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Postembryonic Polarity Modification in the Acoel Convolutriloba longifissura

Eric Young
eyoung258@hotmail.com

James M. Sikes Ph.D.
University of San Francisco

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Undergraduate Honors Thesis

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Abstract

Metazoans establish the bilateral body plan early in embryogenesis by patterning orthogonal body axes with polarity that is unaltered during the lifetime of most animals. While some organisms re-establish/modify body axes during regeneration and asexual reproduction, the acoel flatworm *Convolutriloba longifissura* is unusual in its ability to modify left-right (L-R) axis polarity during longitudinal fission. We have developed *C. longifissura* as a model for studying the mechanisms of L-R polarity modification during postembryonic development. Regeneration experiments have elucidated the temporal dynamics of midline re-specification, suggesting that parallel L-R axes replace the pre-existing midline prior to longitudinal fission. We have characterized the spatiotemporal expression of genes encoding ligands and receptors of signaling pathways with conserved functions in polarity specification and axial patterning. Expression domains of BMP, Notch, and Slit/Robo signaling components are dynamic prior to and during longitudinal fission. RNAi-mediated gene knockdown of Notch and Slit/Robo signals disrupt longitudinal fission while BMP disrupted normal midline patterning suggesting a role in modulating changes in L-R axis polarity.

Introduction

Most developmental processes depend on the establishment of axial polarity that organizes the body plan based on anterior-posterior (A-P), dorsal-ventral (D-V), and left-right (L-R) axes. In most metazoans, the establishment of axial polarity is set during embryogenesis by patterning these orthogonal body axes and is rarely modified afterwards. Established axes provide a framework for the remainder of embryonic development to proceed. For example, axial specification in the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* occurs in the early stages of cleavage and is unchanged in adulthood. (Riechmann 2001, Kemphues 2000) Research on the evolutionary origin of axes have shown that highly conserved signal transduction pathways are necessary for the establishment of axial polarity in embryonic developmental processes (Martindale 2005). Embryonic studies in model organisms *C. elegans*, *Drosophila*, *Mus*, and *Danio* have identified key signaling pathways involved in directing axial specification. For instance, cell-cell interactions between follicle cells and the oocyte utilizing the maternal determinants interact with *dpp*, an orthologue of the vertebrate BMP, to produce a D-V axis in Drosophila (Moussian and Roth 2005). Proper embryogenesis would not be possible without axial establishment using these pathways.

In addition, axial re-specification and patterning occurs in the postembryonic processes of regeneration and asexual reproduction. In the planarian model, regeneration occurs via totipotent stem cells known as neoblasts that comprise 20-30% of the cells in its body (Dubois 1949). Amputation experiments performed on the regenerative planarians showed reformation of structures requires restoration of correct tissue polarity (Reddien and Sánchez Alvarado, 2004). Furthermore, normal regenerative ability could be rescued in planarian species limited in the ability by restoring proper axis polarity signals (Sikes & Newmark 2013). Unlike regeneration which occurs along an already established axes, asexual reproduction events such as budding or

fission require modifying or the *de novo* creation of new axes (Sikes & Bely 2008). In budding hydra, buds arise as outgrowths from the parental polyp developing into smaller replicates of the parental polyp before physical separation. (Hughes 1990). The parental individual with an already established body axis sprouts a completely new body axis to yield linked individuals. Some invertebrates such as, *Convolutriloba* acoels, radically modify body axes during fission and budding (Sikes & Bely 2008).

To better understand the process of axial re-specification during postembryonic development, we investigated the molecular mechanisms that underlie asexual reproduction in the acoelomorph *Convolutriloba longifissura*. In a two-step process, *C. longifissura* undergoes a transverse fission followed by a longitudinal fission in the detached posterior half yielding two daughter progeny from the detached portion (Fig 1). Longitudinal fission is an unusual mode of asexual division exclusive to this acoel (Åkesson et al. 2001) and a species of tapeworm (Specht and Voge, 1965. During longitudinal fission, *C. longifissura* modifies L-R axis polarity and establishes parallel midlines *de novo*, a process thought to require redeployment of developmental genes to modify and re-specify the established body axis. However, the underlying mechanisms by which this occurs are not known.

