What's Got Zebrafish in a Twist? Characterizing *twist1b* Cis-Regulatory Activity in the Neural Crest of *Danio rerio*

By

Louis Balas

University of Colorado Boulder

A thesis submitted to the

University of Colorado Boulder

In partial fulfillment

Of the requirements to receive

Honors designation in

Department of Molecular, Cellular, and Developmental Biology

Defense date: April 12, 2023

Thesis Advisor

Daniel Medeiros, Ph.D., Department of Ecology and Evolutionary Biology

Honors Council Representative

Kristin Moore Ph.D., Department of Molecular and Cellular Developmental Biology

Outside Reader

Abby Hickcox Ph.D., Arts and Sciences Honors Program

Abstract

Neural Crest Cells (NCC) and their derivatives form many craniofacial structures unique to vertebrates. These structures allowed vertebrates to transition from filter-feeders to predators and are thought to be some of the main drivers of vertebrates' evolutionary success. How NCC evolved in ancestral vertebrates remains unclear. However, it is thought that the recruitment of *twist* genes to the underlying gene-regulatory network (GRN) of the neural plate border (NPB) drove NCC evolution. We hypothesize that mutations in cis-regulatory elements (CREs) of ancestral *twist* genes may have facilitated their specific expression at the NPB. This study identified and tested two potential CREs of *twist1b* in a representative vertebrate, *Danio rerio* (zebrafish), using enhancer detection vectors mediated by tol2 transposon. We also compared the efficiency of two different types of these detection vectors, pGreenE and Twist1b-pGreenE, for resolving enhancer activity *in-vivo*. Zebrafish transfected with candidate CREs in pGreenE demonstrated low frequencies of *twist1b*-like expression patterns in neural-crest-derived tissue during early development, while no reporter activity was detected in zebrafish transfected with CREs in the Twist1b-pGreenE vector. These results suggest that the tested CREs may be involved in regulating *twist1b* expression, specifically in the neural crest. However, future efforts should be oriented towards confirming the role these CREs play in the regulation of *twist1b.*

Introduction

It has long been thought that the emergence of neural crest cells (NCC) drove the origin and diversification of the vertebrate clade (Gans and Northcutt 1983). This proposal, coined the 'new head' hypothesis, is based on the neural crest's (NC) role in forming many craniofacial structures and sensory systems unique to vertebrates. These structures and their derivatives are

believed to have facilitated the evolutionary transition from a filter-feeder lifestyle to the predatory lifestyle that distinguishes vertebrates from their inveterate chordate relatives (Martik and Bronner 2021; Sauka-Spengler and Bronner-Fraser 2008). In support of this notion, definitive NCC have been identified in the most basal extant vertebrates but not in their living invertebrate relatives, urochordates (tunicates) and cephalochordates (amphioxus) (Meulemans, McCauley, and Bronner-Fraser 2003; York and McCauley 2020; Yu et al. 2008). Thus, it appears that the NC is a uniquely vertebrate innovation. Understanding the evolutionary and molecular origins of the NC is essential to understanding vertebrate evolution.

The NC describes a group of multipotent, migratory cells that originate at the dorsal-most aspect of the forming embryonic central nervous system, from which they then detach, undergo an epithelial-to-mesenchymal transition (EMT), and then migrate extensively throughout the embryo to form a multitude of different cell types (Munoz and Trainor 2015; York and McCauley 2020). These cell types give rise to structures such as the bone and cartilage of the facial skeleton, neurons and glia of the cranial ganglia, smooth muscle and pigment cells, components of the heart and endocrine systems, and much more (Munoz and Trainor 2015). A combination of interacting molecular signals, transcription factors, and downstream effector genes regulate each stage of NCC development and specification in a series of circuits and feedback loops collectively anointed the neural crest gene regulatory network (NC-GRN). The NC-GRN initiates NC development with inductive signals that establish the neural plate border (NPB) and indirectly upregulate a key set of genes called NC-specifiers. These NC-specifiers, including *Snail, FoxD3, AP-2, Id, Sox9/10*, and *Twist,* encode for transcriptional regulators that confer multipotency and migratory properties to NCC cells; defining features of NCC (Martik and Bronner 2021; Sauka-Spengler and Bronner-Fraser 2008).

One particularly influential component of the NC-GRN is the neural-crest specifier *twist*. *Twist* genes encode transcription factors essential for embryonic development and are conserved widely across the metazoa (Germanguz et al. 2007). In most vertebrates, *twist* transcripts are expressed early in development at the neural plate border and mediate the EMT, confer migratory potential to NCC, and later help reconfigure the cytoskeleton (Barriga et al. 2013). Homologs of *twist* genes have also been found in the invertebrate chordates, where they are expressed in the mesoderm. However, *twist* expression is missing at the NPB and most of the ectoderm of both tunicates and amphioxus (Cheung et al. 2019; Meulemans and Bronner-Fraser 2005, 2007). This absence of *twist* expression at the NPB of the invertebrate chordates, and the lack of definitive neural crest in these organisms, suggests that integration of *twist* into the underlying regulatory network at the NPB facilitated the evolution of the neural crest. In support of this notion, forced expression of *twist* in tunicate embryos induced long-range, NC-like migration of the a9.49 melanocyte lineage and reprogramed melanocyte progenitors into mesodermal derivatives (Abitua et al. 2012). Thus, the co-option of *twist* to the vertebrate NC-GRN represents a possible way NCC evolved their capacity for long-range migration.

