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Towards understanding the function of an ETS-like gene in Nematostella vectensis: generation of a knockout mutant line and a transgenic reporter line

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Towards understanding the function of an ETS-like gene in *Nematostella vectensis*: generation of a knockout mutant line and a transgenic reporter line

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By

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Acknowledgements

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Abstract

Due to their unique phylogenic position as sister to Bilateria, Cnidaria are often credited with the utility of allowing for reconstruction of ancestral biology based on characteristics shared with bilaterians and other animals. This factor makes investigation into the nervous systems of cnidarians critical in understanding early neural evolution. Wamides, a class of neuropeptides, have been shown to play a regulatory role in life cycle transitions across many different species. The cnidarian specific Wamide neuropeptide, GLWamide, has previously been identified to play an accelerator role in the metamorphic timing of a specific species of sea anemone, *Nematostella vectensis*. However, the mechanism by which GLWamide signaling regulates developmental timing remains unknown. Preliminary data has established the ETS gene as a particular gene of interest. This transcription factor-encoding gene was identified as a downstream target for GLWamide signaling. Thus, an ETS-like transcription factor serves as a candidate molecule that controls life cycle transition upon the activation of GLWamide signaling in this sea anemone species. Here, we used CRISPR/Cas 9-mediated targeted mutagenesis to create ETS knock-out mutant lines in order to evaluate phenotypic differences that were induced by this partial loss-of-function gene. We established two heterozygous mutant lines. The first line carries an allele of a targeted transgenic cassette via meganuclease (I-SceI)-assisted integration, while the other carries an allele containing ETS site-specific DNA modifications induced through CRISPR/Cas 9-mediated transgenesis. In this line, we found two distinct mutation patterns within our ETS F1 samples that could potentially alter the protein synthesis occurring in the *N. vectensis* genome. In addition, in-situ hybridization was used to evaluate the expression pattern of ETS transcription factor during *N. vectensis* development. Early life cycle stages (namely the planula stage) demonstrated little to no expression. However, expression increased throughout later life stages, with endodermal expression seen in the tentacle budding stage, and endodermal and region-specific expression seen in the primary polyp stage. Upon analysis of the expression pattern seen, evidence suggests this transcription factor could be heavily involved in the tentacle formation and development processes in *N. vectensis*. Based on these results, we hypothesize that this ETS-like transcription factor could control life cycle transitions in *N. vectensis* upon activation of GLWamide signaling, though further research is required to verify our findings. The establishment of the two mutant lines discussed here will set the foundation for the generation of homozygous null mutants, which will be found in the F2 generations. These mutants will be examined for phenotypic differences in developmental timing, the pattern of tentacular morphogenesis, and/or the pattern of postembryonic cell type development to better assess the ETS gene function in *N. vectensis*.

Introduction

With the advancement of ongoing research in nervous system development in Metazoan evolution, the sister group relationship between Cnidaria and Bilateria within the phylogenic tree remains relatively unchanged[1]. Thus, Cnidaria, a group consisting of predominantly marine invertebrates such as sea anemones and corals, is considered to share a common ancestor with Bilateria (a group consisting largely of bilaterally symmetrical animals, including humans)[2]. This ancestor is thought to possess deeply conserved mechanisms of nervous system development as genomic data revealed that cnidarians possess a more complex genome than expected with surprising similarities to that of bilaterians[3]. The sister group relationship and the genomic similarities establishes cnidarians as the most closely related nonbilaterian animals (Figure 1)[4, 5]. Therefore, to better understand and reconstruct early events in nervous system evolution, comparison of the neural development between Cnidaria and Bilateria must be investigated[6].

One comparison that can be drawn is the presence of neuropeptides in both Cnidaria and Bilateria sister groups. Neuropeptides are evolutionary ancient molecules released by neurons that act as modulators or hormones to regulate a variety of biological processes, including behavior, physiology, and development[8,9]. They have been found to be present in all metazoan groups possessing a nervous system, and comparative genomic evidence suggests that several neuropeptides are shared across Bilateria and Cnidaria[8,9]. These conserved neuropeptides are thought by many to be early neurotransmitters in the evolution of the nervous system, making understanding their roles in neural development of great importance[9].

Neuropeptide signaling typically affects target cells by binding and activating specific G-protein-coupled receptors, which trigger G-protein-mediated activation or inhibition of downstream effectors[10]. The last common ancestor among Cnidaria and Bilateria had various small amidated peptides, including Wamide[11]. In cnidarians, external stimuli are directly translated into neuropeptide signals by chemosensory-neurosecretory cells releasing small peptides to regulate growth and metamorphosis, specifically GLWamide. GLWamide is a cnidarian-specific neuropeptide belonging to the Wamide neuropeptide superfamily[8,11]. Research has shown that GLWamide plays a modulatory role in determining metamorphic timing in cnidarians, but the mechanism in which this modulation occurs remains poorly resolved[8,9].

Nematostella vectensis, an estuarine sea anemone species, is one of the most important models for the study for cnidarian embryonic and postembryonic development, as well as neurobiology. Its genome contains an unexpected degree of genetic diversity[7], including numerous ancient neuropeptides. They are diploblastic animals, in that they possess two germ layers, consisting of an internal endoderm and an outer ectoderm[4]. They move through their life cycle relatively quickly, becoming adult polyps in approximately 12 weeks (Figure 2)[4].

Preliminary data suggests that, upon performing RNA-seq to generate transciptome data, an ETS-like transcription factor-encoding gene (Joint Genome Institute Nv gene ID 92405) was significantly upregulated in specific response to GLWamide signaling[9]. Thus, this gene was selected as the gene is of interest for our research. ETS transcription factors, belonging to the ETS gene family (Figure 3)[12,25], act as positive or negative regulators of the expression of genes (known as ETS targets) that are involved in various biological processes, including control of cellular proliferation, differentiation, hematopoiesis, apoptosis, tissue remodeling, angiogenesis,

and transformation[13]. In *N. vectensis,* the ETS gene was identified as a downstream target for GLWamide signaling.

Here, we used an ETS-like transcription factor as a candidate molecule that controls initiation of life cycle transition upon activation of GLWamide signaling. We established two heterozygous mutant lines to test the mechanism by which GLWamide signaling regulates developmental timing in *N. vectensis*. The first line, named ETS Kaede (transgenic reporter line), carries a mutated allele by using meganuclease I-SceI-assisted genomic integration. This line allowed us to visualize and monitor cells expressing the ETS reporter gene under the control of a gene-specific promoter during the life cycle transitions in *N. vectensis*[14]. The second line, the ETS line (knockout mutant line), carries an allele containing ETS site-specific DNA modifications induced through CRISPR/Cas 9-mediated transgenesis[7]. This mutant line will allow for the comparison of phenotypic differences of partial-knockout mutant animals- that is mutants that are heterozygous at the target gene loci, possessing both a mutated allele and a wildtype (unmutated) allele- seen in the first generation of this line (denoted ETS F1)- and wildtype animals. Further development of this line will also allow for comparison of phenotypic differences of full knockout, or null, mutants that are homozygous for the mutated allele at the target loci (denoted ETS F2). The purpose of these two lines is to assess the expression pattern of the ETS gene to determine its function, or lack thereof, in *N. vectensis* life cycle transitions.

	CRISPR/CAS 9 MUTANTS (KNOCKOUT LINE)	**MEGANUCLEASE I-SCEI MUTANTS (REPORTER LINE)**
PARENTS (PREVIOUSLY MADE) GENERATION	**ETS F0**; mosaic (i.e. some cells may be heterozygous, others may be wildtype or homozygous for mutant alleles)	**ETS Kaede F0**; mosaic (i.e. some cells may be heterozygous, others may be wildtype or homozygous for mutant alleles)
FIRST GENERATION (USED IN RESEARCH HERE)	**ETS F1**; heterozygosity (i.e. mutants with one mutated allele and one wildtype allele) of interest	**ETS Kaede F1**; heterozygosity (i.e. mutants with one mutated allele and one wildtype allele) of interest
SECOND GENERATION (FUTURE RESEARCH)	**ETS F2**; homozygosity (i.e. mutants with 2 mutated alleles) of interest	**ETS Kaede F2**; homozygosity (i.e. mutants with 2 mutated alleles) of interest

Table 1. The table illustrates the generations discussed of each line (knockout mutant line and reporter line), as well as their designated name and the mutants of interest in each generation.

