THE MYSTERY OF THE DELTA PHENOTYPE: THE ROLE OF THE NOTCH SIGNALING PATHWAY IN *TRIBOLIUM CASTANEUM* EMBRYOGENESIS

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2017

STATEMENT BY AUTHOR

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Acknowledgments:

I would like to thank Anthony Seago and Tara Coalter for carrying out the Delta eRNAi injections; Samantha Pluta for helping with data analysis; Nipam Patel for providing the Pax 3/7 antibody; Greg Davis for helping me get Nipam Patel to send the Pax 3/7 antibody; Terri Williams for providing analysis on the *Thamnocephalus* orthologs as well as insight for the *Tribolium* orthologs; Lisa Nagy for helping with all aspects of my project from experimental design through data analysis and discussion and comments on the manuscript; and all members of the Nagy lab over the past 3 years for all of their support.

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ABSTRACT

Vertebrates, annelids, and arthropods have evolved to form their body plans via segmentation. The question is whether this process stems from a common, segmented ancestor or if segmentation in these three phyla evolved from a series of independent events. To determine which of these theories is true, we look to determining whether these phyla share any pathways in the development of their segments. The Notch signaling pathway is a well-known pathway that vertebrates utilize for segmentation. Without it, somitogenesis does not occur properly as the segmentation oscillator is not functioning. *Drosophila* does not use this pathway for segmentation, but several other arthropods have recently been found to utilize it in the formation and maintenance of their segments (17-24). There has been debate as to whether *Tribolium castaneum* also uses the Notch pathway during segmentation as previous knockdowns of the Notch and Delta genes have led to a loss of segments and appendages/mouthparts (25-27). To determine this pathway's involvement in *Tribolium* segmentation, I knocked down the Delta gene via eRNAi and attempted to determine Notch and Delta expression patterns via *in situ*

hybridization. My results were inconclusive for determining the role of the Notch signaling pathway in segmentation. In the Delta dsRNA embryos, a loss of the labial segment, head and mouthpart defects, a loss of leg formation, and midline defects were seen. Future experiments need to be performed to determine whether an overexpression of mesoderm, ectoderm, or both is the cause of the defective ventral midline and whether this could lead to a loss of segments later in development. Overall, I can conclude that the Notch signaling pathway plays a role in mouthpart/leg development, the labial segment, and what I believe to be lateral inhibition between mesoderm and ectoderm determination.

I. INTRODUCTION

A. The Evolution of Segmentation

Every organism has evolved to form their body plans through unique yet similar mechanisms. Of the 30 well described phyla, metameric segmentation of the anterior-posterior axis occurs in just a minority (1). This minority of organisms, however, have exhibited high morphological diversity, which argues for some sort of functional benefits to utilizing repetitive building blocks (2-4). This repetition allows for the organisms to have some flexibility in differentiating their body regions depending on the task that each space must perform (1). When we think of this type of advantageous design, we generally think of convergent evolution among these different organisms such as the emergence of distinct fingerprints in koalas and humans. This begs the question as to whether there are multiple evolutionary and/or molecular origins of segmentation (1). There are two commonly held, yet opposing theories: 1. The common ancestor between these organisms built its body plan via segmentation. 2. Through a series of

independent events, these taxa have evolved to build their body plans through segmentation as that is the developmental tool that was most advantageous. The answer to this evolutionary question could lie within the discovery of pathways that are shared in the development of segmentation amongst these three taxa.

The three major taxa that exhibit metameric segmentation that have been well studied: arthropods, annelids, and vertebrates. When researchers first began studying these three taxa, there appeared to be very distinct molecular mechanisms that each utilized to generate their segments (1). When investigators tried to use the well described segmentation gene network known from *Drosophila melanogaster* to uncover a common link to segmentation they did not get anywhere (1). Rather recently, however, there has been some headway in finding a molecular link between the taxa in terms of pair-rule patterning and the formation of segment borders (1). There is also evidence for an ancestral Notch-driven oscillator that has become a strong candidate for the basis of a molecular link in segmentation between the three taxa.

B. The Notch/Delta Signaling Pathway

The Notch/Delta signaling pathway is involved in several developmental processes that regulate cell fate (5). For example, *Notch* and *Delta* form a boundary in the wing imaginal disc in *Drosophila melanogaster* that allows for the proper formation of the dorsal-ventral axis (5). Without this boundary, a proper wing will not form. The formation of the compound eye in *Drosophila* is regulated in a similar way by this signaling pathway (5). In fact, many imaginal discs in *Drosophila* utilize the Notch signaling pathway to regulate axis patterning, such as in the leg imaginal disc (5). Notch/Delta signaling is also crucial in the mechanism of lateral inhibition in neural development in *Drosophila* embryos (5).

Notch itself is a transmembrane protein with its ligand also being a transmembrane protein. *Delta* is one of the most common ligands for *Notch*, but *Serrate* and *Jagged* are both known to be ligands for this receptor as well. As the ligands are also membrane-bound proteins, *Notch* signaling requires direct cell-to-cell contact (5). When the ligand on one cell binds to *Notch* on an adjacent cell, a proteolytic event occurs at the intracellular portion of the *Notch* protein. This Notch intracellular domain is cleaved and translocated to the nucleus of the cell (5). It is there that it can bind to and activate *Suppressor of Hairless* (*Su*(*H*)) and act as a co-transcription factor for genes such as *Hairy*. Depending on the desired outcome of Notch signaling, a wide range of genes can be switched on and off (5).

C. Notch and Vertebrate Segmentation

Perhaps one of the first indications of the importance of Notch signaling in vertebrates came from studying *Notch1* null mutants in mice (6). This homozygous null mutant produced embryos that exhibited severe defects in somitogenesis. Without *Notch1*, the somitogenesis was delayed and disorganized in the embryos (6). It was also found that *Notch1* gene function was required for the embryo to survive during the second half of gestation, whereas the mutation had no effect on the first half of gestation (6). It was from these results that it was concluded that Notch1 normally coordinates the process of somitogenesis (6). In homozygous *Su(H)* null embryos, a slightly more severe phenotype was observed with somitogenesis failing earlier than what was seen in the *Notch1* null mutants (7). This result shows the significance of the Notch signaling pathway in somitogenesis.

In *Xenopus* and zebrafish embryos, Notch activity was also demonstrated to be important for somitogenesis. These embryos were injected with mRNAs that encoded proteins that either ubiquitously deregulated Notch signaling activation or inhibited signaling altogether (8-10). In both cases, aberrant somite formation occurred. These results indicate that Notch signaling plays a role in segmentation by way of mediating the cell-to-cell interactions that occur in forming the segmental boundaries for somitogenesis (9). These experiments concluded that tight control of Notch signaling is crucial for the proper implementation of somitogenesis (8-10). Notch signaling was also found to act as a segmentation clock in these experiments with cyclic expression of Notch corresponding to a formation of segments during somitogenesis (8-10). Altogether, these studies implicate Notch signaling in the regulation of segmentation in vertebrates (11).

D. Notch and Annelid Segmentation

Knowing how Notch signaling affects segmentation in vertebrates, investigators sought to test its function in other segmented phyla, such as annelids (12). Notch homologs in the leech *Helobdella robusta* are expressed in the posterior progress zone (PPZ), which is also known as the posterior growth zone, as well as in many different cell types in the early embryo, (12). In order to knockdown Notch function, stage 5 embryos were bathed in a Notch inhibitor (DAPT) for 36 hours (12). DAPT inhibits that proteolytic event that translocates the Notch intracellular portion of the protein, thereby inhibiting Notch signaling (12). This treatment was also combined with an injection of a morpholino for As *Hro-hes*, a downstream target of Notch (12).

This combination produced a phenotype in which segmentation was disrupted (12). The severity of the disruption ranged from mild to severe, depending on the amount of morpholino injected into the subject (12). These results indicate that disrupting Notch signaling leads to a disruption of segmentation. This means that Notch signaling could function in either the maintenance of the PPZ and/or the overall patterning process of segmentation, thereby providing evidence for the Notch signaling pathway's action in segmentation in this taxa (12).

E. Notch and Arthropods

In *Drosophila melanogaster*, Notch function is important for cell-to-cell interactions that are necessary for proper epidermal, mesodermal and neural cell fate determinations, but Notch does not function is segmentation (1, 13, 14) (Introductory Figure). *Drosophila* embryos, however, show a very derived mechanism of segmentation, where all segments form nearly simultaneously. By contrast, nearly all other arthropods add their segments sequentially, in an anterior-posterior progression. *Drosophila* is one of the most well studied organisms to date. It is used as the comparison and example for arthropod development, which has lead to the discovery that many of the mechanisms and networks utilized by *Drosophila* for segmentation were found to work in other arthropods as well (i.e., pair-rule genes).

Based on its role in *Drosophila*, it was assumed that Notch would not be involved in arthropod segmentation, until Stollewerk et al., 2003 performed a RNAi knockdown of Delta in the spider *Cupiennius salei*. This particular experiment and subsequent paper blew the lid off of the role of Notch signaling in arthropod segmentation. When Notch and Delta were knocked down through embryonic RNAi (eRNAi), similarities were found among this arthropod and vertebrate somitogenesis (17). The embryos exhibited defects in segment patterning, such as a

malformation of segments with varying width, size, and shape, and in the formation of the segment borders (17) (Introductory Figure). The posterior growth zone was also found to be enlarged and malformed (17). When the expression patterns of both Notch and Delta were evaluated in *Cupiennius*, it was found that they were similar to that seen in vertebrate somitogenesis (17).