It is unclear how *twist* was integrated into the NC-GRN. We hypothesize that mutations in cis-regulatory elements (CREs) of ancestral twist genes directed the expression of *twist* at the neural plate border. CREs are composed of sequences of non-coding DNA that regulate the expression of neighboring genes. These CREs can, in a highly specific manner, limit the expression of a gene to certain tissue types or developmental stages and are now thought to be the most prevalent cause of morphological divergence (Wittkopp and Kalay 2012). As such, mutations in these CREs (CRE divergence) can direct the expression of an associated gene in new cells or tissues. Transgenic and whole-genome regulatory comparisons between amphioxus

and vertebrates have revealed that amphioxus lacks the CREs necessary to direct the expression of NC-specifiers SoxE and FoxD3 in the vertebrate NC (Jandzik et al. 2015; Marlétaz et al. 2018; Yu, Holland, and Holland 2004). This suggests these CREs may have evolved during early vertebrate evolution to direct SoxE and FoxD3 expression in the NC. Thus, there is a precedent for the role of CRE divergence in the evolution of NCC.

Investigating the role of CRE divergence in the co-option of *twist* to the NC border ultimately involves identifying CREs in an invertebrate chordate (amphioxus), a more basal vertebrate (lamprey), and a more derived vertebrate (zebrafish), and comparing the expression patterns of those CREs in those organisms. We expect that vertebrates possess CREs not found in amphioxus that evolved to drive expression of *twist* at the NPB. These CREs could have evolved via many different avenues. A completely new NC CRE could have emerged near the *twist* locus - via transposition or point mutations - to drive the expression of *twist* in the ectoderm. Or, a CRE found in amphioxus could have mutated to drive *twist* NPB expression while retaining its old expression patterns. Through multiple rounds of genome duplication, a CRE regulating *twist* in amphioxus could also have acquired enough paralogues to produce enough of a synergistic effect on the *twist* gene to enforce its expression at the NPB. Alternatively, no CRE evolution could have occurred, and some other gene-regulating *twist* could have moved to the ectoderm and brought *twist* with it. Comparing the different *twist* CREs in these three organisms will help determine which of these "avenues" occurred in the context of the NC. Additionally, it will give us insight into how CREs evolve.

My project aims to initiate this comparison process by discovering and characterizing the activity of *twist1b* CREs in a more derived vertebrate, *Danio rerio* (zebrafish). Four different twist genes (*twist1a*, 1b, 2, 3) are found in zebrafish and are thought to have arisen through three

rounds of gene duplication. Some of these genes retained more ancestral expression patterns, while others diverged significantly. *Twist1b* captures more ancestral patterns of *twist* found in the basal vertebrate lamprey and thus serves as a good candidate to investigate the evolution of *twist* CREs during early vertebrate history (Martik and Bronner 2021). If the expression of candidate *twist1b* CREs recapitulates similar expression patterns of native *twist1b* transcripts in the NC-derived tissue (ectoderm) of the developing zebrafish embryo, these CREs most likely play a role in directing the expression of *twist1b* to the NC.

Experimental Approach

To identify candidate *twist1b* CREs in zebrafish, we leveraged data generated by a genomic analysis tool called ChromHMM that uses DNA sequencing data to predict the probability of a DNA sequence being a CRE. Candidate *twist1b* CREs were selected, synthesized, and tested for their expression patterns in zebrafish using two different enhancer detection vectors mediated by tol2. Enhancers are a type of CRE that increase the expression of neighboring genes. These enhancer-detection vectors contain a candidate CRE, a reporter gene, and a gene encoding for the tol2 transposon. These vectors can be integrated into the genome of zebrafish via their injection into the single-cell stage of a zebrafish embryo with tol2 transposase mRNA. After the establishment of transgenic zebrafish, some sort of fluorescent protein will be produced in tissues where that CRE is active. Typically, enhancer detection vectors rely on using some non-specific, minimal promoter (Gata, Myc, Krt4, cFos) to drive the expression of the chosen reporter gene.

