A genome-wide assessment of conserved SNP alleles reveals a panel of regulatory SNPs relevant to the peripheral nerve

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Abstract

**Background:** Identifying functional non-coding variation is critical for defining the genetic contributions to human disease. While single-nucleotide polymorphisms (SNPs) within cis-acting transcriptional regulatory elements have been implicated in disease pathogenesis, not all cell types have been assessed and functional validations have been limited. In particular, the cells of the peripheral nervous system have been excluded from genome-wide efforts to link non-coding SNPs to altered gene function. Addressing this gap is essential for defining the genetic architecture of diseases that affect the peripheral nerve. We developed a computational pipeline to identify SNPs that affect regulatory function (rSNPs) and evaluated our predictions on a set of 144 regions in Schwann cells, motor neurons, and muscle cells.

**Results:** We identified 28 regions that display regulatory activity in at least one cell type and 13 SNPs that affect regulatory function. We then tailored our pipeline to one peripheral nerve cell type by incorporating SOX10 ChIP-Seq data; SOX10 is essential for Schwann cells. We prioritized 22 putative SOX10 response elements harboring a SNP and rapidly validated two rSNPs. We then selected one of these elements for further characterization to assess the biological relevance of our approach. Deletion of the element from the genome of cultured Schwann cells—followed by differential gene expression studies—revealed Tubb2b as a candidate target gene. Studying the enhancer in developing mouse embryos revealed activity in SOX10-positive cells including the dorsal root ganglia and melanoblasts.

**Conclusions:** Our efforts provide insight into the utility of employing strict conservation for rSNP discovery. This strategy, combined with functional analyses, can yield candidate target genes. In support of this, our efforts suggest that investigating the role of Tubb2b in SOX10-positive cells may reveal novel biology within these cell populations.

**Keywords:** SOX10, Schwann cells, Peripheral nerve, Transcriptional regulation, Neuropathy, TUBB2B, Enhancer

Background

The identification of functional genetic variation is critical for understanding the allelic, locus, and clinical heterogeneity of human inherited disease. Genome-wide association studies (GWAS) have identified common variants in non-coding sequences—including transcriptional regulatory elements—that have implications for defining disease-causing and disease-modifying variants [1]. A major challenge remains in understanding the functional consequences of the implicated variants, which are frequently single-nucleotide polymorphisms (SNPs). Predicting the functional effects of SNPs is difficult due to many factors including limited knowledge of cis-acting regulatory elements (CREs) and an incomplete vocabulary of transcription factor binding sites (TFBS), especially in understudied cell populations. Furthermore, it is difficult to predict the location of CREs as they may reside anywhere in the genome and millions of base pairs distal to the genes that they regulate [2]. While ENCODE improved our TFBS vocabulary and, as a consequence, our understanding of functional genetic variation,
many tissue types have not been assessed and many of these predictions have not been functionally validated [3]. The ability to predict the effect of non-coding variation on gene expression and rapidly validate genomic regions for cis-regulatory activity will aid the identification of modifiers of human disease [4].

Charcot-Marie-Tooth (CMT) disease displays vast clinical heterogeneity and variable penetrance. CMT is an inherited peripheral neuropathy that affects 1 in 2500 individuals worldwide and is characterized by impaired motor and sensory nerve function in the distal extremities [5]. CMT is subdivided into two major classes: demyelinating (CMT1) and axonal (CMT2) [6]. CMT1 affects Schwann cell myelination and, predictably, genes associated with CMT1, including myelin protein zero (MPZ) [7, 8] and peripheral myelin protein 22 (PMP22) [9, 10], are critical for that process. Similarly, the genes implicated in CMT2, which affects motor and sensory axons, include neurofilament light chain (NEFL) [11] and mitofusin 2 (MFN2) [12], which are critical for axon function. Interestingly, while over 80 genes have been implicated in CMT disease [13], phenotypic variability is observed even among patients with a molecularly indistinguishable mutation. For example, patients with a duplication of PMP22 have variable age of onset (3-73 years of age), variable motor and sensory nerve involvement, and display a broad spectrum of severity, ranging from mild difficulty in walking or running to impairment requiring a wheelchair [14].

Among genetic variants that may modify human disease phenotypes, regulatory SNPs (rSNPs) present a unique challenge. Single-base-pair changes within sequence space that lacks a vocabulary leave few clues to their influence. rSNPs typically reside within transcriptional regulatory elements and have allele-specific effects on gene expression. An rSNP may increase regulatory activity as demonstrated by the rSNP within an enhancer at NOS1AP that elevates risk of cardiac arrhythmias [15]. Conversely, rSNPs may decrease regulatory activity as observed at SH3TC2, where the minor allele greatly reduces the binding ability of CREB [16]. Finally, an rSNP may create a novel TFBS as observed within the TNF promoter region, which results in the establishment of an OCT-1 binding site and increased gene expression [17]. While the cause of clinical variability within CMT disease is unknown, one possibility is that rSNPs, likely within TFBSs, may affect gene function in the cells of the peripheral nerve and exacerbate or alleviate the disease phenotype. Consistent with this notion, mutations in the promoter of GJB1 cause CMT disease [18]. A subset of GJB1 promoter mutations disrupt binding of the transcription factor SOX10, which is critical for Schwann cell development and function; SOX10 directly regulates genes important for Schwann cells and that have been implicated in demyelinating CMT disease (e.g., MPZ, GJB1, and PMP22) [19–21].

Here, we describe a genome-wide search for rSNPs with potential relevance to the peripheral nerve. We developed a computational pipeline to identify human non-coding genomic sequences that harbor a SNP with a major allele that is conserved among diverse vertebrate species: human, mouse, and chicken. Subsequently, we undertook a pilot functional evaluation of a subset of conserved genomic segments via luciferase assays in three cell lines relevant to the peripheral nerve and CMT disease: Schwann cells, motor neurons, and muscle cells. Finally, we deeply characterized one validated rSNP, demonstrating that it resides in a SOX10-responsive CRE that may regulate Tubb2b, which is mutated in patients with asymmetric polymicrogyria [22]. Interestingly, our results show that strict conservation analyses may not consistently enrich for CREs and associated rSNPs, thus underscoring the employment of complementary approaches.

Results

Genome-wide computational predictions of regulatory SNPs (rSNPs)

To identify and prioritize a set of putative CREs that harbor rSNPs, we developed a novel computational pipeline (Fig. 1). First, the human (hg18), mouse (mm9), and chicken (Gal3) genomes were downloaded from the UCSC Genome Browser [23], aligned using MultiPipMaker [24], and the alignments analyzed using ExactPlus [25] to identify genomic segments that are identical among the three species and at least five base pairs in length [26]. Next, conserved regions overlapping a SNP that was ‘validated by-frequency’ [i.e., SNPs that were submitted (dbSNP 130) with allele frequency information] were identified to generate a panel of conserved regions harboring a SNP. A similar analysis was performed using dbSNP 142 (Additional file 1: Figure S1). Finally, regions that overlapped exons (≥1 bp) were removed using the RefSeq database. The above process established a panel of 6197 SNPs that reside within 6164 conserved, non-coding sequences (Additional file 2).

