Apoptosis as Potential Cause for Notochord Cell Loss in *Molgula occulta*

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Abstract

The evolution of the chordate body plan is still unresolved. Notochord evolution can be studied with two tunicates—the tailed *M. oculata* and the tailless *M. occulta.* The tailed *M. oculata* has 40 notochord cells that are converged and extended. The tailless *M. occulta* does not form a tail in their larval stage as it only has 20 notochord cells that have not converged or extended. A hypothesis for the loss of notochord cells in *M. occulta* is that the notochord cells are being destroyed by apoptosis (programmed cell death). In this study, preparations for studying programmed cell death (PCD) using two genes *Programmed cell death 2 (PDCD2)* and *Programmed cell death interacting protein 6 (PCDIP6)* were made for *in situ* hybridizations. A cell death assay and *in situ* hybridizations will be done in the future on a variety of developmental stages of both the tailed and tailless species to assess differential regulation of the PCD genes. These studies elucidate if differential regulation of PCD is potentially the cellular mechanism for the loss of a tail in *M. occulta*.

Introduction

A fundamental question in the evolution of development is the origin of the chordate body plan. Evolution of chordate features such as notochords, dorsal nerve cords, and pharyngeal slits can provide insights into how complex evolutionary traits are created and/or lost. Evolution of the notochord in particular can be studied using a unique pair of organisms—*Molgula oculata* (a tailed ascidian) and *Molgula occulta* (a tailless ascidian). These species are found sympatrically in sand flats near the Roscoff Marine Lab Station Biologique in France.

Ascidians are tunicates/urochordates, a subphylum of chordata. Ascidians have all of the chordate features mentioned above during their larval phase (Terajima, *et al*, 2003). Like the majority of ascidians, *M. oculata* undergoes tailed larval development and has 40 notochord cells that have converged and extended (Figure 1A). In contrast, its sister species *M. occulta* undergoes a modified larval development in which the tail does not develop, and it only has 20 notochord cells which have not converged or extended (Figure 1C). As a result of the tailless larval development, *M. occulta* and other tailless ascidians lack chordate features like the neural sensory organ known as the otolith, and a tail with both a notochord and striated muscle (Swalla, *et al*, 1999). The two species develop similarly until neurulation, when the tailed species undergoes convergence and makes a long tail. After hatching, the tailed *M. oculata* has a free-swimming stage, then absorbs the tail and undergoes metamorphosis. The tailless species, however, goes straight to metamorphosis after hatching.

The absence of a tail in *M. occulta* has been shown to be a result of loss of function mutations, rather than a tailless species being the ancestral chordate muscle (Swalla, *et al*, 1999). The tailed and tailless species can be cross-hybridized to create an intermediate hybrid phenotype (Figure 1B). If sperm from the tailed species fertilizes an egg from the tailless species, some of the resulting larvae show the missing chordate features: both the otolith and the tail with the notochord and the striated muscle cells—however, the tail is shortened (Swalla, *et al*, 1999).

A potential cellular mechanism for the loss of notochord cells may be apoptosis, which is programmed cell death (PCD). PCD may be the cause as the tailless species and the hybrid have less notochord cells, which could have been the result of cell death. PCD is a cell suicide mechanism that aids in the normal development of organisms and maintenance of

tissue. Disruption of PCD, however, can cause developmental delays and lethality in certain embryos (Mu, *et al*, 2010). PCD has been seen to occur in ascidians during metamorphosis, and in general, PCD occurs in all multicellular organisms (Terajima, *et al*, 2003). Studying PCD in ascidians may provide insights into the evolution of programmed cell death networks and shed light on the mechanisms by which PCD can cause developmental abnormalities like the loss of notochord cells.

PCD might be differentially regulated to cause the loss of a tail. Takada, *et al,* previously showed that the gene *Brachyury* is expressed in notochord lineage cells, and expression of this gene disappears prematurely in the tailless *M. occulta* compared to the tailed *M. oculata* (Takada, *et al*, 2002). *Brachyury* specifies the notochord and activates the transcription of downstream genes (Takada, *et al*, 2002). PCD genes are downstream of *Brachyury* (Takada, *et al*, 2002), and the change in *Brachyury* expression may be causing differential regulation of PCD genes, which in turn causes the loss of a tail/notochord cells by killing the cells. Research on the expression of PCD genes in the tailless species and the *M. oculata* x *M. occulta* hybrid will look to see if notochord cells are being created only to be destroyed by apoptosis.

Previous Work

Hausch and Swalla have data suggesting that there is a heterochronic shift in metamorphosis in *Molgula* to make it earlier as compared to *Boltenia villosa* (Hausch and Swalla, 2011). They tested this by assessing expression levels of metamorphosis genes at different stages of development from transcriptome data obtained from Michigan State University through collaboration between the Brown lab and the Swalla lab at University of Washington (Hausch and Swalla, 2011). The results from the transcriptome data show that *Molgula* had metamorphosis genes expressed earlier in developmental stages compared to *Boltenia* (Hausch and Swalla, 2011). Their hypothesis is that if *Molgula* enter metamorphosis early and their larval stage is cut short compared to *Boltenia*, there may be less time for development, which may make it easier for the tail loss (Hausch and Swalla, 2011). The tail loss may be caused by a shift in timing for the PCD genes. Hausch studied PCD with two genes – *PDCD2* (programmed cell death gene 2) and *PCDIP6* (programmed cell death interacting protein 6).

PDCD2 is an evolutionarily conserved gene in chordates that is expressed in many larval and embryonic tissues (Baron, *et al*, 2010). In *Drosophila*, *PDCD2* promotes cell proliferation and self-renewal of pluripotent embryonic cells, while *PDCD2* mutants have developmental delays (Mu, *et al*, 2010). In humans, *PDCD2* is associated with the pathogenesis of tumors, and it was found to induce apoptosis via a caspase cascade (caspase inhibitors can block apoptosis though) (Baron, *et al*, 2010).

