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Joshua Buksbaum April 1, 2019

Id Genes role in Chordamesoderm and Notochord Development in Zebrafish (*Danio rerio)*

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Abstract

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Notochord development is essential for chordate development and viability. The notochord develops from the chordamesoderm a population of proliferating stem cells in the tail bud region. We have previously shown that both BMP and Nodal signals are essential to maintaining the proliferative state of the tail bud, and ensuring proper notochord development. *Id* or inhibitor of differentiation genes are known regulators of proliferation and differentiaon. There are three *id* genes expressed in the zebrafish notochord and chordamesoderm. We hypothesized that these *Id* genes play an integral role in this process by integrating BMP and Nodal signals to facilitate the development of the notochord. We used antisense morpholine oligonucleotide to inhibit the expression of *Id2a* and *Id3* in developing zebrafish embryos. The results of these experiments were visualized with RNA insitu hybridizations. Our initial live photographs suggested that morpholino injections were responsible for producing a consistent phenotype of axial shortening and possibly disrupting the maintenance of the tail bud. However, in situ hybridizations did not show disturbed notochord development or tail bud proliferation, and thus didn't resemble our earlier work with BMP and Nodal signaling. Further work is necessary to determine the cause of the phenotypic appearance of the injected embryos, and why we did not see a disturbance of notochord and tail bud signaling similar to our work with BMP and Nodal signaling.

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I would like to thank Dr. Andreas Fritz for his continued and essential mentorship and advising throughout this entire undertaking. It has been a privilege to perform research in his laboratory and I'm eternally grateful for this experience. Additionally, I would like to thank Dr. Iain Shepherd and Dr. Kenneth Moberg for their generosity with their time in committing to serve on my Honor's Committee, in addition to their valuable comments and contributions to my research. I would also like to acknowledge the other students who assisted in training me, Ana Dobrita (C '18), and in our shared responsibilities of caring for the zebrafish, Rushay Amarath-Madav (Ox '17 C'19) and Ria Brown (C '19). Lastly, I would like to thank Emory University and the Emory Department of Biology for the excellent education and experiences afforded to me inside and out of the classroom.

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Background and Introduction

The notochord is the first organ to fully differentiate during vertebrate embryogenesis and is a defining feature of phylum chordata (Stemple 2005). The posterior notochord develops through the processes of axis elongation through the tail bud of vertebrate embryo (Glickman 2003). The chordamesoderm is a subset of the mesoderm and is the precursor cell population that will form the notochord. These progenitor cells are maintained in the tail bud region of the embryo, at the posterior end of the chordamesoderm and developing notochord. The maintenance of these MPC's is critical to populating the developing notochord with differentiated cells and for the continuation of axis elongation (Row 2016).

In previous work, we have shown that notochord development is regulated by BMP and Nodal pathways originating from opposite ends of the embryo (Esterberg et al. 2008, unpublished results from Esterberg and Fritz, Figures 2, 3, 4). At the bud stage and the beginning of gastrulation, the shield and ventral gastrula margin act as dorsal and ventral signaling centers with respectively high Nodal and BMP signals. The shield and ventral gastrula margins and their respective Nodal and BMP signals converge at the end of gastrulation and populate the tail bud region with precursor cells (Agathon et al. 2003, Figure 1). We have shown that in the chordamesoderm the proliferation and differentiation of mesodermal progenitor cells (MPC's) into the notochord is balanced through both BMP and Nodal signals. The Nodal and BMP pathways act on a shared regulatory region of the *id,* or inhibitors of differentiation, genes as evidenced by the presence of regulatory elements for both BMP and Nodal signals. The i*d* genes act as regulators of differentiation and bind DNA in vertebrates through their protein's conserved basic-helix-loop-helix (bHLH) structure (Chong et al. 2005). The *id* proteins are known to inhibit cell differentiation and keep differentiation factors inactive, as seen for example in developing

muscle cells as they act as negative regulators of myogenesis (Yokoyama 2011). *Id* proteins also play a role in neurogenesis as their down-regulation corresponds to ectomesenchyme specification from cranio-neural crest cell precursors (Das 2011).

The zebrafish genome contains five *id* genes. *Id* expression has been studied in the context of adult neurogenesis in the zebrafish brain (Diotel et. al 2015). *Id2a* and *id3* function has been analyzed using morpholino knock-down. Uribe et al. focused on retinal development in zebrafish. They reported that *id2a* inhibition caused retinal precursors to fail to differentiate and suggested a connection between *id2a* and notch signaling. Li et al. investigated the role of *id3* in the developing zebrafish diencephalon, and proposed that it modulated development of dopaminergic neurons. They used the *id3* morpholino to assess brain development, and characterized the resulting morphant phenotype as having reduced brain and head size. Of the five zebrafish *id* genes, two of them, *id1* and *id3*, are expressed within tail precursors during gastrulation. Following gastrulation, *id1*, *id2a*, and *id3* are expressed in the axial and presomitic mesoderm in addition to the tail bud, where their expression persists until approximately 24 hours post fertilization (Esterberg and Fritz, unpublished). *Id1, id2a,* and *id3* are also all expressed in the developing notochord (Figure 5). Our research focuses on a subset of these three *id* genes, specifically *id2a* and *id3*. Based on our previous work, we have proposed a mechanism for the control of *id* gene expression, namely that it is activated directly by BMP signals and indirectly Nodal signaling acting through its effector Cited3ab, and analyzed the resulting phenotypes from manipulation of these BMP and Nodal signals (Figures 2 and 3: Esterberg et al. 2008 and unpublished results). However, we have yet to study the phenotypes that result from the inhibition of *id* gene expression itself. The results of our experiments will further our understanding of how *id* genes facilitate chordamesoderm and notochord development in

