Investigating Notch Signaling and Sequential Segmentation in the Fairy Shrimp, Thamnocephalus platyurus

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INVESTIGATING NOTCH SIGNALING AND SEQUENTIAL SEGMENTATION IN THE FAIRY SHRIMP, *Thamnocephalus platyurus*

BY

SARA I. KHALIL

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INVESTIGATING NOTCH SIGNALING AND SEQUENTIAL SEGMENTATION IN THE FAIRY SHRIMP, *THAMNOCEPHALUS PLATYURUS*

BY

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ABSTRACT

Segmentation is a key feature of arthropod diversity and evolution. In the standard model for arthropod development, Drosophila melanogaster, segments develop simultaneously by a progressive subdivision of the embryo. By contrast, most arthropods add segments sequentially from a posterior region called the growth zone and in a manner similar to vertebrates.

Recent work, mainly focused on insects, suggests that Notch signaling might play a role in arthropods that segment sequentially. These studies document a potential regulatory similarity between sequentially segmenting arthropods and vertebrates. In vertebrates, somite formation involves a molecular oscillator that functions as a pacemaker, driving periodic expression of genes along the anterior-posterior axis.

Here we focus on segmentation in crustaceans, the sister taxon to insects. We investigate the relationship between Notch signaling and segmentation in the fairy shrimp, Thamnocephalus platyurus. In order to explore gene regulation of segmentation in these animals, we will be tracing expression of Notch pathway genes using in situ hybridization and investigating their functions by using a chemical inhibitor of the Notch pathway.
INTRODUCTION

Segmentation is widespread among metazoans. Three major clades of complex animals share a segmented body plan: the annelids, arthropods and vertebrates (Couso 2009). Segmentation occurs during development; the body is physically divided into repeated units called segments. This process is initiated at early stages of development by several cellular and molecular signaling pathways. Most segmented animals develop their segments sequentially from a posterior region called the growth zone (Kume and Dan 1988; Anderson 1973; Davis and Patel 2002). New segments are added in the posterior, forming a series of older to younger segments from anterior to posterior. The best studied group of sequentially segmenting animals is vertebrates. In vertebrate development, segment formation, also called somitogenesis, involves a molecular oscillator (Aulehla and Herrmann 2004). This oscillator is known as a somitogenesis clock and functions as a pacemaker, driving periodic expression of genes along the anterior-posterior axis. Each somite is formed by a wave of gene expression that arises within cells (Oates et al. 2012). A wavefront translates the temporal periodic patterns into a special periodic series of segments (Gomez and Pourquie 2009).

The Notch signaling pathway was found to play a crucial role in the vertebrate somitogenesis clock. Two important components of the pathway, the Notch receptor and the Delta ligand, were observed to be involved in the formation of inter- and intra-somatic boundaries (Barrantes et al. 1999). Also, a downstream effector of the Notch pathway, hairy, was identified as a main molecular oscillator that is periodically expressed during segmentation. Each wave of hairy gene expression originates in the posterior region and moves anteriorly. A new somite forms when each wave reaches the anterior (Oates et al. 2012). Notch synchronizes
these oscillations of gene expression and is important for proper posterior segmentation (Jiang et al. 2000).

Segmentation was believed to have evolved independently in the three major phyla based on phylogenetic distance and varied types of segmental morphogenesis. In arthropods, for example, understanding of segmental mechanisms was based on the fruit fly *Drosophila melanogaster*, a model organism used in numerous genetic and developmental studies. However, *Drosophila* is an atypical representative because unlike most arthropods, they form their segments simultaneously, using a cascade of transcription factors to subdivide the eggs into progressively smaller sub-regions (Peel et al. 2005). By contrast, most arthropods add their segments sequentially from a posterior growth zone and in a manner similar to vertebrates. Therefore, most arthropods make segments differently from *Drosophila*: during early development and prior to segment patterning, their segments are cellularized rather than patterned in a syncytium (Peel et al. 2005).

The first paper to document a potential regulatory similarity between sequentially segmenting arthropods and vertebrates was a study on spiders. Stollewerk et al. (2003) showed that disrupting components of the Notch signaling pathway interrupts normal gene expression and affects segment shape and polarity. In vertebrates, Notch malfunction disrupts somite formation (Ozbudak and Pourquié 2008). Similarly, *Notch* and *Delta* knockdown experiments in spider embryos disrupted *hairy* expression and showed severe segmentation defects in size, width, borders and patterning (Stollewerk et al. 2003; Schoppmeier and Damen 2005).

Subsequent studies have extended this initial finding to a small number of other arthropods, mainly insects (Stollewerk et al. 2003; Schoppmeier and Damen 2005; Pueyo et al.
2008). These studies explored the role of Notch and whether a similar and conserved segmentation mechanism exists for arthropods and vertebrates.

Although Notch signaling has been found to play a role in some chelicerates and insects, its importance in crustacean segmentation rests on two limited studies. Pharmacological experiments inhibiting Notch signaling were performed in two branchiopod crustaceans *Thamnocephalus platyurus* and *Artemia franciscana*. In these experiments, Notch signaling was disrupted by applying a pharmacological inhibitor, N-[N-(3,5-difluorophenacyl-L-alanyl)]-(S)-phenylglycine t-butyler ester (DAPT). DAPT blocks the Notch pathway by inhibiting \(\gamma\)-secretase. Normally, \(\gamma\)-secretase mediates the cleavage of the intracellular domain of the Notch receptor that then translocates to the nucleus and associates with transcription factors. DAPT is a known inhibitor of Notch function (Geling et al. 2002; Micchelli et al. 2003). In previous experiments, Notch function was disrupted when DAPT was applied to cockroach, fruit fly and zebrafish embryos (Pueyo et al. 2008; Geling et al. 2002; Micchelli et al. 2003). The total number of segments was reduced in DAPT-treated embryos indicating an involvement in the segmentation process (Williams et al. 2012). A parallel study in the branchiopod, *Daphnia*, gave similar results whereby normal expression of Notch and its downstream effector, hairy, was disrupted by DAPT (Eriksson et al. 2013). In all crustaceans, DAPT was used to block the Notch signaling pathway. However, DAPT blocks all \(\gamma\)–secretase activity and therefore its action is less specific than direct gene knockdown. Only Eriksson et al. (2013) looked at the effect of DAPT on Notch pathway gene expression. Furthermore, both studies did not explore the function of specific Notch pathway genes through RNAi gene knockdown experiments.

Of particular interest are the functions of five important Notch pathway genes: *Notch*, *Delta*, *Serrate*, *hairy* and *Suppressor of Hairless*. Delta and Serrate products are important
ligands of the Notch signaling pathway that interact with extracellular regions of the Notch receptor and activate it (Fig. 1). Once Notch is activated, proteases mediate an extracellular and intramembrane cleavage (Kwon et al. 2012). The cleaved intracellular domain translocates to the nucleus and interacts with other factors such as Suppressor of Hairless and hairy to activate transcription (Artavanis-Tsakonas et al. 1999). Although Notch signaling is implicated in arthropod segmentation, the actual role of the Notch genes across various species is only beginning to be understood.