Identification of enhancers relevant to the peripheral nerve

To validate the efficacy of our computational approach, we selected a set of 160 regions (~2.5% of the total dataset; Additional file 3), which includes all genomic segments harboring a SNP that we identified on chromosome X (94 regions), chromosome 22 (29 regions), and chromosome 21 (37 regions). To functionally assess these 160 genomic segments, we tested each for the ability to direct luciferase reporter gene expression in vitro using immortalized cell lines relevant to the peripheral nerve: Schwann cells (S16), motor neurons (MN1), and muscle cells (myoblasts;
C2C12). S16 cells are an immortalized rat Schwann cell line that expresses myelin-associated genes (e.g., MAG, MPZ, and PMP22) and transcription factors (e.g. SOX10 and EGR2) [27, 28]. MN1 cells were generated by somatic cell fusion between mouse spinal motor neurons and mouse neuroblastoma cells and exhibit traits similar to motor neurons including the ability to form neurite projections [29]. C2C12 cells are a mouse muscle progenitor cell line [30] that can be differentiated into muscle cells [31]. This process is incomplete, however, resulting in a heterogeneous population of cells and yielding transfection data that are difficult to interpret. Therefore, we focused our analyses on S16 and MN1 cell lines, but include the C2C12 data as supplemental material since they represent a critical tissue for peripheral nerve diseases. Briefly, each putative enhancer harboring the major SNP allele (Additional file 3) was cloned upstream of a minimal promoter directing luciferase gene expression [25]. Sequences surrounding each conserved region were selected based on the PhastCons 17-way vertebrate alignment dataset (mean = 885 bp, range = 105 bp to 2497 bp) [32]. The selected sequences were then separately transfected into each cell line, and luciferase activity was measured relative to a control vector with no genomic insert. Regions demonstrating a greater than five-fold increase in luciferase activity relative to the control vector were considered to have ‘strong’ activity and were selected for further analyses.

We successfully cloned 144 of the 160 genomic segments in our pilot study. Of the 16 regions lost: one SNP was removed in updated databases and was excluded from further analysis, six were amplified along with another region in the same PCR product, eight could not be amplified, and one could not be cloned. The regions were named SNP Conservation ("SC") followed by the chromosome and were numbered from the p-arm to the q-arm. For example, SCX-1 is the most distal region identified on the p-arm of chromosome X. Each of the 144 putative enhancers was subjected to Sanger sequencing to verify the presence of the major allele and then transfected into S16, MN1, and C2C12 cell lines. The activity of each region was assessed in both the forward and reverse orientation with respect to the minimal promoter. Of the 144 regions tested in S16 cells, 13 demonstrated ‘strong’ luciferase activity in at least one orientation: SCX-3, SCX-4, SCX-21, SCX-39, SCX-58, SCX-60, SCX-65, SCX-67, SCX-78, SCX-81, SCX-13, SCX-116, and SCX-20 (Fig. 2a and Table 1). In experiments using MN1 cells, 11 of the 144 regions demonstrated ‘strong’ luciferase activity in at least one orientation: SCX-3, SCX-4, SCX-21, SCX-45, SCX-58, SCX-60, SCX-63, SCX-10, SCX-12, SCX-1, and SCX-8 (Fig. 2b and Table 1). In experiments using differentiated C2C12 cells, 21 of the 144 regions demonstrated ‘strong’ luciferase activity in at least one orientation: SCX-10, SCX-16, SCX-18, SCX-27, SCX-33, SCX-34, SCX-21, SCX-8, SCX-14, SCX-3, SCX-4, SCX-18, SCX-20, SCX-21, SCX-33, SCX-45, SCX-52, SCX-58, SCX-60, SCX-63, and SCX-67 (Additional file 4: Figure S2 and Table 1). In summary, we identified 28 unique regions with strong luciferase activity in at least one orientation in Schwann cells, motor neurons, and/or muscle cells. While we did not exclude conserved regions harboring multiple SNPs, no such element exceeded our threshold for activity in the above experiments.

The minor alleles of eight peripheral nerve enhancers significantly reduce regulatory activity

The 13 genomic segments active in Schwann cells, the 11 genomic segments active in motor neurons, and the 21 genomic segments active in muscle cells were mutagenized to the minor allele and reassessed in the respective cell line to test for allele-specific differences in regulatory activity. Each allele was tested in both orientations (forward and reverse, relative to the minimal promoter) regardless of the endogenous orientation with respect to the predicted target gene. In reporting these data, the more active allele of each
region was set to ‘100%’, and the activity of the less active allele was expressed relative to the more active allele. In Schwann cells, SC21-13, SCX-4, SCX-58, SCX-60, SCX-67, SCX-78, and SCX-81 displayed allele-specific differences in luciferase activity (Fig. 3a, b, and Table 1). In motor neurons, SC21-10, SCX-4, SCX-58, and SCX-60 displayed allele-specific differences in luciferase activity (Fig. 3c, d, and Table 1). In C2C12 cells, SC21-18, SC21-27, SC22-8, SCX-4, SCX-21, SCX-45, and SCX-67 displayed allel-especific differences in luciferase activity (Additional file 5: Figure S3). Combined, we identified 13 unique regions displaying significant allele-specific differences in regulatory activity between the major and minor alleles. Three regions were unique to S16 cells, one region was unique to MN1 cells, and five regions were unique to C2C12 cells, while the remaining four regions demonstrated reduced regulatory activity in a combination of cells (Table 1).

Predicting differential transcription factor binding to active regions

One possible explanation for the allele-specific differences described above is that the SNP alleles
display differential affinity for transcription factors. To test this possibility, we used an in silico transcription factor binding site prediction program, TRANSFAC [33]. Sequence fragments of 30 base pairs centered around each major SNP allele were generated. The major allele was swapped for the

| Region   | Fold-increase Forward<sup>a</sup> | Fold-increase Reverse<sup>a</sup> | Coordinates (hg18) | rs Number |
|----------|-----------------------------|-----------------------------|------------------|-----------|
| SC21-10  | 5.91 (M)                    | 0.25 (M)                    | chr21:21,313,182-21,313,188 | rs7277262 |
| SC21-12  | 9.95 (M)                    | 5.34 (M)                    | chr21:22,353,874-22,353,880 | rs2827297 |
| SC21-13  | 11.42 (S)                   | 1.04 (S)                    | chr21:27,318,695-27,318,702 | rs233616  |
| SC21-16  | 28.67 (S)                   | 2.73 (S)                    | chr21:29,424,534-29,424,540 | rs2832203 |
| SC21-18  | 1.37 (C)                    | 8.90 (C)                    | chr21:33,139,128-33,139,135 | rs2833975 |
| SC21-20  | 5.81 (S)                    | 5.04 (S)                    | chr21:33,273,214-33,273,219 | rs2834040 |
| SC21-27  | 16.05 (C)                   | 4.71 (C)                    | chr21:36,269,198-36,269,214 | rs2835196 |
| SC21-33  | 13.85 (C)                   | 3.40 (C)                    | chr21:38,940,551-38,940,556 | rs16996658|
| SC21-34  | 6.22 (C)                    | 0.33 (C)                    | chr21:38,958,107-38,958,118 | rs8130434 |
| SC22-1   | 2.58 (M)                    | 6.68 (M)                    | chr22:16,689,437-16,689,442 | rs5992119 |
| SC22-8   | 1.00 (M)                    | 5.73 (M)                    | chr22:25,678,449-25,678,472 | rs5761863 |
| SC22-14  | 4.70 (C)                    | 13.83 (C)                   | chr22:26,146,779-26,146,784 | rs733164  |
| SCX-3    | 0.20 (S)                    | 6.97 (S)                    | chrX:15,529,186-15,529,192 | rs4646115 |
| SCX-4    | 0.17 (S)                    | 6.85 (S)                    | chrX:17,730,099-17,730,104 | rs2187846 |
| SCX-18   | 14.59 (C)                   | 0.98 (C)                    | chrX:31,252,044-31,252,049 | rs7884417 |
| SCX-21   | 8.63 (S)                    | 0.18 (S)                    | chrX:31,764,702-31,764,707 | rs1379871 |
| SCX-33   | 0.67 (C)                    | 13.92 (C)                   | chrX:31,854,058-31,854,064 | rs6623642 |
| SCX-39   | 5.84 (S)                    | 0.50 (S)                    | chrX:86,429,198-86,429,205 | rs16980794|
| SCX-45   | 0.37 (M)                    | 10.62 (M)                   | chrX:92,656,893-92,656,900 | rs12687113|
| SCX-52   | 0.50 (C)                    | 5.02 (C)                    | chrX:99,454,087-99,454,093 | rs7064056 |
| SCX-58   | 6.33 (S)                    | 0.64 (S)                    | chrX:121,682,472-121,682,478 | rs17273301|
| SCX-60   | 0.43 (S)                    | 5.73 (S)                    | chrX:123,382,405-123,382,410 | rs2076164 |
| SCX-63   | 21.68 (M)                   | 1.04 (M)                    | chrX:125,247,137-125,247,140 | rs16998722|
| SCX-65   | 15.05 (S)                   | 0.41 (S)                    | chrX:125,925,884-125,925,893 | rs5930055 |
| SCX-67   | 8.97 (S)                    | 6.90 (S)                    | chrX:127,229,700-127,229,720 | rs17266605|
| SCX-78   | 5.21 (S)                    | 5.80 (S)                    | chrX:146,960,885-146,960,891 | rs6525876 |
| SCX-81   | 1.09 (S)                    | 12.85 (S)                   | chrX:147,430,625-147,430,635 | rs17252118|