addition to the role of bHLH genes in the development of the notochord. Based on our model the known role of *id* genes, we expect that *id* genes are involved in balancing the proliferation and differentiation of the chordamesoderm. Specifically, we hypothesized that the *id* genes play a significant role in promoting the proliferation of the chordamesoderm while inhibiting the premature differentiation of the notochord. We expected that our experimental manipulation of *id* gene expression to result in early differentiation of the chordamesoderm into the notochord, leading to a truncated overall notochord and premature depletion of the mesodermal progenitor cells in the tail bud region.

We have an antisense splice-blocking morphopline oligonucleotide (Morpholino/MO) for *id2a* and a translation-blocking MO for *id3* to study the roles of the *id2a* and *id3* genes in the zebrafish chordamesoderm and developing notochord. Our experimental set up relies on *id2a* and *id3* morpholino injections to block expression of *id2a* and *id3* in the embryo, and observation of the resulting knockdown phenotypes. Our experiments have demonstrated that *id2a* and *id3* morphants yield promising results in affecting the development of the notochord as evidenced by altered morphology of the notochord. We have performed RNA in situ hybridizations to help illustrate the developmental effects behind these altered morphologies. As seen in Figure 3 and unpublished images from our lab in Figure 4, in situ hybridizations using probes for specific genes known to be expressed in the notochord such as *sonic hedgehog* (*shh*) and the zebrafish *brachyury* orthologue, *notail* (*ntl*) allow us to visualize the effects of *id2a* and *id3* MO injections on the developing notochord, analogous to our analysis of the effects of manipulating BMP and Nodal signaling.

Methods

Morpholino injection:

Embryos were collected immediately after fertilization and injected at 1-4 cell stages using standard morpholino injection procedures (Rosen 2009). The 25 nucleotide sequence of the splice-blocking *id2a* morpholino is as follows: 5'GCTTCATGTTGACAGCAG-GATTTC 3'. The 25 nucleotide sequence of the translation-blocking *id3* morpholino is as follows: 5' CTGA-TCGCCTTCATTCTGGGTCGG 3'. Approximately 8 ng of morpholino was injected into each embryo. Morpholinos were diluted from 300 nmol/1mL stock solutions. The injection solution for the *id2a* morpholino was obtained by performing a 1:33.3 dilution from the stock solution resulting in a final concentration of 30 uM. The injection solution for the *id3* morpholino was obtained by performing a 1:15 dilution from the stock solution resulting in a final concentration of 50 uM. Additionally, we co-injected our *id2a* and *id3* morpholinos using a 1:1 dilution of the 30 uM *id2a* morpholino and the 50 uM *id3* morpholino.

In situ hybridization:

The *ntl* sequence was linearized with HindIII restriction enzyme and the probe was synthesized using T7 RNA polymerase and digoxigenin was added as a label. The *shh* sequence was linearized with XhoI restriction enzyme and the probe was synthesized using T7 RNA polymerase and a digoxigenin.

Results

Morpholino Injections, Live Photographs

We spent a great deal of time attempting to assess the proper dosage of the *id2a* and *id3* morpholinos. We tested many doses and obtained a wide spectrum of phenotypes, and were eventually able to ascertain certain concentrations that yielded reasonably consistent phenotypes when assessed under a light microscope. We determined that concentrations of 30 uM for the *id2a* morpholino and 50 uM for the *id3* morpholino produced a consistently affected morphant phenotype. 20 somite (20S), *id2a, id3,* and our co-injected, 1:1 dilution injected embryos exhibited disrupted axis elongation suggesting a possible disruption of the tail bud progenitor cells and the chordamesoderm, and possible early differentiation of the notochord (Figure 6). At approximately 36 hours post fertilization, injected embryos continued to display consistent disruption of axis elongation as evidenced by the moderate to more dramatic bending of their tails, again potentially suggesting a disturbance of the balance of proliferation and differentiation in the chordamesoderm and notochord (Figure 7).

Morpholino Injections, In Situ Hybridization

We also spent a great deal of time attempting to perform in situ hybridizations to assess our claims about the causes of these visual results. We have recently been able to synthesize new *ntl* and *shh* probes, and have confirmed their effectiveness in control in situ hybridizations. Using these new probes, the results of these in situ hybridizations did not conclusively suggest that *id* gene inhibition is sufficient to disrupt the development of the notochord and to disturb the proliferative state of the tail bud (Figure 8, 9).

Our experiments have demonstrated that *id2a* and *id3* displayed consistently affected morphants phenotypes as characterized by disrupted axis elongation (Figures 6, 7). However, our in situ hybridizations did not resemble those of our prior work with BMP and Nodal signaling, and were not able to illustrate disrupted development of the notochord and maintenance of the proliferation of the tail bud as observed with disruption of BMP and Nodal signaling (Figures 3, 4, 8, 9).