Here, I extend earlier work by cloning and examining the expression patterns and function of Notch genes in the fairy shrimp, Thamnocephalus, through in situ hybridization. These studies are necessary to support the hypothesis about the role of Notch in Thamnocephalus segmentation. As a first step to performing gene knockdown experiments in crustaceans, I made dsRNA for RNA interference.
Figure 1. The Notch signaling pathway. An overview showing key processes involved in the Notch signaling pathway: (1) intramembrane cleavage (2) nuclear translocation and (3) transcriptional activation (after Kwon et al. 2012). The genes that will be focused on in this study are highlighted by a red font: \textit{Notch, Delta, Serrate, hairy} and \textit{Suppressor of Hairless} (after Artavanis-Tsakonas et al. 1999).

MATERIALS AND METHODS

The overall workflow of the present study involves three major experiments: isolating and cloning Notch pathway genes of interest (GOI), synthesizing probes for \textit{in situ} hybridization and knocking down Notch function using a pharmacological inhibitor and RNAi experiments (Fig. 2).

We focused on five Notch pathway genes: \textit{Notch, Delta, Serrate, hairy} and \textit{Suppressor of Hairless}. 
Figure 2. Experimental approach for studying Notch genes in *Thamnocephalus*. A general overview for the different experiments performed in order to study the expression patterns and function of specific Notch pathway genes: (A) isolating and cloning Notch pathway genes, (B) synthesizing RNA probe for the genes of interest to be used in *in situ* hybridization experiments and (C) inhibiting the Notch pathway by either using a chemical inhibitor, DAPT, or synthesizing dsRNA for gene knockdown experiments.

A. Isolating Homologs of Notch Pathway Genes

Figure 3. Experimental approach for isolating and cloning genes of interest. The overall workflow for isolating and cloning the Notch pathway genes involved sequence alignments, PCR, ligation/transformation, DNA purification and sequencing.

To clone *Notch*, *Delta*, *Serrate*, *hairy*, and *Suppressor of Hairless* from the *Thamnocephalus*, I followed the workflow in Fig. 3. A transcriptome of larval *Thamnocephalus* was used to identify orthologs of Notch pathway genes. The identified contigs, continuous
sequences of DNA, were trimmed down to include only the coding region of the gene of interest. The NCBI primer design tool was used to design primers specific to the trimmed gene sequence. The best choice primer was selected based on several factors. First, the clone sequence needed to be about 500 bp long and the selected primers had to include all or as much as possible of the previously identified high homology regions. Additional parameters considered were having a G at the 3’ end, a length of ~20-24 amino acids, low self-complementation, low repeated G sequence (3 or fewer sequential G’s), 40-60% C-G content, Tₘ values (related to C-G content) between 50-70°C, and a low tertiary structure complementarity.

*Thamnocephalus* cDNA was amplified using the selected reverse and forward primers:

**Notch**
*Primer 2 (665 bp target)*
Forward: 5’-AGACGGCACTCAAGACTGTG-3’  
Reverse: 5’-CTTCCCAGCAGCGGATAGAG-3’

*Primer 9 (628 bp target)*
Forward: 5’-TGCCAACTCCGAATCAACGA-3’  
Reverse: 5’-AGAGGATTCCTCCAAAGTGCCG-3’

**Suppressor of Hairless**
*Primer 7 (554 bp target)*
Forward: 5’-CAATGGGGAGCCTTTCACTCAGAT-3’  
Reverse: 5’-GACTCCTCGCATTCAACGACAT-3’

**Serrate**
*Primer 2 (606 bp target)*
Forward: 5’-GCATGGCTCTTTGTAATGGCG-3’  
Reverse: 5’-TTACTGCACGGCTGTTCTT-3’

*Primer 3 (510 bp target)*
Forward: 5’-TTGCCTTCCGGACAGTTCAA-3’  
Reverse: 5’-GCCTGCAACAAGTCATGCAA-3’

**hairy**
*Primer 8 (422 bp target)*
Forward: 5’-ACAAGCTCAGGCTAGGAAGC-3’  
Reverse: 5’-GTTCGACAACTGGCTGCTG-3’

**Delta**
*Primer 1 (405 bp target)*
Forward: 5’-CGAGCCAGGGATGACAAGTT-3’  
Reverse: 5’-AATCTCGCAGTGTTCCAG-3’

*Primer 3 (484 bp target)*
Forward: 5’-GCACATTGAGGATGTGAGC-3’  
Reverse: 5’-TGTTACAGGAGCGCTGGTTTC-3’

*Primer 4 (411 bp target)*
Forward: 5’-ATCGTGCGTGGACAAAGTGA-3’  
Reverse: 5’-TCACATACGCTGCGGAAATA-3’
The PCR results were verified by gel electrophoresis (see Appendix I). The PCR products were used to make clones containing the GOI by following the StrataClone Cloning Kit (Agilent Technologies) and using the PCR-cloning vector pSC-A-amp (Fig. 4). A ligation reaction mixture was added to StrataClone SoloPack competent cells and this transformation mixture was plated on to Luria broth agar (LBA), ampicillin\(^+\) (100 \(\mu\)g/mL)/X-gal\(^+\) plates.

A standard white/blue colony screening test was performed. Individual white colonies were selected and streaked on to new LB amp and Xgal plates. From these plates, individual colonies were used as DNA template for additional PCR reactions with M13 forward and reverse primers. These PCR reactions determined if the selected colony had the proper insert since the M13 primers amplify regions of interest. Gel electrophoresis was used to verify the presence of the Notch, Delta, Serrate, hairy and Suppressor of Hairless genes in the amplified product (see Appendix I).

**Figure 4. A map of the StrataClone PCR Cloning Vector.** A pSC-A-amp/kan vector was used in the ligation/transformation experiments. The insertion site for the PCR products of the genes of interest is highlighted in red.
After experimentally isolating the Notch pathway genes, the samples were purified with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) and sent to Genewiz (http://www.genewiz.com/public/DNA-sequencing-services.aspx) for sequencing in order to verify that the obtained gene sequences match with those of the cDNA isolates. The sequence results were aligned to the theoretical amplified sequence using the Molecular Evolutionary Genetics Analysis (MEGA) software.

**Glycerol Stock and Miniprep**

After cloning the Notch genes and verifying their presence by sequencing, a glycerol stock of cells was made using the plates with the bacteria carrying the genes of interest that had been saved. The glycerol stocks were stored in the -80°C freezer for future use.

Plasmid DNA was isolated using the QIAPrep Miniprep Kit (Qiagen). Bacterial cells from the colony were grown in broth to a high density and then removed and isolated for later use. The yield and purity were determined by using the nanodrop.

**B. Studying Gene Expression- Riboprobe Synthesis and in situ Hybridization**

The purified PCR product containing the GOI was used to generate both sense and antisense labelled RNA probes for *in situ* hybridization if the match was close to 100% between the sequences of the cloned genes and those in the original transcriptome for *Thamnocephalus*.

