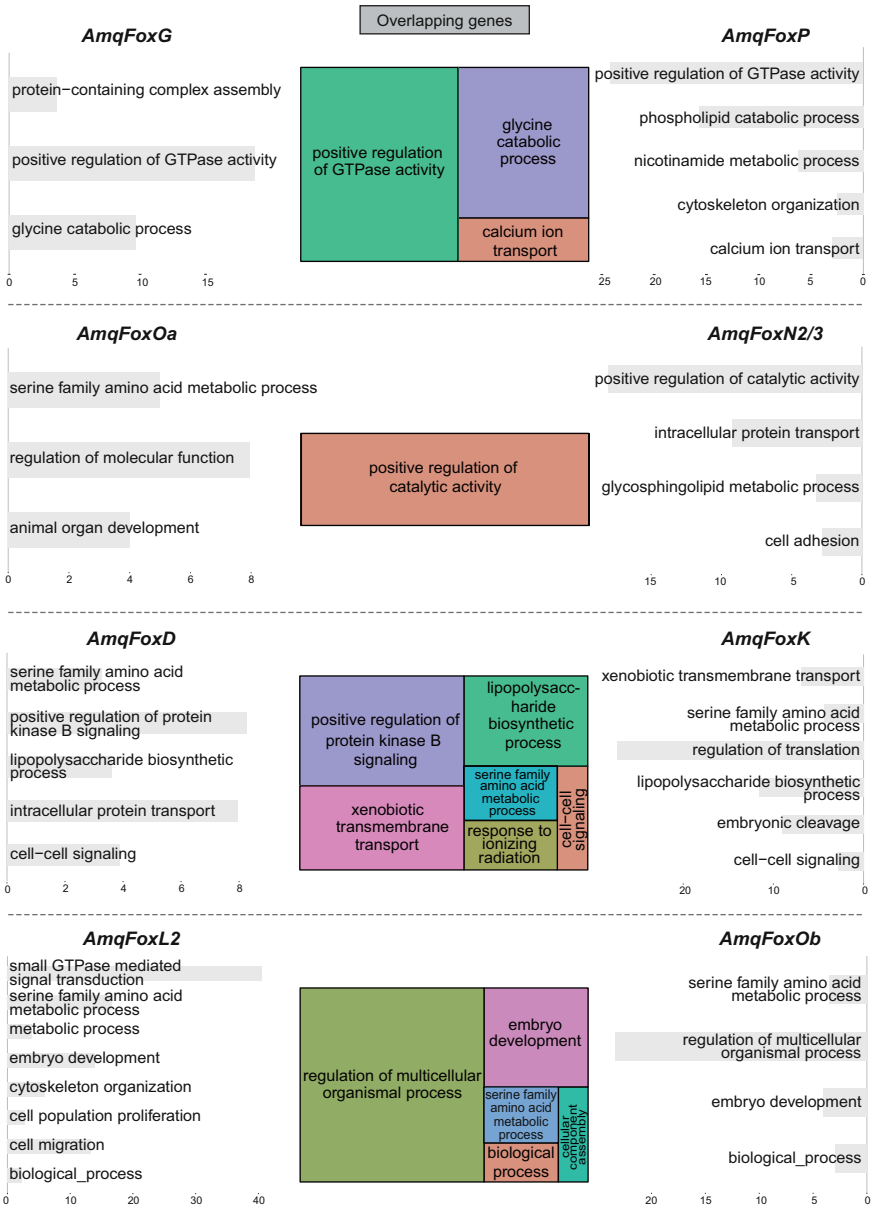


FIGURE 5 GO enrichments of co-expressed genes. Left and right bar plots show GO enrichment results of co-expressed gene suites with each *Fox* gene; the scale indicates the adjusted *P*-value ($-\log_{10}$(*P*-value)) of the enriched GO term relative to the whole *Amphimedon queenslandica* genome (Table S4). The central panel shows GO enrichments that overlap between the co-expression gene suites, with rectangles being the clustered representative GO items. Size of the rectangles represents adjusted *P*-value and colors of rectangles are random.

between *AmqFoxOa* and *AmqFoxN2/3* networks (Figure 4; Table S3). These co-expression networks are enriched in genes involved in metabolic activities, including serine and glycosphingolipid metabolism (Figure 5), and genes involved in morphogenesis, including animal organ development and cell adhesion (Table S4).

