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Role of *cdx4* and *sp5l* in zebrafish development

by

Wesley Tsai

Honors Thesis

in

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University of Richmond
Richmond, VA

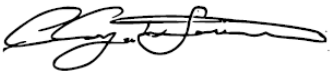
April 28th, 2023

Advisor: Dr. Isaac Skromne

This thesis has been accepted as part of the honors requirements
in the Program in Biochemistry and Molecular Biology.

_____ Wednesday, April 26, 2023

(advisor signature) *(date)*



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ABSTRACT

The Caudal Type Homeobox transcription factors *cdx* are a family of genes found in vertebrates that regulates body regionalization and anterior-posterior patterning. They are also responsible for regulating axial elongation, but the mechanisms behind this behavior are not known. Previous studies in mouse embryonic stem cells have shown that the *cdx* genes are necessary for upregulating the gene *sp5* which may be linked to axial elongation. Sp5 is a zinc-finger transcription factor belonging to the specificity protein (sp) family. Our group has used in-situ hybridization experiments on zebrafish embryos to show that *sp5-like* (*sp5l*) is transcribed within tailbud tissues that develop into paraxial mesoderm and ultimately give rise to the somites. We also found that *sp5l* expression in the tailbud and paraxial mesoderm is diminished when *cdx4* or the signaling factor Wnt is inhibited. Lastly, we found that inhibiting Wnt and upregulating *cdx4* can rescue *sp5l* expression. Additionally, using CRISPR gene editing we found that *sp5l* knockouts in zebrafish cause distortions in the somites and deformations in the tail and spinal column. Together, our results show that *sp5l* regulation by Wnt signaling is mediated by *cdx4*, and that this regulation is necessary for tailbud posteriorization and somitogenesis. These results shed new light on the mechanisms of axial elongation.

INTRODUCTION

The vertebrate body trunk and tail develop in part through the proliferation, specification, and segmentation of the paraxial mesoderm into somites that give rise to the vertebral column, associated skeletal muscles, and supporting tissues. This process requires the coordination of specification and axial pattering mechanisms that, while individually well understood, their coordination is unclear. Axial tissue identity is known to be controlled by the Hox family of transcription factors, while tissue patterning is known to be regulated by various signaling factors including Wnt, FGF, and retinoic acid (Deschamps and Nes, 2005). However, these signaling factors are also necessary for tissue specification, and it is unclear how they function simultaneously and in coordination in both processes.

The Cdx transcription factors are promising candidates to coordinate tissue specification and patterning processes. The *cdx* gene family code for homeobox-containing factors that are regulated by Wnt, FGF, and retinoic acid—the signaling factors that are necessary for both specification and patterning of the paraxial mesoderm. Previous studies have shown that *cdx* and its derivatives genes are expressed along the anterior-posterior axis in zebrafish, with high levels in the posterior side that taper off anteriorly. This places *cdx* expression in the correct time and place for regulating paraxial mesoderm specification and patterning. Deletions of the *cdx* genes in zebrafish embryos have been shown to lead to a shortening of the tail and spine (Skromne et al., 2007). Cdx is also upstream of the HOX gene family, which imparts trunk and tail tissues their axial identity. Prior studies have shown that with each successive knockout of the *cdx* genes, the HOX gene cascade timing was disrupted, which would lead to more severe vertebral defects (Neijts et al., 2016). As the *cdx* genes are conserved within all vertebrates, the study of *cdx* and its associated pathways could reveal insights into evolutionary mechanisms, shared

histories, and developmental processes. Additionally, as many diseases such as colorectal cancer (Bai et al., 2003) and adenocarcinoma (Kaimaktchiev et al., 2004) are associated with the *cdx* genes, examining its mechanisms could lead to valuable medical information.

This study aims to examine one of the candidate genes downstream of Cdx, the sp-like transcription factor *sp5l*. This gene was selected based on gene ontology screens of Cdx targets in mice and zebrafish embryos. Previous studies have suggested that *sp5l* signals FGF to regulate somitogenesis, and that *sp5l* is necessary for proper tailbud formation (Sun et al., 2006). Other studies have shown that *sp5l* is regulated by Wnt and regulates mesoderm and neuroectoderm patterning (Weidinger et al., 2005). As such, we aimed to examine and evaluate this potential connection between *cdx* and *sp5l* as an explanation of how *cdx* can regulate axial elongation. We also wanted to see how other signaling factors such as Wnt impact this relationship, as various papers have stated that the signaling factor Wnt upregulates both *cdx* and *sp5l*.

This present study reports that zebrafish *sp5l* is transcribed in posterior tissues during axial elongation and that *sp5l* inactivation results in trunk and tail abnormalities. Considering the possible relationships between *sp5l* and *cdx*, we show that *sp5l* is downstream of Cdx4 and that downregulating Cdx4 leads to a decrease in *sp5l* expression. Additionally, we show that Wnt regulates *sp5l* expression through Cdx4. Considering the studies on the activity of *sp5l*, we propose that *sp5l* is necessary for the formation of the paraxial mesoderm and somitogenesis, working as a Cdx4 effector gene.

