Georgia Journal of Science

Volume 81 *No. 2 Scholarly Contributions from the Membership and Others* Article 14

2023

Brown Anole (Anolis sagrei) Hoxa5: Insights into the Divergence of Hoxa5 Gene Expression and Regulation Across Evolutionarily Divergent Gnathostome Vertebrates

Jennifer A. Lange
University of North Georgia, jalang1045@ung.edu

Amber L. Rittgers
University of Georgia, amber.rittgers@uga.edu

Adam Davis
University of North Georgia, adam.davis@ung.edu

Brown Anole (Anolis sagrei) Hoxa5: Insights into the Divergence of Hoxa5 Gene Expression and Regulation Across Evolutionarily Divergent Gnathostome Vertebrates

Acknowledgements

We gratefully acknowledge Dr. Doug Menke from the Department of Genetics of University of Georgia for providing brown anole lizard embryos and the brown anole lizard genome for this research project. This research was made possible by the funding from the University of North Georgia College of Science and Mathematics Faculty-Student Research Grant.

Brown Anole (*Anolis sagrei*) *Hoxa5*: Insights into the Divergence of *Hoxa5* Gene Expression and Regulation Across Evolutionarily Divergent Gnathostome Vertebrates

Jennifer Ally Lange[1], Amber Lynn Rittgers[2], and Adam Davis[3]*
[1]Current graduate student address, Carolinas College of Health Sciences, Charlotte, NC, 28217, USA
[2]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, 30322, USA
[3]Department of Biology, University of North Georgia, Oakwood, Georgia, 30566, USA
*Corresponding author: adam.davis@ung.edu; 678-717-2320

ABSTRACT

Hox genes are evolutionarily conserved developmental regulatory genes that function, in part, to pattern the anterior-posterior (AP) axis of organs and organ systems during animal embryonic development. *Hoxa5*, specifically, is shown to be expressed in the spinal cord, somites, or transient compartments giving rise to the vertebrae and ribs, developing gut, lungs, and limbs of the mouse (*Mus musculus*). The *cis*-regulatory elements (CREs), or short DNA sequences, that direct *Hoxa5* expression in these embryonic domains have been mapped and functionally tested in the mouse as well. Similar *Hoxa5* expression patterns have been observed in chicken (*Gallus gallus*), American alligator (*Alligator mississipiensis*), and dogfish shark (*Scyliorhinus canicular*), but have shown divergence in the anterior limit of expression within the somites. Specifically, while mouse expression begins in somite 3, chicken, alligator, and shark begin in 8, 9, and 9, respectively. Further, no *hoxa5* expression has been observed in the somites for teleost fish. Here, we present the embryonic *Hoxa5* expression pattern within brown anole lizard (*Anolis sagrei*). Our data shows that *Hoxa5* within the lizard has an anterior limit of expression in somite 6 and exhibits a more similar expression pattern to that of mouse, chicken, alligator, and shark than to teleost fishes. Furthermore, our comparative genomic DNA sequence analyses display that the functional CREs mapped in the mouse are conserved among the tetrapods, but not with the shark or teleost fishes. Our analyses suggest that divergent *Hoxa5* expression patterns result from divergence within their respective CREs.

INTRODUCTION

The vertebrate embryonic trunk is composed of a series of somites, or transient mesoderm-derived compartments that give rise to the vertebrae, ribs and associated tendons and musculature (Tajbakhsh and Spörle, 1998). The anterior-posterior arrangement of somites and their derived structures is determined, in part, by *Hox* genes, which are evolutionarily conserved and encode transcription factors that function to regulate the expression of downstream genes (McGinnis and Krumlauf, 1992; Krumlauf, 1994). *Hoxa5*, in particular, has been shown in mouse (*Mus musculus*) to be integral in the development of the cervical and thoracic vertebrae as well as many other organs from several other body systems, including the spinal cord, respiratory, digestive, endocrine, and reproductive systems (Aubin et al., 1997, 1998, 1999, and 2002; Boucherat et al., 2012 and 2013; Garin et al., 2006; Gendronneau et al., 2012; Jeannotte et al., 1993 and 2016; Kinkead et al., 2004; Krieger et al., 2004; Mandeville et

al., 2006; Meunier et al., 2003; Philippidou et al., 2012;). Several *cis*-regulatory elements (CREs), or short DNA sequences that function to bind transcription factors and regulate gene expression, for *Hoxa5* have been mapped and functionally tested within the mouse model system as well. Several enhancer modules containing these CREs and located downstream of mouse *Hoxa5* have been shown to direct this gene within the mesoderm (somites and other structures) and spinal cord (Moreau and Jeannotte, 2002; Tabariès et al., 2005). Specifically, several caudal-related homeobox (CDX) sites specific to mesoderm and retinoic acid response elements (RARE) for the spinal cord, were identified (Moreau and Jeannotte, 2002; Tabariès et al., 2005). However, outside of the mouse genome, it is unknown if these CREs are present for other vertebrates.