<sup>a</sup>Activity only shown for cell line(s) where region was active
S = S16, M = MN1, C = C2C12
minor allele and both sequences were submitted to TRANSFAC. The sequences were assessed for TFBS predictions using the TRANSFAC Match algorithm and the vertebrate database of transcription factors, minimizing the sum of false positive and false negative errors. Results were filtered to display only unique differences in predicted TFBSs, which may explain the differential activity.

For the seven genomic segments differentially active in Schwann cells (Fig. 4a-d and f-h), each had at least one allele-specific TFBS prediction. Interestingly, none of the four Schwann cell specific regions [SC21-13, SCX-67, SCX-78, and SCX-81 (Fig. 4a-d)] harbored predicted TFBSs known to be important for these cells. While these results may be due to the limitations of TRANSFAC, they may also illustrate potentially novel roles of the predicted transcription factors in Schwann cells. For the four genomic segments differentially active in motor neurons (Fig. 4e-h), one (SC21-10) displayed no allele-specific predictions (Fig. 4e). This result may indicate that the SNP does not ablate or alter TFBS binding affinity or it may reflect the incomplete nature of TFBS databases. For the seven genomic segments differentially active in muscle cells (Additional file 6: Figure S4), each had at least one allele-specific TFBS prediction; two of these segments demonstrated regulatory activity only in muscle cells (SC21-18 and SC21-27). Strikingly, a LEF-1 binding site prediction is unique to the minor allele of SC21-27. Lef-1 expression increases in mouse muscle cells following muscle injury [34]. Our data show that the minor allele of SC21-27 demonstrates significantly lower regulatory activity compared to the major allele (Additional file 5: Figure S3). Indeed, LEF-1 can act as a transcriptional repressor [35, 36] suggesting that the increased activity of the major allele is due to decreased LEF-1 binding; however, further study is needed to determine the role of the putative LEF-1 binding site in regulatory activity. Taken together, TRANSFAC revealed differential TFBS predictions across many of the genomic segments showing allele-specific differences in regulatory activity. While the significance of these predictions vary with respect to what is known about Schwann cell, motor neuron, and muscle cell biology, some may represent novel findings relevant to gene regulation in these cells.
Identification of two rSNPs within SOX10 response elements

Our pipeline successfully identified a small panel of putative rSNPs in a transcription-factor-independent approach. We next wanted to evaluate our computational predictions in the context of TFBS information (i.e., motif analysis and ChIP-Seq data). To this end, we evaluated the conserved regions, identified above, harboring a SNP from dbSNP 130 (6164 regions; Fig. 1 and Additional file 2) for the presence of a predicted SOX10 binding site (Fig. 5a). SOX10 was chosen because it is critical for Schwann cell function and regulates genes involved in peripheral nerve myelination [19–21]. SOX10 also directly binds to DNA via a well-defined consensus sequence as either a monomer or as a dimer when two monomeric sequences are oriented in a head-to-head fashion [37–39]. We first identified all SOX10 monomeric consensus sequences within the human genome [5′ to 3′: ACAA, ACACA, ACAAT, or ACAAG (and the reverse complement of each)]. We then overlapped these data with the conserved regions harboring a SNP, which revealed 224 putative monomeric SOX10 binding sites containing a SNP and conserved among human, mouse, and chicken (Additional file 7). This dataset was further prioritized by only including sequences that overlap with SOX10 ChIP-Seq data [39] (resulting in nine regions, referred to as rSNP-containing and SOX10-relevant (rSOX) 1 through 9 in Fig. 5a and Table 2) and by separately assessing for the presence of a dimeric SOX10 consensus sequence where both monomers were conserved, but only one monomer was required to contain a SNP (resulting in 13 regions, referred to as rSOX-10 through 22 in Fig. 5a and Table 2).

The major allele of each of the 22 rSOX regions was cloned and evaluated for regulatory activity in S16 cells via luciferase assays as described above; SOX10 and SOX10 target genes are expressed in S16 cells [40]. Sequences surrounding each conserved rSOX region was selected for cloning based on the PhastCons 17-way vertebrate alignment dataset (mean = 729 bp, range = 319 bp to 1834 bp) [32]. These efforts revealed four genomic segments that exceeded a five-fold threshold of activity in at least one orientation: rSOX-1, rSOX-4, rSOX-6, and rSOX-22 (Fig. 5b and c, and Table 3). To assess for allele-specific differences in regulatory activity, the four genomic segments with strong activity were mutagenized to the minor allele and reassessed in luciferase assays in S16 cells. Two regions, rSOX-4 and rSOX-22, showed a significant difference in luciferase activity between the two alleles (Fig. 6a). The minor allele of rSOX-4 demonstrated 45.8% of the activity compared to the major allele, and the minor allele of rSOX-22 demonstrated 57.0% of the activity of the major allele.

We further assessed the role of SOX10 in regulating rSOX-4 and rSOX-22 by deleting the putative SOX10 binding sites harboring a SNP (a single monomeric sequence for rSOX-4 and a single dimeric sequence for rSOX-22) and performing luciferase activity assays in S16 cells. In each case we observed an even more dramatic decrease in regulatory activity upon deleting the SOX10 binding site. Relative to the major alleles, rSOX-4 ΔSOX demonstrated 36.5% activity, and rSOX-22 ΔSOX demonstrated 10.9% activity.
activity (Fig. 6b). We also tested each SNP allele and ΔSOX10 genomic segment for activity in MN1 cells, which do not express endogenous SOX10. Neither region harboring the major allele showed strong activity in MN1 cells, consistent with SOX10 being required for the observed regulatory activities (Fig. 6b). Furthermore, neither the minor SNP allele nor deletion of the SOX10 consensus sequence had an effect on regulatory activity in SOX10-negative MN1 cells.

We next evaluated the role of SOX10 in regulating rSOX-4 and rSOX-22 by studying the activity of each allele in SOX10-negative MN1 cells co-transfected with a construct to overexpress wild-type SOX10. In the absence of SOX10, neither allele of the two regions demonstrated activity in MN1 cells, consistent with the results above. In contrast, in the presence of SOX10, both regions displayed strong activity, approximately 200-fold and 89-fold higher for rSOX-4 and rSOX-22, respectively (Fig. 6c). Conversely, when we performed luciferase assays with a construct to express a dominant-negative SOX10 protein (E189X) to deplete endogenous SOX10 function in S16 cells [41], both regions displayed severely decreased activity (~ 9%) compared to the respective major alleles (Fig. 6d). Taken together, our data indicate that SOX10 is both necessary and sufficient for the in vitro regulatory activity of rSOX-4 and rSOX-22 in cultured Schwann cells.