MATERIALS AND METHODS

Selection of Target Gene

To find novel genes that were directly down regulated in *cdx* triple null mice, we compared RNA-seq and ChIP-seq databases from Amin et al., 2016. To categorize gene function we conducted a gene ontology analysis using the GOrilla program (http://cbl-gorilla.cs.technion.ac.il/). To determine the expression pattern of candidate genes in zebrafish we used the Zebrafish Information Network (ZFIN, http://zfin.org). The gene *sp5l* was chosen as it had prominent expression in the tail bud. The *sp5l* cDNA sequence was downloaded from the zebrafish genome browser Ensembl (https://useast.ensembl.org/Danio_rerio/Info/Index), which was viewed and annotated in the program Snapgene (Figure 1A). Lastly, the database Alphafold (https://alphafold.ebi.ac.uk/) was used to analyze the structure of Sp5l (Figure 1B).

PCR Primer Selection and gene cloning

Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) was used with the *sp5l* coding sequence to identify a pair of primers that would generate a sequence between 600-1000 base pairs long.

The two primers that were chosen were ATTCGAGCTTTCCCCGGTTA (left primer) and TGGGTCTTCACATGCTTGGA (right primer). At the 5'-end of the right primer, we added the Sp6 RNA polymerase recognition site (in italics) to generate the final right primer *GATATTTAGGTGACACTATAGAAGAG*TGGGTCTTCACATGCTTGGA. These primer sequences were validated by comparing them to the zebrafish genome via BLAST to ensure single-target recognition. Gene amplification was done by PCR using 2x Taq (NEB) following manufacturer instructions, at an annealing temperature of 60°C. Gel electrophoresis was conducted to verify the PCR product (Figure 1C).

Gene Expression Analysis

Multiple rounds of in-situ hybridization (standard procedure with synthesized antisense primer as previously described (Skromne et al., 2007)) were used to validate and analyze the expression patterns of *sp5l* in zebrafish embryos under various conditions. The following embryos and conditions were used in each round:

Round 1: Wildtype embryos at shield stage (6 hpf), tailbud stage (10 hpf), 10-somite stage (14 hpf), and 18-somite stage (18 hpf). Kgg (*cdx4* single mutant) embryos at shield stage and tailbud stage.

Round 2: Wildtype embryos and wildtype embryos injected with *cdx4* morpholino during the one-cell stage. In-situ hybridization was conducted at the shield stage, tailbud stage, 10-somite stage, and 18-somite stage.

Round 3: Wildtype embryos and Wnt-inhibited wildtype embryos at shield stage, tailbud stage, 10 somite stage, and 18 somite stage. Wnt inhibitor (IWR-1-endo, 4 μM) was added to the embryos at the 1 cell stage for the embryos grown to shield or tailbud stage and added to the embryos at the shield stage for the embryos grown to 10 or 18 somite stage.

Round 4: Wildtype embryos, Wnt-inhibited embryos, hsp70:*cdx4* heat-shocked embryos, and hsp70:*cdx4* heat-shocked embryos that were also Wnt inhibited. Embryos that were inhibited with Wnt inhibitor were inhibited at the shield stage. Embryos that were heat-shocked were placed in a 37°C hot water bath for 1 hour at 12 hpf and then cooled down for an additional hour. All embryos were processed with in-situ hybridization at the 10-somite stage (14 hpf). Hsp70:*cdx4* are transgenic fish that will over-express *cdx4* after heat-shocking.

CRISPR gRNA Selection

CRISPR gRNA was selected by using UCSC's genome viewer (http://genome.uscs.edu) and the Burgess lab zebrafish genomics gRNA database. The gRNA was selected by using the genome viewer's GA/GG target finder. The chosen 5' gRNA was on the first exon, and the 3' gRNA was on the second exon. The gRNAs were modified to have promoters on their 5' ends and invariant sequences on their 3' ends to be able to synthesize RNA. Both gRNAs were GG.

G1:

TAATACGACTCACTATAGGATAGCAAAGTAGTAGCGCGTTTTAGAGCTAGAAATAGC

G2:

TAATACGACTCACTATAGAAATTGTCCGCTCTGGATAGTTTTAGAGCTAGAAATAGC

Gel electrophoresis was conducted to verify the guides were successfully synthesized (Figure 1D).

CRISPR Gene Knockout Analysis

The standard CRISPR preparation and injection protocol for zebrafish was used (Varshney et al., 2015). Zebrafish embryos (n=214) were injected with the synthesized oligo in their 1-cell stage and then examined for changes at 24 hpf. This experiment was carried out twice, with 121 embryos getting injected in the first round and 93 embryos in the second round. During the second round, zebrafish with the transgene Actin-GFP were used instead of wildtype fish to better visualize the somites. PCR melting curve analysis was conducted to verify successful CRISPR gene editing (data not shown).

RESULTS

Gene Selection and analysis

Previous research has shown by chromatin immunoprecipitation and sequencing (ChIP-seq) that the mouse *cdx*2 gene binds to 3,970 gene targets, 74 of which were observed to be down regulated in *cdx* triple null mutant embryonic stem cells (Amin et al., 2016). Of these 74 genes, gene ontology analysis revealed that 25 had molecular functions in known biological processes (e.g., HOX genes, FGF signaling), leaving 49 novel genes to explore further. To determine which of the 49 genes could contribute to zebrafish embryonic posterior structure development, we analyzed published expression zebrafish databases (ZFIN) with the rationale that most genes that regulate trunk and tail formation would be present in the tail bud during development. As such, 11 genes that were prominently expressed in the tail buds of zebrafish embryos were chosen for consideration. For this research, the gene *sp5l* was chosen as it had expression in various tissues, most prominently in the tail bud (Thorpe et al., 2005).