**Riboprobe Synthesis**

Sense and antisense RNA probes were synthesized using a MAXIscript DIG-Labeled RNA Probe Synthesis (Ambion) as per the company’s instructions. This was done using dig UTP *in vitro* by the action of T3 and T7 RNA polymerases. The antisense RNA probe binds to the
mRNA of the GOI and the sense RNA probe acts as a control. A whole mount in situ hybridization (WISH) experimental procedure was carried out using staged animals and both RNA probes to track the expression patterns of each GOI. In situ hybridization tells us where a gene is expressed.

**Riboprobe Hydrolysis**

Initially, antisense probes for hairy, Serrate, and Suppressor of Hairless gave clear results for in situ hybridization. For Delta and Notch, the in situ expression patterns were very hard to interpret. As a result, riboprobes only for Delta and Notch were hydrolyzed. This was done in order to maximize chances of hybridization, to improve penetration into larval tissue, and to optimize the expression signal. Hydrolyzed riboprobes for Serrate, Suppressor of Hairless and hairy were not tested.

For hydrolysis, the riboprobe for Notch and Delta were incubated in a sodium carbonate buffer. An estimated time of incubation in the hydrolysis buffer was calculated based on the following formula:

\[
Time \ (min) = \frac{(L_i - L_f)}{(0.11 \times L_i \times L_f)}
\]

where: Li = initial probe length (in kb), Lf = final probe length (in kb) and 0.11 = number of cuts per kb per min.

The probe was incubated at 60°C for the calculated amount of time. RNA was precipitated by adding LiCl (10 µL of 4M LiCl) and 100% ice cold ethanol. After at least two hours, the sample was centrifuged at 4°C and 12,300 rpm. An RNAse-free gel was used to check the size and intensity of the hydrolyzed probe.
Whole Mount In situ Hybridization (WISH)

Our basic protocol follows the WISH protocol in Nulsen and Nagy (1999) with buffers replaced by Roche buffers. Staged animals were first rehydrated from 100% methanol to PBTw (1X PBS with 0.1% Tween 20) and then sonicated until ~10% of each animal showed signs of damage, indicating that the cuticle of the other specimens had broken enough to enable penetration. The animals were left in a 60°C waterbath in prehybridization buffer (hybridization buffer, denatured salmon sperm DNA (100 µg/mL), yeast tRNA (200 µg/mL) for at least 4 hr after which the buffer was replaced with RNA probe/hyb buffer and left to hybridize overnight. An RNAsе-free environment was maintained throughout these initial steps of the in situ hybridization.

The RNA probe/hyb buffer solution was removed and the embryos were washed in hybridization buffer, 2X SSC+0.1% CHAPS, 10X WISH Roche Block solution and 1X Roche washing buffer. After these washes, the animals were stored overnight in an anti-DIG antibody/block solution.

The antibody solution was removed and the animals were washed with Roche washing buffer and 1X Roche detection buffer prior to addition of 1-Step NBT/BCIP (pH 9.5) developer solution. The color reaction was stopped by rinsing 1X Roche washing buffer and then counter-staining the nuclei with Hoechst (Sigma) in Roche washing buffer. Animals were then stored in glycerol and left overnight before mounting them and viewing them on a Nikon E600 Epi-fluorescence and photographed using Spot camera and software (Diagnostics).
C. Treating Staged Animals with a Pharmacological Inhibitor, DAPT

Animals were collected within 15 min of hatching and placed into small culture dishes in a temperature and light controlled environment chamber (818 low temp. illuminated incubator, Precision Scientific). They were reared in a 6-well cell culture dish and were fed 1 µl of yeast/algae mixture at the time of collection. Three treatment groups were chosen: 100 µM DAPT in 1% Dimethyl sulfoxide (DMSO), DMSO control and a no treatment negative control. Animals were fixed for 30 min in 9% formaldehyde in phosphate buffered saline (PBS) with 50mM ethylene glycol tetra-acetic acid (EGTA) and stored in 100% methanol.

D. Knockdown Experiments- dsRNA Synthesis and RNAi Experiments

Once the RNA probe gave us successful in situ results, we used the gene sequence to develop tools to determine gene function through knockdown experiments. The clones developed for RNA probe templates were used as a dsRNA template for later use in RNAi. The MEGAscript Kit (Ambion) was used to synthesize dsRNA for each GOI. *Thamnocephalus* were injected with dsRNA for each of Suppressor of Hairless, Delta and hairy genes. Result verifying dsRNA synthesis are shown in Appendix I. However, only very preliminary injection results were obtained and are therefore not reported in this thesis.

RESULTS

*Isolating Notch Pathway Orthologs*

Previously, a 20 million base pair (bp) transcriptome from mixed larval stage was sequenced and a database was assembled (Stangl et al., in prep). This transcriptome represents over 35,000 unique protein-coding genes among which are the Notch pathway genes. The sequences of five Notch pathway genes were isolated from the *Thamnocephalus* cDNA database
using nucleotide sequences for the GOI in closely related species such as brine shrimp, flour beetle, fruit fly and cricket were obtained from the National Center for Biotechnology Information (NCBI) website.

We identified a *Notch* orthologue that was 7311 bp long. Within this sequence, we amplified two regions that were 665 bp and 628 bp long. Both regions have homology to Epidermal Growth Factor (EGF) repeats 26-33 (Fig. 5a).

We identified a 2286 bp *Delta* orthologue. Initial amplification for cloning resulted in 405 bp and 484 bp long regions that included an N terminus of Notch ligand (MNNL) region and EGF repeats 1-8. However, this region never produced the correct sequence after cloning. A literature search was done to evaluate other studies that focused on *Delta* in arthropods and to determine which specific domain were successfully targeted. Based on this evaluation, a new primer set was designed to incorporate the Delta Serrate Ligand (DSL) domain and amplify a 411 bp region (Fig. 5b).

We identified a *Serrate* orthologue that was a 1392 bp fragment. A maximum likelihood phylogenetic tree confirmed that the isolated sequence, despite being a fragment, is closely related to *Serrate* (Fig. 6). Within this sequence, we amplified two regions that were 510 bp and 606 bp long. One region has homology to the DSL domain and EGF repeats 1-3 while the other included part of the MNNL domain (Fig. 5c).

We identified a *hairy* orthologue that was 807 bp long. Within this sequence, we amplified a region that was 422 bp long having homology to the helix-loop-helix (HLH) domain (Fig. 5d).
We identified a *Suppressor of Hairless* orthologue that was 2052 bp long. Within this sequence, we amplified a region that was 544 bp long having homology to the immunoglobulin-like fold, plexins and transcription factors (IPT) domain (Fig. 5b).