Ablation of rSOX-4 in cultured Schwann cells reduces Tubb2b expression

Identifying the target gene(s) of cis-acting regulatory elements is essential for understanding the biological significance of rSNPs that reside within these elements. To identify the target gene of an enhancer/rSNP pair identified in this study, we selected rSOX-4 and deleted it from S16 cells using CRISPR/Cas9 [42] technology. We then performed RNA-Seq analysis in an unbiased attempt to identify candidate target genes with altered expression. The rSOX-4 element is a 651-base-pair region that resides in a ~ 1 Mb interval devoid of annotated genes (Additional file 8: Figure S5A) and that displayed genomic features indicating that it is an active, SOX10-bound enhancer, including: H3K27 acetylation from adult rat peripheral nerve [43], SOX10 ChIP-Seq from P15 rat sciatic nerve [39], and DNase hypersensitivity from the S16 cell line [26] (Additional file 8: Figure S5A). Briefly, repair templates were generated with ~ 1 Kb arms of homology to the 5’ and 3’ regions surrounding the 651-base-pair rSOX-4 element, and flanking a floxed blastocidin (or neomycin) resistance cassette (Fig. 7a). S16 cells were transfected with the blastocidin repair template, human codon optimized Cas9 (hCas9), and one of two gRNAs targeting rSOX-4 (Additional file 9: Figure S6). Next, cells were selected
Table 2 Twenty-two conserved SOX10 consensus sequences containing SNPs

| Name  | Conserved Region (hg18) | rs Number |
|-------|--------------------------|-----------|
| rSOX-1 | chr2:44834620-44834625 | rs3738980 |
| rSOX-2 | chr3:62462974-62462979 | rs6445273 |
| rSOX-3 | chr4:146183931-146183936 | rs34577920 |
| rSOX-4 | chr6:22818474-22818479 | rs16866790 |
| rSOX-5 | chr6:98577359-98577364 | rs12524696 |
| rSOX-6 | chr6:98602222-98692227 | rs17814604 |
| rSOX-7 | chr7:131601713-131601718 | rs1364510 |
| rSOX-8 | chr13:66589464-66589469 | rs17082112 |
| rSOX-9 | chr15:55214627-55214632 | rs2703617 |
| rSOX-10 | chr1:48904340-48904354 | rs1966247 |
| rSOX-11 | chr1:168449895-168449935 | rs16863114 |
| rSOX-12 | chr6:118599910-118599921 | rs17335828 |
| rSOX-13 | chr7:9853948-9853966 | rs12702949 |
| rSOX-14 | chr7:95350538-95350566 | rs10249566 |
| rSOX-15 | chr8:37281347-37281384 | rs17333409 |
| rSOX-16 | chr8:138460036-138460047 | rs16907090 |
| rSOX-17 | chr9:80243161-80243189 | rs17788061 |
| rSOX-18 | chr10:78070796-78070831 | rs17460556 |
| rSOX-19 | chr10:130596685-130596718 | rs11819115 |
| rSOX-20 | chr11:31400150-31400183 | rs1376362 |
| rSOX-21 | chr11:125354123-125354139 | rs11607720 |
| rSOX-22 | chr16:53169712-53169728 | rs1186802 |

rSOX-1 through rSOX-9 are conserved monomeric SOX10 consensus sequences that overlap with SOX10 ChIP-Seq data.

rSOX-10 through rSOX-22 are conserved dimeric SOX10 consensus sequences.

Table 3 Four genomic segments harboring a SOX10 consensus sequence display regulatory activity in Schwann cells

| Region | Fold-increase Forward | Fold-increase Reverse | Coordinates (hg18) | rs Number | MAFa |
|--------|-----------------------|-----------------------|--------------------|-----------|------|
| rSOX-1 | 2.57                  | 8.29                  | chr1:244834620-44834625 | rs3738980 | 0.3035 |
| rSOX-4 | 4.83                  | 25.96                 | chr6:22818474-22818479 | rs16866790 | 0.2001 |
| rSOX-6 | 0.33                  | 11.01                 | chr6:98692222-98692227 | rs17814604 | 0.1014 |
| rSOX-22 | 14.12               | 4.44                  | chr16:53169712-53169728 | rs1186802 | 0.4219 |

Coordinates for the conserved SOX10 monomeric sites for rSOX-1, –4, and –6 and the dimeric site for rSOX-22

*MAF is the minor allele frequency from the 1000 Genomes Project [72].

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from five clonal cell populations generated from unmodified S16 cells, performed RNA-Seq as described above, and reanalyzed for differential gene expression between the rSOX-4 mutant S16 cells and all unmodified S16 cell lines. In agreement with the ddPCR validation studies, Tubb2b, but not Gmnn, was significantly decreased in expression in the rSOX-4 mutant cells compared to all unmodified S16 samples (Additional file 10: Figure S7). Finally, we performed capped analysis gene expression (CAGE) [47] on MN1 cells with and without a construct to over-express SOX10 (see Methods for details; Fogarty and Antonellis, manuscript in preparation). In the presence of SOX10, the known SOX10 target genes Mpz and Mitf show increased reads per million (Fig. 8c and d). Importantly, in the presence of SOX10, Tubb2b shows a dramatic increase in expression while Gmnn does not. Taken together, these results support the conclusion that Tubb2b is regulated by SOX10 activity at rSOX-4.

rSOX-4 directs LacZ expression in SOX10-positive cells at E11.5

To determine the physiological relevance of rSOX-4 in vivo, we assessed the ability of the genomic segment harboring the major allele to direct LacZ expression during mouse development. Because the regulatory activity of rSOX-4 is dependent on SOX10, we anticipated that rSOX-4 would demonstrate activity in tissues in which SOX10 is expressed. Based on the RNA-Seq data indicating that Tubb2b is the target gene of this enhancer, we selected embryonic day 11.5 (E11.5) due to the known role of Tubb2b in neuronal migration [22]. We reasoned that rSOX-4 and Tubb2b may be performing a similar role in migratory neural crest cells and neural crest derivatives; Schwann cells are derived from the neural crest. By E11.5 in mice, the neural tube has closed, and neural crest cells and derivatives have started to migrate [48–51].
We cloned rSOX-4 upstream of the minimal Hsp68 promoter directing LacZ expression [52] in the reverse orientation because this was the more active orientation in luciferase assays. The rSOX-4:LacZ transgene was liberated from the plasmid backbone, gel purified, and injected into mouse zygotes. Zygotes were implanted into pseudo-pregnant female mice, and embryos were allowed to develop until E11.5 and then harvested to examine LacZ expression. We isolated 54 embryos from ten mice and identified six LacZ positive mice (Fig. 9a-f). Five embryos demonstrated LacZ staining in the dorsal root ganglia and migrating melanoblasts (Fig. 9a, b, and d-f), both tissues in which SOX10 is expressed at E11.5 [53, 54]. A single embryo displayed ubiquitous blue LacZ staining throughout the entire embryo, likely a consequence of position effect (Fig. 9c). These experiments demonstrate that rSOX-4 is active in SOX10-positive cells during mouse development and suggest that Tubb2b plays an important role in neural crest cell function.

