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The Effects of *pknox1.1* Knockdown on *hoxb1a* Expression in r4 of *Danio rerio* Hindbrain

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Abstract

Zebrafish hindbrain development begins with the folding of the neural plate into the neural tube that gives rise to segments in the hindbrain known as rhombomeres. Each rhombomere gives rise to important structures, such as the otic vesicle and various craniofacial nerves. The first of the rhombomeres to develop is r4—modulated by proper development of *hoxb1a*. Proper development of r4 triggers a cascade of gene expression of other genes such as fgfs, *krox-20*, and many other genes required for proper segmentation of the rest of the hindbrain. Another one of these genes responsible for proper rhombomere segregation is *pknox1.1*. The interaction between *pknox1.1* and *hoxb1a* has not been extensively studied at 19 hpf, thus there could be potential interaction between *pknox1.1* and *hoxb1a* at this time due to similar functionality in the hindbrain. To study this interaction, *pknox1.1* expression was knocked down via morpholino technology at the unicellular zygotic stage of zebrafish development and probed with antisense DIG-labeled in situ *hoxb1a* probe at 19 hpf. The flat-mounted images of the embryos showed identical *hoxb1a* staining in r4 between wild-type embryos and morpholino-injected embryos. The identical patterns in staining indicate *pknox1.1* is not essential for proper *hoxb1a* expression in r4 which is most likely due to proper activity of other hox cofactors, such as *pbx1, lzr/pbx4,* and *meis1*. In order to make the study more cohesive, examining *hoxb1a* expression at multiple stages of development (16, 18, and 24 hpf) along with knocking down these other *hox* cofactors and examining their effect on *hoxb1a* expression could be performed. Phenotypic confirmation via ChIP or immunostaining could have also strengthened the cohesiveness of the study by demonstrating the presence of an interaction between *hoxb1a* and *pknox1.1*.

Introduction

Danio rerio as a Model Organism

The zebrafish (*Danio rerio*) is a small freshwater vertebrate fish that originated in India, but can now be found in any pet store and is more accustomed to survive in brackish environments (Briggs, 2002). Its emergence as a model organism began with the discovery of its short generation time, large numbers of eggs produced with each mating, relatively cheap in terms of cost and maintenance, relatively easy to maintain, and all stages of development can be manipulated due to external fertilization (Briggs, 2002; Tavara & Lopes, 2013). The transparency of the zebrafish also allows for great ease of studying their early developmental

stages; embryogenesis, many of the first divisions, and even organelle formation can be seen by the naked eye with little to no staining throughout the first 5 days of development (Briggs, 2002). This principle allows for effective visualization of changes in gene expression via in situ hybridizations which can be easily looked at in whole mount (Briggs, 2002).

As zebrafish became more prominently used as model organisms, the molecular information about its genome has accumulated as well, making genetic studies more capable as well (Briggs, 2002). The entire zebrafish genome has been successfully sequenced thus every single gene within zebrafish is known and available on NCBI, thus we can compare homologies between zebrafish and a variety of different organisms; most importantly humans (Howe et al., 2013). About 70% of protein-coding genes in humans are related to genes found in zebrafish and 84% of genes associated with human disease have a zebrafish counterpart, such as the *parkin, pink1, dj-1,* and *irrk2* genes (Howe et al., 2013; Tavara & Lopes, 2013). In zebrafish, these genes have been shown to have conserved functions in development of dopaminergic neurons and mutants of these genes have been shown to result in severe neurodegeneration (Tavara & Lopes, 2013). The homologs of these genes in humans have been found to be associated with Parkinson's disease, thus zebrafish provide greater insight on how different diseases such as Parkinson's originate. Many cancers, such as melanoma, have also been studied in zebrafish through mutations in homologous genes such as *braf*, elucidating the functionality of this gene in the role is plays with skin cancer which in this is case *braf* mutants were shown to demonstrate large lesions in proliferating melanocytes (Tavares & Lopes, 2013). By studying zebrafish, it is possible to elucidate the functionality of mutations or diseases in zebrafish and apply these findings to medical treatments or gene therapies in humans (Howe et al., 2013; Briggs, 2002). For example, it is known that *braf* is responsible for signaling MEK which further

phosphorylates ERK to encourage cellular proliferation (Tavares & Lopes, 2013). Since this gene and its subsequent protein BRAF is responsible for initiating this pathway, in a severe melanoma this protein is being produced at an alarming rate, causing this pathway to occur at a faster rate, stimulating the uncontrolled cellular proliferation of the melanoma (Nijenhuis et al., 2013). Thus, we can develop drugs to target and inhibit the pathway by creating chemicals with high specificity to the BRAF protein, rendering it nonfunctional, such as vemurafenib (Nijenhuis et al., 2013).

Not only can we examine homologous genes in zebrafish, but zebrafish are known for their transgenic capabilities as well. Transgenesis is the ability to take genetic information from a separate organism and incorporation of this genetic information into the genome of the model organism, in this case the zebrafish (Thijs et al., 2012). In Thijs et al., 2012, the CD41 gene involved in thrombocyte development is fused with GFP and then transplanted back into the zebrafish. Even though the GFP is a foreign gene, it is readily incorporated into the zebrafish genome when fused with another gene (Thijs et al., 2012). This transgenic capacity makes it possible to examine gene expression of a wide variety of different genes since it is possible to fuse fluorescent proteins, such as GFP, to these genes and observe expression dynamically throughout development based on the strength of the fluorescence as seen with CD41 (Thijs et al., 2012).

One of the greatest advantages of utilizing zebrafish as model organisms is their relatively short life cycle. Zebrafish undergo organogenesis as early as 24 hpf, fully hatch from their eggs as early as 48-72 hpf (3 days), reach adolescence at approximately 14-21 days (2-3 weeks) and reach sexual maturity in a matter of 90 days (3 months) (Kimmel et al., 1995). Such a short reproductive maturation time frame allows these organisms to be easily and readily

studied in a shorter time frame since crossing and embryonic studies can begin once the zebrafish are capable of reproducing (Briggs, 2002). The combinations of their short generation time, transgenic capacity, relatively cheap cost and maintenance, transparent bodies, and the knowledge of the sequenced genome/ homologous genes with humans make zebrafish ideal model organisms. These characteristics allow for manipulation such as altering genes involved with organ development or cellular differentiation such as *braf*, consensus sequences such as homeobox genes, and appropriate development of important structures—such as the pharyngeal arches or portions of the brain such as the hindbrain with little to no cost, financially and time-wise (Briggs, 2002).