The *sp5l* gene codes for a transcription factor and is located on the 23rd chromosome. The gene spans 2853 bp, has 2 exons, and its coding sequence has 1074 bp (Figure 1A). The gene codes for a protein comprised mostly of loose chains and alpha helices (Figure 1B) according to the Alphafold protein database (https://alphafold.ebi.ac.uk/). This protein has zinc finger domains that, according to the RCSB protein data bank (https://www.rcsb.org/), should allow it to interact with DNA to regulate gene transcription.

Sp5l expression in the tailbud is under *cdx4* control in zebrafish

To determine the timing and placement of *Sp5l* expression in the embryo we carried out in situ hybridization in wildtype embryos at different developmental stages. Our analysis repeatedly showed that *sp5l* is primarily expressed in the tailbud regions regardless of the growth

stage of the zebrafish embryo. During the shield stage, the embryos had *sp5l* expression around the margin and the organizer shield of the embryo (Figure 2). At the tailbud stage, the embryos had *sp5l* expression in the lateral plate mesoderm, the paraxial mesoderm, and the axial mesoderm (Figure 3). During the 10 somite and 18 somite stages, there were multiple stripes of *sp5l* expression above the tailbud, as well as expression in the tailbud. The expression of *sp5l* in the tailbud tissue places this gene in the right location to mediate *cdx* gene activity.

To determine if *sp5l* was under *cdx4* regulation, we repeated the in-situ hybridization experiments on *cdx4*-deficient embryos. *Cdx4*-deficient embryos were obtained either from *cdx4*$^{-/-}$ mutant fish or with *cdx4* antisense morpholino injections. Embryos that were deficient for *cdx4* showed a marked downregulation of *sp5l* in posterior tissues, including the tailbud. At the onset of gastrulation, there was a reduction in the expression of *sp5l* in the shield of the embryo, forming a gap of expression around the margin (Figure 2). During the tailbud stage, the expression in the paraxial mesoderm was missing, leaving only the expression in the lateral plate mesoderm and the axial mesoderm (Figure 3). At the 10-somite and 18-somite stages, the expression in the tailbud was greatly reduced, and the stripes of expression above the tailbud are completely absent (Figure 4). Together, these results suggest that *cdx4* is necessary for proper *sp5l* transcription.

Wnt regulation of Sp5l is under *Cdx4* control

Previous work has shown that *sp5l* is under Wnt signaling control (Thorpe et al., 2005). To test this, we exposed embryos to the Wnt inhibitor IWR-1-endo and then analyzed *sp5l* expression at the shield, tailbud, 10-somite, and 18-somite stages. Our in-situ hybridization analysis on Wnt-inhibited embryos showed a down regulation of *sp5l* transcription at shield and

tailbud stages, with faint expression in the tailbud at the 10-somite and 18-somite stages compared to the controls (Figure 5). This result confirms that *sp5l* is under Wnt control.

To test if Wnt regulation of *sp5l* is mediated by *cdx4*, a direct Wnt target gene (Ro and Dawid, 2011), we over-expressed *cdx4* from a transgene in the presence and absence of the Wnt inhibitor. Heat-shocking hsp70:*cdx4* transgenic embryos lead to over expression of *sp5l* in the tailbud region, with greater expression above the tailbud relative to non heat-shocked controls. Significantly, in the embryos that were both heat-shocked and Wnt inhibited, *sp5l* was also expressed in a similar manner as embryos not exposed to the Wnt inhibitor. These results suggest that Wnt regulation of *sp5l* transcription is mediated by *cdx4*.

Sp5l loss of function leads to tail defects

To determine the function of *sp5l* in development, we eliminated *sp5l* activity using a CRISPR gene editing approach. A subset of the CRISPR *sp5l* knockout zebrafish embryos displayed various deformities in the tail and somites at 24 hours after injection (n=68). Two phenotypes were observed: 1) Tail defects (n=41) or 2) Complete body deformation (n=27) (Figure 7). In fish that had tail defects, the fish had curved, distorted tails, and some of the somites near the tail were deformed. In fish that had complete body deformation, the fish were shriveled, misshapen, and had disrupted or nonexistent somite patterning. Taken together, these results indicate that *sp5l* has an important role in tail development.

DISCUSSION

Cdx4 mediates posterior mesoderm growth through sp5l

The expression patterns of *sp5l* in the posterior tissues suggest that *sp5l* is strongly involved in the formation of the paraxial mesoderm and segmentation. Expression analysis experiments show that *sp5l* is present in the gastrula margin and all mesodermal derivatives of the tailbud (Figure 3). Therefore, is not surprising that tailbud derivatives are affected in *sp5l*-deficient embryos (Figure 7), as these mesoderm derivatives give rise to notochord, somites, and blood. Significantly, during the 10-somite and 18-somite stages, *sp5l* is present in the tailbud as well as in several faint stripes above the tailbud (Figure 4). These stripes of expression are similar to other genes known to regulate somitogenesis, such as *mesp-a* (Sawada et al., 2000). As such, these stripes in the expression pattern of *sp5l* suggest that *sp5l* may be regulating somitogenesis. This genetic relation remains to be tested. Altogether, *sp5l* is likely to be involved with the growth of the posterior mesoderm, particularly of the somites.