AmqFoxD and *AmqFoxK* are activated together at 6–7 hps and remain relatively highly expressed until about 24 hps; they are co-expressed with 434 and 383 other genes, respectively, 209 (34.4%) of which are shared (Figure 4; Table S3). GO terms enriched in these co-expressed genes are again consistent with metabolic activities (e.g., serine metabolism, lipopolysaccharide biosynthesis, and xenobiotic transport) and developmental processes (e.g., intercellular and protein kinase B signaling) (Figure 5; Table S4).

AmqFoxOb and *AmqFoxL2* are co-expressed and activated early in metamorphosis, but become more highly expressed during later metamorphosis. We found a larger number of genes co-expressed with this pair compared to earlier in metamorphosis, with 402 and 769 co-expressed genes, respectively (Figure 4; Table S3). Of these, 381 (48.5%) genes are co-expressed with both *Fox* genes, with *AmqFoxL2* having only 21 genes unique to its network (Figure 4). Although metabolic processes are still enriched amongst this set of co-expressed genes, GO analysis reveals a larger number of terms related to the regulation of development, including regulation of multicellular processes, embryo development, and cell proliferation, migration, and signaling (Figure 5; Table S4).

4 | DISCUSSION

The *A. queenslandica Fox* gene family is comprised of some genes that arose before the divergence of animal and choanoflagellate lineages and others that arose after (Larroux et al., 2008; Shimeld, Degnan, &

Luke, 2010b; Sebé-Pedrós et al., 2011; this study). Using a revised assembly of the *A. queenslandica* genome (Xiang, 2021), we have identified a new gene in the *FoxM* subfamily. This observation, along with the detection of *FoxM* in other sponge genomes, lends support to this being an ancestral Clade II group with perhaps a premetazoan origin (Figure 1; Sebé-Pedrós et al., 2011). Comparison of the *A. queenslandica Fox* genes to those in other animals and sponges suggests that the *A. queenslandica* gene repertoire is identical to the ancestral metazoan condition, with one possible exception. A member of the *FoxQ2* subfamily appears to be present in the *O. carmela* genome (*O.car m.311996*); the ctenophore *Mnemiopsis leidyi* also appears to have a *FoxQ2* gene (*M.lei ML216314a-PA*). Thus, we infer the last common ancestor to contemporary animals had 12, possibly 13, *Fox* genes, comprised of four premetazoan Clade II genes (*FoxJ1*, *FoxJ2/3*, *FoxN1/4*, and *FoxM*), four metazoan Clade II genes (*FoxK*, *FoxN2/3*, *FoxP*, and *FoxO*), and four or five metazoan Clade I genes (*FoxD*, *FoxG*, *FoxL1*, and *FoxL2*, and possibly *FoxQ2*).

Comparison between different poriferan species suggests that the *Fox* gene family is highly conserved across the phylum, with little gene loss or gain. *Fox* genes previously reported to be *A. queenslandica*-specific (*AmqFox1*, *AmqFox2*, and *AmqFox3*; Larroux et al., 2008) now appear to have arisen earlier in poriferan evolution. *AmqFox1*, *AmqFox2*, and *AmqFox3* appear to be restricted to haplosclerid demosponges, demosponges, and poriferans, respectively. In contrast, *AmqFoxOa* and *AmqFoxOb*, and *AmqFoxN1/4a* and *AmqFoxN1/4b* paralogs appear to be unique to *A. queenslandica*, arising after the divergence of the *Xestospongia* and *Amphimedon* lineages. Although both *AmqFoxN1/4* paralogs are similar to the ancestral *FoxN1/4*, the domain architecture of *AmqFoxOb* is markedly different from those of *AmqFoxOa* and the ancestral *FoxO*, providing an opportunity to investigate how lineage-specific duplication and diversification affect sponge gene evolution and function. With the exception of these lineage-specific *Fox* genes, it appears the repertoire of *Fox* genes present in *A. queenslandica* was also present in the last common ancestor of sponges and other animals.