Danio rerio Hindbrain Development

The vertebrate hindbrain is responsible for controlling essential functions such as heartbeat, respiration, and gives rise to essential cranial nerves that coordinate balance, jaw movement, eye movement, and sensory nerves (Moens & Prince, 2002). The development of the hindbrain stems from the folding of the neural plate into the neural tube, followed by an activation of a cascade of a variety of genes—primarily hox genes, fgfs, *pbx* genes and other specific genes such as *krox-20, pitx2, twist1, vhnf1, val,* and so on (Bohnsack & Kahana, 2013; Maves & Kimmel, 2005). The neural plate begins formation once gastrulation is complete in the zebrafish (approximately at 10 hpf) (Kimmel et al., 1995; Blader & Strähl, 2000). The neural plate formation is induced by ectoderm BMP signals (class of growth factors) that are encoded in genes such as *snailhouse* and *swirl*, triggering neurons to form and organized into a multi-layer plate—hence the name neural plate (Blader & Strähl, 2000). Zebrafish orthologs to *sonic hedgehog* and *tiggywinkle hedgehog* (*syu and yot* respectively) are expressed in the lateral and medial sides of the neural plate at around 11-12 hpf and begin the process of initiating the

folding of the neural plate into the neural tube (Blader & Strähl, 2000). The *cyclops* gene also facilitates this migration of the neural cells within the plate into the tube, causing the medial portion of the plate to fold upwards and connect into the neural tube structure (Blader & Strähl, 2000). Wnt11, Dsh2, Pk1a, and Pk1b facilitate the neuroepithelial convergence as the neural tube begins to close while ensuring proper neural tube lumen formation (Blader & Strähl, 2000; Zigman et al., 2011). By 16 hpf, the neural tube is fully formed and further segmentation of the hindbrain begins (formation of rhombomeres) (Kimmel et al., 1995). What signals these genes to be expressed, however, is the important Vitamin A derivative retinoic acid (RA) (Maves & Kimmel, 2005). Retinal, also known as retinaldehyde, is present within the developing embryo and signals for the expression of *raldh2* at the 30% epiboly stage (~4.7 hpf) (Maves & Kimmel, 2005). The *raldh2* gene produces an enzyme capable of synthesizing retinoic acid from retinal which can now act as a transcriptional co-activator by recruiting enzymes such as histone acetyltransferases that can encourage transcription of the genes mentioned above, allowing for proper fusion of the neural tube (Maves & Kimmel, 2005). RA is capable of recruiting these enzymes by binding to retinoic acid receptors (RARs) alpha and gamma in zebrafish (encoded by *raraa, rarab, rarga,* and *rargb*) which subsequently signals for transcriptional activators and transcription factors to activate the cascade of genes by binding to retinoic acid response elements (RAREs) (Linville et al., 2009). RA also binds potentially antagonistic proteins, such as corepressors, to prevent them from binding to important classes of genes, such as homeobox genes, to facilitate their expression (Oliveira et al., 2013).

Retinoic acid is also responsible for activating gene expression in genes specific for forming the hindbrain as well once the neural tube is fully developed. *raldh2* expression is present within the neural tube and the somites as the embryo develops (Maves & Kimmel, 2005;

Linville et al., 2009). RA activates transcription by recruiting enzymes such as the histone acetyltransferases, but RA control the extent to which genes are expressed by acting upstream of the transcription factors for the gene cascade that ensues as the result of RA being present (Maves & Kimmel, 2005). The time frame at which RA is present to express certain genes, however, varies. For example, RA in a concentration dependent fashion binds to the appropriate alpha and gamma receptors in the promoters of hox genes, *vhnf1*, *fgf3* and *fgf8* to recruit the subsequent transcription factors associated with those genes, allowing transcription of these genes to occur at approximately 11.6-12.5 hpf (Maves et al., 2002; Oliveira et al., 2013). Yet at approximately 14.5 hpf, the RA is repressed by *cyp26* and these genes will not be expressed in this particular segment known as a rhombomere (Linville et al., 2009).

The hindbrain develops into a series of rhombomeres—which are responsible for the proper organization of nerves throughout the entire hindbrain at approximately 16 hpf (Riley et al., 2004; Kimmel et al., 1995). These rhombomeres serve to organize the hindbrain along the anterior-posterior axis and numerous cell types and gene expression patterns are repeated in successive rhombomeres; yet, each segment produces specialized structures (Riley et al., 2004). The vertebrate hindbrain is composed of seven rhomobomeres: r1, r2, r3, r4, r5, r6, and r7 (Purves et al., 2001). RA is responsible for activating the genes that form each rhombomere in varying concentrations at different time frames, that way each rhombomere serves as a marker for each successive rhombomere to develop properly as well (Linville et al., 2009). Rhombomere r1 gives rise to the trochlear nerve which is responsible for innervating the superior oblique muscle in the eye, allowing for rotation of the eye to be possible (Purves et al., 2002). r2 is responsible for proper formation of trigeminal ganglion, allowing for proper development of the nerves involved with movement of the face such as biting and chewing while r3 does not

necessarily give rise to specific ganglia or other nerves, but rather serves as a barrier between r2 and r4 rhombomeres and facilitates proper development of the structures associated with r2 and r4 rhombomeres (Purves et al., 2002). r4 and r5 give rise to the spiral and scarpa's ganglia which form synaptic contact with the hair cells in the ear and the vestibular nerve, allowing for proper sensory of the position of the head in relation to the body which is crucial for proper balance (allows proper orientation of the entire body) (Purves et al., 2002). Branchiomotor neurons also differentiate within r4, which enhances the functionality of the trigeminal ganglion (Rohrschneider et al., 2007). The scarpa's ganglion is connected to the otic vesicle, overlapping on both r5 and r6 (Purves et al., 2002). The otic vesicle is the structure containing the hair cells that detect vibrations and transmits vibrations to the spiral and scarpa's ganglia to allow the zebrafish to orient itself appropriately (Purves et al., 2002). The final rhombomere, r7, is responsible for giving rise to the jugular/ nodose ganglia which coordinate sensory impulses to the gasotrintestinal tract from the brain, such as olfaction and gustation and can also coordinate pain or reflex sensations from blood vessels to the central nervous system (Purves et al., 2002). The order in which the rhombomeres develop is erratic; r4 develops first at approximately 16 hpf, followed by the boundaries between r3/r4 and r4/r5 and then the r1/r2, r2/r3, and r6/r7 boundaries develop (Moens & Prince, 2002; Kimmel et al., 1995). This full segmentation pattern can be observed as early as 18 somites (approximately 18-20 hours poster-fertilization) (Moens & Prince, 2002). RA activates *hox* genes to develop r4 first, *fgf3, fgf8, krox-20*, and many other genes are then activated by RA once r4 is fully developed to form r3 and r5, and the remaining rhombomere boundaries are able to be formed in a similar manner (Linville et al., 2009). The classes of these genes are typically *hox* genes, pbx genes, fgfs, and more specific genes such as *kreisler, mafb, vhnf1, val, efnb2a*, and many more genes as well (Maves & Kimmel, 2005).

Functionality of *hoxb1a* in Hindbrain Development

Because cell types and gene expression patterns in the each rhombomere can be repetitive, the importance of a variety of *hox* genes, *pbx* genes, and various other proteins such as fgfs tightly regulate the boundaries of the rhombomeres to ensure that genes for each appropriate rhombomere are being expressed where appropriate (Riley et al., 2004; Maves et al., 2002). Riley et al., 2004, for example, demonstrates that various *wnt* are exhibit high expression at rhombomere boundaries and that various *delta* genes flank the boundary sites of rhombomeres (Riley et al., 2004). In zebrafish, the first of the rhombomeres to differentiate is rhombomere 4 (Rohrschneider et al., 2007; Maves et al., 2002). In this early differentiation, *hox* genes play a major role in proper determination of the rhombomere 4 boundary and the boundaries of the subsequent rhombomeres will be based on how rhombomere 4 is differentiated (Choe et al., 2011; Rohrschneider et al., 2007). Each rhombomere contains the same set of *hox* genes, yet the availability of the *hox* binding domains varies in each rhombomere (Guthrie, 1996). The availability of the binding domains is typically due to varying concentrations of retinoic acid in each rhombomere (Guthrie, 1996). RA, as mentioned previously, is known to induce *hox* expression—the closer a given *hox* gene is to the 3' end the more likely retinoic acid is to induce expression (Guthrie, 1996). This principle is what allows for varied expression of *hox* genes throughout each rhombomere.