These clones of the Notch pathway genes were then used to make riboprobes in order to determine mRNA expression. The *in situ* hybridization for *Serrate* did not work and *Suppressor of Hairless* showed ubiquitous expression (data not shown). The expression of the other three genes (*Notch, Delta, and hairy*) is detailed below.
Figure 5. Orthologs of Notch pathway genes in *Thamnocephalus*. (a) *Notch*, (b) *Delta*, (c) *Serrate*, (d) *hairy* and (e) *Suppressor of Hairless* orthologs in *Thamnocephalus*. The gene structures of the *Thamnocephalus* Notch pathway genes are compared to those of *Drosophila*. Primers (shown as a red line) that were designed to amplify specific regions of each gene.
Normal Development in Thamnocephalus and expression of Notch pathway genes

At the larval stage of the fairy shrimp, segments form in a sequential manner from a posterior region called the growth zone (Anderson 1973). This does not occur in the embryo, as is the case with many arthropods, but occurs during larval development after hatching. When the fairy shrimp hatch from their cyst, they have no morphologically visible segments. Instead, two to three molecularly specified segments are indicated by the expression of the transcription factor, *engrailed*. Fig. 7 shows a larval stage of the fairy shrimp, seven hours after hatching, with older segments appearing in the more anterior regions of the animal. The growth zone is considered to be the region located posterior to the last Engrailed stripe.

Analysis of the expression patterns of Notch pathway genes focuses on the growth zone and regions just anterior to it since this is where segment addition occurs. Previously,
experiments were done to determine the position of the growth zone relative to Engrailed (Fig. 7b). However, our *in situ* hybridization experiments did not involve any double labelling for both the Notch genes and *engrailed*. Therefore, we are not certain about the position of the growth zone and the anterior extent of this region is still ambiguous. For the *in situ* hybridization results below, we described our findings by inferring the general region where new segments are typically specified.
Figure 7. Normal development in *Thamnocephalus*. (a) General morphology of an adult stage of a fairy shrimp showing three main regions: the head, thorax and abdomen (modified from Williams et al. 2012). (b) Larval stage of the fairy shrimp for normal segmentation in a 7 hr fairy shrimp. Cells expressing the segmental marker Engrailed appear pink. The most posterior non-segmental region is called the telson. The last Engrailed stripe defines the anterior border of the growth zone (indicated by the arrow). The growth zone extends from the last Engrailed striped to the telson (solid line, double-headed arrow) and is therefore the region posterior to the last enagelled stripe. Segments are added sequentially such that newer ones occur more posterior (dotted line, double-headed arrow).

**Notch and Delta are expressed in segmentally repeated stripes but not in the most posterior growth zone**

*Notch* was expressed in repeated stripes in the general region where new segments are being specified. However, no expression was seen throughout the broad posterior growth zone. At 1 hr, larvae showed weak expression in two to three broad stripes in the posterior (Fig. 8A, B, C). This segmental expression was mostly maintained at later larval stages. At 7 hr, three narrow and equally spaced stripes of *Notch* expression were very clear in the posterior region (Fig. 8D,
E, F). However, a fourth incomplete stripe appears to be transitioning into the more anterior regions (Fig. 8D).

*Delta* was also expressed in repeated stripes in the general region where new segments are being specified but not throughout the broad posterior growth zone. At 1 hr, a segmental pattern was visible in the posterior (Fig. 8G, H). These expression patterns were variable between larvae (Fig. 8G, H). Two clear and complete stripes formed posterior to two incomplete anterior stripes (Fig. 8G). Another larva only showed two faint complete posterior stripes (Fig. 8H). This segmental expression was mostly maintained at later larval stages. At 7 hr, all animals showed three complete stripes with two (Fig. 8I) or one (Fig. 8J) incomplete anterior stripes.
Figure 8. Expression of Notch and Delta in Thamnocephalus. Notch expression for 1 hr (A, B, C) and more developed stages 6 hr (D, E, F) of the fairy shrimp. Arrows in A and B indicate two stripes that are visible in the posterior at 1 hr. (C) is a higher magnification of the posterior region of (B) and the arrows point at the two stripes of Notch expression. The arrows in D and E indicate three segmental stripes of Notch expression in the posterior region at 7 hr. The arrowhead indicates an incomplete stripe found anterior to the three complete posterior stripes. (F) is a higher magnification of the posterior region of (E) and the arrows point at the three stripes of Notch expression. An arrowhead located most anteriorly in (D) points at an incomplete stripe present anterior to the three posterior stripes. Delta expression of 1 hr fairy shrimp showing two slightly different patterns (G, H). In (G) arrows in the posterior indicate two complete stripes. However, the arrowheads in the anterior indicate two more anterior stripes that are incomplete stripes. In (H) arrows point at only two complete yet very faint stripes that are present in the posterior. For Delta expression patterns of 7 hr fairy shrimp, arrows in (I) and (J) point at three complete...
posterior stripes. Arrowheads in the more anterior regions indicate two (I) or one (J) incomplete stripe. Scale bar: in A, 100µm for A, B, D,E, G-J and in C, 250µm for C, F.

**Hairy expression in the growth zone and segmental stripes**

*Hairy* was expressed in repeated stripes in the general posterior region where new segments are being specified. At 0 hr, a broad band of expression appeared in the growth zone of many larvae (Fig. 9A, C). However, expression was variable between larvae: some larvae show no expression in the growth zone (Figs. 9B, E) while others have expression only at the anterior border of the growth zone (Fig. 9 D). These expression patterns were generally maintained at later larval stages. At 7 hr, *hairy* was expressed in stripes with (Fig. 9F, H) or without (Fig. 9G, I) additional expression at the anterior border of this general region of segment specification.

There was an interesting additional expression pattern in the limb bud of 7 hr larva (Fig. 9J, K). Repeated stripes of expression were seen parallel to the anterior/posterior body axis, repeating transversely, medial to lateral. These patterns were visible at a time when early limb buds subdivide to form their many branches (Williams 2007).
Figure 9. Expression of *hairy* in *Thamnocephalus*. For 0 hr animals, (A) arrowheads show segmental stripes in the anterior and the arrow shows a broad band of expression in the posterior. This band of expression is absent in (B). (C), (D), (E) are higher magnification of the posterior region. In (C), a double-headed arrow shows a broad band of posterior expression. The arrow in (D) shows expression at what could be the anterior border of the general region for segment specification and (E) shows no expression at that posterior region. At 7 hr, arrows in (F) and (G) point at stripes in the posterior growth zone region.
(H) is a higher magnification of the posterior region of (F) and reveals possible staining of what could be the anterior border of the general region where new segments are specified. This staining is not visible in (I), a higher magnification of the posterior region of (G). Also at 7 hr, arrows indicate repeated expression patterns that are oriented parallel to the axis of the body and occur in the limb buds (J, K). Scale bar: in A, 100µm for A, B, F, G and in I, 250µm for C, D, E, H, I.

**Expression of hairy in Notch knockdown experiments**

DAPT is a chemical inhibitor that disrupts the Notch pathway by blocking Y–secretase and preventing the normal cleavage of the intracellular domain of the Notch receptor. *Hairy* expression was examined in order to study the effect of DAPT on segment formation. Since *hairy* expression patterns were the clearest, only hairy *in situ* hybridization experiments were performed with DAPT-treated animals.

