Identification of Neural Crest and Glial Enhancers at the Mouse Sox10 Locus through Transgenesis in Zebrafish

Anthony Antonellis1, Jimmy L. Huynh2, Shih-Queen Lee-Lin1, Ryan M. Vinton2, Gabriel Renaud1, Stacie K. Loftus3, Gene Elliot3, Tyra G. Wolfsberg1, Eric D. Green1, Andrew S. McCallion2, William J. Pavan3

1 Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 2 McKusick–Nathans Institute of Genetic Medicine, Department of Molecular and Comparative Pathobiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 3 Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Sox10 is a dynamically regulated transcription factor gene that is essential for the development of neural crest–derived and oligodendroglioblast populations. Developmental genes often require multiple regulatory sequences that integrate discrete and overlapping functions to coordinate their expression. To identify Sox10 cis-regulatory elements, we integrated multiple model systems, including cell-based screens and transposon-mediated transgenesis in zebrafish, to scrutinize mammalian conserved, noncoding genomic segments at the mouse Sox10 locus. We demonstrate that eight of 11 Sox10 genomic elements direct reporter gene expression in transgenic zebrafish similar to patterns observed in transgenic mice, despite an absence of observable sequence conservation between mice and zebrafish. Multiple segments direct expression in overlapping populations of neural crest derivatives and glial cells, ranging from pan-Sox10 and pan-neural crest regulatory control to the modulation of expression in subpopulations of Sox10-expressing cells, including developing melanocytes and Schwann cells. Several sequences demonstrate overlapping spatial control, yet direct expression in incompletely overlapping developmental intervals. We were able to partially explain neural crest expression patterns by the presence of head-to-head Sox family binding sites within two of the elements. Moreover, we were able to use this transcription factor binding site signature to identify the corresponding zebrafish enhancers in the absence of overall sequence homology. We demonstrate the utility of zebrafish transgenesis as a high-fidelity surrogate in the dissection of mammalian gene regulation, especially those with dynamically controlled developmental expression.

Introduction

The neural crest is a transient migratory population of cells that arise at the dorsal aspect of the neural tube as it closes and that give rise to myriad structures including, but not limited to, melanocytes, enteric nervous system (ENS), and myelinating Schwann cells [1]. Sox10 (SRY-box containing gene 10) encodes a critical transcription factor in neural crest development [2,3]. All neural crest cells express Sox10 upon emigration throughout the early stages of their respective journeys. Subsequently, expression is down-regulated in all neural crest–derived cells with population-specific timing. Sox10 expression is maintained in melanocytes (mouse) and Schwann cells (mouse and zebrafish) [2,3,4,5,6,7,8]. Additionally, Sox10 is expressed in presumptive oligodendrocytes; signal increases in concert with the onset of myelination and is maintained thereafter [2,6,9,10]. Naturally occurring and induced mutations in animal models and spontaneous mutations in the human population exemplify the developmental requirement for Sox10 during development. Human loss-of-function SOX10 mutations result in Waardenburg-Shah Syndrome (WS4; OMIM Accession No. 277580), a disorder characterized by impaired pigmentation and aganglionic megacolon consistent with Hirschsprung's Disease [11,12,13,14]. Furthermore, dominant-negative SOX10 mutations have been identified in patients with PCWH (OMIM Accession No. 609136), a complex syndrome characterized by the co-presentation of a WS4-like phenotype with central dysmyelinating leukodystrophy and demyelinating peripheral neuropathy consistent with Charcot-Marie-Tooth disease type I [11,15].

Similarly, studies in zebrafish and mice demonstrate the broad need for SOX10 during vertebrate development [4,8,16,17]. A missense mutation in the zebrafish sox10 coding sequence results in dramatic hypopigmentation accompanied by a reduction in enteric nervous system neuronal and Schwann cell populations [16,17]. In a similar fashion, mutations in the mouse Sox10 gene yield mice with impaired pigmentation and megacolon, and such models have been critical for understanding the expression and function of Sox10 [4,18]. Indeed, in a recent report we identified a...
deletion of approximately 16 kb residing approximately 47 kb upstream of Sox10, resulting in the Sox10Hry mouse model of WS4 [19]. Although these mice present with enteric and pigmentary deficits, this deletion severely disrupts, but does not ablate, developmental expression of Sox10. These data suggested that the deletion harbors a subset of cis-acting transcriptional regulatory elements necessary for Sox10 expression, and our functional analyses revealed at least one highly conserved segment within the deleted region that directs reporter gene expression in cultured melanocytes [19]. Comprehensive dissection of the cis-acting transcriptional regulatory sequences at Sox10 will be important for understanding the dynamic control of this transcription factor in specific neural crest derivatives and in oligodendrocytes, as well as for assessing the role of non-coding mutations in disease susceptibility in which Sox10 may play a role. Furthermore, these efforts will provide a significant first step in establishing the sequence substrates necessary for the development of cell-specific regulatory vocabularies.

Here, we report a comprehensive analysis of highly conserved, non-coding sequences at the mouse Sox10 locus, integrating in vitro cell studies in multiple cell lines and transgenesis in developing zebrafish and mice. Our results indicate that regulatory control at this locus coordinates information from a wide array of sequences whose independent regulatory contributions range from discrete sub-populations of crest derivatives to near pan-neural crest or pan-Sox10 expression. Critically, our data highlight the power of our zebrafish transgenic assay, demonstrating that it provides a high fidelity read out for the regulatory function of conserved non-coding Sox10 mouse sequences even in the absence of overt sequence conservation. Our results are largely consistent with recent preliminary analysis of a subset of Sox10 sequences in mouse, including the identification of regulatory elements that direct expression in peripheral neuronal populations and non-ectomesenchymal crest derivatives. Where differences are evident, they are largely accounted for by incomplete overlap among assayed sequences or the ability of dynamic analyses in zebrafish to uncover reporter expression that is not evident in Sox10 positive populations during mammalian development. Consequently, we also provide the first report of sequences directing expression in developing melanocytes and myelinating glia, for which no previous report exists. Additionally, our data also revealed regulatory segments that function in the same cell types but within different developmental time intervals. Finally, we demonstrate that two identified regulatory sequences with pan-neural crest regulatory control harbor dimeric SoxE binding sites that account for a significant fraction of their regulatory integrity, and use this data to identify two regulatory sequences at the zebrafish Sox10 locus that we propose to be their functional orthologs.

Results

Conserved, Non-Coding Sequences at Sox10 Display Enhancer Activity in Neural Crest–Derived Cells

Evolutionary sequence conservation is a robust metric for the identification of putatively functional sequences [20,21,22]. In a recent study, we identified nine highly-conserved genomic segments 5′ to the mouse Sox10 coding exons [19]. We termed these Sox10-MCSs (for Multiple-species Conserved Sequences; Figure 1A and Table 1). To determine their regulatory potential, we have assayed each segment in vitro, and in vivo through transgenesis in vertebrate systems. In addition, to more closely examine the genomic region surrounding the Sox10 transcriptional start site (TSS), we also assayed a genomic segment that harbors a conserved intron one element (Sox10-MCS1, Figure 1B), another that extends 5′ to the TSS (Sox10-MCS1B, Figure 1B), and another harboring only sequences upstream of the TSS (Sox10-MCS1C, Figure 1B).

