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Elucidation of the Pax/Six gene regulatory network in *Ephydatia muelleri*

by

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Honors Thesis

in

Program in Biochemistry and Molecular Biology

University of Richmond

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This thesis has been accepted as part of the honors requirements
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Table of Contents

- Acknowledgments
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- Figures and Tables

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Abstract

The evolution of gene regulatory networks accounts for much of the diversity we observe in the animal kingdom. In particular, the Pax/Six/Eya/Dac gene network has been known to play critical roles in development of all bilaterians and cnidarians. However, this network has not been characterized in Porifera. These studies focus on tracing the evolutionary history of the PSED network back to the most basal organism in the metazoan phylogeny through demonstrating the presence of a direct gene regulatory network between PaxB and Six1/2 orthologs in sponges. To this end, putative Six1/2 cis-regulatory elements were identified while the PaxBprd transcription factor was isolated and purified. Binding assays demonstrated that the PaxBprd protein bound directly to upstream cis-regulatory regions of the Six1/2 gene, as well as a conserved Six1/2 intron site in two different species of sponges. Additionally, we focused on broadening the Pax gene regulatory network in sponges by looking at additional downstream gene targets of

Pax. We identified a possible indirect regulatory network between Pax and four novel target genes through the use of RNAi and qRT-PCR techniques. This study helps to elucidate the origins of the PSED network as well as provide the beginnings of a whole gene regulatory network in sponges.

Introduction

Scientists have long puzzled over the question of how organisms have evolved into the complex and diverse metazoans we see today. While basal organisms were once viewed as primitive, less genetically complex organisms, recent studies have shown that the last common ancestor of the metazoans possessed all but one of the major signal transduction protein-coding gene families that are essential for development (Ryan et al., 2006). Therefore, the huge diversity of complex animals and subsequent morphological simplicity of basal organisms must arise from differences in spatiotemporal deployment of these genes through gene regulatory networks, as well as divergences of regulatory machinery (Erwin et al., 2011). Some key questions about gene regulatory networks involve looking at the evolution of complex body plans and sensory organs. The emergence of complex body plans complete with nervous systems, tissues, and organs appeared for the first time about 540 million years ago during the Cambrian explosion (Degnan 2009). However, some of the genes that regulate complex body plan development, such as the *Pax* and *Six* genes, are found in pre-Cambrian animal genomes and are thus buried deep within the metazoan phylogeny. These basal organisms can provide important clues to help answer over-arching questions about animal evolution and diversity (Peterson, 2000).

The most basally branching group of metazoans are sponges, which can trace their lineage back for over 600 million years (Li, 1998; Nichols, 2005). Sponges contain most of the animal specific genetic toolkit genes including transcription factors and structural gene families even though they lack major animal traits (Srivastava et al., 2010). Interestingly, the progenitor to sponges, choanoflagellates, does not possess most of these important genes families (King et al., 2008). Thus, it is clear that sponges represent a key step in evolution essential for developmental pathways involved in diverse processes such as growth, differentiation, cell specification, and adhesion.

One of the key conserved gene regulatory networks is the Pax/Six/Eya/Dac (PSED) network, which is involved in complex developmental processes such as eye development, nervous systems (Galliot, 2009), muscles (Relaix, 1999) and kidneys (Dressler, 2006) in bilaterian animals. While the Eya orthologs are only found in cnidarians and bilaterians and Dac orthologs are found only in bilaterians, the genomes of demosponges *Amphimedon queenslandica* and *Chalinula loosanoffi* contain one Pax gene (PaxB) and one Six gene (Six1/2) (Hill, 2010). Thus, sponges can be used to study the origins of the PSED network at the base of the metazoan phylogeny.

The Pax and Six families have been well characterized in more complex metazoans. Humans have six Six genes and nine Pax genes, which likely arose via gene duplication and divergence events. Six genes are grouped into *Six1/2*, *Six4/5* and *Six3/6* subfamilies, with the demosponge ortholog from the Six1/2 family (Seo, 1999; Hill et al. 2010). Pax genes have been divided into *Pax2/5/8*, *Pax3/7*, *Pax1/9* and *Pax4/6* subfamilies (Bopp 1986 and 1989; Burri, 1989; Gruss and Walther, 1992). These Pax families are involved in a variety of processes such as kidney, eye, and inner ear

development (Torres et al., 1996), as well as midbrain and cerebellum development (Urbanek et al., 1997; Schwarz et al., 1997). Pax genes are conserved throughout the bilaterian phylogeny, most notably through the conserved role of *Pax6* in bilaterian eye development (Quiring, 1994). While *Pax6* has been considered the “universal eye gene” (Gehring and Ikeo, 1999; Gehring, 2002), recent studies in Cnidaria have shown that PaxB is the progenitor gene for eye development, which likely gave rise to the Pax6 and Pax2 genes through gene duplication and divergence (Kozmik, 2003). The PaxB protein is comprised of a Pax2-like paired domain and octapeptide domain with a Pax6-like homeodomain (Hoshiyama et al., 1998), which further supports the role of PaxB as the primordial eye development gene.