**Discussion**

The identification and characterization of regulatory variation (e.g., rSNPs) will improve our understanding of human biology and disease. To study regulatory variation in the human genome we developed a computational pipeline to identify genomic sequences that harbor a SNP.
and that are conserved among human, mouse, and chicken. We functionally evaluated a pilot set of 144 conserved genomic segments in luciferase assays in three cell lines relevant to the peripheral nerve: Schwann cells, motor neurons, and muscle cells. We identified 28 (19% of the 144 elements) putative enhancers, including 13 (9% of the 144 elements) SNPs that demonstrated significant allele-specific differences in regulatory activity. We further assessed our pipeline by separately incorporating conserved sequence information and ChIP-Seq data for SOX10—a transcription factor essential for Schwann cells. We prioritized 22 conserved genomic segments harboring a SNP predicted to disrupt a SOX10 binding site and identified two (9% of the 22 elements) rSNPs with a significant effect on regulatory activity. Finally, we deeply characterized one rSNP-containing SOX10 response element by deleting the region in cultured Schwann cells to identify dysregulated genes and by generating transient transgenic mice to study in vivo enhancer activity. Our findings revealed a far-upstream regulatory variant that effects SOX10 function at Tubb2b, thus displaying the ability of our approach to find biologically relevant transcriptional regulatory elements and associated rSNPs.

The computational and functional studies described here yielded a large panel of SNPs that reside in highly conserved regions, RNA-Seq data from cultured Schwann cells, and a MA plot demonstrating differential gene expression between the rSOX-4 deleted cells and untreated S16 cells. Each dot represents a gene and red dots indicate a significant difference in expression between the two cell populations (adjusted p < 0.05). Genes with a positive or negative log2 fold change demonstrated higher or lower expression in the rSOX-4 cells line compared to the untreated S16 cells, respectively. Tubb2b and Gmnn are indicated by arrows. Data from the untreated cells are shown by the blue bars, and data from rSOX-4 deleted cells are shown by the red bars. Aldh5a1 was used as a control gene, which showed no expression changes between the two cell populations. SOX10 increases Tubb2b expression in heterologous cells. MN1 cells were transfected with a construct to express GFP-SOX10, sorted into GFP-positive and GFP-negative populations, and subjected to cap analysis gene expression (CAGE). CAGE reads mapping to the Mpz (c), Mitf (d), Tubb2b (e), and Gmnn (f) loci were normalized to reads per million and are indicated in red (SOX10-positive cells) and blue (SOX10-negative cells). RefSeq-annotated transcripts at each locus in the mouse genome (mm10) are shown in black and, in each case, are transcribed from left to right.

Table 4: Six genes on chromosome 17 are differentially expressed in Schwann cells deleted for rSOX-4

| Gene     | p-valuea | Fold Changeb | Distance from rSOX-4c |
|----------|----------|--------------|-----------------------|
| Tubb2b   | 1.48E-10 | -7.41        | 8.9                   |
| Rbm24    | 0.025    | 2.63         | 23                    |
| Gmnn     | 0.042    | -1.61        | 1.9                   |
| Eif4     | 0.044    | 1.73         | 47                    |
| Mylpa    | 0.044    | 1.59         | 20.5                  |
| Akr1c19  | 0.045    | 2.50         | 29                    |

aNBenjamini-Hochberg adjusted p-value
bFold change calculated relative to unmodified S16 cells (negative values indicate lower expression in rSOX-4 mutant cells, positive values indicate higher expression in rSOX-4 mutant cells)
cDistances are given in million basepairs
cells, and small panels of regulatory SNPs (rSNPs) and cis-regulatory elements (CREs) useful for investigators studying peripheral nerve and SOX10 biology. Furthermore, the pipeline developed here is flexible and may accommodate additional CRE information when it becomes available; for example, we combined our pipeline with previously unavailable SOX10 ChIP-Seq [39], S16 DNase hypersensitivity [26], and H3K27 acetylation [43] datasets to prioritize putative SOX10 binding sites for functional validation. There are, however, several inherent limitations to our approach that could be improved upon. First, our computational predictions of rSNPs relied on non-coding sequence conservation among diverse vertebrate species. This limitation is problematic because rSNPs may reside in less well-conserved genomic regions [55]. Indeed, our efforts suggest that the employment of strict conservation analyses does not dramatically enrich for functional regulatory elements and associated rSNPs. To address this problem, one could overlap available SOX10 ChIP-Seq data [39] with our data set of SOX10 consensus sequences [26] and study SNPs that map to overlapping genomic sequences, regardless of sequence conservation. Second, we tested a small subset of the 6164 regions predicted by our computational approach. Recent advances in massively parallel reporter assays would allow for functional interpretation of most, if not all, of our predictions [56, 57]. The use of a minimally active promoter also restricts our interpretation because it only allows for detection of major alleles which direct high levels of reporter activity; using an active basal promoter could enable the identification of repressive elements. Finally, the rSNPs identified here were studied outside of the proper genomic context (i.e., a small genomic segment surround each SNP allele was studied for regulatory activity in a vector containing a luciferase reporter gene). Interestingly, the majority of the genomic segments and rSNPs that we studied showed orientation-specific activity and an orientation-specific effect of the rSNP on regulatory activity. With respect to the orientation-specific effects, while the classical definition of an enhancer is that it should act in an orientation-independent manner, this likely depends on the specific sequence of the element and how it is cloned into a reporter gene vector; indeed, enhancers with orientation-dependent activity in vitro and in vivo have been reported [58, 59]. With respect to the orientation-specific effect of the rSNP, one possible explanation for this is that the position of the rSNP in the reporter gene construct has orientation-specific effects on transcription factor and transcriptional machinery binding in vitro. While one genomic segment harboring an rSNP (rSOX-4) was evaluated via CRISPR-mediated deletion of the regulatory element, future analysis of rSNPs important for peripheral nerve biology and disease should include studying allele-specific effects at the endogenous locus in an appropriate cell or animal model.

Characterization of rSOX-4 via CRISPR/Cas9 [42] mediated deletion revealed Tubb2b as a strong candidate target gene of this enhancer. TUBB2B is a critical component of microtubules, and mutations in human TUBB2B result in polymicrogyria [22]. Interestingly, our transgenic mouse studies revealed that rSOX-4 directs LacZ expression in the dorsal root ganglia and migratory melanoblasts. A
In this study, we developed a computational and functional pipeline that identified a small panel of cis-acting regulatory elements harboring regulatory SNPs (rSNPs). This pipeline revealed rSNPs in both a transcription factor independent and dependent manner. While our computational approach may be less relevant for biological systems with well-developed genomic data sets, we believe it allows a less-biased strategy for identifying rSNPs important for developmental stages and tissues for which ChIP-Seq and related genomic analyses are not feasible. We then deeply characterized an enhancer that is regulated by SOX10 (rSOX-4) through CRISPR-mediated deletion and in vivo transient transgenic mouse reporter experiments. Through these studies, Tubb2b was identified as a strong candidate target gene of rSOX-4. While additional experiments are necessary to determine the role of rSOX-4 in SOX10-positive cells, the SNP within rSOX-4, the rSOX-4 enhancer, and the Tubb2b locus all represent excellent candidate modifiers of neurocrustopathies. Additionally, understanding and characterizing the role of Tubb2b in neural crest-derived cells—in particular the dorsal root ganglia and melanoblasts—will provide insight into the biology of these cell populations.

### Methods

**PCR and cloning of putative regulatory elements**

PCR was performed utilizing primers containing attB1 and attB2 Gateway cloning (Invitrogen) sequences to amplify a region surrounding each of the 144 conserved, non-coding regions with SNPs from human DNA (Additional file 3) and the 22 SOX10 regions (Table 2). The total amplified region was based on general conservation using the PhastCons 17-way vertebrate alignment [32]. If a region fell within a PhastCons track, the primers were designed to amplify the entire region defined by the track. If a region did not reside within a PhastCons track, then primers were designed to amplify a region conserved between human and mouse only (~ 500 bp). Each amplified region was BP cloned into pDONR221 using the Gateway cloning technology (Invitrogen). The regions were sequenced to verify the SNP allele and absence of additional mutations. These regions were subsequently LR cloned (Invitrogen) upstream of a minimal E1B promoter directing luciferase expression [25] in both the forward (pE1B Forward) and reverse (pE1B Reverse) orientations. Active regions were converted to the alternative allele, or SOX10 binding sites were deleted using site-directed mutagenesis. Mutagenic primers (sequences available upon request) were designed, and mutagenesis was performed on the regions cloned into pDONR221 using the QuikChange II Mutagenesis Kit (Agilent Technologies). The resulting constructs were sequenced to verify specificity and then LR cloned into pE1B as described above.