In this experiment, three treatment groups were chosen: DAPT in a DMSO solvent, DMSO control and a no treatment negative control. *Hairy* expression was very faint in DAPT-treated 3 hr animals. Therefore, it was difficult to conclude with certainty that there are fewer stripes when the animals are treated with DAPT. However, the distance between the last two posterior stripes appears to be reduced in the DAPT-treated animals (Fig. 10A, D). These last two posterior stripes are farther apart in the DMSO-treated (Fig. 10B, E) and the negative control animals (Fig. 10C, F). Also, the DAPT-treated animals tend to have a more swollen and less elongated abdominal region (Fig. 10A, D).

The results of this experiment are inconclusive since DAPT might have an effect on either stripe development or swelling of the posterior, or both. It is difficult to account for differences in the total number of stripes and the distance between them with faint *hairy* expression. Also, swelling of the posterior region requires further investigation in order to be certain about specific effects of DAPT.
Figure 10. *Hairy* expression in larvae with blocked $\Upsilon$–secretase activity. DAPT-treated 3 hr animals show almost fused posterior stripes and a swollen abdominal region (A, D). Arrows in (A) indicate the two posterior stripes. In the two control groups, one with the DAPT solvent DMSO (B, E) and the other with no treatment (C, F), arrows point at the two posterior stripes that seem to be located farther apart (B, C). Figs. (E) and (F) are higher magnifications that focus on the posterior regions of (B) and (C), respectively.

DISCUSSION

We isolated and cloned *Notch, Delta, Serrate, hairy* and *Suppressor of Hairless* from the branchiopod crustacean *Thamnocephalus* and investigated their expression at different stages of development. Our results demonstrate that Notch signaling pathway genes are present in the posterior region where segments are added. *Notch, Delta* and *hairy* are expressed in repeated stripes in the general region where new segments are specified, supporting previous work about the involvement of Notch signaling in segmentation of *Thamnocephalus*. In addition, the dynamic *hairy* expression patterns are suggestive of oscillations in the growth zone.
These results support the emerging hypothesis of the role of Notch signaling throughout sequentially segmented arthropods.

**Evidence for Notch signaling in crustacean segmentation**

The importance of Notch signaling in crustacean segmentation rests on two studies. Pharmacological experiments inhibiting Notch signaling were performed in three branchiopod crustaceans *Thamnocephalus platyurus, Artemia francescana,* and *Daphnia magna.* In these experiments, Notch signaling was blocked using DAPT, an inhibitor of the pathway that acts at the level of γ–secretase. The study on *Thamnocephalus* and *Artemia* demonstrated that blocking γ–secretase activity caused repeatable and specific effects on segmentation (Williams et al. 2012). However, no data was provided as to whether Notch pathway genes were present. My study identified Notch orthologs, demonstrated their expression in posterior segmental stripes through in situ hybridization, and also looked at the effects of DAPT on hairy expression. These experiments are important in understanding the role of Notch in segmentation of *Thamnocephalus.*

Normally, *Thamnocephalus* hatch with two segmental stripes and then add segments sequentially with time. These two stripes are always present regardless of DAPT treatment. However, once exposed to DAPT, segmentation slows in a dose-dependent manner and affects the total number of segments that form (Williams et al. 2012). In the branchiopod, *Daphnia,* Notch signaling genes were expressed in the growth zone and in segmental stripes. Expression of Notch and its downstream effector, hairy, was severely reduced and in some cases absent in DAPT-treated embryos (Eriksso et al. 2013). Our in situ hybridization data shows that Notch genes are expressed in posterior regions involved in segmentation and therefore suggest that they
most likely play a role in *Thamnocephalus* segmentation. These expression patterns were generally maintained at different larval stages. Furthermore, our DAPT experiments were coupled with *hairy in situ* hybridization that showed differences in the expression patterns rather than total number of stripes. In DAPT-treated animals, *hairy* was expressed in stripes, which appeared to be very close to each other in comparison to the evenly spaced stripes of animals in the DMSO and negative controls. The overall morphology of the posterior region of DAPT-treated animals was also altered, resulting in a swollen appearance. Therefore, similar to previous findings of Williams et al. (2012) and Eriksson et al. (2013), DAPT seems to have an effect on segmentation. However, the results of our DAPT experiments are not very conclusive due to the weak *in situ* staining. As a result, these experiments need to be repeated in the future.

The two studies that focused on *Thamnocephalus* and *Daphnia* revealed that γ–secretase plays a role in segmentation. Despite being a known inhibitor, DAPT blocks all γ–secretase activity and therefore could be a less precise block of Notch signaling. Ideally, knockdown experiments of Notch pathway genes would be most helpful in understanding the role that Notch plays in forming sequential segments in *Thamnocephalus*. Delta, SuH and hairy dsRNA were synthesized as a prelude for these knockdown experiments.

**Evidence that Notch signaling plays a role in sequentially segmenting arthropods**

Crustaceans and insects are sister taxa and are expected to share similar patterns of development and segmentation. Notch signaling was examined in insects (Pueyo et al. 2008; Kainz et al. 2011). *Delta, Notch* and *hairy* in the cockroach *Periplaneta americana* showed expression in segmental stripes and in the growth zone that were similar to those in *T.platyurus*. Gene knockdown experiments were used to study the functional role of Notch genes. Upon
inhibiting the Notch pathway, Notch expression was lost and segmental defects were observed with Delta and hairy (Pueyo et al. 2008).

In the cricket Gryllus bimaculatus, Delta and Notch genes were found to be essential for the formation of posterior segments at different developmental stages (Mito et al. 2011). Delta expression patterns were observed along segmental stripes and in the posterior growth zone and Notch was uniformly expressed during early stages of development. However, another study in cricket revealed that these segmental observations might be misleading. Notch genes also play a role in neurogenesis, and defects in the morphology of segments were correlated with developmental delays in neuronal germ layer formation (Kainz et al. 2011).

A study on the honeybee Apis mellifera, a member of the most basal group of insects that are early evolving, also found that Notch plays a role in segmental patterning rather than segmentation (Wilson et al. 2010). In Delta knockdown experiments, a segmental patterning defect was observed. The segments were not missing, fused or malformed; instead, they appeared to be reduced in size. These observations suggested that Notch might play a role in patterning segments after they have been defined. Since Notch signaling does not regulate segmentation in fruit fly, tribolium and the honey bee it is possible that Notch signaling control of segmentation might be lost in more derived insects.

Based on these findings, crustaceans and most insects appear to share a Notch requirement. Holometabolous insects, insects that undergo complete metamorphosis, seem to have lost this requirement.