Notch signaling was then investigated in another spider, *Parasteatoda tepidariorum* (formerly *Achaearanea tepidariorum*). When Delta was knocked down in this animal, defects were found in the formation of the caudal lobe of the embryos of the female spiders following pRNAi injections (18). These embryos displayed an unusually large invagination of the germ disc which resulted in thicker cells in the emerging caudal region (18). The most severely affected embryos had a significant reduction of the prosoma, disorganized germband configuration, and a loss of the entire opisthosoma (18) (Introductory Figure). The researchers also found that when Delta was knocked down, an overproduction of twist-expressing mesoderm cells occurred as a result of the reduction in caudal-expressing ectoderm cells (18). This all led to the conclusion that progressive activation of the Notch signaling pathway from the blastopore leads to stochastic cell fate decisions between either mesoderm or caudal ectoderm fates via lateral inhibition in order to set up a functional caudal lobe (18). Without this lobe, the animal will not form its segments properly; therefore, without Notch signaling that sets up this functional lobe the animal would not be able to undergo segmentation (18).

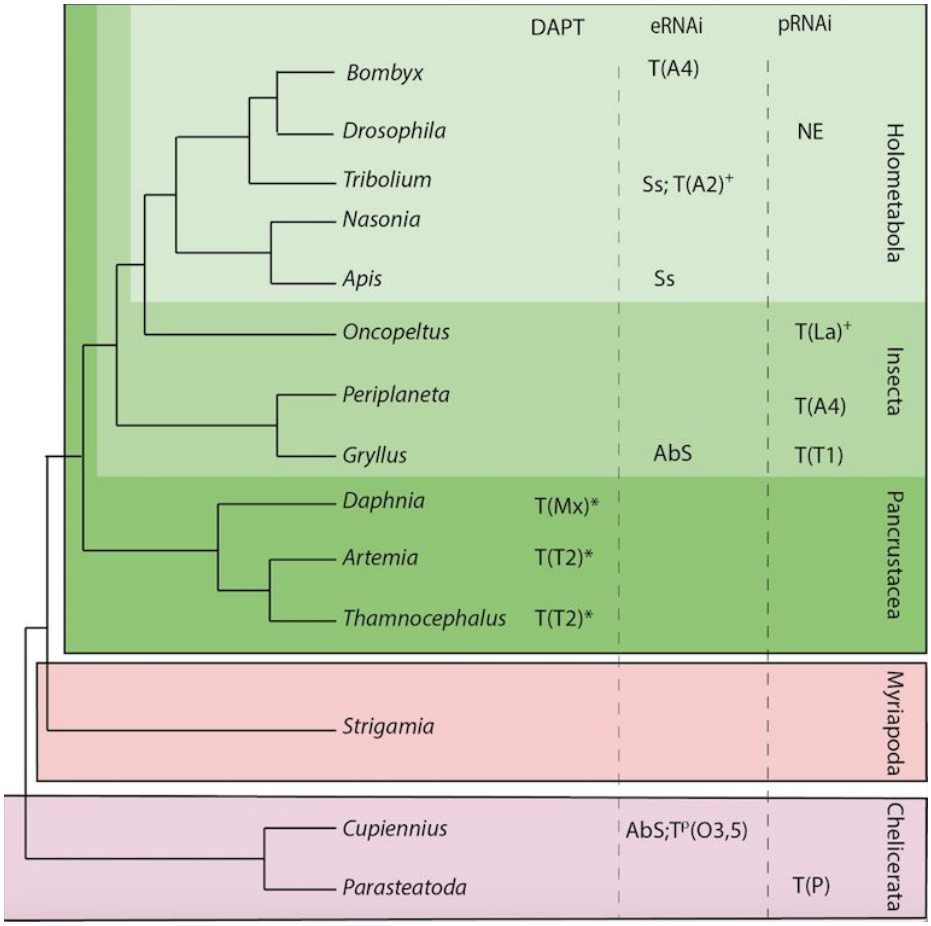
With the basal arthropods displaying segmentation defects in the absence of Notch signaling, a big question was left concerning the evolution of segmentation: given the functional role of Notch signaling in spiders, when during the evolution of arthropods was this function

lost? This led to an investigation Notch signaling in more derived arthropods. In the branchiopod crustacean *Thamnocephalus platyurus*, embryonic knockdown of Notch via chemical inhibition (DAPT) resulted in an unexpected phenotype : segmentation was truncated at thoracic segment 2 but elongation of the animal was unaffected (19). This phenotype of loss of segmentation but continued elongation was observed in several other branchiopod crustaceans (20), which makes this a conserved phenotype in this branch of the arthropod tree (Introductory Figure). Thus, in contrast to spider, where both segmentation and elongation were affected by loss of Notch signaling, in the brachiopods the result to knocking down Notch signaling showed for the first time that segmentation could be separated mechanistically from elongation.

When Notch signaling was knocked down in the cricket *Gryllus bimaculatus*, conflicting results were found. In one experiment, pRNAi of Delta in *Gryllus* led to a loss of both body and leg segments (21). This allowed researchers to conclude that Notch signaling is required for segmentation in this arthropod. When Delta was knocked down via eRNAi, however, the animals displayed fused and malformed segments but are actually a result of disruption of neuroectoderm and mesectoderm formation (22) (Introductory Figure). In these experiments, the loss of Delta lead to an overexpression of *twist* in the ventral midline of the animals, and an overexpression of neural cells as detected by antibody staining with Pax 3/7 (22). This leads the authors to conclude that Delta is required for neuroectoderm and mesectoderm formation, and defects in this process lead to secondary anomalies in segmentation.

There is strong evidence for a Notch/Delta function in segmentation of the cockroach *Periplaneta americana* (23). In this organism, Notch and Delta form a cyclic wave of expression that acts with a Wnt organizer to drive segmentation (23). In the late germband stages, Delta

expression can be seen in the posterior growth zone, and then it emerges from this zone in segmentally iterated stripes (20, 23). When Notch is knocked down via pRNAi, embryos are truncated posterior to the fourth abdominal segment (Introductory Figure). Similar defects were seen when embryos were treated with DAPT (23). In *Oncopeltus fasciatus,* Delta pRNAi results showed severe posterior segmentation defects (24) (Introductory Figure). Invagination proceeded normally in the embryos, and the expression pattern of other segmentation genes such as *wingless*, *even-skipped*, and *invected* remained unaffected (24). We can infer that segmentation proceeded normally in the embryos and the larvae post-hatching exhibited the severe posterior defects (24). The most severe phenotype had a fully formed head but a shapeless, fused trunk throughout its posterior (24). We can conclude from this data that Notch signaling is evolving throughout the more derived arthropods. In the cricket, secondary anomalies occurred in segmentation but this pathway was found to be required for mesoderm and neuroectoderm boundary formation (22). In the cockroach, segment truncation occurred posterior to the fourth abdominal segment which means it functions in segmentation (23). Finally, the milkweed bug managed to form segments properly during embryogenesis but then lost the posterior segments sometime before hatching which suggests a function for the Notch signaling pathway in maintaining segments (24).



Introductory Figure: Phylogenetic tree of Notch signaling. This tree shows the effect of knocking down the Notch signaling pathway in several arthropods from Chelicerata to Holometabola. Each column indicates the different method used to knock the pathway down: DAPT = chemical inhibition; eRNAi = embryonic RNAI; pRNAi = parental RNAi. NE = no effect on segmentation; T = truncation; Ss = smaller segments; Abs = abnormal segments, not truncated; O = opisthosomal segment; P = prosomal segment; A = abdominal segment; T = thoracic segment; * = disruption of segmentation but not elongation (20).

F. Notch and *Tribolium castaneum*

There has been a long held understanding that the Notch signaling pathway is not utilized in the segmentation process of *Tribolium castaneum* (1, 25, 26). When you look at the data, however, there appears to be a loss of function concerning the organism's segments when the pathway is knocked down. When Delta was knocked down via eRNAi (Aranda, PhD thesis) a

late loss of segments occurred (25). Despite this result, Aranda nonetheless concluded that the Notch signaling pathway is not involved in segmentation because knockdowns of Su(H) were normal (25). However, these results are not shown and there is no mechanistic explanation given for the late loss of segments. Delta knockdowns were also reported to have small heads, deformed mouthparts missing labial segment (25). Aranda's *in situ* analysis predicts a function for Delta in the early mesoderm, early posterior growth zone, mouthparts, and nervous system (25). His phenotypic analysis only uncovers defects in the mouthparts and head - the smaller head implying a defect in the nervous system/brain development (25). There is no analysis of mesodermal or mesectodermal defects or developmental sequence in loss of function embryos to uncover the cause of the late loss of segments.

In Kux et al. 2013, a Notch knockdown led to the reduction in length of the space between segments as determined by marking the segments with an Engrailed antibody (27). The overall length of the embryos was also reduced and in the most severe mutants engrailed expression was interrupted or barely visible (27). These eRNAi embryos were also stained with the antibody for Snail, which is a neural marker, and it was found that loss of Notch lead to an upregulation of snail expression in the cephalic and ventral neuroectoderm (27). Snail positive cells also formed clusters rather than singularized cells as seen in wild type embryos, which shows that loss of Notch function leads to a loss of neuroblast singularization (27). Snail expression, which is thought to be of mesodermal origin, was also abolished in the posterior growth zone, indicating a role for Notch in growth zone development (27). Caudal expression in the ectoderm of the posterior growth zone, however, was not affected by the Notch knockdown (27).

Taken together the Kux (27) and Aranda (25, 26) data suggest a previously ignored function for the Notch signaling pathway in *Tribolium castaneum*. This led me to investigate the role of this signaling pathway in this organisms during embryogenesis via knockdowns of an integral gene in the pathway, Delta. I attempted to determine the expression pattern of both genes at different time points as well to find correlative evidence for their function based on when and where they were expressed. I found that Delta knockdowns resulted in a loss of the labial segment and midline defects, but I was not able to replicate the late loss of segment phenotype reported by Aranda (2008). Some Delta knockdowns also show a loss of engrailed staining in the ventral nervous system.