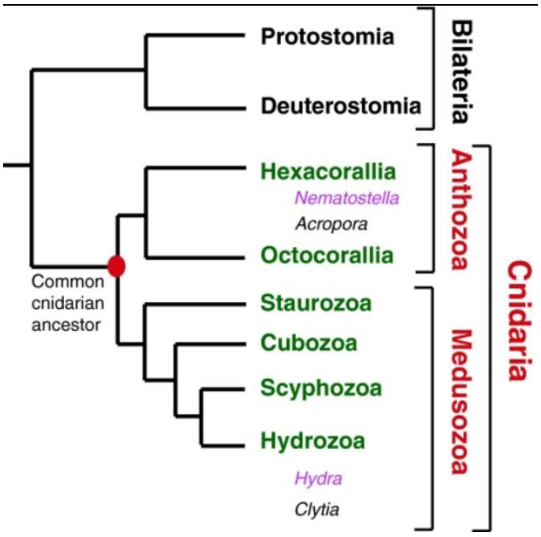

Figure 1. Phylogenic tree showing the sister relationship between Cnidaria and Bilateria and the common ancestor they are thought to share. It also indicates where *Nematostella* is relative to other tree relations[5].

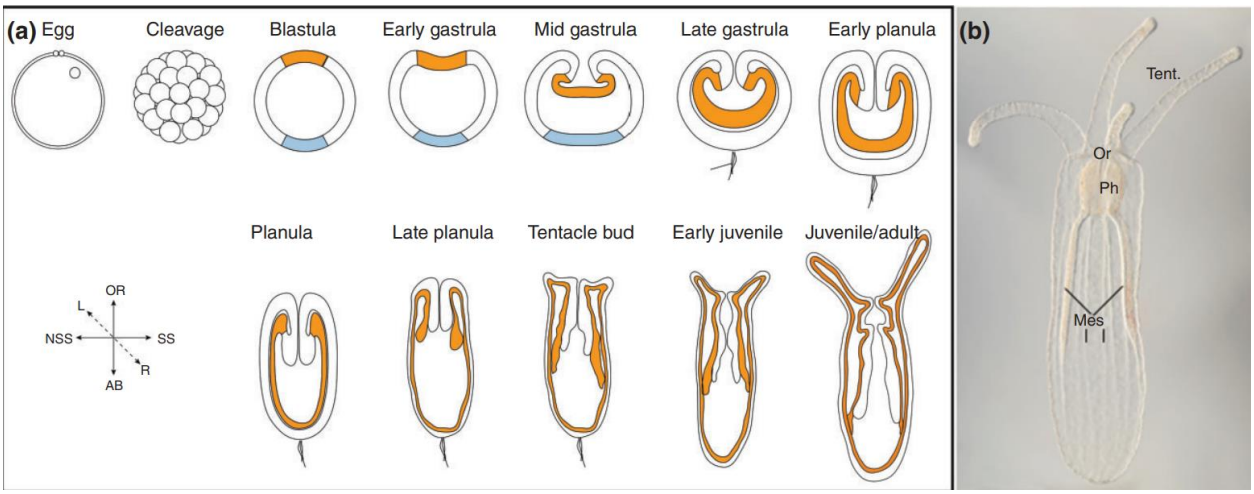

Figure 2. The life cycle stages and morphology in *Nematostella.* (a) *Nematostella* life/developmental stages where the endoderm is indicated in orange, the outer ectoderm is white, and the ectodermal domain is in blue. (b) The morphology of an adult polyp where the mouth region is indicated by "Or", the tentacles by "Tent.", the pharynx region by "Ph", and the mesenteries by "Mes".

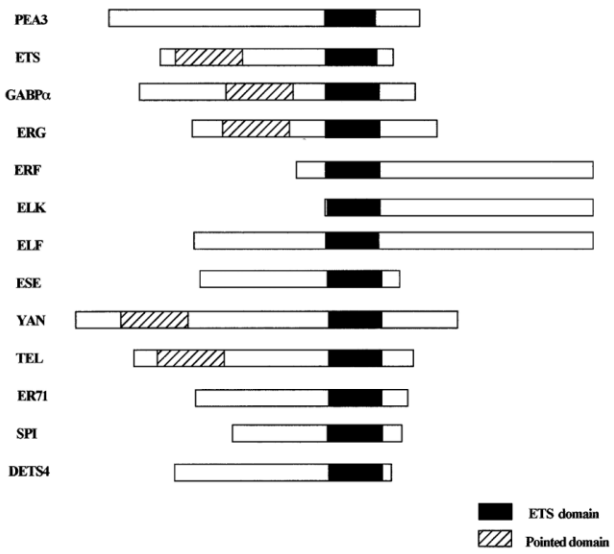

Figure 3. The ETS gene family of transcription factors can be divided into 13 subfamilies based on the sequence similarity of their ETS domains. Schematic diagrams of a representative from each ETS subfamily protein. Most ETS proteins have a carboxyl-terminal domain (black), while only some have a Pointed domain (striped)[25].

Materials and Methods

Animal Culture

Knockout Mutant Line

The ETS F0 parents were previously generated via embryonic microinjection using CRISPR/Cas9-mediated gene editing, creating mosaic ETS F0 knockout mutants, and raised to sexual maturity[5]. CRISPR/Cas9-mediated gene editing is achieved when CRISPR (clustered regularly interspaced short palindromic repeats) sgRNAs, or short-guide RNAs, for a specific gene are introduced into cells along with Cas9 (CRISPR associated enzyme 9)[24]. The Cas9 protein is then guided by CRISPR to the target gene and causes a double stranded break in the DNA, making this technique highly successful in creating gene mutations[24]. To create ETS F1 offspring, six identified mutant ETS F0 males were crossed with wildtype females (ETS ♂ x WT ♀) following the published spawning protocol[15]. The six groups of ETS F1 offspring were raised separately under the same conditions. Each group was fed *Artemia* (brine shrimp) every 2-3 days, kept at room temperature (around 20ºC) and raised in 1/3 seawater (salinity roughly 12%) that was cleaned out and changed upon feeding[15,16].

Transgenic Reporter Line

The ETS Kadae F0 animals were previously made by microinjecting fertilized eggs based on meganuclease (I-SceI)-assisted integration of a transgenic cassette cocktails. The key steps of the procedure included cloning and preparation of the transgenesis vector, spawning, digestion of

plasmid DNA, microinjection of the digested construct, and raising of selected embryos to sexual maturity (approximately 4-6 months)[14]. The F0 parents were mosaic transgenic and may or may not demonstrate germ line transmission. Thus, ETS Kaede F0 females were crossed with ETS Kaede F0 males to create ETS Kaede F1 offspring. ETS Kaede F1 offspring were raised together at room temperature in 1/3 seawater, which was changed weekly, and fed *Artemia* 1-2 times per week[15,16].

Genomic DNA Extraction

Genomic DNA was extracted from both the previously generated ETS F0 mutant parents and six groups ETS F1 offspring. The single ETS F0 female and 12 selected ETS F0 males were transferred to individual petri dishes containing a 2.43% MgCl in 1/3 seawater solution and placed on an orbital shaker for 15 minutes to relax tentacles for surgical removal of the tissue. After relaxation, genomic DNA was extracted from each sample as per a previously published protocol[5]. Each sample animal (ETS F0 ♀1, ETS F0 ♂ 1-12) was then placed in individual well plate with previous Culture conditions. Genomic DNA was extracted from 8 selected embryos per group (6 groups; 48 total samples) using a published protocol[5].

PCR

Genotyping PCR and Gel Electrophoresis

Both ETS F0 and ETS F1 samples underwent two distinct polymerase chain reactions (PCR), primary genotyping PCR and then secondary genotyping PCR. This sequence of reactions exponentially amplified the region of interest, the target ETS-like locus, within the newly extracted gDNA. Primary and secondary PCR was completed following the recommended guidelines of the Phusion® High-Fidelity DNA Polymerase protocol (Figure 4 & Figure 5). The primers used in primary PCR were the ETS 1º F1 forward primer with a sequence of 5'-GCGATGACCTTACCGCTGAT-3' and ETS 1º R1 reverse primer with a sequence of 5'-GCGACCTTCCAAGGCTTCAA-3' (Figure 4, C). The primers used in secondary PCR were ETS Genotyping F1 forward primer with a sequence of 5'-CTGTGCAGTGGCGGCAACAC-3' and ETS Genotyping R1 reverse primer with a sequence of 5'-GGGCCTCAACTTAGACGTAAAAGCC-3' (Figure 5, C). While other steps in the PCR cycles remained the same, the annealing temperature and duration were adjusted using the recommendations in the Phusion® High-Fidelity DNA Polymerase protocol (Figure 4 & Figure 5). A 1.5% agarose gel was made, and gel electrophoresis was performed using the PCR products. Gel electrophoresis works by applying a current to previously loaded wells containing DNA fragments. As DNA is negatively charged, it moves away from the negatively charged end (the top here) of the gel, with smaller DNA fragments moving farther through the gel[17]. The ETS-like gene fragment found in wildtype *N. vectensis* is expected to be 1310 base pairs

following secondary PCR. Guide RNAs for CRISPR/Cas-9 mutagenesis are shown within the wildtype genome. These guide RNA cleave their corresponding target sites and, thus, create ETS F1 knockout mutants with smaller bands of DNA depicted in the gel, indicating possible deletion mutations[5].