The earliest rhombomere to develop and differentiate, r4, is controlled primarily by the expression of the *hox* gene, *hoxb1a* (Rohrschneider et al., 2007). *hoxb1a* is responsible for giving rise to the branchiomotor neurons, neurons that assist with the trigeminal ganglion and ultimately help coordinate cranio-facial movement, in the r4 rhombomere (Rohrschneider et al., 2007). The expression of *hoxb1a* affects the expression of various other genes expressed in r4, such as *pk1b*,

which are expressed in the branchiomotor neurons as they migrate throughout the r4 rhombomere and towards the posterior of the hindbrain (Rohrschneider et al., 2007). When this gene is nonfunctional, the r4 rhombomere demonstrates similar functionality to that of the r2 rhombomere and thus the migration of the branchiomotor neurons is not properly carried out and coordination of cranio-facial movement is hindered as a result (Rohrschneider et al., 2007; McClintock et al., 2002). A nonfunctional *hoxb1a* gene also results in an inhibited patterning of the otic vesicle because the boundaries of r4 are not as pronounced and thus the boundaries of subsequent surrounding rhombomeres are less pronounced as well (McClintock et al., 2002). Because the otic vesicle is differentiated through r5 and r6, this structure cannot be properly differentiated due to a lack of boundary determination and a lack of gene expression organization; proper r4 differentiation is critical for subsequent rhombomere differentiation as well (McClintock et al., 2002).

Functionality of *pknox1.1* in Hindbrain Development

The expression of *hox* genes is not solely affected by varying concentrations of retinoic acid, but also through genes coding for proteins that bind with the proteins encoded by the hox genes such as *hoxb1a* (Deflorian et al., 2003). One of the more ubiquitously expressed proteins that forms a heterotrimeric complex with many different *hox* genes is *pknox1.1* also known as *prep1.1* (Deflorian et al., 2003). *pknox1.1* has been demonstrated to promote proper rhombomere segregation and formation, proper migration of facial nerve motor neurons, and formation of the pharyngeal arches (Deflorian et al., 2003). When *pknox1.1* is rendered nonfunctional via morpholino-based injections, apoptosis in the hindbrain along with lack of jaw formation, smaller head size, smaller eye-size, and an enlarged swim bladder were seen in zebrafish as demonstrated in Deflorian et al., 2003. *pknox1.1* knockdown has also been shown to interfere

with proper segmentation of the rhombomeres in the hindbrain based on inhibited expression of *fox1.2/mariposa* and *pax6.1*: genes that depict hindbrain segmentation by outlining rhombomere boundaries (Deflorian et al., 2003). Because *pknox1.1* has been shown to inhibit proper rhombomere segmentation, and *hoxb1a* is a critical gene required for proper r4 formation and therefore overall rhombomere segregation and formation, there could potentially be an interaction between these two different genes in regards to hindbrain development. However, *pknox1.1* is one of many hox cofactors known to interact with hox genes, including *hoxb1a,* and promote proper rhombomeric differentiation (Moens & Selleri, 2006; Erickson et al., 2007). *pknox1.1* is a member of a class of proteins known as MEIS proteins—homeodomain proteins that collectively enhance expression of different hox genes in vertebrates and are responsible for organizing the hindbrain as well (Moens & Selleri, 2006). In addition to *pknox*, *homothorax (hth),* and *meis* are included in this class of homeodomains (Moens & Selleri, 2006). Another class of homeodomains known as PBCs which contain primarily pbx genes and *extradenticle (exd)* that have been classified to induce and promote gene *hoxb1a* expression in r4, along with expression of *fgf3, fgf8*, and *vhnf1* required for proper r3 and r5 formation (Moens & Salleri, 2006; Erickson et al., 2007). All of these proteins act similarly as *pknox,* forming homdimeric, heterodimeric, and heterotrimeric complexes with *hoxb1a* and other hox genes expressed throughout hindbrain development (such as *hoxb1b, hoxb5a* etc.) (Oliveira et al., 2013). *hoxb1a* is also known to auto-regulate its expression when associated with *pbx* proteins, thus *pknox1.1* is not the only gene interacting and promoting *hoxb1a* expression (Pöpperl et al., 2000). Since there are so many different homeodomains responsible and associated with hox gene expression in general, the magnitude to which *pknox1.1* affects rhombomere formation in regards to *hoxb1a* expression has not been classified extensively. Understanding how these genes interact or if

these genes even interact all could provide greater insight on how the differentiation of rhombomere 4 begins in the hindbrain.

Morpholino Technology as a Tool to Knockdown Gene Expression

Determining the effects of knocking down *pknox1.*1 as previously described in Deflorian et al., 2003 was performed through morpholino-based injection technology. Morpholino antisense oligonucleotides differ from the typical DNA nucleotide structure and typical antisense oligonucleotides in that they do not have a phosphodiester backbone. Typically, the phosphorus in the phosphate group is bound to four oxygen heteroatoms, but with morpholinos the phosphorus is bonded to two oxygen and two nitrogen heteroatoms (Corey & Abrams, 2001). Despite the different backbones, morpholinos complementary bind to nucleic acid sequences by the standard Watson-Crick base pairing (hydrogen bonding) (Corey & Abrams, 2001). These oligonucleotides do not bind any more tightly than analogous DNA or RNA oligonucleotides but their different backbone structures are resistant to degradation by various nucleases (endo and exonucleases), making them more stable (Corey & Abrams, 2001). The backbones in morpholinos also do not carry a negative charge unlike in DNA and RNA nucleotide sequences, making them less likely to react with other proteins and biological molecules within the organism (Corey & Abrams, 2001). Regular antisense oligonucleotides are able to form DNA-RNA hybrids that can act as a substrate for RNase H which promotes cleavage and cutting of a desired mRNA target (Corey & Abrams, 2001). The degradation of the mRNA allows for the antisense oligonucleotides to bind to any sequence within the coding region (Corey & Abrams, 2001). Morpholinos, however, form only RNA-morpholino hybrids that are incapable of facilitating RNase H substrate activity, thus preventing mRNA degradation (Corey & Abrams, 2001). Because of this, ribosomes are easily displaced by the morpholinos during translation as

long the morpholino is targeted for the 5' untranslated region (UTR) of the gene of interest or the start codon and first exons of the gene of interest (Corey & Abrams, 2001). Morpholinos designed to specifically target the 5' UTR or beginning exons are able to efficiently inhibit translation because the entire ribosome will have much greater difficulty binding due to the displacement (Bill et al., 2009). Morpholinos also function by preventing alternative splicing through inhibition of proper spliceosome binding (Bill et al., 2009). In order for proper splicing to occur, the snRNPs must be able to bind in the following order: U1, U2, followed by the U4/U5/U6 trimeric complex. Most morpholinos engineered to block alternative splicing to knock down a variant of a gene (such as *mrf4tv1* vs. *mrf4tv2*) bind so that the U2 snRNP is unable to bind, thereby preventing the lariat formed to excise the intronic sequence from forming and ultimately inhibiting proper splicing of the variant (Bill et al., 2009). Morpholinos are typically introduced via injection at the 1 to 8-cell stage in zebrafish embryos to ensure for optimal functionality (Bill et al., 2009). Cytoplasmic bridges connecting these early embryonic cells allow for rapid diffusion of the morpholinos, resulting in ubiquitous delivery (Bill et al., 2009). Most importantly, morpholino injections allow for knockdown of gene expression without being lethal, unlike chemicals such as 2'-O-methyl oligonucleotides and locked nucleic oligonoclueotides (Kloosterman et al., 2007).

