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Development of a genetic over-expression system for the freshwater sponge Ephydatia muelleri

Joe Walsh
University of Richmond

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by

Joe Walsh

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Introduction

Porifera, an ancient animal phylum, diverged from its sister protistan group *Choanoflagellata* to form one of the first branches in the metazoan phylogeny (Nosenko et al., 2013). As the earliest diverging metazoans, the sponges have maintained the same basic physiologies and gene families as found in their metazoan relatives (Leys et al., 2012; Srivastava et al., 2010). With similar structure and function to the earliest metazoans as shown by Precambrian fossil records, extant sponges can shed light on the development of animals and animal-specific gene networks (Zongjun et al., 2015). The genomes of sponges are extraordinary because they possess an extensive set of animal-specific genes despite the lack of major animal traits (e.g., organ systems, tissue) (Rivera et al., 2013). In later animals, these genes expanded and diversified as increasing levels of complexity emerged (Holstein et al., 2010; Funayama et al., 2010). Sponges possess genes that are crucial for setting up important developmental pathways for growth, body plan formation, stem cell maintenance, differentiation, cell specification, adhesion and innate immunity (Hill et al., 2010; Riesgo et al., 2014). Given sponges' evolutionary importance, we can study how these fundamental elements interact and function in sponges and begin to deduce how they were modified, leading to more complex animals.

The freshwater sponge, *Ephydatia muelleri,* is an emerging basal animal model system to study the genetic and developmental regulatory networks that played fundamental roles in the evolution of animals. *Ephydatia* are prevalent throughout all major land masses allowing for worldwide access to the sponges' preserved early metazoan gene interactions (Rivera et al., 2013). Defined methods for typical growth and development in a laboratory setting make *Ephydatia* an appealing organism for functional genetic studies. Totipotent stem cell containing

gemmules are easily harvested from lakes, rivers, and streams. Gemmules can be stored long term at 4°C (and even frozen at -80°C stored in DMSO like other animal cell types), hatched, and grown into healthy, fully functional adult sponges in small volumes of specialized media (Rivera et al., 2011). *Ephydatia muelleri* tissue is transparent allowing for easy visualization for whole organism and cellular expression studies. The ability to perform targeted gene expression studies in *Ephydatia* will allow us and others to study both basic developmental processes fundamental to all animals, and to employ a model system that will inform studies on disease-related processes associated with multicellularity.

With a recently sequenced *Ephydatia* genome and transcriptome, as well as methodologies for knocking down gene expression (Rivera et al., 2011), a necessity for future genetic studies is to be able to induce targeted gene expression in cells of this organism. Previous RNAi studies in sponges have shown an ability of cells to take up nucleic acids from their growth media resulting in phenotypes due to decreasing expression levels of developmental transcription factors (Rivera et al., 2013). Complimenting this work with over-expression of developmental transcription factors and analysis of the resulting phenotypic changes would give us insight to how these key animal conserved genes function. Over-expression systems produce essential data to compliment RNAi studies investigating gene functions at the base of the metazoan phylogeny.

The aim of this work is to develop an over-expression system for *Ephydatia muelleri*. Preliminary work by Klaske Schippers (Schippers, 2013) showed sponges were induced to transcribe reporter genes driven by the widely used cytomegalovirus promoter (CMV). Building off Schippers work, newly identified native *Ephydatia* promoters were identified from the genome and verified to drive expression of reporter genes. Validated over-expression vectors and

transfection technology will allow researchers around the world who are working with this organism to ask questions about cell specification, gene regulation and animal development.

Methods

Identification of putative promoter regions

Genes in the *Ephydatia muelleri* genome were annotated in collaboration with the S-Four consortium using beta versions of the Apollo annotation tool and the GBrowse genome browser. Gene annotations were conducted using the search function in the GBrowse genome browser, the annotation function of Apollo as well as BLASTN, BLASTX, and TBLASTX in NCBI's non-redundant database. Results for each scaffold were saved in Apollo and immediately available for search in GBrowse software. There is not a function in this software to BLAST against the *Ephydatia muelleri* genome. All annotations were conducted for a scaffold and therefore options for which genes were available were limited. The *Ephydatia muelleri* genome was searched for genes with promoters of interest using the GBrowse genome browser. The genome was in draft form, so it contained many unassigned regions. If gene models/predictions were found they were searched in a local genome file using the CLC main workbench software (Qiagen). Target genes were evaluated for sufficient base pairs (~1kb) upstream of the gene. The promoter regions were saved in another file and primers were designed to amplify putative promoter regions (For EF1a F:ACCGGTCATTAATCGAATTCTGCAGGGGAAACTAGAACATCATGTAACAA and R:GACCGGTGGATCCCGGGCCCGGCGCATGCGATTTTTCTAAGC, For Piwi F:TAGTCCATTCGAATTCTGCAGCCTCGCGCAATGTAATCTTT and R:GACCGGTGGATCCCGGGCCCGGAAGCCTGGAGGGAGGTAAC , For DAD F:ATGAGCTCATATGATTAATAGATCTGA and R:GCGCGGCCGCGGATCCCGGGCTGCA).

Genomic DNA isolation

DNA was isolated using a modified protocol from the Wizard® SV Genomic DNA Purification System (Promega). Stage 5 sponges were harvested by cutting a p1000 pipette tip about 1mm and scraping the sponge tissue of 10+ sponges off about a 12 well plate. Sponge tissue was digested overnight (16 - 18 hours) at 55°C, lysed, and genomic DNA was isolated according to the Purification of Genomic DNA from Mouse Tail Clippings or Animal Tissues using a microcentrifuge. Alternatively, genomic DNA was also isolated using a modified CTAB Genomic DNA purification. Scrapped sponge tissue was scraped as described above and put in 350µL of 2x CTAB buffer (Hill et al., 2010). Sponge tissue was homogenized with a pestle and an additional 350µL of 2x CTAB buffer was added. Proteinase K was added to a concentration of 280mg/mL and the solution was digested for an hour at 65°C vortexing frequently. After digestion 500µL of 24:1 chloroform to isoamyl alcohol solution was added for 5 minutes. The contents were centrifuged at 13,000 rpm for 2 minutes. The top layer of the liquid was transferred to a new tube with 600µL of isopropanol. The tube was placed in the -20°C freezer overnight. The tube was centrifuged for 15 minutes at 13,000 rpm and the supernatant was poured off. The pellet was washed twice with 450µL of 70% ethanol. The washes were poured off and the sample was air dried for 20 minutes. Elution buffer (Qiagen) was added to the tube and incubated for 2 hours at room temperature. DNA quantities were measured using a Nanodrop.

Promoter amplification

Promoters were amplified from 100 - 200ng of *Ephydatia muelleri* genomic DNA using primers described above. Piwi and Ef1α were amplified using an adjusted protocol from GoTaq® Green

Master Mix (Promega) for 25μL using the following protocol: 1) 94°C for 5 minutes; 2) 94°C for 30 seconds; 3) 56°C for 30 seconds; 4) 72°C for 70 seconds; 5) Repeat steps 2-4 thirty-four more times; 6) 72°C for 5 minutes; 7) The samples were cooled to 4°C for storage. DAD was also amplified using an adjusted protocol from GoTaq® Green Master Mix (Promega) for 25μL using the following protocol: 1) 94°C for 3 minutes; 2) 94°C for 45 seconds; 3) 58°C for 30 seconds; 4) 72°C for 2 minutes and 45 seconds; 5) Repeat steps 2-4 thirty-two more times; 6) 72°C for 5 minutes; 7) The samples were cooled to 4°C for storage.