II. MATERIALS AND METHODs

RNA Extraction

A 24 hour egg collection was done in order to collect embryos from a wide range of time points for the isolation of mRNA transcripts. The embryos were then collected in an eppendorf tube and 1 mL of TRIzol (Invitrogen) was added to the tube. The Tissue Terror (a metal homogenizer) was then used to homogenize the embryos and all of their cells. The cells then incubated at room temperature for 5 minutes to allow for the complete dissociation of the nucleoproteins. 0.2 mL of chloroform was added to the tube and incubated for 2 minutes. The sample was then centrifuged for 15 minutes at maximum speed at 4°C. This allowed for the mixture to separate into three different layers: the lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA was in the upper aqueous phase, which was carefully transferred to a clean eppendorf tube. 0.5 mL of isopropanol was then

added to this aqueous phase and incubated at room temperature for 10 minutes. The sample was then centrifuged for 10 minutes at maximum speed at 4°C. A glass pipette and rubber bulb was used to remove the supernatant from the pellet. The pellet was resuspended in 70% ethanol and centrifuged for 5 minutes at maximum speed at 4°C. The supernatant was removed using a glass pipette and a rubber bulb, and the pellet air-dried for 10-15 minutes. The pellet was then resuspended in 50 ul of RNase-free water by gently pipetting the pellet up and down. This solution was then incubated on a heat block at 55°C for 10 minutes. The concentration of the RNA was determined by using the Nanodrop to measure its absorbance at 260 nm.

cDNA Synthesis

The SMART MMLV Reverse Transcriptase protocol and kit were used. 2.5 ul of 20 uM oligo dT primers or random decamer primers (Invitrogen) were added to about 1 ug of the extracted RNA sample before 11.5 ul of RNase-free water was added. This mixture was then heated at 70° C for 3 minutes, after which it was immediately cooled on ice. The sample was then briefly centrifuged. The following solutions were then added to the sample: 4 ul 5X First Strand Buffer, 2 ul dNTPs, and 2 ul 100 mM DTT. The contents of the tube were mixed by gently pipetting the solution up and down before 0.5 ul of the SMART MMLV RT was added and the solution was mixed again. This mixture was then incubated at 42° C for one hour. The reaction was then terminated by heating the solution at 70° C for 15 minutes. The concentration of the cDNA was determined using the Nanodrop.

PCR Amplification

Specific primers were designed to amplify a 794 bp fragment of the Notch gene from cDNA and a 189 bp fragment and 1.6 kb fragment of the Delta gene (Fig. 1A & B). The primers used for

Notch were nested primers: N-F1: ATGCGACATTGACATCGACGAAT;

N-R1:CTTTGGGGCATTTACACGAGTAGGAA;

N-F2:GCAACCAGTACGTCGATTCATACAC;

N-R2: GAATCACATTCGTTGATTTCCGTCT

The first set of primers used for the 189 bp Delta fragment were provided by the Jockush lab:

Dl-F1: AACTCCGAATGCGAGGGAATGTGT;

Dl-R1: AAGTGAAGGGAAAAGGCAAAGCGA

The second set of primers for the 1.6 kb Delta fragment were designed using Primer3 Bio Tools:

Dl-F2: TGCAGTGAGCGGAATTGACT

DL-R2 - CTCCGTTGGCACAAGGTTTG

The primers were resuspended in water to 100 mM. The components for PCR were all mixed together in a thin walled PCR tube in accordance with the specifications for the One Taq Polymerase (New England Bio Labs). The program included an initial denaturation (95°C for 30 seconds), 30 cycles of denaturation (95°C for 30 seconds), primer annealing (45°-68°C for 15 seconds), and extension (68°C for 1 minute/kb), and a final extension (68°C for 5 minutes) as its steps. PCR products were checked on a 1% agarose gel and stored at -20°C.

Cloning

The gene was cloned into a Strataclone pSC - B-amp/kan PCR cloning vector. Using One Taq Polymerase allowed for "sticky ends" (poly-A rich ends) to be added to the amplified gene, which allowed the gene to be cloned into this vector. On either side of the gene insert site sits an Eco RI site with M13 F/R primer sites on either side of the gene. The cloning processes includes a ligation and a transformation step. The gene of interest is mixed with a Strataclone cloning

buffer and a Strataclone vector mix. This ligation mixture is mixed thoroughly and incubated at room temperature for five minutes, after which the mix is immediately put on ice. A tube of Strataclone SoloPack competent cells (*E. coli* cells) is thawed on ice, and the ligation mix is added to the cells. These cells are incubated for 20 minutes on ice, at which point the LB medium is pre-warmed to 42°C. The transformation mixture is heat shocked at 42°C for 45 seconds. This process makes holes in the cell membranes that allows the gene insert-vector to move into the cells. The mixture is incubated on ice for 2 minutes, after which 250 ul of prewarmed LB medium is added to the mix. The competent cells must recover for at least 1 hour at 37°C with shaking. Pre-made X-gal LB-kanamycin plates, which will allow for blue-white selection of the colonies, are warmed for this time period as well. The entire transformation mixture is plated on the plate and allowed to grow overnight at 37°C.

Colony PCR/Sequencing

After allowing the cells to grow into colonies overnight, selection of appropriate colonies can be done. Colonies that should have incorporated the vector will be white or light blue. Colony PCR is done to ensure that the gene of interest is in the selected colony. For each colony that will be checked, make a PCR mixture in accordance with the specifications for the One Taq Polymerase. This time, however, M13 F/R primers (annealing temperature: 55°C) are used to amplify the gene of interest. These primers add 260 bp to the size of the gene as it shows up on a 1% agarose gel. Using a sterile pipette tip, carefully pick up a colony and gently swirl it in the PCR mixture. Make sure no liquid has entered the pipette tip. If this does occur, apply pressure to the open end of the tip with a finger to push the liquid back into the PCR tube. Afterwards, take the pipette tip and streak it on a section of a pre-made X-gal LB-kanamycin plate. Once all

colonies are set up in their appropriate PCR tubes and streaked on the plate, place the plate in the 37°C incubator overnight to allow the colonies to grow. This allows for individual colonies to grow from the streak, allowing easier isolation of single colonies in later uses. Colony PCR is run and the product is checked on a 1% agarose gel.

This gel determines whether the product is the correct size as the gene of interest. To confirm the sequence of the cloned gene , a sample of the PCR product is sent for sequencing at a separate facility. The product goes through a PCR clean up process (illustra GFX PCR DNA and Gel Band Purification Kit) that will remove any excess nucleotides, primers, etc. and leave just the amplified product. Once the clean up process is complete, the product can be evaluated for its concentration (ng/ul) . With the concentration in hand, a sample of the product (no more than 8 ul) is placed in a separate eppendorf tube and labeled appropriately with its name and concentration. The UA sequencing facility will sequence the PCR product . The sequence can then be evaluated using BLAST to ensure that it is the correct gene.

Plasmid Mini-prep

The PureLink Quick Plasmid MiniPrep Kit (Invitrogen) was used for all plasmid preparations. The isolated bacterial colony that contained the correct gene sequence was grown overnight in LB medium. 1-5 ul of this culture was then briefly centrifuged at room temperature at maximum speed after which all LB medium was removed. 250 ul of the Resuspension Buffer with RNase-A was added the cell pellet and the pellet was resuspended until the solution was homogeneous. 250 ul of the Lysis Buffer was then added. The tube was gently inverted in order to mix the solution until it was homogeneous, and then the tube was incubated at room temperature for 5 minutes. 350 ul of Precipitation Buffer was then added and the tube was

gently inverted once again until the solution was homogeneous. The lysate was centrifuged for 10 minutes at maximum speed. The supernatant was loaded onto a spin column in a wash tube and centrifuged at 12,000xg for 1 minute. The flow-through was discarded and 500 ul of Wash Buffer W10 (with ethanol) was added to the column. The column was incubated at room temperature for 1 minute and then centrifuged at 12,000xg for 1 minute. The flow-through was discarded again and 700 ul of Wash Buffer W9 (with ethanol) was added to the column. This was centrifuged at 12,000xg for 1 minute after which the flow-through was discarded. This step was repeated once more to ensure all wash buffer was removed from the column, and the wash tube was discarded with the flow-through this time with the column being placed in a fresh eppendorf tube. 75 ul of preheated TE Buffer was added to the column and incubated at room temperature for 1 minute. The column was centrifuged at 12,000xg for 2 minutes, after which the column was discarded and the concentration of the purified plasmid was determined using the Nanodrop.

Riboprobe Synthesis

Delta, Notch, and Wingless T3 and T7 digoxygenin labelled riboprobes were synthesized using an Ambion MAXIscript kit. Template preparation for riboprobe synthesis was performed via PCR amplification with M13 F/R primers. Probes were hydrolyzed to 150 bp with 2X HCO2 Buffer to facilitate entry into the cell membranes of the specimen. Riboprobe concentrations were determined using RNase-free gels and Dot Blots against a control RNA with a known concentration (Roche).

***In situ* Hybridization**

For *in situ* hybridization, embryos were collected between 0 – 30 hours after egg lay. I followed a method used by Benton et al. 2016 that was an adaptation of the method used by Schinko et al.2009. Embryos were stepped out of 100% methanol using a 75%, 50%, and 25% methanol series, at which point the embryos were washed in PBTween. The embryos were then rinsed with Hyb B (50% formamide, 5x SSC (pH 5.5) (30)), and then set for pre-hybridization in Hyb A (50% deionized formamide; 5x SSC (pH 5.5); 10 mg/mL boiled sonicated salmon testis DNA; 20 mg/mL yeast tRNA; 50 mg/mL heparin (30)) for 1 hour at 65°C. Riboprobes were diluted to 50 ng/ul in Hyb A and heated at 80° C for 5 minutes. Embryos were hybridized overnight at 65° C in Hyb A. Embryos were washed with Hyb B and PBTween for 15 minutes at 65°C and washed in blocking reagent composed of PBTween + 0.1% BSA, and incubated (room temperature) overnight in anti-DIG antibody (1 : 1000 dilution). Excess antibody was washed away with PBTween for 20 and 30 (x2) minute increments. Embryos were rinsed with freshly prepared Alkaline Phosphatase developing solution quickly twice. 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP/X-Phos; Roche) and 100 mg/ml 4-nitro blue tetrazolium chloride (NBT; Roche) were then added to the developing solution to create the staining solution, and embryos were incubated in staining solution in the dark for 10-20 minutes. The embryos were checked, periodically, to monitor staining. Staining was stopped with several rinses of PBTween followed by 1:2000 ul DAPI (1 mg/ml): PBTween staining. Embryos were stored in 80% glycerol with 4% propyl-gallate.