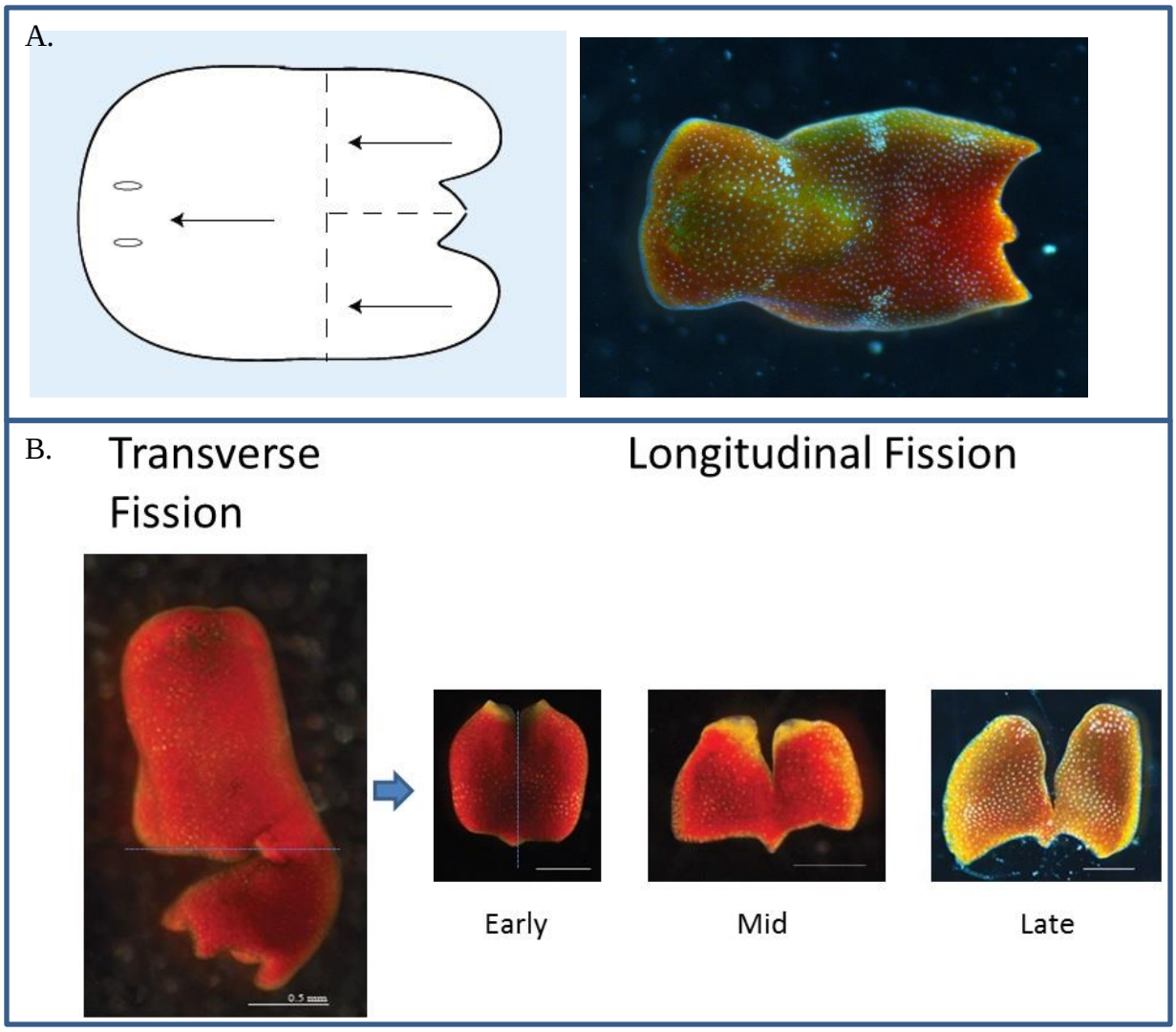

Figure 1: Asexual reproduction in *C. longifissura* occurs via a 2-step fission process: transverse followed by longitudinal. (A) Midline re-specification occurs in the parental body during asexual reproduction. (B) Transverse fission occurs first followed by progression of longitudinal fission

Previous work in model organisms have determined pathways involved in patterning midline and L-R axis during regeneration or embryogenesis, specifically: Notch, BMP, and Slit/Robo. In *Danio*, Notch signaling regulates fate decisions in midline precursors (Latimer and Appel 2006) In planarians, BMP signals are expressed in the lateral plane of injury and function in patterning D-V axes as evidenced by RNAi mediated knockdown of *BMPs* resulting in the regeneration of dorsal structures in place of normally ventral tissues (Reddien et al. 2007). The Slit/Robo pathway is vital for maintenance of the planarian midline. RNAi mediated knockdown of *slit* results in the collapse of many newly regenerated tissues at the midline (Cebrià et al. 2007). Given the highly conserved nature of these pathways in both embryonic and postembryonic developmental processes, acoels may utilize similar signals for axial re-specification during asexual reproduction.

Since the molecular mechanisms that drive the unique re-specification of midline identity and L-R axis polarity in *C. longifissura* are not well understood, we have developed this species as a model to better understand the mechanisms that drive postembryonic axial re-specification. By utilizing the regenerative properties of *C. longifissura*, we have characterized the spatiotemporal patterns of L-R axis re-specification. Amputation experiments and quantitative PCR revealed the transient nature and temporal dynamics of axes specification. RNA interference elucidated function of candidate genes in the asexual reproductive process of axial re-specification. Our experiments revealed that active re-patterning occurs prior to fission initiation. Functional studies and qPCR analysis identified key signaling pathways that mediate both re-specication and patterning of L-R axes.

Results

Axial re-specification occurs prior to initiation of fission

Utilizing the regenerative capabilities of *C. longifissura*, we investigated the temporal dynamics of axial re-specification in the posterior region of actively budding adult worms. Amputation experiments allowed inferences as to the current state of axis polarity since regenerated tissues develop according to the underlying axis polarity inherent in the body. Animals amputated via transverse lesion along future transverse fission plane (N=60), regenerated bifurcated anterior structures before progressing to complete longitudinal fission (Fig. 2A). Animals amputated at a more anterior site (N=48), were unable to complete transverse fission likely due to the loss of anterior neural structures that prevented movement and active tearing of the body. However, longitudinal fission proceeded in these headless animals without the prerequisite transverse fission resulting in two independent tail structures in the posterior region (Fig 2B). Finally, tissues excised from the posterior region along the middle lobe bounded by the inferred location of novel midline development (N=40) regenerated bifurcated heads (Fig. 2C).