Beyond mouse, *Hoxa5* embryonic gene expression has been observed in several other vertebrate organisms. These include other tetrapods, such as chicken (*Gallus gallus*) and American alligator (*Alligator mississippiensis*), the chondrichthyan dogfish shark (*Scyliorhinus canicula*), and several teleost fishes, such as zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), and Nile tilapia (*Oreochromis niloticus*) (Davis and Stellwag, 2010; Hortopan and Baraban, 2011; Lyon et al., 2013; Mansfield and Abzhanov, 2010; Oulion et al., 2011). While all genes mentioned are expressed within the neural tube that gives rise to the spinal cord, there is divergence in expression in the somites. For instance, while mouse *Hoxa5* is expressed most anteriorly in somite 3, *Hoxa5* of dogfish shark, alligator, and chicken show anterior limits of expression in somites 9, 9, and 8, respectively (Mansfield and Abzhanov, 2010; Oulion et al., 2011). Further, no teleost *hoxa5a* gene was observed to be expressed within the somites (Davis and Stellwag, 2010; Hortopan and Baraban, 2011; Lyon et al., 2013). Interestingly, the lineage leading to teleost fishes underwent a whole genome duplication, thereby giving rise to two *Hoxa5* genes, *hoxa5a* and *hoxa5b* (Amores et al., 1998 and 2004; Crow et al., 2006; Hoegg et al., 2007; Moghadam et al., 2005; Prince, 2002; Stellwag, 1999). However, independent gene loss post-genome duplication has resulted in the presence of just *hoxa5a* in teleosts.

Here, we present the expression pattern of *Hoxa5* within the brown anole lizard (*Anolis sagrei*), an emerging vertebrate model for gene editing and biomedical research (Rasys et al., 2019). We show that brown anole *Hoxa5* exhibits an expression pattern that is more conserved with dogfish shark and other tetrapods than with teleost fishes. Namely, we found that brown anole *Hoxa5* is expressed within the somites, limb buds, and neural tube. However, we found that brown anole *Hoxa5* shows an anterior somite limit of expression at somite 6, which is divergent from mouse, chicken, alligator, and shark. We also show through bioinformatics analyses, that both enhancer modules characterized downstream of mouse *Hoxa5* are conserved among tetrapods, but that the mesoderm-specific CREs have diverged for shark and teleost fishes. While the loss of the mesoderm-specific CREs were not surprising for teleost fishes, these results suggest that other sequences are necessary for directing shark *Hoxa5* within the developing somites.

MATERIALS AND METHODS

Brown anole *Hoxa5* cDNA cloning

Brown anole lizard embryos were obtained from the Genetics Department of University of Georgia (kind gift from Dr. Doug Menke). RNA was extracted from stage 2

and 3 lizard embryos using TRIzol (Invitrogen, Carlsbad.CA) (Sanger et al., 2008). Lizard *Hoxa5* complimentary DNA (cDNA) was generated from RNAs using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The primers used for amplification of the entire coding sequence of *Hoxa5* cDNA were designed based on the sequenced genome of *Anolis sagrei* (kind gift from Dr. Doug Menke) (LizA5For: 5'-ATGAGCTCTTATTTTGTAAACTCC-3' and LizA5Rev: 5'-TTAGGGCGAAAGGCTCCTC-3'). PCR was performed in a 50 μL volume containing 25 μL of One Taq 2x Master mix with Standard Buffer (New England Biolabs, Ipswich, MA), 5 μL of 3 pmol/μL for both forward and reverse primers, 1 μL cDNA, 14 μL nuclease-free molecular grade water (ThermoFisher Scientific, Waltham, MA). PCR conditions were as follows: 1 min at 94 °C, 34 cycles of 45 sec at 94 °C, 30 sec at 55 °C, and 45 sec at 72 °C, and 10 min at 72 °C. PCR products were subcloned into PCRII TOPO TA plasmid vectors (Invitrogen, Carlsbad, CA) and cloned into One Shot Top10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA), according to the manufacturers instructions. Plasmid DNAs were isolated from *E. coli* using Plasmid DNA Miniprep kits (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Confirmation and orientation of PCR product corresponding to the insert from the plasmid cDNA clone was determined by enzyme restriction digestion analysis (New England Biolabs, Ipswich, MA) and DNA sequencing (GENEWIZ, South Plainfield, NJ).

Whole-mount *In Situ* Hybridization

Lizard embryos were extracted from eggs using microdissection foreceps and scissors (ThermoFisher Scientific, Waltham, MA) on a Motic SMZ-171 Stereo Zoom Microscope (Motic, Feasterville, PA) and developmentally staged according to Sanger et al. (2008). Stage 1-3 lizards were fixed overnight at 4 °C in 4% paraformaldehyde (ThermoFisher Scientific, Waltham, MA), dehydrated in a graded series of methanol (ThermoFisher Scientific, Waltham, MA) with 0.1% phosphate buffered saline with 0.1% Tween 20 (ThermoFisher Scientific, Waltham, MA), and stored in 100% methanol at -20 °C until use.