While previous work has identified PaxB and Six1/2 orthologs in sponges (Hill et al. 2010), no studies have yet elucidated the presence of a regulatory network between these two genes in sponges, an animal without eyes. This paper focuses on determining the origins and evolutionary history of the PSED network by examining the relationship between the PaxB and Six1/2 genes in the sponge *Ephydatia mulleri* (*Em*). We were interested in determining whether or not a direct gene regulatory network between these two genes is established in sponges. To this end, early data suggested the existence of a regulatory association between PaxB and Six1/2. In qRT-PCR experiments, the expression of *EmSix1/2* was significantly decreased when *EmPaxB* is knocked down through RNAi treatment, (Rivera et al., manuscript in progress). My thesis work shows that PaxB is in a direct regulatory relationship with Six1/2 in Porifera. We were also interested in establishing the first basal gene regulatory network in sponges by searching for additional putative downstream genes of PaxB. My thesis work demonstrates

preliminary data to support indirect regulation of several genetic toolkit genes by PaxB. By examining these gene regulatory networks in basal organisms, clues to the evolutionary history of complex and diverse metazoans can be elucidated.

Materials and Methods

Identification of putative *EmSix1/2* cis-regulatory elements

Putative EmPaxB protein binding sites were identified by searching the 5' upstream and intron regions of *EmSix1/2* and *AqSix1/2* gene sequences for a Pax2/5/8 consensus binding sequence RNGMANTSAWGCGRMM (Czerny, 1995). The *Amphimedon queenslandica* genome has been fully sequenced and is available at <http://spongezome.metazome.net/cgi-bin/gbrowse/amohimedon/>. The motif search was completed using the Regulatory Sequence Search tool under EBI's Alternative Splicing Database (ASD) (www.ebi.ac.uk/asd-srv/wb.cgi?method=3) allowing for a 25% mismatch for *EmSix1/2*, and done by hand for *AqSix1/2*.

EmPaxBprd protein cloning, over-expression and purification

The 127 amino acid paired domain of EmPaxB protein (EmPaxBprd) was PCR amplified with primers designed with restriction sites at each end (Table 1). PCR products were restriction digested at the 5' and 3' *XhoI* and *NcoI* sites, and cloned into a similarly cut pET-GQ plasmid (a gift from Eric Gounaux, Oregon Health and Sciences University). The recombinant plasmid was used to transform chemically competent Origami 2(DE3) cells (Novagen), and colonies were screened by PCR with the positive colonies being sequenced to confirm the presence of EmPaxBprd in the correct reading frame. Glycerol

stocks of pGQ/*PaxB* in Origami 2(DE3) cells were used to start 50 mL overnights at 37 °C in LB-kanamycin (kan). The overnights were used to seed 1.5 L LB-kan, and growth was continued until an OD₆₀₀ of 0.8 was reached. The temperature of growth was then lowered to 20 °C for 30 min followed by induction with 1 mM IPTG. Growth was continued for 16-18 h at 20 °C, after which the cells were harvested by centrifugation using a Sorval RC-5B Refrigerated Superspeed centrifuge for 10 min at 4 °C and 4,000 rpm. The cell pellet was then resuspended in 50 mL buffer A (20 mM Tris, 50 mM NaCl, pH 8), 5 mM MgSO₄, 0.5 mM PMSF, 0.3 mM deoxycolic acid, and 16 μM lysozyme, and lysed by sonication using a Branson Digital Sonifier for one minute. Lysed cells were centrifuged at 12,500 rpm for 30 min at 4 °C on an Sorval RC-5B Refrigerated Superspeed centrifuge, and the high speed supernatant was loaded onto a nickel affinity column (~20 mL, Novagen) and rotated overnight at 4 °C. The column was rinsed with 100 mL buffer A followed by 30 mL washes with 10 mM, 100 mM, 250 mM, 500 mM, and 1M imidazole in buffer A. All elution fractions (except 500mM) were collected in 30 mL of buffer A since high concentrations of imidazole caused protein precipitation. Collection of the fractions with 500 mM imidazole were separated into 6 x 5 mL fractions collected in 15mL buffer A. Pure fractions (500 mM imidazole elutions), as determined by SDS-PAGE, were dialyzed in buffer A at pH 7.5. The *PaxB* paired domain was concentrated to 128 μM [14,186 g/mole, $\epsilon(280) = 12,840 \text{ M}^{-1} \text{ cm}^{-1}$].