Analysis

Embryos were mounted in 80% glycerol with 4% propyl-gallate on glass slides and imaged using a Zeiss Axioplan microscope at 10X and 20X magnification with an AxioVision camera captured on the AxioVision software (Release 4.8.2).

dsRNA Synthesis

Delta and Notch double-stranded RNA (dsRNA) molecules were synthesized using an Ambion MEGAscript kit. Template preparation for dsRNA was performed via PCR amplification with a pSCA-T7 primer. This primer was made in order to recognize the Eco-RI site that flanks the insert and adds a T7 promoter on both strands allowing for the synthesis of dsRNA from a single template. Concentrations of the dsRNA were determined using the Nanodrop to measure concentration in ng/ul and absorbance at 260 nm.

RNAi Microinjections

Embryonic RNAi (eRNAi): A 1 hour egg lay was done, after which the embryos were collected and incubated at 30°C in a petri dish for 3-4 hours. A 1% agarose plate was poured and allowed to solidify before 200 micron nitex strips were cut and placed onto the agarose for lining up the embryos. Embryos were dechorionated in 3% bleach solution for 2 minutes before being thoroughly rinsed with deionized water. A 0000 paintbrush was used to carefully pick up the embryos from the nitex mesh and transfer them to the strips of nitex. The embryos were arranged so that all posterior ends were pointing to one side of the dish. Glass capillary tubes were used in a Sutter micro-pipette puller (Model P-97) under the following conditions: Heat - 295, Pull - 200, Velocity - 100, Del - 30, P - 500. The injection buffer was made as follows: 1 ul Fast Green Dye (5%), 9 ul injection buffer. 1 ul of this injection buffer was then added to 9 ul of dsRNA for the RNA injections. The rounded end of the needle was placed in the appropriate

solution and capillary action allowed the needle to fill up. The needle was then placed in the injection arm of the injection apparatus. The nitrogen tank was opened a quarter turn, the balance for the apparatus was set to 0.5 - 1, and the P-inject was set to about 5. The needles were broken using sharp tweezers under the injection microscope at 6 - 8X magnification. The injection arm was then arranged at a 45 degree angle before the microscope was focused on the embryos at 2X magnification. The needle was then dropped into focus. The needle was inserted into the embryo's side in the middle of the specimen. 1 or 2 pumps of the injection pedal were done for each embryo to inject a small volume into each specimen. After injections, the plates were placed in the 30°C incubator.

Pupal RNAi (pRNAi): This protocol was derived from the embryo injection protocol. Pupae were collected anywhere from the day of injections to 3 days before injections. If the injection took place a few days after collection, the pupae were placed in the 25°C incubator to slow their development. The pupae were sexed and separated using flat, rounded tweezers. The males and non-injected females were placed in separate petri dishes with flour and put in the 30°C incubator. For the injected females, double-sided tape was placed on glass slides and then sprinkled with flour. The flour was then scraped off of one edge with the round tweezers and the posterior ends of the pupae were carefully pressed down onto the tape. The injection buffer and dsRNA were made as described for eRNAi. Glass capillary tubes were pulled in the micropipette puller under the following conditions: Heat - 290, Pull - 150, Velocity - 100, Del - 100, P - 500. The injection procedure was carried out as described above for eRNAi. The needle was inserted into the lateral side of the 6th abdominal segment. After injecting, a fine

paintbrush was used to gently remove the pupae from the tape. The injected females were then placed in a petri dish with flour in the 30°C incubator.

Antibody Staining and Analysis

Embryos were carefully dissected from their vitelline membranes by placing them in a drop of PBTriton on a slide with double-stick tape . The vitelline membrane was carefully peeled away from the embryos using #5 Dumont forceps. In some cases, the vitelline membranes came off during the fixation process. In many other cases, the embryos were too fragile for dissection and were only subjected to DAPI staining (1:200). Dissected embryos were stained overnight in primary antibody for either Engrailed (1:150; 4D9 Developmental Studies Hybridoma Bank) or Pax 3/7 (1:150; gift from Nipam Patel). The next day the embryos were placed in secondary antibody (Cy3 conjugated anti-mouse IgG, 1:200; Jackson Labs) for 2 hours before DAPI (100 ng/ml) staining as the final step. Embryos were mounted in 80% glycerol/4% propyl-gallate on glass slides and imaged using a Zeiss Axioplan microscope at 10X and 20X magnification and an AxioVision camera captured on the AxioVision software (Release 4.8.2).

III. RESULTS

Cloning

A 189 bp fragment of *Tc-Delta* was cloned into a pSC-a Strataclone plasmid vector. (Fig. 1A). This fragment for *Tribolium* was used for all RNAi experiments. A larger piece of the Delta gene (1.6 kb) was cloned into a pSC-A Strataclone plasmid vector to increase the signal for *in situ* hybridization (Fig. 1A). This fragment spans the EGF repeats rather than the DSL domain, which was a successful domain for *Thamnocephalus* experiments. The larger

fragment also includes sequence that was previously used for *in situ* hybridization for Delta in *Tribolium* (25, 26). A single section of the Notch gene (794 bp) was cloned into a pSC-A Strataclone plasmid vector. This fragment was used for *in situ* hybridization (Fig. 1B).

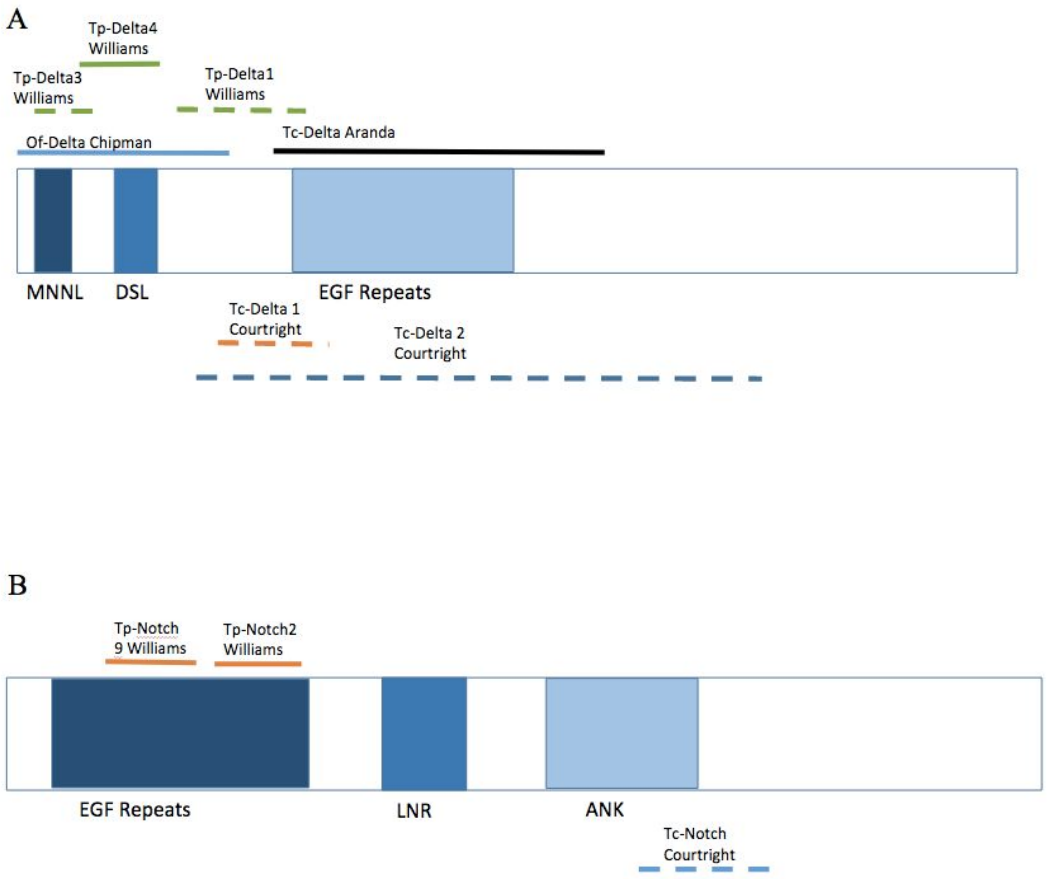


Figure 1: Delta and Notch orthologs in *Tribolium castaneum*. (A) The *Tribolium* Delta ortholog contains a conserved MNNL domain, DSL domain, and several EGF-like repeats. The diagram shows where fragments of the gene have been successfully cloned in *Thamnocephalus platyurus (Tp)*, *Oncopeltus fasciatus (Of)*, and *Tribolium castaneum(Tc)*. The dashed lines indicate clones that were not successful for *in situ* hybridization experiments in Tp. (B) The *Tribolium* Notch ortholog contains several EGF-like repeats and conserved LNR and ANK domains. The diagram shows where fragments of the gene have been successfully cloned in *Thamnocephalus platyurus (Tp)* and *Tribolium castaneum (Tc)*.

In situ hybridization

Aranda et al. 2006, 2008 published the expression pattern for Delta and Notch stages of embryogenesis. They report that Delta is expressed in a circumferential stripe in the mandibular segment, the posterior growth zone and the midline along the length of the embryo. They report Notch having ubiquitous expression. Given our interest in the function of Delta in posterior growth, I sought to further define the expression in the posterior growth zone and along the ventral midline. I was also interested as to whether the early midline expression was in mesodermal or neural precursors, so proposed to double-label embryos with Delta and twist expression, a marker for early mesoderm. There were some promising preliminary results (Fig. 2), but all subsequent *in situ* hybridization experiments yielded embryos with high background and little to no signal (Supplementary Table 1&2). The controls using probes for *wingless* and *twist* consistently worked, , so the procedure for *in situ* hybridization is good.