Constructing over-expression vectors

The DAD promoter in our constructed plasmid drives the expression of an intron containing GFP from the pPD187.49 plasmid courtesy of Dr. Andrew Fire. The GFP was amplified (with promoters F:TATTAATAGATCTGAGCTCGGATCCCGGGAGACCCAAGCTTGGTA and R:AGCGGCCGCGCTAGCGCTACTGCAGTCGACTAGGGCCCTCTAGCGAAT) under the adjusted protocol from GoTaq® Green Master Mix (Promega) for 25μL as follows: 1) 94°C for 3 minutes; 2) 94°C for 45 seconds; 3) 54°C for 30 seconds; 4) 72°C for 1 minute and 15 seconds; 5) Repeat steps 2-4 thirty-five more times; 6) 72°C for 5 minutes; 7) The samples were cooled to 4°C for storage. The pEGFP-N1 vector and GFP PCR product were digested with BglII and NotI at 37°C for one hour following the NEB restriction enzyme protocol using buffer 3 and BSA in a double digest. The digests were run on a gel and the band of appropriate size was extracted following the MinElute™ gel extraction kit (Qiagen). The GFP and plasmid backbone were ligated together in a 3:1 insert to vector ratio using the T4 DNA ligase protocol. The 20μL reaction was run at room temperature for 3 hours. The ligation was transformed into DH5α

competent cells, plasmid prepped using the QIAprep® Spin Miniprep Kit (Qiagen), and digested with BglII and AseI restriction enzymes and run on an agarose gel to confirm size. This new plasmid pIGFP was sequenced (with the primer: GGCGTGGATAGCGGTTTGACTCAC) and confirmed to contain the correct GFP insert.

To insert the DAD promoter into this new plasmid, pIGFP, the frozen stock was grown overnight in LB broth with ampicillin and plasmid prepped using the QIAprep® Spin Miniprep Kit (Qiagen). The plasmid and promoter were cut with AseI and XmaI in a NEB double digest in buffer 2 at 37°C for 5 hours. The promoter was purified using the QIAquick® PCR Purification Kit (Qiagen) and the backbone was run on an agarose gel, cut out and purified using the QIAquick® Gel Extraction Kit (Qiagen). The DAD promoter was ligated to the pIGFP backbone in a 3:1 insert to vector ratio using the T4 DNA ligase protocol. The 20μL reaction was run at room temperature for 3 hours. The ligation was transformed into DH5α competent cells, plasmid prepped using the QIAprep® Spin Miniprep Kit (Qiagen), and digested with the single cutter StuI and run on an agarose gel to confirm size. This new plasmid pIGFP was sequenced (with the primer: GTGACATGGGACGCTTCCACAAAG) and confirmed to contain the correct DAD promoter insert.

The promoters for EF1α and Piwi as well as pEGFP-N1 were cut with AseI using NEB buffer 3 at 37°C for 2 hours in a 50μL reaction. The products were purified using the QIAquick® PCR Purification Kit (Qiagen). The products were digested with XmaI using NEB CutSmart® buffer at 37°C for 2 hours in a 50μL reaction. The promoters were purified using the QIAquick® PCR Purification Kit (Qiagen) and the backbone was run on an agarose gel, cut out and purified using the QIAquick® Gel Extraction Kit (Qiagen). The EF1α and Piwi promoters were ligated to

the pEGFP backbone in a 3:1 insert to vector ratio using the T4 DNA ligase protocol. The 20µL reaction was run at room temperature for 3 hours. The ligation was transformed into DH5α competent cells, plasmid prepped using the QIAprep® Spin Miniprep Kit (Qiagen), and part of the original colony was used in a colony PCR that confirmed our insert in each vector. The 20µL reactions were adjusted from the protocol of GoTaq® Green Master Mix (Promega) using the following protocol: 1) 94°C for 5 minutes; 2) 94°C for 30 seconds; 3) 56°C for 30 seconds; 4) 72°C for 70 seconds; 5) Repeat steps 2-4 thirty-four more times; 6) 72°C for 5 minutes; 7) The samples were cooled to 4°C for storage. These new plasmids, pEGFP-EF1α and pEGFP-Piwi, were sequenced (with the primer: CGAACGACCTACACCGAACT) and confirmed to contain the correct EF1α and Piwi promoters.

ptdTomato-CMV was constructed from removal of the ttC7B gene from a CMV and tandem tomato fluorescent protein containing plasmid (ttC7B-tdTomato) provided by Dr. Omar Quintero. The restriction enzyme XhoI, a double cutter, cut out the gene ttC7B. The results were visualized and the desired band was purified using the QIAquick® Gel Extraction Kit (Qiagen). The plasmid was ligated back together with T4 DNA ligase protocol for 3 hours at room temperature. The ligation was transformed into DH5α competent cells, plasmid prepped using the QIAprep® Spin Miniprep Kit (Qiagen), and digested with StuI and run on an agarose gel to confirm size. The new plasmid ptdTomato-CMV was sequenced (with the primer: GGCGTGGATAGCGGTTTGACTCAC) and confirmed to contain the correct juncture.

Collection and culturing of sponges

Protocols are described in (Schenkelaars et al., 2014). Sponges were collected during late winter from Prince William Forest National Park, VA. Samples were stored at 4°C in foil for

several months before use. Gemmules were picked from sponge tissue and washed with a 1.5% hydrogen peroxide solution diluted with Strekal's freshwater medium (Strekal et al., 1974) for 5 minutes. The solution was removed and the gemmules were washed 8 times with Strekal's medium and stored before use at 4°C wrapped in foil. Three to five gemmules were spaced apart from each other in a 12 well plate on a glass 22mm cover slip with each well containing 2 mL of Strekal's medium. Sponges were cultured in the dark and newly developed juveniles hatched from gemmules for two to three days before their Strekal's medium were changed (after attachment to plate was confirmed) on a daily basis until further use.

Transfection of tissue

Methods adopted from Lipofectamine™ 2000 (Life Technologies) plasmid DNA transfection and prior work from Schippers (2013). Sponges were hatched for 2 - 3 days until stage three (Funayama et al., 2005). Media was changed to 1mL M-med (Funayama et al., 2005). Vector (800 ng) was added to M-med (50μL) and 2μL of Lipofectamine™ 2000 was added to a separate 50μL of M-med for 5 minutes. The two tubes were combined and lipid/DNA complexes were formed for 30 minutes at room temperature. Complex solution was added (100μL) to each well. Sponges were treated for three days in the dark at room temperature.

For transfection of Cascade Blue® hydrazide (Life Technologies), medium was changed to 0.5mL of Strekal's medium per well and 2μL of Lipofectamine was incubated with 40μL in purified water before being combined with 60μLof 10mM Cascade Blue® hydrazide. The remaining protocol was held constant.