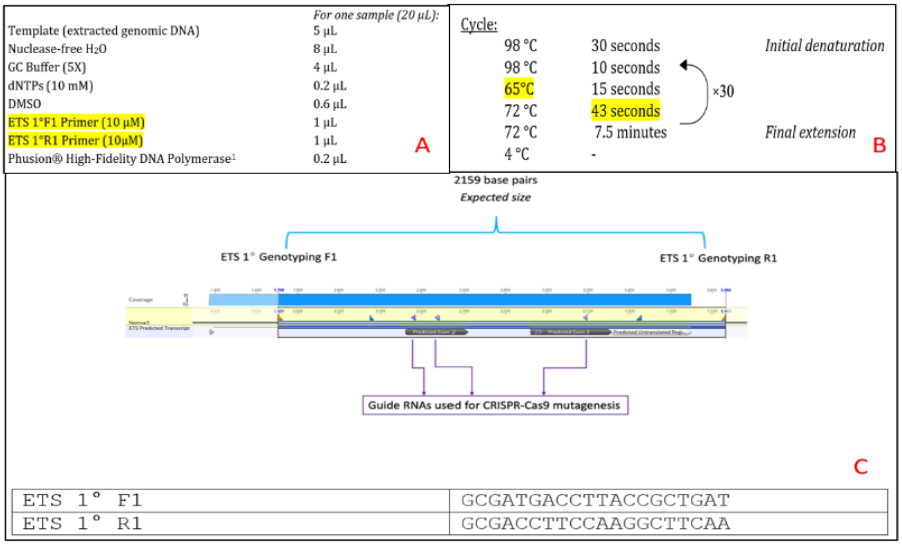

Figure 4. Primary PCR Protocol. A) PCR mix, indicating reagents used and the corresponding amounts. B) Cycle used in the reaction. C) Map of genome with expected size and effects of primers, along with expected placement of sgRNAs used in CRISPR/Cas9 mutagenesis.

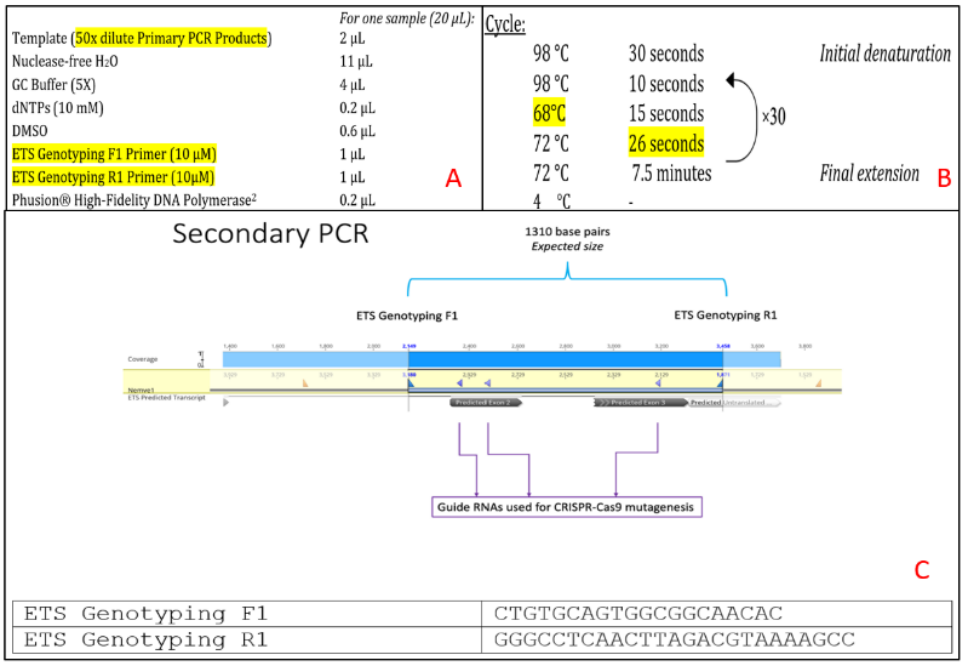

Figure 5. Secondary PCR Protocol. A) PCR mix, indicating reagents used and the corresponding amounts. B) Cycle used in the reaction. C) Map of genome with expected size and effects of primers, along with expected placement of sgRNAs used in CRISPR/Cas9 mutagenesis.

RACE PCR and Gel Electrophoresis

RACE, or Rapid Amplification of cDNA Ends, was conducted using the SMARTer® RACE 5'/3' Kit. RACE allows for the generation of full length cDNAs, DNA that is complementary to mRNA, by using reverse transcription and PCR to amplify copies of the region between the 3' or 5' end. The process begins with RNA extraction, followed by the synthesis of cDNA from an RNA template (first strand cDNA synthesis). Finally, two polymerase chain reactions (PCR).

cDNA Synthesis

The synthesis of cDNA was conducted by the Nakanishi lab (Julia Baranyk) prior to the beginning of my research using the SMARTer® RACE 5'/3' Kit (Section V). RNA extractions from *N. vectensis* individuals, ranging from 1-7 days post fertilization (gastrula-tentacle bud/early primary polyp stage), were conducted following the protocol described in the Direct-zol™ RNA Microprep Kit. Extracted total RNA served as a template for cDNA synthesis. This template RNA has directionality indicated by the 5' end (5' cap) to a 3' end (polyA tail). The SMARTScribe Reverse Transcriptase (RT) enzyme binds to the polyA tail of the mRNA, which had been primed using a modified oligo primer prior to RT binding and began reading the mRNA template in the 3' to 5' (reverse) direction. This allowed for the synthesis of the first-strand cDNA. Once the RT enzyme reached the 5' end of the RNA, its terminal transferase activity attached a small number of additional nucleotides to the 3' end of the newly created first-strand cDNA. The SMARTer II A Oligonucleotide then annealed to the extended 3' tail of the cDNA and the oligo served as a template for the RT. Upon the RT switching templates from the mRNA to the oligo, the generation of a 5' RACE ready cDNA copy of the original mRNA (with additional SMARTer sequence at the end) is completed. The procedure for synthesis of the 3' RACE ready cDNA following the same protocol as 5', except that there was no SMARTer II A Oligonucleotide added to 3' cDNA reactions. This allowed the polyA tail to remain unprimed, causing SMARTScribe Reverse Transcriptase (RT) enzyme to bind to the 5' end and began reading the mRNA template in the 5' to 3' direction.

Primary and Secondary RACE PCR and Gel Electrophoresis

Primary RACE PCR was completed to generate the 5' and 3' cDNA fragments and secondary RACE PCR was done following primary RACE PCR to amplify its products. The reactions were done following the SMARTer® RACE 5'/3' Kit (Section VI) protocol. The protocols were relatively similar for both reactions, however, there was variability in the gene specific primers and PCR cycles used (see *Appendix* for reaction-specific protocols). A 1.5% agarose gel was made, and gel electrophoresis was performed using the PCR products following both reactions, but the secondary RACE PCR products gel was used to continue with gel extractions.

Gel Extraction, Cloning, and Sample Preparation for Sequencing

To extract PCR products following the conclusion of corresponding gel electrophoresis, the QIAquick® PCR & Gel Cleanup Kit protocol was followed. After extracting the DNA, gene cloning was conducted using the TOPO® TA Cloning® Kit for Sequencing. This kit allows for obtained PCR products, extracted from the gel, to be inserted into a plasmid vector. *Taq* polymerase adds an A nucleotide to the 3' ends of the specified PCR products (ETS F1 mutated DNA or RACE cDNA products) and the TOPO vector has overhanging 3' T nucleotide residues, allowing for the targeted PCR product to ligate with the TOPO vector. Once ligated, competent *E. coli* cells were transformed and allowed to grow on nutrient, ampicillin- containing agar plates overnight to amplify PCR-TOPO vector plasmid products containing the DNA of interest. Colonies were formed on agar plates overnight. Sample colonies were selected for colony PCR (*See* Appendix) using M13 gene-specific primers, followed by gel electrophoresis, to verify the cloning had been successful. Successful cloning was indicated by analyzing the agarose gel to be sure that there was only one band present (we did not was to have varying sized DNA fragments) and that the size matched the expected size previously determined for that cDNA strand (*See* Figure 5). Once verified, overnight culture (*See* Appendix) and ZymoPURE™ Plasmid Miniprep protocol was conducted to purify plasmids from positive clones so that they can be sequenced. The ZymoPURE™ purification process uses a modified alkaline lysis method and binding chemistry to separate the target DNA from the transformed cells, producing highly concentrated plasmid DNA samples. These DNA samples were then tested for purity and concentration using the NANODrop Microvolume Spectrophotometer[19] and, after obtaining sufficient measurements of sample purity and concentration, were prepared for sequencing following the guidelines of Eurofins Genomics[20]. Once sequencing samples, containing the template DNA, pure water, and either M-13 forward or reverse primer, were produced, they were sent to Eurofins Genomics for sequencing. Following return of sequencing results, sequencing analysis was done using the Geneious Prime software[21].

in-situ Hybridization

The process of in situ hybridization was performed as described in published protocols[23,24]. Wildtype animals, ranging from 1-9 days post fertilization, were fixed in 4% paraformaldehyde following an established fixation protocol. Upon receiving the RACE sequencing results back from Eurofins Genomics and analyzing and aligning them (Geneious Prime), we determined directionality and analyzed the sequence using the TOPO® TA Cloning® Kit for Sequencing (Appendix B: Vectors). Then we generated RNA detection probe templates using the MEGAscript® Kit protocol. The RNA probes possess mRNA that is complementary to the target gene mRNA, creating "sense" and "antisense" strands. UTP molecules in these RNA probe strands are conjugated with digoxigenin[24]. Since digoxigenin is abnormal in animal cells, it does not interfere with coding properties of the mRNA, but it does make it cell-recognizably different from other RNA[24]. Then, after the in-situ chemicals were made (*See* Appendix *for* chemicals)[23], the procedure allowed for the sample tissue to become permeable to the probes, so

that they moved in and out of sample cells[24]. Once the probe was inside the cells, hybridization occurred between the probe RNA and the target RNA and, upon the application of an antibody that specifically recognizes digoxigenin, visualization was possible[24]. In-situ hybridization imaging was performed by Julia Baranyk.