We first tested the ability of each Sox10-MCS to direct reporter gene expression in two relevant cell lines. Briefly, we cloned each mouse genomic segment (Figure 1A and 1B) upstream of a minimal promoter (pEl1) directing basal luciferase expression and transfected them independently into two cell lines that express Sox10 [cultured melanocytes (mel-an-a cells) and cultured Schwann cells (S16 cells)], and one in which Sox10 is not expressed (NIH-3T3 cells). To enrich for authentic enhancers, we defined “enhancer activity” as segments that enhance reporter gene expression at a level 10-fold or higher compared to the control (pEl1 in Figure 1C through E). One segment (Sox10-MCS7) showed enhancer activity in all three cell lines (Figure 1C through E), three segments (Sox10-MCS4, Sox10-MCS5, and Sox10-MCS9) showed enhancer activity only in the neural crest–derived cultured melanocytes and Schwann cells (Figure 1C and D), and three segments (Sox10-MCS2, Sox10-MCS3, and Sox10-MCS6) showed enhancer activity only in cultured Schwann cells (Figure 1D). Interestingly, while neither Sox10-MCS1 nor Sox10-MCS1B displayed enhancer activity, Sox10-MCS1C directed reporter gene expression in both melanocytes and Schwann cells (Figure 1F). These data suggest that Sox10-MCS1C may represent a proximal Sox10 enhancer element. In total, 8 of the 11 genomic fragments studied displayed enhancer activity in Sox10 expressing cell lines, suggesting that the Sox10 locus does harbor multiple, coordinated cis-acting regulatory elements.

Sox10-MCSs Direct Tissue-Specific Reporter Gene Expression in Transgenic Zebrafish Embryos

The expression of Sox10 in crest-derived populations and in oligodendrocytes of mammals and fish has been well documented [2,3,4,6,8,10,16,17,18,23]. To determine whether the mouse sequences direct expression in a manner consistent with endogenous sox10, we studied the ability of each Sox10-MCS to

Author Summary

The neural crest is a population of embryonic migratory stem cells. They form atop the future spinal cord and migrate throughout developing embryos and form many different cells, including the epidermal pigment cells, bone cells in the head, and nerve cells of the peripheral nervous system. In this study, we studied the genome elements responsible for expression of SOX10, a dynamically expressed gene that is essential for neural crest development. We isolated candidate regulatory elements for SOX10 by identifying the small percentage of genomic DNA around the gene that did not vary as avian and mammalian genomes changed though evolution. We tested these fragments for their ability to regulate gene expression in zebrafish, a model system that is highly efficient for DNA-mediated expression studies and embryology. We found that even though the genome sequences were not similar to the SOX10 gene in fish, the genomic fragments were able to recapitulate the dynamic expression of SOX10 during development. Through computational analysis of the sequences, we identified a transcription factor binding site signature that identified the corresponding zebrafish SOX10 regulatory elements. This study describes a paradigm for dissecting regulation of essential genes that display complex expression patterns during development.

Zebrafish Analysis of Mammalian Sox10 Regulation

PloS Genetics | www.plosgenetics.org

September 2008 | Volume 4 | Issue 9 | e1000174
**Figure 1. Sox10-MCSs display enhancer activity in Neural Crest-derived cell lines.** A) Sox10-MCSs (pale blue bars) identified within and upstream of the Sox10 locus, depicted by a 75 kb interval from the UCSC Genome Browser (genome.ucsc.edu). B) Enlargement of a 2 kb interval encompassing the Sox10 transcriptional start site. Three elements, comprising sequence from within intron one alone (Sox10-MCS1), intron one and sequence 5' of the TSS (Sox10-MCS1B), and sequence only 5' of the TSS (Sox10-MCS1C) were subcloned and tested individually for enhancer activity. C–F) Sox10-MCSs cloned upstream of a minimal promoter driving luciferase reporter expression were assayed for enhancer activity in melan-a cells (C and F), S16 cells (D and F), and NIH-3T3 cells (E). The red line demarcates the 10-fold cut-off for “enhancer activity” (see Results); arrows indicate sequences exceeding that activity threshold. Error bars, SD (standard deviation).

doi:10.1371/journal.pgen.1000174.g001
direct reporter gene expression in developing transgenic zebrafish. Importantly, these sequences are not overly conserved between mammals and teleosts. Furthermore, the limited number of publicly available teleost genomic sequences and the evolutionary distance among them often makes it challenging even to identify sequences conserved within this evolutionary branch (infraclass). Briefly, each mouse segment was cloned upstream of the c-Fos minimal promoter and the enhanced green fluorescent protein (EGFP) coding sequence. The resulting constructs were injected into two-cell zebrafish embryos and evaluated for EGFP reporter expression at selected time points (1 to 5 days post-fertilization; dpf). G0 embryos showing consistent reporter gene expression were raised for germline transmission and reporter expression patterns were further evaluated in subsequent generations in two or more founder lines. In agreement with our in vitro analyses, neither Sox10-MCS1 nor Sox10-MCS1B directed reporter gene expression in zebrafish G0 embryos (n = 400; data not shown). By contrast, however, Sox10-MCS1C not only directed reporter expression in crest-derived cell lines but also directed tissue-specific reporter expression within neural crest populations in vivo, including premigratory neural crest cells, early migrating melanoblasts, cranial ganglia, lateral line ganglia, Schwann cells of the peripheral motor neurons, the sympathetic ganglia, and glial cell populations, including oligodendrocytes (data not shown). Although the former cells are not grossly affected in mutant mice, these data are consistent with developmental expression of Sox10 in mouse [7,10,18,24] and zebrafish [16,17,23]. Sox10-MCS8 directs reporter gene expression in a subpopulation of the enteric nervous system (Figure 2K), Schwann cells of the peripheral nervous system (data not shown), and the sympathetic ganglia (data not shown). But unlike the other constructs that we assayed, which displayed consistent reporter expression patterns among independent founder lines, Sox10-MCS8 showed notable spatial expression differences among multiple lines (n = 4). This was likely a result of position effects. However, while there was variability in expression patterns from the different founders, reporter expression remained limited to sox10 expressing tissues. Moreover, reported sites of expression were consistent among two or more lines. The reporter expression for this construct alone reflects a composite profile from the expression patterns of identified founders. Both Sox10-MCS7 and Sox10-MCS9 also directed expression in forming otic and craniofacial connective tissue. Finally, Sox10-MCS9 directs reporter gene expression in developing melanocytes (data not shown), the lateral line ganglia (data not shown), and cranial ganglia (Figure 2L). Thus, deletion of Sox10-MCS7, Sox10-MCS8, and Sox10-MCS9 likely explain, at least in part, the impaired pigmentation and aganglionic megacolon observed in Sox10<sup>−/−</sup> mice.

Interestingly, among the elements driving reporter expression in oligodendrocytes, some direct reporter expression during incompletely overlapping developmental windows. This temporally distinct control is perhaps most notable for sequence elements Sox10-MCS5 and Sox10-MCS7, as they direct overlapping reporter expression in the forming ventral white matter fiber tracts of the brain and spinal cord at time points between 2 dpf and 5 dpf. Although Sox10-MCS5 is detected early (2 dpf) in immature oligodendrocytes (Figure 2G), signal driven by Sox10-MCS7 is not readily detected in these populations until more than 24 hours later (data not shown); reporter expression driven by this sequence ultimately then overlaps with persistent expression driven by Sox10-MCS5 by 5 dpf (Figure 2H and J). These observations highlight the power of temporally dynamic transgenic analyses in zebrafish, as myelination in mammals is a postnatal event and would escape mid-gestational analyses.