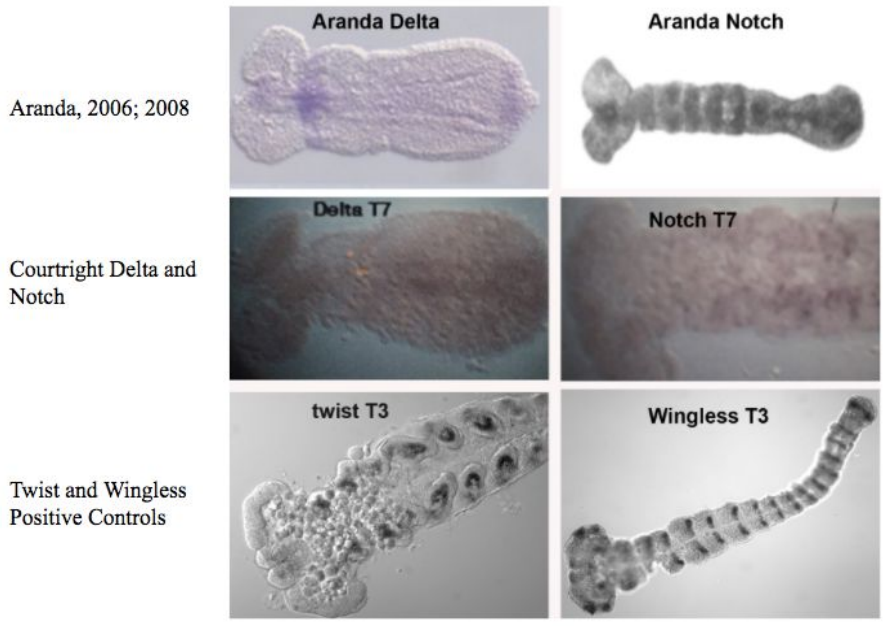


Figure 2: *Tc*-Delta and *Tc*-Notch *in situ* hybridization results. At this stage, Aranda 2006, 2008 report that Delta is expressed in the mandibular segment, the posterior growth zone, and weakly in the mesoderm. Notch is expressed ubiquitously, with enhanced expression in segmental stripes and in a midline stripe that extends from the anterior to the posterior. The Delta embryo has some expression down the midline of the animal, in the mesoderm. The background, however, is too high for positive results. The Notch embryo shows expression in the nervous

system, but again the background is too high for positive results. The Twist and Wingless positive controls worked flawlessly.

Analysis of loss of function: embryonic RNAi

To determine the function of Delta (Dl) in *Tribolium* embryogenesis, microinjections of double-stranded RNA (dsRNA) were carried out on embryos roughly 3-4 hours AEL. The same number of embryos were injected for the buffer as were injected for Dl dsRNA (Table 1). When measured, embryo survival post-injection varied between 25-50% but was no different between dsDl injections and buffer injections. Additional loss of embryos occurred during the fixation process (Table 1). This could be because of how fragile the injected embryos are for both Dl dsRNA and buffer injected groups.

dsDlRNA was injected into embryos at 3 different concentrations, approximately 500 ng/ul, 2000 ng/ul and 5000ng/ul (Table 2). Subsequent to injection with ~500 ng/ul, embryos developed with abnormal midlines, reduced heads and irregularly formed segments. At subsequent to injections of 2000 ng/ul (25, 26), I uncovered similar phenotypes as at 500 ng/ul. In addition, 10% of the embryos were missing their labial segment (as described by Aranda 2006). At this concentration, embryos also appeared to be affected at earlier stages of development. 37.5% of the embryos had early arrest phenotype, however a similar percentage of buffer injected embryos arrested pre-blastoderm, so at this time, it is unclear whether Dl serves an early pre-blastoderm function or not (Table 2). 12.5% of the embryos failed to complete germband formation which resulted in truncated embryos (Table 2). An equal percentage of embryos also exhibited a phenotype in which they had small heads, no legs or mouthpart (mp)

formation, and amorphous segments. The concentration of dsRNA was increased to roughly 5000 ng/ul. This resulted in 75% of the eggs arresting in early development (Table 2).

Table 1: *Tc*-Delta eRNAi Survival (* - Tara Coalter's injections; ** - Anthony Seago's injections)

Date	Concentration (ng/ul)	# Injected	# Survived	# Through Fixation
12/4/2015**	498	40	unknown	8
12/4/2015**	Buffer	40	7	7
12/9/2015*	498	25	unknown	5
12/9/2015*	Buffer	25	N/A; all died	N/A; all died
2/17/2016*	492.6	30	unknown	13
2/17/2016*	Buffer	30	unknown	unknown
6/16/2016*	5004.2	30	unknown	4
6/16/2016*	Buffer	unknown	unknown	unknown
3/8/2017*	2043.4	100	30	30
3/8/2017*	Buffer	100	24	12
3/29/2017*	2000	120	60	14
3/29/2017*	Buffer	120	53	11
4/5/17*	2000	88	46	17
4/5/17*	Buffer	94	54	38

Note: The embryos from 4/5/17 injections were left to develop for 5 days after egg lay in order to try and get them to hatching. This led to most of the embryos drying up and the conclusion that we need better ways to culture the embryos if we plan to leave them incubating for so long.

Table 2: *Tc*-Delta eRNAi Concentration Dependence

Approximate Concentration (ng/ul)	# Analyzed	V: Early Arrest	IV: Failed to Complete Germband	III: Small Head, No Legs, No MP, Amorphous Segments	II:A: Missing Labial Segment	IIB: Abnormal Midline/Head	I: Small Head/Irregular Tissue	Normal
500	23					13 (56.5%)	3 (13%)	7 (30.5%)
2000	40	15 (37.5%)	5 (12.5%)	5 (12.5%)	4 (10%)	4 (10%)	3 (7.5%)	4 (10%)
5000	4	3 (75%)						1 (25%)

Analysis of progressive loss of function over developmental time

Although the dataset is small, there is evidence that the midline defect initiates prior to 25hrs of development, as embryos with abnormal midlines were visible at this early time point (Fig. 3, Table 3). 50% of the animals showed delayed development, with fewer segments than the average buffer injected control but at this point I cannot determine whether this is simply a delay in development induced by injection trauma, or a phenotypic effect of the dsDl injections on segmentation (Fig. 3, Table 3). This defect could be explained by a loss of lateral inhibition that affects the determination of mesodermal tissue and ectodermal tissue. A loss of the labial segment was seen in 3 animals (Fig. 4, Table 4). A midline defect was also seen in these animals. Many of the different categories of phenotypes exhibited a head defect as well (Fig. 4, Table 4) which can be attributed to Delta's role in development of the larval brain. The Delta injected specimen also exhibited an early arrest phenotype, but the buffer injected animals also had this phenotype. For this reason, it is unclear whether this phenotype is caused by a lack of Delta or by injection damage.

