Supplemental Information

Spine Patterning Is Guided by Segmentation of the Notochord Sheath

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Supplemental Information

- Supplemental Figures and Legends S1-S6
- Supplemental Experimental Procedures
- Supplemental References
Figure S1. Alternating segments in the notochord sheath are generated in an anteroposterior direction, Related to Figure 2.

Confocal images showing the development of alternating `entpd5a:pkRED+` and `col9a2:GFPCaaX+` segments in the notochord sheath from early (4.0mm SL) to late stages (6.5mm SL).

Scale bars are 100 μm. All images have been digitally stitched. Dotted line (bottom panel) indicates where two images were manually stitched together due to specimen displacement.
Figure S2. Transcript expression in col9a2+, double+, and entpd5a+ cell populations shows consistent replicates and distinct enrichment signatures for various segmentation genes, Related to Figure 3.

(A) Principal component analysis shows clustering of cell populations based on expression of all genes.

(B) Heat map visualization of somite segmentation genes show enrichment in the double+ transitional population (top heat map) for mespba, notch1a, ripply1, and her1; whereas, notch2, notch3, ifng, and dkk1b were specifically up-regulated in the entpd5a+ population (bottom heat map).
Figure S3. *in situ* hybridization of *mespbb* reveals segmented expression in the notochord sheath, Related to Figure 3.

Cryo-sections of 10 dpf larvae show expression of the *mespbb* transcript in notochord sheath cells in a segmented pattern (arrows) that correlates with somite boundaries (dotted lines).

Scale bars are 50 µm. Red dotted line (bottom panel) indicates where two images were manually stitched together.
Figure S4. Somite boundaries are unaffected by genetic manipulations in the notochord, Related to Figure 5.

(A) Brightfield imaging of *col9a2*:QF2 x QUAS:nlsVenus-V2a-notch1aICD (QUAS:NICD) or QUAS:nVenus-V2a-SuHDN (QUAS:SuHDN) crosses show that QUAS expression does not affect somite patterning or boundary formation at 48 hpf.

(B) Phalloidin staining of 48 hpf embryos did not show defects in somite organization compared to clutch-mate controls. White (*) denotes pigmentation. Scale bars for (A) and (B) are 100 µm.

(C) Control siblings from QF2 x QUAS crosses, i.e. expressing one or no transgene, did not present defects in notochord or spine segmentation. Scale bars are 500 µm.
Figure S5. Alteration of notochord sheath segmentation produces defects in centra shape, size, and average number, Related to Figure 5.

(A) Graphs depicting dorsal/ventral length ratios of centra in col9a2:QF2 x QUAS:GFP (n=19) and col9a2:QF2 x QUAS:nlsVenus-V2a-notch1aICD (n=20) fish. To characterize vertebral shape changes, we measured the length of individual centra along the dorsal and ventral sides as shown before (Hayes et al., 2013). Horizontal dotted lines demarcate ±0.1 ratio spreads, determined by the maximum deviation observed in col9a2:QF2 x QUAS:GFP controls. Deviation from a ratio of 1±0.1 is apparent for multiple centra along the anterior portion of the spine in col9a2:QF2 x QUAS:nlsVenus-V2a-notch1aICD fish. Individual fish are represented by a unique color and each individual centra is represented by a unique point. The graph’s x-axis extends in the anterior (A) posterior (P) direction, starting with the first rib-bearing vertebrae and ending with the last caudal vertebrae.

(B) Graph depicting the number of centra for misexpression crosses. Expression of QUAS:nVenus-V2a-SuHDN results in a statistically significant increase in the number of centra (n=16; p=0.0093). Whereas the mean number of centra in controls expressing QUAS:GFP was 26, fish expressing QUAS:nVenus-V2a-SuHDN on average had 27 centra. Conversely, expression of QUAS:mespbb-p2A-eGFP led to a decrease in the mean number of centra (n=22; p=0.0292). Manipulations with QUAS:nlsVenus-V2a-notch1aICD (NICD) did not lead to a significant increase or decrease in the number of
segments. *p* values for number of centra were determined via Welch’s unequal variances *t*-test.

(C) Centra length comparisons between manipulations show that significantly longer centra compared to controls were generated upon expression of *NICD* (*n*=20, *p*=0.0001) and *mespbb* (*n*=22, *p*=0.0104). *p* values for vertebrae length comparisons were calculated from a 1-way ANOVA with Dunnett’s multiple comparisons test. For graphs (B) and (C), error bars denote mean and SEM. All analyses exclude the Weberian and caudal tail vertebrae.
Figure S6. Delayed expression of QUAS:mespbb-p2A-eGFP bypasses early embryonic defects, Related to Figure 5. Compared to siblings lacking transgenic expression, col9a2:QF2;QUAS:mespbb-p2A-eGFP fish expressing both transgenes had severe defects. Injection of 50 pg or 100 pg of QS RNA (Potter et al., 2010) delayed onset of mespbb-p2A-eGFP expression and partially rescued early developmental defects. Scale bars are 100 µm.
Supplemental Experimental Procedures

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Animal experiments were approved by the Duke Institutional Animal Care and Use Committee (IACUC).