Discussion

As suggested by our previous research, we believe that the three *id* genes expressed in the developing notochord mediate the balance between proliferation and differentiation. As shown, the *id* genes are regulated by BMP and Nodal signaling; loss of BMP or Nodal signaling leads to loss of *id* expression and a premature loss of precursor cells (MPCs), while increase of BMP signal leads to an excess of proliferation and notochord formation (Figures 3, 4). In these previous experiments, in situ hybridizations with *shh* and *ntl* probes were used to visualize the notochord and to assess how much notochord tissue was present, while also seeing how many or how dense the precursor cells were in the tail bud region (Figures 3, 4). These experiments showed that knocking down BMP or Nodal signaling led to a change in the amount of precursor cells present in the tail bud.

Our goal was to ascertain whether or not knocking down *id* gene signaling would have a similar effect to inhibiting BMP and Nodal signaling as seen in Figures 3 and 4. This would confirm the model we previously proposed where *id* genes integrate the transcriptional signals of BMP and Nodal signals and act as their effectors (Figure 2). Photographs of injected embryos, especially co-injection of *id2a* and *id3*, displayed a consistent phenotype characterized by a shortened axis. However, in situ hybridizations (Figures 8 and 9) did not clearly resemble the results from our prior research with BMP and Nodal signals (Figure 4). Our co -injected *id2a* and *id3* embryos have slight resemblance to the phenotype of the prior *cited3a/b* morpholino experiments (Figure 4, 8, 9). Overall, our injections of *id2a, id3,* and co-injection were insufficient to produce conclusive results and consistent phenotypes resembling our prior experiments with BMP and Nodal signaling. However, there are reasons to assume that our results so far may be inconclusive. We have only performed one set of in situ hybridizations on

one group of morphant embryos, and these experiments will need to be repeated now that we have working probes for *shh* and *ntl.* There are also more control experiments that will need to be performed, especially to address the efficacy of the *id2a* and *id3* morpholinos. In addition to performing control morpholino injections, we could assess the effectiveness of our splice-blocking *id2a* with RT-PCR, and of the translation-blocking *id3* morpholino with antibody staining if an antibody is available. We could also compare our morpholino injected embryos to a true knockout transgenic line if one was available. Lastly, we could attempt to use injection of *id* gene mRNA to rescue the development of our morpholino injected embryos, as detailed by Li et al. and their use of *id3* mRNA to rescue the phenotype of *id3* morpholino injection.

There are many additional areas for investigation into the role of *id* genes in notochord development. We have not had access to an *id1* morpholino, and have only been knocking down *id2a* and *id3*. As discussed previously, *id1* is also expressed in the developing notochord and it is thus possible that our inability to inhibit *id1* expression in the developing notochord and tail bud led to our results not matching those of our previous experiments with BMP and Nodal signaling. It is possible that cross talk between the three *id* genes in the notochord resulted in compensatory up-regulation of *id1*expression in the notochord when *id2a* and *id3* are knocked down, which would have prevented us from observing the phenotype of inhibition of the *id* signaling in the notochord. This could be investigated by in situ hybridization with an *id1* probe to see if *id1* signaling is increased in our injected *id2a, id3*, or co-injected embryos compared to a control embryo. Lastly, we can assess the *id* genes in the context of our proposed model, and see if there are enhanced or synergistic phenotypes when both *id* signaling and BMP or Nodal signaling are inhibited. As shown, inhibition of BMP signaling is possible through the use of dominant negative heat shock receptors and morpholinos, while Nodal signaling inhibition is possible

either through drug treatments or the use of a morpholino for its effector Cited3ab (Figures 3, 4). We would expect that inhibiting BMP or Nodal signaling should act synergistically with *id* gene knockdown. Conversely, we would expect that up regulation of BMP, for example by using the *fstl1/2* morpholinos, to rescue or ameliorate the phenotype caused by *id* gene inhibition. Such additional experiments should clarify the role of *id* genes in notochord development.