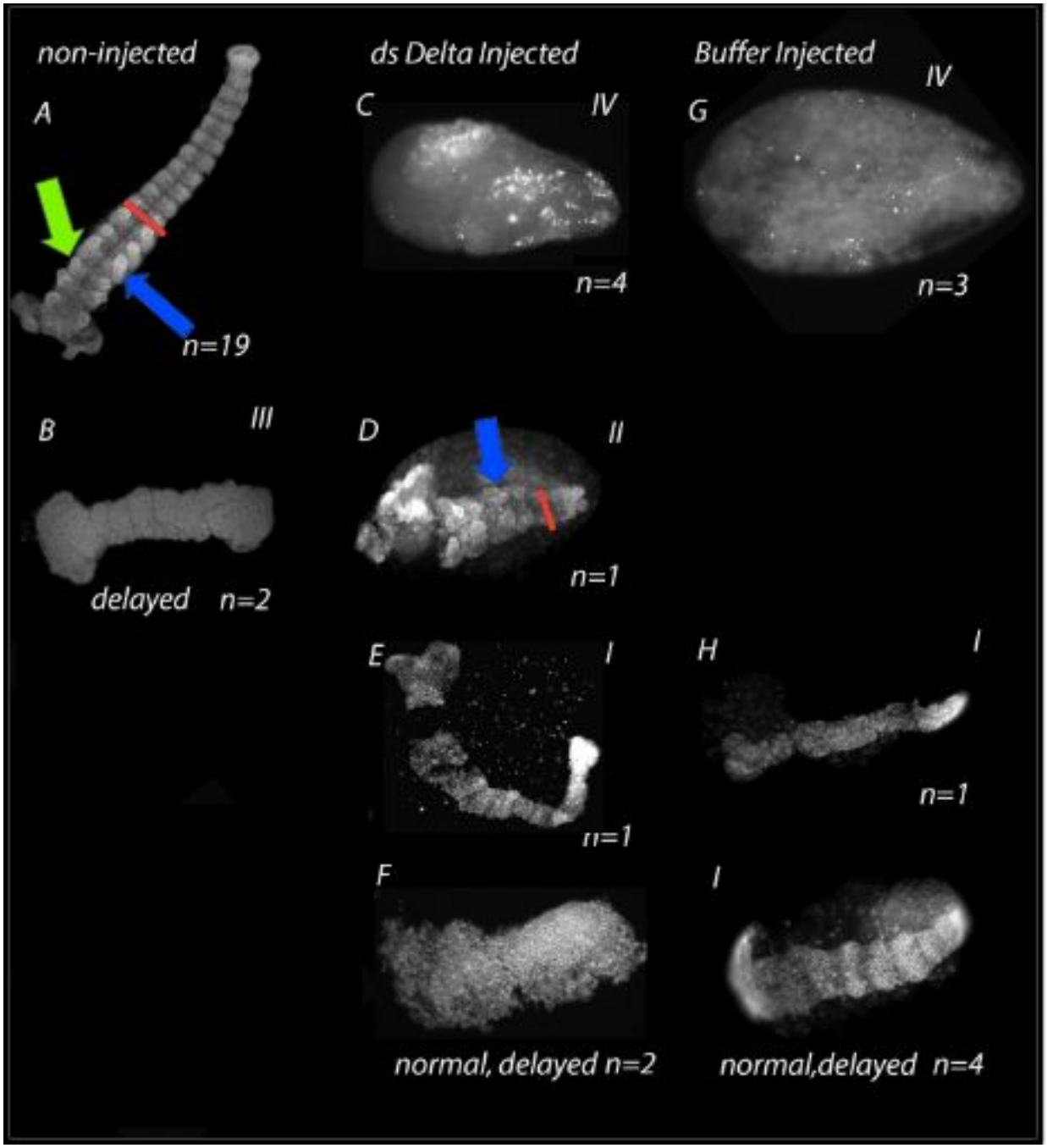


Figure 3: 20-25 hour *Tc*-Delta eRNAi phenotypes. (A-B) Examples of non-injected embryos. (A) Normal 20-25 hour embryos with 16 segments. (B) Delayed, abnormal phenotype observed in only two non-injected animals. (C-F) Examples of Delta dsRNA injected embryos. (C) A category IV (early arrest) mutant, of which the majority of the dsRNA injected exhibited. (D) Example of a category II (reduced segment length/midline defect) mutant. (E) A category I (segment defect/small head/no legs) mutant. (F) Example of a normal but delayed Delta dsRNA injected embryo. (G-I) Examples of buffer injected embryos. (G) A category IV mutant, which was the second most observed phenotype in the buffer injected animals at this stage. (H) Example of a category I mutant in the buffer injected group. (I) A normal but delayed buffer injected embryo. It is normal for embryos that have been injected to be delayed in their development as they have been put through physical trauma. Blue arrows = first leg (T1) segment; green arrow = labial segment; red line indicates segment width, which is reduced in (D).

Table 3: 20-25 hour *Tc*-Delta eRNAi phenotypes.

	total imaged	IV: early arrest	III: delayed	II: reduced segment width/midline defect	I: segment defect/small head/no legs	normal
dsDelta	8	4 (50%)		1 (12.5%)	1 (12.5%)	2 (25%)
Buffer	8	3 (37.5%)			1 (12.5%)	4 (50%)
Non-injected	21		2 (9.5%)			19 (90.5%)

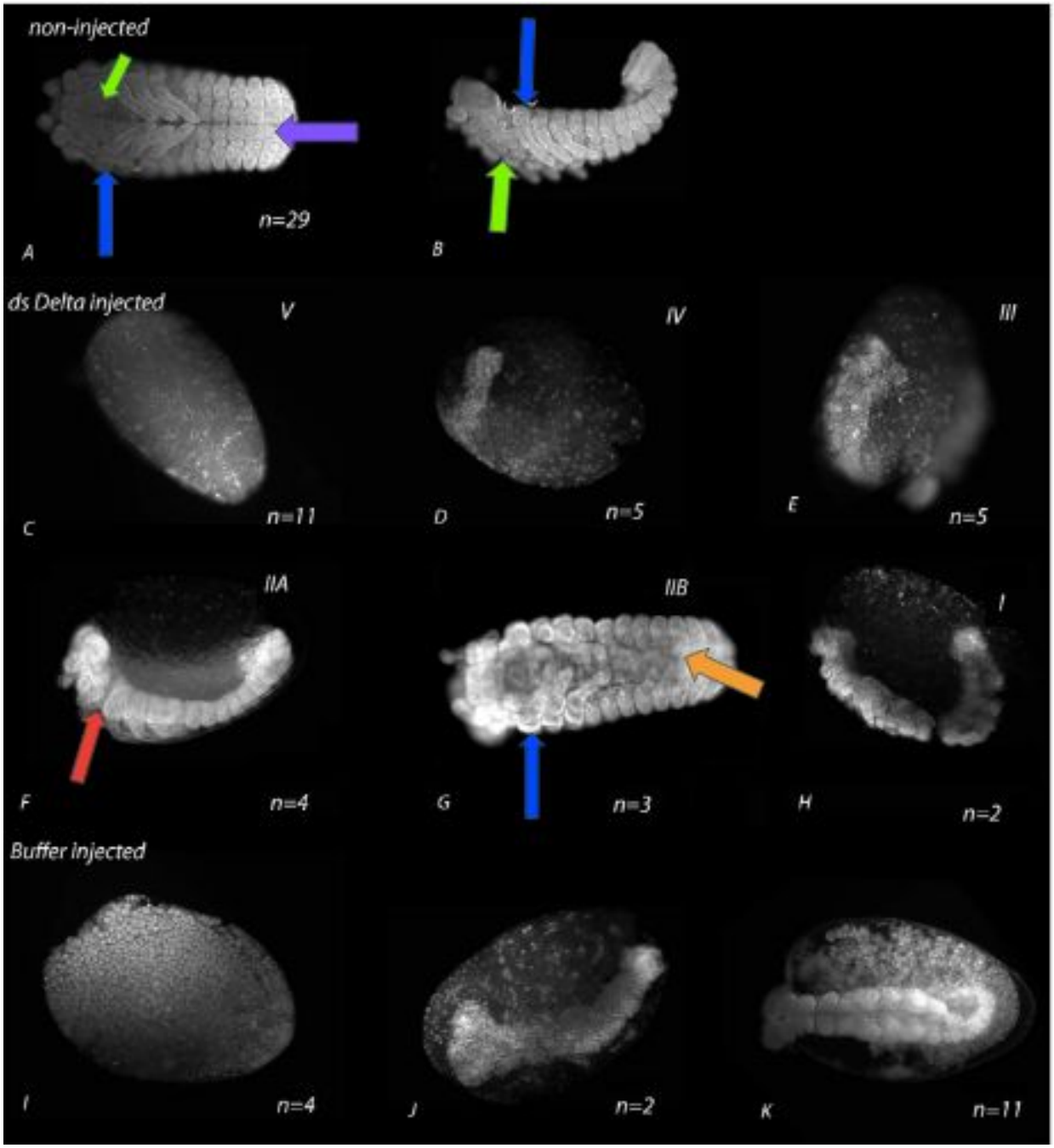


Figure 4: 36-44 hour *Tc*-Delta eRNAi phenotypes. (A-B) Examples of normal non-injected 36-44 hour embryos. (A) A ventral view of a normal 36-44 hour embryo with all appropriate mouthparts, 9-10 thoracic segments, and legs. (B) A lateral view of a normal 36-44 hour embryo which allows you to view the mouthparts, legs, and segments from the side. All non-injected embryos from this collection were normal. (C-H) Examples of Delta dsRNA injected embryos. (C) A category V (early arrest) mutant, of which the majority of the Delta embryos exhibited. (D) Example of a category IV (failed to complete germband) mutant. (E) A category III (small/no head, no legs, no mouthparts (mp), amorphous segments) mutant, which was seen as much as the category IV mutants in this collection. (F) Example of a category IIA (missing labial segment) mutant. This loss of function with the Delta knockdown is consistent with the reported phenotype (25). (G) A category IIB (midline defect) mutant, which is consistent with previously reported Delta phenotypes in the cricket (18). (H) Example of a category I (small

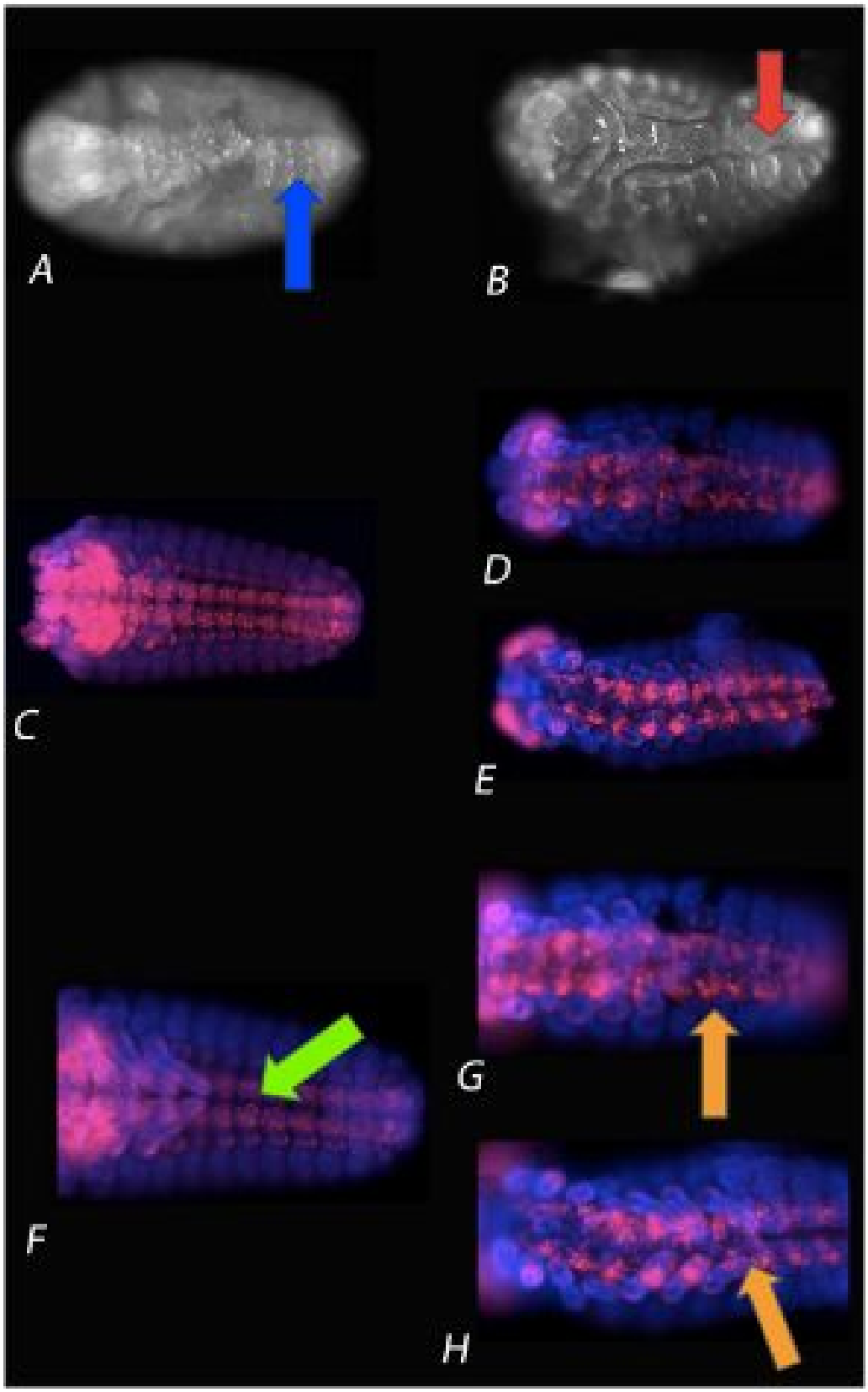
head/irregular tissue) mutant. (I-K) Examples of buffer injected embryos. (I) A category V mutant, which was the second most common phenotype. (J) Example of a category IV mutant. (K) A normal buffer injected embryo, which was the most common phenotype at this time point. Blue arrows = first leg (T1) segment; green arrows = labial segment; red arrow = missing labial segment; purple arrow = normal ventral midline; orange arrow = ventral midline defect.