Spawning and sorting of ETS Kaede positive (transgenic reporter line) F1 animals

To generate the ETS Kaede F1 reporter line animals, previously microinjected parents (ETS Kaede F0) possessed mosaic transgenic mutation via meganuclease I-SceI-assisted genomic integration. ETS Kaede F0 females were crossed with ETS Kaede F0 males. Sorting of the ETS Kaede F1 animals was done using an inverted fluorescence microscope. "Positive" animals displayed the fluorescent marker (tagged target gene passed from F0 parents) throughout their body over their growth period. Positive animals were separated from non-displaying animals ("negative") and raised in Culture conditions so that they may grow until they are sexually mature and can be used to produce the F2 generation.

Results

Prior to my research, using microinjection of CRISPR/Cas-9 and cocktails specifically designed to induce deletions and transgenesis, the Nakanishi lab (specifically Julia Baranyk) made ETS F0 and ETS Kaede F0 mutants to serve as the parents to the lines I would be creating and working with. The ETS F0 mosaic mutants had site-specific deletion mutations of the ETS target gene in *N. vectensis*, resulting in loss of function of the gene product. Due to mosaicism, we cannot be sure whether there is germline transmission of mutant alleles in F0 animals. This is why they were crossed with wildtype animals, and we worked to identify F1 mutant allele carriers. ETS Kaede F0 animals were created through created by meganuclease-mediated transgenesis, allowing us to visualize the expression patterns of this gene in wildtype *N. vectensis*. In-situ hybridization was used to stain certain mRNAs, thereby observing regions of gene expression throughout life cycle transitions in *Nematostella.* Here, through research with these two lines, we analyze the expression patterns of this ETS gene in hopes of further understanding the function of the ETS-like transcription factor and its role in the *Nematostella vectensis* life cycle.

RACE PCR

The synthesis of 5' and 3' cDNA was completed by Julia Baranyk and Dr. Nakanishi prior to the beginning of my research. Using the previously made cDNA, RACE procedure was performed to obtain 5' and 3' amplified copies of the ETS target gene of interest (Figure 6). Julia previously denoted work with the 5' cDNA as "2A" and "2B", and the 3' cDNA as "4" (Figure

6). RACE procedure and cloning protocol (*See* Methods *above*) were performed numerous times to generate the correctly amplified fragments.

The 5’ and 3’ cDNA sample was created by extracting specific RNA from wildtype *N. vectensis* animals. The extracted RNA was then made into corresponding cDNA (*See* Methods *for detailed description*) and cDNA was amplified during RACE PCR. These amplified fragments of DNA underwent cloning, colony PCR, overnight culture, plasmid preparation, and sequencing (Figure 7-9).

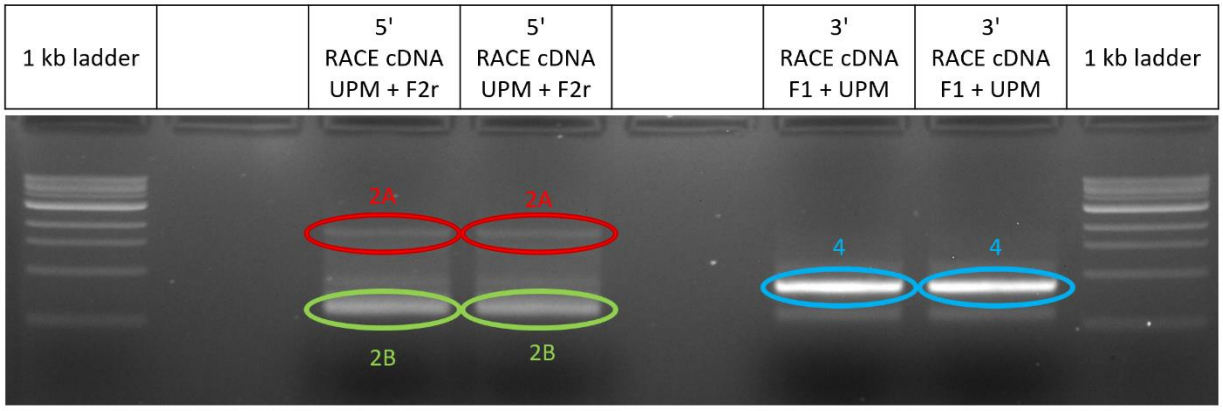

Figure 6. The 5’ (2A and 2B) and 3’ (4) RACE PCR products and their expected sizes. Following sequencing, the sequences will determine directionality so that we may proceed with RNA probe synthesis for in-situ hybridization.

Figure 7. The gel electrophoresis shows 5’ cDNA samples (2B) that were sent for sequencing.

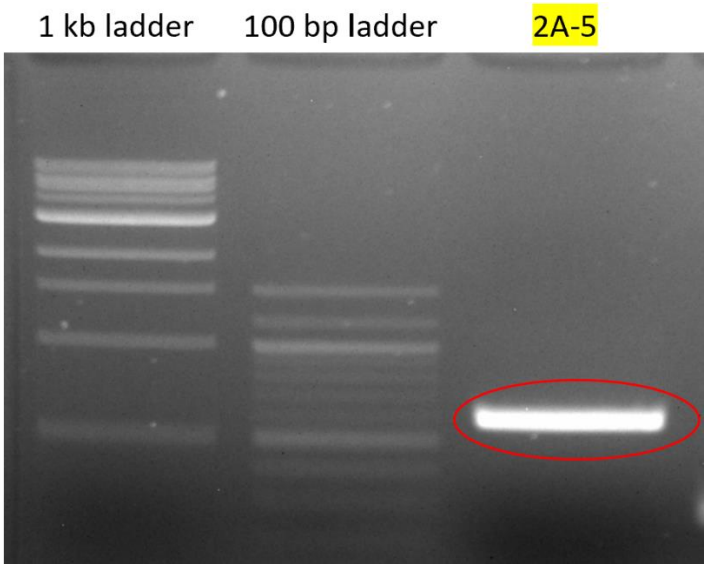

Figure 8. The gel electrophoresis shows a 5' cDNA sample (2A-5) that was sent for sequencing.

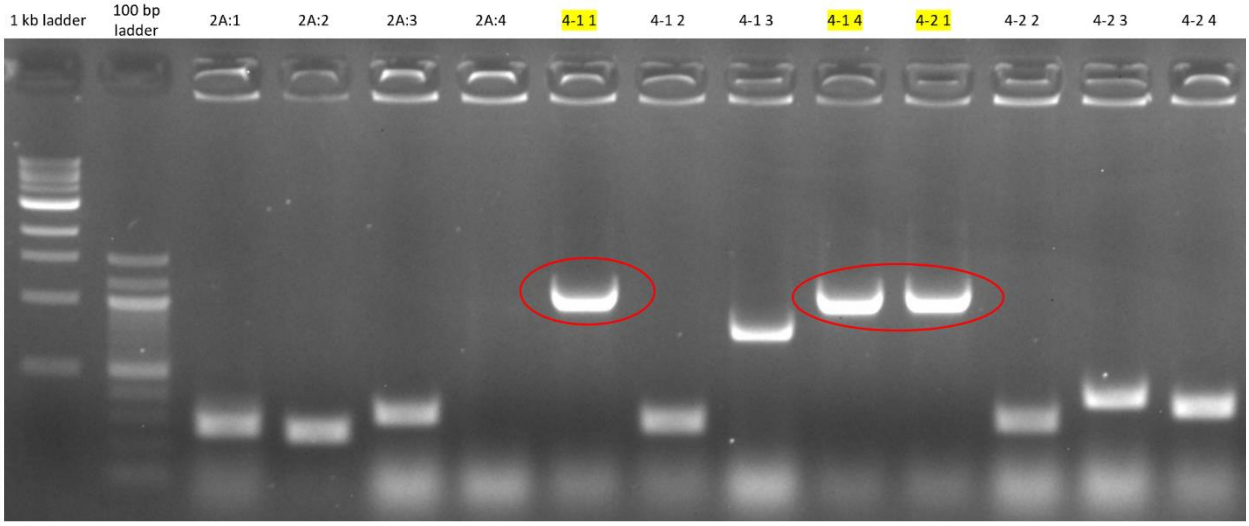

Figure 9. The gel electrophoresis shows 3' cDNA samples (4) that were sent for sequencing.