RNA isolation and qPCR or RT PCR analysis

RNA was isolated from sponge tissue using the RNeasy® Mini Kit (Qiagen). First all Strekal's medium was removed. The sponges were washed with 1mL of Strekal's medium three times to remove free plasmid from the tissue. Sponges were harvested by cutting a p1000 pipette tip about 1mm and scraping the sponge tissue of 3+ sponges off about a 12 well plate. The tissue was introduced into RLT buffer with 2-Mercaptoethanol. The isolation continued to follow the RNeasy® Mini Kit (Qiagen) protocol with the use of RnaseAWAY® (Molecular BioProducts) and the addition of the DNase I on column digestion for 40 minutes. RNA was quantified using a Nanodrop in preparation for cDNA synthesis.

For RT-PCR experiments cDNA was synthesized with SuperScript® III reverse transcriptase (Life Technologies). The cDNA was synthesized using 125ng of RNA per reaction and oligodT primers. The RT-PCR experiments were performed using 1μL of cDNA reactions were adjusted from the protocol of GoTaq® Green Master Mix (Promega) using the following protocol: 1. 94°C for 2 minutes and 30 seconds. 2. 94°C for 30 seconds. 3. 60°C for 30 seconds. 4. 72°C for 30 seconds. 5. Repeat steps 2-4 twenty-nine more times. 6. 72°C for 5 minutes. 7. The samples were cooled to 4°C for storage. For RT PCR of EGFP: F:CGTAAACGGCCACAAGTTCAG and R:TGCTCAGGTAGTGGTTGTCG or F:ACGTAAACGGCCACAAGTTC and R:AAGCACTGCACGCCGTAG. For RT PCR of the intron containing GFP: F:AAAATGTCAAAAGGAGAGGAATTG and R:TGTGGTGCAAATGAATTTAAGG. For RT PCR of EF1α: F:GCGGAGGTATCGACAAGCGT and R:AGCGCAATCGGCCTGTGAG. The samples were visualized in even volumes on an agarose gel.

For qRT-PCR analysis cDNA was synthesized using the SuperScript® VILO™ MasterMix (Life Technologies) with consistent amounts of RNA. SYBR® GreenER™ qPCR SuperMix (Invitrogen) was used with the Chromo4 thermocycler (BioRad). The reaction proceeded as follows: 1) 94°C for 3 minutes; 2) 94°C for 30 seconds; 3) 60°C for 30 seconds; 4) 72°C for 30 seconds; 5) Repeat steps 2-4 thirty-four more times. For qRT-PCR of DAD: F:GCTAAAGCTGGTGGATGCCT and R:ACACGAGATGAAGCCCGAAA. For qRT-PCR of Piwi F:ATCGCTCCATCCTAACGACC and R:ACCTTGATAGCCAGTGCAGG. For qRT-PCR of EF1α: F:GCGGAGGTATCGACAAGCGT and R:AGCGCAATCGGCCTGTGAG. The fluorescence threshold for calculations was selected while the samples were in the exponential growth phase of the reaction. These reactions were compared to serial dilutions of plasmid using the Opticon software to calculate relative initial concentrations. The qRT-PCR was run in duplicate and the values were averaged.

Fluorescent imaging

Images were taken with the Olympus BX61 microscope with a DG4 lamp and ProgRes® camera. Fluorescent images were taken under the TXRed, FITC or DAPI filters under the 10x objective.

Results

Testing transfection of Ephydatia muelleri

Ephydatia muelleri was transfected with Cascade Blue® (Fig. 1) using Lipofectamine™ 2000 to determine the efficacy of this transfection reagent. The control sponge shows no evidence of the fluorescent macromolecule Cascade Blue®. There is some autofluorescence

localized to the gemmule, however it is significantly lower than the Cascade Blue® present in the treated sponge tissue. Sponges treated with Lipofectamine™ 2000 reagent had fluorescence throughout the sponge tissue and from microscopic observation, appeared to be present in all cell types. FuGene® HD (Roche), FuGene® HD (Promega), Lipofectamine™ LTX (Life Technologies), X-tremeGENE HP (Roche) and Lipofectamine™ 3000 (Life Technologies) were also tested as transfection reagents. Efficiency of the reactions varied and Lipofectamine™ 2000 was selected due to its consistency.

Ephydatia muelleri promoter driven expression

Promoters for EF1α, DAD, and Piwi were successfully identified from the *Ephydatia muelleri* genome (Fig. 2). These new promoter regions were amplified, ligated into new expression vectors (Fig. 3) and partially sequenced to confirm the correct sequence and orientation.

For each gene whose promoter was cloned into expression vectors, we identified the overall gene expression levels over the life stages of *Ephydatia muelleri* by using qRT-PCR (Fig. 4). The results show that each identified gene is expressed throughout each of the developmental life stages, confirming that the promoters for these genes must drive gene expression across many developmental stages.

Over-expression vector driven expression in Ephydatia muelleri

Our newly constructed expression vectors were transfected into stage 3-5 *Ephydatia muelleri* (Fig. 5). RNA was isolated from the tissue and analyzed through RT-PCR (Fig. 6). Our vectors drove expression in the presence of Lipofectamine™ 2000 for the promoters CMV, EF1α,

DAD, and Piwi. The vectors didn't drive expression without the Lipofectamine™ 2000 reagent suggesting that a transfection reagent is necessary for DNA plasmids to enter sponge cells.

Ephydatia muelleri transfected with the expression vectors pEGFP-N1 and ptdTomato-CMV were visualized under a fluorescent microscope (Fig. 7). Live *Ephydatia* sponges have high levels of autofluorescence in their tissues regardless of whether or not they are transfected with GFP (Fig. 8) or tdTomato (data not shown). We see the highest levels of autofluorescence in the gemmule. Exposure was adjusted to account for the autofluorescence in control treated sponges, which made evaluation of the promoter driven expression constructs difficult as the images were relatively dark after subtracting background (Fig. 7). Close examination of images (Fig. 7) indicate some evidence that tdTomato expression is driven in the tissue of 7F compared to 7E and that GFP expression is driven in 7H compared to 7G. Nonetheless, technical limitations made the statistical and complete evaluation of the results nearly impossible. We were unable, due to high levels of tissue and gemmule autofluorescence to confidently use this methodology (i.e., fluorescent microscopy) to assess expression of the constructs.

Discussion

The earliest group to branch from the metazoan lineage was the sponge (Porifera). For this reason, sponges are an important system to study the development of early animal gene networks. Their genetic networks may provide insights into early animal evolution. To study these potentially homologous genes in ancient animals, knocking down expression of key genes and overexpressing key genes are essential methods of study. The freshwater sponge, *Ephydatia muelleri*, is a readily available organism to study genes in Porifera. RNAi methods of gene knockdown have been developed for *Ephydatia muelleri* (Rivera et al., 2013) but, over-expression methodology is a necessary, but yet to be developed, tool to infer gene function. By

adapting over-expression methodologies from other animal systems, I attempted to develop the first overexpression system for the freshwater sponge *Ephydatia muelleri.*

