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Characterizing the role of Pax and Six in an emerging model system, the freshwater sponge, *E. muelleri*

by

Ian P Winters

Honors Thesis

in

Department of Biology
University of Richmond
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Advisor: April L Hill

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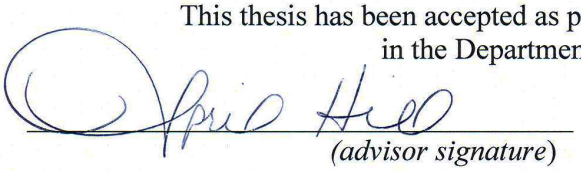

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Honors Thesis

Characterizing the role of Pax and Six in an emerging model system, the freshwater sponge, *E. muelleri*

Ian Winters

Sponges can be viewed as a remnant branch of the earliest successful experiments in metazoan multi-cellularity. As such, these organisms hold many clues into the genetic elements fundamental to the formation of complex animalian life. Two of these elements are the transcription factor encoding genes *PaxB* and *Six1/2*. Homologs of these genes in animals more complex than sponges have been shown to be members of a gene regulatory network involved in organ development. This is of particular intrigue since sponges do not possess such organ systems. Here, I discuss the putative function of *PaxB* and *Six1/2* in sponges, and whether the Pax-Six regulatory network is present in these ancestrally derived metazoans.

INTRODUCTION

The most ancestrally derived of all animal phylums is that of Porifera, the sponges. It is likely that the common ancestors between the sponges and the rest of the metazoan lineage were the first multi-cellular animals to ever inhabit the planet. Sponges contain many of the basic genes used by all animals in the development of their bodies from embryo to adult (Strivastava et al., 2010) Interestingly, these "animal genetic toolkit genes," which are critical to important developmental processes such as stem cell differentiation, growth, cell-to-cell communication, and the formation of sensory systems, are present in sponges even though they lack many of the specific animal traits such as organ systems and a nervous system. Given their evolutionary position, sponges contain the most basic set of these animal specific genes, which expanded and diversified over time as more complex body plans emerged. Thus, the study of Porifera may reveal novel insights into the most fundamental aspects of the developmental processes that are common to all animals. Although sponges do not contain major animal traits, they do share certain features with the rest of the metazoans that make the phylum of further interest.

Sponges are filter feeders and thus many, if not most, of their morphological structures are dedicated to this task. Ostia are small holes that dot the outer tissue of the sponge and allow water and potential food sources into the sponge. This water is directed along canals that are lined by thin elongated epithelial cells known as pinacocytes. Pinacocytes also make up the pinacoderm, the epithelial lining of the sponge, and the sponge osculla, a chimney like structure where filtered water is ejected from the sponge. These cells are further involved in compartmentalization, controlling ionic conditions, immune recognition, spicule formation, contractile function, and structural support in sponges (Leys and Hill, 2012). The generation of water flow into and out of sponges is generated by choanocytes (Weissenfels, 1990; Nickel, 2004; Elliot and Leys, 2007). These are flagellated cells that form together into spherical like structures known as choanocyte chambers that pump water in a given direction

along the canals of the sponge. A substantial portion of the sponge is comprised of tissue with a high density of choanocyte chambers, called choanosome. The choanosome has been shown to aid the sponge in sensing subtle changes in water pressure and flow, which allows the organism to make necessary structural changes to its canal system and optimize contractile activity to changes in water flow (Leys and Hill, 2012).

Furthermore, sponges contain a highly active stem cell population, primitive sensory perception, and the ability to respond to the surrounding environment (Boury-Esnault et al., 1999; Ellwanger and Nickel, 2006; Leys and Degan, 2001; Maldonado 2004). These features suggest that proper sponge development is reliant on the same basic molecular and cellular elements that regulate cell proliferation, differentiation, communication, and migration in all animals. Understanding these fundamental processes in an ancestral animal will undoubtedly reveal key factors at play in the development and evolution of the metazoan lineage.

The animal genetic toolkit expanded substantially during the evolution of the animals along with animal complexity. However, in studying the genetic factors that were essential to the rise of the metazoans, it is important to focus on the animal genetic toolkit genes that were present at their origin. Two such gene families that are of particular interest are Pax and Six. Both families code for transcription factors proteins that act by regulating networks of other genes involved in multiple aspects of organogenesis such as in the development of sensory organs (Kawakami et al., 2000; Chi and Epstein, 2002). In complex animals such as the vertebrates, there are nine known Pax genes and six known Six genes. However, only one Pax and one Six ortholog are present in sponges, known as *PaxB* and *Six1/2*, respectively. Since sponges are directly descended from the common ancestor of all animals, it is likely that *PaxB* and *Six1/2* that are found in sponges gave rise to all of the subsequent Pax and Six genes through gene duplication events over the course of evolution.