**Sox10-MCSs Direct Reporter Gene Expression in a Manner Consistent with Sox10 in Transgenic Mice**

Our analyses represent the first report of mammalian non-coding sequences at Sox10 directing reporter gene expression in zebrafish and

---

**Table 1. Genomic segments analyzed in this study.**

| Genomic Segment | Coordinates at UCSC Genome Browser<sup>1</sup> |
|-----------------|-----------------------------------------------|
| Sox10-MCS1      | chr15:78,991,037–78,991,472                  |
| Sox10-MCS1B     | chr15:78,991,037–78,991,759                  |
| Sox10-MCS1C     | chr15:78,991,602–78,992,260                  |
| Sox10-MCS2      | chr15:78,992,786–78,993,476                  |
| Sox10-MCS3      | chr15:79,001,113–79,001,872                  |
| Sox10-MCS4      | chr15:79,020,080–79,020,899                  |
| Sox10-MCS5      | chr15:79,028,761–79,029,301                  |
| Sox10-MCS6      | chr15:79,034,832–79,035,411                  |
| Sox10-MCS7      | chr15:79,040,170–79,040,404                  |
| Sox10-MCS8      | chr15:79,046,712–79,047,379                  |
| Sox10-MCS9      | chr15:79,050,690–79,051,355                  |
| zf-sox10-E1     | chr13:15,933,435–15,933,953                  |
| zf-sox10-E2     | chr13:15,949,868–15,950,386                  |
| zf-sox10-E3     | chr13:15,992,412–15,992,923                  |

<sup>1</sup>Coordinates are from the February 2006 UCSC Genome Browser Mouse assembly and the March 2006 UCSC Genome Browser Zebrafish assembly for mouse and zebrafish genomic sequences, respectively. doi:10.1371/journal.pgen.1000174.t001
display success consistent with recent analyses of the RET locus. To determine the fidelity of these observations, we assayed a subset of the identified enhancers in mice, prioritizing two genomic segments whose regulatory control can independently account for a significant fraction of, if not all, Sox10 expression. First, Sox10-MCS4 modulated expression in nearly every sox10 expressing structure in zebrafish. Second, Sox10-MCS7, which is deleted in the Sox10Hry mouse model of WS4 [19], directed similarly broad sox10-appropriate expression, including in developing melanocytes and the enteric nervous system (data not shown), and in melanoblasts (Figure 4D, inset). The only relevant structure in which reporter gene expression was not observed was the otic vesicle, consistent with our observations in Figure 2.

Figure 2. Mouse Sox10-MCSs direct EGFP reporter expression in zebrafish embryos consistent with endogenous sox10. A–C) Sox10-MCS1C directs reporter gene expression to the cranial ganglia (A; white arrows) and within premigratory neural crest (A; open arrowheads) at 24 hpf (A). By 72 hpf (B), signal is detected in scattered oligodendrocytes (asterisks) along the spinal column and descending Schwann cells (white arrowheads) surrounding peripheral motor neurons. Weak reporter expression is also detected along the sympathetic chain (B; white arrow). At 5 dpf (C), signal is clearly detected in the ENS (C; white arrows). D) Sox10-MCS2 directs weak EGFP expression in glial cells of the CNS (white arrowheads) and PNS (white arrow). E and F) Sox10-MCS4 directs reporter gene expression in presumptive oligodendroglial cells at 48 hpf (E; white arrowhead). By 5 dpf (F), robust signal is detected in several neural crest lineages, including Schwann cells (F; white arrowheads), sympathetic chain ganglia (open arrowheads), and the enteric nervous system (white arrows). As with endogenous sox10, EGFP expression is maintained in mature oligodendrocytes (asterisks). G and H) Sox10-MCS5 also directs signal in early oligodendroglial cells (G; white arrowheads) and sustains signal in mature oligodendrocytes (asterisks). Glial cells of the PNS (H; white arrowheads) are clearly detected at 5 dpf (H). I) In situ hybridization using GFP riboprobe detects early migrating melanoblasts (black arrows) in Sox10-MCS7 transgenic embryos at 24 hpf. J) By 5 dpf, Sox10-MCS7 directs signal to oligodendrocytes (white arrowheads) along the ventral column. K) Sox10-MCS8 directs weak reporter to the ENS (white arrows) at 5 dpf. L) at 72 hpf, sustained reporter gene expression appears in the cranial ganglia (white arrows) of Sox10-MCS9 transgenic zebrafish embryos.

doi:10.1371/journal.pgen.1000174.t002
zebrafish. Similarly, at E13.5 reporter expression directed by Sox10-MCS4 provided a near complete recapitulation of the endogenous Sox10 gene product (n = 3); for example, expression was observed in sensory neurons, sympathetic ganglia, the enteric nervous system, and melanoblasts (data not shown). Given the absence of sequence conservation between the corresponding sequences from these species, data resulting from the analysis of the mouse Sox10-MCS4 sequence in mice and fish display a remarkable concordance.

Sox10-MCS7 likewise directed reporter gene expression consistent with both endogenous Sox10 expression at E11.5 (n = 1) and with our analyses in transgenic zebrafish. LacZ expression was detected in the cranial ganglia (Figure 4E), dorsal root ganglia (Figure 4E), otic vesicle (Figure 4E), melanoblasts (Figure 4F), sympathetic ganglia (Figure 4E), and in the enteric nervous system (Figure 4F, inset). These data underscore the potential role for Sox10-MCS7, among others, in the developmental regulation of Sox10 transcription. In particular, they suggest that this segment plays a role in Sox10 expression during melanocyte migration and development, and support the hypothesis that deletion of this element may contribute to the impaired pigmentation observed in the Sox10Hry mouse model of WS4 (see above). Furthermore, Sox10-MCS7 also directs expression in the mouse enteric nervous system, suggesting that deletion of this region may also contribute to the aganglionic megacolon observed in Sox10MCS7 mice. Taken together, these data suggest that analysis of mouse Sox10 regulatory sequences in zebrafish serves as a reliable surrogate for their analysis in mice. These studies revealed two enhancers with near pan-neural crest regulatory control, including sequences directing expression in melanoblasts.

Targeted Mutagenesis of SoxE Consensus Sequences within Sox10-MCS4 and Sox10-MCS7 Dramatically Alters Enhancer Activity

Members of the SoxE transcription factor family (Sox8, Sox9, and Sox10) bind DNA as either monomers, wherein they bind to single SRY-like consensus sequences (e.g., ACAAA), or as dimers that bind to two SRY-like binding sites oriented in a head-to-head fashion (hereafter referred to as dimeric SoxE consensus sequences) [25]. Our previous computational and functional analyses revealed highly-conserved dimeric SoxE consensus sequences in Sox10-MCS7 [19]. Furthermore, it has previously been hypothesized that SoxE proteins (e.g., SOX9) directly regulate the transcription of SOX10 [26]. To determine if SoxE proteins might regulate other genomic segments at Sox10, we searched each Sox10-MCS for similar consensus sequences. Interestingly, both Sox10-MCS4 and Sox10-MCS7 contain dimeric SoxE consensus sequences that are perfectly conserved between mammals and chicken (Figure 5A), consistent with their demonstrated biological functions and with the suggestion that these sequences have an important role in the transcriptional regulation of Sox10.