PCDIP6 is also known as *Bv-Aip* in *Boltenia villosa* (Vito, *et al*, 1999). *Bv-Aip* has a role in the regulation of apoptosis. *Aip* (Alg-2 interacting protein) is associated with the *Alg2*, a gene that is directly involved with controlling programmed cell death (Vito, *et al*, 1999). Together, they need calcium to become active for apoptosis (Vito, *et al*, 1999). Studies by Vito, *et al* have shown that overexpression of *Aip* deletion mutants can protect a cell from apoptosis (Vito, *et al*, 1999).

Hausch used transcriptome data from *M. oculata* and *M. occulta* to do protein alignments for PDCD2 and PCDIP6 with 5 other species: *Saccoglossus kowalevskii*

(hemichordate), *Danio rerio* (Actinopterygii), *Ciona intestinalis* (ascidian), *Mus musculus* (mammal), and *Homo sapiens* (mammal) (Hausch and Swalla, 2011). Regions of high conservation were used to make primers for PCR (discussed in methods section). Hausch has shown that both genes are expressed in the embryos of the *Molgula* species based on transcriptome data.

In order to study the programmed cell death genes, *in situ* hybridizations need to be done to view the expression of the two genes (*PDCD2* and *PCDIP6*) at various time points. This manuscript will discuss the preparations for *in situ* hybridizations as well as preparation and results from morphological staining the *Molgula oculata*, *Molgula occulta*, and hybrid embryos.

Materials and Methods

Embryo Collection

Molgula oculata and *Molgula occulta* were collected from sand flats near the Roscoff Marine Lab Station Biologique in France by Dr. Billie Swalla. The gonads of the molgulids were dissected to retrieve gametes from both species. The gametes were fertilized with sperm from the same species. Hybrids were also created using *M. oculata* sperm and *M. occulta* eggs. Embryos developed to a certain stage and were then dechorionated and fixed in 4% paraformaldehyde. Some embryos were then embedded into polyester wax. Gastrula stage cDNA libraries were created from for *M. oculata* and *M. occulta* embryos, and genomic DNA was obtained. Swalla collected and fixed all embryos.

Primer Creation for PCR

Hausch created two sets of primers for *PDCD2* and *PCDIP6* using two different methods: the program primer3 and manually, by choosing highly evolutionarily conserved regions of the gene sequence from transcriptome data (Hausch and Swalla, 2011). A third set of primers for PCDIP6 was created using highly conserved regions between the molgulid transcriptome data and sequences of *Ciona Intestinalis* from NCBI. Primers for PDCD2 are shown in figure 3 and primers for PCDIP6 are shown in figure 4.

Polymerase Chain Reaction (PCR)

PCR was done using gastrula stage cDNA libraries from *M. oculata* and *M. occulta* and genomic DNA from *M. oculata.* The program used was as follows:

Denature	94°	4 minutes	
Denature	94°	1 minute	35 cycles
Anneal	38°	1 minute	
Extend	72°	1 minute	
Extend	72°	10 minutes	
Stay	6°	hold	

DNA extraction

PCR products were run on a 1% agarose gel. Bands were cut out, and the DNA was extracted using Illustra GFX PCR gel band purification kit. Purified DNA was then ligated into a TOPO TA vector at 12° overnight. Ligated DNA was then transformed into competent cells, and then plated on LB plates with kanamycin and X-gal. White colonies were picked

and were grown overnight in LB. DNA was extracted from cultures via the 5 Prime miniprep kit. To determine if the PCR product was in the plasmid, the DNA was verified by restriction digest with *EcoRI*. DNA that had the right fragment size from the restriction digest was then sent to sequencing using primers T7 and SP6 to obtain sense and antisense sequences.

Sequence Alignment

The transcriptome data from Brown and Swalla was used as input for BLAST. Sequences were first translated using ExPASy to determine the correct reading frame (Gasteiger *et al,* 2003). Protein BLAST was then used, and sequences that matched to the gene of interest (either PDCD2 or PCDIP6) were then obtained for alignment. Sequences from NCBI from the following organisms were included: *Ciona intestinalis* (Tunicate), *Branchiostoma floridae* (Cephalochordate), *Strongylocentrotus purpuratus* (Echinoderm), *Saccoglossus kowalevskii* (Hemichordate), *Gallus gallus* (Vertebrate), *Mus musculus* (Vertebrate), *Homo sapiens* (Vertebrate), *Xenopus* species (Vertebrate), *Danio rerio* (Vertebrate), and *Drosophila* species (Outgroup). The transcriptome sequences and the sequences from the clones containing the PCR were grouped together as they were identical except for one base pair. The transcriptome sequences/the sequences from the PCR and the sequences from NCBI were then aligned with Mafft (Katoh, *et al*, 2002). Gblocks was used to eliminate poorly aligned positions and divergent regions of the alignment as it makes the dataset more suitable for phylogenetic analysis (Talavera and Castresana, 2007).

Gene Trees

Mega5 (Molecular Evolutionary Genetics Analysis) was used to create maximum likelihood trees for both genes (Tamura, 2011). The model used for the tree was the WAG with frequencies model (WAG is a model of globular protein evolution). The bootstrap method was used with 100 bootstrap replications (this method judges the strength of support for nodes).

Morphological Staining

Wax-embedded embryos from the F+6 stage of *M. oculata*, *M. occulta*, and the hybrid were sectioned into 5 micron sections using a microtome and placed on gel subbed slides. Embryos were dewaxed in absolute alcohol and then stained with Milligan's Trichrome staining (Presnell and Schreibman, 1997). Connective tissue was stained green/blue while muscle cells were stained in magenta/purple.

Results

PCR

For *PDCD2*, repeated PCRs failed to produce a band on an agarose gel (gel not shown). For *PCDIP6*, primer set #2 produced two different sized bands from the *M. occulta* cDNA library. One was the expected size at ~400 base pairs (Figure 4A). The other band was larger, near ~800 base pairs (Figure 4B). The PCR using genomic DNA from *M. oculata* as a template produced a band that was near ~1000 base pairs in length (Figure 4C). From the *M. oculata* cDNA library, there was a band on the gel near ~900 base pairs (Figure 4D). There were multiple bands for the control which was expected as the primers for the control were

optimized for a higher annealing temperature, and the lower annealing temperature used in this program decreased specificity for primer binding (Figure 4E).