Whole mount *in situ* hybridization (WISH) was performed in 4 mL glass vials containing 2-4 embryos. All steps outlined in the standard operating procedure (SOP) published by Davis et al. (2019) were used with modifications. The volumes used for fish embryos in the SOP were doubled due to the size of the lizard embryos. The lizard embryos were also digested with proteinase K (ThermoFisher Scientific, Waltham, MA) for 30 min at room temperature. Production and purification of sense and antisense digoxigenin-labeled riboprobes for negative control and experimental embryos, respectively, were performed using a SP6/T7 Transcription Kit (Roche, Indianapolis, IN) and a QIAquick PCR Purification Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The embryos were mounted on microscope slides using a Motic SMZ-171 Stereo Zoom Microscope (Motic, Feasterville, PA) and photographed using an Amscope B490 compound microscope and associated 10 megapixel camera (Amscope, Irvine, CA). Images were further processed using Adobe Photoshop (Adobe, San Jose, CA). Several morphological landmarks were used to determine the expression pattern of *Hoxa5*, including the rhombomeres of the hindbrain, otic vesicle (or developing ear), and the somites.

Comparative Genomic DNA Sequence Analysis

Hoxa5 Bioinformatics analyses using mVISTA (http://genome.lbl.gov/vista/index.shtml) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) were used to examine the functionally tested enhancer regions in mouse with other vertebrates with known *Hoxa5* or *hoxa5a* expression patterns (Frazer et al 2004; Madeira et al., 2019; Mayor et al. 2000). Sequences spanning from the *Hoxa5* start codon to the *Hoxa4* stop codon (or *hoxa5a* and *hoxa4a* for teleost fishes) were retrieved from Genbank and Ensembl for analysis of the known downstream enhancer regions of *Hoxa5*. Analyzed sequences include Alligator (Accession #: NW_017712875.1), Chicken (NC_052574.1), Brown Anole Lizard (Genomic DNA sequence supplied by Dr. Doug Menke), Mouse (NC_000072.7), Dogfish shark (FQ032658.1), Zebrafish (NC_007130.7), Japanese Medaka (AB232918.1), and Nile tilapia (GCA_001858045.3). The Shuffle-LAGAN option in mVISTA was used for detecting rearrangements and inversions for the genomic DNA sequence alignment. The parameters used for sequence analysis in mVISTA were as follows: window 100 bp, minimum conservation width of 100 bp, and conservation identity of 70 %. Mouse *Hoxa5* was used as the reference sequence in the mVISTA analysis since the regulatory enhancer regions were characterized in this system (Moreau and Jeannotte, 2002; Tabariès et al., 2005). Genomic DNA sequence regions of other species that were observed to be conserved with the regions of mouse *Hoxa5* that contain the characterized CREs in mVISTA were further aligned and analyzed using Clustal Omega. All default parameters were used for the Clustal Omega sequence alignments. Other genomic regions that showed conservation with mouse *Hoxa5* outside of the known enhancer regions were not analyzed, as these regions were not functionally tested in mouse or any other model organism.

RESULTS

Brown Anole Lizard *Hoxa5* Expression Patterns

We observed brown anole *Hoxa5* to show anterior limits of expression in the neural tube at the level of somite 3 and within the somites themselves at somite6 (Fig. 1A). We also observed *Hoxa5* to be expressed in several other developing tissues, including the lateral plate mesoderm ventral to the somites and the fore and hindlimbs (Fig. 1A). Excluding the somite expression, brown anole *Hoxa5* showed a conserved expression pattern with other tetrapod vertebrates and shark. However, the anterior limit of expression in the somites was shown to be divergent from mouse, chicken, alligator, and shark. No expression within the embryonic tissues mentioned above was observed within the negative control embryos (Fig. 1B).