To test this hypothesis, we established a deletion series across Sox10-MCS4 (Figure 5B) to identify sequence intervals that are essential for aspects of its regulatory control. We assayed each resulting genomic segment for the ability to direct reporter gene expression in vitro and in vivo as before. In cultured melanocytes and Schwann cells, the assayed constructs modulated reporter gene expression with each deletion (Figure 5C). Specifically, removal of 5’ sequences to create Sox10-MCS4.1 caused a ~75% and ~60% reduction of enhancer activity in melanocytes and Schwann cells,
respectively. By contrast, removal of additional 5‘ sequences to create Sox10-MCS4.2 resulted in a ~4.5-fold and ~2-fold increase in enhancer activity compared to Sox10-MCS4 in melanocytes and Schwann cells, respectively. This significant increase in activity may result from the removal of sequences adjacent to the dimeric SoxE consensus sequences in Sox10-MCS4.2 (Figure 5B). This observation is consistent with analysis of Sox10-MCS7, wherein in vitro activity is increased upon the deletion of sequences flanking a core 95-basepair fragment containing the dimeric SoxE consensus sequences [19]. Further deletion of sequences from the 5‘ end of Sox10-MCS4.2 to create Sox10-MCS4.3 reduced enhancer activity below levels observed for Sox10-MCS4.2 in both cell lines. Interestingly, this deletion removes the dimeric SoxE consensus sequence, but does not disrupt a highly conserved, monomeric SoxE consensus sequence (Figure 5B), suggesting that the interval containing the dimeric sequence contributes significantly to the observed activity. The remaining SoxE consensus sequence may also explain the less dramatic effect of deleting the dimeric SoxE consensus sequences in Sox10-MCS4 compared to Sox10-MCS7 (see below). Finally, the removal of sequences encompassing this consensus sequence to create Sox10-MCS4.4 severely reduced enhancer activity below that of Sox10-MCS4, especially in cultured melanocytes (Figure 5C). These data suggest that the intervals containing SoxE binding sites play a critical role in the activation of reporter expression controlled by Sox10-MCS4 and Sox10-MCS7 in these cell lines. We thus directly assayed their necessity through independent deletion and mutation of the dimeric SoxE consensus sequences in both genomic segments (Figure 5G) and compared the ability of each wild-type and mutated genomic segment to direct reporter gene expression in the above cell lines. Consistent with this postulate, deletion or mutation of the dimeric SoxE consensus sequences significantly reduced the enhancer activity in both cell lines (Figure 5G; p-values between 5.98×10^{-11} and 2.11×10^{-7}). This effect was highly significant for both deletion and mutation of these sequences within both elements, albeit more severe for Sox10-MCS7 (≥80% reduction in enhancer activity, p=5.42×10^{-7}) than for Sox10-MCS4 (50–80% reduction in activity, p=2.11×10^{-7}). Together these data support the hypothesis that the dimeric SoxE consensus sequences are important for the function of both genomic segments in these...
Figure 5. Analyses of a dimeric SoxE consensus sequence within Sox10-MCS4 and Sox10-MCS7. A) Consensus SoxE family binding sites are oriented in a head-to-head fashion within Sox10-MCS4 and Sox10-MCS7. B) A deletion series across Sox10-MCS4 (pale blue bars) is depicted in the UCSC Genome Browser. The position and sequence of a monomeric and a dimeric SoxE consensus sequence is shown below the deletion series. C) In vitro enhancer activity for each fragment of the deletion series was tested individually in melan-a cells (blue bars) and S16 cells (red bars). The results for Sox10-MCS4 are included for cross-comparison of modular enhancer activity. Error bars, SD. D–F) In vivo enhancer activity was compared at 72 hpf for Sox10-MCS4.1 through Sox10-MCS4.3 in transgenic zebrafish embryos. Sox10-MCS4.4 failed to direct reporter expression in G0 embryos and was not raised for germline transmission. Sox10-MCS4.1 (D) directed reporter expression to Schwann cells (white arrows) and weak reporter expression to sympathetic ganglia (white arrowheads). Sox10-MCS4.2 (E) directed reporter expression in an opposite fashion, as signal appeared weaker in Schwann cells and more robust in sympathetic ganglia. Sox10-MCS4.3 (F) directed an extremely low level of reporter expression to these two neural crest–derived populations, and the arrow and arrowheads show the relative position of where the Schwann cells and sympathetic ganglia is normally positioned. G) Site-directed mutagenesis was used to delete and mutate the dimeric SoxE consensus sequence within Sox10-MCS4 and Sox10-MCS7. These mutagenized constructs were tested for in vitro enhancer activity in melanocytes (blue bars) and Schwann cells (red bars), and compared against their wild-type sequences. P-values are given above each tested construct and error bars indicate the standard deviation. H and I) Sox10-MCS4 with a deleted head-to-head SoxE family binding site was selected for transmission through the germline. Analysis of two different founder lines (H and I, respectively) revealed a decrease in signal in oligodendrocytes (asterisks) and scattered reporter expression in a subset of the ENS (white arrowheads). Schwann cells (white arrow), however, appear to be unaffected.

doi:10.1371/journal.pgen.1000174.g005
two cell types (see above). They further suggest that although Sox10-MCS7 appears to be highly dependent on the dimeric SoxE consensus sequences for its function, domains in addition to this motif also contribute aspects of the biological potential demonstrated by Sox10-MCS4.

To determine the in vivo functional requirement for each of the Sox10-MCS4 sequence intervals described above, including the dimeric SoxE sequences, we studied each Sox10-MCS4 fragment (wild type and mutant) in transgenic zebrafish embryos. Once again all constructs were passed through the germline and evaluated in multiple founder lines to ensure the integrity of our analyses. Sox10-MCS4.1 directed expression in a similar manner to that observed for Sox10-MCS4, however with significantly reduced intensity. For example, low levels of expression were observed in Schwann cells surrounding spinal motor neurons, and almost no signal was observed in the sympathetic ganglia (Figure 5D). In contrast, Sox10-MCS4.2 directed reporter gene expression at higher levels in sympathetic chain ganglia and at reduced levels in glia surrounding spinal motor neurons when compared to Sox10-MCS4.1 (Figure 5E). Interestingly, this observed flux in signal intensity is completely consistent with our in vitro observations. A more dramatic loss of reporter gene expression was evident upon analysis of Sox10-MCS4.3; expression was severely reduced in populations such as the cranial and sympathetic ganglia at 24 hpf (data not shown), as well as in oligodendrocytes and Schwann cells at 72 hpf (Figure 5F). This is similarly consistent with our in vitro observations and with the hypothesis that the dimeric SoxE motif is important for Sox10-MCS4 function. Finally, injection of over 400 G0 zebrafish embryos with Sox10-MCS4.4 failed to direct detectable reporter gene expression in any structures, indicating that all sequences necessary to direct tissue-specific reporter gene expression had been deleted. These data are also consistent with our in vitro analyses and may be explained by the removal of the monomeric SoxE consensus sequence (see above).

To determine whether the observed reporter deficits in fish injected with Sox10-MCS4.3 were the result of removing the dimeric SoxE consensus sequences, we next evaluated transgene reporter expression directed by Sox10-MCS4 harboring a deletion of the dimeric consensus sequences alone. The resulting embryos displayed a decrease in EGFP-positive enteric ganglia (compare Figure 5H and I to Figure 2F); reporter gene expression was only detected in dorsally located ganglia, suggesting that the consensus sequence may be critical for a subset of cells in the enteric nervous system. Expression was also severely reduced in oligodendrocytes (compare Figure 5H and I to Figure 2E and F). Interestingly, expression in Schwann cells was not disrupted (compare Figure 5H and I to Figure 2F). These data were consistent upon analysis of at least two independent founder lines (Figure 5H and I) and suggest that the dimeric SoxE consensus sequences in Sox10-MCS4 play a more critical role regulating Sox10 expression during oligodendrocyte and ENS development than in Schwann cell development at the evaluated time points.