Figures

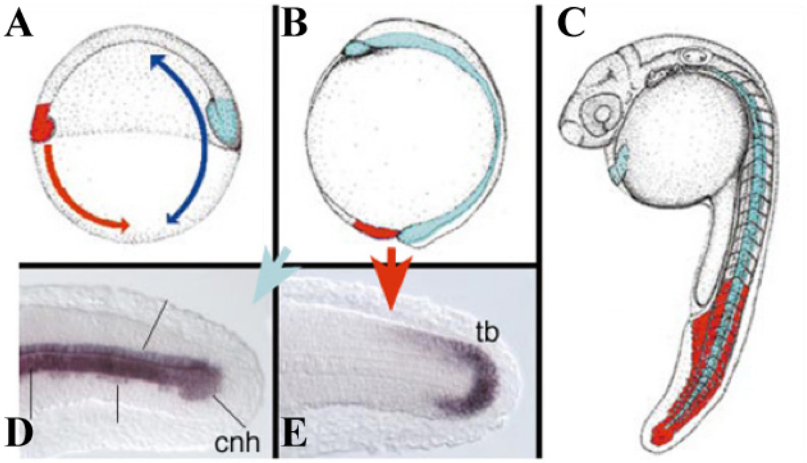
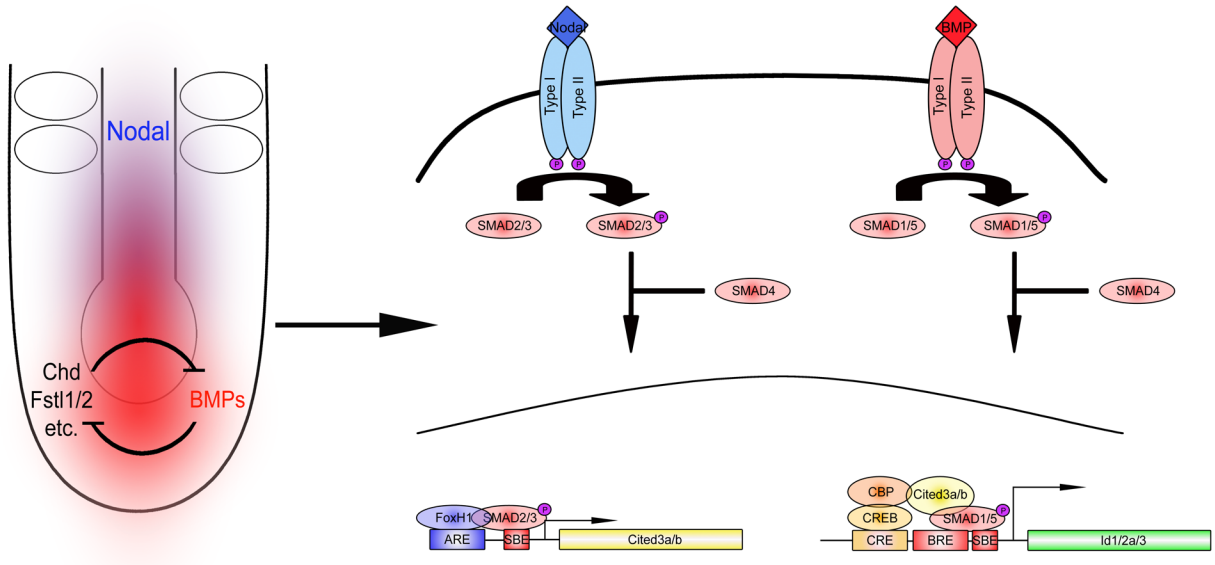


Figure 1: A, B, C: During gastrulation, descendants of the dorsal signaling center (blue) and the ventral signaling center (red) migrate into proximity to each other and ultimately give rise to the axial and presomitic mesoderm. The tail bud precursor pool supplies the outgrowth of these tissues. D, E: Axial precursors contribute to the axial mesoderm and neural ectoderm (lower left panel, CNH labeled with *shh*), but do not contribute to paraxial mesoderm (lower right panel, tail bud labeled with *eve*), indicating the presence of two distinct populations of precursor cells within the tail bud, each with limited and defined contributory capabilities. Adapted from Agathon et al. 2003.