The native *Ephydatia muelleri* promoters EF1α, DAD, and Piwi were identified and isolated from a draft genome. EF1α or Elongation factor 1 alpha is a commonly used housekeeping gene involved in translation that has been used in sponges to normalize data due to its constitutive expression across all developmental stages (Rivera et al., 2013). Using the EF1α promoter for expression of other genes allows for high levels of expression to evaluate phenotypic change and deduce functions of genes. DAD, DAD1 or defender against death 1 is an inhibitory protein for apoptosis and is a constitutively expressed housekeeping gene (Nakashima et al., 1993). DAD was cited for being expressed at high levels throughout all tissues in humans and the gene is highly conserved with homologues present in *O. Sativa, C. elegans* and many other organisms (Tanaka et al., 1997). The start codon and transcript of DAD are not known in *Ephydatia muelleri* as this gene was annotated by members of the Four-S consortium. The isolated promoter for DAD does drive expression in sponges, indicating it does contain key elements of the promoter region. Further work needs to be done to evaluate the coding portion of DAD in relation to the isolated promoter. Piwi is known to be expressed exclusively in sponge stem cells (Funayama et al., 2010). By driving expression with a stem cell specific promoter, future experiments will be able to express target genes only in stem cells. This method could be instrumental in studying cell differentiation in *Ephydatia muelleri.* The Piwi promoter has most of Piwi's 5' UTR which may cause unexpected protein localization. There needs to be further work as to the function of Piwi in sponge genetic networks and where the protein is localized in cells. Nearly all of the upstream region to Piwi that was available was included in the promoter. EF1α, DAD, and Piwi are expressed in each development stage in *Ephydatia muelleri*. The

identified promoters should drive the target gene of interest's expression throughout development of *Ephydatia muelleri*. The ability to drive expression of a gene of interest in each stage is essential to study the development of sponges and the genes involved.

The CMV promoter is a widely used promoter and was suggested to drive expression in sponges (Schippers, 2013). CMV, EF1α, DAD, and Piwi drove mRNA expression in fully grown juvenile *Ephydatia.* The DAD promoter and the CMV promoter drove mRNA expression of the intron containing GFP from the pPD187.49 plasmid. The pPD187.49 GFP wasn't spliced in the sponge tissue as agarose gel analysis showed GFP at its full length of 700 bp instead of the spliced GFP at 500 bp. Any protein resulting from this transcript wouldn't be functional. So even though expression was possible, processing of GFP did not take place. The CMV promoter already exists in a non-splicable form of GFP but, the DAD promoter would need to be moved to a different vector or the GFP would have to be exchanged for DAD to drive expression of a functional GFP.

It was confirmed by RT-PCR analysis that the CMV, EF1α, DAD, and Piwi promoters drive expression of GFP. Further, the plasmid ptdTomato-CMV was transfected into a human cell line and results were visualized (results not shown) by fluorescent microscopy. Results confirmed that our plasmid induced detectable expression of tdTomato. These results confirm that our expression constructs are able to drive gene expression in transfected cells. After expression of a gene of interest is confirmed it is important to know what cell types are expressing the gene of interest and how efficient expression is in the tissue. When visualized for protein expression, our fluorescent protein didn't reach high levels above background fluorescence that would allow us to consistently evaluate cell type expression patterns. Preliminary work from Schippers (2013) suggested that detectable fluorescence was transient

and difficult to replicate. In the experiments conducted here, there was some evidence of different levels of fluorescence from replicate to replicate. Fluorescence in the sponge tissue furthest from the gemmule (in the growing pinacoderm) of the treated sponges was observed to be higher in several samples compared to control sponges. This indicates that some cells may have been expressing GFP at higher levels and the brightness could have been due to GFP expression and not only due to tissue density (given that the cell density is low in the growing pinacoderm). However, due to native autofluorescence in all live *Ephydatia* tissue, this result wasn't consistent or replicable enough to conclude that GFP was expressed at high levels. When we tested the sponge for autofluorescence we found high levels of autofluorescence across the visible spectrum. In the far-red there was considerably less autofluorescence while the red had the highest level of autofluorescence followed by green then blue with significantly high levels. The far-red fluorescent proteins were not used because they are substantially less bright than GFP or tdTomato. Due to issues visualizing GFP and tdTomato, far-red fluorescent proteins (mPlum) were not studied further with the concern that their expression wouldn't be above autofluorescence either. Thus, while we could demonstrate that our vectors drive over-expression of target genes (i.e., GFP, tdTomato), technical limitations prevented evaluation of cell and/or tissue type expression of the target gene.

To assay for protein expression western blots were conducted for GFP according to existing methods for sponge tissue. After repeated trials GFP protein was not detected from the sponge samples. This indicates that the production of GFP protein production was either not efficient enough to detect above background or that in spite of the fact that GFP mRNA was made, the protein was not translated. Furthermore, β-gal expression experiments were

inconclusive; further optimization is needed to evaluate reporter gene expression by cell type in the sponge tissue, where overexpression is driven.

With proven transfection technology and validated promoters, increasing efficiency is the next step to develop an overexpression system. Recently the CRISPR methods have come to the forefront of molecular biology for their potential efficiency (Gilbert et al., 2013). This system requires transfection technology for the model organism as well as knowledge of promoters to enhance protein expression (Perez-Pinera et al., 2013). This project has developed the background knowledge to develop the CRISPR overexpression and CRISPRi systems. Future work in developing an overexpression system in *Ephydatia muelleri* should focus on the CRISPR system.

Appendix

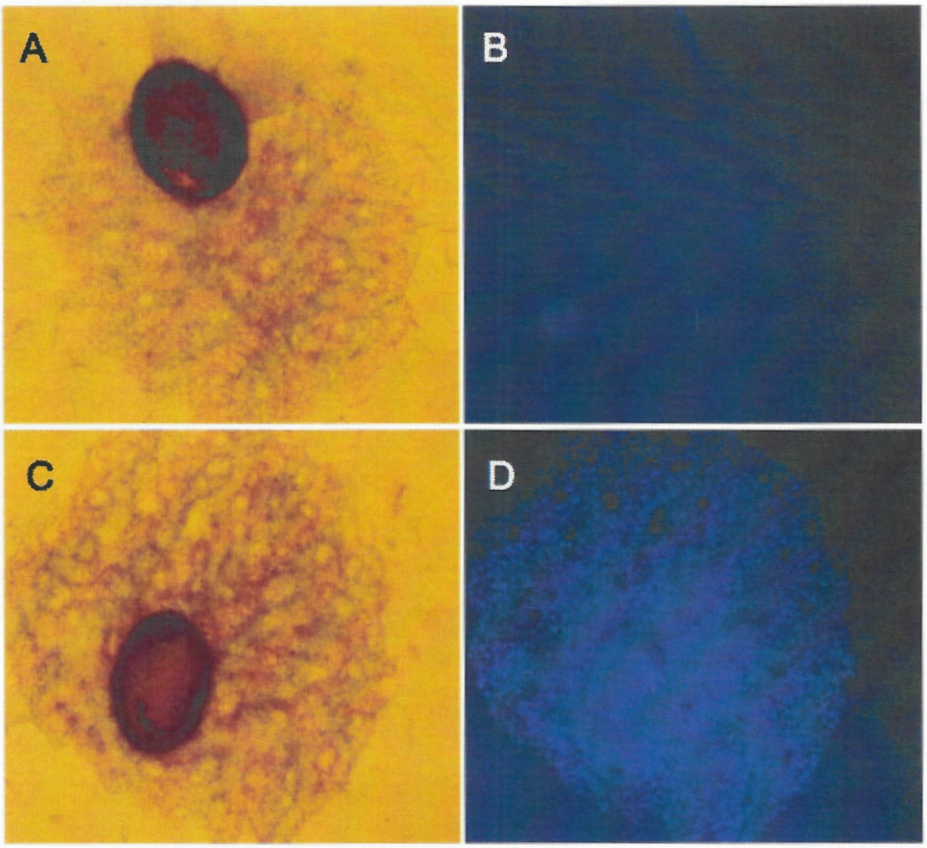


Figure 1. Transfection of *Ephydatia muelleri* with Cascade Blue®.
Control (A, B) and treated sponges (C, D) with Lipofectamine™ 2000 and Cascade Blue®. Bright field images (A, C) show the whole sponge. The fluorescent images (B, D) are visualized under a DAPI filter.