Genotyping and Sequencing

Mutations in the ETS F0 and ETS F1 knockout animals

The results of gel electrophoresis (following both secondary genotyping PCR) demonstrate that we were successful in extracting genomic DNA from the selected samples. From the ETS F0 genotyping gel (Figure 10), we saw that CRISPR/Cas-9 microinjection had been successful in inducing deletion mutations at the ETS gene target. As previously discussed, (See *Materials and Methods*), CRISPR/Cas-9 causes DNA mutations via double-strand break(s) in DNA. As cells try to fix DNA damage via the imperfect process of non-homologous end

joining (NHEJ), indels (insertions or deletion mutations of DNA bases) emerge[24]. Often, these indels cause a frameshift in the gene, resulting in a premature stop codon downstream of the mutation. Thus, we expect the mutant alleles to be smaller than wildtype. Individuals that only displayed the expected wildtype band size, 1310 base pairs (Figure 10; indicated by blue arrows), were considered wildtype, and did not contain a mutated allele of interest and, therefore, were not crossed to create the ETS F1 animals. Individuals that displayed obvious heterozygosity and possessed DNA much smaller than that of the wildtype size (Figure 10; indicated by red circles), were successful CRISPR/Cas-9 mosaic ETS F0 knockout mutants. These mutants were crossed with wildtype female animals (WT♀ C14, WT♀ C15, and WT♀ C16) to generate the ETS F1 offspring.

Following the successful creation of the ETS F1 offspring, the animals were screened to see if they possessed mutations that were induced in their parents. As mosaic ETS F0 mutants were crossed with wildtype animals, the F1 offspring had a relatively lower chance of inheriting this allele from its mutated parent, indicating that we will most likely see a mixture of mutated heterozygous ETS F1 animals and homozygous wildtype F1 animals following gel electrophoresis (Figure 11). The expected wildtype allele for secondary PCR is 1310 base pairs (Figure 11; indicated by the blue arrows) and, using the 1 kb ladder, the expected size of secondary PCR bands should be between the second and third bands from the bottom (1.0 and 1.5 kilobases). Deviation from this expected size, especially those samples containing smaller DNA (Figure 11, an example is indicated by the red arrow), could be considered a mutated allele and thus, possess the knockout mutation induced in the F0 generation.

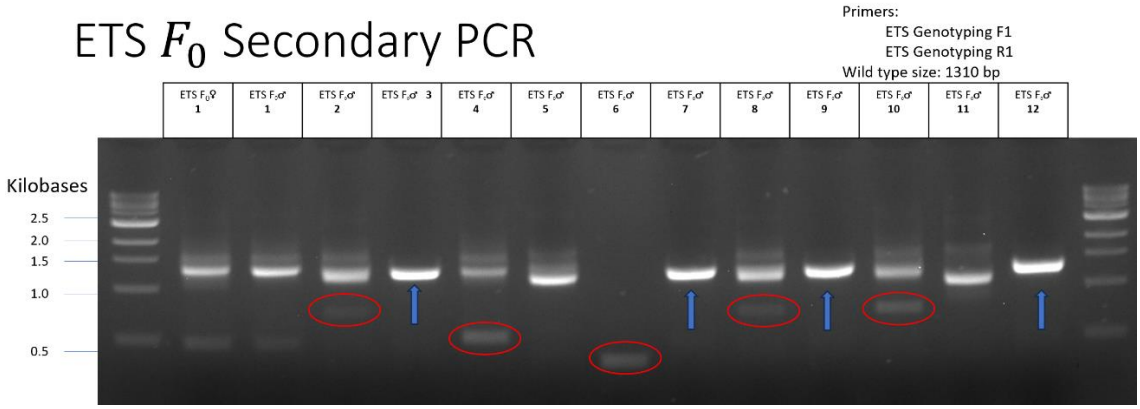

Figure 10. Gel electrophoresis results from secondary genotyping PCR products indicating the presence of a CRISPR/Cas-9- induced mutations in F0 animals. The suspected mutants (indicated by red circles) possess smaller DNA at this locus than the wildtype allele (indicated by blue arrows). This image suggests deletion mutations within mosaic F0 animals. However, the differing sizes of suspected deletion mutations could indicate that there is more than one type of mutation present in these samples.

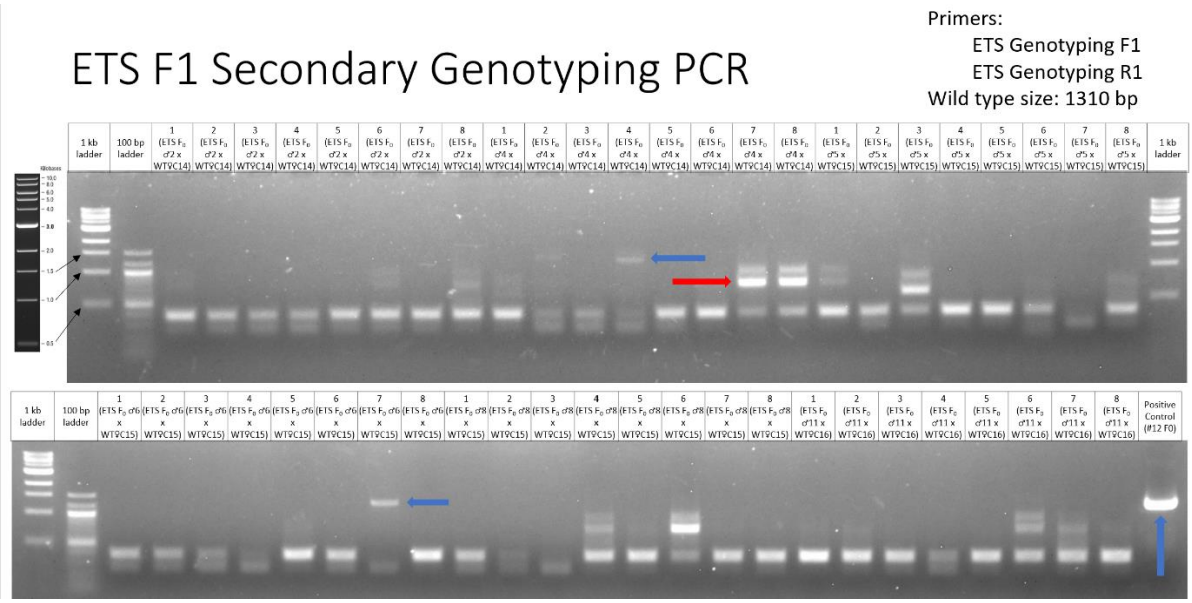

Figure 11. Gel electrophoresis results from secondary genotyping PCR products indicating the presence of F0 inherited mutations in the ETS F1 offspring (i.e. that they are heterozygous mutants). The suspected mutants (example shown by the red arrow) possess smaller DNA than wildtype DNA (indicated by blue arrows) at this locus, indicating deletion mutations within the ETS F1 animals. However, the differing sizes of suspected deletion mutations could indicate that there is more than one type of mutation present in these samples. F1 heterozygotes possess than two bands per sample as a result of crossing F0 mosaic mutants with wildtype animals.

Sequencing DNA results of ETS F1 offspring

Gel Extraction, cloning, and sample preparation for sequencing the DNA of our samples was performed (*See* Methods and Materials). Upon receiving sequencing samples back, they were analyzed in hopes of gaining a better understanding of the mutations seen in the gel PCR products. After removing certain sequences that were much too ambiguous, as they contained far too many "N" nucleotide distinctions and would not provide any relevant information upon interpretation, we removed the vectors from our sequences and viewed DNA sequences of our PCR Product samples via annotating in Geneious Prime software (as instructed in the TOPO® TA Cloning® Kit for Sequencing). Mutant allele sequences were identified by annotating/aligning them to the wildtype *N. vectensis* reference genome and ETS predicted transcript. Our analysis revealed that our samples contained two distinct mutant alleles, classified as mutation type A (Figure 12) and B. In mutated A samples, there was an 8 base-pair insertion mutation- from base pair 2368 to 2375- within the sequence that aligns with ETS sgRNA1 within the reference (Figure 13). There was also a 2-base pair insertion mutation within samples containing the "A" mutations around 2490-2491 base pairs, aligning with sgRNA2 within the reference (Figure 14), as well as a large deletion mutation, roughly 710 base pairs, from 2498-3208 base pairs (Figure 15). Since the size of these insertion and deletion mutations is not multiples of 3, as codons encoding for specific amino acids are made of 3 nucleotides, it can be inferred that these mutations cause a frameshift. While protein synthesis could still be possible with this frameshift mutation, it is highly likely that different amino acids are encoded and, thus,

different proteins are being synthesized here. Since this frameshift often causes a premature stop codon downstream of the mutation, we can assume that the DNA-binding domain was not encoded correctly and there was a partial (because sample is heterozygous for mutant allele) loss of gene function[24].