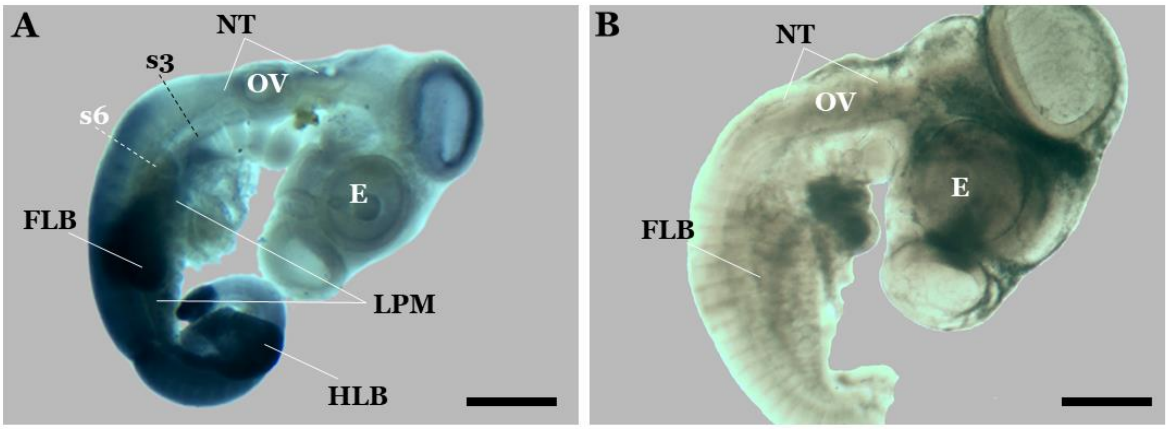

Figure 1: Gene expression pattern results of brown anole *Hoxa5* in experimental (A) and control (B) embryos. Both embryos shown are representative images of the experimental and control embryos tested. Stage 3 embryos are mounted such that the lateral side is facing the reader and the anterior is facing right. (A) S3 and the black dashed line represent the anterior limit of expression in the neural tube at the level of somite 3 in the experimental embryo. S6 and the white dashed line represent the anterior limit of expression within the somites themselves. E, eye; FLB, forelimb bud; HLB, hindlimb bud; LPM, lateral plate mesoderm; NT, neural tube; OV, otic vesicle; S, somite. Scale bar = 1 mm.

Comparative Genomic DNA Sequence Analysis

Several enhancer regions downstream of *Hoxa5* have been identified in the mouse model system. These include a mesoderm-specific enhancer region that contains several CDX transcription factor binding sites and a spinal cord region that contains RARE binding sites (Moreau and Jeannotte, 2002; Tabariès et al., 2005). From our mVISTA and Clustal alignment data, we observed that the mesoderm enhancer region showed conservation only among the tetrapods analyzed in this study (Fig. 2A and B). Specifically, we observed the presence of a conserved peak specific to the mesoderm enhancer region of mouse in chicken, alligator, and brown anole lizard but not in shark or teleost fishes (Fig. 2A). Further, all CDX binding sites shown to be functional for mouse were conserved between mouse, chicken, alligator, and brown anole lizard (Fig. 2B). However, no conservation with these elements was observed for shark or teleost fishes. By contrast, we observed there to be higher conservation with the spinal cord enhancer region among all organisms assayed in this study (Fig. 2A and C). All species assayed contained a conserved peak corresponding to the mouse spinal cord enhancer region and conserved RARE sites (Fig. 2A and C). Further, both RARE sites were observed to contain complete sequence conservation between all tetrapods and shark, but some divergence in teleost fishes (Fig. 2C).