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides for the Pax2/5/8 consensus sequence and all identified potential EmPaxBprd binding sites were synthesized (Invitrogen) and labeled using the DIG DNA

Labeling Kit[®] (Roche Diagnostics). EMSA reactions were set up with 41 μ M PaxB paired domain, as described in the DIG gel shift kit, and incubated on ice (5-7 min) before being loaded into the 6% DNA Retardation gel (Invitrogen). Gels were run for 70 min at 70V. Samples were transferred for 90 min at 30V and the membrane was cross-linked using UV stratalinker 1800 (Stratagene). Detection was performed as described in the DIG Gel Shift Kit, 2nd Generation[®] (Roche Diagnostics). A non-specific DIG-labeled control oligonucleotide for Oct2A as given in the DIG Gel Shift Kit was used as a negative control for the PaxB protein.

Identification of Pax gene downstream targets

Hefalmp software was used to search for potential downstream genes of Pax 2, 5, 8, and 6. Identified Homo sapiens putative gene sequences were then searched in the *Ephydatia muelleri* transcriptome for target gene orthologs. These sequences were verified on the NCBI data bank, as well as on the Amphimedon genome. Based on these results, primers were designed using Primer3 software for the putative Pax gene targets (Table 1).

RNA isolation and qRT-PCR analysis

RNA was isolated from *E. muelleri* sponge tissue harvested at developmental stages 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/ μ l) 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 (Table 1). Gene expression levels were normalized to *Efla*, 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 *Efla* values.

Results

***cis*-regulatory element identification**

In order to study the regulatory interaction of the PaxB transcription factor with putative upstream and intron regions of the *Six1/2* gene, putative PaxB binding sites were identified through genome walking and bioinformatic techniques. The resulting data identified eleven putative binding sites upstream of the *Six1/2* gene for the PaxB protein in *Ephydatia muelleri*, while one putative binding site was positioned in the intron region of that gene (Figure 1B-C). Additionally, two putative PaxB binding sites were identified in the *Amphimedon queenslandica* *Six1/2* gene (*AqSix1/2*), while an additional putative binding site was also found in the conserved intron region of this gene.

EMSA

EMSA demonstrated that the PaxBprd transcription factor binds directly to regulatory sites found near (in cis) to the *Six1/2* gene. A nonspecific DNA-binding oligonucleotide confirmed that PaxBprd was not a low affinity protein, as there was no shift (Figure 1A). As a positive control, the known Pax 2/5/8 consensus sequence (Crezny, 1995) bound specifically to the PaxBprd, thus indicating that the PaxB protein has been conserved across evolutionary history, linked most closely to the Pax 2/5/8 family (Figure 1A). Additionally, the EmPaxBpd transcription factor binds directly to two upstream *cis*-regulatory sequences in the *Six1/2* gene of both *Ephydatia muelleri* and *Amphimedon queenslandica* (Figure 1A-B). The EmPaxBpd also binds directly to a site within the conserved intron of both species of sponges (Figure 1A-B). A sponge-specific PaxB binding motif was generated based on the positive and negative EMSA data using MEME, a motif-based sequence analysis tool (Figure 1C). This indicates that EmPaxBprd acts as a regulatory transcription factor for the *Six1/2* gene, suggesting that these two genes are involved in a gene regulatory network. .

Identification and qPCR analysis of Pax gene downstream targets.

Based on HEFaIMP genome functional mapping, ten putative Pax downstream targets were identified; MAPK8, SOX21, RXRG, NPY2R, BMP7, TLE4, RUNX1, SOX2, SOX3, CDX2. Each putative target gene was searched in the draft transcriptome of *Ephydatia muelleri* and followed with gene specific RT-PCR to ensure that each gene was expressed in *Ephydatia* tissue at developmental times that PaxB is known to be expressed. Expression levels of MAPK8, SOX21, RXRG, and BMP7 were then evaluated via qRT-PCR in sponges that were treated with dsRNA for *PaxB*. This experiment could establish whether or not these genes would be decreased in expression

if PaxB levels were decreased. If these genes showed decreased expression levels in sponges after RNAi treatment for PaxB, then a potential regulatory relationship could be established. The resulting percent knockdowns ranged from 20.22% to 38.61% (Table 2). These results suggest there could be an indirect regulatory relationship between PaxB and the putative downstream targets MAPK8, SOX21, RXRG, and BMP7.