pknox1.1 **Knockdown via Morpholino-Injections in** ***Danio rerio***

Previous studies such as Deflorian et al., 2003, Kloosterman et al., 2007, Maves et al., 2002, Moens & Selleri, 2006 and Rohrschneider et al., 2007 have provided valuable insight in the functionality of *pknox1.1*, *hoxb1a*, other genes that affect *hoxb1a* expression (i.e. pbx genes), and morpholinos as a useful technology to knockdown gene expression without lethal effects as seen in other antisense oligonucleotides. In order to study the potential interaction between

hoxb1a and *pknox1.1* in the developing hindbrain in zebrafish, expression of *pknox1.1* was knocked down via morpholino injections at the unicellular zygotic stage of development. The morpholinos MOa and MOb bind to exon 2 of *pknox1.1* and inhibit ribosomal binding, resulting in little to no translation of *pknox1.1*. Expression of *hoxb1a* was examined via in situ hybridization using a *hoxb1a* antisense probe at 19 hpf. Here we find that *pknox1.1* does not seem to affect *hoxb1a* expression in r4 at 19 hpf due to similarities in *hoxb1a* expression between wild-type and *pknox1.1* morpholino-injected zebrafish embryos.

Results

RNA in situ probes were prepared by transforming recombinant pWI plasmids containing *hoxb1a* DNA flanked with NotI and KpnI restriction sites donated by the Sagerström Lab into *E. coli* bacteria, grown and purified, digested, and cleaned. Labeled complementary probewith Digoxigenin UTP was generated and purified. Zebrafish embryos were injected with *pknox1.1* morpholinos designed to knockdown *pknox1.1* expression. Embryos were subjected to in situ hybridization with the *hoxb1a* anti-sense in situ probe and imaged.

Preparation of Anti-Sense *hoxb1a* In Situ Probe

pWI plasmids donated from University of Massachusetts Medical School via the Sagerström Lab contained *hoxb1a* DNA that was successfully flanked with NotI and KpnI restriction sites at the 5' and 3' ends respectively and ligated into the plasmid pWI (Figure 1).

A

5'GCGGCCGC(NotI)ATGGGGTATGAACAGTTCCGGATGAATCTTTCTTGGGAGTACACAATTTGCAACCGTGGGA
CGAACGCCTACTCGCCCAAGGCTGGATACCACCACTTGGACCAGGCGTTCCCGGGCCCTTTCCACACTGGACACGCT
AGTGACAGCTATAACGCTGATGGACGACTTTACGTAGGGGGGAGCAATCAGCCACCAACAGCAGCAGCACAACATCG
GCACCAGAACGGCATCTACGCGCATCACCAGCACCAAAATCAAACTGGCATGGGCCTTACCTATGGTGGAACTGGGA
CAACAAGTTATGGGACACAGGCCTGCGCCAACTCGGACTATGCTCAACACCAGTATTTTATCAACCCTGAGCAGGAT
GGGATGTATTATCACTCATCAGGTTTTTCAACATCAAATGCCAGTCCACACTATGGCTCTATGGCCGGTGCGTACTG

CGGGGCACAGGGAGCCGTTCCAGCCGCACCTTATCAGCATCATGGATGCGAAGGCCAGGATCACCAGCGAGCATATT
CACAAGGCACCTACGCTGACTTATCGGCCTCTCAAGGAACGGAGAAGGACACGGATCAGCCGCCACCTGGGAAGACA
TTCGATTGGATGAAAGTCAAAAGGAATCCCCCCAAAACAGGTAAAGTGGCTGAGTACGGACTAGGGCCGCAAAACAC
TATTCGGACAAATTTCACAACCAAACAACTGACAGAGCTCGAAAAAGAATTTCACTTCAGCAAGTATCTGACGCGAG
CGCGGCGTGTGGAGATTGCTGCCACACTTGAGCTTAACGAGACGCAGGTTAAGATTTGGTTTCAAAACCGCCGAATG
AAACAGAAGAAGCGAGAGAAGGAGGGACTCGCGCCTGCTTCCTCCACTTCGTCTAAAGACCTCGAGGATCAATCTGA
TCACTCAACTTCAACATCTCCAGAAGCCTCTCCAAGTCCGGATTCCTAA**GGTACC(KpnI)**3’

B

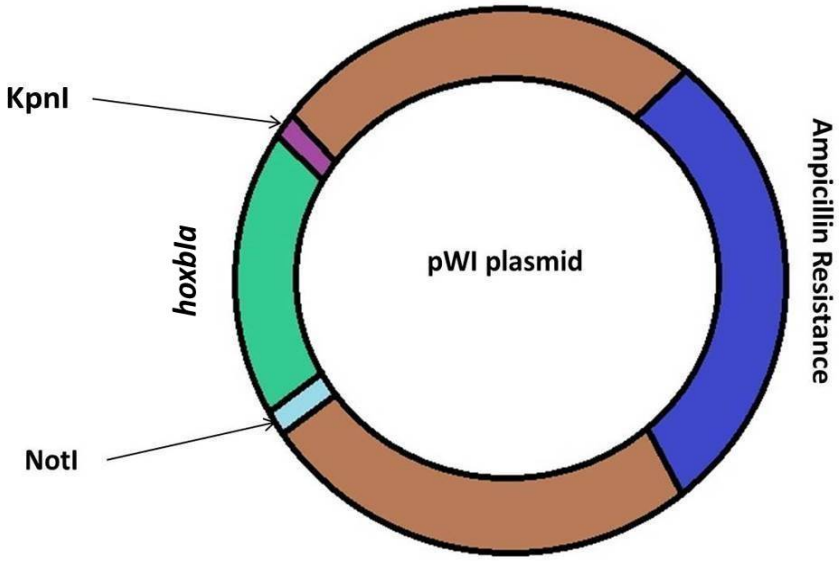

Figure 1. Restriction enzyme sequences and *hoxb1a* sequence in the pWI plasmid. A. The nucleotide sequence of *hoxbIa* noting the restriction enzyme sequences of 5’ Not1 and 3’ KpnI sequence attained **B.** the pWI plasmid containing ampicillin resistance for selection purposes and the *hoxbIa* gene flanked with 5’ NotI and 3’ KpnI sites.

The transformed *E. coli* cells containing the recombinant plasmid (Figure 1) demonstrated little growth when 25 µL of bacteria was plated on Agar/Ampicillin/LB plates, but greater growth when 75 µL of bacteria was plated on the same medium (Figure 2). However, minimal growth was seen.