It has been well established that Pax and Six genes interact with each other in a genetic circuit know as the PSED network, named after the four major gene families involved, Pax, Six, Eyesabsent, and Dachsund. This network is implicated in the formation of many important animal specific traits such as eyes, muscles, endocrine glands, placodes, kidneys, and pharyngeal pouches (e.g., Hanson, 2001; Bassham and Postelthwait, 2005 ; Kozmik, 2005; Mazet et al., 2005; Silver and Rebay, 2005; Kozmik et al., 2007). Of the PSED network, sponges only contain a Pax and a Six gene. Thus there is the question of whether a portion of the PSED network, Pax-Six (PS), was established early in development in sponges, before other genes were recruited into the circuit later in evolution.

An emerging model system for the study of metazoan evolutionary developmental biology is the freshwater sponge, *Ephydatia muelleri*. Like other freshwater sponges, *E. muelleri* reproduces asexually by producing many dense spherical cysts of totipotent stem cells covered in a protective protein coat. These cysts are known as gemmules, and they can bear the burden of winter to hatch into entirely new sponges when the correct conditions are met. Because of this, gemmules can be collected from gemmulating sponge tissue, and stored at 4°C for over a year. These gemmules can be made to hatch at room temperature (~22°C - 25°C) to be used in experimentation. All of the tissue that makes up a new sponge is derived from the pluripotent stem cells of the gemmule known as archeocytes. This makes *E. muelleri* an intereting model for stem cell proliferation and differentiation. The clear appearance of the resulting sponge tissue also aids in the visualization of certain aspects of sponge development. Furthermore, *E. muelleri* can be collected from rivers and ponds in every continent of the world (Manconi and Pronzato, 2002). Currently, the genome and transcriptome of this emerging model organism are being processed. Recently, our lab published a paper on a technique to induce RNAi in sponges thereby knocking down the expression of target genes (Rivera et al. 2011).

Here, I discuss the putative functions of *PaxB* and *Six*1/2 in *E. muelleri*. Our results show that these two genes may be involved in the formation of choanocytes, which serve as pumping chambers of the sponge, and in the epithelial cells lining the canals of the sponge. Additionally, our data points to the potential regulation of *Six1/2* by PaxB in this model organism, implying that indeed, a simplified version of the PSED network, namely PS, was established as early in development as the sponges.

METHODS

Sponge culture. Gemmules embedded in adult *Ephydatia muelleri* tissue during reproductive stage were collected from three freshwater rivers, one in Montana, one in Connecticut, and the other in Virginia. This tissue was maintained at about 4°C during shipment to the Hill Lab. Once in the lab the gemmules were separated from surrounding adult tissue, washed once in 1% Hydrogen Peroxide, and then rinsed 8 times in cold 1x Strekals Medium (Strekal and McDiffet, 1974). Rinsed gemmules were stored in the dark at 4°C in a 15ml conical tube containing 1x Strekals. For treatment, 3-4 gemmules were plated in each well of a 12 or 24 well tissue culture plate and hatched in ~1ml 1x Strekals at room temperature (RT) and in the dark. Gemmules requiring biological imaging were plated/hatched on 18mm circular cover glass placed in each well of the tissue culture plates. Spent 1x Strekals media was replaced with fresh 1x Strekals once daily for treatments starting more than a day after the gemmules hatched.

In Situ Hybridization. In situ hybridization to whole mount sponges was essentially performed as described in Funayama et al (2005) with the addition of several steps. Sponges were fixed with 4% paraformaldehyde in 1/4 Holtfreter's solution (HS) overnight followed by a dehydration series (25% ethanol in 1/4 HS; 50% ethanol in 1/4 HS, 75% ethanol in 1/4 HS, 100% ethanol) and stored at -80°C until use. Prior to hybridization, tissues were rehydrated through an ethanol/PBT (phosphate buffered saline with 0.1% Tween-20) series followed by a 10 min wash in PBT. Tissue was treated with 1µg/ml Proteinase K for 1 min at room temperature and immediately washed in 2mg/ml glycine to stop the reaction. Tissue was washed once in PBT and then post-fixed for 30 min in 4% paraformaldehyde/PBS at 4°C. After fixation, tissue was washed twice in 0.1M triethanolamine. After the second transfer, 2.5 µl of acetic acid was mixed well with 1 mL of 0.1M triethanolamine and added to the tissue for 5 min (without rotation). This solution was changed for a solution with 5 µl of acetic acid in 1 mL of 0.1M triethanolamine and incubated for 5 min. Tissue was washed twice for 10 min in PBT prior to prehybridization. Tissue was prehybridized at 55-60°C for at least two hours (up to overnight) in (50% formamide, 5X SSC at pH 4.5, 50 µg/mL heparin, 0.1% Tween-20, 10 mM DTT, 1X Denhart's and 100 µg/mL sheared salmon sperm DNA). Hybridization was carried out overnight with digoxygenin labeled RNA probes (10-0.5 ng/µl) at 55-60°C. Probes were recovered and used multiple times. Results with lowest background hybridization were achieved with probes that had been re-used more than two times. After hybridization, tissues were washed three times in 50% formamide/5X SSC/0.1% Tween-20 for 20 min, followed by three additional washes in the same solution at hybridization temperature. Following, tissues were washed two times for 20 min each in 0.1M maleic acid buffer (Roche Diagnostics)/0.1% Tween-20 before pre-blocking for 1 hour in 0.1M maleic acid buffer/0.1% Tween-20 with 1% blocking reagent (Roche Diagnostics). Tissue was subsequently incubated overnight at 4°C in 1% blocking reagent with alkaline phosphatase conjugated antidigoxigenin antibody (Roche Diagnostics) diluted 1:3000. Tissues were washed six times for 30 min in 0.1M maleic acid buffer/0.1% Tween-20 at room temperature. Tissues were processed through fresh alkaline phosphatase buffer two times before incubating in AP buffer containing 175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphatase and 180 mg/mL nitroblue-tetrazolium chloride (Roche Diagnostics). After color reaction was completed (2 hours to overnight), tissue was washed in 0.1 M maleic acid buffer, post-fixed in 4% paraformaldehyde/PBT, washed in PBS, stained with DAPI and visualized by light microscopy (get microscopes). After whole mount pictures were taken, tissue was processed in epon and sectioned on a Leica Ultracut UCT ultramicrotome and pictures were taken using DIC imaging on an Olympus BX61 microscope with a ProgRes C14 plus (Jenoptik) camera.