Fish stocks
Adult zebrafish of the Ekkwill strain (EK) were maintained and bred as previously described (Ellis et al., 2013; Garcia et al., 2017). All experiments with animals were approved by Duke University. Individual fish were used for genetic manipulation experiments and compared to siblings and experimental control fish of similar size and age. Independent experiments were repeated using separate clutches of animals. Strains generated for this study: Tg(col9a2:QF2)pd1163, Tg(QUAS:nlsVenus-V2a-notch1alCD)pd1164, Tg(QUAS:nVenus-V2a-SuHDN)pd1165, Tg(QUAS:mespbb-p2A-eGFP)pd1166, Tg(id2a:GFPCaaX)pd1167, and TgBAC(entpd5a:pkRED)hu7478. Previously published strains: Tg(col9a2:GFPCaaX)pd1151 (Garcia et al., 2017), Tg(osx:mcherry-NTR)pd43, Tg(osx:mTagBFP-2A-CreER)pd45 (Singh et al., 2012), Tg(her1:her1-Venus)bk15 (Delaune et al., 2012), Tg(TP1:VenusPEST)s940 (Ninov et al., 2012), and Tg(-2421/+29sox9b:EGFP)uw2 (Plavicki et al., 2014), referred to in this paper as sox9b:eGFP. Transgenic lines were generated using the Tol2 system as described before (Kawakami, 2007).

METHOD DETAILS

Transgenesis
All constructs for transgenic fish were generated using the same cloning strategies as before (Ellis et al., 2013; Garcia et al., 2017; Rodriguez-Fraticelli et al., 2015) The p5E-QUAS and pME-QF2 vectors were gifts from Marnie Halpern (Subedi et al., 2014). The pME-nlsVenus-V2a-notch1alCD and p3E-nVenus-V2a-SuHDN plasmids were gifts from David Parichy (Eom et al., 2015). The pBS-SK-QS plasmid was a gift from Christopher J. Potter and Liqun Luo (Potter et al., 2010).

To generate pME-nVenus-V2a-SuHDN, we amplified nVenus-V2a-SuHDN using the primers: forward, 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGGCTCCAAAGAAGAAG 3'; reverse, 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTCAGAGGCTCGAGA 3'.

pME-mespbb-p2A-eGP was generated by amplifying cDNA using primers: forward, 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGGCTCCAAAGAAGAAG 3'; reverse, 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTCAGAGGCTCGAGA 3'.

To generate p5E-id2a, a 1kb sequence from id2a promoter was amplified from genomic DNA using primers: forward, 5'AAGCTTCATCGTGCAAACGT 3'; reverse, 5'CCCACAGTGAGTTCAGAAAGC 3'.

BAC recombineering strategies (Bussmann and Schulte-Merker, 2011) were used to generate TgBAC(entpd5a:pkRED)hu7478.
Calcein staining and skeletal preparations
Calcein staining and skeletal preparations were done as previously described (Garcia et al., 2017). ImageJ was used to count and measure vertebrae using the multipoint and line tools, respectively. Measurements for the length and wedge analyses were individually recorded from lines drawn either through the center of each centra or along the dorsal/ventral sides. Values calculated for each analysis were either normalized to the standard length of the fish (length) or were calculated from ratios of dorsal/ventral length measurements (wedge) (Hayes et al., 2013). Images taken of Alizarin skeletal preparations were converted to black-and-white images and inverted in ImageJ software to better highlight defects.

Drug treatments
**Early DAPT treatment:** Transgenic embryos with entpd5a:pkRED expression were incubated at 28°C for exactly 7 hours. At this stage, embryos were treated with a concentration of 10mg/mL of pronase in egg water to remove chorions for subsequent DAPT treatment. Using an established protocol (Ozbudak and Lewis, 2008), embryos were treated with a 100µM concentration of DAPT or a DMSO control for three hours. DAPT and DMSO treatments were washed out using three rinses of egg water and embryos were left to develop at 28°C until they were transferred to the aquaculture system. Larvae were then imaged at 14 dpf using the AX10 Zeiss microscope as described above.

**Late DAPT treatment:** 7 dpf larvae expressing entpd5a:pkRED, were imaged using the AX10 Zoom V116 Zeiss microscope as described above. Following imaging, larvae were treated with either 100µM DAPT or a DMSO control for 24 hours. Fresh DAPT and DMSO solutions were replaced after 12 hours. Larvae were removed from treatment, imaged, and placed back on to the aquaculture system to recover for 48 hours. After 48 hours of recovery, larvae were imaged again. Images of the same larva before treatment and after treatment were compared for quantifying expression levels.

**In situ** hybridization and histological methods
Twelve µm sagittal cryo-sections of 4% paraformaldehyde-fixed 10 dpf larvae were generated for **in situ** hybridizations. Tissue sections were imaged using a Leica DM6000 compound microscope.