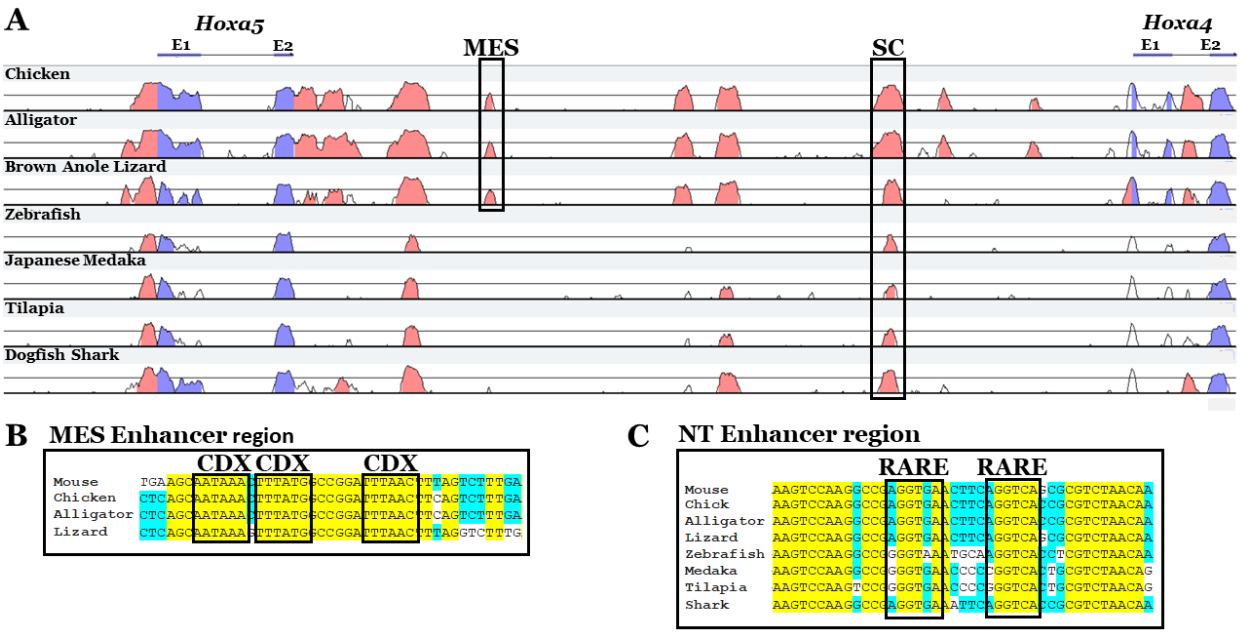

Figure 2: Genomic DNA sequence analysis of the intergenic region downstream of *Hoxa5* and upstream of *Hoxa4*. (A) mVISTA analysis of the intergenic region. All peaks correspond to DNA sequences that are conserved with the mouse genomic DNA reference sequence. Blue-shaded peaks and red-shaded peaks correspond to exons of *Hoxa5* and *Hoxa4* and conserved noncoding DNA sequences, respectively, that are at or above 70% conservation identity with respect to the mouse genomic DNA reference sequence. Uncolored peaks correspond to coding or noncoding sequences that are below 70% conservation identity with respect to the reference sequence. Regions of containing the mesoderm (MES) and spinal cord (SC) CREs were identified using mVISTA. Other conserved noncoding DNA peaks were not analyzed as these regions were not functionally tested in mouse. (B and C) Clustal Omega sequence alignment analysis of the conserved MES and SC enhancer regions. Boxed-in sequences correspond to functional CDX and RARE CREs characterized in mouse. Yellow coloring corresponds to 100% sequence conservation at specific nucleotide sites across all species analyzed. Blue coloring corresponds to nucleotides at specific sites that show less than 100% sequence conservation among all species but greater than 50% sequence conservation. Uncolored nucleotides correspond to divergent sequences. No sequences for shark or teleost fishes were added in 2B because no corresponding sequence was observed from the mVISTA analysis shown in 2A. CDX, caudal related homeobox *cis*-regulatory element; E1, exon 1; E2, exon 2; MES, Mesodermal enhancer region; RARE, retinoic acid response element; SC, spinal cord enhancer region;

DISCUSSION

Our expression pattern and comparative genomic DNA sequence analyses of brown anole *Hoxa5* show that this gene exhibits higher conservation in both gene expression and genomic DNA sequence with *Hoxa5* of other tetrapods than with shark and teleost fishes. Specifically, this gene is expressed within the spinal cord, somites, lateral plate mesoderm, and the fore and hindlimb buds, as seen in other tetrapod species. Further, while the spinal cord-specific RARE CREs show high sequence conservation among all species assayed, only the tetrapod genomic sequences exhibited conservation with the mesoderm-specific CDX sites. The presence of mesoderm-specific expression of shark *Hoxa5* but the lack of conservation with the mesdoderm-specific enhancer site for this gene suggests that divergent genomic DNA sequences are required for its expression (Oulion et al., 2011). However, CDX sites may still be functional in driving shark *Hoxa5* expression in the mesoderm but may be functioning from genomic regions that are divergent from tetrapods. Such results were observed for *Hoxb2*,

wherein regulatory elements that were conserved with their sequence were shown to be divergent in location and orientation between teleost fishes and tetrapods (Scemama et al., 2002). The lack of the mesoderm enhancer region was less surprising for *hoxa5a* in teleost fishes than for shark *Hoxa5*, as no *hoxa5a* genes have been observed to be expressed within the somites or lateral plate mesoderm (Davis and Stellwag, 2010; Hortopan and Baraban, 2011; Lyon et al., 2013). The loss of teleost *hoxa5a* mesodermal expression may be due to relaxed selection on the mesoderm enhancer following the whole genome duplication event in the lineage leading to the teleost fishes (Amores et al., 1998 and 2004; Crow et al., 2006; Hoegg et al., 2007; Moghadam et al., 2005; Prince, 2002; Stellwag, 1999). Such an event would have led to two teleost *Hoxa5* genes, *hoxa5a* and *hoxa5b*. Based on the gene expression data between teleost fishes and other gnathostome vertebrates, it is tantalizing to speculate that a subfunctionalization event occurred within the incipient *hoxa5a* and *hoxa5b* genes. This would have resulted in the partitioning of functions between these gene paralogs, such that the combined expression and function of both would resemble that of the ancestral *Hoxa5* gene (Force et al., 1999). Subfunctionalization has been shown to occur in other teleost gene duplicates, including gene paralogs *hoxb1a* and *hoxb1b* and *hoxb5a* and *hoxb5b* (Bruce et al., 2001; Hurley et al., 2007; McClintock et al., 2001 and 2002). In these instances post-genome duplication, complementary and degenerative mutations would have allowed for the preservation of gene duplicates since they recapitulated the expression and function of their respective ancestral *Hox* genes (Force et al., 1999). Consistent with this mechanism, the mesoderm-specific expression may have been allocated to *hoxa5b* prior to its deletion from the teleost genome. However, the loss of *hoxa5b* and its mesoderm-specific expression would not be detrimental to teleost development. *Hox* genes exhibit nested expression patterns along the anterior-posterior axis and have been shown to be involved in auto- and cross-regulatory activities in maintaining proper development of this axis (McGinnis and Krumlauf, 1992). Further, the occurrence of multiple *Hox* clusters, with four in non-teleost gnathostome vertebrates and at least seven in teleosts, has allowed for the redundancy of *Hox* gene expression along the anterior-posterior axis (Amores et al., 1998 and 2004; Hoegg et al., 2007; Moghadam et al., 2005; Prince, 2002; Stellwag, 1999). Therefore, the loss of mesoderm-expressing *hoxa2b* would not disrupt somite development or other mesoderm-specific structures in teleost fishes.