RNAi. RNAi constructs were synthesized as described in Rivera et al., (2011). For feeding *E. muelleri* dsRNA expressing HT115 bacteria, 5 mL overnight cultures in LB broth with ampicillin were inoculated with L440-Em-PaxB or L440-Em-Six1/2 one day prior to the sponge feeding stage. The next day, each overnight culture was inoculated in 100 mL LB broth with ampicillin and maintained at 37°C with shaking. Bacterial cultures were induced with 19 mg/100 mL of Isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{595} 0.4 (~3 hrs

after scale up) to begin bacterial production of dsRNA. Cells were induced for 1.5 hours. 50 mL of cells from each overnight culture were centrifuged in conical tubes for 8 minutes at 4000 rpm at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 50 mL 1x Strekal's media. This constituted the feeding stock. From this stock dilutions were made for each treatment to a concentration of ~1 × 10^8 cells/mL. The 1x Strekal's culture medium was removed from each sponge and 1 mL of the appropriate dilution of bacteria producing dsRNA was added. Sponges were cultured for 24 hours with bacteria producing dsRNA. After 24 hours, sponges were washed several times in 1X Strekal's medium, photographed on an Olympus SZX12 stereomicroscope and then harvested for RNA after 1-2 hours. qRT-PCR and Western Blot Analysis was used to confirm knockdown of PaxB and Six1/2 as described in (Rivera et al., 2011).

To assess cellular and tissue level differences in RNAi treated sponges, treated and control tissues were post-fixed in 4% paraformaldehyde in PBS, dehydrated and subsequently embedded in epon plastic. Epon sections were taken using an ultramicrotome followed by staining in toluidine blue and visualization on an Olympus BX61 microscope.

RNA isolation and q-PCR. RNA was isolated from *E. muelleri* sponge tissue harvested at developmental stages or after RNAi treatment using the RNeasy® Mini Kit (Qiagen), limiting genomic DNA contamination through an additional on-column DNase I treatment. Equal amounts of cDNA (125-200 ng/µl) were synthesized from sponge mRNA using Superscript III reverse transcriptase (Invitrogen) and oligodT primer. SYBR Green (Invitrogen) chemistry and the Chromo4 (BioRad) were used for qRT-PCR using cycling conditions of 94°C for 3 min followed by 30s at 94°C, 30s at 55-61°C, 1 min at 72°C for 35 cycles with gene-specific primers (For *PaxB*, F: 5'-GCGCACCAGGACCCAGCAG-3'; R: 5'-GGACGGGGGGCACCATGAAG-3' and for *Six1/2*, F: 5'-CGGCGAGGAGACGAGCTATT-3'; R: 5'-CGTGGTCGTAAGCCCGGTTTGT-3'). *PaxB* and *Six1/2* levels were normalized to *Ef1a*, a 'housekeeping' gene in *E. muelleri* that is consistently expressed at high levels throughout development. For all qRT-PCR experiments, duplicates were performed from master mixes. Threshold values for Ct calculation were selected by hand to optimize efficiency for all samples. Standard curves using plasmid dilutions as templates were made for each gene in each qPCR experiment and efficiency-corrected Ct values were compared to these curves to calculate relative concentrations using Opticon Monitor software. The relative concentration values of duplicates were averaged and experimental averages were normalized to *Ef1a* values.

RESULTS

Previous research conducted in Dr. April Hill's lab has shown that *PaxB* and *Six1/2* are expressed for at least the duration of sponge development from the gemmule to young adult stage. Furthermore, both genes seem to increase slightly in expression as development progresses.

Spatiotemporal expression of PaxB and Six1/2. *In situ* hybridization using probes complimentary to *PaxB* and *Six1/2* mRNA was conducted to further elucidate their spatiotemporal expression patterns in young adult *E. muelleri* sponges, which already have all of the specialized cell types seen in the species. The *in situ* hybridizations indicate that *PaxB* and *Six1/2* mRNA expression patterns are remarkably similar. mRNA expression of each gene seems to be particularly localized to regions near the gemmule coat (*PaxB*: Figure 1B; *Six1/2*: Figure 1F). DAPI staining of cell nuclei shows that this region is the developing choanoderm, where a dense concentration of choanocyte chambers in the sponge tissue is produced (*PaxB*: Figure 1A; *Six1/2*: Figure 1E). Farther away from the gemmule coat towards the developing edge of the young adult sponges, *PaxB* and *Six1/2* mRNA is seen in large cells characteristic of archaeocytes, which are pluripotent stem cells that can likely differentiate into all of the cell types of *E. muelleri* (*PaxB*: Figure 1C; *Six1/2*: Figure 1G).