However, there is evidence that there is a degree of compatibility between different enhancers and minimal promoters and that, therefore, minimal promoters may not accurately

capture endogenous patterns of enhancer activity (Bessa et al. 2009; Chan et al. 2019). Additionally, to identify differences in orthologous enhancers across species (as we aim to do with *twist* enhancers), the enhancer detection vector must have high degrees of accuracy, as differences between orthologous enhancers may be subtle. Thus, we tested the activity of *twist1b* CREs using two different vectors. Both vectors contain genes encoding for the tol2 transposon and a gene encoding for green fluorescent protein (GFP). The first vector, pGreenE, is driven by the minimal cFos promoter. The second vector, the novel Twist1b-pGreenE, is driven by the native *twist1b* promoter. We developed Twist1b-pGreenE to investigate whether using a promoter specific to the CRE being tested improves the fidelity and specificity of captured expression patterns. Candidate CREs were integrated into each vector and injected into the embryos of developing zebrafish, and zebrafish were assayed for patterns of GFP expression.

Results

Two candidate CREs were computationally selected to be tested *in vivo*

We began identifying candidate *twist1b* CREs by establishing a "putative regulatory landscape" that would best represent the genomic regions most likely to house CREs related to *twist1b.* The boundaries of these regulatory landscapes are thought to be established by the interactions of topologically associated domains (TADs), which are regions of DNA that preferentially contact each other to facilitate the interaction of CREs (Tena and Santos-Pereira 2021). The boundaries of these TADs are often demarcated by the binding of CTCF transcription factors (Kentepozidou et al. 2020). Using a combination of CTCF CHIP-Seq data and Hi-C sequencing tracks from the UCSC zebrafish genome browser, we identified the boundaries of TADs to characterize this regulatory landscape (Figure 1A). Peaks in the CTCF track represent

CTCF binding at that sequence. Transitions from red to blue in the Hi-C tracks represent potential TAD boundaries. The regulatory region can be located on the UCSC genome browser by inputting chr16: 19,951,883 - 19,503,828 into the BLAT function.

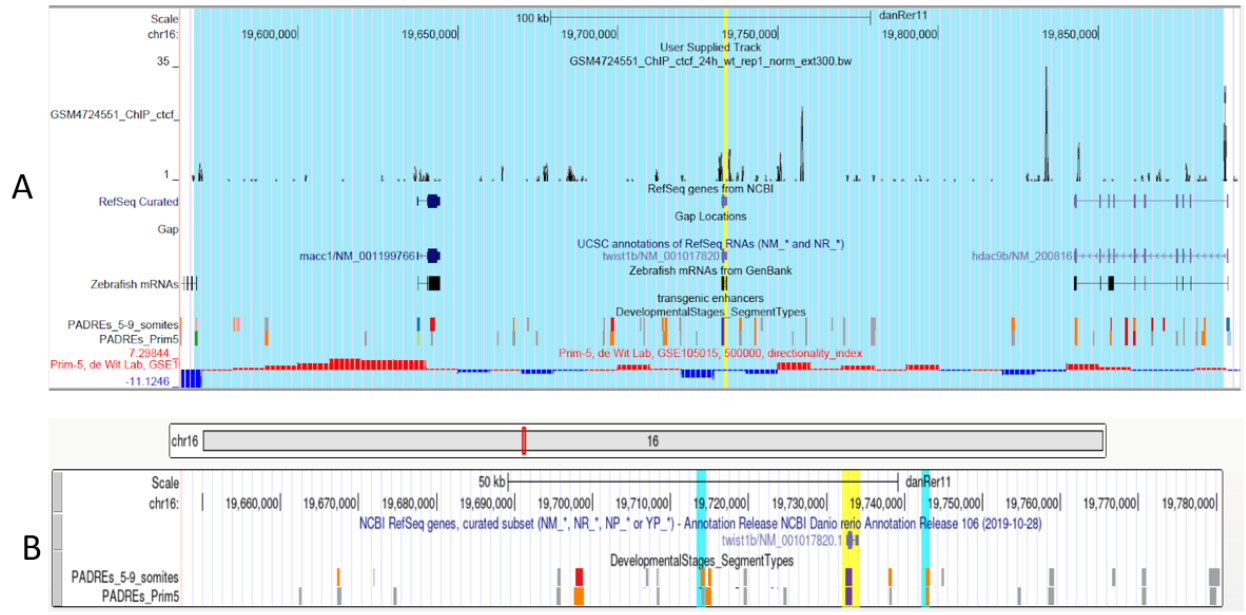

Figure 1. Identification of CRE candidates within the *twist1b* regulatory landscape.
A) Putative *twist1b* regulatory landscape mapped in light blue in the UCSC genome browser. The *twist1b* locus is highlighted in yellow. CTCF peaks are represented in the top tracks, and Hi-C peaks in the bottom track. B) Selected candidate enhancers are highlighted in light blue on the UCSC genome browser. The light blue highlighted regions demonstrate the overlap between predicated CRE sequences and PADRE annotations. The *twist1b* locus is highlighted in yellow.