Intriguingly, only specific regions of the *sp5l* expression domain seem to be dependent on the presence of *cdx4*. In *cdx4*-deficient embryos, *sp5l* expression was absent in the shield of the embryo during the shield stage, but the expression was still present around the margin (Figure 2). Likewise, during the tailbud stage, *sp5l* expression was absent in the paraxial mesoderm but still present within the lateral plate mesoderm and the axial mesoderm (Figure 3). This result in particular suggests that *cdx4* is regulating the formation of the paraxial mesoderm through *sp5l*, but other factors may be important for *sp5l* transcription in the axial and lateral plate mesoderm. Lastly, in the 10-somite and 18-somite stage embryos, inhibition of *cdx4* with morpholino leads to no stripe patterns of *sp5l* expression and nearly nonexistent expression in the tailbud (Figure 4). These results suggest that *cdx4* regulation of somitogenesis is in part dependent on *sp5l*

function. However, as *sp5l* expression is not completely lost in *cdx4* deficient embryos, other factors such as the other *cdx* genes may be regulating *sp5l* in these regions.

Wnt mediates sp5l through *cdx*

Our present study reveals novel data on the regulation of *sp5l*. According to literature, it is unclear if both Wnt and *cdx4* are directly regulating *sp5l* expression, or if Wnt is regulating *sp5l* through *cdx4*. By supplying an excess of *cdx4* and inhibiting Wnt, we aimed to elucidate this pathway. The in-situ hybridization experiments on Wnt-inhibited embryos, hsp70:*cdx4* heat-shocked embryos, and hsp70:*cdx4* embryos that were both Wnt-inhibited and heat-shocked (Figure 6) showed that inhibiting Wnt lowered *sp5l* expression, while over-expressing *cdx4* increased and expanded *sp5l* expression. More importantly, when Wnt was inhibited and *cdx4* was over-expressed, *sp5l* expression was still increased and expanded. This result shows that Wnt is not necessary for *sp5l* expression if *cdx4* is supplied. As such, it shows that Wnt is regulating *sp5l* levels indirectly through *cdx4*. This is supported by previous studies that stated that Wnt regulates *cdx* (Deschamps and Nes, 2005), and our previous results that show that *cdx* regulates *sp5l*.

Sp5l is necessary for proper somite formation

Functional analyses of sp5 in humans have shown that it is responsible for bone formation and post-anal tail morphogenesis. Our CRISPR-induced *sp5l*-deficient fish brings new data to the function of *sp5l* in zebrafish. *Sp5l* deficient zebrafish that had visible defects exhibited two different phenotypes: tail defects and complete deformation (Figure 7). Embryos with tail defects had short, bent tail buds with some disruptions in the somites, while embryos with complete deformation were completely misshapen and had distorted or absent somite structures. These results show that *sp5l* is necessary for tail development, tailbud

posteriorization, and proper somite formation. This data supports previous studies that state that *sp5l* regulates somitogenesis in mouse homologs (Sun et al., 2006).

Future Directions

This study opens up several questions related to the relationship between *cdx4* and *sp5l*, and their role in somite formation. One possible follow-up study could involve the relationship between *sp5l* and some of the other related signaling factors, such as retinoic acid and FGF, with further expression analysis experiments. This is because previous studies have suggested that *sp5l* is regulating FGF to control somite formation. Another potential inquiry could be examining if successive *cdx* knockouts would lead to greater reductions in *sp5l*, as only certain expression regions of *sp5l* were absent in *cdx4* deficient embryos. Lastly, future experiments could investigate if the stripe pattern expression of *sp5l* overlaps with other genes related to somitogenesis, such as *mesp-a*. These experiments would increase our understanding of the gene regulatory networks that lead to the development and growth of the vertebrate trunk and tail.

Acknowledgments and Attributions

I would like to show my gratitude to Dr. Isaac Skromne, who has been the most outstanding mentor ever since he welcomed me into their class and lab. He has been an unwavering source of inspiration and knowledge throughout my endeavors. I would also like to give thanks to the laboratory technicians Quan Chau and Zach Perkins, whose support and friendship have greatly aided this project. Lastly, I would like to thank the University of Richmond Department of Biology and the University of Richmond Summer Fellowship program for giving me the opportunity and the resources to accomplish this project.