DNA extraction

Figure 5, 6, and 7 show the results of a restriction digest using *EcoRI* on the DNA from the miniprep. This was done to verify that the correct sequence was cloned into the TA vector. Six clones from the each set of PCR products from the cDNA libraries and two clones from the PCR products from the *M. oculata* genomic DNA were used. The restriction digests of those from the cDNA libraries have similar sizes to the expected. The restriction digest of the two clones from the *M. oculata* genomic DNA produced bands that were smaller than expected.

Sequence Analysis

The clones were sent for sequencing with two different primers – T7 and SP6. These two primers flank the PCR product in the TA vector. Due to an error, clones were either sequenced twice with the primer for T7 or twice with the primer for SP6, but never sequenced with both primers for the same clone. Five of the six sequences from the clones from the ~400 base pair band from the *M. occulta* cDNA were almost perfect matches to the sequence from the transcriptome data as shown in figure 8. All but one of the sequences from the clones with the other PCR products (~800 base pair sequence from *M. occulta* cDNA, ~900 base pair sequence from *M. oculata* cDNA, and the ~1000 base pair sequence from the *M. oculata* genomic DNA) did not match to sequences from any eukaryote, but instead to proteins in *Escherichia coli*. The remaining sequence was from the PCR product from the *M. oculata* genomic DNA, and it matched to a replicase/helicase/endonuclease in *Saccoglossus kowalevskii* and *Danio rerio*.

Gene Trees

Figure 9 shows the maximum likelihood gene tree created by Mega from protein alignments for PDCD2. In this gene tree, the tunicates all group together. The vertebrates all group together as well. The respective representative from the echinoderms, the hemichordates, and the cephalochordates are sister groups to each other.

Figure 10 shows the maximum likelihood gene tree created by Mega from protein alignments for PCDIP6. The four transcriptome sequences group together. The sequences from the clones were not included as they were only one basepair off from the sequence of *Molgula occulta* 4176 (sequence in figure 3). The molgulid sequences were the sister group to the fellow tunicates of *Ciona intestinalis*. The tunicates relate to others with a long branch, indicating that there may be less sequence similarity to other organisms. The vertebrates clustered together as predicted.

Morphological Analyses

Milligan's Trichrome staining dyes connective tissue blue and muscle cells purple. Figure 11 shows the staining on *M. oculata*, the hybrid, and *M. occulta* from the F+6 stage. Muscle cells flank the notochord cells normally. As seen in the first picture of *M. oculata*, the muscle cells are in two places: around the neural tube and around the posterior side of the embryo. In the hybrid, the muscle cells are mainly seen around the edges of the posterior side

of the embryo. In contrast, the muscle cells in the *M. occulta* embryo are towards the middle on the posterior side.

Discussion

Programmed cell death genes may be responsible for the evolution of the anural (tailless) *Molgula* species, in particular *M. occulta.* Differential regulation of PCD can alter development of embryos. Due to time constraints, the results of this study are inconclusive on the expression of PCD genes, but the preparations for *in situ* hybridizations will be discussed as well as the morphological staining and future work stemming from this data.

The transcriptome data from Brown and Swalla showed that there was no expression of PDCD2 at the gastrula stage. This suggests that there was little to no expression of PDCD2 in the gastrula stage embryos (transcriptome data was good but not absolutely complete, suggesting that there may be low expression levels of genes that are absent from the transcriptome data). The cDNA libraries used for PCR were from the gastrula stage, and *PDCD2* could not be amplified from this. cDNA libraries from later stages should be used in future PCRs. Genomic DNA from the molgulids was not used to amplify *PDCD2* as the genomic DNA would contain the sequences from introns, and the intron sequences should not be included in the creation of RNA probes for *in situ* hybridizations.

The transcriptome data for PCDIP6 showed that there was similar expression in the hybrids compared to *M. occulta.* Expression level comparisons between the tailed and tailless species can give insight into the possibility that PCDIP6 is differentially regulated. This can be confirmed by quantitative PCR using cDNA libraries from a variety of developmental stages to see the relative abundance of gene expression between *M. oculata*, *M. occulta*, and the hybrid. If the PCD genes have higher expression in *M. occulta* and the hybrid at the stage when the notochord cells divide from 20 cells to 40 cells, PCD may be correlated with the loss of notochord cells.

Four PCR products were obtained from the primers for *PCDIP6* (~400 base pair band from *M. occulta* gastrula cDNA library, ~800 base pairs from *M. occulta* gastrula cDNA library, ~900 base pair band from *M. oculata* gastrula cDNA library, and ~1000 base pairs band from *M. oculata* genomic DNA, as shown in figure 4). The three PCR bands that were not the expected size of 400 base pairs may have been the result of the annealing temperature being low at 38°. This low annealing temperature may have lead to low specificity of primer binding, and as a result, different DNA sections may have been amplified.

From the sequencing, results that did not BLAST to a match for PCDIP6 (after translating the nucleotide sequence to protein sequence) also did not have the correct primer sequence in it either. One particular result from the genomic DNA did not have the primer sequence but did have a BLAST hit to a replicase/helicase/endonuclease in *Saccoglossus kowalevskii* and *Danio rerio*. A replicase is an enzyme that catalyzes the replication of RNA from RNA, a helicase separates two strands of nucleic acid, and an endonuclease cleaves phosphodiester bonds. These are all important enzymes, and this sequence should be further studied to see if it appears in the transcriptome data. This sequence could code for an endonuclease that cleaves DNA in apoptotic cells, which would be important for the study of PCD genes.

The clones that did have BLAST results that matched PDCIP6 were nearly identical to the sequence from the transcriptome data. RNA probes should be made from these clones

for PCDIP6. After that, *in situ* hybridizations should be performed at various time points—gastrula and neurula stages, the tailbud stage, and early metamorphosis (the stages in which the tail should be formed/the notochord cells should be dividing). The *in situ* hybridizations should be done on the tailed *Molgula oculata*, the tailless *Molgula occulta*, and the hybrid. By doing the *in situ* hybridizations on both species, the tailed *Molgula oculata* can be used as a comparison for normal PCD expression.