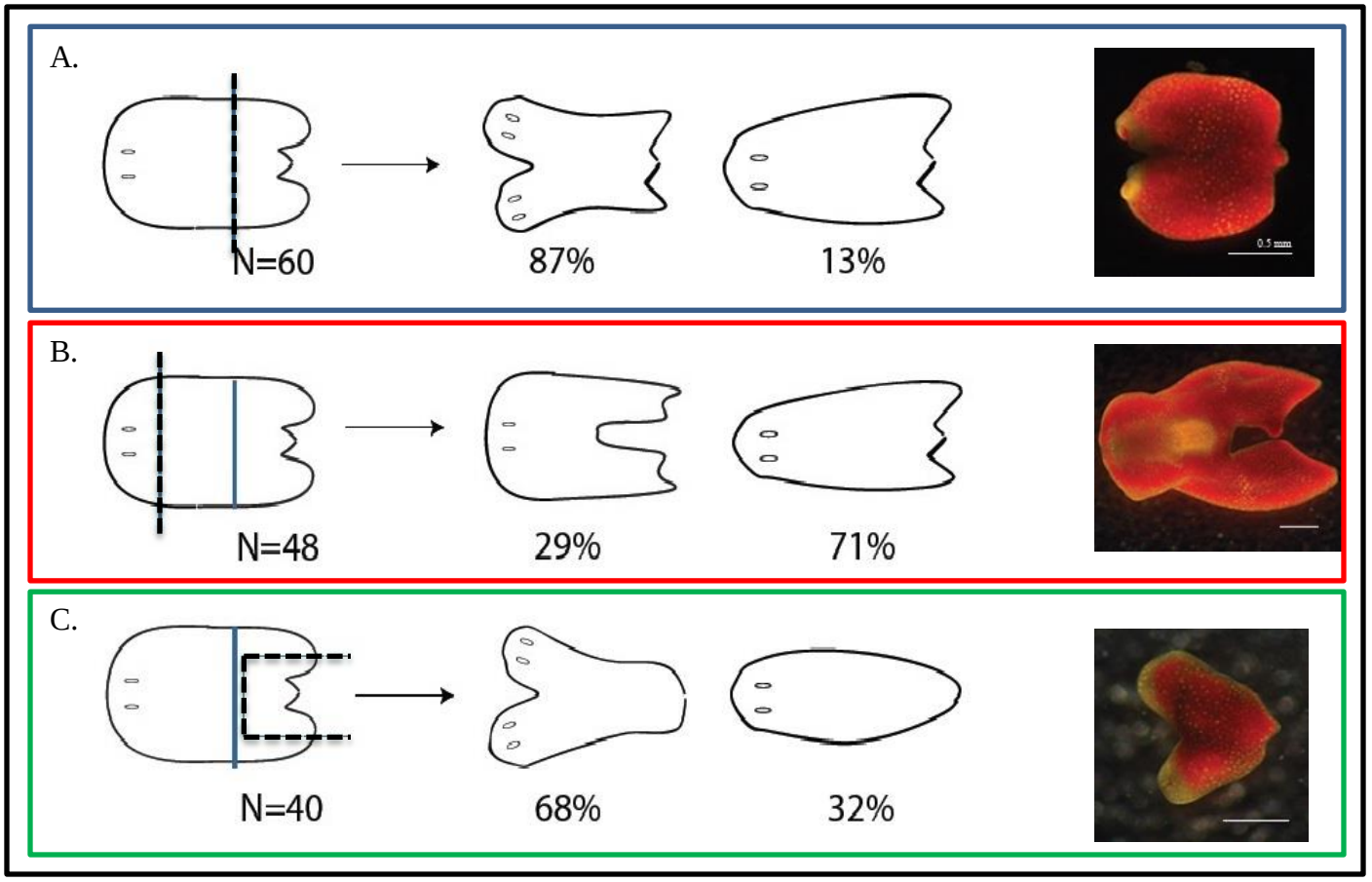

Figure 2: Amputation experiments provide inferences of underlying axis polarity. Solid blue line denotes transverse fission site while black dotted line denotes experimental amputations. (A) Amputation along the transverse fission plane produces posterior fragments that regenerate bifurcated heads before undergoing subsequent longitudinal fission. (B) Amputation along a more anterior transverse plane leads to initiation of longitudinal fission in the posterior tissues without prior transverse fission. (C) Excision of tissues bounded by the location of inferred newly patterned midlines and below the transverse fission plane leads to regeneration of bifurcated heads.

Conserved signal transduction during longitudinal fission

Given the conserved function of Notch, Slit, and BMP signaling pathways in establishing axis polarity during embryogenesis and regeneration, we investigated the temporal dynamics of gene expression of ligands, receptors, and intracellular effectors. We utilized quantitative RT-PCR (qPCR) to assess temporal changes in gene expression that occur when undergoing longitudinal fission. We found significant changes in the level of gene expression between control (non-fissioning) tissues and tissue undergoing longitudinal fission for all 3 signaling pathways (Fig. 3). To better elucidate the temporal nature of signaling throughout the longitudinal fission process, we next evaluated levels of gene expression during early and later fission stages. Slit/Robo signaling demonstrated differing expression patterns in ligands and receptors during fission. While expression of the *slit3* ligand and *robo3* receptor were significantly downregulated in late fission stages, *slit1* and *robo1* were significantly upregulated during all stages of fission (Fig. 4A). Most homologs of Notch ligands as well as BMP ligands and receptors were downregulated; *notch2* was significantly downregulated during late stage budding while *notch3* expression was significantly reduced throughout the fission process (Fig. 4B). BMP ligands *bmp2* and *bmp3* were downregulated during late stage while *bmp4* demonstrated no change in expression compared to throughout longitudinal fission. Both BMP receptors and the intracellular effector *smad3* were downregulated during all fission stages (Fig. 4C).

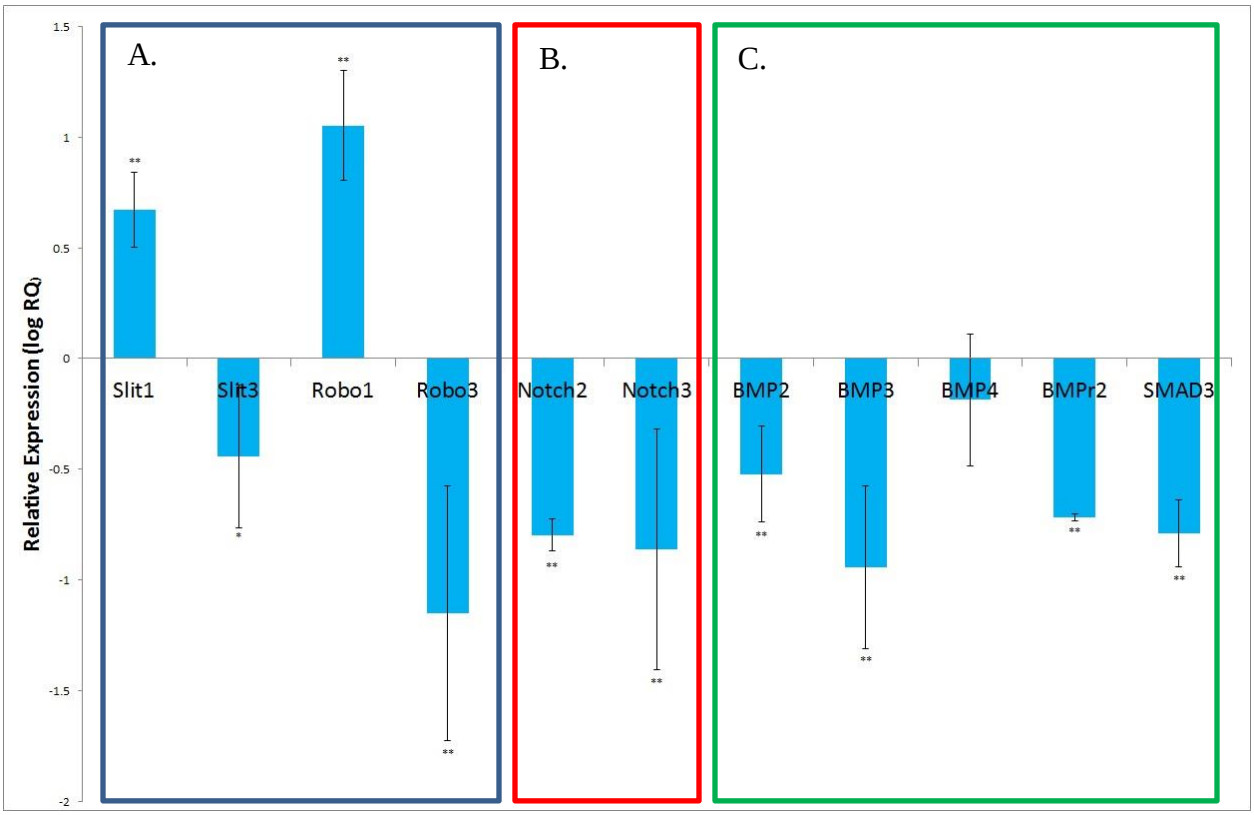

Figure 3: Candidate signaling pathway components are differentially regulated in actively fissioning animals. Expression was standardized to GAPDH and compared to non-fissioning adult posterior tissue. * denotes significant change between control and sample where $P \leq 0.05$ while ** denotes significance to a $P \leq 0.025$ value. (N=15)