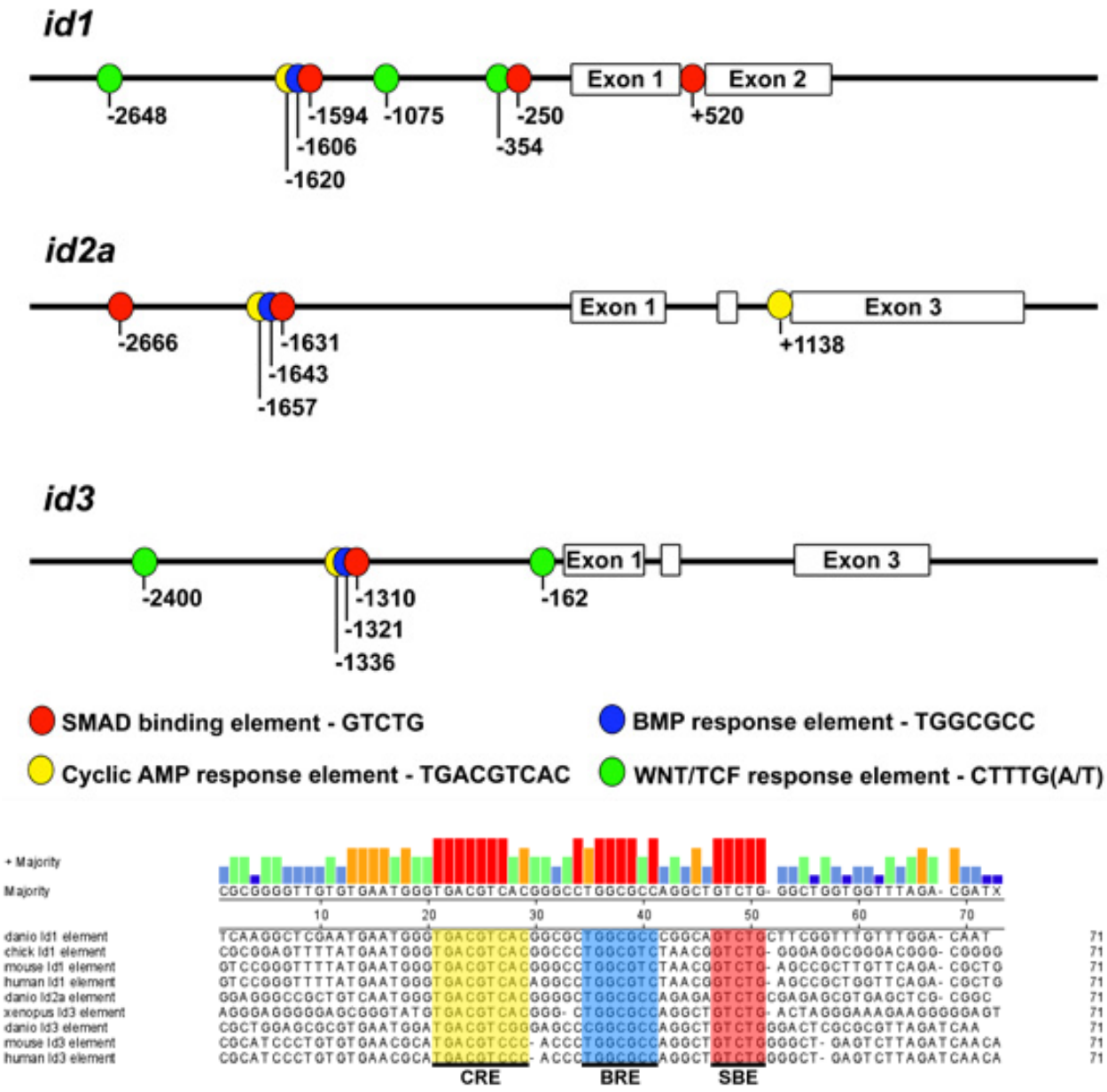


Figure 2: (A) Proposed model of BMP and Nodal signaling and their interactions with the *id* genes. As a result of gastrulation and migration of the BMP rich ventral signaling center, axial precursors must integrate BMP signaling in addition to existing nodal signals. One strategy these cells undertake to modulate these signals is the regulation of *id1, id2a,* and *id3*. Nodal signaling directly activates the expression of p300/CBP interacting proteins Cited3a/b, which complex with BMP activated SMADs and p300 to regulate transcription of *id1*, *id2a*, and *id3* within axial derivatives. Loss of BMP, Nodal, or Cited3a/b signaling causes axial precursors to lose their proliferative abilities and leave the precursor pool. (B, C) Conserved BMP and Nodal response elements present in *id1, id2a*, and *id3*

promoters, allowing each of these three *id* genes to respond to BMP and Nodal signals. Adapted from Esterberg and Fritz, unpublished.

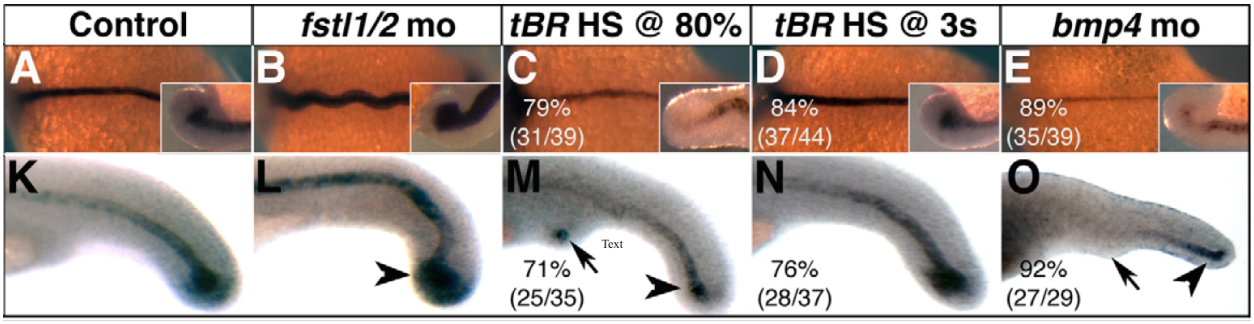


Figure 3: A-E: dorsal view of *shh*a expression in 14S embryos, with lateral view of chordo-neural hinge inset. K-O: lateral view of *ntl* signaling in 24 hpf embryos, with arrowheads indicating the tail bud and complete arrows in M and O indicating ectopic tail formation. *Fstl1* and *Fstl2* are known inhibitors of BMP signaling, the *fstl1/2* column is exhibiting upregulation of BMP signaling and thus shows an increase in tail bud and notochord signaling. *tBR* HS is a dominant negative BMP receptor under heat shock control. When heat shock is activated at 80% epiboly and BMP signaling is inhibited, there is significant reduction in notochord and tail bud signaling. Heat shock at the later 3S stage does not produce similar results, and instead more closely resembles the control figures. Lastly, the *bmp4* morpholino figures dramatic reduction of notochord and tail bud signaling as well. From Esterberg and Fritz 2008.