EF1α Promoter:

AATGCTGCTANNNNNNNNNNNNNNNNNNGGGAAACTAGAACATCATGTAACAAGTTACTTAACATTCTAC
CTCTTTGCTGGTGTATACAATTCAAGCTTTTCAGTTATGGTCTCGAGAACCATCTCCCATTGATTCATGA
TGATTCGAGGCAGGAGTACCCTTGCTCCTCGAAATCGCTCATCTCCATTTCGAAAGACACTGATAAGTAA
ATGCATAAGAGAAGGGACAAGGTTGTGTTTTTCTTACTTACTGTATAACTAGAGGCTTTTCAACAGGTTT
TTTGTATCTTGCAGATGCAGCAACTGCCCGCTGGAACTTCGTAGCGGGAGTTCCAAGCTAGGATGACTGA
ATACATTTAACTTTAGTGTATGTGAGCTAAACATTATTACCTCATTAGCTGGTCTTCTTGGCAGTAGTCT
TCGTTGGTATTGATAGCTATGTGCATAGTGAGCGGTAAATATTGCTATAAGACATGTGCGCTGAAGTTAC
TATATTGTACCGCCAATAATTTGGATACCTTATTTTCTTAAACTGCTCCCCTCCACTTGCAACATAGTAG
GATCCGGTCTTTAGGTCACCAATGTTCTGGATTCGCTGCCCACCATTAGGTGTATATATAGTTCTAACGG
CGACAGGGGCTTTTATGCTGCTCGTCACGCGTCCAAGGAAAGTAGCAAAGTCACGAACTTGACGGTCGTT
GACAACAAGAAGCTTTGGTTCAGCATTTACATCGCCATTGCGGTAAATATATATCTTCTTTGCAGGCTTA

GGTTCGTACTGGGTCATTACTGTGTTATCCATACACAATTCGCTTCACCATCCATTTGTGTTTACATGCG
TTTCCATGGTACCGAGGTCTTACTTATTTTTAGAATGTACCCTCTCAGGCCCATATAAATTTTTTTAAAA
TTAAGACGGTCAGAAGTGCATCATCCACGTGTTCAAGTGCTTAGAAAATTCGCATGCGCAAAAGAACATT
CCTTTCAGAGCGCGTGTACGGTTGACTTAAGTTGGTCGGACTTTTGATCTGAAAACATCGGAACGTCTCT
GCTACTTTTTGGTGAGTGGTAGCTGCGGTATGACACAATTCTGCTGTCCTGTGTATAATGTCACTGTGAT
TTTACAATGCTTCGACTTATCCTTTTAGCGCAAAAAATCAAAC**ATG**GGAAAGGAAAAGACACACATTAAC
ATCGTCGTCGTTGGCCATGTCGACTCTGG

DAD Promoter:

AGAGAGGGTACTCCTTGTTGATTCTGAGTGGTTTGGTTATGGTCTGCTTGGAGCTTCTTTCTGAGTGCAG
GAACAGGTGGGGAAGGGGCTCTTTGGACTGGTGGTGCAGCCTGGTATGGTTGTGGTCGAGGGGGAACCTG
TTGCAGTGGTGGGGATGGTGGCAGCNTGAGNANNNNNNTTNTNNNGGTGCAACNGNTGCTTCAGAGCTGG
CACAGGAGGTGACGAAGCCCTGTACTGCTGCCCACCACCAATCGAACTATCCAGGGCAGACATTTGGTCA
AGATTTTGGTGCACCCTAGCCCCACTTGCTTCCTCCTGCTTCTTCGATGTTTCCATTGCACGTTTTCCCT
CTGCCTCCTTCTTTAGTNGNNCATNCCTNNNANGTNCNTNCNCTTCCCTCTTCCTTGCCATCTCCTGCTC
CCTCTTGTAGTTCTCAGCTATCCTGTCCTGCTCGCTCTTGATCCTCTCCAGTTCCTTTATGTCCTCCAGC
TTCTTCGCTTCCTTCTCCTTCTTCTTAATAGCTTCCCTCTCCTCCATCTGCTTCCTCAGGGACTCGCGAT
AGTCCTCAAGGCCTTCCTGCTGATTGTTTTCTTCAAGGACCAGTGTTGCAACGACGGGACCATTCTCCTT
GTTGGGGCTTGCTTCAGTGTTAGGCAATGGCTTGTCAGAAGGGGCTGTGTTCTCACCACGAGGGGACGTC
AGACTGCTCTTGAGGAGCTTCTCATTGTTGACCAACCTCATCTTCCTCAGGTCCGCTACAAGCCGCCCAC
GCTCATCTCTCATTGGTGCCCCACCCCCTCCTTTCCCCCAGGGATTGTACTCCTGGACTTCGTCGACCGC
CTTTGTCTTTCTGTGCTCCTCCTTCTCTTTCTTCTCCTCCATTTGCTGCTTCAACTGCAATCTGTAGGCA
CTTTTATCCTTCTTTGGGTCTTTGCCATTGGCAGCAGGGACCCCAGGTTGCTGTCGCCCGTTTCCCTGTC
TCCCGTCTCTCTCCCCCTCCCCCCTCTGGCTGTCCTGCACTTGACCCCCCTCATTTTCTGACTGTTGCCT
GTGTGGTGGGGCATAGCTGTCCCGTGGAAAAGATGGCGGATAGTAGGGATTTGGACCGTAGGTGTGAGGG
GGGTGGAGGGCAGGCGGGTAGAAGGGAGATGGCCACTGGGGCGGAGCAAATGCGCCGTACTCGGGCCTAT
ACATATAGGGAGGGTACTGAGGGGGATAGGGGTAGTGTGGGTCCAAAGGGGCAGTGGGATAATGATAGCT
AGGTCCTGGGTACTGGTGGTCACCAGGGCGTGCGTGATGAGGACCAGGCGGATTGTAAGGGTCAGGTGGT
GAATAAGGGCNNTGGCNNNAGGNTNTGNNTGGNNNTCATGTGGTGACCATCTCCTGTGAGGTGGTTCTTG
CGCCATCCTCCCTTCCGCTTTCCGTTGAGACTCCCTGTTTTCACGCAGTGGGGGTGGGGGTGAAGTGTCG
CTTCTCTGTGGCCTAGCCCTCGTCTGGACATTCTCATCTCCAGACACCTCTAAGGGTACTCCCTTTTCTC
TTTGCCTGGCCAANGTTTTGCTTCTTATCTGAGCCTTGAGCTCTTCAGCGTACTGCTTCTGCTTGTTCCG
CTTTTCCACGTCACTTTCTTTAACCCCTATAGCTGCCATCCCCATTAAAACAGGAGCGGATATTGACCTT
GTCCCCTTGGACACTTCCTGCTTCCCTGGAGAGAGGTGTGATGGTGGAGTTTTAAGGTTATCACGATTGC
CATTTTGTGCCATCTGAGAGTAGGCTCTTCCCTTCGCCCACTGCATCAAATGCACCTCATCATCTTCCCA
CCTCTGCGCAGCAAATGGTGGTCCAGGAGCGGGACCGATTGGAGGCCGTCCCGGACCATTTGGTGGTGGT
CTTGCATCATCATNGTCCCCCCAGCGCTTCCTTCGCTTGTAAAAGTCTTGATCCGCCGGGAGTTGATGGT
AGCGCCTTTCCTCTGCCAGCCTCCGTTCCTTGATCTCTAAATAAGGGTCTCTAGCAGCCACTCCCGATGG
AGGCCTGAAGCCAACATCTGCACCCGGTCCAACCTCGCCAATTTCTCTGTCTCTTTGCAGCGACATTTCT
CTTCGTAACTCTGCAATTGATGGTTTTCCAGAAGCTAGAATTTTTTCGTTTTTAATTTCAACCGTTTTTC
TTCCTTCCCTCAGACTGTCCGTTCTCGAACTACGAAAAACATTTATAGGACATGTGTGTGACATGGGACG
CTTCCACAAAGCGCGTACCTATTTTGTTGTGCATCTGAATACAGTCTCCCTTTCTCCTCGTTCAACCTAT
TCCTATGGGAAGCTATTAAGCTCTGCAGATCTACATTACTCATGTTTACCGATTTTTAAAAGTGGTAACC
TTGGAGCGCTCGTGCCGCAAATCGGGTTCTAATAACTCGCAACAAGGGCACGTTCGAAGGAAGAAACAAA