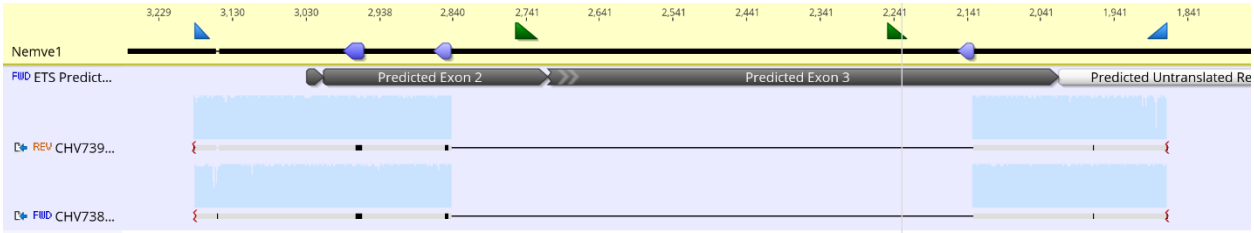

Figure 12. The mutated "A" pattern genomic sequence mapped to the "Nemve1" reference (wildtype) genomic sequence. The blue area indicates there the sequences (mutated and wildtype) align, while the black lines/small bands indicate areas where the mutated and wildtype sequences do not align, indicating a possible mutation. The sgRNA target sites are indicated by the purple triangle/box shaped arrows along the black "Nemve1" bar.

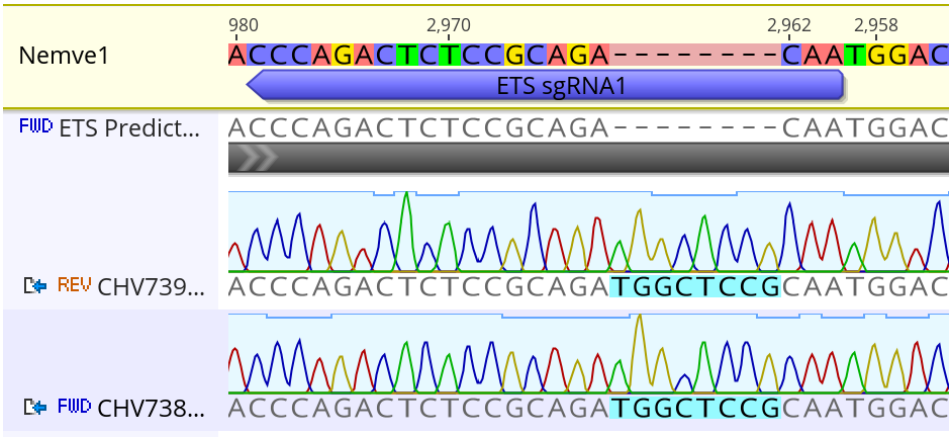

Figure 13. An 8 base-pair insertion mutation- from base pair 2368 to 2375- within the sequence that aligns with ETS sgRNA1 within the reference. The inserted base pairs are highlighted blue in the sample sequences.

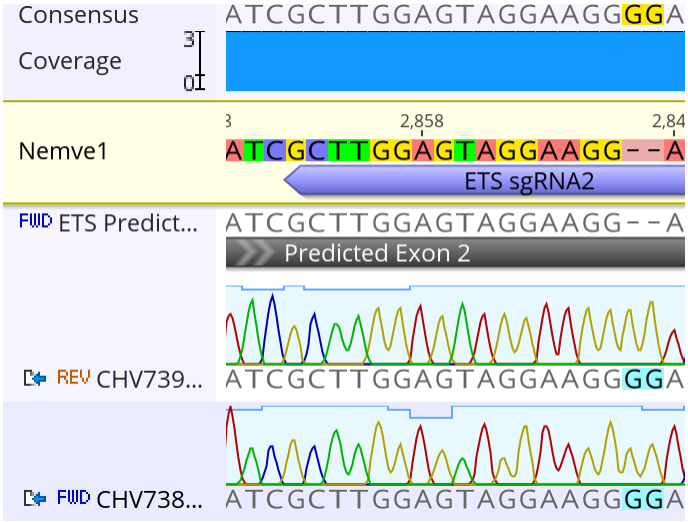

Figure 14. A 2-base pair insertion mutation, the highlighted light blue "GG" base pairs, around 2490-2491 base pairs, aligning with sgRNA2 within the "Nemve1" reference.

Figure 15. A large deletion mutation, roughly 710 base pairs, within the mutant "A", from 2498-3208 base pairs mapped to the reference. The lack of blue, along with the large red line in this part of the sequences, indicate the deleted area.

RNA Probe Synthesis and in-situ Hybridization

The in-situ hybridization process was completed with the combined efforts of myself and Julia Baranyk. Prior to the start of the in-situ hybridization reaction, RNA probe synthesis, animal fixation, and making in-situ chemicals[23] was completed. Julia completed the Anemone in-situ Hybridization (*See* Appendix) reaction and imaging. During the in-situ reaction, fixed wildtype *N. vectensis* animals (1-9 dpf) were stained for the targeted ETS- like mRNAs. This occurred due to the introduction of a complementary sequence, the digoxigenin mRNA detection probes (Figure 16 & Figure 17), to the target mRNAs. The results depicted the ETS expression patterns of 3 early life stages, planula, tentacle budding stage, and primary polyp, in the *N. vectensis* life cycle (Figure 18). As seen in the image, the ETS-like transcription factor had changing expression throughout the *N. vectensis* life cycle stages, showing little to no expression in the early planula stage (Figure 18, A), while having primarily endodermal

expression during the tentacle budding stage of development (Figure 18, B). Interestingly, during the primary polyp stage (Figure 18, C), there was not only expression throughout the endoderm, but also distinguishable expression within the pharynx region, as indicated in Figure 18.

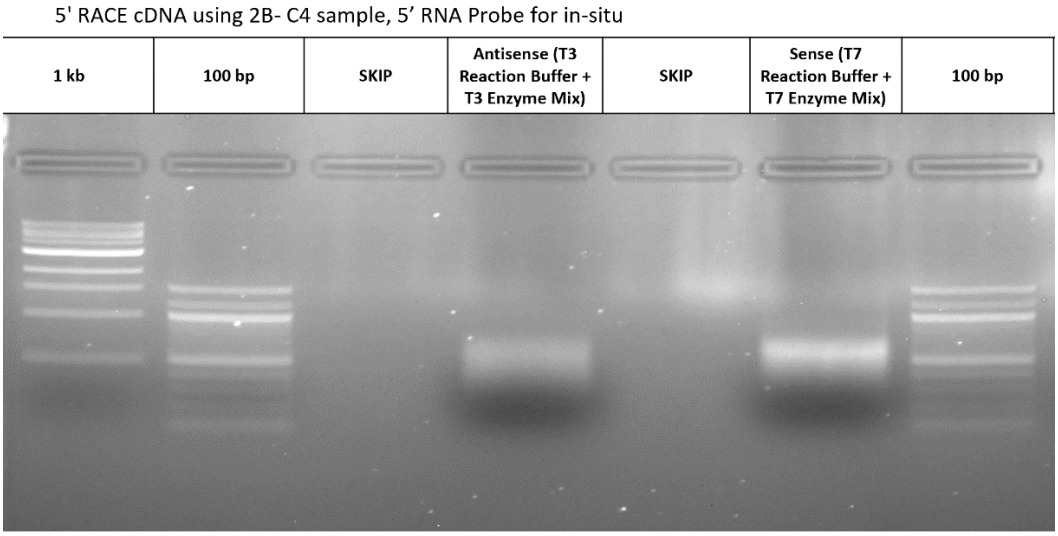

Figure 16. The gel electrophoresis shows the completed 5’ mRNA detection probe, with both “sense” and “antisense” strands, that was used to complete in-situ hybridization. Note: The unclear quality of the bands is normal and not due to error. Probes were tested for purity and concentration using NANODrop and found to be well within the suggested range.