In contrast to the conserved spinal cord and mesoderm enhancer regions with tetrapods and shark and tetrapods only, respectively, brown anole *Hoxa5* exhibits a divergent anterior limit of expression within the somites from mouse, chicken, alligator, and shark. For instance, while brown anole *Hoxa5* is expressed most anteriorly within somite 6, *Hoxa5* genes of mouse, chicken, alligator, and shark are expressed most anteriorly in somites 3, 8, 9, and 9, respectively (Fig. 3) (Mansfield and Abzhanov, 2010; Oulion et al., 2011). The divergence in the somite-specific expression suggests that there are divergent sequences outside of the CDX sites responsible for directing these expression patterns. From our mVISTA analysis, we observed the presence of several conserved intergenic noncoding DNA regions outside of the mesoderm and spinal cord enhancer regions. It may be possible that one or several of these regions function in conjunction with the CDX sites in determining the exact location of the anterior limits of somite expression in mouse and other tetrapod species. In support, it has been shown that several genomic DNA sequences upstream of *Hoxa2* function in conjunction with a

Krox20 CRE in directing *Hoxa2* gene expression within rhombomeres 3 and 5 of the embryonic hindbrain (Maconochie et al., 2001). Similar genetic mechanisms may be employed for *Hoxa5* directed expression within the somites. Future functional genomic analyses should be performed to detect the CREs involved in driving these divergent gene expression patterns.

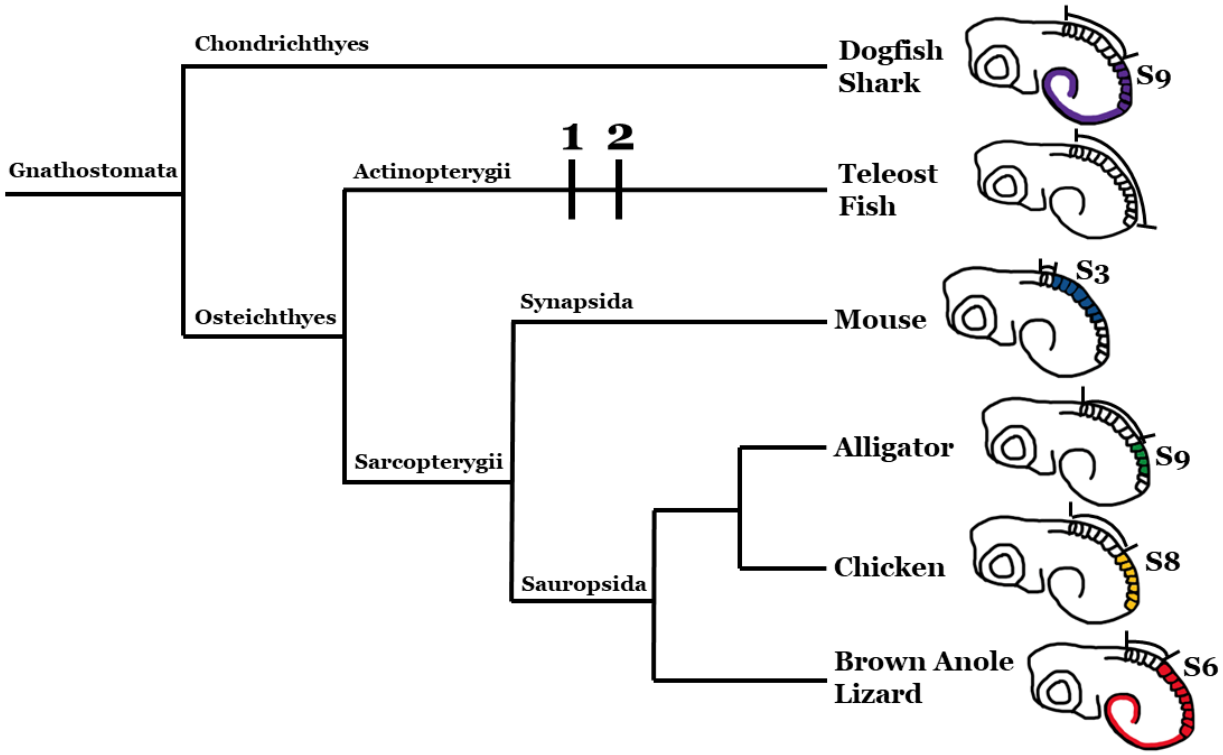

Figure 3: *Hoxa5* somite expression evolution in the Gnathostomata. Drawings represent left-facing pharyngula-stage embryos in organisms in which *Hoxa5* expression has been tested. Coloring and text associated with embryos represent the anterior limits of *Hoxa5* expression in the somites. Curved black lines with brackets over embryos denote non-*Hoxa5* expressing somites. (1) whole genome duplication event; (2) loss of *hoxa2b*. Phylogeny based on Pough et al. (2019). S; Somite.

References

Amores, A., A. Force, Y.L. Yan, L. Joly, C. Amemiya, A. Fritz, R.K. Ho, J. Langeland, V.E. Prince, and Y.L. Wang, M. Westerfield, M. Ekker, and J.H. Postlethwait. 1998. Zebrafish *hox* clusters and vertebrate genome evolution. Science 282(5394), 1711-1714.