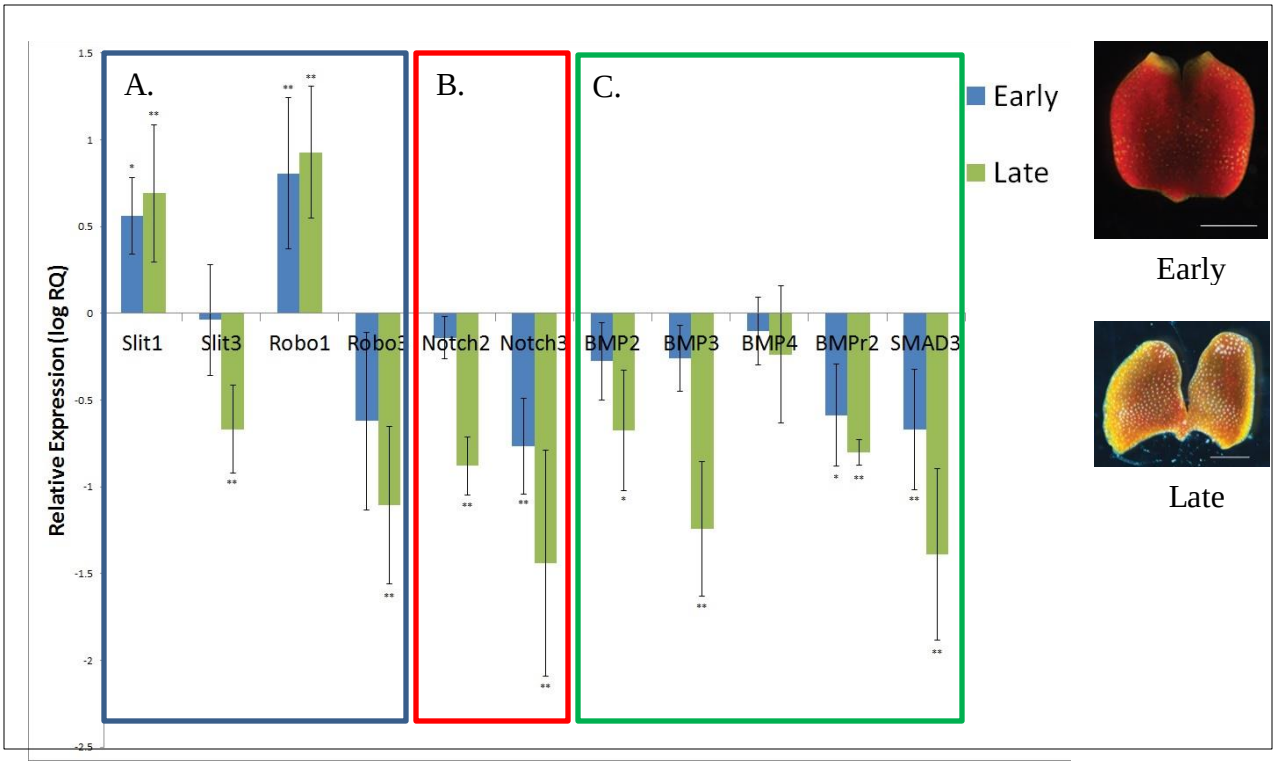

Figure 4: Candidate signaling pathway components are differentially regulated during longitudinal fission. Expression was standardized to GAPDH and compared to non-fissioning adult posterior tissue. * denotes significant change between control and sample where $P \leq 0.05$ while ** denotes $P \leq 0.01$. (N=15)

Conserved Signaling Pathways Function in Longitudinal Fission

To elucidate the function of these pathways, during longitudinal fission, we utilized RNA interference to knockdown the expression of signaling pathway ligands. Following knockdown of all slit homologs (*slit1*, *slit2*, and *slit3*) worms significantly increased the rate of fission by 40% (Fig. 5). Progeny produced from longitudinal fission were smaller than either control or other experimental progeny since the increased rate of fission led to smaller animals fissioning successively (Fig. 8). Conversely, combinatorial knockdown of all notch homologs (*notch1*, *notch2*, and *notch3*) resulted in a 52% decrease in fission rate.

Given the differential expression of slit homologs in our qPCR analysis (Fig 3A), single gene knockdowns were performed to clarify the specific role of each gene in generating the altered fission rate phenotype. Knockdown of *slit1* resulted in no effect on fission rate or noticeable phenotype. Both *slit2and slit3* knockdown worms showed an increase in fission rate. (Fig. 6) Subsequent qPCR analysis on experimental animals confirmed RNAi-mediated knockdowns were successful. Knockdowns from *slit1(RNAi*) resulted in reduction of both *slit1*and *slit2* expression while *slit2(RNAi)* and *slit3(RNAi)* knockdown animals only showed reduction of *slit2* and *slit3* expression respectively. (Fig. 7)

While bmp4 knockdown did not affect fission rate, both the anterior fission product and both progeny of longitudinal fission failed to regenerate a complete posterior developing a single lobe rather than a trilobed tail typical of controls (Fig. 8).