Identification of the Zebrafish Functional Orthologs of Sox10-MCS4 and Sox10-MCS7

We have shown that highly conserved mouse sequences at Sox10 direct tissue-specific reporter gene expression in developing transgenic zebrafish. These observations belie the fact that there is no observable non-coding sequence conservation between mammals and zebrafish at Sox10 (Figure 1A), and are consistent with previous analyses at the RET locus [27]. To identify the zebrafish orthologs of Sox10-MCS4 and Sox10-MCS7, we exploited the observation that these two genomic segments harbor highly conserved dimeric SoxE consensus sequences. Briefly, we computationally identified all dimeric SoxE consensus sequences in a 100 kb window upstream of the zebrafish sox10 locus. These analyses identified three such consensus sequences (Figure 6A). We hypothesized that sequences encompassing these motifs would function as in vitro and in vivo enhancers in a manner overlapping Sox10-MCS4 and/or Sox10-MCS7. Thus, we PCR amplified 500 bp surrounding each of these consensus sequences (zf-sox10-E1, zf-sox10-E2, and zf-sox10-E3; Figure 6A and Table 1) from zebrafish genomic DNA and tested each in vitro and in vivo as above. zf-sox10-E1 displays strong enhancer activity in Schwann cells, directing reporter gene expression ∼12-fold higher than the control vector (Figure 6B). In contrast, neither zf-sox10-E2 nor zf-sox10-E3 displays strong enhancer activity in either cell line. However, it is important to note that these cells do not fully represent the range of cell types or stages in cell maturation in which sox10 is expressed during development. Indeed, when assayed in vitro, both zf-sox10-E1 and zf-sox10-E3 but not zf-sox10-E2 directly express EGFP in cell populations consistent with endogenous sox10 expression. zf-sox10-E1 directed reporter expression in scattered cells in zebrafish G0 embryos (24 hpf) in a manner consistent with migrating neural crest, appearing to be positioned at multiple points along the anterior posterior axis both dorso-laterally and along a ventral path (Figure 6C). Additionally, although zf-sox10-E3 did not display enhancer activity in Schwann and melanocyte cell lines, it did direct reporter gene expression in forming oligodendrocytes of the central nervous system (Figure 6D) of mosaic G0 embryos. Importantly, these observations underscore the benefit of evaluating regulatory control within an intact organism.

Taken together, the presence of such a small number of conserved dimeric SoxE consensus sequences in the evaluated interval and the data from in vitro and in vivo functional assays strongly suggest that zf-sox10-E1 and zf-sox10-E3 are the zebrafish orthologs of Sox10-MCS4 and Sox10-MCS7, respectively. This is further supported by the relative position of each genomic segment in the mouse and zebrafish genomes (compare Figure 1A to 6A) and their associated regulatory control. However, one might expect that the simple identification of conserved dimeric SoxE consensus sequences would yield a high number of elements with in vitro enhancer activity, and that these data may reflect a high false positive rate. However, note that zf-sox10-E2 did not direct reporter gene expression in cultured cells (Figure 6B) or developing zebrafish (data not shown). To determine the frequency with which sequences encompassing a highly conserved SoxE consensus sequence will identify enhancers that function in a subset of SoxE positive tissues, we computationally identified all dimeric SoxE consensus sequences in the mouse genome. We then identified the subset of these consensus sequences that have a conservation profile consistent with Sox10-MCS4 and Sox10-MCS7 and that reside within intronic regions of genes expressed in melanocytes (see methods for details). We chose to examine intronic sequences because: (1) they provide a definable genomic element in which to search; (2) introns are known to frequently harbor enhancer sequences; and (3) functional intronic SoxE binding sites have been identified within introns of other SOX10 target genes [e.g., myelin protein zero (MPZ) [28]]. These analyses provided a dataset of 44 genomic segments (Table S1), which were then tested for enhancer activity in cultured melanocytes and Schwann cells as described above. Only two of the 44 genomic segments (4.5%) increased reporter gene expression at least 10-fold higher in either cell line; both directed reporter gene expression in cultured melanocytes (Figure 6E) and Schwann cells (Figure 6F), suggesting they may be true SoxE target enhancers. Although we anticipate that some degree of the low frequency of success may
Figure 6. Motif-based search for functional zebrafish orthologs and SoxE positive enhancers. A) A computational search for dimeric SoxE consensus sequences in a 100 kb window upstream of the zebrafish sox10 gene resulted in three hits (pale blue bars). The distance from the sox10 TSS is denoted above each enhancer (E1–E3). B) A 500 bp region surrounding each dimeric SoxE consensus sequence was tested for in vitro enhancer activity in a melanocyte cell line (blue bars) and a Schwann cell line (red bars). Each fragment is compared against a control (pE1b with no insert) and error bars indicate the standard deviation. C) zf-sox10-E1 directs reporter expression in scattered cells throughout the 24 hpf, G0 embryo, consistent with migrating neural crest cells (white arrows). D) zf-sox10-E3 directs reporter expression in cranial oligodendrocytes (asterisks) at 5 dpf in G0 embryos. E and F) Data mining of the mouse genome for highly conserved dimeric SoxE consensus sequences in the intron of genes expressed in melanocytes identified 44 genomic segments. In vivo enhancer activity was tested in cultured melanocytes (E) and Schwann cells (F). Only two segments drove luciferase expression above a 10-fold threshold in both cell lines (black arrows). Error bars, SD. doi:10.1371/journal.pgen.1000174.g006
arise from the amplification and assay of incomplete regulatory modules or from their assay in inappropriate crest derivatives, we anticipate that many are simply false negatives. Thus, our data suggest that implementing this approach at orthologous loci may enrich for correctly identified crest enhancers, and on a genomic scale, greater information regarding the composition and configuration of critical binding site motifs will be necessary.

Discussion

SOX10 encodes a developmentally critical transcription factor whose roles in development and disease have been well described. Mutations in *SOX10* underlie defects in several neural crest–derived populations, resulting in epidermal hypopigmentation, enteric aganglionosis, and demyelinating peripheral neuropathy; outside neural crest–derived populations, they can also result in dysmyelinating pathologies of the central nervous system oligodendrocytes [11,13,14,15]. The pleiotropic roles of Sox10 are facilitated by tightly controlled and dynamic regulatory control, encoded by sequences at this locus but until recently, little was known of the cis-acting sequences that modulate its dynamic expression in vertebrates [29,30]. The first step in deciphering this regulatory lexicon is the identification of cis-acting regulatory sequences within and flanking *SOX10*. We directly addressed this challenge, assaying the regulatory control exerted by highly conserved, non-coding sequences at the mouse Sox10 locus in cultured cells and in vivo through transgenesis in zebrafish and mice.

Data generated in vitro and in vivo was highly concordant, and critically identified regulatory elements were consistent with all aspects of Sox10 expression during embryogenesis, in both NC-derived and non-NC-derived structures. For example, six genomic segments (*Sox10*-MCS1C, *Sox10*-MCS2, *Sox10*-MCS4, *Sox10*-MCS5, *Sox10*-MCS7, and *Sox10*-MCS9) directed EGFP expression in developing melanocytes, and six genomic segments (*Sox10*-MCS1C, *Sox10*-MCS2, *Sox10*-MCS4, *Sox10*-MCS5, *Sox10*-MCS7, and *Sox10*-MCS8) directed EGFP expression in Schwann cells during zebrafish embryogenesis. These are the first data showing that non-coding sequences at Sox10 direct expression in these NC derivatives. Furthermore, six genomic segments (*Sox10*-MCS1C, *Sox10*-MCS2, *Sox10*-MCS4, *Sox10*-MCS5, *Sox10*-MCS7, and *Sox10*-MCS8) directed EGFP expression in oligodendrocytes; these represent the first data showing that non-coding sequences at Sox10 direct expression in this non-NC-derived cell population. Importantly, these data were generated in teleosts using mouse genomic sequence with which we detected no overt sequence conservation.