Discussion

In order to look at the origins of the PSED network, we are interested in the relationship between *PaxB* and *Six1/2*, which are the only PSED members present in sponges at the base of the metazoan phylogeny. RNAi knockdown experiments demonstrated a possible indirect regulatory relationship between *PaxB* and *Six1/2*, as *Six1/2* expression is significantly decreased when *PaxB* is knocked down. In order to establish a direct regulatory network however, it is necessary to look at the actual binding sites for PaxB protein and the *Six1/2* gene. To this end, a canonical Pax consensus binding sequence for the Pax 2/5/8 families has already been established (Czerny, 1995). Using this sequence, we found 14 putative *Six1/2* cis-regulatory elements in both the *Amiphedon queenslandica* and *Ephydatia muelleri* species (Figure 1). Through EMSA experiments, we were able to determine that two upstream cis-regulatory elements of the *Six1/2* gene bound to the EmPaxBprd transcription factor in both species of sponges (Figure 1). Interestingly, these two binding sequences are located in different places on the upstream region of *Six1/2*. The differences in placement of these regulatory sequences could result in distinctive spatial and temporal gene patterning between these two species, thus accounting for morphological divergences that provide the basis for evolution.

Additionally, binding assays showed that the EmPaxBprd transcription factor bound to a *Six1/2* intron sequence in both species of sponges (Figure 1). This intron is known to be conserved in *Six2* and *Six6* genes through bilaterians (Gallardo et al., 1999; Boucher et al., 2000) but has never yet been characterized in sponges before. The presence of this intron in sponges suggests that this sequence has been conserved throughout evolutionary history from the most basal group of the metazoan tree. Furthermore, it seems likely that selection acted upon this intron sequence given its conservation and the presence of cis-regulatory sequences within.

Through these binding studies, we have been able to elucidate the presence of a direct gene regulatory network between the Pax and Six genes in sponges. As Pax and Six are the only members of the PSED network present in sponges, and are not found outside of the animal phyla, we can place the origin of the PSED network at the base of animal evolution. This provides a hypothesis that the *Eyes absent* and *Dachshund* genes evolved later and were added into the network throughout the course of evolutionary history.

We were also interested in broadening the Pax gene regulatory network in sponges by characterizing additional putative downstream target genes of Pax. Using bioinformatic techniques, we identified ten novel Pax target genes in sponges that are known targets in more complex animals such as humans. Through RNAi knockdown experiments of PaxB, we were able to test expression levels for four of these putative Pax target genes. MAPK8, RXRG, BMP7, and SOX21 all showed a significant decrease in expression in the presence of a PaxB knockdown. This suggests the presence of an indirect regulatory network between these genes. Interestingly, these genes are all

important genetic toolkit elements in development. MAPK8 is a mitogen-activated kinase involved in embryonic development, cell proliferation, apoptosis, and tissue morphogenesis (Ip and Davis, 1998; Whitmarsh *et al.*, 1998), while RXRG is a retinoic acid receptor implicated in embryological development processes such as cell proliferation and differentiation (Reichrath *et al.*, 1997). BMP7 is a morphogen involved in body plan development through gradient signaling, which regulates cell fate specification (Myers *et al.*, 2002), while SOX21 plays a critical role in cell fate and differentiation (Lefebvre *et al.*, 2007). These results indicate that Pax could play a role in regulating other key developmental genes in sponges. These additional Pax downstream gene targets begin to establish a broader whole gene regulatory network in basal organisms.