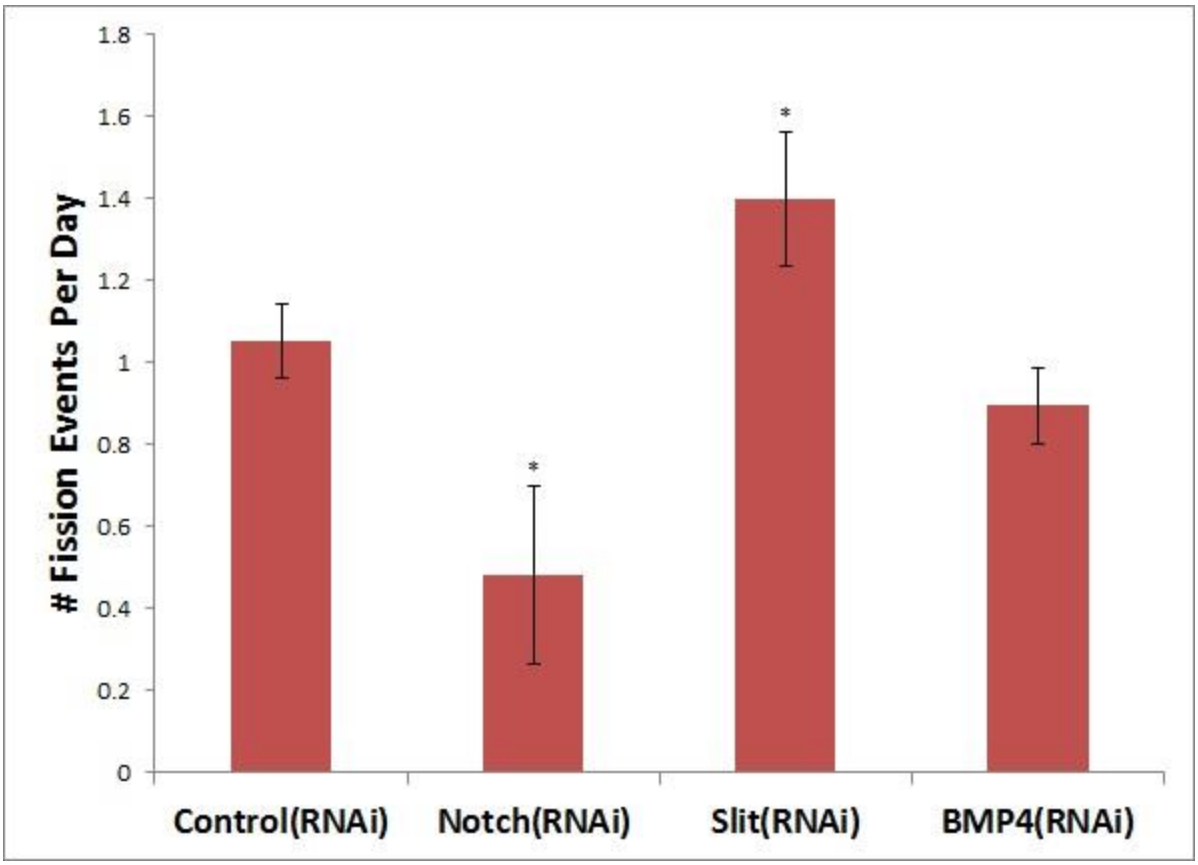

Figure 5: RNAi mediated knockdown of *notch* and *slit* ligands affect fission rate. Control(RNAi) and bmp4(RNAi) worms fissioned on average about once daily. (N=36)

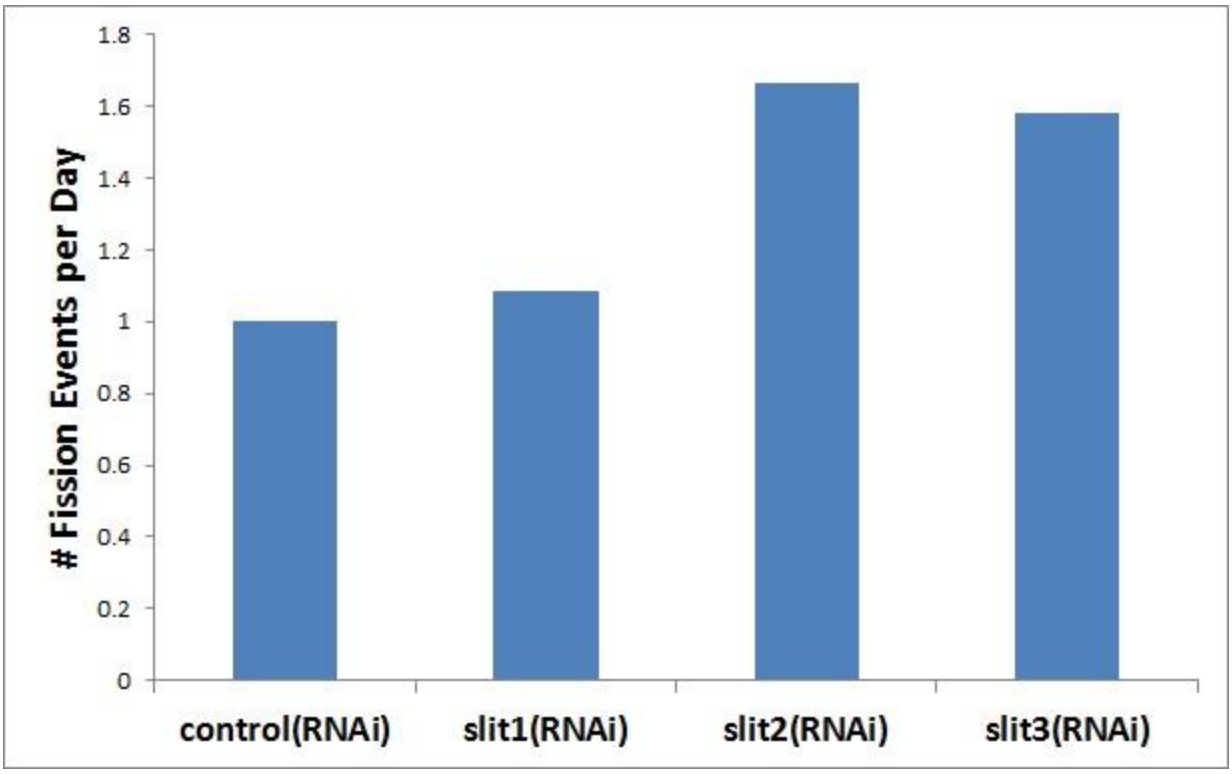

Figure 6: Knockdowns of *slit2* and *slit3* yielded changes in fission rate. *Slit1* knockdown resulted in fission rate similar to control worms. Both *slit 2* and *slit3* knockdowns resulted in marked increases in fission rate. (Data are based on a single experimental trial, N=8)