In an effort to evaluate the fidelity of our observations in zebrafish, we likewise assayed two genomic segments using transgenesis in developing mice, prioritizing sequences that displayed broad Sox10-appropriate regulatory control in teleosts (*Sox10*-MCS4 and *Sox10*-MCS7). The resulting data strongly suggest that zebrafish transgenesis serves as a reliable surrogate for the assayed sequences; *Sox10*-MCS4 and *Sox10*-MCS7 both directed reporter gene expression in nearly all endogenous sites of Sox10 expression during key stages of mouse development (Figure 4). Taken together, one of the most striking observations is the degree of functional overlap shared among identified regulatory sequences. This appears to be an increasingly common observation at developmental genes [31,32,33,34] and suggests a degree of functional redundancy in their regulation. However, in this case, we know that this is unlikely; our recent Sox10<sup>[10]<sup>th</sup> mouse model of Waardenburg-Shah Syndrome demonstrated that homozygous mutant mice harboring carry a deletion that encompasses *Sox10*-MCS7, *Sox10*-MCS8, and *Sox10*-MCS9 display severe hypopigmentation and enteric aganglionosis. Data presented in this study strongly suggests that the deletion of these three regulatory elements contribute to the enteric and pigmentary phenotype of the *Sox10<sup>[10]<sup>th</sup> mice [19]. However, these are not the only regulatory elements contributing to expression in the affected cell types; *Sox10*-MCS1C, *Sox10*-MCS4, and *Sox10*-MCS5, among others, are not deleted in the *Sox10<sup>[10]<sup>th</sup> mice yet they also direct reporter gene expression in developing melanocytes and/or the enteric nervous system (Figure 5). Thus, a parsimonious explanation for the observed data is that multiple regulatory sequences are coordinately required for the proper spatial and temporal expression of Sox10 during development, each contributing to expression in certain subsets of tissues in an additive or interactive manner. To fully address this issue, it will be necessary to perform cell- and tissue-specific ChIP, as well as deletion and mutation analysis of each *Sox10*-MCS within the context of the entire gene (e.g., in BAC transgenic mice).

Consistent with our data, Werner et al. [30] made similar observations in their preliminary evaluation of a small number of overlapping fragments in mice. However, there are a number of notable discrepancies, especially when considering regulatory control in developing melanocytes, sympathetic ganglia and glial cells. First, our analyses revealed six genomic segments (*Sox10*-MCS1C, *Sox10*-MCS2, *Sox10*-MCS4, *Sox10*-MCS5, *Sox10*-MCS7, and *Sox10*-MCS9) that direct reporter gene expression in developing melanocytes, consistent with the known role of Sox10 in these cells. Werner and colleagues [30] assayed three genomic segments that partially overlap with sequences that we assayed. (Their sequences, termed U1, U2, and U3 correspond to *Sox10*-MCS7, *Sox10*-MCS5 and *Sox10*-MCS4 respectively). None of the “U” elements directed reporter gene expression in melanoblasts, including U1 and U3, which correspond to *Sox10*-MCS7 and *Sox10*-MCS4, respectively. However, our analyses of these genomic segments in developing mouse embryos revealed clear expression in melanoblasts of mice at E11, E11.5 and E13.5 (Figure 4 and data not shown), consistent with our observations in transgenic zebrafish embryos (Figure 2). Importantly, U3 lacks 170 bp present in *Sox10*-MCS4, suggesting that the additional sequences in *Sox10*-MCS4 may be important for expression in developing melanocytes. In contrast, U1 and U2 both encompass and are 38% and 49% larger than *Sox10*-MCS7 and *Sox10*-MCS5, respectively, yet in our assays both genomic segments directed reporter gene expression in developing melanocytes; importantly, in the case of *Sox10*-MCS7, this was substantiated by reporter expression in melanocyte cell lines as well as reporter expression in melanoblasts of transgenic zebrafish and mouse embryos. One possible explanation is that these U elements also harbor local repressor sequences that are active in developing melanocytes. Another possibility is that position-related effects altered the activity of U1, U2, and U3 such that expression in melanoblasts was not detectable. Importantly, our data strongly suggest that *Sox10*-MCS4 and *Sox10*-MCS7 are important for expression during mammalian melanocyte development, and represent the first report of transcriptional regulatory elements important for Sox10 expression in melanoblasts.

Second, similarly in our evaluation of *Sox10*-MCS4 in transgenic zebrafish and mouse, we observed expression in the sympathetic ganglia. Once again, this contrasts with observations for U3 [30]. Interestingly, U3 ceases sequence overlap with *Sox10*-MCS4 at a point where our assayed deletion fragment *Sox10*-MCS4.4 begins; critical motifs contained within this fragment may work in conjunction with other transcription factor binding sites found throughout the *Sox10*-MCS4 sequence but are not sufficient to independently direct reporter gene expression to the sympa-
thetic ganglia. Another possibility is that U3 harbors a local repressor within the 32 bp that it extends beyond our Sox10-MCS4 element.

Third, Sox10 expression in developing oligodendrocytes and Schwann cells of the central and peripheral nervous system, respectively, is critical for the formation of all white matter fiber tracts [6,7,9,10,18]. Indeed, six of the genomic segments at Sox10 analyzed in zebrafish revealed expression in oligodendrocytes and Schwann cells (Sox10-MCS1C, Sox10-MCS2, Sox10-MCS4, Sox10-MCS5, Sox10-MCS7, and Sox10-MCS8). However, although U1-3 overlap Sox10-MCS4-2 as described above, the authors were unable to detect reporter expression in these populations because myelination is a post-natal developmental event in mammals [6,8,10]. By contrast, myelination in zebrafish begins at 2–3 days post fertilization in zebrafish and thus, the in vivo enhancer activity of genomic segments important for expression in myelinating cells can be readily detected. Our data underscore another major strength of employing zebrafish to study the transcriptional regulation of Sox10. Indeed, this is the first report of transcriptional regulatory elements important for Sox10 expression in Schwann cells and oligodendrocytes. Additionally, we demonstrate that the same elements exert regulatory control in both populations.

Additionally, in a recent report of BAC-based transgenic rescue of Sox10 deficient mouse phenotypes, Dehal and colleagues [29] report the evaluation of a BAC transgene termed DelA, wherein most sequence 5’ to the Sox10 coding sequence has been deleted. Although, this BAC appears to retain the regulatory sequences we have termed Sox10-MCS1C and Sox10-MCS2, it directs reporter expression only in the otic canals and melanoblasts, and weakly in the superior cervical ganglia. Given our observation that when assayed independently these sequences direct expression in many neural crest derivatives, one parsimonious explanation is that, in context, these sequences must behave coordinately and/or overcome local repression in order to elicit its requisite control. Taken in combination, these observations may explain previous failures to generate effective Sox10 reporter transgenes using conventional “promoter bashing” strategies. Consequently, Sox10-MCS1C may perhaps be more readily considered a proximal enhancer than a regulatory promoter of Sox10.