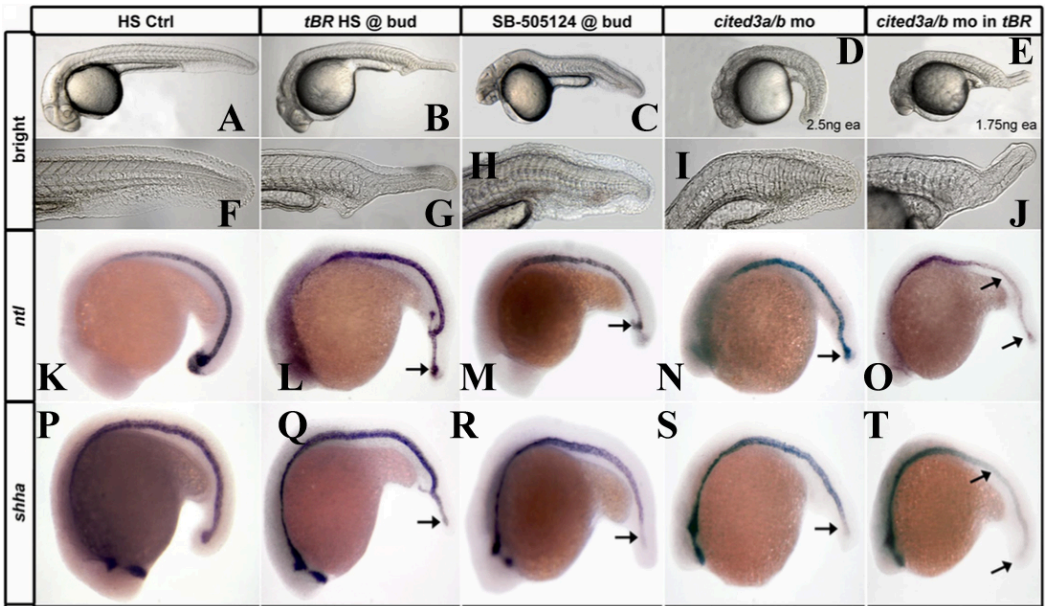


Figure 4: *Cited3a/b* interacts with the BMP signaling pathway to balance proliferation and fate assignment within axial precursors. B, G, L, Q: Inhibition of BMP signaling at the end of gastrulation with a dominant negative BMP receptor under heat shock control causes patterning defects (B, G) in addition to reduction of the CNH as evidenced through *ntl* (L) and *shh* (Q) expression. C, D, H, I, M, N, R, S: The tail and CNH phenotypes of embryos with inhibited Nodal signaling through treatment with SB-505124 (C, H, M, R) and *cited3a/b* morpholino resemble each other. E, J, O, T: A more severe tail phenotype is seen in *tBR* embryos injected with sub-optimal doses of *cited3a/b* morpholinos (E, J). The CNH is molecularly undetectable as evidenced by *ntl* (O) and *shh* (T) expression, which is also sharply reduced in the posterior notochord. A-J: 30 hpf embryos. K-T: 18S embryos. All views are lateral with anterior to the left. Adapted from Esterberg and Fritz unpublished.

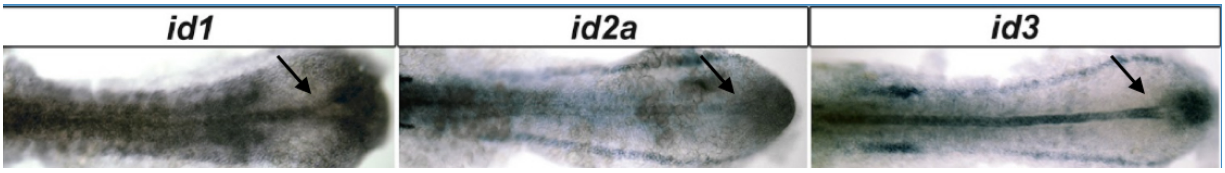


Figure 5: In situ hybridizations for *id1, id2a*, and *id3*, detailing their expression pattern in the notochord and tail bud, in addition to other axial structures such as the somites. Arrows indicating notochord and chordamesoderm. Views from flat mounts of 9 somite embryos with anterior to the left. Adapted from Esterberg and Fritz unpublished research.

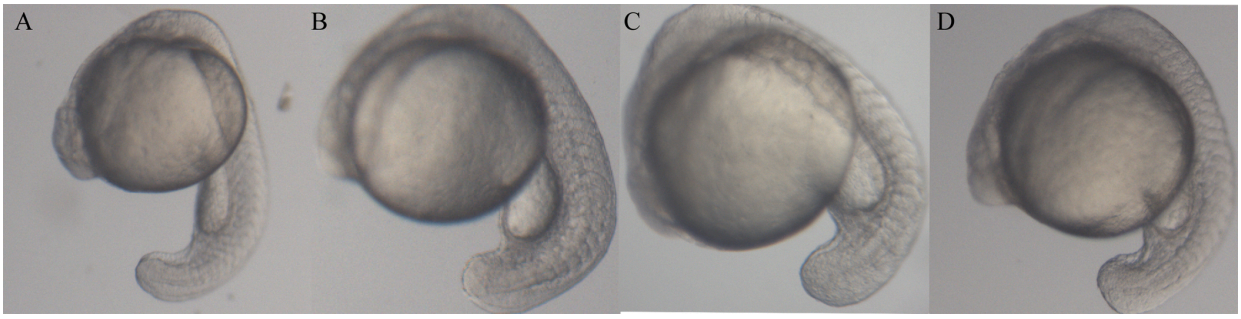


Figure 6: A: 20 S uninjected control. B: 20S *id2a* 30 uM morpholino injected embryo. C: 20S *id3* 50 uM morpholino injected embryo. D: 20 S 1:1 dilution of *id2a* 30 uM and *id3* 50 uM morpholinos injected embryo. B-D: phenotypic disturbance of the tail bud in addition to axial shortening. All views are lateral with anterior to the left.