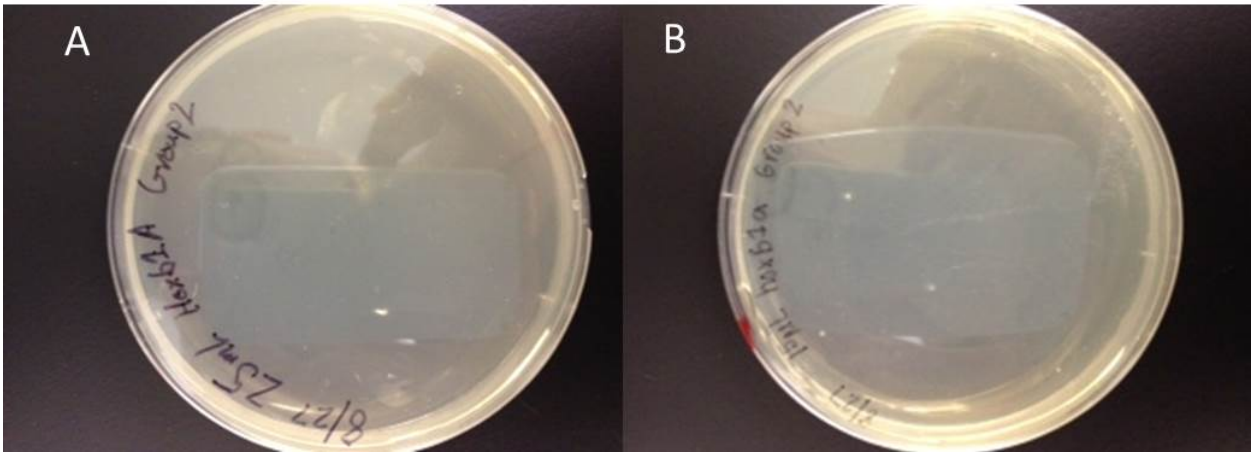

Figure 2. pWI transformed bacterial culture plates. A. 25 µL of plated transformed *E. coli* cells and **B.** 75 µL of plated transformed *E. coli* after overnight growth at 37°C on Agar/Ampicillin/LB plates.

The electrophoresed linearized pWI DNA samples had molecular weights of approximately 5500 bp and showed little degradation (Figure 3).

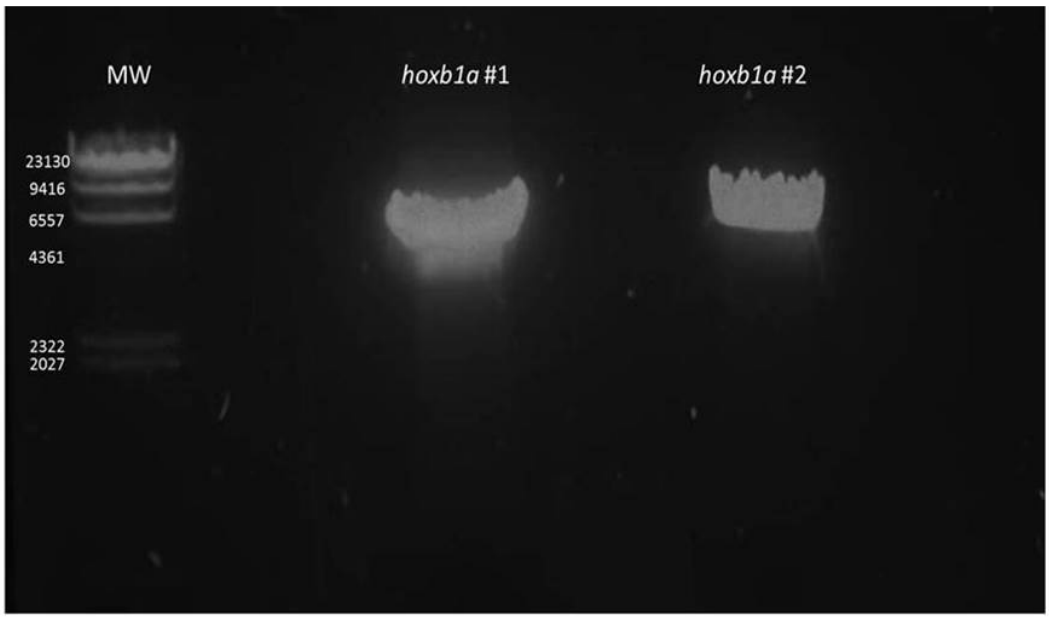

Figure 3. 1% Agarose gel electrophoresis of purified linear pWI DNA samples. MW: molecular weight (λ Hind III) shown in bp. *hoxb1a* has a molecular weight of approximately 5500 bp and both linear DNA samples demonstrate successful linearization

Purified *hoxb1a* antisense in situ probes were electrophoresed; sample *hoxb1a* #1 demonstrated decent purity with little degradation, while sample *hoxb1a* #2 demonstrated little

RNA isolation with degradation (Figure 4). Both samples ran at approximately 5500 bp as well (Figure 4). Degradation product can be visualized at approximately 2174 bp (Figure 4).

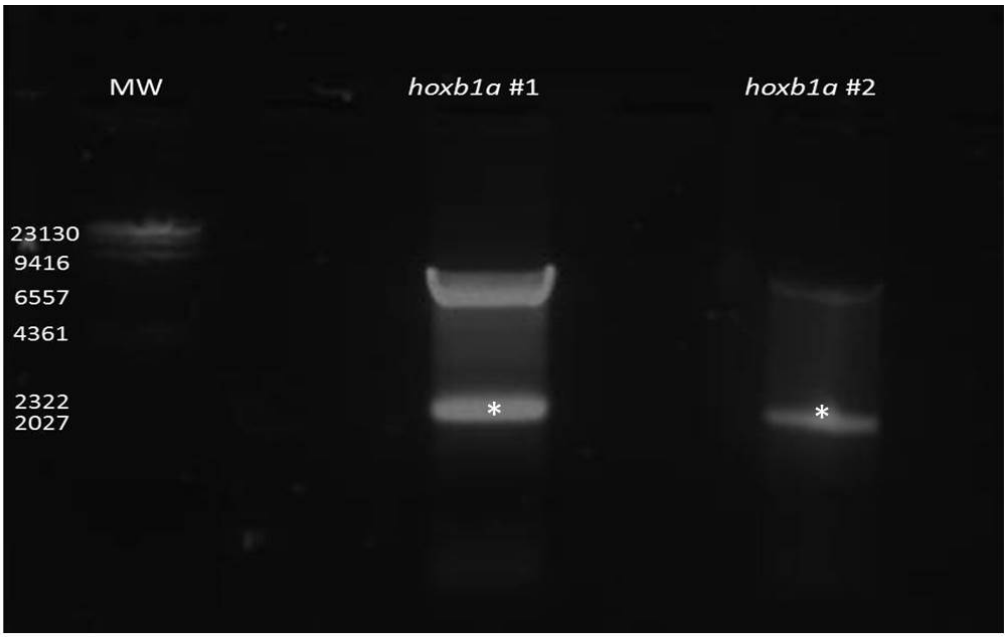

Figure 4. 1% Agarose gel electorphoresis of cleaned anti-sense in situ *hoxb1a* probes. MW: molecular weight (λ Hind III) with base pairs shown at left; visualization not seen due to error in loading. *hoxb1a #1* probe has a molecular weight of 5500 bp. *hoxb1a* #2 generated no probe. Note: * denotes degradation in product.