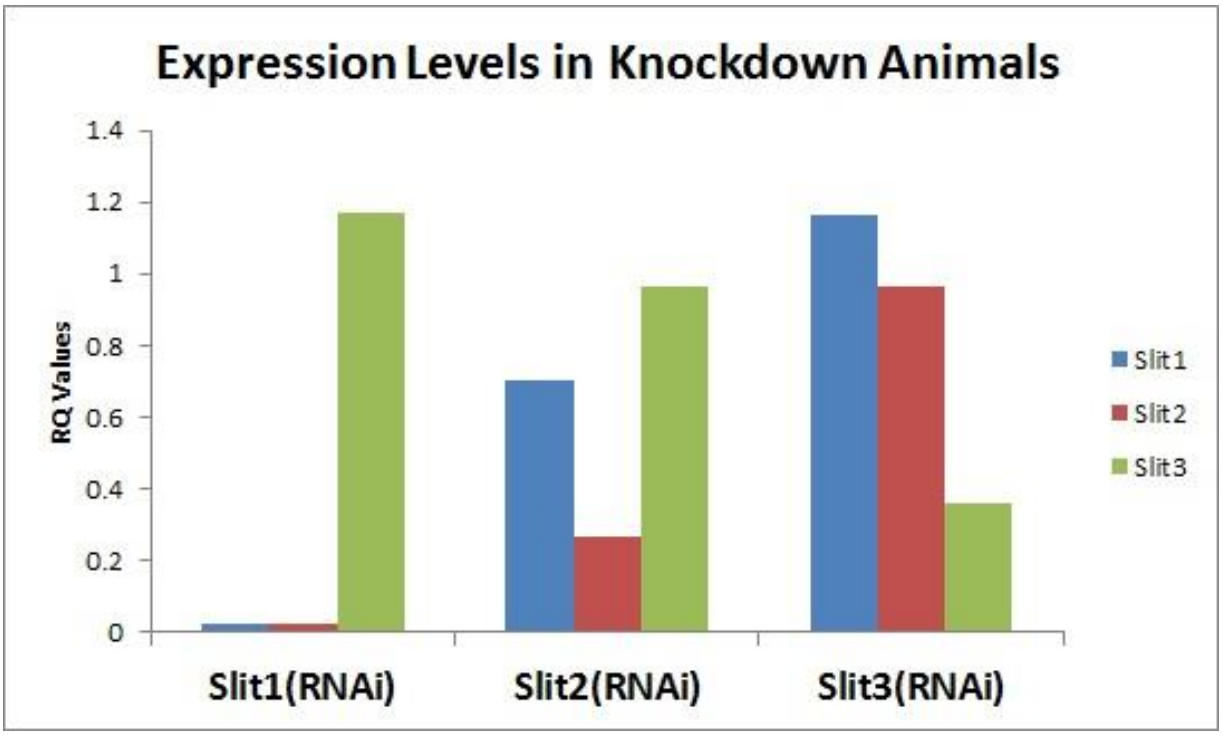

Fig 7: Expression of slit homologs after RNAi-mediated knockdown. Analysis confirms successful gene knockdowns. and potential internal regulation of Slit signals. (Data are based on a single experimental trial, N=8).

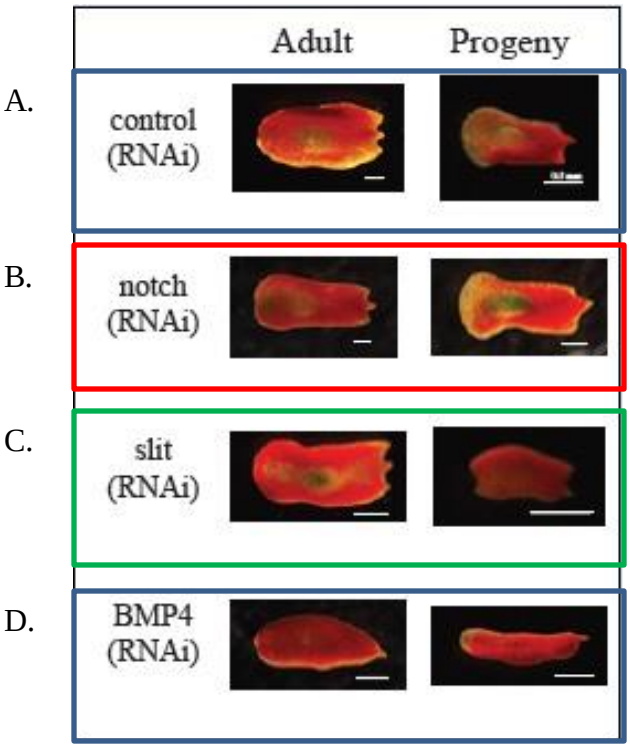

Figure 8: RNAi mediated knockdown of *notch*, *slit*, and *BMP* ligands affect size and morphology of asexual progeny.(A) *Control(RNAi)* adult and progeny. (B) *notch(RNAi)* adult and progeny show no noticeable physical abnormality (C) *slit(RNAi)* adult and progeny are smaller than either the control or other RNAi progeny. (D) *BMP4(RNAi)* anterior fission products and progeny that develop after longitudinal fission fail to regenerate a trilobed posterior margin. Scale bars = 0.5 um

Discussion

Axial re-specification occurs via internal cues

Bilaterian animals require axial specification during development to act as a coordinate system for the proper positioning of body structures. In most animals, modifications do not occur after embryonic development however; this is not the case for *C. longifissura*. Asexual reproduction in *C. longifissura* requires the re-specification of axial polarity that includes deleting the adult midline and establishing parallel midlines *de novo*. The timing and molecular mechanism of this re-specification have not been characterized and offer insights into the developmental processes that drive radical postembryonic development.

Two primary modes of asexual fission exist within metazoans. During architomic fission, animals first split into two or more fragments and then subsequently replace missing structures. In paratomic fission, new anterior and posterior structures develop, at least partially, prior to physical separation. (Bely 1999) Since architomic fission is not preceded by the intercalation of novel tissues, limited developmental processes are thought to occur prior to fission aside from the initiation of the fission furrow (Hughes 1990). While paratomic fission is observed in many animal phyla including acoels, *C. longifissura* appears to utilize architomic fission mechanisms during asexual reproduction with no tissue growth or development before fission begins and regeneration of structures post-fission.

However, our data detailing the temporal dynamics of axis modification during longitudinal fission suggest that axial re-specification occurs via intrinsic cues before the morphological features of fission manifest. Amputations of *C. longifissura* at the transverse fission plane and tissues excised from the region bordering the location of the new midlines of the asexual progeny frequently develop as two-headed regenerates, a characteristic typical of

dual re-specified midlines, suggesting the body axes are transient in the adult body prior to fission initiation. Amputation at more anterior regions eliminates the capacity for *C. longifissura* to actively move while adhering to the substrate and thus inhibits transverse fission. Despite not undergoing the first step in the normal fission process, the animal still initiates and completes longitudinal fission. Together these data suggest that *C. longifissura* repolarize existing body tissues prior to fission events with no intercalation of new tissues within the adult soma.

C. longifissura likely utilizes an internal mechanism similar to the early initiation of paratomic fission to begin signaling that will eventually re-specify axis polarity in progeny. However, unlike paratomic fission, *C. longifissura* completes patterning along the midline and structural restoration subsequent to the completion of fission. Therefore, we propose a stepwise model by which longitudinal fission occurs in *C. longifissura* where internal signals initiate the elimination of the parental midline and LR body axis polarity in posterior tissues and parallel novel midlines are established *de novo* before transverse fission. Prior to transverse fission, axis re-specification seems to be complete, yet development and patterning of tissues at anterior regions and along the pre-established midline occurs at the late stages of longitudinal fission and following tissue separation. Subsequent morphallactic reorganization of posterior tissues results in the formation of trilobed tails in each fission product.