**Cell culture and transfection for luciferase experiments**

We obtained immortalized rat Schwann (S16) cells in 2006 from Richard Quarles (NIH/NINDS) who originally established these cells [27]. We obtained mouse MN1 cells in 2004 from Kurt Fischbeck (NIH/NINDS), who obtained them directly from the laboratory that generated these cells (H. Kim, University of Chicago) [29]. The C2C12 [30] cells were obtained from ATCC (CRL-1772) in 2011. We routinely test our cells for mycoplasma contamination. All cell lines were verified for...
authenticity using gene expression experiments including RT-PCR and sequencing of products (all three cell lines), RNA-Seq (S16 cells), and CAGE (MN1 cells). S16 and MN1 cells were cultured at 37 °C in 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen), 1X glutamine (Gibco), and 1X Penicillin/Streptavidin (ThermoFisher). S16 and MN1 cells were plated at 10,000 cells/well of a 96-well plate. The cells were transfected the following day using Lipofectamine 2000 (Life Sciences). Each well was transfected with 200 ng of pE1B [25] plasmid containing the region of interest and 2 ng of a renilla expression construct. Cells were harvested 48 h after transfection and assessed using the Dual-Luciferase Reporter system (Promega). Luciferase activity was normalized to renilla activity, and all regions of interest were compared to a control vector with no human genomic insert (‘Empty’ in figures). For transcription factor overexpression assays in S16 and MN1 cells, an additional 100 ng of the overexpression plasmid was added per well. C2C12 cells were assessed as described above with the following changes [65]: they were plated at a concentration of 5000 cells/well in a 96-well plate, and 24 h after transfection the media was changed to differentiation media: Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5% horse serum (FBS; Invitrogen), 1X glutamine (Gibco), and 1X Penicillin/Streptavidin (ThermoFisher). For all luciferase assays, at least eight technical replicates across three biological replicates (n = 24) were performed.

**TRANSFAC analysis**

The major allele of each SNP was centered within 30 bp of genomic sequence and extracted using the UCSC Human Genome Browser. The minor allele nucleotide of the candidate rSNP was substituted into the above sequence to generate the minor allele sequence. The major and minor allele sequences were then submitted to TRANSFAC using the Match tool [66]; the ‘vertebrate, non-redundant’ and ‘minimize the sum of false positive and negative error rates’ options were used. Results were visually examined and manually filtered to exclude any predicted binding sites that were identical between the major and minor allele, regardless of the core or matrix scores.

**CRISPR-mediated deletion of rSOX-4**

Two guide RNAs (gRNAs) were designed and cloned into a gRNA expression plasmid (Addgene plasmid #41824) as previously described [42]. The repair template was constructed using Gibson assembly [67] and cloned into pUC19. The 5’ and 3’ homology arms were PCR-amplified from S16 genomic DNA, the blasticidin resistance cassette was PCR amplified from pCMV/Bsd (ThermoFisher - Cat no. V510-20), and the neomycin resistance cassette was PCR amplified from the hCas9 backbone (Addgene plasmid #41815) [42]. Wild-type S16 cells were cultured as described above and plated at 100,000 cells/well in a 6-well dish 24 h prior to transfection. The cells were transfected with 3 µg of total DNA [1 µg of hCas9, 1 µg of gRNA expression plasmid, and 1 µg of linearized repair template (~ 1:1:1 M ratio)] using Lipofectamine 2000. Standard growth media was replaced after 4 h of transfection, and the cells were grown for 72 h at 37 °C and 5% CO₂. The growth media was removed, and selection media (standard growth media with 4 µg/mL of blasticidin and/or 700 µg/mL of neomycin) was added. After selection (~ 3 days for blasticidin and ~ 14 days for neomycin) the cells were expanded to a T-75 flask before flow sorting into a 96-well plate to generate clonal populations. The cells were gradually expanded over ~1 month, genomic DNA was harvested, and diagnostic PCR was performed using genomic- and drug-specific primers. The procedure was repeated to target any remaining wild-type alleles using a neomycin resistance repair template; however, the gRNAs had to be reversed due to indels in the remaining wild-type alleles after blasticidin selection (Additional file 9: Figure S6; i.e. gRNA1 was used in rSOX-4 Clone 2-B and gRNA2 was used in rSOX-4 Clone 1-B). Additionally, we were unable to amplify the blasticidin repair template from rSOX-4 Clone 2 following the second round of clone generation. Double-resistant clones (blasticidin and neomycin) were expanded in T-75 flasks, transiently transfected with a Cre-GFP expression plasmid (Addgene plasmid #13776) to remove the floxed drug cassettes, and flow sorted to generate clonal GFP-positive cells.

**RNA-Seq analysis of wild-type and rSOX-4 mutant cells**

RNA was isolated from the three rSOX-4 mutant S16 lines, two unmodified S16 lines [one parental cell line of the rSOX-4 mutants (passage 9) and one older passage (passage 39)], and five clonal expansions of unmodified S16 cells using TRIzol extraction (ThermoFisher). mRNA libraries were generated using TruSeq (Illumina) and subjected to 50 bp single-end sequencing on a HiSeq2000. Two technical replicates of the rSOX-4 mutant and two unmodified S16 lines cells were pooled and run across two sequencing lanes which resulted in ~21.5 million reads per cell line. The five clonally expanded S16 cells were only sequenced once. Read quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were aligned to the rat Rnor_5.0 (Ensembl) reference genome using STAR [44]. Default parameters were used, except only uniquely mapped reads were allowed (~82% of total reads mapped uniquely). HTSeq [45] was used to count the number of reads per
gene using default parameters with the stranded reads function disabled. Finally, differential gene expression between the rSOX-4 mutant and unmodified S16 cells was determined using DESeq2 [46]. All programs were run on the ARCTS flux servers at the University of Michigan. An identical analysis for the five clonally expanded unmodified S16 cells was performed as described above. Data for the two technical and two biological replicates of unmodified S16 cells are located on NCBI GEO (GSE81709).

Digital droplet PCR
cDNA was synthesized from RNA extracted from the rSOX-4 mutant and unmodified S16 cells using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each reaction contained 3.2 μL ultrapure water, 2 μL 10X random primers, 2 μL 10X RT buffer, 1 μL RNase inhibitor, 0.8 μL dNTPs, 1 μL RNA in 10 μL of water, and 1 μL MultiScribe reverse transcriptase. The reaction was incubated for 10 min at 25 °C, then 2 h at 37 °C, then 85 °C for 5 s, and finally held at 4 °C. The resulting cDNA was diluted 1:333 in ultrapure water prior to the digital droplet PCR (ddPCR). Each ddPCR contained 11.5 μL 2X ddPCR supermix (BioRad), 1 μL FAM probe, 1 μL HEX probe, 6 μL cDNA template (diluted 1:333), and 3.5 μL water. FAM probes were designed against the gene of interest while HEX probes were against the control gene (Gapdh). Probes and primers were ordered from IDT PrimeTime as prede-