In Situ Hybridization of *hoxb1a* in *pknox1.1*-morpholino injected embryos

Expression of *pknox1.1* was knocked down via morpholino-injections specific for binding sites in exon 2 (Figure 5).

5′_GTTTAATTTGTAACTATAATAATTAATTTTATTGAATAATTTTTATTAATAATGCATAAAT
ACTGTGAGCCTTGGAAAATAATAAAACTGATTTTTTTAATTGCTTTTTCATTGTATTGCTCAAT
GCTTATAGAGGGTTTTATTATATTTTATGCAGATGCACTTCTCTACAGAATTATCCCAATCAAT
ACAAAGTATTAAATCTTTCTAAATAGAGATATTCAACAAGTCAACTGGTGAAATTGTATTCATA
TCACCAAAATATATTGCAGAATTGGAAAATATTGCAATGTTAGATTTGTCCAAAATCAGTAGCT
CTAACTGGACGGTTCAAGTGACCAAAAAATCCCAAGTGGAGAATTGTTAATATTTTTTTAGAGC
AAAAGAAAACTGATAATATTATTAGTGGAGGCCTTTTTTTAAATAAAGATTTAATGTTACCTTT
AGGTCCTCTCCTGCTGCCTGTCCATATAATGTCCCAGTGTTGGCAGTGGCCATTTTGAATATGA
TGGCTGCCCAGTCTGTGTCCATAGACAAATACCCAGAGGGAGACCAGCAG_3′

Figure 5. Nucleotide sequence of exon 2 in zebrafish *pknox1.1*. Morpholino binding sites are noted in yellow (MOa) and green (MOb). *pknox1.1* sequence obtained from Genbank ID # NM_131891.2

Wild-type embryos and morpholino-injected embryos demonstrated *hoxb1a* expression in rhombomere (r) r4 (Figure 6, A-D). Some morpholino-injected embryos demonstrated slightly decreased in *hoxb1a* expression in r4 (Figure 6, E). Wild-type embryos were larger in size in comparison to the morpholino-injected embryos.

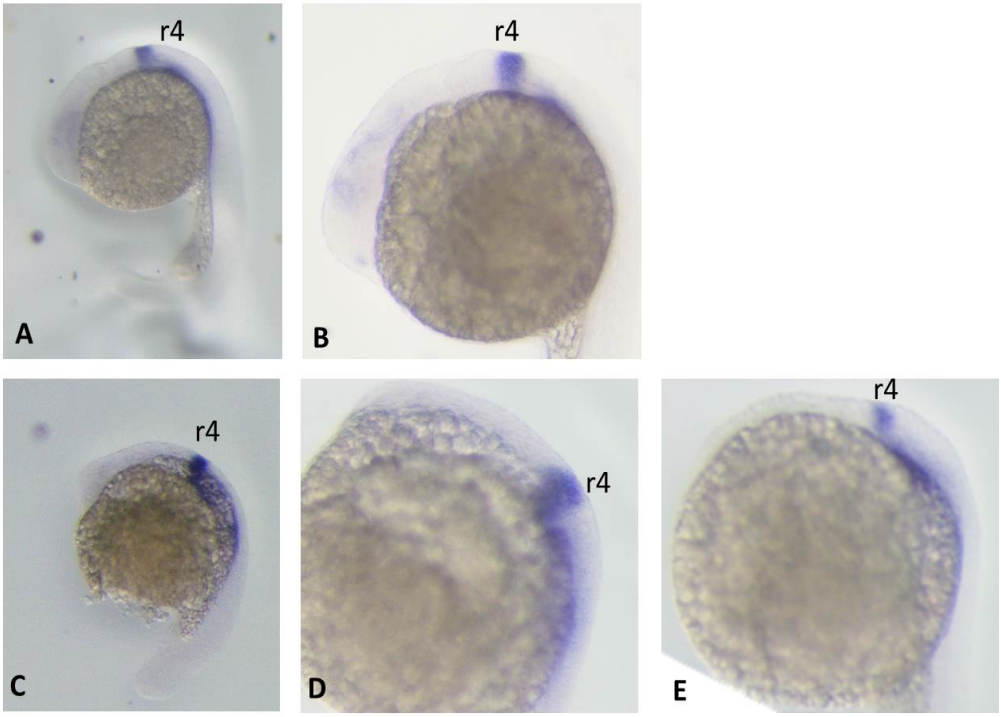

Figure 6. Control and morpholino-injected r4 staining of *hoxb1a*. A. Lateral view of whole control embryo. **B.** Lateral hindbrain magnification of control embryo. **C.** Whole morpholino-injected embryo. **D.** Lateral hindbrain magnification of morpholino-injected embryo. **E.** Lateral hindbrain magnification of morpholino-injected embryo with slightly decreased r4 staining.

Average r4 length was slightly greater in morpholino-injected embryos than in normal embryos, while average r4 area was similar between morpholino-injected embryos and normal embryos (Figure 7, Table 1). The 2 tail T-Test values for r4 length and r4 area were 0.135 and 0.934 respectively, demonstrating no statistical significance.

Table 1. Average r4 length and area of control and morpholino-injected embryos.

	Average r4 Length	Average r4 Area	Paired T-Test Value for r4 Length	Paired T-Test Value for r4 Area
Control	0.1314	0.0259	0.135	0.934
Morpholino	0.1405	0.0257		

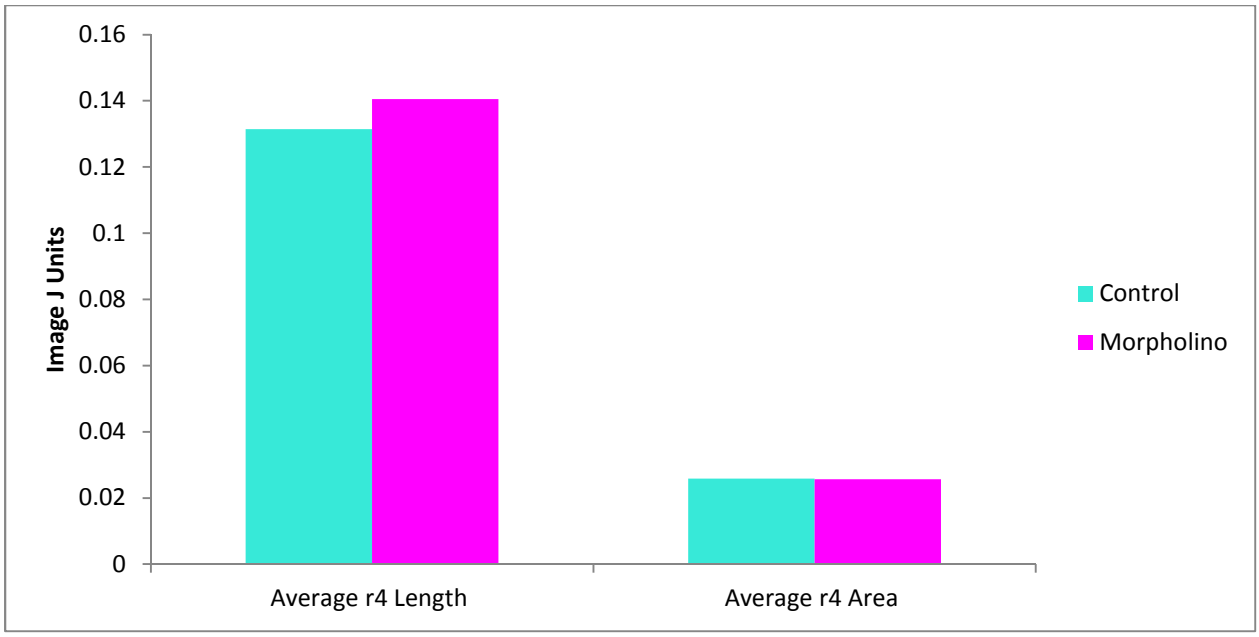

Figure 7. Control and morpholino-injected average embryo r4 length and area. Paired T-Test values ($T\text{-}Test_{Length}$ = 0.135, $T\text{-}Test_{Area}$ = 0.934) indicate no significant differences in average r4 length and area of the control vs. morpholino-injected embryos.