Sections of the post-*in situ* sponge tissue were taken after epon embedding to examine the expression patterns of *PaxB* and *Six1/2* at the cellular level. These sections reveal that once again mRNA expression of *PaxB* and *Six1/2* occurred in similar cell types; both showed expression in pinacocytes (epithelial cells, most noticeably lining canals), in some of the choanocyte chambers, and in particularly, in cells between the choanocyte chambers, named corner cells, that will likely

develop into new choanocytes (*PaxB*: Figure 1D; *Six1/2*: Figure 1H).

Knockdown of PaxB and Six1/2 expression by RNAi. Sponges fed bacteria at the young adult stage (i.e. when adult structures are already defined) expressing PaxB or Six1/2 dsRNA had visible phenotypic defects. The aquiferous system was deformed with ill-defined canal systems, smaller ostial openings, and a degradation of the osculla (*PaxB:* Figure 2). To examine this phenotype in greater detail at the cellular level RNAi treated and control sponges were embedded in epon resin and sectioned. Each section was stained with toluidine blue, a potent tissue stain, for ease of visualization. Compared to untreated sponges, where endopinacocytes clearly comprise the endothelial lining of the canal system, both PaxB and Six1/2 dsRNA treated sponges result in a qualitative decrease in their number of pinacocytes and thus have a much less well-defined canal system. Furthermore, choanocyte chambers seen in control tissue are consistently spherical, well defined, and present throughout the sponge tissue in an organized manner. However, in treated sponges choanocyte chambers are often poorly defined, and occur sporadically throughout the sponge's tissue. Also, it appears that there are more undifferentiated archaeocytes in the treated versus the control tissue (Control: Figure 3A,B *PaxB*: Figure 3C,D; *Six1/2*: Figure 3E,F). It is possible that additional morphological changes may occur in other cell types, however given the lack of cell specific markers this is difficult to asses.

Determining the regulatory relationship between PaxB and Six1/2. After feeding sponges bacteria expressing PaxB dsRNA, the mRNA expression levels of *Six1/2* were assessed by quantitative-PCR. This was done to determine whether a decrease in PaxB protein would have an affect on the expression of *Six1/2* mRNA levels. Interestingly, *Six1/2* expression was significantly decreased post-PaxB RNAi (Figure 4). This suggests that PaxB positively regulates the *Six1/2* gene in some way, although further analysis of this regulatory relationship is required to confirm this finding (see Rued, Anna: Honors Thesis).

DISCUSSION

The vast diversity of animal body plans that have graced the planet is astounding. Traditionally, the evolution of this diversity has been thought to occur through changes to protein coding DNA sequences. Research over the last decade, however, supports the idea that changes in gene regulatory elements may drive the modification of phenotypic traits and body plans of animals over time (Gompel et al., 2005). Gene regulatory networks regulate gene expression, and are therefore critical to the proper development of animal body plans. Thus, the incredible diversification of body plans over the course of evolution is likely due in part to the acquisition of novel functions of genes in evolutionarily conserved regulatory networks (Davidson and Erwin, 2006). Since sponges are an ancient metazoan lineage, they provide an ideal model in which to study the early evolutionary function of genes from important gene regulatory networks.

One highly conserved gene regulatory network that has been well studied in both invertebrates and vertebrates more complex than sponges is the retinal determination gene network (PSED). This network was originally found to play a critical role in the eye development of insects and vertebrates (Treisman, 1999; Relaix and Buckingham, 1999), but is now know to be present in all animal phylums more evolutionarily recent than Porifera. To date only two main members of the PSED network have been found in sponges, one Pax ortholog, *PaxB,* and one Six ortholog, *Six1/2.* Thus, there is interest in whether *PaxB* and *Six1/2* are in a regulatory relationship in sponges, which would indicate that at least a portion of the PSED network was in place earlier in animal evolution than previously though. Furthermore, the Pax and Six genes as well as other members of the PSED network are known to function in many important aspects of organogenesis in higher metazoans (Kawakami et al., 2000;; Hanson, 2001; Chi and Epstein, 2002; Bassham and Postelthwait, 2005 ; Kozmik, 2005; Mazet et al., 2005; Silver and Rebay, 2005; Kozmik et al., 2007). Since sponges do not contain these organ systems, the function of *PaxB* and *Six1/2* in sponges is particularly intriguing. These Pax and Six orthologs likely represent the first ever iterations of these genes, and therefore provide clues to their original function. Elucidating

the function of *PaxB* and *Six1/2* in sponges provides insights into not only the evolution of the PSED network, but into how individual genes gain novel functions over the course of evolution. Here, we provide evidence on the function of *PaxB* and *Six1/2*, and the possibility of a regulatory connection between these two genes, in an emerging model system, the freshwater sponge *E. muelleri*.