Contrary to previous thought, the mechanism seen in *C. longifissura* opens the possibility that architomy may be preceded by dramatic developmental changes prior to fission. Could it be possible organisms that employ architomic fission utilize similar intrinsic cues to undergo dramatic developmental change prior to fission? *C. longifissura* exhibits post-fission patterning characteristic of architomic fission; however, most animals undergoing architomic fission need only to re-establish A-P axis polarity while *C. longifissura* must re-specify its midline. The

relative rarity of axial re-specification supports that an intrinsic signaling cue mechanism might be inherent to the process. Further research investigating possible similar preparatory signals in architomic fissioning animals could deduce whether intrinsic mechanisms exist in all postembryonic developmental processes or only in organisms that must re-specify axes and thus why the relative rarity of the ability in nature.

Slit and Notch signals function in axial re-specification

To elucidate the internal cues that control axis modification prior to fission, we investigated the expression and role of conserved pathways involved in midline identity and patterning left-right axis polarity.

Planarian and mammalian neurodevelopment studies have identified the role of Slit/Robo signaling in midline patterning. Knockdowns of Slit/Robo signals in regenerating planarians resulted in the collapse of tissue along the midline with failure to pattern the bilobed ganglion and improper placement of photoreceptors on either side of the anterior midline (Cebria et al. 2007). . In mammalian embryogenesis, axonal growth and extension along the midline has been shown to be regulated by Slit/Robo signaling. (Cebrià et al. 2007, Bagri et al., 2002) Knockdown of Slit signaling resulted in significantly increased fission rates which likely implicates its role in maintaining the midline. We speculate that as fission initiates, slit signaling is reduced in expression as the parental midline undergoes modification. Given that longitudinal fission happens only in the posterior third of the body in the absence of complex patterned organ structures, Slit signaling mediated midline modification could be sufficient to drive the initiation of fission.

Notch signaling plays key roles in regulating cell specification and proliferation along the midline in zebrafish (Latimer and Appel 2006) and controls expression of midline enhancers

during *Drosophila* embryogenesis. (Pearson and Crews 2014). Knockdowns of Notch signal transduction leads to a significant decrease in fission rates. These data suggest that Notch is necessary for the initiation of the fission process most likely by directly modulating changes in axial polarity in pre-fission tissues. Similar to the aforementioned systems, we infer Notch signaling initiates midline development in *C. longifissura* allowing for the progression of longitudinal fission. The role of Notch signaling in midline development would require an increase in expression to initiate the fission process while reduction in expression would inhibit the process from occurring.

Our functional data suggest that Slit/Robo and Notch signals alternatively regulate changes in midline polarity and thus the initiation of fission. Functional data complement the divergent alterations in expression of signaling ligands suggesting that Notch and Slit/Robo pathways work as antagonistic midline regulatory pathways where Slit/Robo maintain the parental midline and Notch signals initiate re-specification and *de novo* patterning of altered LR axis polarity in the future site of longitudinal fission. To elucidate a possible modulatory mechanism in midline modification between the two pathways, quantification of Slit signals expression via qPCR could be performed in animals subjected to notch RNAi-mediated knockdown and vice versa.

BMP signals have been shown to pattern D-V axes in regenerative tissue. (Reddien et al. 2007) Previous studies on the acoelomorph *Hofstenia miamia* determined perturbation of Bmp-Admp resulted in abnormal regenerates displaying a bloated, rounded tail. (Srivastav 2014) While *BMP4* expression levels did not change during longitudinal fission, knockdowns resulted in abnormal posterior patterning after the completion of fission. Worms demonstrating this phenotype failed to develop lateral lobes producing a single posterior projection along the

midline in both the anterior fragment of transverse fission and the dual progeny of longitudinal fission. Our data suggests that BMP4 signals are likely upregulated after the completion of longitudinal fission and function in medial-lateral axial patterning. *In-Situ* hybridizations on animals prior and subsequent to the completion of fission should be performed to confirm changes in BMP expression domain.

With a clear void in available animal models that can dramatically modify their axes after embryogenesis, we have further developed *C. longifissura* as a feasible model for studying *in vivo* axial re-specification during asexual reproduction. Characterization of the asexual reproductive process has allowed us to elucidate the functional role of Slit/Robo, Notch, and BMP signaling in re-specifying and patterning axes during and after the fission process. Preliminary data from *slit1(RNAi)* knockdowns (Fig 7) suggest possible a possible modulatory system within the Slit/Robo pathway. Furthermore, we speculate a possible regulatory mechanism could exist between the antagonistically functioning Notch and Slit/Robo pathways. Using our model, we raise the possibility that preparatory intrinsic cues could exist in all postembryonic processes including architomic fission. Understanding the developmental mechanisms that allow the radical modification of axial polarity displayed in these organisms could potentially be an invaluable tool in furthering the field of regenerative medicine. As a prerequisite for tissue regeneration, correct tissue polarity must be established. *C. longifissura*'s unique ability to modify and re-establish axial polarity provides an opportunity to better understand the process and hopefully one day harness it.

Methods and Materials

Animal Culture

Acoels were kept in 12 gallon aquaria at 24 degrees C that ran on 12h dark-light cycles in 34ppt artificial seawater (ASW, Instant Ocean). Animals were fed twice a week with recently hatched *Artemia* nauplii.

Regeneration Experiments

To assess the temporal nature of axial re-specification, regeneration experiments were performed using live *C. longifissura*. Amputations were performed on sufficiently developed worms with the assistance of a cold block in place of anesthesia. All experimental animals were maintained in ~2 mL ASW in 24-well plates at room temperature under full spectrum illumination running on 12h dark-light cycles.

Amputations were performed at various locations of the animal. The specimen was severed at the plane of transverse fission (n=60), anterior to the plane (n=48), and posterior to the plane between the two lateral lobes (n=40). Regeneration polarity was used to deduce axis polarity of adult tissue following amputations.

Gene Isolation

Candidate genes were isolated by extracting RNA from *C. longifissura* using TRIzol reagent (Ambion) or Direct-zol RNA Miniprep kit (Zymo) under manufacturer instructions. The RNA was then reverse transcribed with Protoscript II Reverse Transcriptase (New England Biolabs) and amplified using PCR with primer sequences in Table 1 designed based on contigs assembled from a transcriptome (sequenced using Illumina HiSeq). PCR was performed under the following conditions: 1.) 5 min at 94°C, 2.) 30 sec. at 94°C, 3.) 30 sec. at 55°C, 4.) 1.5 min at

72°C, 5.) repeat from step 2 35x, 6.) 5 mins at 72°C. PCR products were purified using spin columns (Zymo DNA Clean and Concentrator; Zymo Corporation) then 2–3 μl of PCR product was ligated with 70ng of Eam1105I-digested pJC53.2 plasmid (Collins et al. 2010) and cloned into E. coli strain JM109.