Figures and Tables

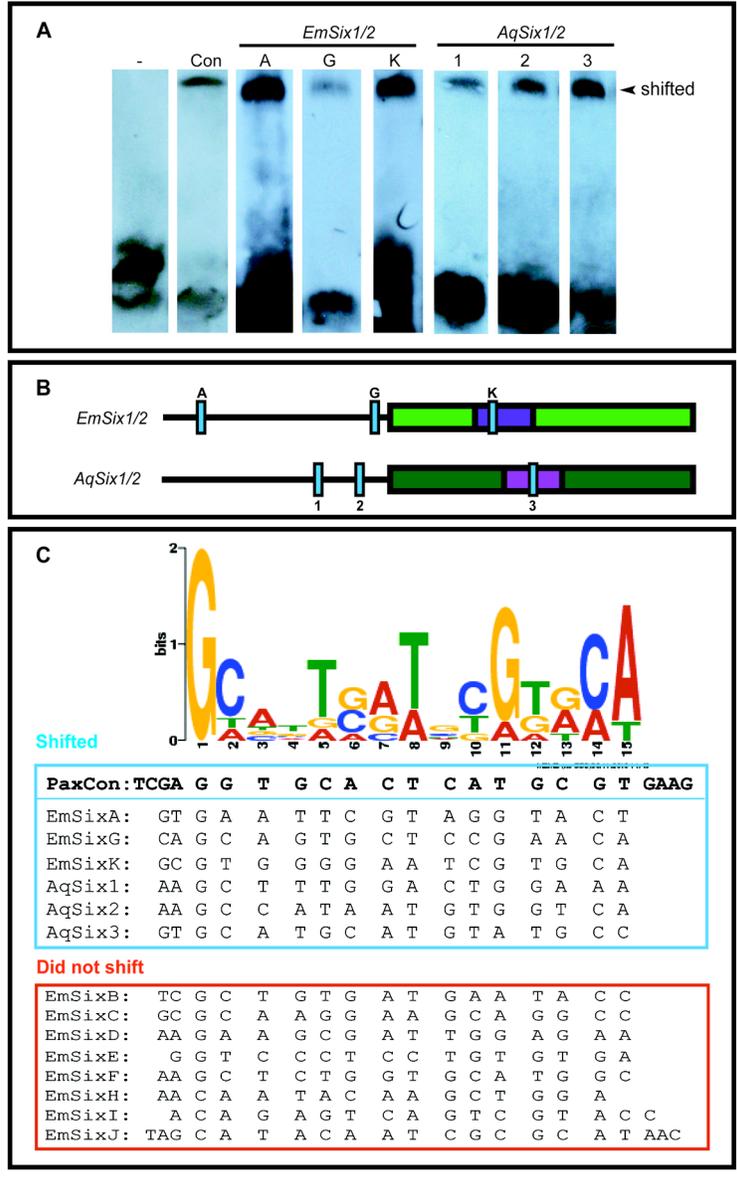


Figure 1: PaxB protein directly regulates the *Six1/2* gene in sponge species *Ephydatia muelleri* and *Amphimedon queenslandica*. A) EMSA data shows that EmPaxB paired domain protein binds to the Pax2/5/8 consensus binding sequence as well as three *cis*-regulatory elements in both *EmSix1/2* and *AqSix1/2*. This causes a shift in DNA migration through a 6% DNA Retardation Gel (Invitrogen) as indicated by the arrow, which is visualized through DIG labelling. B) Schematic view of the *cis*-regulatory elements in the *EmSix1/2* and *AqSix1/2* regulatory regions that were bound by EmPaxB. C) The sponge-specific PaxB paired domain binding motif generated from the positive and negative EMSA data.

Table 1: Oligonucleotides and Primers used

Protein Purification	Em PaxB	5' - GCTCGGGTTCTCCTGCTTGTAGTC - 3' 5' - CGCCCGTTGACGAAAAGCCCG - 3'
EMSA	PaxCon	RNGMANTSAWGCGRMM
	Negative control	5' GTACGGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG 3' 3' CCTCATAGGTCGAGGCATCGTACGTTTAGGAGACCAGCT 5'
qRT-PCR	PaxB	5'-GCGCACCAGGACCCAGCAG-3' 5'-GGACGGGGGGCACCATGAAG-3'
	MAPK8	5'-CACTCAGCCAACGTTCTTCA -3' 5'-CATGATTTCTGGAGCCCTGT -3'
	SOX21	5'-ATGATCCCAATGCTCGAAAG -3' 5'-AGTGGTGGAGGTTACAAGG -3'
	BMP7	5'-GTCCAACCCGCATCTTATGT -3' 5'-CACGATCACTTGCTGCAGTT -3'
	RXRG	5'-GGGACTCAAACCACAAGGA -3' 5'-TTCTGAAGGCTAGCTGGTGT -3'

Table 2: qRT-PCR percent knockdowns for MAPK8, SOX21, RXRG, and BMP7 using PaxB RNAi cDNA and HT115 cDNA. Genes were normalized with house keeping gene EF1 α .

Gene	Percent Knockdown
MAPK8	38.61 %
SOX21	20.22 %
RXRG	29.67 %
BMP7	25.31 %

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