Amores, A., T. Suzuki, Y.L. Yan, J. Pomeroy, A. Singer, C. Amemiya, and J.H. Postlethwait. 2004. Developmental roles of pufferfish *Hox* clusters and genome evolution in ray-fin fish. Genome Research, 14(1), 1-10.

Aubin J., M. Lemieux, M. Tremblay, J. Bérard, and L. Jeannotte. 1997. Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects. Developmental Biology 192(2), 432–445.

Aubin, J., M. Lemieux, M. Tremblay, R.R. Behringer, and L. Jeannotte . 1998. Transcriptional interferences at the *Hoxa4/Hoxa5* locus: Importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. Developmental Dynamics 212(1), 141–156.

Aubin, J., P. Chailler, D. Ménard, and L. Jeannotte. 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. American Journal of Phsiology 277(5), 965-973.

Aubin, J., U. Déry, M. Lemieux, P. Chailler, and L. Jeannotte. 2002. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. Development 129(17), 4075-4087.

Boucherat, O., J. Chakir, and L. Jeannotte. 2012. The loss of *Hoxa5* function promotes Notch-dependent goblet cell metaplasia in lung airways. Biology Open 1(7), 677-691.

Boucherat, O., S. Montaron, F.A. Bérubé-Simard, J. Aubin, P. Philippidou, D.M. Wellik, J.S. Dasen, and L. Jeannotte. 2013. Partial functional redundancy between *Hoxa5* and *Hoxb5* paralog genes during lung morphogenesis. American Journal of Physiology 304(12), 817-830.

Bruce, A.E., A.C. Oates, V.E. Prince, and R.K. Ho. 2001. Additional hox clusters in the zebrafish: divergent expression patterns belie equivalent activities of duplicate HoxB5 genes. Evolution and Development 3(3), 127-144.

Crow, K.D., P.F. Stadler, V.J. Lynch, C. Amemiya, and G.P. Wagner. 2006. The "fish-specific" Hox cluster duplication is coincident with the origin of teleosts. Molecular Biology and Evolution 23(1), 121–136.

Davis, A. and E.J. Stellwag. 2010. Spatio-temporal patterns of *Hox* paralog group 3-6 gene expression during Japanese medaka (*Oryzias latipes*) embryonic development. Gene Expression Patterns, 10(6), 244-250.

Davis, A., H. Nguyen, and J. Qian. 2019. Zebrafish embryos and bioinformatics: useful and marketable exercises for students enrolled in upper-level undergraduate courses. Eastern Biologist, Special Issue, 1, 47-63.

Force, A., M. Lynch, F.B. Pickett, A. Amores, Y.L. Yan, and J. Postlethwait. 1999. Preservation of duplicate genes by complimentary, degenerative mutations. Genetics 151(4), 1531-1545.

Frazer, K.A., L. Pachter, A. Poliakov, E.M. Rubin, and I. Dubchak. 2004. VISTA: computational tools for comparative genomics. Nucleic Acids Research 1(32), W273-279.

Garin, É., M. Lemieux, Y. Coulombe, G.W. Robinson, and L. Jeannotte. 2006. Stromal *Hoxa5* function controls the growth and differentiation of mammary alveolar epithelium. Developmental Dynamics 235(7), 1858-1871.

Gendronneau, G., O. Boucherat, J. Aubin, M. Lemieux, and > Jeannotte. 2012. The loss of *Hoxa5* function causes estrous acyclicity and ovarian epithelial inclusion cysts. Endocrinology 153(3), 1484-1497.

Hoegg, S., J.L. Boore, J.V. Kuehl, and A. Meyer. 2007. Comparative phylogenomic analyses of teleost fish Hox gene clusters: Lessons from the cichlid fish *Astatotilapia burtoni*. BMC Genomics 8, 317.

Hurley, I.A., J. Scemama, and V.E. Prince. 2007. Consequences of *Hoxb1* duplication in teleost fish. Evolution and Development 9(6), 540-554.

Hortopan, G.A. and S.C. Baraban. 2011. Aberrant expression of genes necessary for neuronal development and Notch signaling in in epileptic *mind bomb* zebrafish. Developmental Dynamics, 240(8), 1964–1976.

Jeannotte, L., M. Lemieux, J. Charron, F. Poirier, and E.J. Robertson. 1993. Specification of axial identity in the mouse: Role of the Hoxa-5 (Hox1.3) gene. Genes and Development 7(11), 2085-2096.

Jeannotte, L., F. Gotti, and K. Landry-Truchon. 2016. Hoxa5: A Key Player in Development and Disease. Journal of Developmental Biology 4(2), 13.

Kinkead, R., M. Leblanc, R. Gulemetova, M. Lalancette-Hebért, M. Lemieux, I. Mandeville, and L. Jeannotte. 2004. Respiratory adaptations to lung morphological defects in adult mice lacking *Hoxa5* gene function. Pediatric Research 56(4), 553-562.