Generation of transient transgenic mice harboring rSOX-4: LacZ
rSOX-4 was LR cloned upstream of a LacZ reporter gene directed by the HSP68 minimal promoter [52]. The plasmid was sequence verified, and the rSOX-4:LacZ transgene fragment was excised from the vector backbone by digestion with SalI. The transgene was isolated and injected into mouse zygotes by the University of Michigan transgenic animal core, and zygotes were implanted into pseudo-pregnant mice. Pregnant female mice were sacrificed (CO2 vapor was employed because it is easy to use and is the recommended choice of the Panel of the American Veterinarian Medical Association) at embryonic day 11.5 (E11.5), and embryos were dissected and placed into 15 mL of ice cold fixative solution: 0.2% glutaralde-

Cap analysis gene expression (CAGE)
The human SOX10 open-reading frame in pDONR221 (Addgene plasmid #24749) was obtained and SOX10 was cloned into pcDNA-DEST53 using LR Clonase (Thermo-Fisher) to generate a GFP-SOX10 expression construct. MN1 cells [29] were plated at a density of 100,000 cells per mL and transfected with the GFP-SOX10 expression construct using Lipofectamine 2000 (ThermoFisher). After 48 h, cells were harvested, suspended in PBS, and sorted into GFP-positive and GFP-negative populations using a SY3200 Cell Sorter (Sony). RNA was isolated from each cell population using the RNeasy Mini Kit (QUIAGEN). A CAGE library was generated for each sample as described [47], with modifications made for compatibility with current generation Illumina sequencing platforms. Each library was sequenced on an Illumina MiSeq (4 million reads generated per library). The sequencing data was analyzed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), filtered using TagDust2 [68], mapped using BWA [69], clustered using Paraclu [70], and visualized using BEDTools [71] and the UCSC Genome Browser [23].
Additional file 2: List of the 6197 SNPs residing within 6164 conserved, non-coding sequences. Columns 1 through 3 are BED file formatted hg18 coordinates of the conserved, non-coding regions. Columns 4 through 6 are BED file formatted hg18 coordinates of the SNPs residing within the identified regions. The final column is the rs ID number for each SNP. (TXT 352 kb)

Additional file 3: List of the pilot set of regions and SNPs from chromosome 21, 22, and X. Columns 1 through 3 are BED file formatted hg18 coordinates of the conserved, non-coding regions. Columns 4 through 6 are BED file formatted hg18 coordinates of the SNPs residing within the identified regions. The final column is the rs ID number for each SNP. (TXT 9 kb)

Additional file 4: Figure S2. Activity of a set of putative regulatory elements on chromosomes 21, 22, and X in muscle (C2C12) cells. 144 genomic regions containing the major SNP allele were cloned upstream of a luciferase reporter gene and tested in the forward (blue bars; upper) or reverse (red bars; lower) orientation in C2C12 cells. The activity of each genomic segment is expressed relative to a control vector with no insert (first column) set to “1”. Dashed lines indicate a five-fold increase in activity over the control vector, and error bars show standard deviations. (AI 1152 kb)

Additional file 5: Figure S3. Seven regions display allele-specific differences in luciferase activity in muscle (C2C12) cells. (A) The activity of the major (black bars) and minor (grey bars) alleles of the 21 regions active in muscle cells (Additional file 4: Figure S2) were evaluated in the forward (A) or reverse (B) orientation. In both panels, the allele with higher luciferase activity was set to “100”, error bars represent standard deviations, bold and underlined text indicate the orientation(s) that were active in experiments shown in Additional file 4: Figure S2, and asterisks indicate a significant change in activity (p ≤ 0.05). (AI 1169 kb)

Additional file 6: Figure S4. TRANSFAC predictions of transcription factor binding sites within elements active in muscle cells. TRANSFAC was used to assess for allele-specific TFBS predictions between the major and minor alleles of SNP alleles that had a significant effect on luciferase activity. Results are shown for seven regions active in C2C12 cells: SC21-18 (A), SC21-27 (B), SC22-8 (C), SCX-4 (D), SCX-21 (E), SCX-45 (F), and SCX-67 (G). Thirty-base-pairs surrounding each SNP allele was submitted to TRANSFAC. Dashed arrows indicate the position and direction of the predicted TFBS, the name of the transcription factor is indicated above each arrow, and the core and matrix scores are indicated at the right. Only allele-specific TFBS predictions are displayed. Underlined base pairs indicate conserved bases, and the SNP alleles are highlighted in red and bold text. (AI 1331 kb)

Additional file 7: List of the 224 SOX10 consensus sites harboring SNPs. Columns 1 through 3 are BED file formatted hg18 coordinates of the conserved, non-coding SOX10 consensus sites. Columns 4 through 6 are BED file formatted hg18 coordinates of the SNPs residing within the identified regions. The final column is the rs ID number for each SNP. (TXT 14 kb)

Additional file 8: Figure S5. rSOX-4 overlaps genomic features associated with enhancers. (A; Top) rSOX-4 (red box) overlaps three lyase 3 gene acylation peaks (H3K27Ac; purple track), SOX10 Chip-Seq peaks from sciatic nerve (pink track), and S16 Dnase hypersensitivity peaks (S16 Dnase HSS; black track). (A; Bottom) Zoomed-out browser from above to show surrounding m5 RefSeq genes. Dashed lines to the green bar indicate the position of S0X4-4 within a gene desert. (B; Top) SOX4-22 (red box) does not overlap any genomic feature assessed. (B; Bottom) Zoomed-out browser from above to show surrounding m5 RefSeq genes. Dashed lines to the green bar indicate the position of S0X4-22 within a gene desert. In all panels, track names are at the left, the scale for each track is indicated, and the width of each browser window is noted at the top (kb = kilobase pairs and Mb = megabase pairs). (AI 1851 kb)

Additional file 9: Figure S6. CRISPR cuts all alleles in S16 cells. A wild-type specific diagnostic PCR was performed on rSOX-4 Clone 1-8 and 2-8, and PCR products were sequenced. (A) A single base pair insertion (yellow box) of an adenine was identified in the remaining non-recombined alleles of rSOX-4 Clone 1-8 that resides within the gRNA-1 recognition site. (B) A 79 base-pair deletion was detected encompassing the gRNA-2 cut site in rSOX-4 Clone 2-8. The yellow box represents the expected 5’ sequence, and the blue box represents the 3’ sequence. In both panels, the m5 genome sequence is the expected sequence, and gRNA-1, SOX10 consensus site, and gRNA-2 are labeled and indicated by lines under the nucleotide sequences. (AI 1471 kb)

Additional file 10: Figure S7. Tub2b2b expression is significantly reduced in rSOX-4 mutant S16 cells. An MA plot of the mean expression of every gene (dots) against the log2-fold change is shown. The mean expression is calculated as the mean of the normalized counts across all samples, and the log2 fold change is relative to unmodified S16 cells. Genes above the red line (“GT”) indicate higher expression in rSOX-4 mutant cells, and genes below the red line indicate lower expression in rSOX-4 mutant cells. Red dots indicate genes significantly differentially expressed between rSOX-4 mutant and unmodified S16 cells (p < 0.05). Gmnn and Tub2b2b are labeled and indicated by arrows. (AI 17314 kb)

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Availability of data and materials
All reagents, and custom Perl scripts will be available upon request to the corresponding author. All bioinformatic and genome-wide functional data have been provided as additional material. RNA-Seq datasets generated in this study are uploaded to GEO (GSE81709).

Authors’ contributions
WDL and AA wrote Perl scripts to identify SOX10 binding sites; EAF performed all experiments and analysis relevant to CAGE. WDL and AV performed initial cloning, maternogenesis, and luciferase analysis. WDL and AA designed, performed, and analyzed all of the remaining data; WDL, EAF, and AA wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
The mouse transgenic experiments were performed by the University of Michigan Transgenic Animal Core and were approved by the University of Michigan Committee on the Use and Care of Animals.