The *in situ* hybridizations may show that the programmed cell death genes are on during the late gastula and neurula stages and early tailbud stages in the tailless species and the hybrid (the notochord would be dividing around this time). The expected expression for the PCD genes is that the apoptotic cells would be around the notochord cells in the hybrid and the tailless *M. occulta.* If the programmed cell death genes were expressed more in the tailless species compared to the tailed species, the programmed cell death genes may be correlated with the lack of notochord cells and therefore, the cellular mechanism for the loss of the tail.

The gene trees for PDCD2 and PCDIP6 show different relationships between the phyla. In figure 9, for the gene tree for PDCD2, vertebrates are closer to the phyla of hemichordates, echinoderms, and cephalochordates than to tunicates, whereas in the gene tree for PCDIP6 in figure 10, vertebrates and tunicates are sister groups. However, in the gene tree for PCDIP6, tunicates are suffering from long-branch attraction, which indicates that the group is rapidly evolving (Bergsten, 2002). The bootstrap values for the gene tree with PDCD2 were low, meaning that there was high sequence divergence, and phylogenetic links are weak. However, the bootstrap values between the vertebrate sequences were high, suggesting that there may be functional conservation with the PCD genes in vertebrates.

Morphological analysis of the embryos from F+6 showed differential placement of muscle cells. In figure 11 on the left, the embryos stained with trichrome can be compared to the pictorial representations of the molgulids on the right. In the images on the left of figure 11, the dark orange cells are notochord cells and the red and white cells are muscle cells. The trichrome staining shows that the muscle cells flank the notochord cells (using the pictures on the left as a guide). Notably, the trichrome staining shows that the muscle cells of the *M. occulta* are gathered towards the middle of the posterior region, similar to the location of the notochord cells on the pictorial representation. Trichrome staining needs to be done on later developmental stages of the molgulid embryos to track the movement of the muscle cells and the notochord cells.

In addition, an *in situ* cell death kit should be used to identify apoptotic cells to verify that the programmed cell death genes are expressed and are indeed targeting cells for destruction. Apoptosis is usually activated by a caspase cascade; however, activation of caspases do not necessarily mean that the cell will undergo apoptosis (sometimes, caspase activity is needed to protect a cell from apoptosis) (Vito, *et al*, 1999). It is possible that the cell has PCD gene expression at some level but other genes are also being expressed to protect the cell from destruction. The cell death kit should be used on molgulid embryos at a variety of stages including late gastrula, neurula, and early tailbud. The different stages can show differential regulation of PCD genes if cells are targeted for apoptosis at different times between the species.

Further studies could focus on other *Molgula* species that lacking a tail—*M. tectiformis, M. bleizi, M. retortiformis,* and *M. provisionalis* (Tagawa K, *et al*, 1997). These

species can provide insight if abnormal expression of the programmed cell death genes leads to the loss of tail in other species as well or if there are other reasons for the loss of tail.

In addition, PCD can also be knocked down in the tailless *Molgula* embryos to assess if it will lead to the reemergence of a tail. If the tail formed, then misregulation of the PCD genes in the tailless species may be the cause for the loss of a tail/notochord cells. Knocking down PCD in the tailed *Molgula* embryos would elucidate the necessity of PCD at those stages in development. These studies would characterize the normal versus abnormal role of PCD in development as well as provide insight on the evolution of the notochord.

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Figure 1

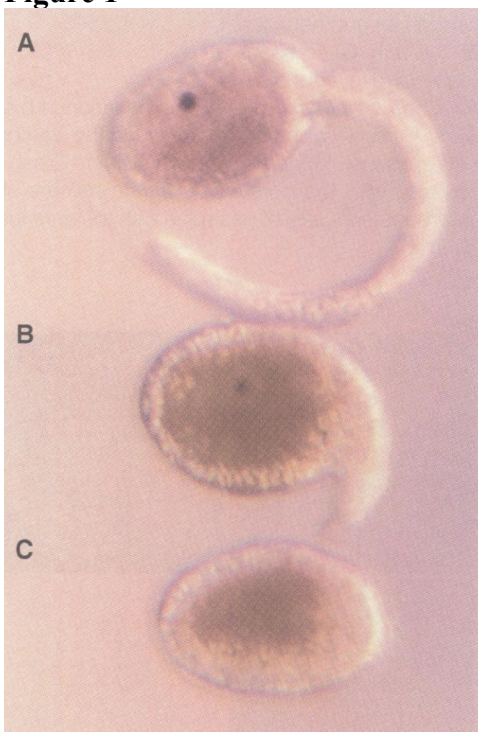


Figure 1: A shows *Molgula oculata* with the notochord with 40 cells that have converged and extended. **B** shows the hybrid species created via crossfertilization of *M. oculata* and *M. occulta*. It has 20 notochord cells that have converged and extended. **C** shows *Molgula occulta*, which has 20 notochord cells, not converged nor extended. (Figure from Swalla and Jeffery, 1996).