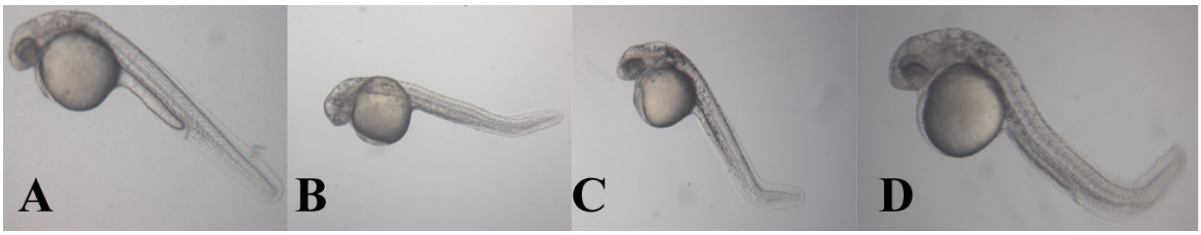


Figure 7: A: 36 hpf uninjected control. B: 36 hpf *id2a* 30 uM morpholino injected embryo. C: 36 hpf *id3* 50 uM morpholino injected embryo. D: 36 hpf 1:1 dilution of *id2a* 30 uM nd *id3* 50 uM morpholinos injected embryo. B-D: Disturbed axis elongation as evidenced by bending at CNH.

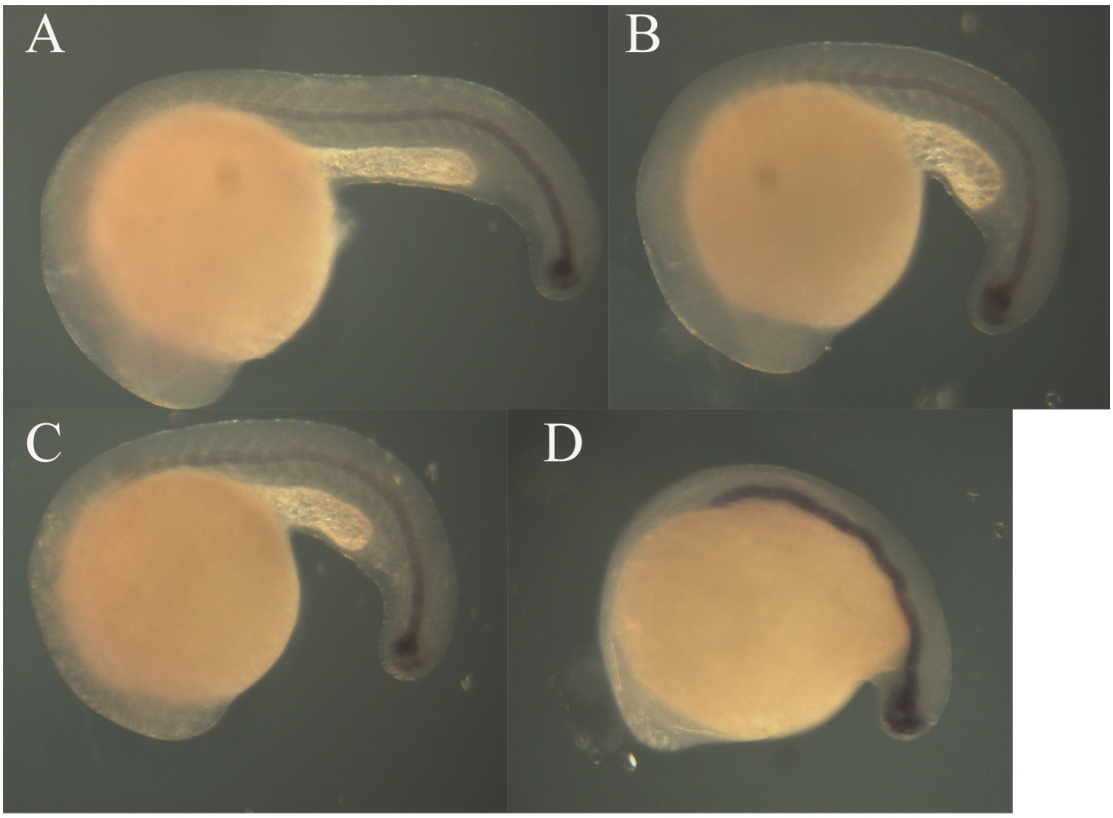


Figure 8: A: 20S uninjected control embryo stained with *ntl* probe. 30 control embryos were stained, all exhibited similar and consistent morphology to the selected embryo. B: *id2a* 30 uM injected 20S embryo, stained with *ntl*. 25 embryos were stained, all exhibited similar and consistent morphology to the selected embryo. C: *id3* 50 uM injected 20S embryo, stained with *ntl*. 28 embryos were stained, all all exhibited similar and consistent morphology to the selected embryo. D: 1:1 dilution of *id2a* 30 uM and *id3* 50 uM injected 20S embryo stained with *ntl*. 26 embryos were stained, approximately %40 percent of them exhibited similar morphology to the one selected. B, C:

Compared to control, axis elongation is disrupted as evidenced by *ntl* staining of shorter notochords. No significant difference in *ntl* staining intensity in posterior notochord or tail bud. D: In addition to dramatic inhibition of axis elongation as compared to controls and *id2a* and *id3* injected embryos, there is darker and more intense *ntl* staining in posterior notochord. Additionally, it appears that there is an increase in intensity in *ntl* staining tail bud.