Our computational and functional analyses of conserved, non-coding sequences at Sox10 have illuminated the potentially important role of SoxE regulation of Sox10. We have previously demonstrated that deletion of a large region harboring a dimeric SoxE consensus sequence in mouse gives rise to a phenotype similar to Sox10 coding mutations [19]. Here, we also report that two genomic segments (Sox10-MCS4 and Sox10-MCS7) harbor highly-conserved dimeric SoxE consensus sequences that are essential for the appropriate control of reporter gene expression in relevant cells and tissues. However, while the dimeric SoxE consensus sequences analyzed in this study are likely important for Sox10 expression, our data also indicate that they are not essential for all regulatory control exerted by the sequences containing them and, indeed, other sequences within these segments are likely to be similarly important. Consistent with this idea we also provide deletion of sequences upstream of the SoxE consensus sequences in Sox10-MCS4 also significantly reduce reporter gene expression (Sox10-MCS4-1, Figure 5C). Thus, it is likely that other sequences within the studied genomic segments also contribute to the appropriate regulatory control of Sox10. Elucidation of this underlying vocabulary will be critical for a full understanding of Sox10 expression during vertebrate development.

Although we successfully implemented the zebrafish transgenic assays using mouse genomic DNA with conservation restricted to mammalian and avian species, the absence of corresponding sequence conservation between mammals and fish prevented us from directly identifying the zebrafish orthologs of each mouse Sox10-MCS. We were able, however, to uncover potential zebrafish orthologs of Sox10-MCS4 and Sox10-MCS7 by cataloging dimeric SoxE consensus sequences upstream of the zebrafish sox10 gene (n = 3). Of the three fragments that harbor these consensus sequences, two (zf-sox10-E1 and zf-sox10-E3) directed reporter gene expression in Sox10 relevant cells and tissues. Furthermore, we demonstrated that the likelihood of stumbling upon a Sox10-like regulatory element by chance based only on the presence of dimeric SoxE consensus sequences was very low; only two of 44 genomic segments that harbor such sequences and that reside within non-coding sequences at genes expressed in melanocytes directed reporter expression in a corresponding cell line. Thus, we propose that zf-sox10-E1 and zf-sox10-E3 are the zebrafish orthologs of Sox10-MCS4 and Sox10-MCS7, respectively. These observations suggest that motif-based strategies may be useful for identifying the functional orthologs of cis-acting transcriptional regulatory elements in the absence of overt non-coding sequence conservation.

In characterizing Sox10-MCS4 and Sox10-MCS7, we have described the first pan-neural crest enhancers; these genomic segments direct reporter gene expression in nearly all tissues in which Sox10 is expressed, including peripheral nervous system neurons, developing melanocytes, and glial cells. As such, the constructs and transgenic zebrafish strains we have developed provide critical tools for studying the role of Sox10, and more generally, the neural crest in human development and disease. Additionally, these genomic segments will now be scrutinized for variants that may cause or convey susceptibility to disease.

The studies reported here further our knowledge of the transcriptional regulation of the Sox10 locus and underscore the complex regulatory control of this developmentally critical transcription factor. Specifically, we make several notable observations. First, multiple genomic segments direct reporter gene expression in overlapping neural crest derivatives and glial cells. Second, at least two genomic segments direct expression in a pan-neural crest manner and SoxE consensus sequences are required for the integrity of their regulatory control. Third, genomic sequences derived from the mouse Sox10 locus direct appropriate reporter gene expression in developing zebrafish, even in the absence of observable conservation and demonstrate that zebrafish provide a high fidelity surrogate for the evaluation of mammalian Sox10 regulatory sequences. Finally, we provide the first report of Sox10 regulatory sequences that direct expression in developing melanocytes and glia. These findings have important implications for studying neural crest development and the etiology of related diseases, and, more generally, provide a paradigm for dissecting the regulatory control of genes implicated in human disease.

Methods

Vector Construction

Expression constructs were generated using Gateway technology (Invitrogen, Carlsbad, CA). Briefly, PCR primers containing flanking attB sites were designed to amplify each genomic region of interest. Subsequent to PCR reactions, purified products were recombined into the pDONR221 vector according to the manufacturer’s specifications (Invitrogen). Each insert was sequenced to ensure the absence of PCR-induced errors. Mutagenesis of MCS4 and MCS7 was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and appropriate mutation-bearing oligonucleotides. Subsequently, plasmid DNA isolated from each entry clone was recombined.
with either pLGF-E1b (for luciferase reporter gene expression in culture cells) [19], pT2fosGW (for EGFP expression in developing zebrafish embryos) [27], or Hsp60-LacZ (for LacZ expression in developing mouse embryos) [35] destination vectors according to the manufacturer’s specifications (Invitrogen). Each resulting construct underwent restriction enzyme digest with BsrGI, EcoRV, or SalI, respectively, to confirm the presence of the appropriately sized insert.

**Cell Culture, Transfections, and Luciferase Activity Assays**

Immortalized melanocytes (melan-a) [36], Schwann cells (S16) [37], and NIH-3T3 cells were cultured under standard conditions. 5×10^5 cells were placed into each well of a 96-well culture plate and transfectioned with luciferase reporter vectors (see above) using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. For each reaction, 0.25 μl of lipofectamine 2000 and 25 μl of OptiMEM I minimal growth medium (Invitrogen) were combined and incubated at room temperature for 10 minutes. Purified luciferase reporter vector (200 ng) or the equivalent volume of water (in the case of DNA-negative controls), and 2 ng of the internal control pCMV-RL renilla expression vector (Promega, Madison, WI) were diluted in 25 μl of OptiMEM I and combined with the lipofectamine-OptiMEM I mixture. The ~50 μl reactions were incubated at room temperature for 20 minutes and then added to a single well of the 96-well culture plate containing cells. After a 3 hour incubation at 37°C, the medium was aspirated and normal growth medium added.

After a 48 hour incubation at 37°C, cells were washed with 1X PBS and lysed at room temperature using 1X Passive Lysis Buffer (Promega). A total of 4 μl of the resulting cell lysate were transferred to a white polystyrene 96-well assay plate (Corning Inc., Corning, NY). Luciferase and renilla activities were determined using the Dual Luciferase Reporter 1000 Assay System (Promega). A total of 4 emissions of firefly luciferase (luciferase reporter vector) and 2 emissions of renilla renilla luciferase (Promega) were diluted in 25 μl of OptiMEM I and combined with the lipofectamine-OptiMEM I mixture. The ~50 μl reactions were incubated at room temperature for 20 minutes and then added to a single well of the 96-well culture plate containing cells. After a 3 hour incubation at 37°C, the medium was aspirated and normal growth medium added.

### Zebrafish Reporter Gene Expression Assays

Zebrafish were raised and bred in accordance with standard conditions [38,39]. Embryos were maintained at 28°C and staged in accordance with standard methods [38,39]. EGFP expression constructs (see above) were injected into AB background G0 embryos (n=200), as previously described [27,40]. Injected embryos were evaluated for reporter expression between 24 hpf and 5 dpf. Embryos displaying consistent EGFP expression were selected and allowed to mature, facilitating germine transmission and evaluation of reporter expression. Embryos were analyzed and imaged using a Carl Zeiss Lumar V12 Stereo microscope with AxioVision version 4.5 software.

### In Situ Hybridization

Embryos selected for in situ hybridization were raised in embryo medium containing 0.003% phenylthiocarbamide to prevent pigmentation and fixed for in situ hybridization using standard protocols. Probes were labeled with digoxigenin using DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN) and detected with the appropriate antibody. A blue precipitate was formed by incubating with BCIP and NBT (Roche Applied Science). Green fluorescent protein (GFP) probe was used as previously described [27].
Author Contributions

Conceived and designed the experiments: AA JLH ASM WJP. Performed the experiments: AA JLH SQLL RMV GR SKL GE TGW ASM.

References

1. Kalcheim C, LeDouarin NM (1986) Requirement of a neural tube signal for the differentiation of neural crest cells into dorsal root ganglia. Dev Biol 116: 451–466.