Discussion

The morpholino-injected zebrafish embryos demonstrated similar if not identical staining as the wild-type embryos when subjected to in situ hybridization (Figure 6 A-D) with some embryos showing slightly decreased *hoxb1a* expression (Figure 6 E). The similar areas and length of r4 *hoxb1a* staining (Figure 7, Table 1) in both wild-type embryos and morpholino-injected embryos suggests that a.) the morpholino injections were unsuccessful at knocking down *pknox1.1* or b.) knocking out *pknox1.1* does not necessarily inhibit *hoxb1a* expression in r4 due multiple hox cofactors that also play a role in modulating *hoxb1a* expression in r4. While it is definitely a possibility that some of the morpholino injections may not have been successful due to experimental error, the likelihood that the majority of the morpholino injections were unsuccessful seems fairly unlikely and thus the lack of inhibited *hoxb1a* expression in r4 of the morpholino-injected embryos is most likely due to the presence of multiple cofactors that facilitate *hoxb1a* expression in r4—cofactors that are not necessarily affected by *pknox1.1*

expression. Had *hoxb1a* expression been consistently less intense in the morpholino-injected embryos (consistently smaller r4 areas and lengths) than the wild-type embryos, then *pknox1.1* is most likely prominent and paramount hox cofactor and therefore essential in proper r4 development, assuming the r4 staining area and length are significantly different. The morpholino-injected embryos appeared to have developed differently than the wild-type embryos (Figure 6 A, C) because the morpholino-injected embryos showed greater curvature in the posterior end, whereas the wild-type embryos demonstrated greater posterior alignment i.e. the back and somites were straighter vs. curved in the morpholino-injected embryos. The degradation seen in the antisense in situ probes (Figure 4) may have potentially contributed to improper binding to *hoxb1a* mRNA in r4, yet staining of *hoxb1a* in r4 was present (Figure 6 A-E). Although *pknox1.1* is essential to zebrafish embryonic development as described in Deflorian et al., 2003, *pknox1.1* is not the only gene associated with proper *hoxb1a* expression in r4 therefore knocking down *pknox1.1* expression only will not produce a noticeable different in *hoxb1a* expression.

Cofactors Associated with Proper *hoxb1a* Expression in r4

lzr/pbx4 are important cofactors also associated with *hoxb1a* expression in r4 (Pöpperl et al., 2000). These genes interact directly with *hoxb1a*; the proteins of these genes cross-regulate *hoxb1a* and encourage expression of *hoxb1a* and many other hox genes through their homeodomain binding capabilities (Pöpperl et al., 2000). *hoxb1a* produces homeodomain transcription factors that function to bind with *lzr/pbx4* products to auto-regulate and enhance *hoxb1a* transcription (Pöpperl et al., 2000; Moens & Selleri, 2006). *meis1, pbx1, pbx4/lzr,* and *prep1.1* all act as *hoxb1a* cofactors (Meons & Selleri, 2006). Despite knocking down *pknox1.1* via morpholinos, these others cofactors are still present within the developing zebrafish

hindbrain and bind to the promoter of *hoxb1a* to encourage its expression in r4 (Moens & Selleri, 2006). If multiple cofactors had been targeted via morpholinos, such as *lzr/pbx4* and *meis1*, perhaps decreased expression in *hoxb1a* expression would have been noticeable in the in situ hybridized morpholino-injected embryos. By knocking out only *pknox1.1*, an effect analogous to knocking out only a single miRNA in liver regeneration as seen in Dippold et al., 2013 where a drastic difference in development is unable to be discerned due to overlapping functionality of miRNAs. The overlapping functions of the homeodomains in promoting *hoxb1a* expression ensure proper *hoxb1a* expression in r4—even if one of the homeodomains are rendered non-functional, such as *pknox1.1* (Moens & Selleri, 2006; Pöpperl et al., 2000). It is probable that the morpholino injections may not have been performed correctly and the embryos examined may not have been successfully injected with the morpholinos for *pknox1.1*, it is more likely that the functional expression of *lzr/pbx4, meis1,* and *pbx1* (along with many other hox cofactors) was enough to still promote successful *hoxb1a* expression in r4 of the hindbrain. Some embryos were able to demonstrate slightly decreased *hoxb1a* expression (Figure 6 E), but not consistently enough to conclusively illustrate *pknox1.1* is one of the only hox cofactors or one of the more prominent cofactors affecting *hoxb1a* expression in r4. In order to obtain a more in depth loop as to which of these cofactors play a larger role in modulating *hoxb1a* expression, these genes would ideally need to be targeted via morpholinos in the same manner as *pknox1.1* and *hoxb1a* expression would be examined via in situ hybridization. When a certain cofactor is knocked down, such as *pbx1* for example, if *hoxb1a* staining were to be significantly less intense (significantly decreased expression) then the gene encoding that cofactor would be more essential for proper *hoxb1a* expression in r4 at 19 hpf.

Potential Genes to Further Examine in Regards to *hoxb1a* Expression in r4

Since the genes *pbx1, lzr/pbx4,* and *meis1* that are all known to act as hox cofactors, their interactions with *hoxb1a* could have been examined in the same manner as *pknox1.1* was. Morpholinos could have been utilized to knockout *pbx1*, *lzr/pbx4,* and *meis1* respectively in different groups of embryos assuming approximately 20-30 embryos per group and in situ antisense *hoxb1a* probe could either be utilized from probe already generated, or by synthesizing new probe as detailed above. Morpholinos for each gene can be ordered from http://www.gene-tools.com/products_and_applications and require little to no preparation aside from the appropriate assembly of the microinjector apparatus. If the cofactors produced by any of these genes are more pivotal in proper *hoxb1a* expression in r4, then there would theoretically be decreased expression or potentially aberrant expression in r4 when *pb1x, lzr/pbx4,* or *meis1* are knocked down via morpholino-injections at the unicellular stage of development. If the knockout of any of these genes still results in the same *hoxb1a* expression seen in wild-type embryos, then most likely none of the cofactors are more crucial to *hoxb1a* expression than the other and the knockout of one cofactor makes little difference due to overlapping effectiveness of the other cofactors.