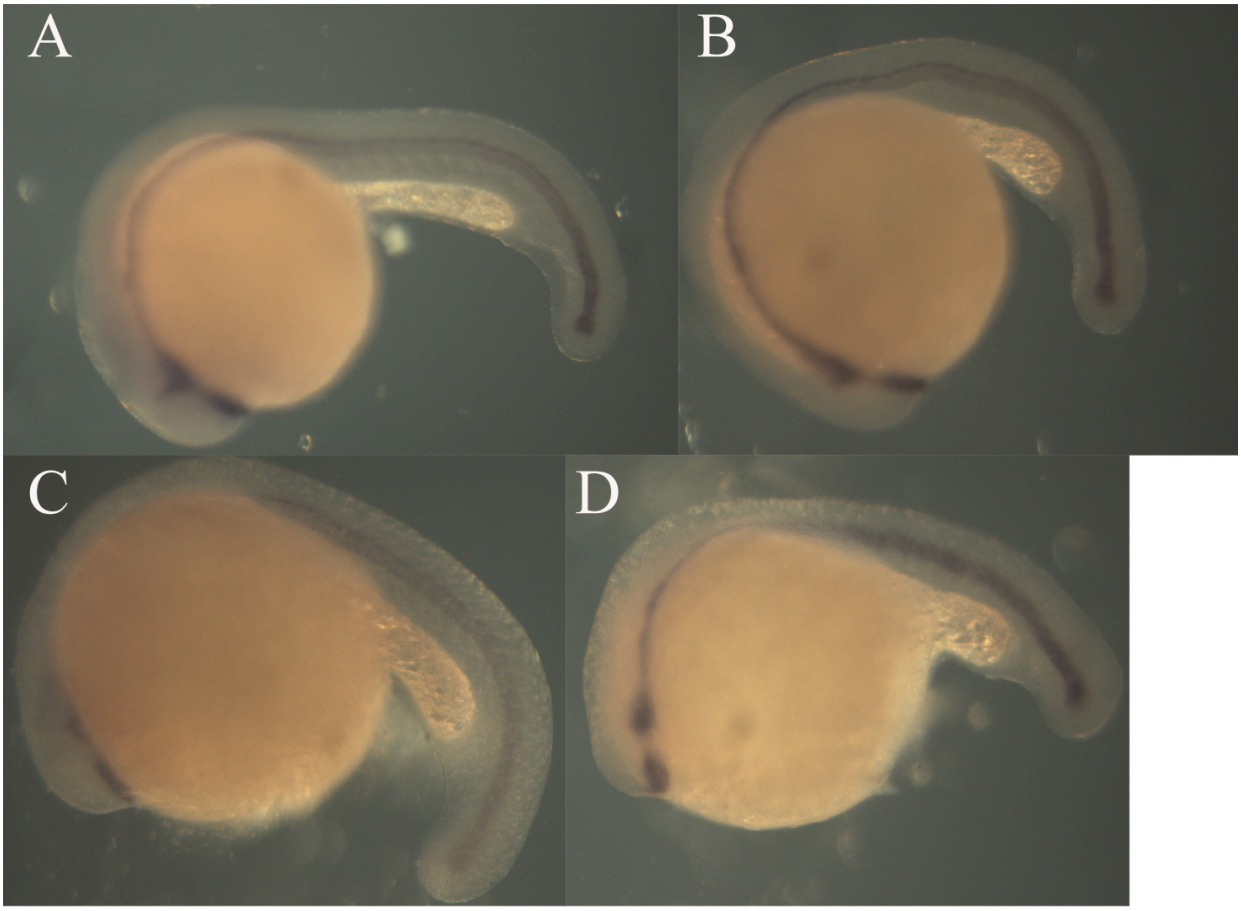


Figure 9: A: 20S uninjected control embryo stained with *shh* probe. 24 control embryos were stained, all exhibited similar and consistent morphology to the selected embryo. B: *id2a* 30 uM injected 20S embryo stained with *shh*. 25 embryos were stained, all exhibited similar and consistent morphology to the selected embryo. C: *id3* 50 uM injected 20S embryo stained with *shh.* 25 embryos weres stained, all exhibited similar and consistent morphology to the selected embryo. D: 1:1 dilution of *id2a* 30 uM and *id3* 50 uM injected 20S embryo stained with *shh.* 25 embryos were stained, approximately 30% percent of them exhibited similar morphology to the one selected. B, C: Compared to control, axis elongation is disrupted as evidenced by *shh* staining of shorter notochords. No significant difference in *shh* staining intensity in posterior notochord or tail bud. D: In addition to more pronounced inhibition

of axis extension as compared to control and *id2a* and *id3* injected embryos, there appears to be a slight increase in intensity of *shh* staining in the posterior notochord, which also extends further anteriorly in the notochord. Tail bud *shh* staining appears similar to control.

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