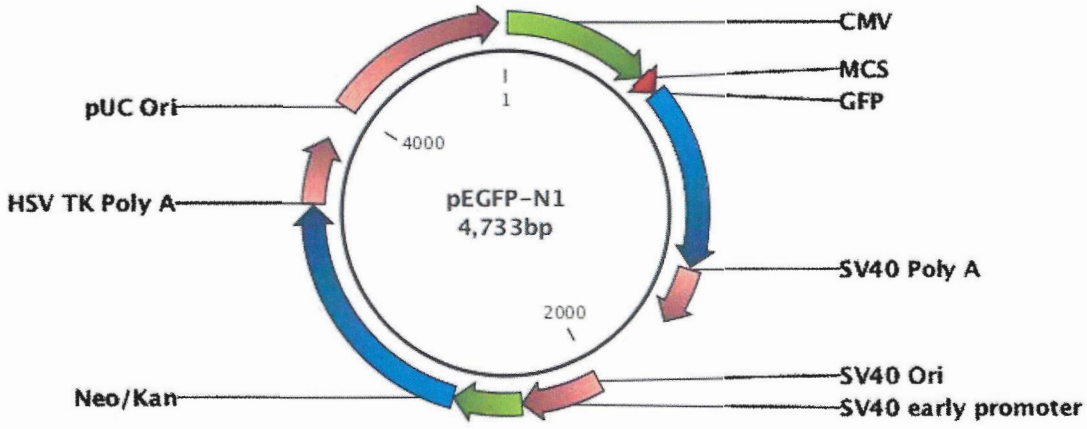
AGAAGGGCTGCGAAGAATGAGCTCCGAGAATCTTATCGTGATAGCTAAGCAGTTCATCAATCAGTATAGC
TACAACACGCCGCAGAAGCTAAAGCTGGTGGATGCCTACCT

Piwi Promoter:

TGATGGGGTCCGTACAGATTTTCTTGCCCCCCCCTCGCGCAATGTAATCTTTTTTCGTTTTGGCACGAAT
TACATGCCATACCAATCTGCTCCAGCGCCTTTCAGTGGCTGTACAAAGGAGAAATGTGTAGGGTACATTT
CCTTTCTTTGCTGACCTCTATGAAGTGTTCATTGGCAGATTATGGTTGTTATCGCATAGATAGTATCTAT
GGTTATCGTAACTTTGATAATGATATTTGCAACTTGGAGTTTGTGGTAGTCCGTGCTTGTGCATTATAAT
AATAATGTGTAGGTGTTCGTGTATTCTTTTAAAAAGTTTTGCCCACCGGCGCATGCGCGTATNCGNNNNN
GTGGAACGTTCGATGTTAGGCATTGCAGCTGTGGTAGCTGTGGAAGCTGTGAACCAACCGTGAAATCCTC
TACGGACGCTTAGTGGGAACTAACTGAAGCACGGAAGAGGTGACGAAAGTCCAGCGTAACACGTTAGTAG
ATAAAATACGAAAACAGGCGTGTGATAACTGCAAGACATAGGAAGTGCAGGGAAAAGAAGGGTGATTTGT
AAAAGGTTGGTCAAATCGTACCTTAGTTTACTCAGAACCCATTATTGTGCTCTGTGTAACGCTGTATATG
CTGAACCGTCCTTAGTGATGTGTCTGTCCGTAAACTTTTAAAAAGATTATCATTGTGTGTCGAACAACCA
GCTAACAGAAAAAATTTGGTTTTTATTTTTAACCCGTCTTCCATAAACTACGGCACCGTAGCTTTTTCCC
CGCTGTGAGGTGGGAACGCTGTCAAATATTCGGTGTCTTTTGTTGCTCATTAGTGCTCTTTAAATAGAAA
TAATCTTTTTTGCGCGCGTATTGTTAATTCTGCAATTTAGAACAGTTGAAGCCAATTAACCTCTTGTACG
CCTATTGTCTAAATAAATACATCGTTACCTCCCTCCAGGCTTCTCTCATCAAAAGACACACAACA**ATG**GC
AGACAAGGTACCCATAGCTC

Figure 2. Identified promoter regions from the *Ephydatia muelleri* genome.
The promoters identified in the genome draft of *Ephydatia muelleri* are shown above. Some bases have not yet been identified and are denoted with "N". The green highlighted portion was amplified as the promoter. The red highlighted section is the start of the 5' UTR. The start codon is bold.

A.