**In situ** hybridization probes for mespbb and her1 were described previously (Cutty et al., 2012; Gajewski et al., 2003). PCR amplification off vectors or linearized vectors were used to generate digoxigenin- 4 labeled cRNA probes. **In situ** hybridizations were performed with the aid of an InSituPro robot (Intavis) as described (Poss et al., 2002).

Resource Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Alexa Fluor® 568 Phalloidin | ThermoFisher | Cat#A12380 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester (DAPT) | Alfa Aesar | Cat#208255-80-5 |
| **Trypsin-EDTA** | ThermoFisher | Cat#25200056 |
|------------------|-------------|--------------|
| **Collagenase**  | Sigma-Aldrich | Cat#C2674 |
| **Calcein**      | Sigma-Aldrich | Cat#0875 |
| **Alizarin Red** | Sigma-Aldrich | Cat#A5533 |
| **Leibovitz’s L-15 media** | ThermoFisher | Cat#21083-027 |
| **Paraformaldehyde** | Fisher | Cat#AC4167802 |
| **Propidium Iodide** | ThermoFisher | Cat#P3566 |
| **DMSO** | Sigma-Aldrich | Cat#67-68-5 |

**Critical Commercial Assays**

RNeasy Plus Micro Kit Qiagen Cat#74034

**Deposited Data**

Raw and analyzed data This paper https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109176

**Experimental Models: Organisms/Strains**

**Zebrafish:** Tg(col9a2:GFPCaaX)pd115 (Garcia et al., 2017) N/A

**Zebrafish:** Tg(osx:mcherry-NTR)pd43 (Singh et al., 2012) N/A

**Zebrafish:** Tg(osx:mTagBFP-2A-CreER)pd45 (Singh et al., 2012) N/A

**Zebrafish:** Tg(her1:her1-Venus)bk15 (Delaune et al., 2012) N/A

**Zebrafish:** Tg(TP1:Venus-PEST)s940 (Ninov et al., 2012) N/A

**Zebrafish:** Tg(-2421/+29sox9b:EGFP)uw2 (Plavicki et al., 2014) N/A

**Zebrafish:** Tg(col9a2:QF2)pd1163 This work N/A

**Zebrafish:** Tg(QUAS:nlsVenus-V2a-notch1aICD)pd1164 This work N/A

**Zebrafish:** Tg(QUAS:nVenus-V2a-SuHDN)pd1165 This work N/A

**Zebrafish:** Tg(QUAS:mespbb-p2A-eGFP)pd1166 This work N/A

**Zebrafish:** Tg(id2a:GFPCaaX)pd1167 This work N/A

**Zebrafish:** TgBAC(entpd5a:pkRED) This work N/A

**Oligonucleotides**

**Primers:** pME-nVenus-V2a-SuHDN forward: 5’GGGGACAAGTTTGTACAAAAAAGCAGGC TC CATGGCTCCAAAGAAGAAG 3’ reverse: 5’GGGGACCACTTTGTACAAGAAAGC TGGTCTAGTTCTAGAGGCTCGAGA 3’

**Primers:** pME-mespbb-p2A-eGP forward: 5’GGGGACAAGTTTGTACAAAAAAGCAGGG TCCATGGACGCATCATCCTCTCCTTTCC 3’ reverse: This paper N/A

**Primers:** pME-mespbb-p2A-eGP forward: 5’GGGGACAAGTTTGTACAAAAAAGCAGGG TCCATGGACGCATCATCCTCTCCTTTCC 3’ reverse: This paper N/A
| 5’GGGGACCACTTTGTACAAGAAAGCTGGG TCTCCCCAGAAACTCTGGTGCGA 3’ | 5’AAGCTTCATCGTGCAAACGT 3’ reverse: 5’CCCACAGTGAGTTCAGAAAGC 3’ | This paper | N/A |
|---|---|---|---|
| **Primers:** | **p5E-id2a** forward: | **Recombinant DNA** | |
| | 5’ | (Subedi et al., 2014) | N/A |
| | | **pME-QF2** | (Subedi et al., 2014) |
| | | **pME-nlsVenus-V2a-notch1aICD** | (Eom et al., 2015) |
| | | **p3E-nVenus-V2a-SuHDN** | (Eom et al., 2015) |
| | | **pBS-SK-QS** | (Potter et al., 2010) |
| **Software and Algorithms** | **DAVID Bioinformatics Resources 6.8** | Huang da et al., 2009a, b) | [https://david.ncifcrf.gov/](https://david.ncifcrf.gov/) |
| | **DESeq2** | (Anders et al., 2015; Love et al., 2014) | [https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/deseq2/deseq2/2.11.39](https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/deseq2/deseq2/2.11.39) |
| | **htseq-count** | (Kim et al., 2015) | [https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/lpons/htseq_count/htseq_count/0.6.1galaxy3](https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/lpons/htseq_count/htseq_count/0.6.1galaxy3) |
| | **HISAT2** | (Kim et al., 2015) | [https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/hisat2/hisat2/2.0.5.2](https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/hisat2/hisat2/2.0.5.2) |
| | **Graphpad** | GraphPad Software, La Jolla California USA | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/); RRID: SCR_002798 |
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