Gene	Forward Primer	Reverse Primer
slit1	GCACATCAAAAACCTCAACCGA	GGCGTAAATTCTGCCACTTGTT
slit2	GAGGCATGTTCAAGGATTTACC	GCA GCA TCA CTG TAG AGC AAA G
slit3	GCTAAAATGTCGCCAAGCTAAC	AGC TCT GCA GTA TAT CGG GGT A
notch1	TGCAGTGAAACTAATGGAGAGG	CAC CCT TGT ATC CAG TGT TGA A
notch2	GAGGATATGAGGGCAAGAATTG	GTT TGA ACA CGA AGA GGA GTT G
notch3	GTGTCGCCATATTTTACCCTGT	ATC CAG GTG TCC TGC AAT TAT C
BMP4	CCCTCCAGGAATTTTGGATCCA	GCGTGGTTAGTGGCATTGTATC

Table 1: Primers utilized to amplify *C. longifissura* cDNA in the cloning procedure.

RNA interference

To generate dsRNA, templates cloned into pJC53.2 were amplified with a modified T7 oligonucleotide (GGATCCTAATACGACTCACTATAGGG), purified using a DNA Clean & Concentrator kit (Zymo Research) and eluted in 15 uL of water. 10.5 uL of each PCR product was used as template for *in vitro* transcription in a reaction containing 5 uL 100 mM mix of ribonucleotide triphosphates (rNTPs) (Promega), 2 uL high-yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl2, 20 mM spermidine, 0.1 M DTT), 1 uL thermostable inorganic pyrophosphatase (New England Biolabs), 0.5 uL murine RNase inhibitor (New England Biolabs), and 1 uL T7 RNA polymerase. Samples were incubated at 37°C for 16 h and then treated with RNase-free DNase (New England Biolab) and cleaned/concentrated via ammonium acetate precipitation. Synthesized RNA was then annealed by heating at 95°C, 75°C and 50°C each for 3 min.

A microinjection technique introduced dsRNA into the syncytial gut that elicited a systemic knockdown of target genes within the *C. longifissura* sample specimen. Triarylmethane or tartrazine dye was added to coat the solution for visualization purposes. To better assess the effects of knocking down a pathway and to reduce the possibility of homolog gene duplications causing rescue effects, combinatorial dsRNA injections of slit (*slit1, slit2, slit3*; n=36) and notch (*notch1, notch2, notch3;* n=36) were performed. Worms were injected with ~50 nL dsRNA solution using a Nanoject II micromanipulator (Drummond Scientific). Injections ran on a schedule of day 1, 3, 5, 8, 10, 12, 15, 17 while observations continued through day 19. A negative control sample was administered by injecting worms from the same tank with dsRNA of ccdB- and camR containing insert of pJC53.2 (Collins et al. 2010) on the same schedule. Animals were scored for any abnormalities in L-R axis and/or any modifications to the rate or timing of longitudinal fission.

qPCR

To determine levels of gene expression during longitudinal fission, quantitative PCR (qPCR) was performed. cDNA libraries from 5 worms at different stages of fission as judged by progress of the longitudinal fission furrow were constructed. Control sampled included posterior tissue from animals that were non-fissioning and were used to compare gene expression before initiation of fission. RNA was extracted using TRIzol (Ambion) reagent or the Direct-zol RNA Miniprep kit (Zymo). cDNA was then synthesized using Protoscript II Reverse Transcriptase (New England Biolabs) under manufacturer protocol.

Quantitative PCR was performed using cDNA from each sample was tested for expression of slit homologs (*slit1, slit3*), robo homologs (*robo1, robo3*), notch homologs (*notch2, notch3*), and BMP pathway ligands and receptors (*BMP2-4, SMAD3, BMPr2*) using

primer sequences in Table 2 and GoTaq SYBR green qPCR mastermix (Promega) and a CFX96 thermocycler (Biorad). qPCR was performed under the following conditions: 1.) 3 min at 95°C, 2) 10 sec at 95°C, 3.) 30 sec at 55°C, 4.) repeat from step 2 39x. Three biological replicates were performed and all samples were measured in triplicate to account for pipetting error. Each trial was standardized to GAPDH. Expression of genes in the samples were compared against the control sample of non-fissioning posterior tissue.

At the conclusion of RNAi experiments, expression levels of slit homologs were assessed via qPCR. RNA extraction and cDNA synthesis followed the same protocol as above. Expression levels of slit (*slit1, slit2, slit3*) and notch (*notch1, notch2, notch3*) ligands were determined using primers listed in table 2. Expression was standardized to GAPDH and compared to cDNA extracted from "control (RNAi)" animals.

Gene	**Forward Primer**	**Reverse Primer**
slit1 RT	TTCAAATGTGCGGCTCCTTC	TCC GCA CAC TCG CAT TTT TC
slit2 RT	CGAAGCTGATGGTCGATTCTTG	AGGCTCCATCTTCGATGTCTTC
slit3 RT	ACCCTTGCCCTAAAGAATGC	AGCTTGGCGACATTTTAGCC
robo1 RT	TCTTTCCGAACTCGAACAGC	TTGTTCACGTGCAAGCTTCC
robo3 RT	TCACAACGGCAGAAAACTCG	CTCTTCCGTTCGTCTGATTTGC
notch1 RT	CTCCAGACTTCCAAAGCTACAC	TCCTCGCATTCGTTCACATC
notch2 RT	TCGTGTTCAAACAGCGGTTC	CAGCGGTCGAAATTGAGTCATC
notch3 RT	AAACCCTTGCCATGCATTCG	TGGTACTCGCATTTGTGACC
BMP2 RT	AACTATTCTCGCAGGCTGTGG	CACGTTGGCATTTTCGGTTC
BMP3 RT	CGCAATATTGTGGCGAAAGG	CCGCTGTTTGCTTGTAAAGGTC
BMP4 RT	ACACTTGCCCGCATTTCTTC	GCA GTG AAA TGC TTC TGT GC
BMPr2 RT	AGTTGCGACCCCAATTCAAC	AGCCTCACAGCCATTCATTC
SMAD3 RT	TGCTCATGACTGCTGCAAAC	TTTCGCAGATAGCACCTCTCTC
GAPDH RT	AACAGCGATTGCAGTTCACG	AGTAGTGAAAACGCCAGTCG

Table 2: Primers used to amplify *C. longifissura* cDNA for qPCR

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