Figure 2

TTTCCGATTTAAAACAGTTAAAATGGTTACGTCTTACAATGAGTATTTAGGTGTC
AGGTAGCCCGGAATAACTTGTGACACAATAACACACCAATCAAGTTTTATTTTCT
TCAGAAAAGTTTTGAACATGCGCATATTCAGATGTATATCCATCAGATTTTATAA
GGCATGAATTCTTGCAAGTGTAAATAGTGATAACACCCCAATCTATGCCATCAGT
GAAACCTTGGTCAATATTCAAATAATTTAACAGTTGTGGCATGATCTCAAACTCA
AATATTCTGCTGCCTCCACAGCTTCCACACAGAGGAACATCTTCAGTTTTTAATTT
CCCGTCACAAGCACACCAAAGTGGCTCCCCACTCTTGTGATAGCGTAAAACTTGT
TCATGGTTTCCATTCAATTTTTCTTTAAATTCAATGAAGTATTTATCAGCTTTATT
CCCAGCATTAGCAAACTGTTCAAGGTCAGCGGCTGATAAAGATGCACTATTGTTT
GGAAGTTCATCTTGTCTCTTACTCTCTGCTTCATTAATTTTTATTTCAGGCTCCATC
ACGATTTCAAAAGTTGCGAAGTTTAAACCATTCAAATCATTTTCAGCTTCCGAAG
ATGAAATTTCAGAGCAAACTTTTTTATGCTGTTTCCAGTGCAACGTCTGATGAAG
TTTGGAACAATAATTAACTTTCTTACATCTGGAACAATGATTGGGCCCTCTGCAT
CCACATACGTCACAGAGCTTTAAAGACGTAGTTTTAATAGTAGAAATGTAATTTT
TATCATCATATTTTGGTGCATCTTCAGGATAGAAATTGTTCTTTTTGGAGAGTTGG
CAACGAAACACTTTAACAGGAACTCCGGCCGAGTTTTTATAACAGGAATCAGTTT
TGCAACAGAATATGAATATTGTTCGATGGAAAGCATTTTCCGAATTTTCATCTGG
GGCGTAAACTTGTAAAAGAAAAACAAGAGGGTTCGAACAAATTTCACACTGAAG

TTCGGATACAGTTGGCAAATTGTCTGGGTTTAACCAGGATGGTTTCCCACCGATT
TTGCTCGGGAAGAAACATCTGTGCAGTTGCCATGGCGACTTCACAGGCTCGGCA
AATCCTAAATCAACATCCGTTTCTCGAGAGCTTTCATTTGATGAAATTGAAGATG
TTTCATCCATACTACAGTATTTAATCTTAATACAAC

Figure 2: Sequences in blue indicate primer set #1, which has an expected size of 531 base pairs. Sequences in green indicate primer set #2, which has an expected size of 1194 base pairs.

Figure 3

CAGAGGGAGTTCGATTCCACGTGGCACCAAACTTACTCCGAAGTTCGTCGTCTGT
TGATTGTTCTTCATTCATCATTCGCATGCACTCATTTAACAATTGTTGGTTCCGGG
TGAGTGATTCTGGTAGTTCTTGAAGTAGATTTTCAATGGATTGCAGCCCACCCTT
CGATATCACGGCCTGTGATTTTTCTAGTATGGATTGTGGTACAGAATCCCCGGAA
ATATCTTCCAACGCTGCAGGAAGATTTAAAGAAGACATCACACCGTTAATCAAT
GTATTTAATTCTCGTATTCTGGCAACTTCACGTTCGATGATTTGCTTACGTCGATT
TTCATACGATTCAATCGCCGTGTGAACGGCAAGCGGAACCAAACTCGTAAAAAC
ATCAACATATTTGCCACCTAATGCCTCTGTAGCGTTAAACGGCATCGCTTTCGCG
ATTGCTGCCTTCCCAATCGCAGATAATTCCTTTGGAATCGGATCGTGATAAATGA
AATCATTTTCTCTTTGAGCTTCCTTTAATTGAACGCTGACTTTCAATGCCAAATCT
TTCATATTAAACCCTTGTATTAACTCCGGATGTTTTCCGAGAGGTTTTAGTGAAG
GTTCGATCTTTTGCAGTCTTCCAATTTCTTCACCAAATTTCTTTGCATTGTGAGCG
ACGCTCGCCATTTGGAAATGAGCCAAAACTTCATAGAGTGATTTCTTTGTATTGC
ACATTGTTGATACGTTGCTAGGTAACATCTGACCGGCTTCAGACAGCGCACTGAC
CGCATCAGCGTACAATGATGCAGCTTGATTCGCAACTTTCGCAAGAATATCAGGT
TTCATTTTATCAGAACTGGCTTTAGAGTAGAAGCACTCTTGTGACTGCGCGATGA
GGATGCAACGAAACGCAGACAAAACATTTGGTGAGAGATCGCTTGTCGGATTTT
GATTAATCGATGCTAATATGTTGTCTTTGATGAAGTCGAGCATCCCGGCAGCTTC
GGCAAAATGTTTAGCGGCACTTTTCAGTTCGTCATCGTTGCTTAGATTGGCTTCG
CTTGCGATTTGCGACTTGAGTGCTGCGATGTTGTATATCGTCATCAAACGTTCAA
ATTCACCGCTTGCCGACGTTATTTTTCCGCTGCCACTGAACAAAGATCCTTTTTCC
AGTGAATCTTTCCAAGTAAACGAAACGTGAACACCGTTGCTTTCACAGAAAGGA
ACTTTGCGATTTAATGAAGAAAATTGATCATAATATTGTAATAAAACATTGAGAG
AAGCAGCACTTTTATCCTGCGCTTTGTAAACAGCGGTTTTCCGAAGTGAATTAAA
GTCTTTCAAATGTTTTTTGAGATCATCATCAACTTTATCGTATGTGTTTTTAATAA
ATGTTTGAAGCGGCTTCACCAAATCAACATCTGTTGTTTTCTTTAATGGTACGAC
GATGAATCGACCAACATCAACAGTGTAAGCCATCCTGCAGCTCAGTATAATAAA
TAGATTATAACTTCCTTATTAAACT

Figure 3: Sequence for PCDIP6 from transcriptome data. Highlights in blue indicate primer set #1, with an expected size of 411 base pairs. Highlights in green indicate primer set #2, with an expected size of 1511 base pairs. Primer set 1 and 2 were created by Hausch (2011). Primer set #3, shown in red, was created based on evolutionarily conserved regions between *Ciona intestinalis* and the *Molgula* transcriptome data. The expected size is 1076 base pairs.