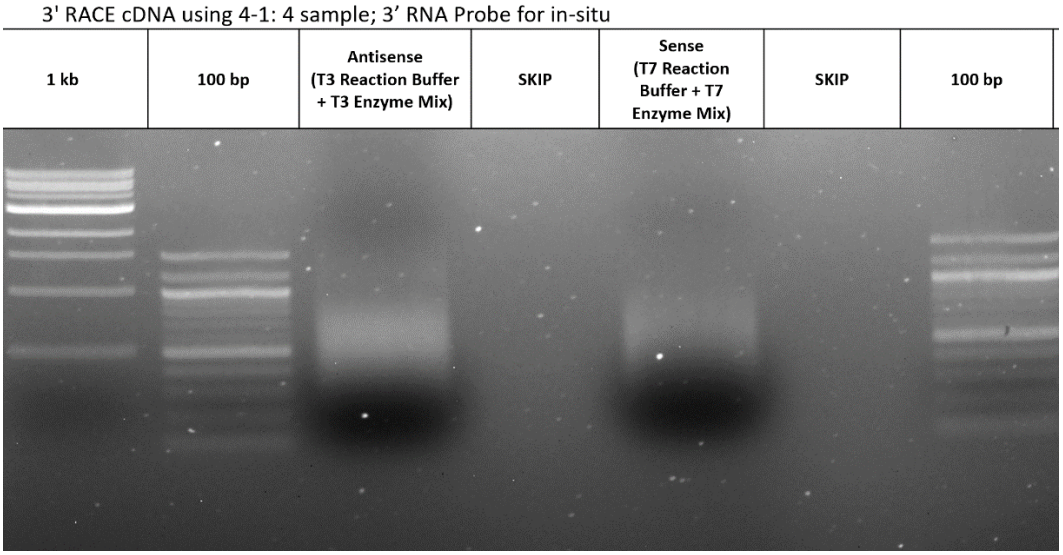

Figure 17. The gel electrophoresis shows the completed 3’ mRNA detection probe, with both “sense” and “antisense” strands, that was used to complete in-situ hybridization. Note: The unclear quality of the bands is normal and not due to error. Probes were tested for purity and concentration using NANODrop and found to be well within the suggested range.

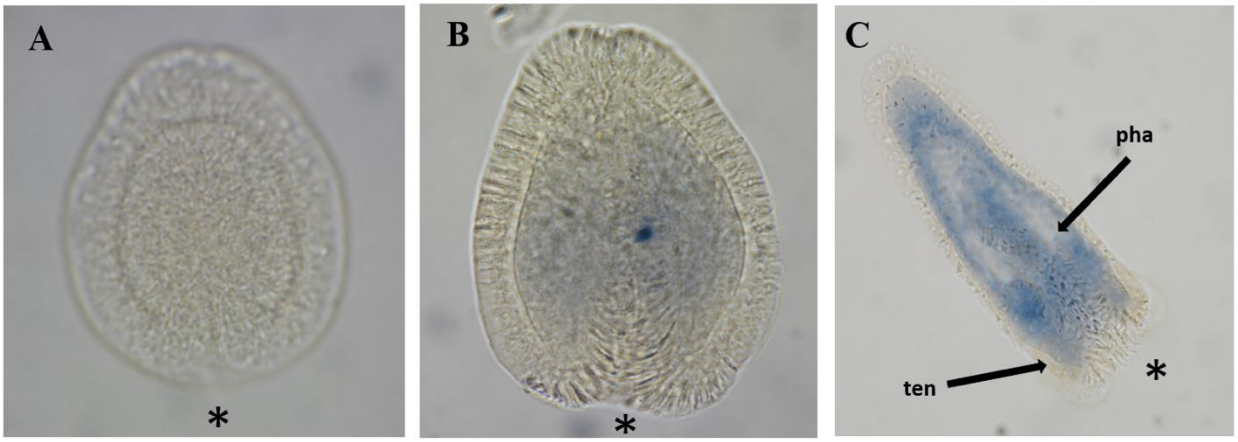

Figure 18. ETS expression pattern in *Nematostella vectensis*. (A) in-situ Hybridization image of an early planula showing no early expression of the ETS-like transcription factor. (B) in-situ Hybridization image of tentacle bud stage depicting expression primarily within the endodermal region. (C) in-situ Hybridization image of a primary polyp showing expression of the ETS-like transcription factor throughout the endoderm, as well as notable expression within the pharynx region (pha). The asterisks (A-C) indicate the site of the blastopore, the future mouth.

ETS F1 transgenic reporter line

The transgenic reporter line carries a mutated allele that is tagged using meganuclease I-SceI-assisted genomic integration. The integration of the target transgenic cassette allowed us to visualize the expression pattern of the ETS reporter gene under the control of a gene-specific promoter during the life cycle transitions in *N. vectensis*[14]. Upon viewing, the ETS Kaede F1 animals demonstrated "true" expression and are not mosaic. The transgene was successfully inherited from the F0 parents to the F1 offspring. After roughly one month post fertilization, the animals expressing fluorescence were transferred to a separate container under the same Culture conditions so that they may continue to grow. They have not been imaged yet, but we continue to see endodermal expression in the positive F1 animals. Moving forward, we plan to continue raising these ETS Kaede F1 animals until they are sexually mature so that they can be crossed to generate ETS Kaede F2.

Discussion

Upon assessing the data collected and analyzing the results, we were able to establish the foundation for functional analysis of this ETS target gene in *Nematostella vectensis*. Also, these results provide evidence to refine a hypothesis regarding the function of the ETS-like transcription factor evaluated here. Based on the GLWamide and RNA-seq data previously mentioned, we hypothesized that the candidate ETS-like transcription factor molecule discussed here could potentially be responsible for explaining the mechanism by which GLWamide

signaling regulates developmental timing and life cycle transitions in *N. vectensis*. Our findings support our initial hypothesis.

First, following the completion of genotyping analysis, we were able to determine that the CRISPR- injected knockout F0 animals had germ line transmission of their mutated ETS allele to the ETS F1 offspring. As the mutation(s) occurring at the target locus could cause a frameshift within the ETS genome and alter protein synthesis, DNA was sequenced. After sequencing, it was not only confirmed that there were induced mutations in the ETS F1 samples, but the type and location of the mutations within the genome was seen. While we did receive sufficient data in the sequence samples to conclude that there were 2 distinct mutation patterns within the samples, further sequencing should be done to validate these results and to screen for different mutation patterns within the ETS genome. These additional efforts will be especially beneficial in the sequencing of the target locus in the ETS F2 null mutants, as they possess complete knockout (i.e. two mutated alleles) of this gene.

Looking at in-situ hybridization images, we see the expression pattern of the ETS-like transcription factor through 3 of the early life cycle transitions in *N. vectensis,* early planula (roughly 2 days post fertilization), tentacle bud (roughly 6 days post fertilization), and primary polyp (8+ days post fertilization). The expression pattern portrayed in these images, with relatively no expression during the planula stage to large endodermal expression throughout the tentacle bud and primary polyp stage, lead us to the belief that this transcription factor could potentially be involved in tentacle formation and development, and thus, supporting our hypothesis. This expression pattern suggests that null mutants (complete knockout this ETS target gene) could develop normally in comparison to wildtype animals during the early *N. vectensis* life stages (i.e. embryo, gastrula, and planula) as little to no expression was seen. However, with increased expression upon entering the tentacle budding stage later in the life cycle, null mutants could have major phenotypic differences in comparison to wildtype animals. To investigate this, further research is needed.

In generating the ETS F2 offspring (crossing ETS F1 heterozygous animals), it can be assumed that the F2 generation should contain null or complete knockout mutants. Establishing null mutants will allow us to see the effects of the completely non-functional ETS-like transcription factor-encoding gene. Analyzing the life cycle transitions and timing of development within these mutants (F2), especially during the tentacle budding stage, is crucial in order to better understand its function in *Nematostella*.

Though evidence has shown that the Wamide neuropeptide family has conserved roles in metamorphosis regulation across Bilateria and Cnidaria, the molecular mechanism of how Wamide neuropeptides regulate life cycle transition remains inconclusive. As such, the emphasis to determine the identity and function of GLWamide receptors remains prevalent. This study established the foundation for functional analysis of a GLWamide response gene, the ETS gene. Future study can build upon these findings with the hope of determining the function of the ETS gene in *Nematostella vectensis* and, thus, shed light on the unknown mechanism of GLWamide signaling in relation to early neural development.