Competing interests
The authors declare that they have no competing interests.
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References
1. Zhang F, Lupski JR. Non-coding genetic variants in human disease. Hum Mol Genet. 2015;24(R1):R102–10.
2. Lettice LA, Heaney SJ, Purdie LA, Li L, de Beer P, Oostra BA, Goode D, Elgar GW, Zuchner S. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489(7415):57–74.
3. Lee D, Gorkin DU, Baker M, Strober BJ, Asoni AL, McCallion AS, Beer MA. A method to predict the impact of regulatory variants from DNA sequence. Nat Genet. 2015;47(8):955–61.
4. Skre H. Clinical and genetic aspects of Charcot-Marie-Tooth’s disease. Cln Genet. 1974;6(3):98–118.
5. Dyck PJ, Lambert EH. Lower motor and primary sensory neuron diseases with peroneal muscular atrophy. I. Neurologic, genetic, and electrophysiological findings in hereditary neuropathies. Arch Neurol. 1968;16(6):603–18.
6. Kulkens T, Bolhuis PA, Wolterman RA, Kemp S, te Nijenhuis S, Valentijn LJ, van der Knaap MS, van den Heuvel L, van de Loo P. SOX10 is mutated in X-linked non-immune myodystrophy. J Med Genet. 2001;38(2):78–83.
7. Hayasaka K, Himoro M, Sato W, Takada G, Uyemura K, Shimizu N, Bird TD, Hayasaka K. SOX10/CREB response element at the Charcot-Marie-tooth disease type 4C locus regulates expression in a mouse model of Waardenburg-Shah (WS4) syndrome. Hum Mol Genet. 2006;15(2):259–71.
8. Gopinath C, Law WD, Rodriguez-Molina JJ, Prasad AB, Song L, Crawford GE, Mullikin JC, Saren J, Antonelli A. Stringent comparative sequence analysis reveals SOX10 as a putative inhibitor of glial cell differentiation. BMC Genomics. 2016;17(1):1887.
9. Goda S, Hammer J, Kobilier C, Quares M. Expression of the myelin-associated glycoprotein in cultures of immortalized Schwann cells. J Neurochem. 1991;56(6):1534–61.
10. Murrey H, Nunez JJ, Howden NJ, Patel PT. Comparative analysis of Schwann cell lines as model systems for myelin gene transcription studies. J Neurosci Res. 2002;69(4):497–508.
11. Saltariz-Guese EF, Kim S, Kim H. Embryonic mouse spinal cord motor neuron hybrid cells. Neuroreport. 1991;2(9):505–8.
12. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature. 1977;270(5593):725–7.
13. Yaffe D, Saxel O. A myogenic cell line with altered serum requirements for growth and differentiation. Differentiation. 1977;7(3):159–66.
14. Amin H, Vachis J, Hamilton A, Steunenwald N, Howden R, Arthur ST. GSK3beta inhibition and LEF1 upregulation in skeletal muscle following a bout of downhill running. J Physiol Sci. 2014;64(1):1–11.
15. Biol IR. Tissue loss and recession in the absence of recombinase. Nat Genet. 2000;28(16):3047–8.
16. Amin H, Vachis J, Hamilton A, Steunenwald N, Howden R, Arthur ST. GSK3beta inhibition and LEF1 upregulation in skeletal muscle following a bout of downhill running. J Physiol Sci. 2014;64(1):1–11.
17. Biol IR. Tissue loss and recession in the absence of recombinase. Nat Genet. 2000;28(16):3047–8.
18. Houlden H, Girard M, Crockrell C, Ingram D, Wood NW, Goossens M, Walker RW, Reilly MM. Connexin 32 promoter P2 mutations: a mechanism of peripheral nerve dysfunction. Ann Neurol. 2004;55(5):730–4.
19. Jones EA, Lopez-Antido C, Srinivasan R, Krueger C, Chang LW, Nagarajan R, Saren J. Regulation of the PMP22 gene through an intronic enhancer. J Neurosci. 2011;31(14):4242–50.
of the SH3-domain kinase binding protein 1 (Sh3kbp1) locus in Schwann cells via an alternative promoter. Mol Cell Neurosci. 2012;49(2):85–96.

41. Inoue K, Khajavi M, Ohyama T, Hiraibayashi S, Wilson J, Reiggl JD, Mancias P, Butler U, Wilkinson MF, Wengen M, et al. Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations. Nat Genet. 2004;36(4):361–9.

42. Mall P, Yang L, Esveldt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science. 2013; 339(6121):823–6.

43. Hung HA, Sun G, Keles S, Sareen J. Dynamic regulation of Schwann cell enhancers after peripheral nerve injury. J Biol Chem. 2015;290(1):16937–50.

44. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15–21.

45. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166–9.

46. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

47. Takahashi H, Lassmann T, Murata M, Carninci P. 5′-end-centered expression profiling using cap-analysis gene expression and next-generation sequencing. Nat Protoc. 2012;7(3):542–61.

48. Serbedzija GN, Fraser SE, Bronner-Fraser M. Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Development. 1990;108(4):605–12.

49. Serbedzija GN, Bronner-Fraser M, Fraser SE. Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development. 1992;116(2):297–307.

50. Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K. The contribution of both forebrain and midbrain crest cells to the mesencephalon in the frontonasal mass of mouse embryos. Dev Biol. 1994;164(2):409–19.

51. Wilson YM, Richards KL, Ford-Perriss ML, Panthier JJ, Murphy M, Nave KA, Birchmeier C, Wegner M. The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. 2001;15(1):66–79.

52. Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, Shoukry M, Minovitsky S, Dubchak I, Holt A, Lewis KD, et al. In vivo enhancer analysis of human conserved non-coding sequences. Nature. 2006;444(7118):499–502.

53. Britsch S, Goericke DH, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M. The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. 1990;4(6):605–12.

54. Arnold CD, Gerlach D, Stelzer C, Boryn LM, Stark A. Genome-wide quantitative enhancer activity maps identified by STARR-seq. Science. 2013;339(6123):1074–8.

55. Cooper GM, Brown CD. Qualifying the relationship between sequence conservation and molecular function. Genome Res. 2008;18(2):201–5.

56. Arnold CD, Gerlach D, Steiner C, Boryn LM, Rathi M, Stark A. Genome-wide quantitative enhancer activity maps identified by STARR-seq. Science. 2013; 339(6123):1074–7.

57. Kwasnieski JC, Mogno I, Myers CA, Corbo JC, Cohen BA. Complex effects of nucleotide variants in a mammalian cis-regulatory element. Proc Natl Acad Sci U S A. 2012;109(47):19498–503.

58. Soil NP, van den Heuvel M, van de Wetering M, de Beer P, Paulissen G, van der Wingen P. MATCH: a tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res. 2003;31(13):3576–9.

59. Gibson DG, Young L, Chiang YM, Venter JC, Hutchison CA 3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2006;5(5):345–53.

60. Lassmann T. TagDust2: a generic method to extract reads from sequencing data. BMC Bioinformatics. 2015;16:24.

61. Li H, Durbin R. Fast and accurate short read alignment with burrows-wheeler transform. Bioinformatics. 2009;25(14):1754–60.

62. Frith MC, Valen E, Krogh A, Hayashizaki Y, Carninci P, Sandelin A. A code for transcription initiation in mammalian genomes. Genome Res. 2008;18(1):1–12.

63. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 2010;26(6):841–2.

64. Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, et al. A global reference for human genetic variation. Nature. 2015;526(7571):68–74.

65. Yang L, Duff MO, Graveley BR, Carmichael GG, Chen LL. Genomewide characterization of non-polyadenylated RNAs. Genome Biol. 2011;12(2):R16.

66. Cheung TH, Barthel KK, Kwan YL, Liu X. Identifying pattern-defined regulatory islands in mammalian genomes. Proc Natl Acad Sci U S A. 2007; 104(24):10116–21.