The relationship between the Notch signaling pathway and segmentation was also examined in myriapods. These studies focused on the expression of Notch pathway genes during phases of segment formation. Specifically, in the centipede Strigamia maritima, Delta and
homologs of the *Drosophila* pair-rule genes, *even-skipped* and *hairy*, showed periodic expression patterns in the posterior region (Chipman and Akam 2008). These patterns were consistent with their involvement in regulating segmentation. However, since no RNAi experiments were performed on myriapods the functions of these genes have not yet been established. Still these findings are consistent with the expression patterns of *Notch* genes in *Thamnocephalus*. In the spiders *Cupiennius salei* and *Achaearanea tepidariorum*, *Notch*, *Delta* and *hairy* are expressed transiently in the growth zone and along segmental stripes (Stollewerk et al. 2003; Oda et al. 2007). Additionally, RNAi experiments with *Delta* and *Notch* revealed defects in segment size and patterning. As a result, findings in basal insects, crustaceans and spiders collectively support the hypothesis that *Notch* plays a role in arthropod segmentation.

**Notch signaling and the presence of a potential segmentation clock in Thamnocephalus**

There seems to be a general consensus about the existence of a relationship between the Notch signaling pathway and sequential segmentation in arthropods. However, the exact role of Notch in segmentation of each species is not clear. In the better studied vertebrate system, Notch plays a role in synchronizing molecular oscillations in the posterior and *hairy*, a downstream effector of Notch, oscillates in a coordinated fashion (Oates et al. 2012). Interestingly, there is additional evidence for the existence of a segmentation clock in sequentially segmenting arthropods. Pueyo et al. (2008) and Stollewerk et al. (2003) show variable expression patterns of the Notch pathway genes that could provide general evidence for the existence of a segmentation clock. Both papers present images of embryos of the same stage but showing different levels of expression. Gene expression seems to be moving anteriorly to form the stripes and could consequently results in a reduction of growth zone staining. This is analogous to what was seen
in early Thamnocephalus specifically with hairy gene expression patterns. Many animals show a broad band expression in the growth zone, while others show no growth zone staining and there is an intermediate stage in which few animals show expression only at the anterior border of the growth zone. Animals with no growth zone expression have an additional stripe. These findings are consistent with our expectations based on the vertebrate segmentation clock. Based on the hairy in situ results, the presence of growth zone staining could be described as the source of the gene expression. The gradual absence of this staining could document the traveling wave of gene expression that will eventually reach the wavefront threshold and form a segment.

We think that Notch and Delta expression in Thamnocephalus occur right where new engrailed stripes are arising. Based on the position of Delta expression, Delta was found to regulate engrailed and play a role in forming stripes in the cockroach (Pueyo et al 2008). Evidence to support this hypothesis in Thamnocephalus could be gather from double-labeling experiments.

**Notch signaling could play additional roles in neurogenesis and limb branching in Thamnocephalus**

Notch, Delta and hairy expressions in Thamnocephalus typically show contiguous stripes in the posterior regions. However, older stripes in more anterior regions, specifically for Notch and Delta expressions, become broken up into what appears to be neural and appendage staining. At these instances, Notch and Delta are most likely involved in other processes, in addition to segmentation. These processes include neuronal germ layer formation and limb bud branching. Apparent segmental expression was shown to control neuronal germ layer formation in the cricket (Mito et al. 2011; Kainz et al. 2011). Similar neuronal staining was observed in more mature Thamnocephalus segments. In addition, the specific pattern of expression seen in older
*Thamnocephalus* segments is correlated with branching of limbs (Williams 2007). Similar expression patterns were seen in figures in Eriksson et al. (2013).

**Figure 11.** Phylogeny showing the loss of Notch signaling in segmentation in *Drosophila*, a more derived arthropod (Pueyo et al. 2008) and highlighting three groups of sequentially segmenting arthropods: chordates (vertebrates), *Cupiennus* (spiders) and *Periplaneta* (cockroaches) in which Notch signaling might play a role in forming their segments.

**CONCLUSION**

My data demonstrates that Notch pathway genes are expressed in repeated segmental stripes in the posterior region where new segments are specified. Also, although not entirely conclusive, DAPT experiments show a disruption to normal *hairy* expression patterns. These findings support the hypothesis that Notch plays a role in *Thamnocephalus* segmentation. Furthermore, dynamic *hairy* expression patterns add to a growing body of evidence for the presence of a potential segmentation clock in sequentially segmented arthropods. The next step is to knockdown specific Notch pathway genes, something that has not yet been done in any crustacean.
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APPENDIX I

Below is supporting documentation for both the cloning of five Notch pathway genes (Notch, Delta, Serrate, hairy and Suppressor of Hairless) in Thamnocephalus and the synthesis of riboprobe and dsRNA. Unless otherwise specified, the gel conditions were: 1.5% agarose gel in 1X TBE buffer and stained with EtBr (for 45 min @112V). For gels of PCR products, 5uL of each product was loaded per lane with 1uL loading dye.

i) Notch

The amino acid sequence for the Thamnocephalus Notch was aligned with a Drosophila Notch homolog to confirm the identity of Notch (Fig. A1). For Notch, we designed two different primer sets 2 and 9 that amplified 665 bp and 628 bp respectively within the complete 7311 bp sequence. After performing PCR, a gel was run to verify the presence of single bands with the expected product size (Fig. A2). Each product was cloned into a 4.3 kb bacterial vector. Individual colonies that resulted from the ligation/transformation procedures were used as DNA template in an additional PCR. In this colony PCR, M13 forward and reverse primers amplify the Notch target as well as a 260 bp fragment that includes both the T3 and T7 promoters necessary for RNA probe and dsRNA synthesis. Gel electrophoresis of the colony PCR was used to confirm that Notch was properly inserted. This gel included the expected band size of the PCR product with a 260 bp vector fragment, resulting in a 925 bp band size for N2 and an 888 bp band size for N9 (Fig. A3 a, b). Subsequently, the samples were purified and sent for sequencing. After analyzing the sequence results and verifying that they were consistent with the initially isolated sequences, an RNA probe and dsRNA were synthesized for in situ hybridization and RNAi gene knockdown experiments, respectively (Fig. A4, A6). The RNA probe for Notch was
hydrolyzed, resulting in the absence of clear single bands (Fig. A5). Instead, a faint smear was only visible for N9, which indicated that probe hydrolysis of N9 was successful. However, the dsRNA synthesis for Notch was unsuccessful since no bands were visible on the gel (Fig. A6).
Figure A1. Amino acid alignment for *Thamnocephalus Notch* with a *Drosophila Notch* homolog.

An alignment showing the amino acid sequence for *Thamnocephalus Notch* (Query) with a *Drosophila Notch* homolog (Sbjct) to confirm the identity of Notch. This alignment focuses on the regions with the highest homology, as indicated by the line in between the Query and Sbjct.
Figure A2. PCR products of cDNA amplified with two Notch primer sets. Lanes 1 and 2 represent duplicate PCR reactions for primer set 2 and show the expected band size of 665 bp. Lanes 3 and 4 represent duplicate PCR reactions for primer set 9 and show the expected band size of 628 bp. Lane 5 is a 50 bp DNA (NEB).
Figure A3. PCR products of individual colonies containing Notch. Each lane, excluding the lanes showing the ladder, represents PCR product for an individual colony grown from a single transformation. (a) Transformation of primer set 2: All colonies showed the expected band size of 925 bp (N2 665 bp + vector fragment 260 bp). (b) Transformation of primer set 9: Lanes 5, 6, 7, 9 and 10 showed the expected band size of 888 bp (N9 628 bp + vector fragment 260 bp). Samples with the correct N2 and N9 inserts were purified and sent for sequencing. L= 50 bp DNA ladder (NEB).