2. Bondurand N, Kobeta A, Pingault V, Lemort N, Encha-Razavi F, et al. (1998) Expression of the SOX10 gene during human development. FEBS Lett 432: 168–172.

3. Pasch C, Hustert E, Pfeifer D, Sudbeck P, Kist R, et al. (1998) The SOX10/ Sox10 gene from human and mouse: sequence, expression, and regulation by the encoded HMG domain transcription factor. Hum Genet 103: 115–123.

4. Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, et al. (1998) Mutation of the Sry-related Sox10 gene in Dominant megalocornea, a mouse model for human Hirschsprung disease. Proc Natl Acad Sci U S A 95: 5161–5165.

5. Kapur RP (1999) Early death of neural crest cells is responsible for total enteric aganglonomus in Sox10(Dom)/Sox10(Dom) mouse embryos. Pediatr Dev Pathol 2: 559–569.

6. Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M (1998) Sox10, a novel transcriptional modulator in glial cells. J Neurosci 18: 237–250.

7. Paratore C, Goerich DE, Suter U, Wegner M, Sommer L (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128: 3949–3961.

8. Southard-Smith EM, Koz L, Pavan WJ (1998) Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat Genet 18: 60–64.

9. Mollaaghababa R, Pavan WJ (2003) The importance of having your SOX on: role of SOX10 in the development of neural crest-derived melanocytes and glia. Oncogene 22: 3024–3034.

10. Stolt CC, Rehberg S, Adler M, Loomes P, Riethmacher D, et al. (2002) Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. Genes Dev 16: 165–176.

11. Inoue K, Khajavi M, Ohyama Y, Hirabayashi S, Wilson J, et al. (2004) Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations. Nat Genet 36: 361–369.

12. Kuhlbrodt K, Schmidt C, Sock E, Pingault V, Bondurand N, et al. (1998) Functional analysis of Sox10 mutations found in human Waardenburg-Hirschsprung patients. J Biol Chem 273: 23033–23038.

13. Pingault V, Bondurand N, Kuhlbrodt K, Goerich DE, Prehu MO, et al. (1998) SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat Genet 18: 171–176.

14. Shah KN, Dalal SJ, Desai MP, Sheth PN, Joshi NC, et al. (1981) White forelock, an unusual variant of Waardenburg syndrome. J Pediatr 99: 432–435.

15. Inoue K, Tanabe Y, Lupski JR (1999) Myelin deficiencies in both the central and the peripheral nervous systems associated with a SOX10 mutation. Am J Hum Neurol 46: 313–318.

16. Dutton KA, Paullyn A, Lopes SS, Elworthy S, Carney TJ, et al. (2001) Zebrafish colourless encodes Sox10 and specifies non-ectomesenchymal neural crest fates. Development 128: 4113–4125.

17. Kelsh RN, Eisen JS (2000) The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. Development 127: 515–525.

18. Britsch S, Goerich DE, Riethmacher D, Perinno RI, Rozmer M, et al. (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev 15: 66–78.

19. Antonellis A, Bennett WR, Menheniott TR, Prasad AB, Lee-Lin SQ, et al. (2006) Deletion of long-range sequences at Sox10 compromises developmental expression in a mouse model of Waardenburg-Shah (WS) syndrome. Hum Mol Genet 15: 259–271.

20. Frazer KA, Elnitski L, Church DM, Dubchak I, Hardison RC (2003) Cross-species sequence comparisons: a review of methods and available resources. Genome Res 13: 1–12.

21. Nardone J, Lee DU, Ansel KM, Rao A (2004) Bioinformatics for the ‘bench biologist’: how to find regulatory regions in genomic DNA. Nat Immunol 5: 768–774.

22. Ramamurthy WV, Palambo M, Thompson W, Fickett JW, Lawrence CE (2000) Human-mouse genome comparisons to locate regulatory sites. Nat Genet 26: 225–228.

23. Carney TJ, Dutton KA, Greenhill E, Delfino-Machin M, Dufourcq P, et al. (2006) A direct role for Sox10 in specification of neural crest-derived sensory neurons. Development 133: 4619–4630.

24. Sommenberg-Riehthamer E, Miehe M, Stolt CG, Goerich DE, Wegner M, et al. (2001) Development and degeneration of dorsal root ganglia in the absence of the HMG-domain transcription factor Sox10. Mech Dev 106: 253–263.

25. Peronan RJ, Wegner M (2000) The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences. Nucleic Acids Res 28: 3047–3053.

26. Cheung M, Briscoe J (2005) Neural crest development is regulated by the transcription factor Sox10. Development 133: 3641–3649.

27. Fisher S, Grice EA, Vinton RM, Blessing SL, McCaIIon AS (2006) Conservation of RET regulatory function from human to zebrafish without sequence similarity. Science 312: 276–279.

28. LeBlanc SE, Jiang SW, Ward RM, Weabates L, Swarren J (2006) Direct regulation of myelin protein zero expression by the Egr2 transactivator. J Biol Chem 281: 5453–5460.

29. DeaI KK, Canitrell VA, Chandler RL, Saunders TL, Mottloclock DP, et al. (2006) Distant regulatory elements in a Sox10-beta GFO BAC transgene are required for expression of Sox10 in the enteric nervous system and other neural crest-derived tissues. Dev Dyn 235: 1413–1432.

30. Werner T, Hammer A, Wahlbali M, Bosl MR, Wegner M (2007) Multiple conserved regulatory elements with overlapping functions determine Sox10 expression in mouse embryogenesis. Nucleic Acids Res 35: 6526–6531.

31. Abassi AA, Paparidis Z, Malik S, Goode DK, Callaway H, et al. (2007) Human GLI1 intragenic conserved non-coding sequences are tissue-specific enhancers. PLoS ONE 2: e366.

32. Aitio N, Zhu Y, Vriel A, Holt A, Alafz V, et al. (2007) Deletion of ultraconserved elements yields viable mice. PLoS Biol 5: e234.

33. de la Calle-Mustienes E, Feijoo CG, Manzanares M, Tena JJ, Rodriguez-Segovius E, et al. (2005) A functional survey of the enhancer activity of conserved non-coding sequences from vertebrate Iroquois cluster gene desomes. Genome Res 15: 1061–1072.

34. McGaughey DM, Vinton RM, Hyndh J, Al-Saif A, Beer MA, et al. (2008) Metrics of sequence constraint overlook regulatory sequences in an exhaustive analysis at phox2b. Genome Res 18: 252–260.

35. Nobrega MA, Ovcharenko I, Afzal V, Rubin EM (2003) Scanning human gene deserts for long-range enhancers. Science 302: 413.

36. Nohrega MA, Ovcharenko I, Alafz V, Rubin EM (2003) Scanning human gene deserts for long-range enhancers. Science 302: 413.

37. Sviderskaya EV, Hill SP, Evans-Whipp TJ, Chin L, Orlow SJ, et al. (2002) Distal regulatory analysis at phox2b. Genome Res 12: 23033–23038.

38. Vistica AV, Bhanwani S, Chin L, Orlow SJ, et al. (2002) Distal regulatory analysis at phox2b. Genome Res 12: 23033–23038.

39. Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). Eugene: University of Oregon Press.

40. Fisher S, Grice EA, Vinton RM, Blessing SL, Ursaki A, et al. (2006) Evaluating the biological relevance of putative enhancers using Toz2 transponson-mediated transgenesis in zebrafish. Nat Protoc 1: 1297–1305.

41. Plagan B, Constantini F, Lacy E (1986) Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Press.