Figures

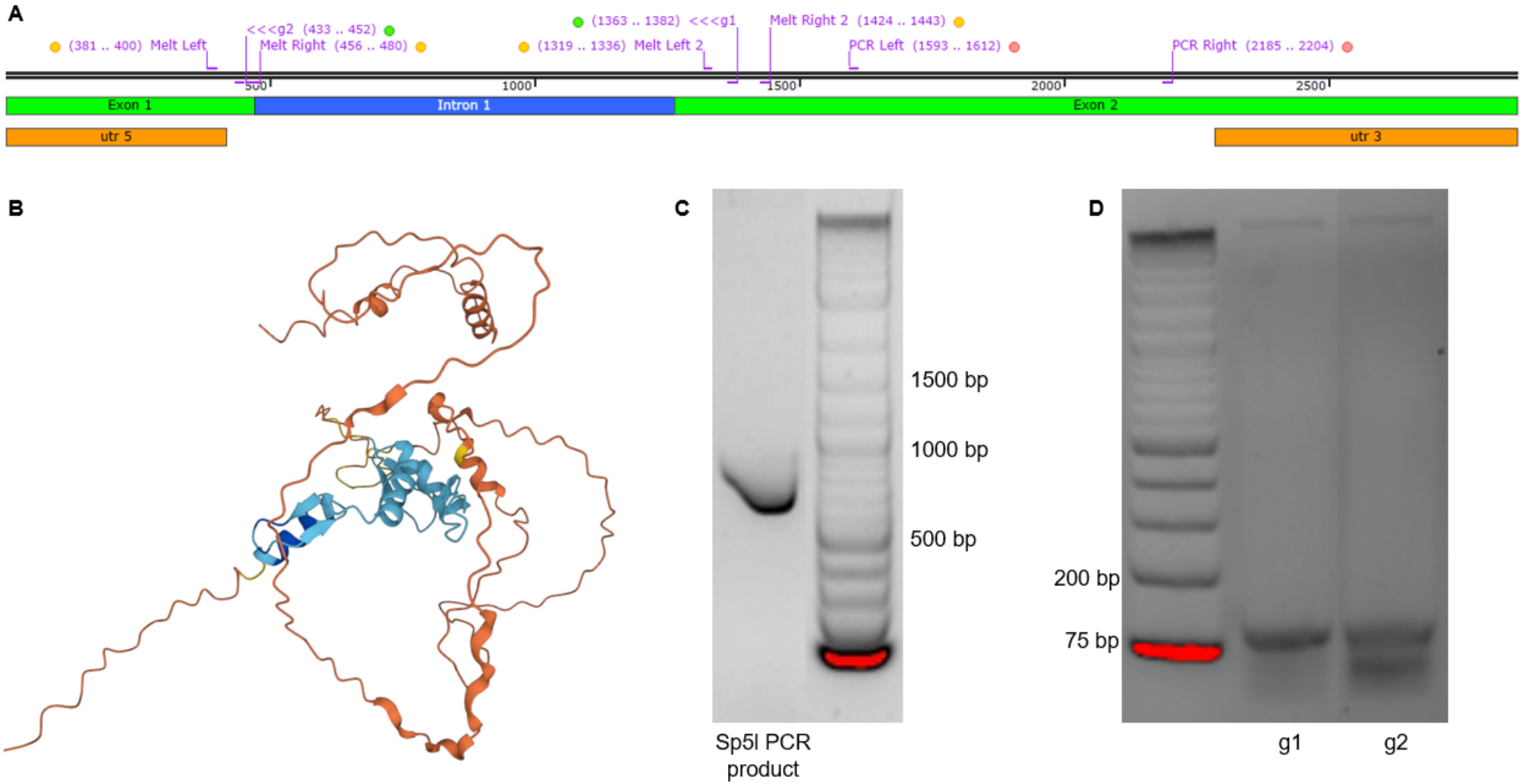

Figure 1: *Sp5l* gene structure in zebrafish. (**A**) Structure of *sp5l* gene transcript. PCR primer used for cloning (in panel C; dark orange circles), guide RNA synthesis (in panel D; green circles), and melting curve PCR analysis (yellow circles; not shown) are indicated on the top strand of the transcript. (**B**) Predicted protein structure of Sp5l from Alphafold. (**C**) Gel electrophoresis showing successful synthesis of *sp5l* from PCR primers. The PCR amplification product (left lane) shows the correct molecular weight of about 700 bp. (**D**) Gel electrophoresis showing the synthesis of guide RNAs g1 and g2 for CRISPR gene editing.

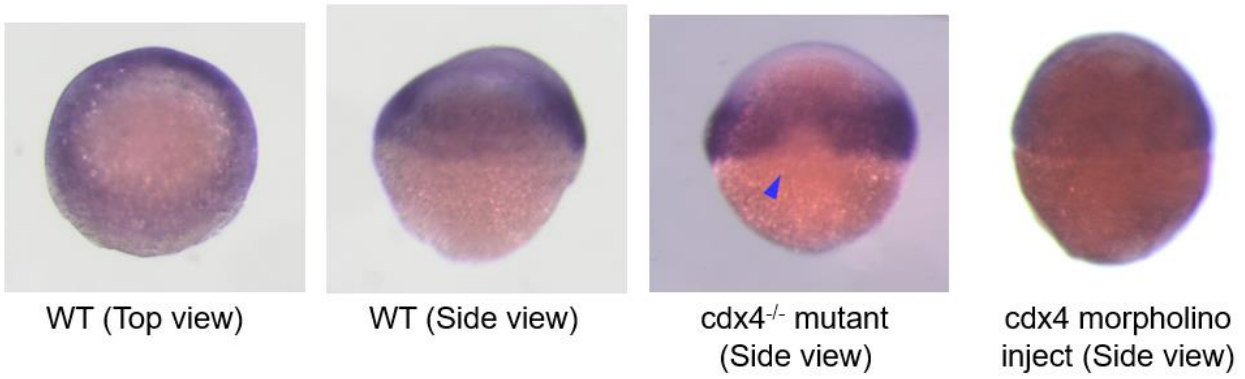

Figure 2. *Sp5l* expression at the onset of gastrulation. Expression in wildtype, *cdx4*$^{-/-}$ mutant, and *cdx4* morpholino injected embryos was analyzed during the shield (6 hpf) stage, as indicated. *Sp5l* expression is absent within the shield of the embryo (blue arrowhead) of the *cdx4*$^{-/-}$ mutants.