In addition to the genes mentioned (*pbx1,lzr/pbx4, meis1*) that code for different *hoxb1a* cofactors, there are other key elements that play a role in modulating *hoxb1a* expression at very early stages of zebrafish embryonic development. The Vitamin A derivative, retinoic acid (RA), is an essential compound that is integral in the development of the entire hindbrain—from the folding of the neural plate into the neural tube to the activation of gene expression in r4 and in the other rhombomeres as well (Linville et al., 2009). RA is essential in gene activation throughout development because retinoic acid binds to retinoic acid receptors (alpha and gamma

in zebrafish) which can then act as a nuclear receptor and ultimately a transcription factor for multiple genes (Oliveira et al., 2013). Because the proper function of RA in hindbrain development is contingent on proper expression of *raraa, rarab, rarga, rargb,* and *raldh2*, it would be interesting to examine how expression of each of these genes affects *hoxb1a* expression in r4 at 19 hpf as well (Linville et al., 2009). In situ antisense probes for *hoxb1a* could still be generated as previously described, but instead of injecting zebrafish embryos at the unicellular stage with morpholinos for *pknox1.1*, different groups of embryos would be injected with morpholinos for *raraa, rarab, rarga, rargb,* and *raldh2*. Only one group of embryos would be injected with one type of morpholino in order to elucidate the effect of each individual gene associated with proper RA function on *hoxb1a* expression. Based on the differences in staining of *hoxb1a* in r4 (if difference were to even be present), these additional experiments could potentially demonstrate which of the receptors are more crucial for proper *hoxb1a* expression or if all of the receptors are equally important and essential for proper *hoxb1a* expression, along with proper expression of *raldh2*. By examining these genes, it would be possible to gain even further insight of the importance of proper RA function, whether or not certain receptors are more important for facilitating RA functionality over others, and whether or not *raldh2* is the only means of retinal being enzymatically processed into retinoic acid (Maves & Kimmel, 2005). If RA cannot be oxidized from retinal via *raldh2* activity, then theoretically RA would not be able to be produced and the neural tube along with the hindbrain would never be able to properly develop and *hoxb1a* expression should be very limited and aberrant assuming *raldh2* is one of the only enzymes capable of modulating retinal oxidation. If other enzymes within the developing zebrafish are capable of facilitating this oxidation, then the knockdown of *raldh2* should theoretically result in little to no change in *hoxb1a* staining in r4.

Critique and Modifications of Current Experimental Design

In terms of the actual experimental design, perhaps examining *hoxb1a* expression at different stages of development would have been beneficial for overall comprehensiveness of the study. For example, examining *hoxb1a* expression when a.) r4 begins differentiation (~16 hpf) b.) when the full segmentation pattern can be fully seen (~18 hpf) and c.) when organogenesis and full innervation of the pharyngeal arches begins to occur (~ 24 hpf) (Kimmel et al., 1995). Perhaps *hoxb1a* expression isn't necessarily impacted at 19 hpf by knockdown of *pknox1.1* and may be impeded when r4 first develops at 16 hpf or when the entire segmentation pattern can be viewed at 18 hpf. Deflorian et al., 2003 eludes to the importance of *pknox1.1* in the patterning of the hindbrain, but gives a general time frame of when *pknox1.1* is really expressed: from 'early development' to about 25 hpf. The stage of development where *hoxb1a* expression was monitored may not have been a stage in development where *pknox1.1* expression is necessarily vital—which may occur at an earlier developmental stage (i.e. 16 hpf) or a later developmental stage (24-25 hpf). Since *pknox1.1* is one of many hox cofactors and *hoxb1a* is known to auto-regulate its expression, perhaps it is required in the pattering of the hindbrain by modulating and regulating *hoxb1a* expression within only r4 and prevents expression from expanding to the other rhombomeres at time intervals past 19 hpf. Although full rhombomeric segregation can be viewed as early as 18 hpf, this does not necessarily mean full modifications and gene expression within the rhombomeres halts at 18 hpf as demonstrated in Deflorian et al., 2003 and Kimmel et al., 1995. By examining *hoxb1a* at multiple stages of development, it would be possible to more conclusively ascertain whether or not *pknox1.1* is as vital to proper hindbrain development via homeodomain interaction as Deflorian et al., 2003 was able to demonstrate or not.

What may have also improved the experiment as a whole would have been to perform knock out some of the genes mentioned, such as *pbx1, meis1, lzr/pbx4,* etc. separately from *pknox1.1* just to gain further insight of some of the other cofactors associated with the expression of *hoxb1a* and to see if the idea that the cofactors affecting *hoxb1a* expression contain overlapping functionality. By knocking out each individual gene separately in different groups of embryos, there would have been a greater cohesiveness of the study to demonstrate whether or not certain *hoxb1a* cofactors affect *hoxb1a* expression more than other cofactors—as detailed in potential genes to further examine.

The experiment also lacked confirmation as to whether or not *pknox1.1* and *hoxb1a* definitively interact. The expected phenotype could have been determined by utilization of ChIP or immunostaining using antibodies specific for the expressed proteins. If *pknox1.1* is one of the co-factors for *hoxb1a* expression, antibodies specific for the *pknox1.1* protein (PREP1) could be utilized to precipitate out the protein bound to the gene sequence (theoretically *hoxb1a*) and then the gene sequence isolated—after proper purification—could be amplified using qPCR via primers specific for the *hoxb1a* gene. If gene amplification does not occur, the gene PREP1 binds to is not *hoxb1a* and therefore may not directly interact with *hoxb1a* expression. Immunostaining would be equally as insightful in terms of examining the protein expression of *hoxb1a* and *pknox1.1*. Primary antibodies and secondary antibodies that produce different fluorescent signals could specifically target the proteins of each of these genes. Based on where the signals are in the developing hindbrain would indicate whether or not *pknox1.1* and *hoxb1a* could be interacting as well. If the signals overlap and coincide within r4 then this may be indicative that *pknox1.1* interacts to some degree with *hoxb1a*—whether in the form of a cofactor or in conjunction with the *hoxb1a* protein to auto-regulate the expression of *hoxb1a*.

Final Conclusions of the Effects of *pknox1.1* Knockdown on *hoxb1a* Expression in r4

Based on Figure 6 A-D and Figure 7, the knockdown of *pknox1.1* did not effect *hoxb1a* expression in r4 of the developing zebrafish hindbrain at 19 hpf. This is most likely due to the functionality and activity of other hox cofactors, such as *pbx1, lzr/pbx4,* and *meis1*. To further study genes in the developing hindbrain that potentially affect *hoxb1a* development, the *pbx1, lzr/pbx4,* and *meis1* cofactors could be knocked down via morpholinos specific for each gene and expression of *hoxb1a* could be examined via in situ hybridization. The same method could be used to study how RA affects *hoxb1a* expression in the hindbrain by targeting the knockdown of *raraa, rarab, rarga, rargb,* and *raldh2* with morpholinos. If *hoxb1a* expression/staining decreases as a result of any of these genes being knocked down, then the given gene would be pivotal in modulating *hoxb1a* expression. In terms of experimental design, the study would have been more conclusive had multiple stages of development been examined, if the genes for the cofactors mentioned above had been studied, and if there was phenotypic confirmation as to whether or not *hoxb1a* and *pknox1.1* interact via ChIP or immunostaining. While improper injection of morpholinos could have been a potential reason why *hoxb1a* expression was similar between wild-type and morpholino-injected embryos and improper hybridization of the antisense in situ *hoxb1a* probe, the proper activity of other hox cofactors such as *pbx1, lzr/pbx4,* and *meis1* most likely allow for proper *hoxb1a* is the absence of *pknox1.1* translation.

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