4.1 | *A. queenslandica Fox* genes are developmentally expressed

Given that *A. queenslandica* and the predicted ancestral metazoan *Fox* gene complements appear to be similar, studying *Fox* expression in this sponge can potentially shed light on the original roles of this transcription factor family in the last common animal ancestor. For instance, bilaterian and cnidarian *FoxO* genes are developmentally expressed and involved in immunity, metabolism, and longevity (Arden, 2008; Boehm et al., 2012; Bosch, 2014; Carlsson & Mahlapuu, 2002; Hedrick et al., 2012; Martins et al., 2016; Tzivion et al., 2011), but it is unclear which of these are ancestral functions.

Analysis of the expression of *Fox* genes during *A. queenslandica* embryogenesis and metamorphosis, and in juveniles and adults – including in three cell types – reveals different expression profiles indicative of diverse roles in regulating sponge development and cell identity. A diversity of developmental roles for *A. queenslandica Fox* genes would be consistent with what has been observed in other animals (e.g., Golson & Kaestner, 2016; Leclère et al., 2019; Magie et al., 2005; Seudre et al., 2022; Shimeld, Boyle, et al., 2010a; Tu et al., 2006).

Both *AmqFoxN1/4* paralogs, along with *AmqFox2* and *AmqFox3*, are upregulated in cleaving embryos. This stage is characterized by unequal cleavage that produces micromeres that are specified early and often express a unique suite of transcription factors and components of developmental signaling pathways (Adamska et al., 2010; Degnan et al., 2015; Larroux et al., 2006; Leys & Degnan, 2002; Richards & Degnan, 2012). The early embryonic expression of *Fox* genes suggests they contribute to early cell specification events. Independently evolved *FoxN1/4* paralogs are maternally expressed and present in the cleaving embryo of the annelid *Owenia fusiformis* (Seudre et al., 2022). *FoxN1/4* is also maternally expressed in an echinoderm (sea urchin) (Tu et al., 2006), raising the possibility of maternal expression being a conserved feature in bilaterians, and possibly metazoans.

AmqFoxJ1, *AmqFoxJ2/3*, *and AmqFoxM* are upregulated at the cloud embryonic stage and through mid-embryogenesis. This a major morphogenetic period in the formation of the larval body plan and includes cell differentiation, migration, and patterning; the larval anterior–posterior axis first appears at the cloud stage (Adamska et al., 2007; Degnan et al., 2015). *FoxJ1* appears to play a role in cilia development in a range of eumetazoans (Marlow et al., 2014; Seudre et al., 2022; Yu et al., 2008). Although we have not determined if *AmqFoxJ1* is expressed in progenitors to the ciliated epithelial cells in *A. queenslandica* larva, we did observe cilia formation at this stage of development (Leys & Degnan, 2002). Supporting this inference of a role in cilia development, we found that *AmqFoxJ1* is upregulated in adult choanocytes, which are ciliated (Figure 3).

4.2 | A role for metazoan-specific *Fox* genes at metamorphosis

In contrast to their limited activity during embryogenesis, there is a pronounced upregulation of multiple *Fox* genes at the start of *A. queenslandica* metamorphosis. This sponge, as is the case for a huge diversity of marine invertebrates, has a planktonic larva that, when competent, settles in response to an inductive benthic cue and commences metamorphosis; for *A. queenslandica*, that cue can be a coralline alga (Say & Degnan, 2020; Song et al., 2021; Ueda et al., 2016). *A. queenslandica* develops into a functional feeding juvenile 3–4 days later. Early metamorphosis involves extensive cell differentiation, transdifferentiation and rearrangement, and apoptosis (Degnan et al., 2015; Nakanishi et al., 2014; Sogabe et al., 2016).

Within 1 h of settling, the elongate *A. queenslandica* larval body plan is flattening against the algae, with larval cells beginning to transdifferentiate, migrate, or undergo apoptosis. Eight *Fox* genes – *AmqFoxG*, *AmqFoxP*, *AmqFoxOa*, *AmqFoxN2/3*, *AmqFoxD*, *AmqFoxK*, *AmqFoxL2*, and *AmqFoxOb* – are upregulated at this time, suggesting

that these genes play a critical role in metamorphosis. Interestingly, all eight *Fox* genes appear to have originated along the metazoan stem, and are the most conserved amongst the sponges surveyed in this study. The only subfamily members to be lost (assuming complete genome coverage) are *FoxG* in *T. wilhelma* and *O. carmela*, *FoxN2/3* in *E. muelleri*, and *FoxK* in *S. ciliatum*. The biphasic metazoan life cycle, of which the pelagobenthic is the most widespread and conserved, also likely evolved along this metazoan stem (Degnan & Degnan, 2010).