Figure 4

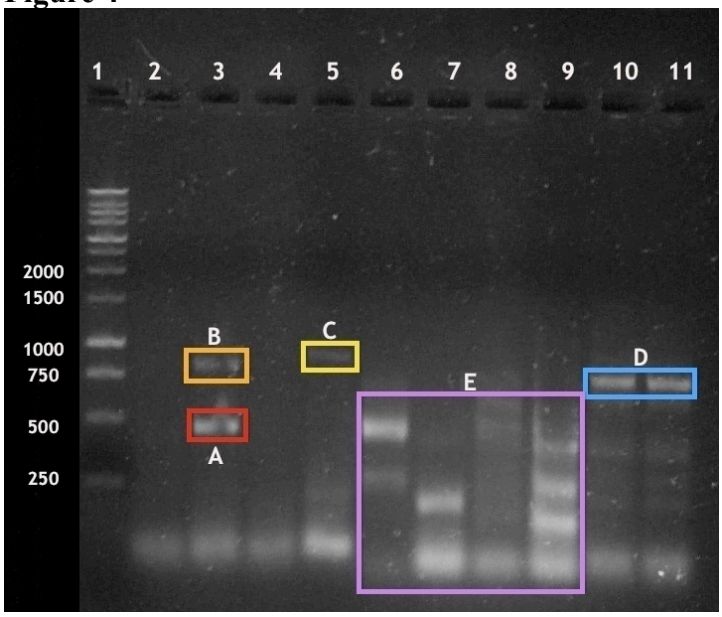


Figure 4: PCR products – A-D PCDIP6 Primer set #1. **A** – ~400 base pairs, *M. occulta* cDNA, **B** - ~800 base pairs, *M. occulta* cDNA, **C** - ~1000 base pairs, *M. oculata* genomic DNA, **D** - ~900 base pairs, *M. oculata* cDNA, **E** – Control PCR, *Prickle*

Figure 5

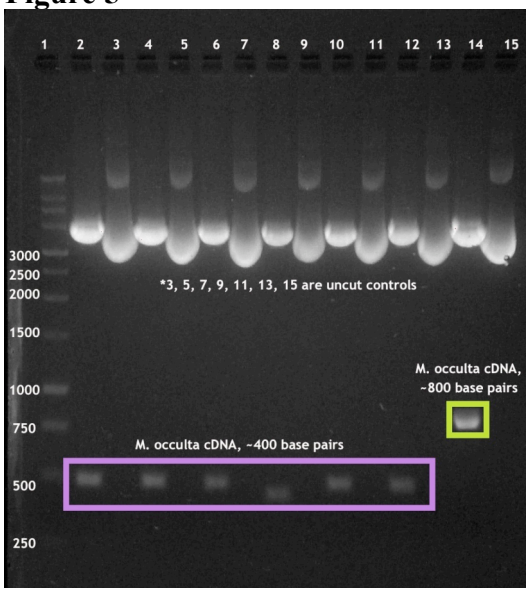
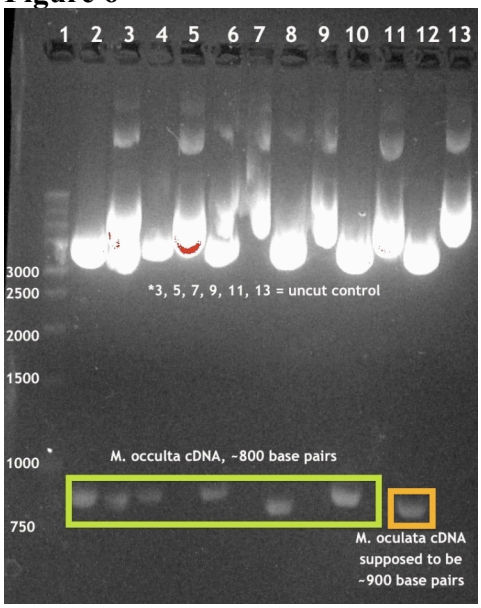


Figure 5: Restriction digest using *EcoRI*

1 – DNA Ladder
2 – *M. occulta* cDNA (400bp) - 1
3 – *M. occulta* cDNA (400bp) – 1 control
4 – *M. occulta* cDNA (400bp) – 2
5 – *M. occulta* cDNA (400bp) – 2 control
6 – *M. occulta* cDNA (400bp) – 3
7 – *M. occulta* cDNA (400bp) – 3 control
8 – *M. occulta* cDNA (400bp) – 4
9 – *M. occulta* cDNA (400bp) – 4 control
10 – *M. occulta* cDNA (400bp) – 5
11 – *M. occulta* cDNA (400bp) – 5 control
12 – *M. occulta* cDNA (400bp) – 6
13 – *M. occulta* cDNA (400bp) – 6 control
14 - *M. occulta* cDNA (800bp) – 6
15 – *M. occulta* cDNA (800bp) – 6 control

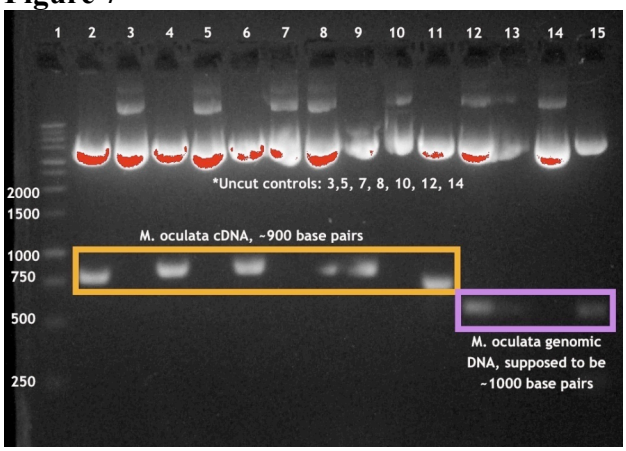
Figure 6



1 – DNA Ladder
2 – *M. occulta* cDNA (800bp) - 2
3 – *M. occulta* cDNA (800bp) – 2 control
4 – *M. occulta* cDNA (800bp) – 3
5 – *M. occulta* cDNA (800bp) – 3 control
6 – *M. occulta* cDNA (800bp) – 4
7 – *M. occulta* cDNA (800bp) – 4 control
8 – *M. occulta* cDNA (800bp) – 5
9 – *M. occulta* cDNA (800bp) – 5 control
10 – *M. occulta* cDNA (800bp) – 6
11 – *M. occulta* cDNA (800bp) – 6 control
12 – *M. oculata* cDNA (900bp) – 1
13 – *M. oculata* cDNA (900bp) – 1 control