Figure A4. RNA probe synthesis for Notch. Probe synthesis gel results for Notch 2 and 3 (each having a sense T7 and antisense T3 strand) showing the expected band sizes: 888 bp for Notch 9 and 925 bp for Notch 2, indicating successful probe synthesis. Wnt T7 was used as a control and L= 50 bp DNA ladder (NEB).
**Figure A5. RNA probe hydrolysis for Notch.** Gel results for the hydrolyzed Notch probe the 888 bp and 925 bp bands are not very clear in this picture. However, N9 sense and antisense probes show a smear indicating successful probe hydrolysis (double-headed arrows). En pp4 T7 was used as a control. (5 uL of each hydrolyzed probe per lane. The probes were denatured for 10min at 70\(^\circ\)C in a mixture of formamide, formaldehyde, and 10X MOPS buffer. N2 and N9= Notch primer sets 2 or 9 (T3: antisense, T7: sense) L= 50 bp DNA ladder (NEB), En pp4 T7= positive control).

**Figure A6. dsRNA synthesis for Notch.** Gel results for Notch dsRNA showing no band. Wnt T7 was used as a control. Despite multiple trials, synthesis of dsRNA for Notch was unsuccessful. L= 50 bp DNA ladder (NEB).
ii) Delta

The amino acid sequence for the *Thamnocephalus Delta* was aligned with a *Drosophila Delta* homolog to confirm the identity of Delta (Fig. A7). For Delta, we designed three different primer sets 1, 3 and 4 that would amplify a small portion of the complete 2286 bp sequence. Initially, primer sets 1 and 3 were designed to amplify 405 bp and 484 bp regions respectively. However, these primer sets did not ultimately yield successful in situ hybridization. Shown below are the gel results for primer set 4 that gave positive in situ staining. After performing PCR with primer set 4, a gel was run to verify the presence of single bands with the 411 bp expected product size (Fig. A8). This product was cloned into a 4.3 kb bacterial vector. Individual colonies that resulted from the ligation/transformation procedures were used as DNA template in an additional PCR. In this colony PCR, M13 primers amplify the Delta target as well as a 260 bp fragment that includes both the T3 and T7 promoters necessary for RNA probe and dsRNA synthesis. Gel electrophoresis of the colony PCR was used to confirm that Delta was properly inserted. This gel included the expected band size of the PCR product with a 260 bp vector fragment, resulting in a 671 bp band (Fig. A9). Subsequently, the samples were purified and sent for sequencing. After analyzing the sequence results and verifying that they were consistent with the initially isolated sequences, an RNA probe and dsRNA were synthesized for in situ hybridization and RNAi gene knockdown experiments, respectively (Fig. A10, A12). The RNA probe for Delta was hydrolyzed and resulted in the presence of clear single band preceded by a smear, indicating successful probe hydrolysis (Fig. A11). The dsRNA synthesis for Delta was also successful since it showed a faint broad band between 500 and 700 bp (Fig. A12).
Figure A7. Amino acid alignment for *Thamnocephalus Delta* with a *Drosophila Delta* homolog.

An alignment showing the amino acid sequence for *Thamnocephalus Delta* (Query) with a *Drosophila Delta* homolog (Sbjct) to confirm the identity of Delta. This alignment focuses on the regions with the highest homology, as indicated by the line in between the Query and Sbjct.
Figure A8. PCR products of cDNA amplified with the Delta primer set. Lanes 1 shows a 50 bp DNA ladder (NEB). Replicate PCR products (Delta A and Delta B) both showed correct band sizes of 411 bp. (Positive control: Human DNA amplified with beta actin primers and negative control: Human DNA amplified with Delta 4 primers).

Figure A9. PCR products of individual colonies containing Delta. Each lane represents PCR product for an individual colony grown from a single transformation. Lanes labeled as 1, 2, 4, 5, and 7 showed the expected band size of 671 bp (411 bp + vector fragment 260 bp). These samples were purified and sent for sequencing. L= 50 bp DNA ladder (NEB).
Figure A10. RNA probe synthesis for Delta. Probe synthesis gel results for Delta (sense T3 and antisense T7 strand) showing the expected band size of 671 bp. Wnt16 T3 was used as a control. L= 100 bp DNA ladder (NEB).

Figure A11. RNA probe hydrolysis for Delta. Gel results for the hydrolyzed Delta probe show a band preceded by a smear for the antisense strand (Delta T7), indicating successful probe hydrolysis. En T7 was used as a control with a band size of 625 bp. (2% agarose gel in 1X TBE buffer and stained with EtBr. 45 min @130V, 5 uL of each hydrolyzed probe per lane. The probe was denatured for 10min at 70°C in a mixture of formamide, formaldehyde, and 10X MOPS buffer. DI= Delta (T3: sense, T7: antisense) L= 50 bp DNA ladder (NEB), En pp4 T7= positive control).
iii) Serrate

The amino acid sequence for the *Thamnocephalus Serrate* fragment was aligned with a *Drosophila Serrate* homolog to confirm the identity of *Serrate* (Fig. A13). For *Serrate*, we designed two different primer sets 2 and 3 that amplified 606 bp and 510 bp respectively within the complete 1392 bp sequence. After performing PCR, a gel was run to verify the presence of single bands with the expected product size (Fig. A14). Each product was cloned into a 4.3 kb bacterial vector. Individual colonies that resulted from the ligation/transformation procedures were used as DNA template in an additional PCR. In this colony PCR, M13 primers amplify the *Serrate* target as well as a 260bp fragment that includes both the T3 and T7 promoters necessary for RNA probe and dsRNA synthesis. Gel electrophoresis of the colony PCR was used to confirm that *Serrate* was properly inserted. This gel included the expected band size of the PCR product with a 260bp vector fragment, resulting in a 1028 bp band size for S2 and an 770 bp band size for S3 (Fig. A15). Subsequently, the samples were purified and sent for sequencing. After analyzing the sequence results and verifying that they were consistent with the initially
isolated sequences, an RNA probe was synthesized for *in situ* hybridization. Gel results for the RNA probe for *Serrate* showed an expected band size of 600 bp (Fig. A16).

**Figure A13. Amino acid alignment for *Thamnocephalus Serrate* with a *Drosophila Serrate* homolog.** An alignment showing the amino acid sequence for *Thamnocephalus Serrate* (Query) with a *Drosophila Serrate* homolog (Sbjct) to confirm the identity of *Serrate*. This alignment focuses on the regions with the highest homology, as indicated by the line in between the Query and Sbjct.
Figure A14. PCR products of cDNA amplified with two Serrate primer sets. The expected band sizes were obtained: 606 bp for Serrate 2 and 510 bp for Serrate 3. An additional 175 bp band appears in the Serrate 2 lane. (Positive control: Human DNA amplified with beta actin primers, negative control: Human DNA amplified with Serrate 2 primers, and L= 50 bp DNA ladder (NEB)).