To identify potential CREs related to *twist1b,* we screened different sequences annotated by the PADRE track in the UCSC zebrafish genome browser within the putative regulatory landscape. The PADRE track represents ATAC-Seq supported predicted developmental regulatory elements annotated by ChromHMM categories (Baranasic et al. 2022). ChromHMM is a tool that can calculate the most probable regulatory state of a segment of non-coding DNA using different DNA sequencing data (Ernst and Kellis 2017). Two different sequences annotated by the PADRE's track were selected to be tested in zebrafish based on their distance from the

twist locus and their annotation score (i.e., the likelihood they represent a regulatory element) (Figure 1B). The two CREs were named CRE1 and CRE7. The DNA sequences of the candidate CREs can be found via the BLAT function. CRE1: chr16: 19713678 - 19714565 and CRE7: chr16: 19742539-19743410.

Embryos transfected with PGreene but not PGreene-Twist1b demonstrate low frequencies of *twist1b-like* expression in the ectoderm

To determine if the candidate CREs are active in a spatiotemporal manner specific to *twist1b* transcripts, CRE1 and CRE7 were injected into the single-cell stage of zebrafish embryos in the pGreenE and Twist1b-pGreenE vector with tol2 mRNA and imaged at 10 and 30 hours post fertilization (hpf). Of the 51 surviving embryos injected with pGreenE-CRE1, 33% showed positive expression for GFP at both developmental stages. However, only 14% of injected embryos demonstrated expression patterns congruent with the expression of *twist1b* transcripts in the ectoderm at 30hpf (Figure 2E). These expression patterns include an increased GFP signal in tissues around the eyes and the forebrain blood vessels (fbv). GFP expression was also seen in the myotomes and yolk of these embryos (2B). In the GFP-positive embryos that did not recapitulate *twist1b* expression patterns, non-specific expression was seen in the developing embryo's gut, yolk, and myotome at 30hpf (Figure 2E). Embryos imaged at 10hpf showed less distinct signs o*f twist1b*-like expression. Predominantly, non-specific GFP expression was seen in the yolk. However, there were consistent but faint signs of GFP in the forming somites, but whether this is related to the activity of *twist1b* is unclear (Figure 2B).