Half of the eight *Fox* genes – *AmqFoxG*, *AmqFoxP*, *AmqFoxOa*, and *AmqFoxN2/3* – have an immediate and marked increase in expression after the larva settles, consistent with a regulatory role in the initiation of metamorphosis. *AmqFoxG* and *AmqFoxP* expression markedly drops by 6 hps (Figure 4), further supporting a role in initiating this transition. Fox proteins can act as transcriptional pioneers and appear responsible for opening chromatin associated with enhancers and promoters. This has been attributed to the winged helix structure of their Forkhead DNA-binding domain being similar to histones H1 and H5, enabling the displacement of histones and the opening of chromatin (Carlsson & Mahlapuu, 2002; Clark et al., 1993; Zaret et al., 2010). For example, FOXA1 and FoxN affect chromatin structure in humans and *Drosophila*, respectively (Cirillo & Zaret, 1999; Strödicke et al., 2000). Given the dramatic morphogenetic change at the commencement of *A. queenslandica* metamorphosis, and the concomitant large-scale rapid change in gene expression, the upregulated *Fox* genes at the beginning of metamorphosis may play a role in facilitating the rapid chromatin state changes that are likely to precede changes in both gene expression and cell state.

4.3 | *A. queenslandica Fox* genes appear to regulate metabolic and developmental processes

Although no two of the eight *Fox* genes are expressed identically at metamorphosis, we do find four pairs with similar expression profiles. *AmqFoxG* and *AmqFoxP* have the most similar expression profiles, including during late embryogenesis and in the larva. They are the only *Fox* genes with substantial expression in the competent larva, and have the most rapid, dramatic, and transient activation at the start of metamorphosis. This shared expression profile suggest that they may autoregulate their own burst of expression at settlement after being activated by an endogenous signaling pathway that has been induced by a signal associated with the coralline alga. Although there appears to be no evidence for specific roles for *Fox* genes in regulating metamorphosis in other species (largely because it has not been studied), it is relevant that *FoxG* is expressed in spiralian and sea urchin larvae, and *FoxP* is expressed in *O. fusiformis* competent larvae and juveniles (Seudre et al., 2022; Tu et al., 2006).

AmqFoxG and *AmqFoxP* are co-expressed with a battery of 577 genes at the start of metamorphosis, suggesting they contribute to regulation of these genes. The shared target genes appear to be largely transiently upregulated at the start of metamorphosis (Figure 4) and are predicted to be involved predominantly in metabolism, cell signaling, and cytoskeletal organization (Figure 5). FoxP has dual activator and repressor functions in other animals, through the formation of homo- and heterodimers, to regulate metabolism and development (reviewed in Golson & Kaestner, 2016).

AmqFoxOa and *AmqFoxN2/3* are also activated within the first hour of metamorphosis, but maintain a relatively high level of expression for at least the first 24 h. This suggests that these two *Fox* genes, along with *AmqFoxG* and *AmqFoxP*, may be under the same regulatory control at the commencement of metamorphosis. The 408 genes co-expressed with *AmqFoxOa* and *AmqFoxN2/3* are also predominantly involved in metabolic processes and morphogenesis, consistent with the rapid body plan reorganization occurring at this time. FoxO in particular is associated with these cellular processes and with the regulation of stress and longevity and metabolism in other animals, which can be influenced by changes in extracellular signals (Carlsson & Mahlapuu, 2002; Golson & Kaestner, 2016; Kwak et al., 2018; Pascual-Carreras et al., 2021; Tan et al., 1998; Yao et al., 2001). For example, FoxO3, FoxG, and FoxA cooperate to regulate cellular processes and stress responses in the sea urchin *Paracentrotus lividus* (Ruocco et al., 2017).