Table 4: 36-44 hour *Tc*-Delta eRNAi phenotypes.

	total imaged	V: early arrest	IV: failed to complete germband	III: small/no head; no legs;no mp;amorphous segments	IIA: missing labial segment	IIB: Midline Defect	I: small head/irregular tissue	normal
dsDelta	32	11 (34.3%)	5 (15.6%)	5 (15.6%)	4 (12.5%)	3 (9.4%)	2 (6.3%)	2 (6.3%)
Buffer	17	4 (23.5%)	2 (11.8%)					11 (64.7%)
Non-injected	29							29 (100%)

Analysis of loss of function in relation to segmentation and neural markers

Although the data set is small, there is evidence that neural expression is disrupted in Delta knockdown embryos. In embryos injected with ~500 ng/ul Delta dsRNA and stained for *engrailed*, neural expression in the ventral nervous system was lost with expression still seen in the segments (Fig. 5). In embryos injected with ~2000 ng/ul Delta dsRNA and stained for *Pax 3/7*, neural expression in the segments along the ventral midline was more rounded and deformed than in wild-type controls (Fig. 5). It does not appear to matter whether the midline defect is more towards the posterior or the anterior of the midline, the same neural patterning defect is observed (Fig. 5).


A
B
C
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E
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Figure 5: Neural defects with loss of Delta function. (A) Wild-type 48 hour embryo showing *engrailed* expression. Break in the middle was caused by mounting damage. *Engrailed* stripes go across the ventral midline for all segments. (B) Mutant 48 hour embryo with *engrailed* staining. Severe midline defect seen in the posterior of the animal along with a loss of *engrailed* expression in the ventral midline. *Engrailed* expression is still present in the segments. Injection: 500 ng/ul. (C) Wild-type 44 hour embryo showing *Pax 3/7* expression, 10X magnification. *Pax 3/7* expression can be seen throughout the ventral midline in the neural cells. Expression also occurs in the head, at the base of the legs, and bordering the midline. (D-E) Mutant 44 hour embryos with *Pax 3/7* staining, 10X magnification. (D) Midline defect occurs more towards the posterior of the animal. Neural staining in the segments along the ventral midline is misshapened and more rounded than the wild-type expression. (E) Midline defect occurs more anterior in this mutant. Same phenotype of neural expression seen as in (D) Injection: 200 ng/ul. (F) Wild-type 44 hour embryo showing *Pax 3/7* expression, 20X magnification. (G-H) Mutant 44 hour embryos with *Pax 3/7* staining, 20X magnification. Blue = DAPI staining; red = Pax 3/7 staining. Blue arrow = normal *engrailed* patterning in the posterior of the ventral midline and segments; red arrow = abnormal midline with missing *engrailed* expression in the ventral midline, but still present in the posterior segments; green arrow = normal shape and distribution of *Pax 3/7* expression; orange arrows = abnormal shape and distribution of *Pax 3/7* expression.

Analysis of loss of function: pupal RNAi

To circumvent the problem with the trauma induced by embryonic injections, I injected female pupae with 2000 ng/ul dsDl RNA following established protocols devised in the Nagy lab. I had avoided this approach previously, as Aranda 2006 describes a complete suppression of egg-laying following pupal injection of 2000 ng/ul dsDl RNA, consistent with the described function of Notch signaling in oogenesis in *Drosophila melanogaster* (5). In my first round of injections I unexpectedly recovered only normal embryos (Table 5). I reasoned I had perhaps injected too little dsRNA, so in my second round of Delta dsRNA injections I over-injected the pupae and only had 9 survivors. This colony displayed diminished egg laying compared to a wild type colony with the same number of adults. The embryos recovered had a range of phenotypes from normal to a missing labial segment (Table 5).

Table 5: *Tc*-Delta pRNAi phenotypes.

	total imaged	VI: Small head, stubby legs, no posterior segment boundaries	V: early arrest	IV: failed to complete germband	III: small/no head; no legs;no mp;amorphous segments	IIA: missing labial segment	IIB: Midline Defect	I: small head/irregular tissue	normal
Dl pRNAi 11.23.16 (23 adults)									27 (100%)
0-48 hr	27								
Dl pRNAi 1.23.17 (9 adults) Week 1									
24 hr	2							1 (50%)	1 (50%)
48 hr	3					1 (33.3%)			2 (66.7%)
72 hr	8								8 (100%)
Dl pRNAi 1.23.17 (9 adults) Week 2									
24 hr	3		1 (33.3%)						2 (66.7%)
48 hr	5	1 (20%)						1 (20%)	3 (60%)

IV. DISCUSSION

Three separate taxa have evolved to build their body plan via segmentation: vertebrates, annelids, and arthropods (28) and there is a long history of a debate surrounding the evolutionary origins of segmentation in the literature (1). The most accepted theory has been that the common ancestor between these three phyla was also a segmented animal. In the past twenty years, however, it has become clear that annelids and arthropods are not monophyletic; rather, they are more closely related to unsegmented phyla than they are to each other (29). This lead to a new theory that the segmented body plan arose independently in these three phyla as the most advantageous developmental process.

So how do we determine which theory is true: independent evolution of segmentation or a common segmented ancestor? The simplest way to determine whether the phyla share a common segmented ancestor is by identifying any molecular mechanisms that are shared among

them. The Notch signaling pathway is known to be necessary for vertebrate somitogenesis (6-11) and is a common pathway to investigate in relation to the segmented body plan. *Drosophila melanogaster* does not use this pathway for segmentation (1), and so the common understanding was that arthropods did not use this pathway for segmentation. This idea was challenged by the discovery that basal arthropods utilize the Notch signaling pathway to build their segments (17, 18). This led to further investigations of the role of Notch signaling in more derived arthropods. Several different arthropod species showed truncation and/or abnormal/shortened segments when the Notch pathway is disrupted (20), suggesting that it is necessary for segmentation to occur.

There is still a debate as to whether the Notch signaling pathway is utilized in *Tribolium castaneum* segmentation (1, 25-27). The reported data on Delta loss of function phenotypes displays a significant loss of segments, and Notch loss of function data shows shortened space between the segments (25-27). Despite these intriguing phenotypes, the authors of these reports have concluded that Notch signaling does not function in *Tribolium* segmentation. To determine the role of the Notch signaling pathway in *Tribolium,* I carried out an investigation into the function of Delta signaling in early *Tribolium* segmentation.

***In situ* Hybridization:**

Early *in situ* hybridization experiments for Delta showed a circumferential band in the mandibular segment, as well as expression down the midline of the embryos (Fig. 2). *Tc*-Notch showed apparent neural expression in late embryos (Fig. 2). Subsequent experiments were carried out to reduce the background and increase the signal for *Delta* expression (Supplementary Table 1), including synthesizing a new *Delta* probe that spanned a greater length

of the *Delta* gene. However I was never able to complete a quality expression analysis despite excellent results from *in situ* hybridizations with riboprobes generated from other genes (Fig. 2). *in situ* hybridization experiments can be repeated with the lab's working procedure and the results should be promising.

Loss of function

Concentration effects:

There is a difference between 500 ng/ul and 2000 ng/ul dsRNA concentrations when injected in the embryos. The midline and head defects were seen at both concentrations, but there were not enough 500 ng/ul injected animals to analyze in order to determine if the other phenotypes were also present but at lower numbers. At 5000 ng/ul I saw dead eggs, meaning this concentration was too high for injections. This implies that there is an effect of concentration on the phenotype of Delta knockdowns in this range. The reported effects on the head/mouthparts (25) were seen at both concentrations. The loss of the labial segment was only seen at 2000 ng/ul, but it is possible that the mouthpart defects detected at 500 ng/ul could lead to this loss of the labial segment in animals at later time points and with more careful collection, mounting, and analysis. I **i**dentified a new mesectoderm effect that is consistent previous reports in other arthropods (22). I observed an abnormal midline that could be caused by a disruption of lateral inhibition between the mesoderm and ectoderm cells along the ventral midline as a consequence of a loss of Delta function. I was not able to replicate the previously reported late loss of segments in *Tribolium*. Many of my animals did not make it to hatching, so it is possible that a loss of segments could have been observed had they been able to reach this point in development. In future experiments, more care must be taken to ensure that the embryos receive

as little physical damage as possible and are kept well hydrated as they incubate to get them to hatching and determine whether a loss of segments occurs.