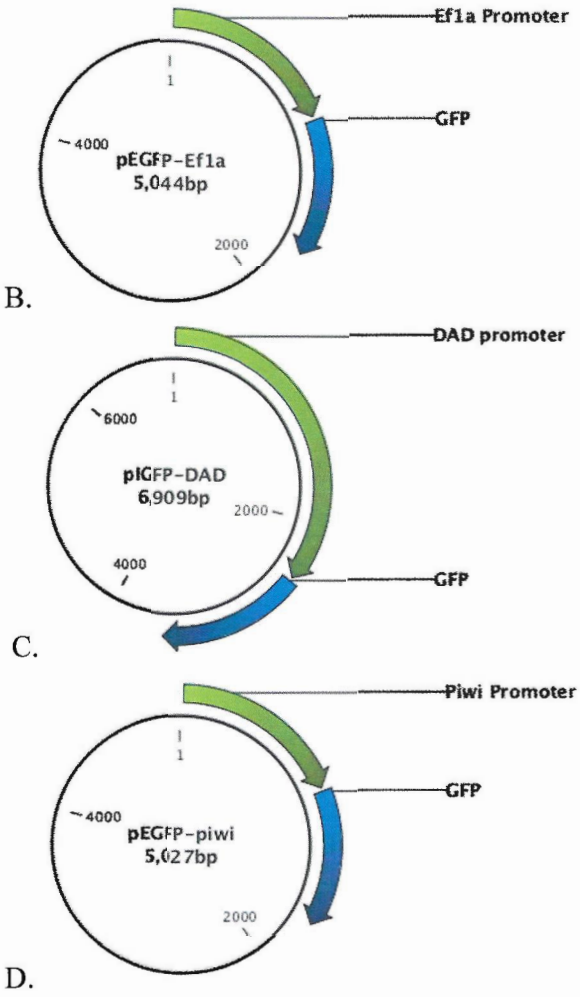
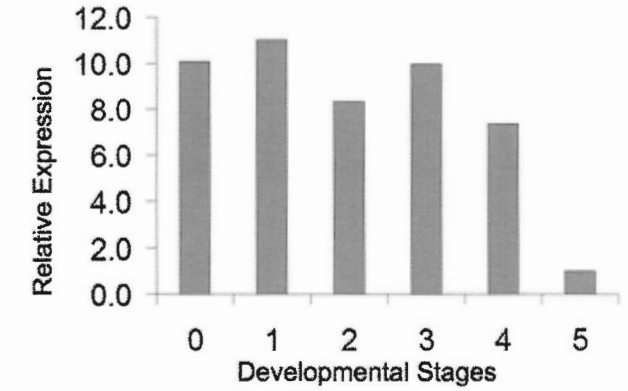
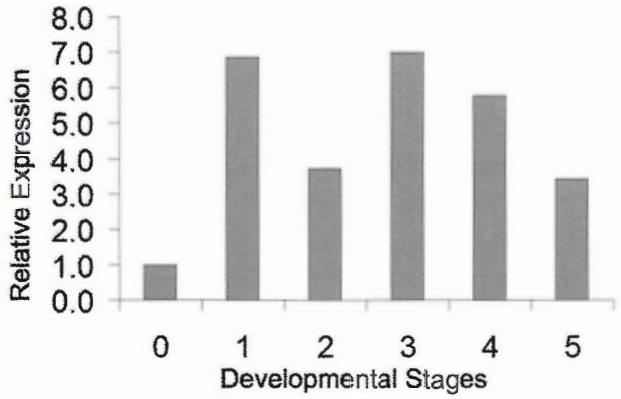


Figure 3. Vector maps of overexpression plasmids.
Visual representations of the vectors used to drive GFP expression in sponges. All plasmids have the same backbone as pEGFP-N1 (A). The only significant difference is the promoter in each vector (B, C, D) is exchanged with the CMV promoter.

A.



B.



C.

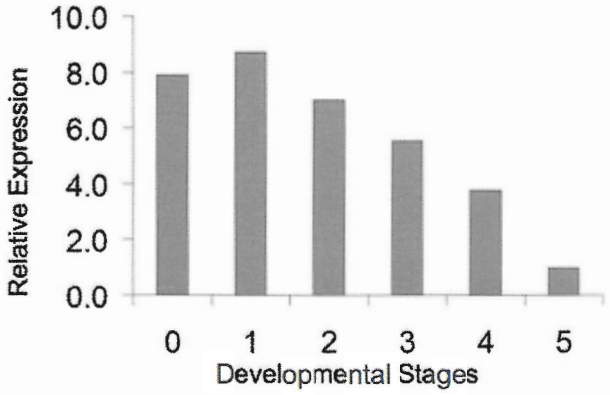


Figure 4. Graphs of promoter of interest expression over the life stages of *Ephydatia muelleri.*

EF1α (A), DAD (B), and Piwi (C) relative expression levels across all developmental stages of the freshwater sponge. The stage with the lowest expression level was adjusted to one and other stages indicate relative expression to that stage.

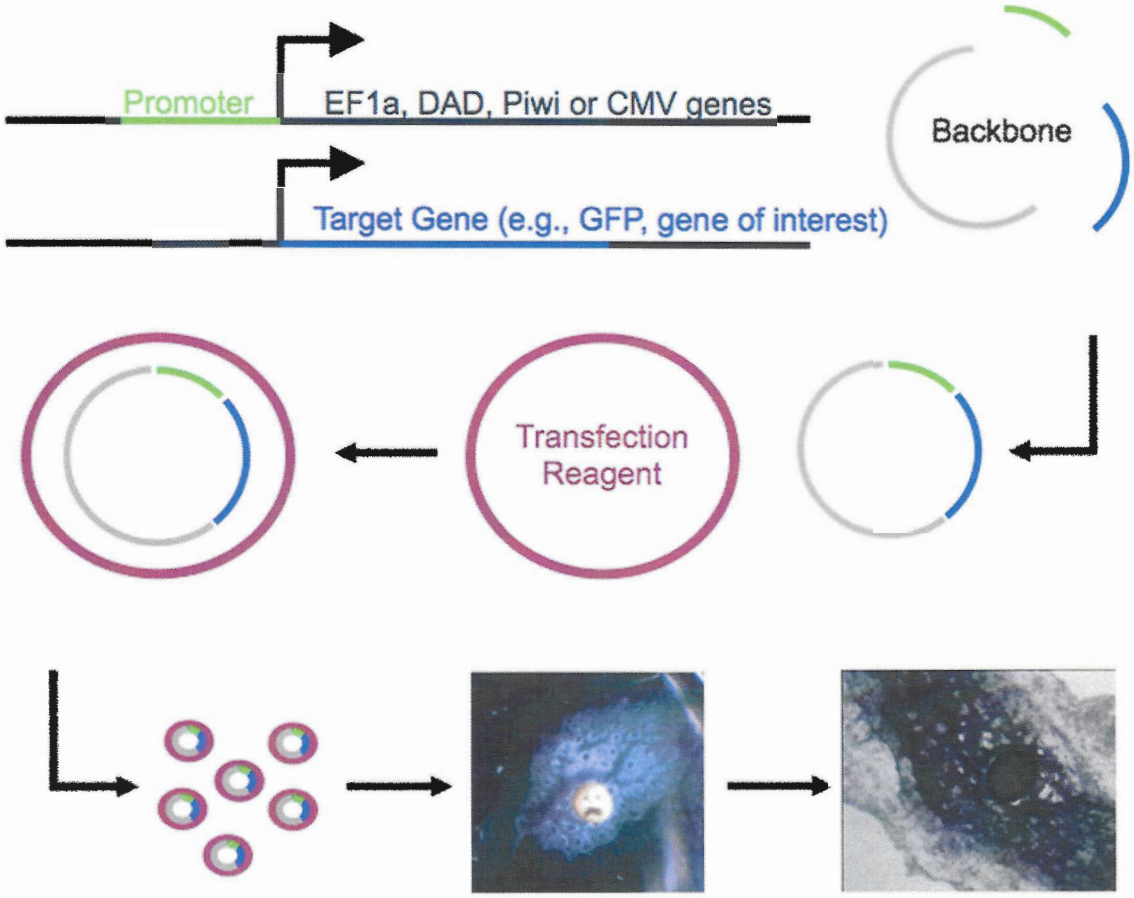


Figure 5. Over-expression schematic.
Identified promoters are amplified and ligated into an expression vector backbone to drive expression of a target gene. The plasmid is complexed with a transfection reagent that allows for the plasmid to enter the sponge cells and drive expression of our target gene.