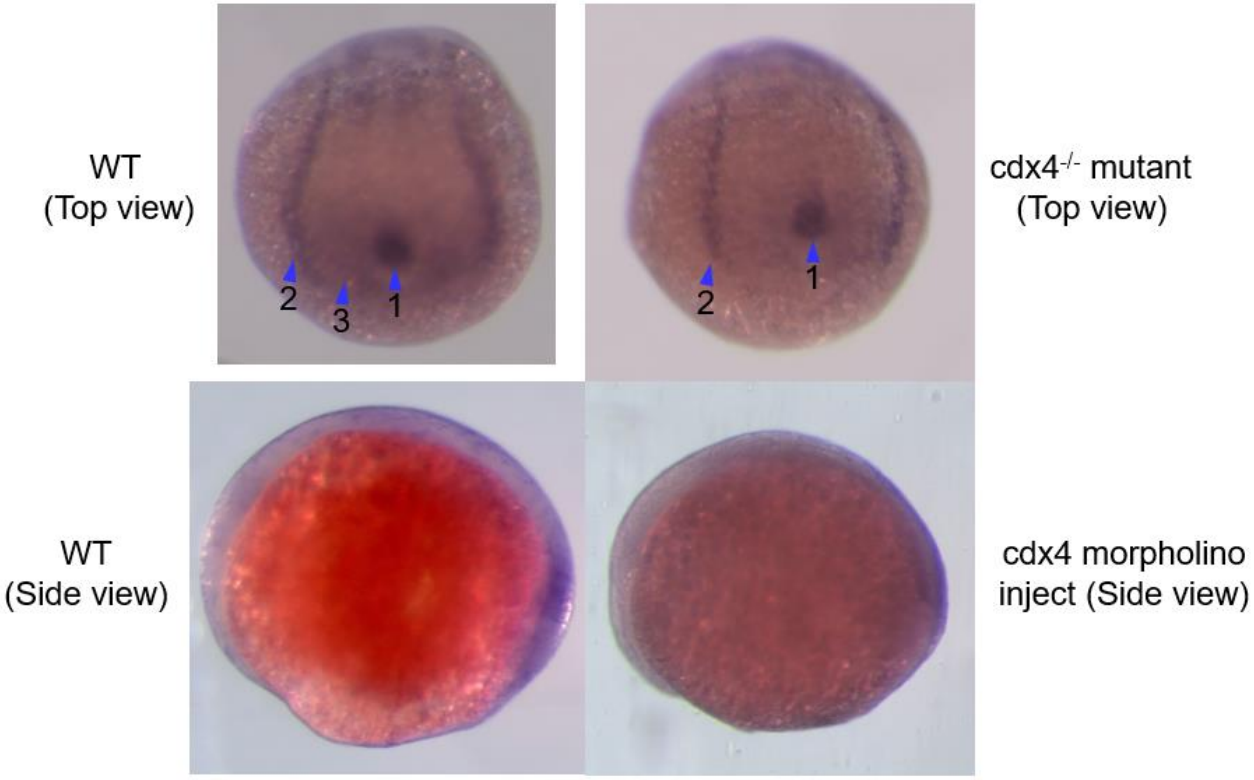

Figure 3. *Sp5l* expression at the end of gastrulation. Expression analysis in wildtype, *cdx4*$^{-/-}$ mutant, and *cdx4* morpholino injected embryos during the tailbud (10 hpf) stage. In wildtype embryos, *sp5l* expression is present within the axial mesoderm (1), the lateral plate mesoderm (2), and the paraxial mesoderm (3). In *cdx4*$^{-/-}$ mutants, only the expression within the axial mesoderm (1) and the lateral plate mesoderm (2) are present, the expression in the paraxial mesoderm is absent.