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23. See *Appendix* for Anemone in-situ Hybridization protocol

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Appendix

ETS Primary RACE PCR

Master Mix	**x 1**	**x 8**
H_2O	34.5 µL	276.0 µL
10X Advantage Buffer	5.0 µL	40.0 µL
dNTPS (10 mM)	1.0 µL	8.0 µL
50X Advantage Polymerase	1.0 µL	8.0 µL
Totals:	41.5 µL	332.0 µL

	cDNA Template	**Primer 1**	**Primer 2**	**Primer 3**	**H_2O**
1 *Positive control*	5' RACE cDNA 2.5 µL	F1 0.5 µL	F2r 0.5 µL		5.0 µL
2	5' RACE cDNA 2.5 µL	UPM 5.0 µL	F2r 1.0 µL		0.0 µL
3 *Positive control*	3' RACE cDNA 2.5 µl	F1 0.5 µL	F2r 0.5 µL		5.0 µL
4	3' RACE cDNA 2.5 µL	F1 1.0 µL	UPM 5.0 µL		0.0 µL
5 *Negative control*	No DNA	F1 0.5 µL	F2r 0.5 µL	UPM 5.0 µL	2.5 µL
6 *Positive control*	5' RACE cDNA 2.5 µL	Pit1 F1 0.5 µL	Pit1F2r 0.5 µL		5.0 µL
7 *Positive control*	3' RACE cDNA 2.5 µL	Pit1 F1 0.5 µL	Pit1F2r 0.5 µL		5.0 µL

Thermal Cycle

94°C – 30 seconds
72°C – 5 minutes
× 5

94°C – 30 seconds
70°C – 30 seconds
72°C – 5 minutes
× 5

94°C – 30 seconds
68°C – 30 seconds
72°C – 5 minutes
× 25

72°C – 7 minutes
4°C – HOLD

ETS Secondary RACE PCR

Master Mix	**x 1**	**x 8**
H_2O	40.0 µL	320.0 µL
10X Advantage Buffer	5.0 µL	40.0 µL
dNTPS (10 mM)	1.0 µL	8.0 µL
50X Advantage Polymerase	1.0 µL	8.0 µL
Totals:	47.0 µL	376.0 µL

	1° PCR Product Template	**Primer 1**	**Primer 2**	**Primer 3**	**Primer 4**	**Primer 5**
1 *Positive control*	**2** 1.0 µL	F1 1.0 µL	F2r 1.0 µL			
2	**2** 1.0 µL	UPMShort 1.0 µL	F1r 1.0 µL			
3 *Positive control*	**4** 1.0 µl	F1 1.0 µl	F2r 1.0 µl			
4	**4** 1.0 µL	F2 1.0 µL	UPMShort 1.0 µL			
5 *Negative control*	No DNA	F1 0.25 µL	F2 0.25 µL	F1r 0.25 µL	F2r 0.25 µL	UPMShort 0.25 µL

Thermal Cycle

94°C – 30 seconds
68°C – 30 seconds
72°C – 3 minutes
× 25
72°C – 7 minutes
4°C – HOLD

Colony PCR

1. Place prepared ampicillin-LB plates and an additional fresh master plate in incubator and warm to 37°C

2. *For each colony (25 µL):*

H_2O	17.25 µL
GoTaq buffer (5X)	5.0 µL
dNTPs	0.5 µL
M13 Forward primer (0.1 µg/µL)	0.5 µL
M13 Reverse primer (0.1 µg/µL)	0.5 µL
GoTaq	1.25 µL

   Transfer 25 µL of solution to each labelled PCR strip tube.

3. Using sterile flame technique to avoid contamination, begin by circling and labelling the colonies that will be used from each plate. Create a grid with corresponding labels on the master plate. Avoiding contamination, touch a sterile pipette tip to the desired colony from the sample plate and dip the pipette tip into the corresponding PCR strip tube, then touch the pipette tip to the appropriate location marked on the master plate and discard. Repeat with remaining samples, then return the sample plates to 4°C and place the master plate in the incubator at 37°C overnight.

4. PCR Cycle:

95 °C	2 minutes	
95 °C	20 seconds	×30
50 °C	30 seconds	
72 °C	75 seconds	
72 °C	5 minutes	
4 °C	-	

Overnight Culture

1. Warm previously prepared master plate to 37°C in an incubator.

2. Label clean glass test tubes for each sample in addition to a negative control sample.

3. Prepare a 50 µg/mL ampicillin-LB liquid solution using sterile flame technique:

   To make one sample (3 mL):

   - LB liquid medium — 3 mL
   - Ampicillin (100 µg/µL) — 1.5 µL

4. Using sterile technique, transfer 3 mL of ampicillin-LB solution into each test tube.

5. Touch a sterile pipette tip to a colony on the master plate and dispense the pipette tip into the correspondingly labelled test tube, maintaining sterile technique. Repeat for all samples and dispense an unused pipette tip into the negative control.

6. Cover tubes and incubate at 37°C while shaking at 200 rpm overnight. Return the master plate to 4°C for storage.

Anemone *in-situ* hybridization protocol

*Use RNAse-free equipment and solutions through hybridization step. All washes are 5 min. at RT on rocker table unless otherwise stated.

-DAY 1-

Pretreatment

- Transfer embryos to a 24 well dish and use 500µl for each wash.
  Rehydrate through: 60% MeOH/40% PTw
  30% MeOH/70% PTw
  4 x PTw washes
- Digest with Proteinase-K (0.01 mg/ml in PTw) for 20 minutes (no shaker) (for early embryos 0.01 mg/mg for 5 min) [*5 µl per 10 ml]*
- Stop digestion with 2 (PTw + mg/ml glycine made up fresh) washes. [*0.1 g per 50 ml]*
- Wash 2x with 1% triethanolamine in PTw (made up fresh), add 1.5 µl acetic anhydride. After5 minutes, add 1.5 µl more acetic anhydride. (The acetic anhydride will not immediately mix with triethanolamine. Swirl and rock to ensure it mixes). [*560 µl per 50 ml]*
- Wash 2 x in PTw
- Refix in 3.7% formaldehyde in PTw for 1 hour at RT. [*1 ml per 10 ml*]
- Wash 5 x in PTw

Prehybe

- Remove as much liquid as possible, add 500 µl hybe buffer – incubate for 10 minutes at RT
- Remove liquid – add 500 µl hybe buffer. Place at hybe temp overnight

Hybe

- Dilute probe to a final concentration of 10-0.05 ng/µl (usually 1.0 ng/µl) in hybe solution (dig-labeled probe should be stored as a 50 ng/µl stock in hybe buffer at – 20 degrees). Denature probe at 80-90°C max for 10 minutes. Remove prehybe and add probe to each well. Hybridize overnight or the weekend.

-DAY 2-

- Remove hybe (can be reused 4-5 times)
- Wash 1 x for 10 minutes and 1 x for 40 minutes with hybe buffer at hybe temp. (Do not forget to prewarm hybe buffer)

Washes (for non-hybe washes, use 900 µl per wash)

- 30 min in 75% hybe + 25% 2X SSC at hybe temp
- 30 min in 50% hybe + 50% 2X SSC at hybe temp
- 30 min in 25% hybe + 75% 2X SSC at hybe temp
- 30 min in 100% 2X SSC at hybe temp

- gloves no longer required -

- 3 x 20 min in 0.05X SSC at hybe temp
- 10 min in 75% 0.05X SSC + 25% PTw at RT
- 10 min in 50% 0.05X SSC + 50% PTw at RT
- 10 min in 25% 0.05X SSC + 75% PTw at RT
- 10 min in 100% PTw at RT

Visualization of Probe

- Wash 5 x with PBT at RT
- Block in Boehringer-Mannheim Blocking buffer (diluted to 1x with maleic acid buffer) 1 hr at RT on rocker (or overnight at 4°C).
- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer to 1:5000) at 4°C overnight on rocker. (Can also incubate 1-4 hrs at room temp)

-DAY3-

- Wash 10x (or more) for 20-30 minutes in PBT.
- Wash 3 x for 10 minutes in AP buffer (embryos tend to stick a lot).
- Develop in AP substrate solution (make fresh) at RT in dark. Monitor color development. (Can also develop slower at 4 degrees)
- Stop reaction by washing 5 x with PTw. Mount in 70% glycerol in PTw.

Hybe Buffer (40 mL)	ADD	FINAL
Formamide	20 mL	50%
20x SSC (pH 4.5)	10 mL	5x
20 mg/mL heparin	0.1 mL	50 μg/mL
*20% Tween-20	0.5 mL	0.1%
20% SDS	2.0 mL	1.0%
10 mg/mL SS DNA	0.2 mL	100 μg/mL
dH_2O	7.5 mL	

10x PBS = 18.6mM NaH_2PO_4 (2.56 g NaH_2PO_4-H_2O per liter dH_2O)
84.1 mM Na_2HPO_4 (11.94 g Na_2HPO_4-H_2O per liter dH_2O)
1,750 mM NaCl (102.2 g NaCl per liter dH_2O)

Mix phosphates in about 800 mL of dH_2O for a 1.0 L volume. Check pH. It should be 7.4 ± 0.4. If more than 0.4 off, start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and rest of dH_2O

PTw = 1xPBS + 0.1% Tween-20 detergent
(100 ml 10x PBS, 895 ml dH_2O, DEPC-treat/autoclave; when cool, add 5 ml 20% Tween)

PBT = 1x PBS + 0.2% Triton X-1-- + -.1% BSA (store at 4 degrees C)
(to 990 mls 1x PBS, add 10 mls 20% Triton X-1--, then add 1g BSA and filter sterilize)

20x SSC = 0.3 M Na citrate + 3 M NaCl
(for 1 L, add 175.3 g NaCl + 88.2 g Na citrate, pH to 7.0, and autoclave)