Figure 6: Restriction digest using *EcoRI*

Figure 7



1 – DNA Ladder
2 – *M. oculata* cDNA (900bp) - 2
3 – *M. oculata* cDNA (900bp) – 2 control
4 – *M. oculata* cDNA (900bp) – 3
5 – *M. oculata* cDNA (900bp) – 3 control
6 – *M. oculata* cDNA (900bp) – 4
7 – *M. oculata* cDNA (900bp) – 4 control
8 – *M. oculata* cDNA (900bp) – 5 control
9 – *M. oculata* cDNA (900bp) – 5
10 – *M. oculata* cDNA (900bp) – 6 control
11 – *M. oculata* cDNA (900bp) – 6
12 – *M. oculata* genomic DNA – 1 control
13 – *M. oculata* genomic DNA (1000bp) – 1
14 – *M. oculata* genomic DNA – 2 control
15 – *M. oculata* genomic DNA – 2 (1000 bp)

Figure 7: Restriction digest using *EcoRI*

Figure 8

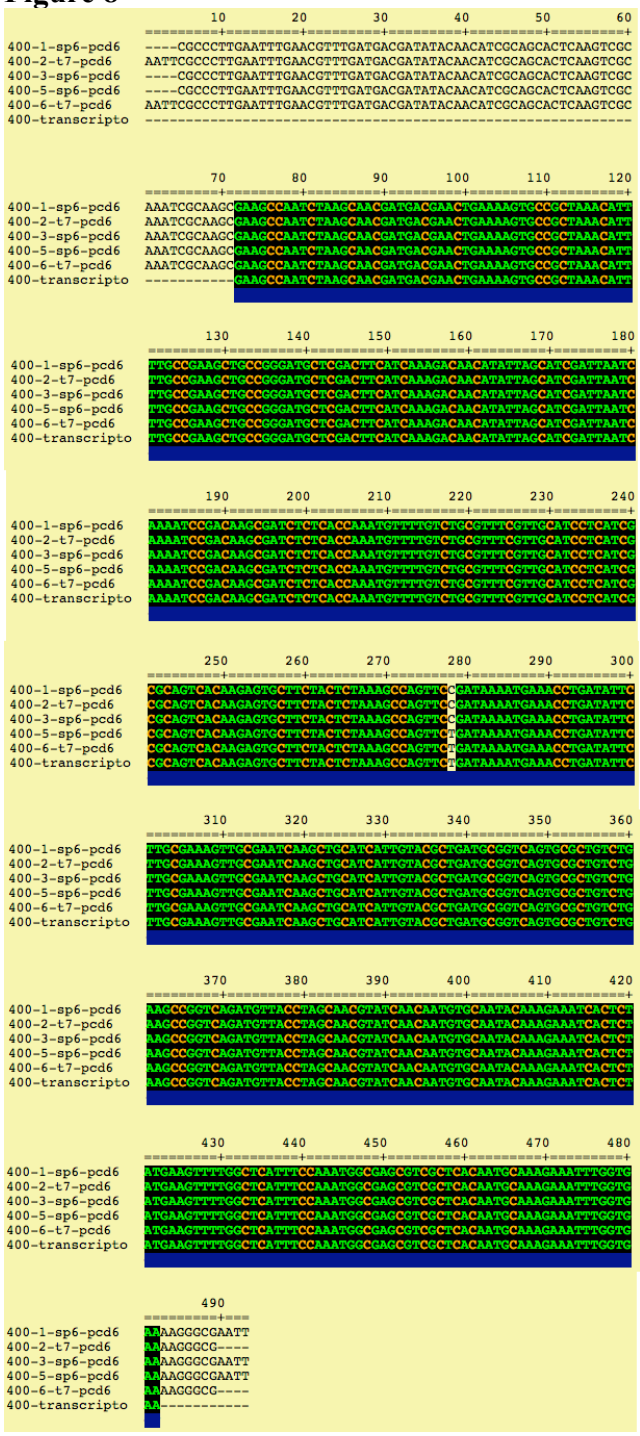


Figure 8: Sequencing data as compared to transcriptome data. Notably, there is only one base pair difference at position 278. The blue under the sequences shows conservation. The beginning of the first five sequences and the end of them are not conserved as they are sequences of the vector itself after the *EcoRI* cut sites.