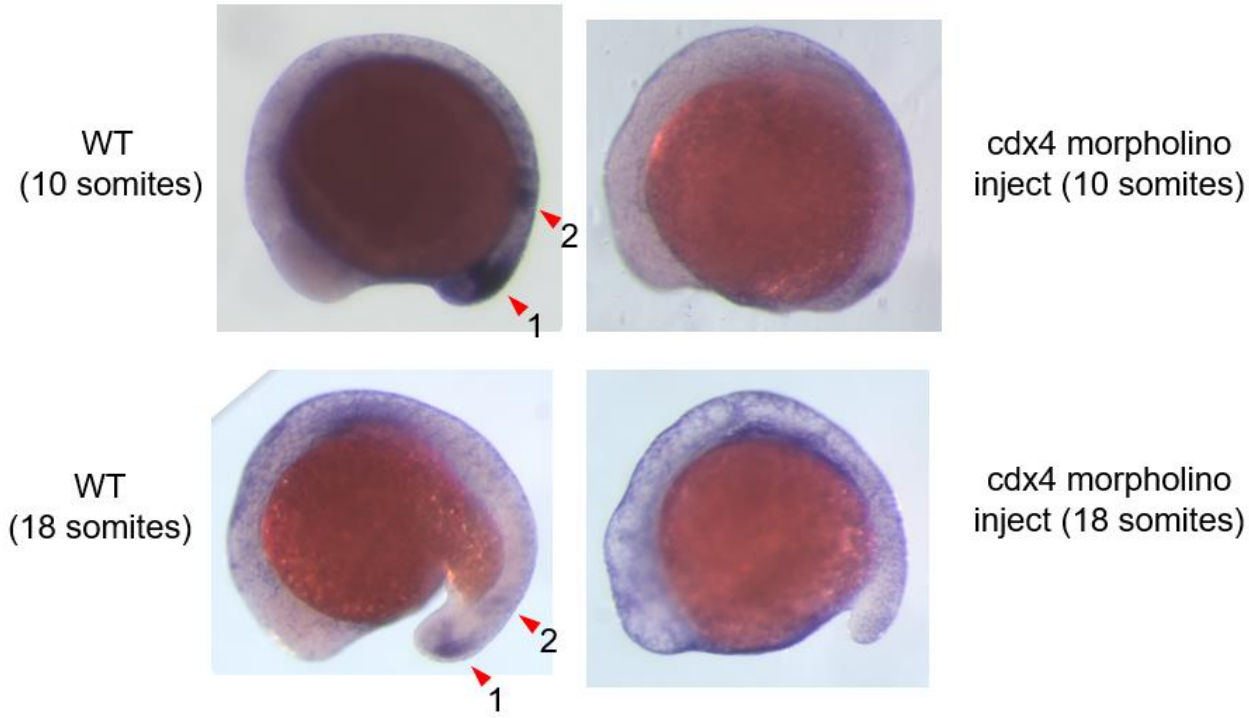

Figure 4. *Sp5l* expression during segmentation stages. Expression analysis in wildtype and *cdx4* morpholino injected embryos during the 10-somite (14 hpf) and the 18-somite (18 hpf) stage. In the wildtype embryos, there is expression in the tailbud (1) and expression within a stripe-like pattern above the tailbud (2) at both stages. The anterior side is on the left and the dorsal side is on the top.

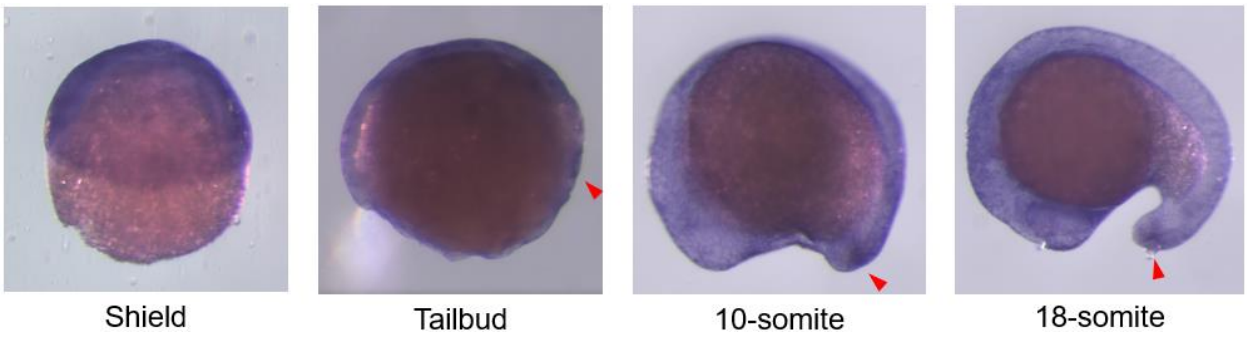

Figure 5. *Sp5l* expression in Wnt-inhibited embryos across various developmental stages. Compared to the *sp5l* expression in wild type embryos (Figs. 2-4), Wnt-inhibited embryos have a greatly reduced expression in the tailbud area (red arrowheads).