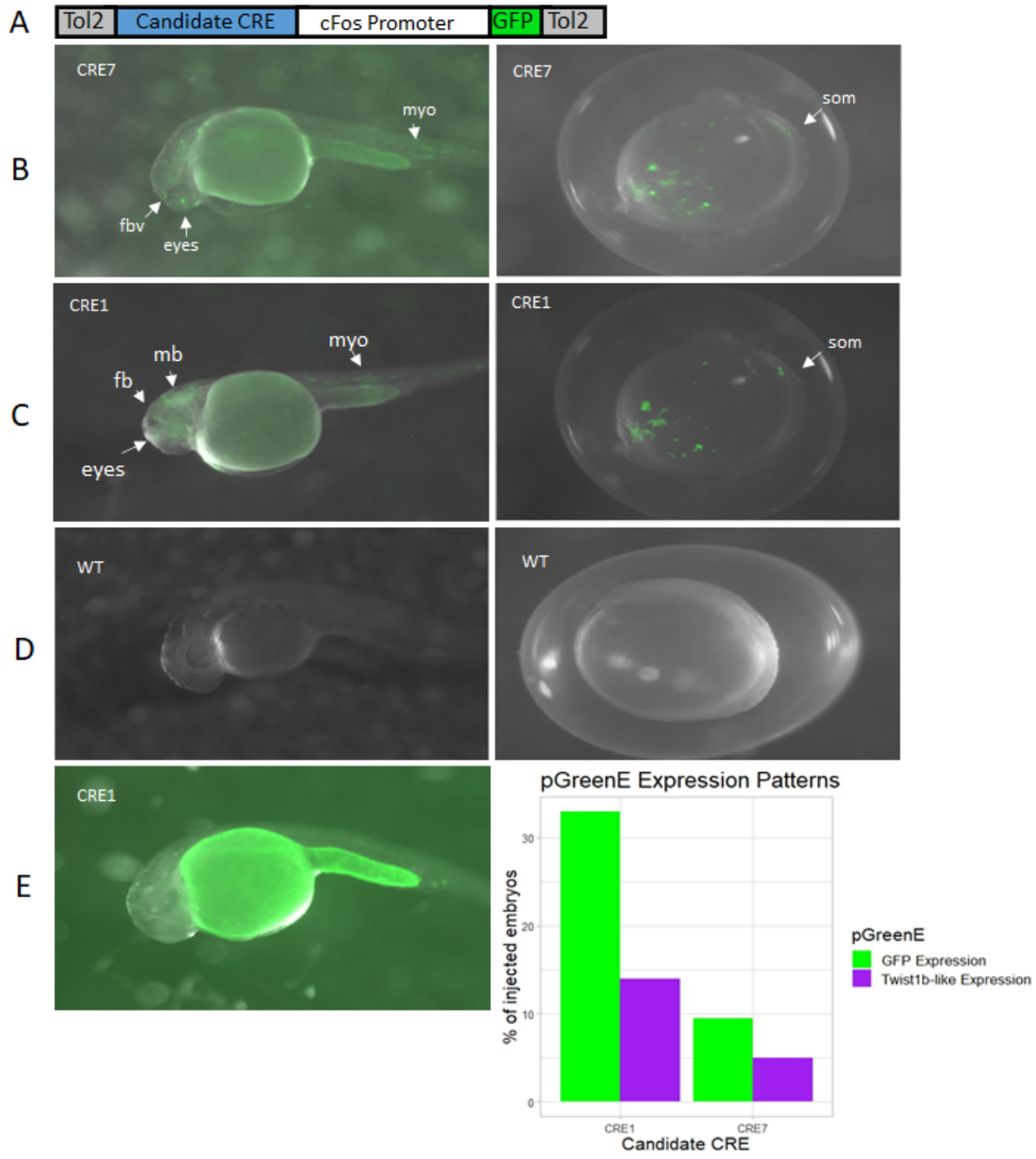

Figure 2. Zebrafish transfected with pGreenE-CRE7 and pGreenE-CRE1 demonstrate low frequencies of *twist1b*-like expression. A) Diagram of pGreenE enhancer detection vector. Views B-D show embryos at 30hpf on the left and at 10hpf on the right. B) pGreenE-CRE7 transgenic zebrafish. C) pGreenE-CRE1 transgenic zebrafish. D) Wildtype zebrafish. E) pGreeneE-CRE1 transgenic zebrafish demonstrating non-specific GFP expression (left). Barchart depicting relative levels of *Twist-1b*-like expression in GFP+ zebrafish (right). Fbv,forebrain blood vessels; myo, myotome; som, somites; fb, forebrain; mb, midbrain.

Of the 21 surviving embryos injected with pGreenE-Cre7, 9% of embryos showed GFP-positive expression, with 5% showing *twist1b*-like expression in the ectoderm (Figure 2E). This includes expression in the forebrain (fb) and midbrain crest (mb), and tissues around the eye. In the other GFP-positive embryo, non-specific expression was seen in the gut and yolk. Embryos injected with CRE7 and visualized at 10hpf recapitulated similar expression patterns to those of CRE1, with predominant expression seen in the yolk and faint levels of expression in the forming somites.

Of the 58 surviving embryos injected with CRE1 in Twist1b-pGreenE, no detectable signs of GFP were observed at 10hpf or 30hpf. Similarly, of the 33 embryos injected with CRE7 in the same vector, no signs of GFP were observed at either developmental stage.