Putative function of PaxB and Six1/2. In situ hybridizations revealed that *PaxB* and *Six1/2* have similar mRNA expression patterns, and are localized in two main cell types: pinacocytes, and choanocytes. Pinacocytes are thin elongated cells that make up the epithelial lining of the sponge called the pinacoderm. These cells line canals and comprise osculla, and are involved in compartmentalization, controlling ionic conditions, immune recognition, spicule formation, contractile function, and structural support (Leys and Hill, 2012). Intriguingly, pinacocytes of *E. muelleri* are ciliated. These cilia likely aid in the aforementioned functions of pinacocytes, as well as in the contractile motion of these cells that moves water through the canals and out the oscullum (Boury-Esnault and Rutzler, 1997). *PaxB* and *Six1/2* expression was also seen with some spatiotemporal variance in choanocytes. Choanocytes are flagellated cells that together form spherical chambers to pump water throughout the aquiferous system of the sponge. By the combined action of the choanocyte's flagella, these chambers generate water flow in the sponge (Weissenfels, 1990; Nickel, 2004; Elliot and Leys, 2007). Furthermore, sponge tissue with a high density of choanocyte chambers, called choanosome, has been implicated in sensing subtle changes in water pressure and flow. This information allows the sponge to make necessary structural changes to its canal system and optimize contractile activity to changes in water flow (Leys and Hill, 2012).

The expression patterns of *PaxB* and *Six1/2* visualized by *in situ* hybridization led to the hypothesis that a knockdown of either gene would result in either defects to the endothelial lining of cell canals and to choanocyte chambers or an overall reduction in these structures. Reducing expression of *PaxB* and *Six1/2* by using a feeding induced RNAi technique support this hypothesis. Knocking down mRNA expression of either *PaxB* or *Six1/2* in young adult sponges resulted in less endothelial cells lining canals (and thus, the loss of a well-defined canal system), and a substantial decrease in the number and density of choanocyte chambers in the sponge.

The expression patterns and loss of function data for *PaxB* and *Six1/2* indicate that both of these genes function primarily in pinacocyte and choanocyte cells in *E. muelleri* sponges, and that they are important to the proper development and proliferation of these cells. Pinacocytes and choanoocytes are both sensory cells, they posess contractile activity and can receive and respond to external stimuli. This is extremely interesting since Pax and Six genes in animals more complex than sponges are also involved in the development of tissues of sensory systems like the CNS and eye development as well as muscle development. Thus, it is likely that *PaxB* and *Six1/2* played a role in the early evolution of sensory function in animals.

Our research indicates that the function of the Pax and Six genes has been conserved very early in animal evolution from sponges to more complex animals such as humans. The remaining question is that of whether an ancestral gene regulatory network between *PaxB* and *Six1/2* was present as early in evolution as the sponges*. In situ* hybridization data shows that *PaxB* and *Six1/2* have similar mRNA expression patterns. Furthermore, knockdown experiments of each gene result in similar phenotypes. This suggests that *PaxB* and *Six1/2* may be co-regulated or involved in a regulatory relationship with each other. To further elucidate the nature of this regulation, we knockdown expression of *PaxB* and quantified the subsequent expression of *Six1/2* via q-RTPCR. If *PaxB* regulates *Six1/2* in some way, a decrease in *PaxB* expression would lead to a change in *Six1/2* expression. The results of this experiment show that a *PaxB* knockdown actually leads to a significant decrease in *Six1/2,* indicating that *PaxB* directly or indirectly up-regulates *Six1/2* expression in *E. muelleri*. This is preliminary evidence that a portion of the PSED network, namely a Pax-Six network, was established earlier in development than previously thought.

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Figure Legends

Figure 1. In situ hybridization analysis with *EmPaxB* (A-D) and *EmSix1/2* (E-H) probes. Whole mount in situ images (A-C & E-G). Epon sections of choanoderm tissue (D,H). B & F have gemmule coat (g) oriented to the right. Expression in choanoderm and expanding growth region is evident. A & E are DAPI stained images of B & F to illustrate choanocyte chamber development in region closest to gemmule coat. Region shown in C is magnified (from B) to show individual cells expressing PaxB in regions of new sponge growth. Region shown in G is magnified (from F) to show Six1/2 expression in cells at regions of tissue expansion/new growth. D & H show epon sections of tissue from B & F. Arrowheads point to pinacocyte cells that line canals while arrows indicate possible archeocyte cells between choanocyte chambers. c, choanocyte chamber; g, gemmule coat.

Figure 2. Control and *EmPaxB* RNAi treated sponges. (A) Control sponges were fed HT115 bacteria alone or HT115 bacteria expressing dsRNA to GFP. (B) Sponges were fed HT115 bacteria expressing dsRNA to *EmPaxB*. Canal structure is less evident in RNAi treated sponges compared to controls.