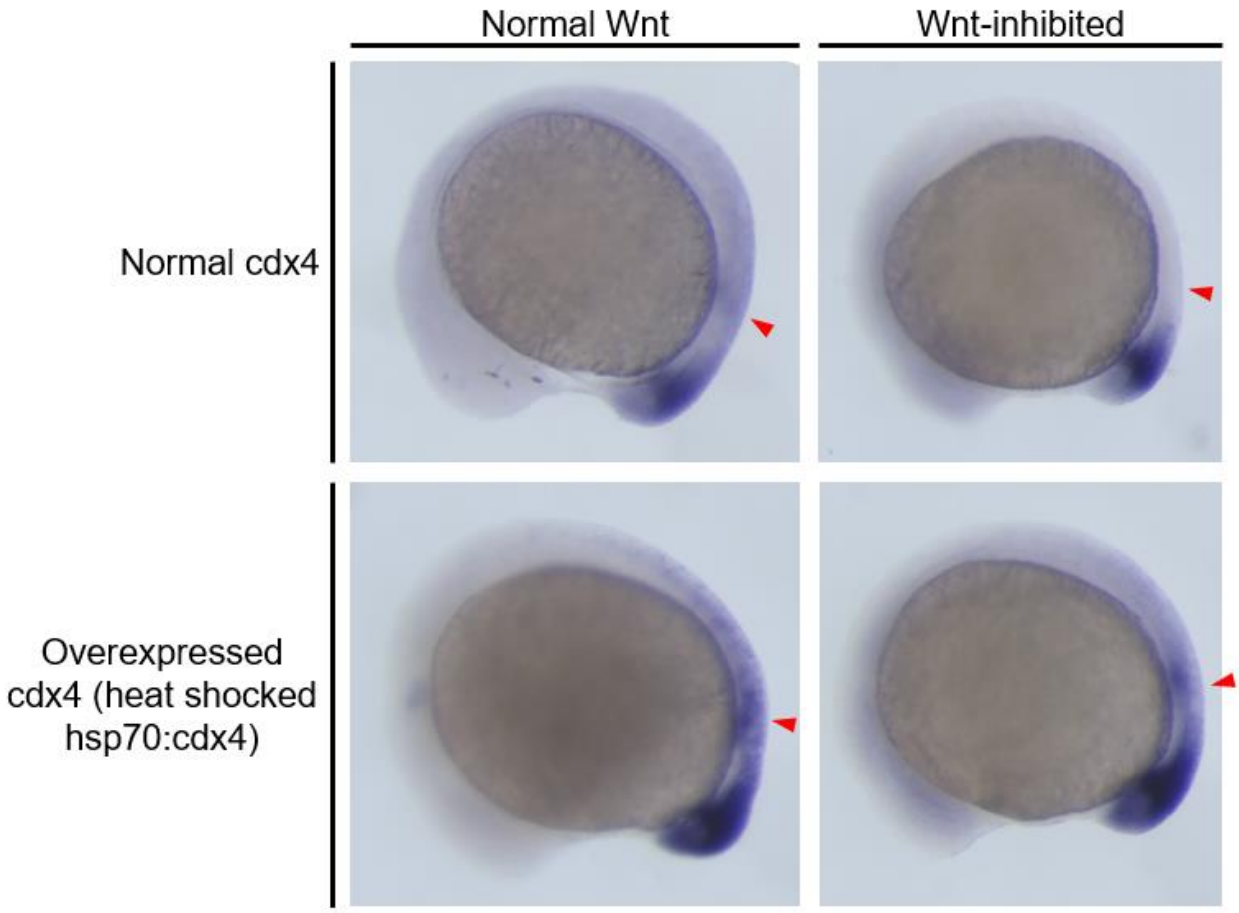

Figure 6. Cdx4 overexpression rescues sp5l defects associated with the loss of Wnt signaling. *Sp5l* expression in 10-somite stage embryos with Wnt inhibitor, *cdx4* overexpression, or both. *Sp5l* expression above the tailbud is reduced in Wnt-inhibited embryos and expanded in *cdx4* overexpressed embryos. *Sp5l* expression is still expanded in embryos that were both Wnt inhibited and *cdx4* overexpressed, indicating that *sp5l* expression is directly downstream of *cdx4* and not Wnt.

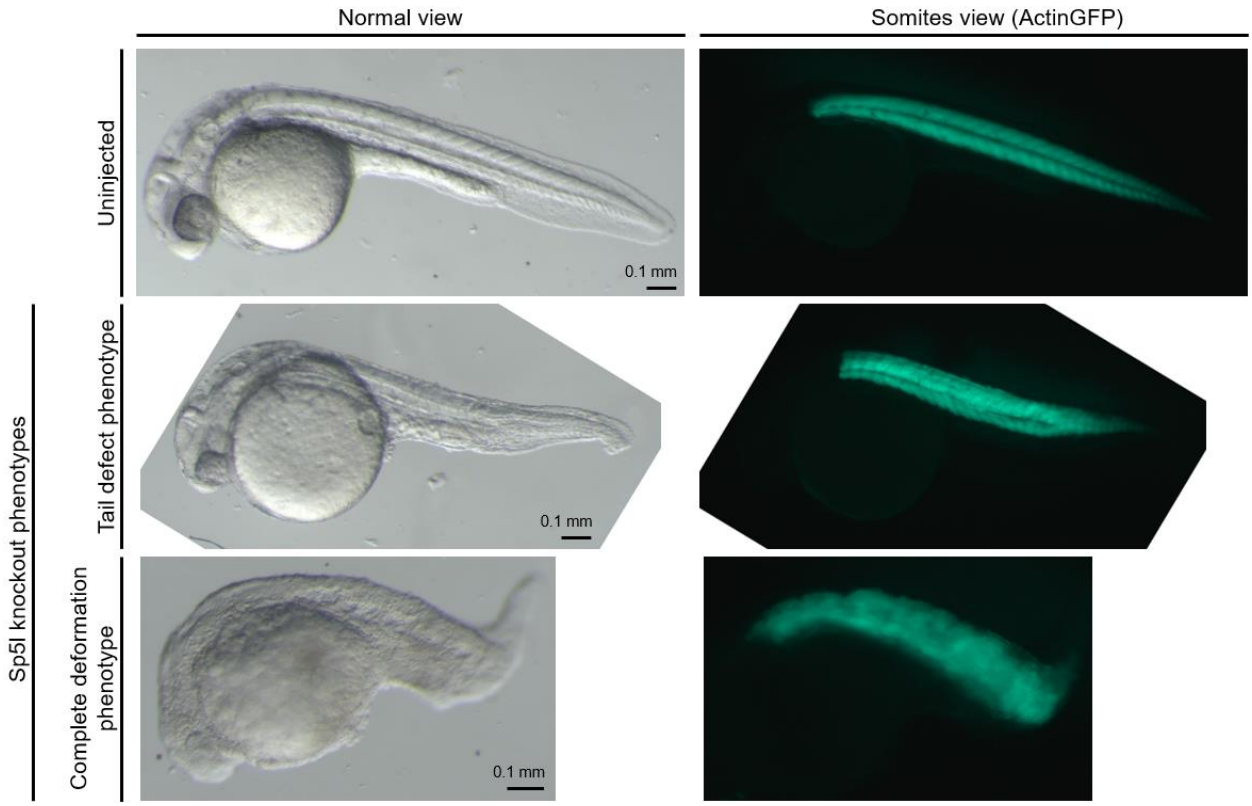

Figure 7. Loss of sp5l causes tail defects. Comparison of phenotypes between uninjected transgenic Actin-GFP embryos and Actin-GFP embryos that were injected with CRISPR *sp5l* knockouts at 24 hpf. Fluorescence photography is provided to observe somite structure. *Sp5l* knockouts were observed to cause two phenotypes: tail defects or complete deformation. Zebrafish with the tail defect phenotype had shortened, hooked tails and some somite disruption. Zebrafish with the complete deformation phenotype were completely misshapen, smaller, and had no discernable somite structure.

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