Figure A15. PCR products of individual colonies containing Serrate. Each lane, excluding the lanes showing the ladder, represents PCR product for an individual colony grown from a single transformation. Lanes labeled as S2-3 and S2-4 show transformation results of primer set 2 with the expected band size 1028 bp (608 bp+ additional band 175 bp + vector fragment 260 bp). Similarly, S3-1, S3-2, S3-3 and S3-4 showed the expected band size of 770 bp (510 bp + vector fragment 260 bp). These samples were purified and sent for sequencing. L= 100 bp DNA ladder (NEB).
iv) hairy

The amino acid sequence for the *Thamnocephalus hairy* was aligned with a *Drosophila hairy* homolog to confirm the identity of *hairy* (Fig. A17). For *hairy*, we designed primer set 4 that amplified 422 bp within the complete 807 bp sequence. After performing PCR, a gel was run to verify the presence of single bands with the expected product size (Fig. A18). The product was cloned into a 4.3 kb bacterial vector. Individual colonies that resulted from the ligation/transformation procedures were used as DNA template in an additional PCR. In this colony PCR, M13 primers amplify the *hairy* target as well as a 260bp fragment that includes both the T3 and T7 promoters necessary for RNA probe and dsRNA synthesis. Gel electrophoresis of the colony PCR was used to confirm that *hairy* was properly inserted. This gel included the expected band size of the PCR product with a 260bp vector fragment, resulting in a 680 bp band size (Fig. A19). Subsequently, the samples were purified and sent for sequencing. After analyzing the sequence results and verifying that they were consistent with the initially isolated sequences, an RNA probe and dsRNA were synthesized for *in situ* hybridization and
RNAi gene knockdown experiments, respectively. Gel results for the RNA probe for *hairy* showed an expected broad band size of 680 bp (Fig. A20). The dsRNA synthesis for *hairy* was also successful since it showed multiple faint bands 700-1200 bp (Fig. A21).

### Figure A17. Amino acid alignment for *Thamnocephalus hairy* with a *Drosophila hairy* homolog.
An alignment showing the amino acid sequence for *Thamnocephalus hairy* (Query) with a *Drosophila hairy* homolog (Sbjct) to confirm the identity of *hairy*. This alignment focuses on the regions with the highest homology, as indicated by the line in between the Query and Sbjct.

### Figure A18. PCR products of cDNA amplified with the *hairy* primer set. Hairy 1, 2 and 3 are PCR products amplified with the same primer set 4 that were used to confirm the results. An expected band size of 422 bp was obtained in addition to a fainter 450 bp band. L= 50 bp DNA ladder (NEB).
Figure A19. **PCR products of individual colonies containing hairy.** Each lane, excluding the lanes showing the ladder, represents PCR product for an individual colony grown from a single transformation. Lanes 1, 3, 4, and 5 show the expected band size of ~680 bp (hairy: 422 bp + vector fragment: 260 bp). Lane 5 shows a very faint 680 bp band in addition to another band. All 4 samples were purified and sent for sequencing. L= 100 bp DNA ladder (NEB).

Figure A20. **RNA probe synthesis for hairy.** Probe synthesis gel results for the antisense strand (T3) with the expected band size of 680 bp. Wnt16 T3 was used as a control. (1.0% agarose gel in 1X TBE buffer and stained with EtBr) L= 100 bp DNA ladder (NEB).
Figure A21. dsRNA synthesis for hairy. Gel results for hairy dsRNA showing multiple faint bands 700-1200 bp and indicating that dsRNA synthesis was successful. Wnt T3 was used as a control (821 bp band). (1.0% agarose gel) L= 100 bp DNA ladder (NEB).

v) Suppressor of Hairless (SuH):

The amino acid sequence for the Thamnocephalus SuH was aligned with a Drosophila SuH homolog to confirm the identity of SuH (Fig. A22). For SuH, we designed primer set 7 that amplified 522 bp within the complete 2052 bp sequence. After performing PCR, a gel was run to verify the presence of single bands with the expected product size (Fig. A23). The product was cloned into a 4.3 kb bacterial vector. Individual colonies that resulted from the ligation/transformation procedures were used as DNA template in an additional PCR. In this colony PCR, M13 primers amplify the SuH target as well as a 260bp fragment that includes both the T3 and T7 promoters necessary for RNA probe and dsRNA synthesis. Gel electrophoresis of the colony PCR was used to confirm that SuH was properly inserted. This gel included the expected band size of the PCR product with a 260bp vector fragment, resulting in an 814 bp band size (Fig. A24). Subsequently, the samples were purified and sent for sequencing. After
analyzing the sequence results and verifying that they were consistent with the initially isolated sequences, an RNA probe and dsRNA were synthesized for \textit{in situ} hybridization and RNAi gene knockdown experiments, respectively. Gel results for the RNA probe for \textit{SuH} showed an expected broad band size of 814 bp (Fig. A25). The dsRNA synthesis for \textit{SuH} was also successful since it showed multiple faint bands 700-1400 bp, including the 814 bp band (Fig. A26).

\textbf{Figure A22. Amino acid alignment for \textit{Thamnocephalus SuH} with a \textit{Drosophila SuH} homolog.} An alignment showing the amino acid sequence for \textit{Thamnocephalus SuH} (Query) with a \textit{Drosophila SuH} homolog (Sbjct) to confirm the identity of \textit{SuH}. This alignment focuses on the regions with the highest homology, as indicated by the line in between the Query and Sbjct.
Figure A23. PCR products of cDNA amplified with the SuH primer set. Lanes 2, 3, 4, 5 represent duplicate PCR reactions for primer set 7. Only lanes 4 and 5 show the expected band size of 554 bp.

L= 50 bp DNA ladder (NEB).

Figure A24. PCR products of individual colonies containing SuH. Each lane, excluding the lanes showing the ladder, represents PCR product for an individual colony grown from a single transformation. Lanes labelled as SuH 4, 5, 6, 8 and 9 show the expected band size of 814 bp (SuH 554 bp + vector fragment 260 bp). These samples were purified and sent for sequencing. L= 50 bp DNA ladder (NEB).
Figure A25. RNA probe synthesis for SuH. Probe synthesis gel results for SuH showing sense (T3) and antisense (T7) strands with the expected band size of 814 bp. Wnt16 T3 was used as a control with an 825 bp band. L = 100 bp DNA ladder (NEB).

Figure A26. dsRNA synthesis for SuH. Gel results for SuH dsRNA showing multiple faint bands 700-1400 bp, including the 814 bp band, and indicating that dsRNA synthesis was successful. L= 100 bp DNA ladder (NEB).
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