Progressive loss of function over developmental time:

The loss of the labial segment and the most severe midline defects were seen in later staged embryos (Fig. 4, Table 4). The early arrest phenotype and some midline defects were seen in earlier time points as well as at later times in development (Fig.3 & 4, Table 3 & 4). Overall, it cannot be determined whether there is a significant difference over developmental time. There are no reports on the loss of function over developmental time, so I wanted to investigate what happens to the embryos over time to lead to a late loss of segments. However, I still cannot conclude why there is a loss of segments. If segments are indeed lost, this loss would have to occur after 36-44 hours as all specimens I examined had a full complement of segments. Future experiments can be done to more carefully observe the progressive loss of function over developmental time as well as to determine when (or if) the segments are lost in Delta knockdown animals by doing live imaging on injected embryos using confocal microscopy.

Loss of the labial segment:

Comparative expression data from *Oncopeltus*, *Gryllus*, and *Tribolium* (21, 24, 25) show a clear circumferential band in the mandibular segment throughout embryogenesis. Oddly, both Aranda and myself see a total loss of the **labial** segment (25). In my early *in situ* experiments I observed what appears to be Delta expression in the mandibular segment (Fig. 2) but not in the labial segment. For Delta expression to effect the development of the labial segment from its expression in the mandibular segment, it would have to signal over a substantial distance. As of now, I have no way to explain this phenotype given reported and experimental results. Perhaps it

has something to do with the head defects that are a consequence of impaired neural development that lead to this loss of the labial segment rather than the mandibular segment, but I cannot be sure as to why this phenotype occurs. It has been reported that all gnathal appendages in some specimen were missing completely (25), so it is possible that this phenotype could be observed in future experiments as well as the loss of the labial segment.

Loss of function in relation to segmentation and neural markers:

One of the most interesting phenotypes observed in the Delta eRNAi knockdowns was a midline defect. The midline of the animals with this phenotype was a mass of cells with no order or alignment visible. This particular phenotype could point to an important role that the Notch signaling pathway plays in *Tribolium* development. In *Drosophila*, Notch has a well known function as a cell surface receptor that is important for the lateral inhibition mechanism that properly spaces the neuronal precursors in the developing animal (13). The Notch signal, however, is important for both ectodermal, neuronal and mesodermal fate (14). When Notch and Delta mutants were assessed with the muscle-specific gene *nautilus* in *Drosophila*, *nautilus* was found to be overexpressed at the expense of neighboring, non-expressing mesodermal cells (14). This mesoderm defect is not a simple consequence of neuronal death, which suggests that Notch and Delta are acting similarly, and independently, in establishing both mesoderm and ectoderm fates in *Drosophila* (14).

Notch was found to have a cell-autonomous function in the mesoderm, as when a Notch minigene was expressed in the mesoderm (and not the ectoderm) in amorphic N^- embryos muscle founder cell hypertrophy was rescued (15). This indicates a requirement for autonomous Notch function in the mesoderm to regulate the proper number of muscle founder cells (15).

Somatic muscle differentiation was only partially normalized, however, which suggests that Notch is required in the ectoderm for the muscle to develop properly in flies (15). A non-autonomous function was also identified as Notch expression in the mesoderm partially rescued epidermal development in the overlying neurogenic ectoderm (15). The truncated Notch protein expressed in the mesoderm lacks the extracellular domain does not, however, rescue the ventral epidermis (15). This suggests that Notch's extracellular domain can non-autonomously rescue epidermal cells from across germ layers (15). These experiments provide evidence for the importance of the Notch signaling pathway in lateral inhibition. Further experiments demonstrated that Delta acts as the signal for this lateral inhibition (16). The evidence for this claim comes from the results of homotopic transplantation of wild type single cells from the ventral neurogenic region (VNR) into hosts that lack the Delta locus (16). All differentiated clones became neural cells (16). Since wild type cells are able to generate epidermal offspring when transplanted into wild type VNR, the signal for lateral inhibition is missing in Delta mutants making it the signal source for this process (16).

In *Tribolium*, Delta has been found to be expressed only in the presumptive mesoderm and not in the neurogenic ectoderm as is seen in flies (31). Upon knockdown of the ventrally expressed genes Twist and Toll (a gene which is crucial for dorsoventral axis formation), Delta becomes downregulated in the mesoderm (31). Whether this is a consequence of both Twist and Toll loss, as early expression patterns indicate that Delta could be affected by either, or just Twist is still unknown (31). This presents an interesting environment for Delta. With its expression in the mesoderm of the ventral midline, this sets up *Tribolium* Delta to act much as it

does in *Drosophila* in a lateral inhibition process that affects both mesoderm and ectodermal patterning.

This reported data could explain the loss of *engrailed* expression along the ventral midline and the irregular expression pattern of *Pax 3/7* that was observed in my experiments. If Delta acts as the signal for lateral inhibition in *Tribolium* as it does in *Drosophila*, then the loss of the signal leads to a loss of cell fate determination between mesoderm and ectoderm. This would lead to perhaps a combination of overexpression and/or underexpression in both neuronal and mesodermal markers. Future experiments that stain for both germ layers would determine whether this is the case or if some other loss/gain of function occurs without Delta function.

Previously reported data for *Gryllus* shows that the Notch signaling pathway does not act directly on segmentation; rather, the pathway works much as described above for *Drosophila*. Delta signal is required for neuroectoderm and mesectoderm formation (22), which implies that Notch signaling is necessary for lateral inhibition in the cricket. This was surprisingly not addressed in their manuscript. The animals had a proper number of segments, but the embryos had abnormal segment morphology (22). The midline of these animals is abnormal, much like was observed in my *Tribolium* animals. It was also found that *Twist* was overexpressed in the midline and *Pax 3/7* had an expression extending through the midline and into the regions flanking it in the segments (22). This could be comparable to the *Tribolium* data after more careful analysis of *Pax 3/7* expression and *Twist* staining in mutant embryos. Overall, the data presented here is quite similar to the previously reported data for *Gryllus*, which could suggest a conserved function for the Notch signaling pathway in lateral inhibition to determine both mesoderm and neural ectoderm.

Loss of function in pupal RNAi:

The results seen in embryos recovered from pRNAi colonies were mostly normal (Table 5), but this could be due to low numbers of embryos scored overall. The labial segment was missing in 33.3% of embryos from the collection that occurred one week after the 9-adult colony was made (Table 5). This suggests that injecting a higher volume of dsRNA into the pupae is sufficient to produce this phenotype. The early arrest phenotype was also observed in 33.3% of embryos in the week 2 collection from the second injected colony (Table 5), suggesting that this phenotype is a consequence of a lack of Delta rather than injection trauma. These results are promising, and pRNAi experiments should be repeated to generate a greater number of injected females to form a larger colony and collect and analyze more embryos. I expect to see a recurrence of the missing labial segment and early arrest phenotypes, and I hope to see the loss of segments phenotype that was previously reported (25). I also hope to see a recurrence of the midline defect that was observed in eRNAi embryos.

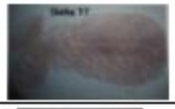
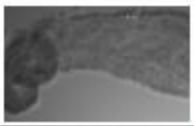
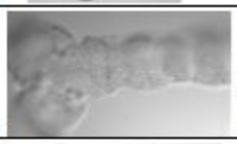
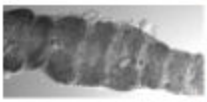
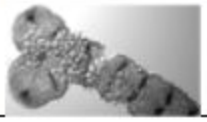
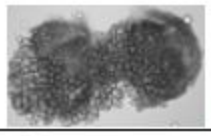
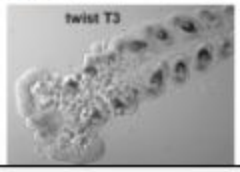
Conclusions:

There was no solid evidence found for a role of the Notch signaling pathway in *Tribolium castaneum* segmentation. Further experiments must be performed to determine whether the reported loss of segments (25) was a novel phenotype or a dominant one. My results can confirm a midline defect that occurs as a consequence of the loss of Delta. Further experiments need to be performed to determine whether this is due lateral inhibition that leads to the determination of mesoderm or ectoderm cell fates. Staining injected embryos or pRNAi embryos with mesoderm and neural markers simultaneously will allow us to determine this. I can also confirm a loss of the labial segment, which is still a mystery considering the multiple

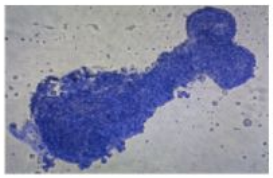
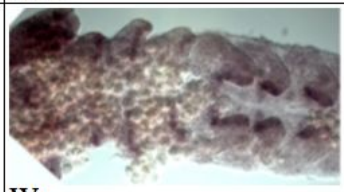
reports of Delta expression in the mandibular segment (21, 24, 25). I can also confirm that Delta plays a role in appendage formation as many of the phenotypes seen included some kind of leg defect. This implies that the Notch signaling pathway has a pleiotropic effect in development. Perhaps the timing of injections matters for the embryos to exhibit different phenotypes. If this is the case, I hope that future experiments can help us determine the full role of the Notch signaling pathway in *Tribolium castaneum* development.

SUPPLEMENTARY MATERIAL

Supplementary Table 1: *Tc*-Delta *in situ* hybridization results.

Date	Delta Results	(+) Control	Test Conditions
9.1.15 Delta Probe 1		N/A	Hyb temp. 65°, unknown concentration of probes
10.13.15 (Delta Probe 1) 2.17.16 (Wg)		 Wg	Hyb temp. 60°, increased washes
3.1.16 Delta Probe 1		 Wg	Hyb Temp. 65°, Pro K treatment, 10-15 minute development
4.5.16 Delta Probe 1		 Wg	Fresh embryos with no MeOH shake
4.19.16 Delta Probe 1		 Wg	Developed for 20 minutes
9.13.16 (New Delta Probe)		N/A	New, longer Delta probe
10.13.16		 Wg	Older embryos
11.1.16		 Wg	No Pro K treatment
3.17.17	N/A		Same conditions as 11.1.16

Supplementary Table 2: *Tc*-Notch *in situ* hybridization results.

Date	Notch Results	(+) Control	Test Conditions
9.1.15		N/A	Hyb temp. 65°, unknown concentration of probes
10.13.15		 Wg	Hyb temp. 60°, increased washes

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