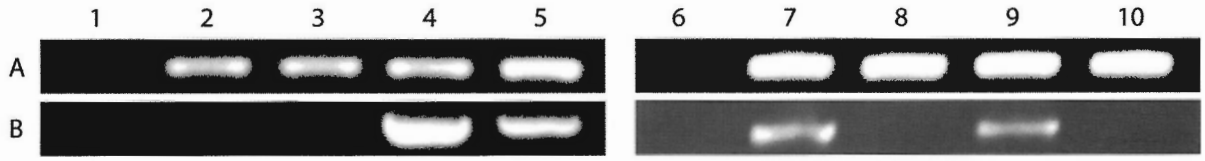


Figure 6. RT-PCR of EF1α and GFP in transfected *Ephydatia muelleri*.
The expression of GFP mRNA in *Ephydatia muelleri* driven by expression vectors. Row A is the EF1α loading control and Row B is GFP expression. Lanes 1 and 6 are negative controls. Significant expression is driven by the CMV promoter (Lane 4) compared to the plasmid control without Lipofectamine™ 2000 (Lane 2). As well as significant expression driven by the DAD promoter (Lane 5) compared the control (Lane 3), Piwi promoter (Lane 7) compared the control (Lane 8), and the EF1α promoter (Lane 9) compared the control (Lane 10).

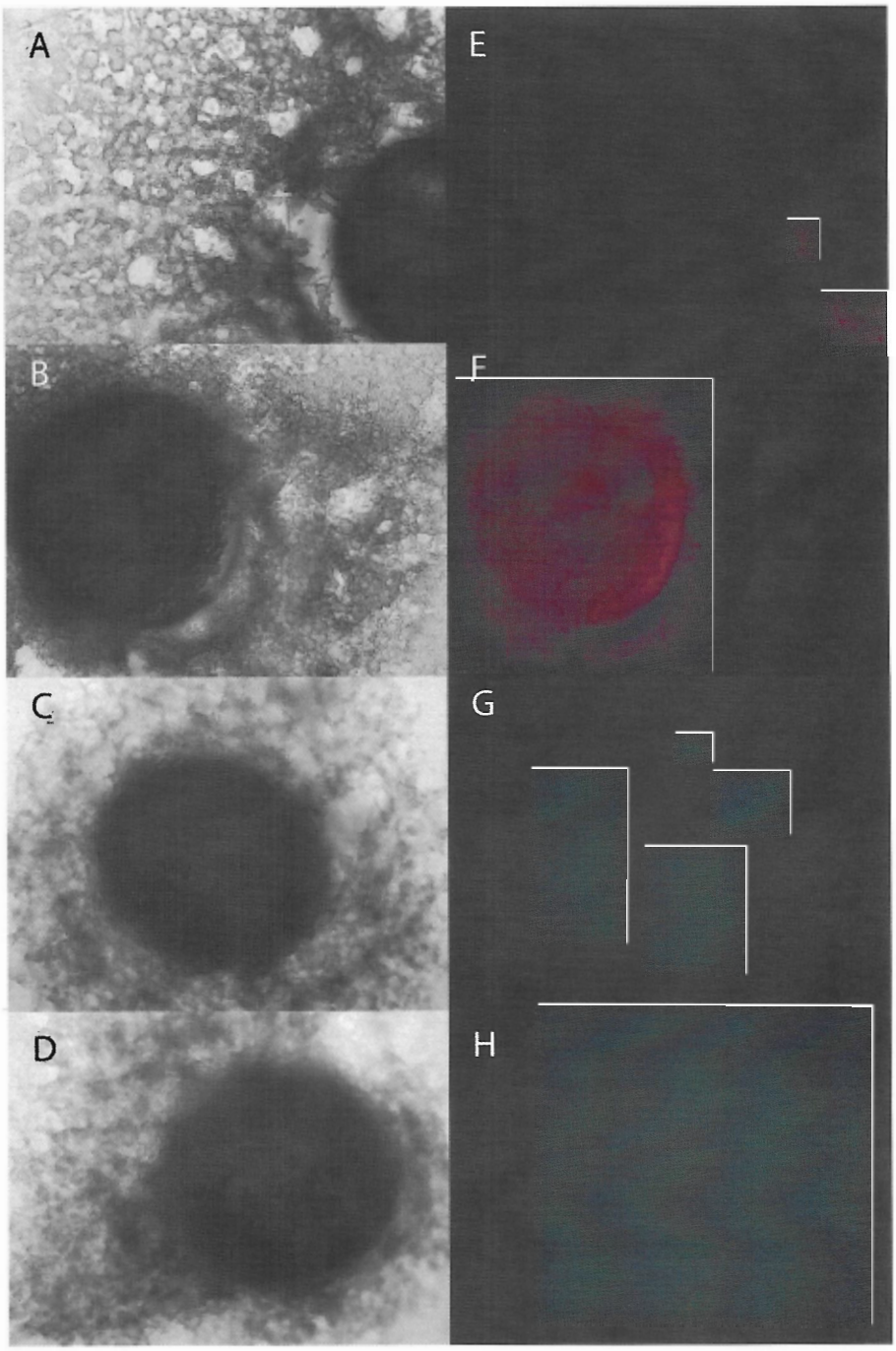


Figure 7. Fluorescence images of GFP and tdTomato expression in *Ephydatia muelleri*. Bright field images (A-D) and fluorescent images (E-H) of control sponges (A, E & C, G) and treated sponges (B, F & D, H) with ptdTomato-CMV (B, F) and pEGFP-N1 (D, H). Sponges were visualized under the TXRed filer (E, F) and the FITC filter (G, H).

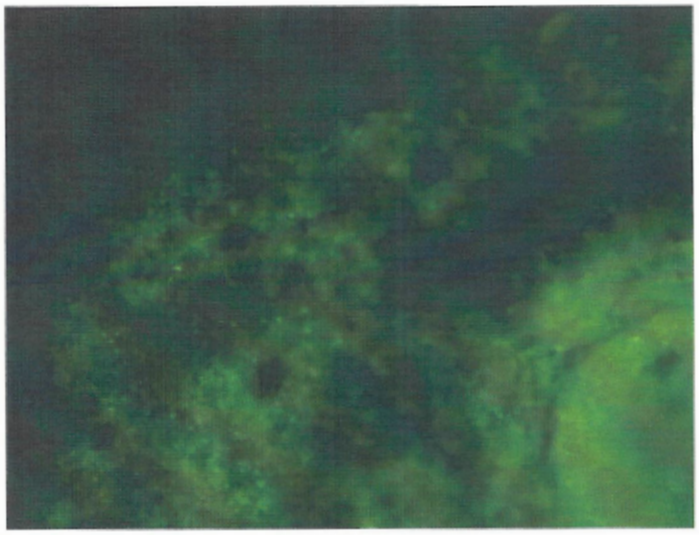


Figure 8. Autofluorescence of *Ephydatia muelleri* with the FITC filter.
Adult control sponges were visualized under the FITC filter.

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