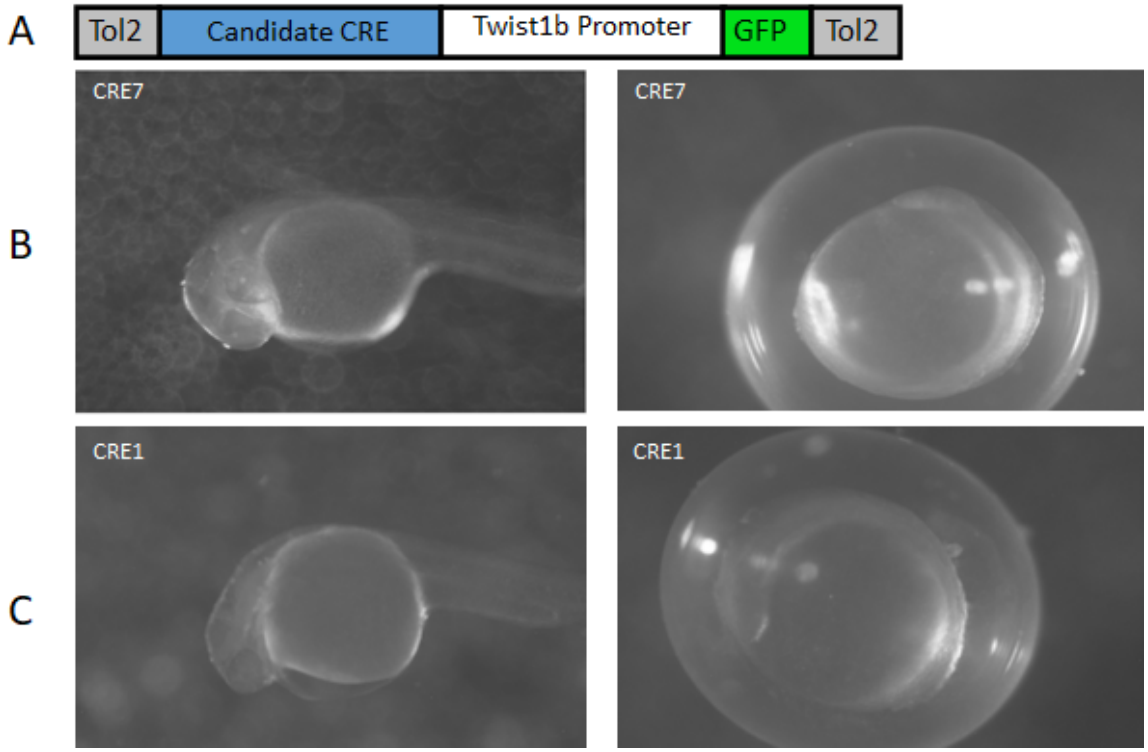

Figure 3. Zebrafish transfected with Twist1b-pGreenE-CRE1 and Twist1b-pGreenE-CRE7 demonstrate no GFP-positive expression. A) Diagram of Twist1b-pGreenE enhancer detection vector. Views B-C show zebrafish at 30hpf on the left and at 10hpf on the right. B) Twist1b-pGreenE-CRE7 transgenic zebrafish. C) Twist1b-pGreenE-CRE1 transgenic zebrafish.

Discussion

This study aimed to identify and test the expression patterns of candidate CREs related to *Twist1b* using two different enhancer-detection vectors: pGreene and pGreene-Twist1b. Two different CRE candidates were identified in the "putative twist*1b* regulatory domain" using ChromHMM-derived PADRE data and functionally tested in zebrafish. Both CREs 1 and 7 demonstrated low frequencies of *twist1b*-like expression patterns in embryos injected with pGreenE 30hpf (>30%) (Figure 2B-C,E). This suggests that these CREs play a role in regulating the expression of *twist1b*. Additionally, since CRE1 and CRE7 recapitulate native *twist1b* expression patterns in the ectoderm to some extent, they may be involved in directing *twist1b* expression in the NC. However, both CREs also exhibited strong GFP signals in the myotome, the compartment that gives rise to skeletal muscle. *Twist1b* transcripts are expressed in the myotome earlier in development, but expression fades after 15hpf (Germanguz et al. 2007). It could be that the GFP persisting in the differentiating muscle fibers is related to *twist1b* activity earlier in development. However, the GFP expression could also be driven by the non-specific activity of the cFos promoter. Indeed, a large proportion of the embryos positive for GFP saw non-specific patterns of GFP expression in the yolk and gut (Figure 2E). Embryos visualized at 10hpf demonstrated similar results, with high levels of GFP expression in the yolk and minimal expression in the forming somites (Figure 2B-C). As such, it is hard to determine if the expression of CRE1 and CRE7 in the pGreenE vector is specific to NC-derived tissues.

Ideally, for statistical significance, at least 100 embryos should be injected per vector in two different injection experiments (Fisher et al. 2006). Unfortunately, due to the spawning habits of the zebrafish we were using, we were unable to obtain more than an average of 40 surviving embryos per construct over the course of the two injection experiments. Therefore, the

expression patterns observed in this experiment should be verified with higher populations of injected embryos. In addition to injecting more embryos, future experiments should incorporate a negative control in which pGreenE is injected without any candidate CRE to determine if the cFos promoter alone is driving significant non-specific expression. This will help confirm whether CRE1 and CRE7 have *twist1b*-like expression patterns during early zebrafish development.

The Twist1b-pGreenE vector was constructed to reduce the non-specific expression seen with enhancer-detection vectors driven by minimal promoters such as cFos. Interestingly, the Twist1b-pGreenE vector failed to promote GFP expression in embryos injected with either CRE. It is possible that a single CRE is insufficient to activate the more selective *twist1b* promoter and that activation of the *twist1b* promoter requires the synergistic influence of multiple enhancers working together. However, it seems improbable that *twist1b* is so tightly regulated that no GFP expression could escape throughout early development, especially given the abundance of *twist1b* transcripts during early development. It is possible that the Twist1b-pGreenE vector has a mutation or is incorrectly constructed so that the tol2 transposon can't properly integrate the target vector into the zebrafish genome. Future efforts will be dedicated to resequencing the vector to ensure deficiencies with the construct itself aren't responsible for the absence of GFP expression in injected zebrafish.

The eventual goal of this project is to develop the capabilities for testing many different *twist* CREs in zebrafish and, eventually, amphioxus and lamprey. This will allow us to address the question of how *twist* was recruited to the NC-GRN and, ultimately, the bigger question: how NCC evolved. Given that CRE1 and CRE7 did recapitulate some degree of NC/*twist1b-like* expression patterns, it seems reasonable to argue that ChromHMM might be a useful tool to

screen and test for other CRE candidates in the future. We've designed the primers for over 20 ChromHMM-identified PADREs related to twist*1b* and *twist2* and aim to synthesize the sequences via PCR for eventual *in vivo* testing in zebrafish. However, our first priority is establishing an adequate enhancer vector that can accurately and specifically capture the expression patterns of different *twist* CREs. This will help us make more confident conclusions about how *twist* was recruited to NC.