Kreiger, K.E., M.A. Abbott, M. Joksimovic, PA. Lueth, I.M. Sonea, L. Jeannotte, and C.K. Tuggle. 2004. Transgenic mice ectopically expressing *Hoxa5* in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Research. Developmental Brain Research 150(2), 125-139.

Krumlauf R. 1994. Hox genes in vertebrate development. Cell 78:191– 201.

Lyon, R.S., A. Davis, and J.L. Scemama. 2013. Spatio-temporal expression patterns of anterior *Hox* genes during Nile tilapia (*Oreochromis niloticus*) embryonic development. Gene Expression Patterns 13(3–4), 104–108.

Maconochie, M.K., S. Nonchev, M. Manzanares, H. Marshall, and R. Krumlauf. 2001. Differences in *Krox20*-dependent regulation of *Hoxa2* and *Hoxb2* during hindbrain development. Developmental Biology 233(2), 468-481.

Madeira, F., Y.M. Park, J. Lee, N Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R.N. Tivey, S.C. Potter, R.D. Finn, R. Lopez. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Research 4(W1), W636-W641.

Mandeville, I., J. Aubin, M. LeBlanc, M. Lalancette-Hébert, M.F. Janelle, G.M. Tremblay, and L. Jeannotte. 2006. Impact of the loss of *Hoxa5* function on lung alveogenesis. Am Journal of Pathology 169(4), 1312-1327.

Mansfield, J. H. and A. Abzhanov. 2010. Hox expression in the American alligator and evolution of archosaurian axial patterning. Journal of Experimental Zoology (Molecular Developmental Evolution), 314B(8), 629–644.

Mayor, C., M. Brudno, J.R. Schwartz, A. Poliakov, E.M. Rubin, K.A. Frazer, L.S. Pachter, and I. Dubchak. 2000. VISTA: visualizing global DNA sequence alignments of arbitrary length. Bioinformatics 16(1), 1046-1047.

McClintock, J.M., R. Carlson, D.M. Mann, and V.E. Prince. 2001. Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes. Development 128(13), 2471-2484.

McClintock, J.M., M.A. Kheirbek, and V.E. Prince. 2002. Knockdown of duplicated hoxb1 genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. Development 129(10), 2339-2354.

McGinnis, W. and R. Krumlauf. 1992. Homeobox genes and axial patterning. Cell 68(2), 283-302.

Moghadam, H., M. Ferguson, and R. Danzmann. 2005. Evolution of Hox clusters in Salmonidae: A comparative analysis between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Journal of Molecular Evolution 61(5), 636-649.

Moreau, J. and L. Jeannotte. 2002. Sequence analysis of a *Hoxa4-Hoxa5* intergenic region including shared regulatory elements. DNA Sequence: the Journal of DNA Sequencing and Mapping 13(4), 203–209.

Meunier, D., J. Aubin, and L. Jeannotte. 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. Developmental Dynamics 227(3), 367-378.

Oulion, S., V. Borday-Birraux, M. Debiais-Thibaud, S. Mazan, P. Laurenti, and D. Casane. 2011. Evolution of repeated structures along the body axis of jawed vertebrates, insights from the *Scyliorhinus canicula* Hox code. Evolution and Development 13(3), 247–259.

Philippidou, P., C.M. Walsh, J. Aubin, L. Jeannotte, and J.S. Dasen. 2012. Sustained *Hox5* gene activity is required for respiratory motor neuron development. Nature Neuroscience 15(12), 1636-1644.

Pough, F.H., W.E. Bemis, B. McGuire, and C.M. Janis. 2019. Diversity, Classification, and Evolution of Vertebrates. Pp. 1-20, *In* F.H. Pough, W.E. Bemis, B. McGuire, and C. M. Janis (Eds.). Vertebrate Life. 11th ed. Oxford University Press, New York, NY. 585 pp.

Prince, V. 2002. The *Hox* paradox: More complex(es) than imagined. Developmental Biology 249(1), 1-15.

Rasys, A.M., S. Park, R.E. Ball, A.J. Alcala, J.D. Lauderdale, and D.B. Menke. 2019. CRISPR-Cas9 gene editing in lizards through microinjection of unfertilized oocytes. Cell Reports 28(9), 2288-2292.

Sanger, T.J., J.B. Losos, and J.J. Gibson-Brown. 2008. A developmental staging series for the lizard genus *Anolis*: A new system for the integration of evolution, development, and ecology. Journal of Morphology 269(2), 129-137.

Stellwag, E.J. 1999. Hox gene duplication in fish. Seminars in Cell and Developmental Biology 10(5), 531-540.

Tabariès, S., J. Lapointe, T. Besch, M. Carter, J. Woollard, C.K. Tuggle, and L. Jeannotte. 2005. Cdx protein interaction with *Hoxa5* regulatory sequences contributes to *Hoxa5* regional expression along the axial skeleton. Molecular and Cellular Biology 25(4), 1389–1401.

Tajbakhsh, S. and R. Spörle. 1998. Somite development: constructing the vertebrate body. Cell 92(1), 9-16.