AmqFoxD and *AmqFoxK*, along with their 608 co-expressed genes, are upregulated a little later in metamorphosis at 6–7, 11–12, and 23–24 hps. These gene batteries again are enriched in genes involved in specific metabolic functions and in a raft of developmental processes. *FoxD* is orally expressed in the postlarvae of cnidarians and has been shown to play a role in larval epithelial–mesenchymal transitions (Fritzenwanker et al., 2004; Leclère et al., 2019; Magie et al., 2005). It is also upregulated in sea urchin larvae (Tu et al., 2006).

AmqFoxL2 and *AmqFoxOb* are upregulated about 1 day after metamorphosis commences and remain activated through the later stages of metamorphosis through to adulthood. AmqFoxOb has a domain architecture that is unique to *A. queenslandica*, suggesting that it plays a different role from AmqFoxOa, which has a domain architecture similar to other FoxO proteins. During this phase of metamorphosis, choanocyte chambers are beginning to form, and choanocytes and archaeocytes are proliferating ("chamber" stage; 24–48 hps). This is followed by the formation of primary spicules, the formation of internal canals, and a general expanding of the body away from the benthic surface ("tent" stage; 48–72 hps), and eventually the formation of a functional aquiferous canal system that allows the sponge to filter feed (juvenile stage; 72–96 hps). Even after developing a functional feeding system, the juvenile body plan remains plastic with continued high levels of cell transdifferentiation, proliferation, and migration (Nakanishi et al., 2014; Sogabe et al., 2016; Sogabe et al., 2019). GO analysis suggests that these two *Fox* genes and their 790 co-expressed genes play important roles in cell differentiation and metabolism.

4.4 | *A. queenslandica Fox* genes are upregulated in choanocytes and pinacocytes

All *A. queenslandica Fox* genes, except *AmqFoxD*, are upregulated in one or both of the two primary epithelial cell types in sponges, choanocytes and pinacocytes. This suggests they may have an ancestral role in the

development or function of this primary metazoan cell type (Belahbib et al., 2018; Fahey & Degnan, 2010; Leys & Riesgo, 2012). *AmqFoxJ1*, *AmqFoxJ2/3*, both *AmqFoxO* and *AmqFoxN* paralogs, *AmqFoxK*, *AmqFoxM*, and *AmqFox2* are upregulated in ciliated choanocytes, which have a primary function in generating water currents through the sponge and capturing microbial food out of these currents; they are one of the major proliferating cell types in *A. queenslandica* (Sogabe et al., 2016; Sogabe et al., 2019). *AmqFoxG*, *AmqFoxP*, *AmqFox3*, and *AmqFoxL1* are upregulated in pinacocytes, which form external and internal epithelial boundaries and express a raft of immunity genes (Sogabe et al., 2019). *AmqFoxL2* is expressed in choanocytes and pinacocytes, similar to that observed for *FoxL* in the demosponge *Suberites domuncula* (Adell & Müller, 2004). Bilaterian orthologs of some of these *Fox* genes regulate epithelial cell behavior involved in immunity and development (Chen et al., 1998; Clevidence et al., 1994; Coffer & Burgering, 2004; Li et al., 2012; Myatt & Lam, 2007; Wang et al., 2009). For example, FoxJ appears to be essential in all organs and structures that contain ciliated epithelial cells (Brody et al., 2000; Chen et al., 1998; Clevidence et al., 1994; Hackett et al., 1995) and FoxN1 plays a role in mammal thymic epithelial cell growth and differentiation (Balciunaite et al., 2002; Coffer & Burgering, 2004).

5 | CONCLUSIONS

Fox genes diversified before animal cladogenesis, giving rise to an important family of transcriptional regulators that further expanded in eumetazoans. The differential expression of *Fox* genes through the *A. queenslandica* life cycle is consistent with the family playing an ancestral role in metazoan development and regulating a range of cellular processes, including differentiation, proliferation, and metabolism. Particularly striking is the rapid and strong activation of eight metazoan-specific *Fox* genes at metamorphosis, a conserved stage of metazoan development that is currently understudied in terms of *Fox* gene expression and function.

AUTHOR CONTRIBUTIONS

HY, SMD, and BMD conceptualized this project and the methodological strategies. HY conducted all bioinformatical analyses. WLH contributed to analysis of gene age. HY prepared the original draft of text and figures, except for Figure 3a, which was prepared by WLH. SMD and BMD made significant contributions to revising the text and figures. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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