Methods

Fish strain and Husbandry

Zebrafish embryos used in microinjection were F1 or F2 progeny of wild-type adults obtained from a commercial supplier. Embryos were raised in tissue culture plates in 30% Daneiu's medium at 28.5°C (Jackman, Draper, and Stock 2004).

Identification of candidate cis-regulatory elements

A putative regulatory landscape was established around the *twist1b* locus using boundaries established by CTCF CHIP-seq and Hi-C tracks on the zebrafish genome browser. Candidate cis-regulatory elements were selected by analyzing DNA sequences annotated by the PADRE track in the UCSC genome browser. PADREs were evaluated for their distance to the *twist1b* locus and their annotation score. The chosen sequences were padded to contain an additional 200bp on the 5' and 3' ends to ensure all potential regulatory activity was captured.

Cloning of regulatory elements into PGreene and PGreene Twist1b

The selected candidate CREs were synthesized by Twist Bioscience with the addition of attB binding sites for cloning into the two plasmids. CRE1 and CRE7 were cloned into the pGreene and pGreenE-Twist1b vectors via the gateway cloning process with DH5 alpha-competent E.coli.

After transformation, colonies were plated on LB agar plates with ampicillin (100ug/ml) and incubated at 37°C overnight. Colony PCR was performed with the sequencing primers 47-cFosRseq: gCTgTgAATggATggACTTCC and 48-3'Tol2Rseq: gCAgAgACTCCCTggTgTCTg to confirm the presence of the constructs in transformed cells. Two colonies were picked from each plate and grown up in LB liquid media overnight at 37°C in a shaking incubator. The colonies were then processed with the Omega BIO-TEK E.Z.N.A Plasmid DNA Miniprep Kit, and DNA concentrations were verified using a Thermo Scientific NANODROP 2000 spectrophotometer. The plasmids with the candidate CREs were then sent to Quintara Biosciences for sequencing. After sequence verification, the vectors were prepared for microinjection via phenol-chloroform extraction.

Transgenic Enhancer Assay

Transgenic lines of zebrafish were created by microinjection of the two enhancer detection vectors with tol2 mRNA using standard procedures (Clark et al. 2011). Two different injection experiments were conducted during two different weeks for redundancy, and all four constructs (CRE1 and CRE7 in pGreenE and Twist1b-pGreeneE) were injected in parallel. Uninjected embryos acted as a control. Following microinjection, embryos were visualized 10 hours post-fertilization for GFP expression using a Zeiss Discovery V8 SteREO Dissecting Microscope. After visualization at 10hpf, embryos were anesthetized using 1ml of tricaine per dish and dechorionated for visualization at 30hpf.

Acknowledgments

Thank you to all members of my thesis committee, including Drs. Daniel Medeiros, Abby Hickcox, and Kristen Moore, for your support and interest in my work. It has been a pleasure to

meet and work with you all during my time at the University of Colorado at Boulder. Thank you to Dr. David Jandzick and (soon-to-be Dr.) Lindsey Ray for your mentorship and hands-on support throughout the thesis process, I couldn't have done it without you!

Works Cited

Abitua, Philip Barron, Eileen Wagner, Ignacio A. Navarrete, and Michael Levine. 2012. "Identification of a Rudimentary Neural Crest in a Non-Vertebrate Chordate." *Nature* 492(7427): 104–7.

Baranasic, Damir et al. 2022. "Multiomic Atlas with Functional Stratification and Developmental Dynamics of Zebrafish Cis-Regulatory Elements." *Nature Genetics* 54(7): 1037–50.

Barriga, Elias H., Patrick H. Maxwell, Ariel E. Reyes, and Roberto Mayor. 2013. "The Hypoxia Factor Hif-1α Controls Neural Crest Chemotaxis and Epithelial to Mesenchymal Transition." *Journal of Cell Biology* 201(5): 759–76.

Bessa, José et al. 2009. "Zebrafish Enhancer Detection (ZED) Vector: A New Tool to Facilitate Transgenesis and the Functional Analysis of Cis-Regulatory Regions in Zebrafish." *Developmental Dynamics: An Official Publication of the American Association of Anatomists* 238(9): 2409–17.

Chan, Shuheng et al. 2019. "Development of Enhancer-Trapping and -Detection Vectors Mediated by the Tol2 Transposon in Zebrafish." *PeerJ* 7: e6862.

Cheung, Martin, Andrew Tai, Peter Jianning Lu, and Kathryn SE Cheah. 2019. "Acquisition of Multipotent and Migratory Neural Crest Cells in Vertebrate Evolution." *Current Opinion in Genetics & Development* 57: 84–90.