Figure 9

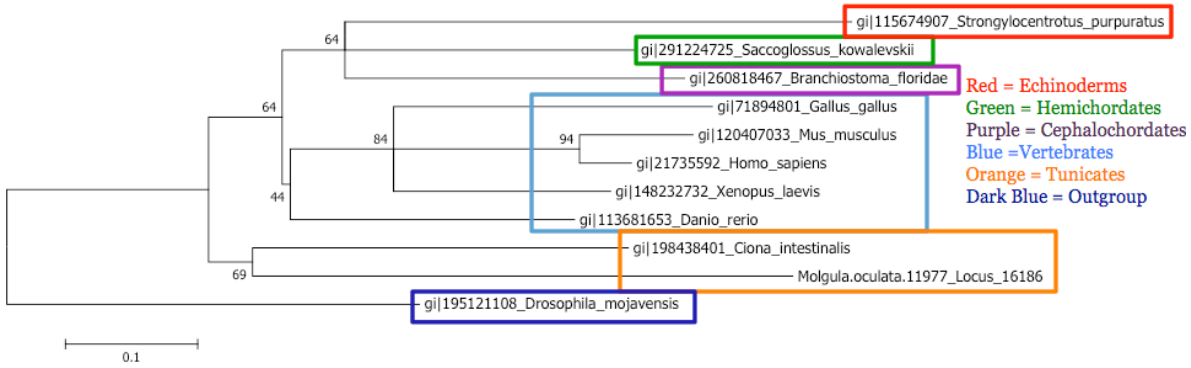


Figure 9: PDCD2 Maximum Likelihood Tree with bootstrap values

Figure 10

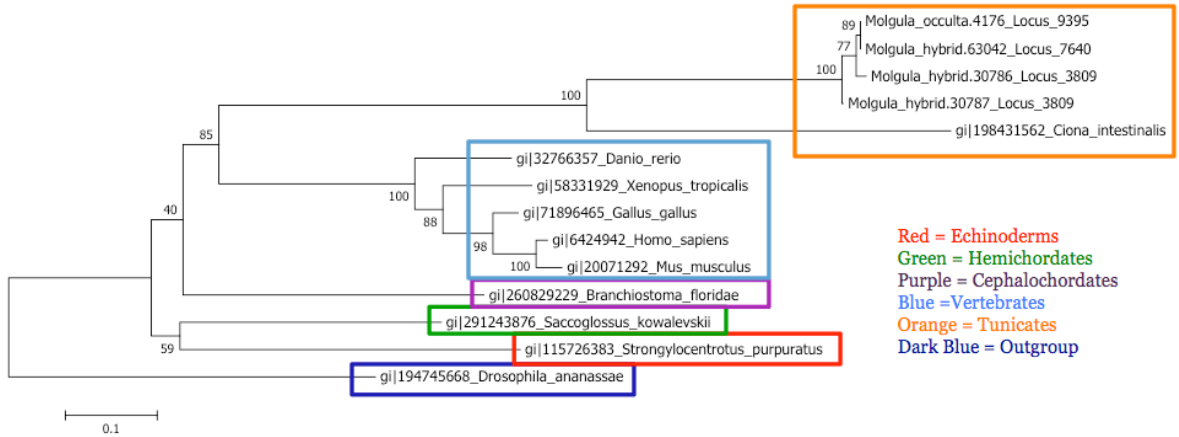


Figure 10: PDCD2 Maximum Likelihood Tree with bootstrap values

Figure 11

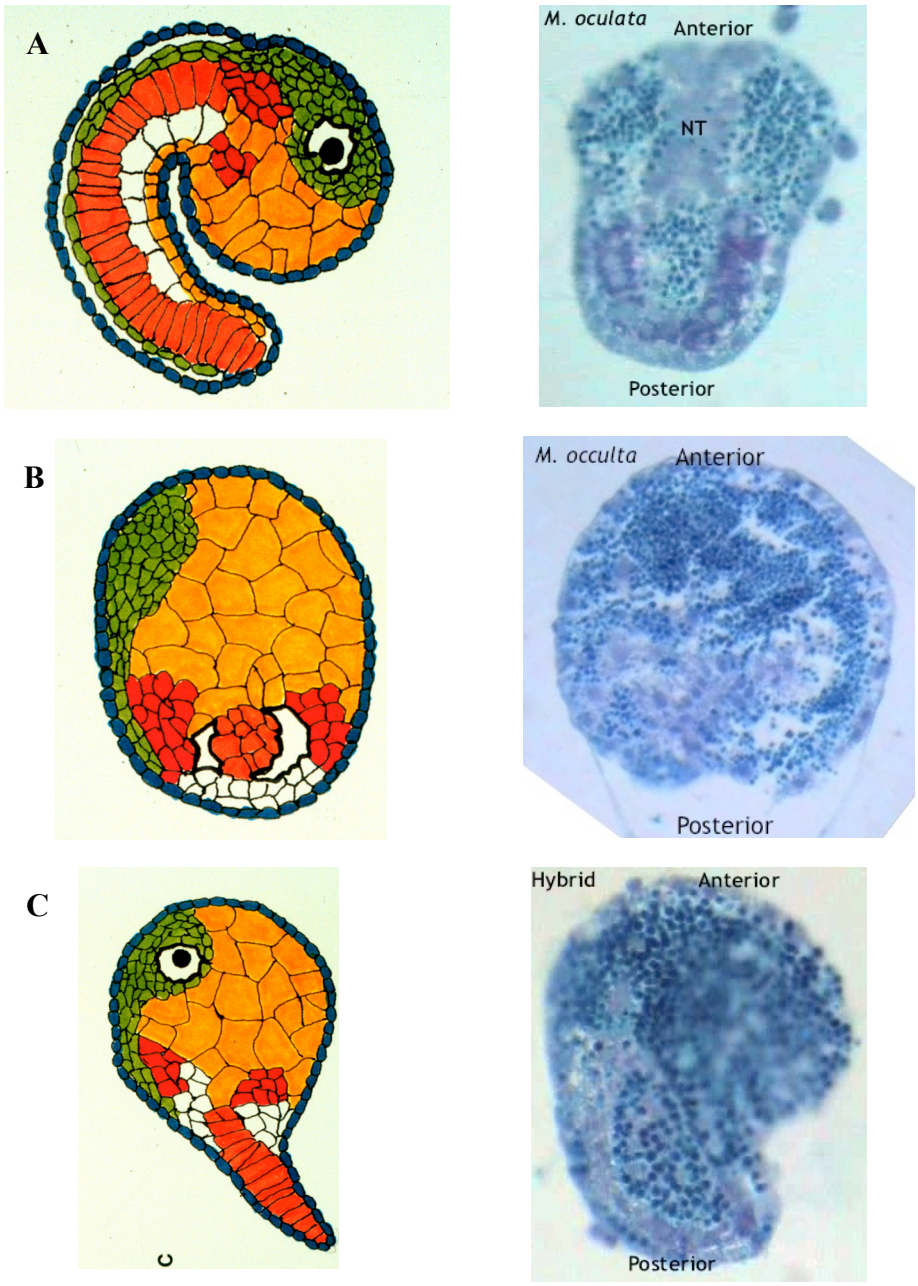


Figure 11: Left: Pictorial representations of *M. oculata* (A), *M. occulta* (B), and the hybrid (C) adapted from Swalla and Jeffery, 1996. Right: Milligan's Trichrome Staining. Blue staining indicates connective tissue. Cells that are purple are muscle cells. NT = neural tube.