Figure 3. RNAi knockdown morphology of juvenile sponges. (A, B) epon sectioned, toluidine blue stained control Stage 5 sponges (fed HT115 bacteria). (C, D) Stage 5 sponges fed bacteria expressing *EmPaxB* dsRNA; (E, F) Stage 5 sponges fed bacteria expressing *EmSix1/2* dsRNA. Red arrowheads indicate location of endopinococytes (or lack thereof) lining canals. Red arrows indicate undifferentiated archeocytes in the choanoderm. All images were photographed in DIC.

Figure 4. qRT-PCR analysis of *EmSix1/2* relative expression levels following treatment of Stage 5 sponges with bacteria expressing for *EmPaxB* dsRNA. Expression levels are normalized to Ef1a.

Figure 1

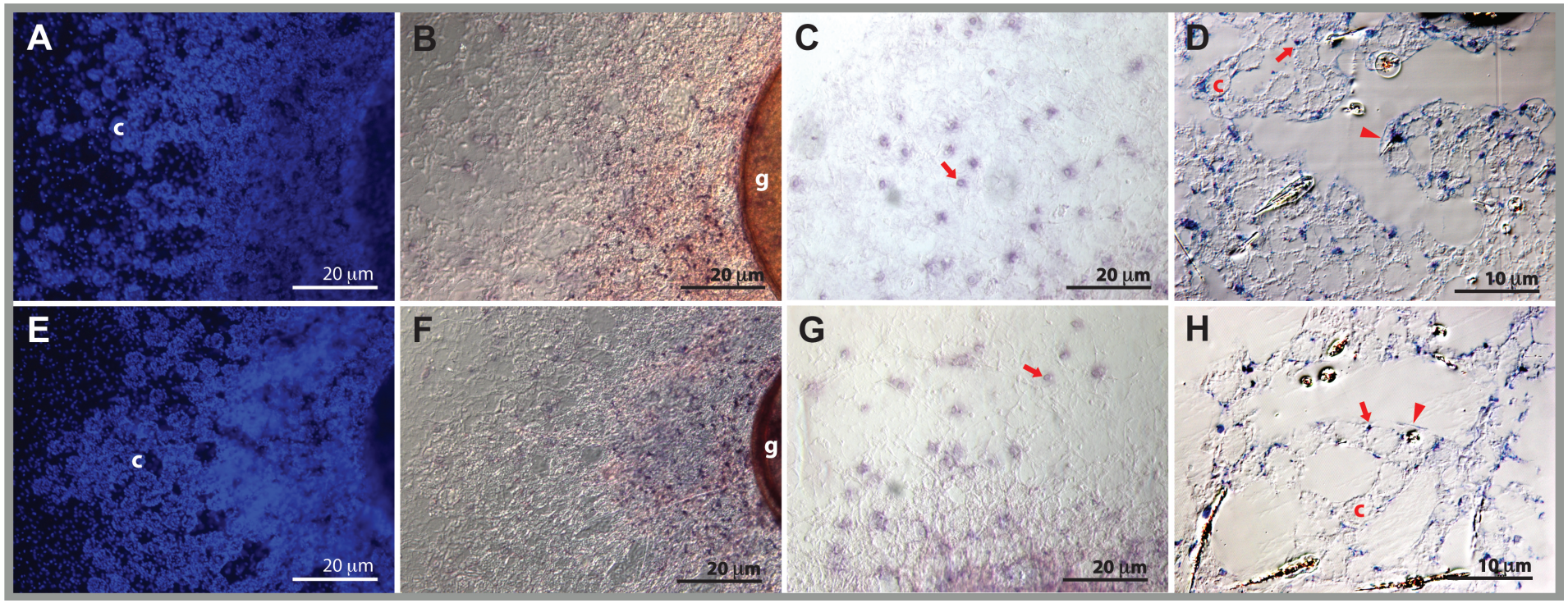


Figure 2

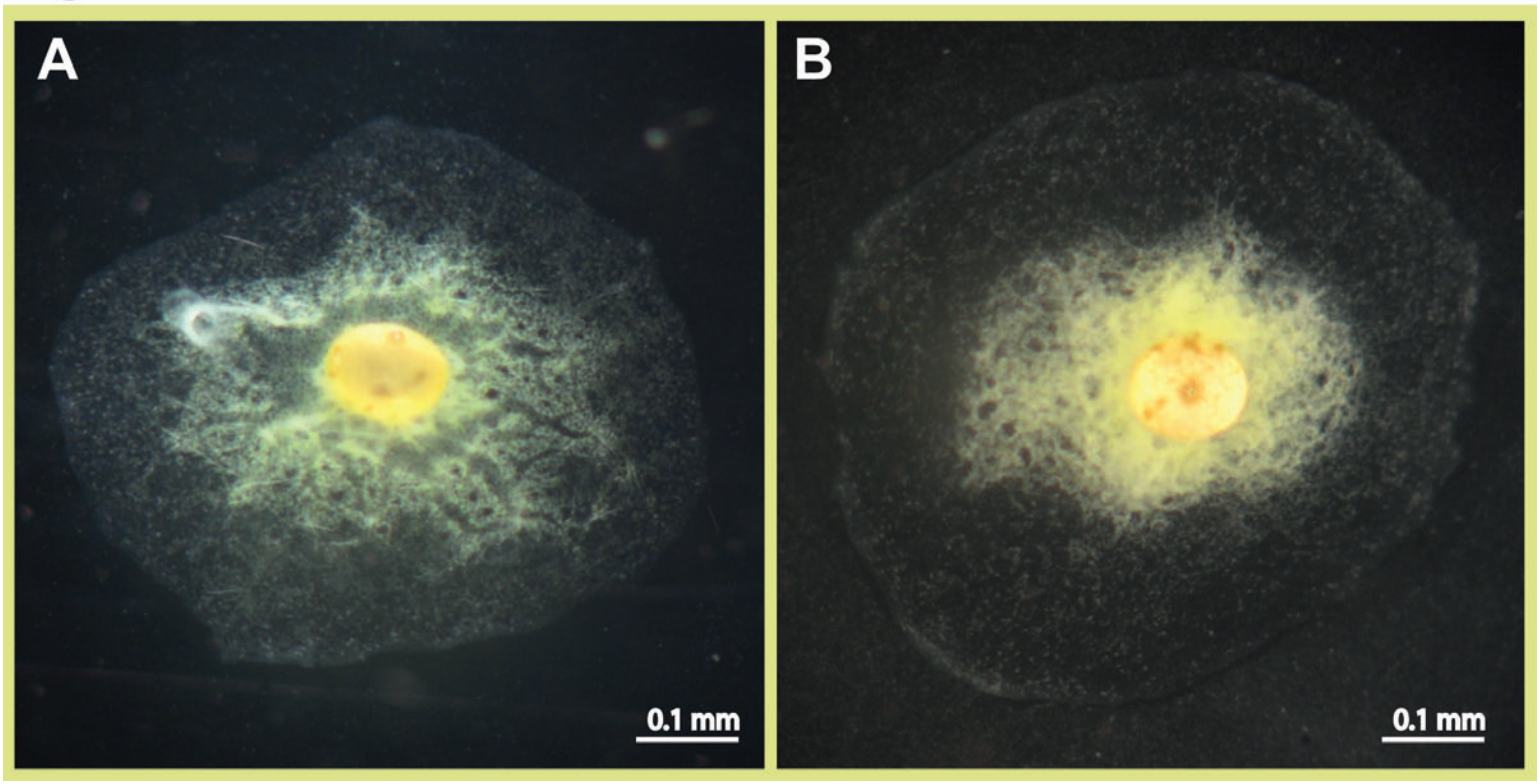


Figure 3

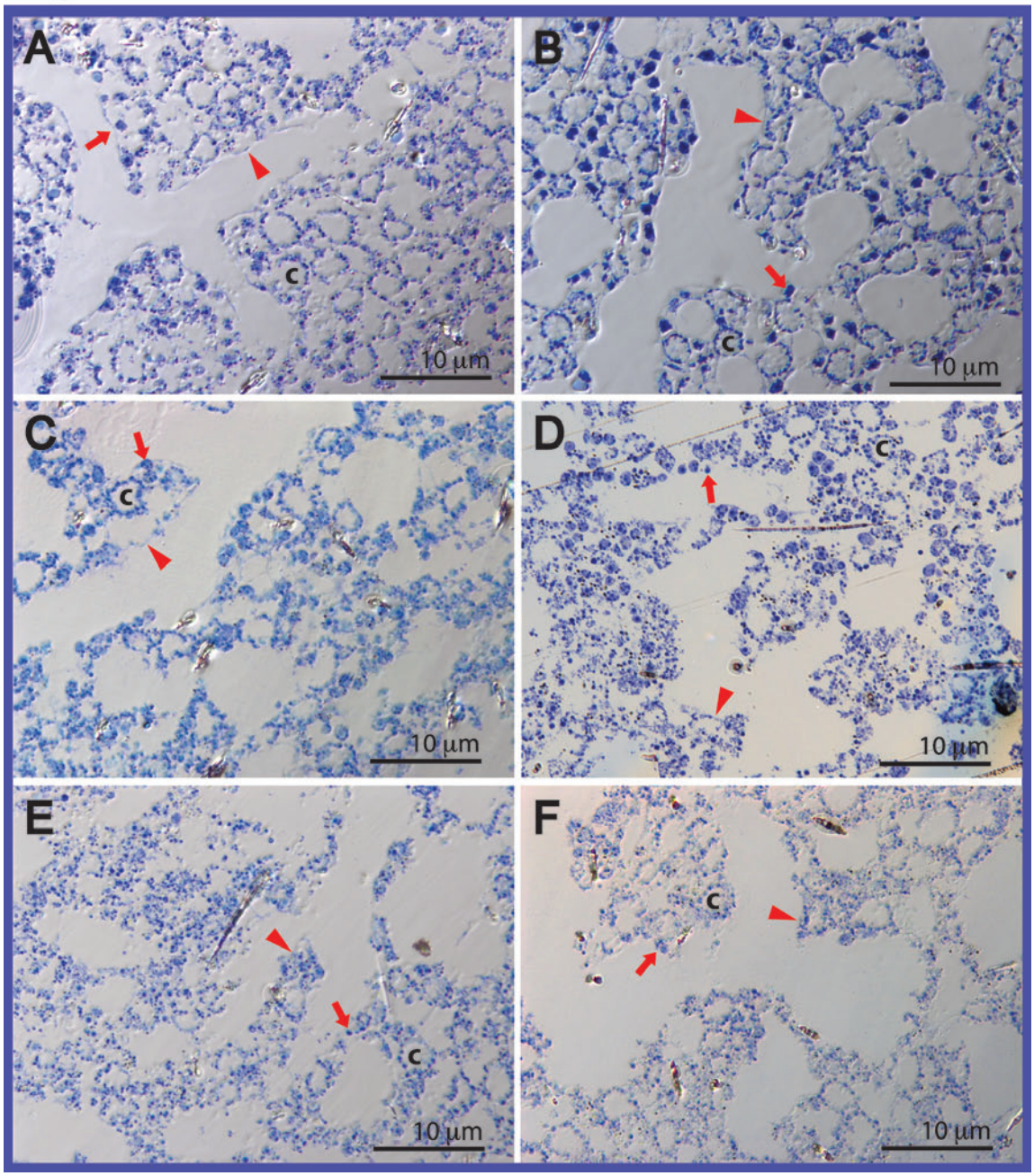
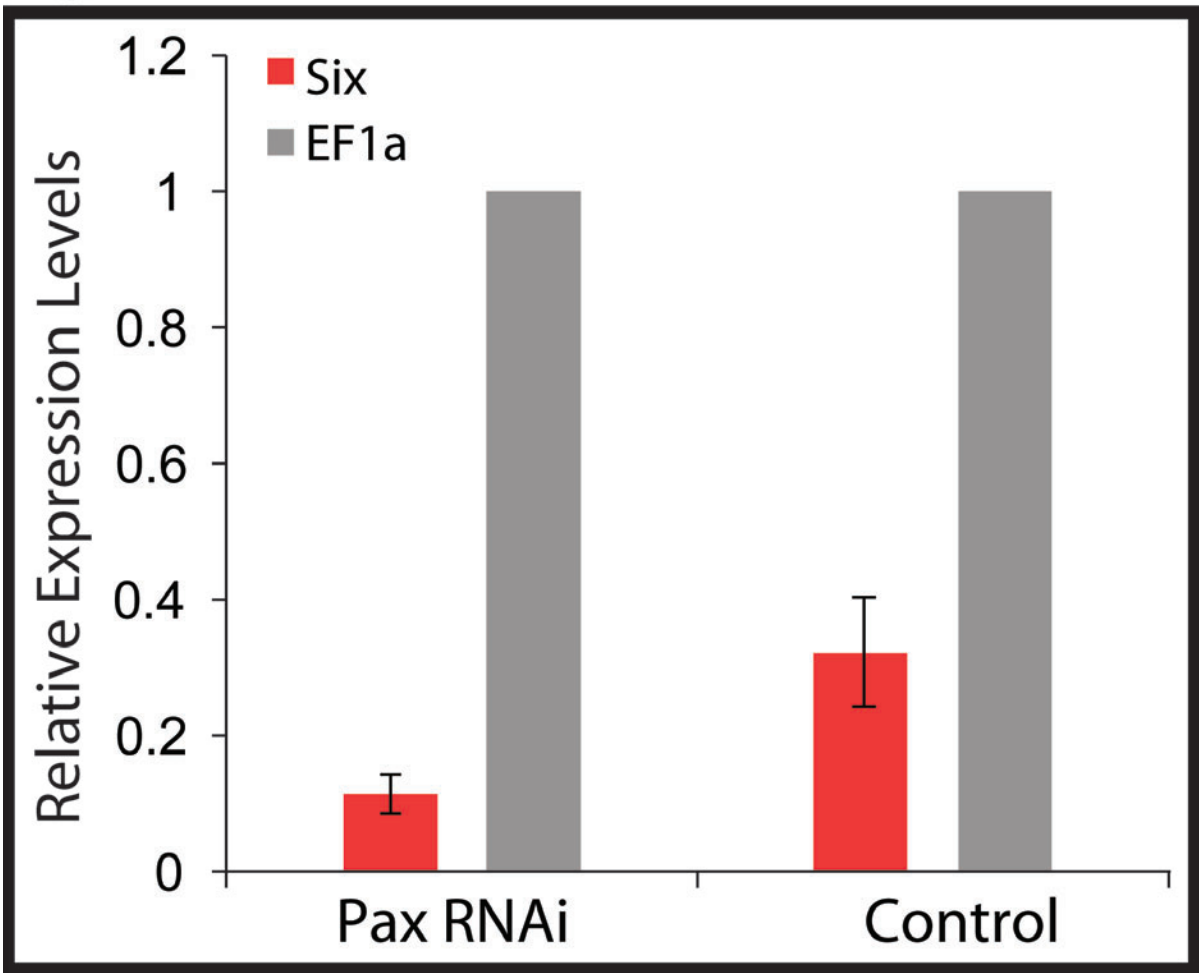


Figure 4


1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression Levels
Six
EF1a
Pax RNAi
Control