Clark, Karl J., Mark D. Urban, Kimberly J. Skuster, and Stephen C. Ekker. 2011. "Chapter 8 - Transgenic Zebrafish Using Transposable Elements." In *Methods in Cell Biology*, The Zebrafish: Genetics, Genomics and Informatics, eds. H. William Detrich, Monte Westerfield, and Leonard I. Zon. Academic Press, 137–49.

https://www.sciencedirect.com/science/article/pii/B9780123748140000082 (April 6, 2023).

Ernst, Jason, and Manolis Kellis. 2017. “Chromatin-State Discovery and Genome Annotation with ChromHMM.” *Nature Protocols* 12(12): 2478–92.

Fisher, Shannon et al. 2006. “Evaluating the Biological Relevance of Putative Enhancers Using Tol2 Transposon-Mediated Transgenesis in Zebrafish.” *Nature Protocols* 1(3): 1297–1306.

Gans, Carl, and R. Glenn Northcutt. 1983. “Neural Crest and the Origin of Vertebrates: A New Head.” *Science* 220(4594): 268–73.

Germanguz, Igal et al. 2007. “Four Twist Genes in Zebrafish, Four Expression Patterns.” *Developmental Dynamics* 236(9): 2615–26.

Jackman, William R., Bruce W. Draper, and David W. Stock. 2004. “Fgf Signaling Is Required for Zebrafish Tooth Development.” *Developmental Biology* 274(1): 139–57.

Jandzik, David et al. 2015. “Evolution of the New Vertebrate Head by Co-Option of an Ancient Chordate Skeletal Tissue.” *Nature* 518(7540): 534–37.

Kentepozidou, Elissavet et al. 2020. “Clustered CTCF Binding Is an Evolutionary Mechanism to Maintain Topologically Associating Domains.” *Genome Biology* 21(1): 5.

Marlétaz, Ferdinand et al. 2018. “Amphioxus Functional Genomics and the Origins of Vertebrate Gene Regulation.” *Nature* 564(7734): 64–70.

Martik, Megan L., and Marianne E. Bronner. 2021. “Riding the Crest to Get a Head: Neural Crest Evolution in Vertebrates.” *Nature Reviews Neuroscience* 22(10): 616–26.

Meulemans, Daniel, and Marianne Bronner-Fraser. 2005. “Central Role of Gene Cooption in Neural Crest Evolution.” *Journal of Experimental Zoology Part B: Molecular and*

Developmental Evolution 304B(4): 298–303.

Meulemans, Daniel, and Marianne Bronner-Fraser. 2007. “Insights from Amphioxus into the Evolution of Vertebrate Cartilage.” *PLoS ONE* 2(8): e787.

Meulemans, Daniel, David McCauley, and Marianne Bronner-Fraser. 2003. “Id Expression in Amphioxus and Lamprey Highlights the Role of Gene Cooption during Neural Crest Evolution.” *Developmental Biology* 264(2): 430–42.

Munoz, William A., and Paul A. Trainor. 2015. “Neural Crest Cell Evolution: How and When Did a Neural Crest Cell Become a Neural Crest Cell.” In *Neural Crest and Placodes*, ed. P. A. Trainor. San Diego: Elsevier Academic Press Inc, 3–26. http://gateway.webofknowledge.com/gateway/Gateway.cgi?GWVersion=2&SrcAuth=DynamicDOIArticle&SrcApp=WOS&KeyAID=10.1016%2Fbs.ctdb.2014.11.001&DestApp=DOI&SrcAppSID=5FieMnf86lrQT3Kshzm&SrcJTitle=NEURAL+CREST+AND+PLACODES&DestDOIRegistrantName=Elsevier (February 21, 2022).

Sauka-Spengler, Tatjana, and Marianne Bronner-Fraser. 2008. “Evolution of the Neural Crest Viewed from a Gene Regulatory Perspective.” *genesis* 46(11): 673–82.

Tena, Juan J., and José M. Santos-Pereira. 2021. “Topologically Associating Domains and Regulatory Landscapes in Development, Evolution and Disease.” *Frontiers in Cell and Developmental Biology* 9. https://www.frontiersin.org/articles/10.3389/fcell.2021.702787 (April 6, 2023).

Wittkopp, Patricia J., and Gizem Kalay. 2012. “Cis-Regulatory Elements: Molecular Mechanisms and Evolutionary Processes Underlying Divergence.” *Nature Reviews Genetics* 13(1): 59–69.

York, Joshua R., and David W. McCauley. 2020. “The Origin and Evolution of Vertebrate Neural

Crest Cells." *Open Biology* 10(1): 190285.

Yu, Jr-Kai, Nicholas D. Holland, and Linda Z. Holland. 2004. "Tissue-Specific Expression of FoxD Reporter Constructs in Amphioxus Embryos." *Developmental Biology* 274(2): 452–61.

Yu, Jr-Kai, Daniel Meulemans, Sonja J. McKeown, and Marianne Bronner-Fraser. 2008. "Insights from the Amphioxus Genome on the Origin of Vertebrate Neural Crest." *Genome Research* 18(7): 1127–32.